The Cerebellum **(MS TC190105)**

Formation of GABAergic synapses in the cerebellum

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Number of text pages: 23, Figures: 3, Table:1

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Abstract

In the adult central nervous system (CNS), γ-amino butyric acid (GABA) is a predominant inhibitory neurotransmitter, and is involved in the expression of various higher brain functions. In the cerebellum, formation of GABAergic synapses is crucial for cerebellar functions. However, it is not fully understood how GABAergic synapses and networks are formed. We are morphologically investigating the developmental changes in GABAergic signaling and the mechanisms underlying the assembly of GABAergic synapses using the cerebellum, which provides an ideal system for the investigation of brain development. Here, I review the anatomy and development of GABAergic synapses and networks in the cerebellar cortex, address the key factors for the formation of GABAergic synapses, and discuss the mechanisms underlying the formation of cerebellar GABAergic networks.

Key Words: glutamic acid decarboxylase, GABA receptor, GABA transporter, reeler mutant mouse

Introduction

In the mammalian CNS, GABA is a predominant inhibitory neurotransmitter which regulates glutamatergic activity [1-3]. Furthermore, GABAergic synaptic transmission also controls experience-dependent plasticity in the visual cortex, induces long-term potentiation, which is the electrophysiological basis of memory and learning, modulates anxiety and generates circadian rhythms [4-6].

In the cerebellar cortex, Purkinje cells and three interneurons, stellate, basket and Golgi cells, are GABAergic. GABAergic innervation regulates the major stream of the cerebellar circuit [7,8], and possibly plays crucial roles in the regulation of movement and motor learning. Elimination of GABAergic input from the Golgi cells in the cerebellar granular layer causes overexcitation of granule cells resulting in severe ataxia during the acute phase [9].

In the first half of this paper, I review the anatomy of the neural circuit and ontogeny of GABAergic synapses in the cerebellar cortex. In the second half, I address the key factors for the assembly of GABAergic synapses and discuss the mechanism underlying the formation of GABAergic networks.

GABAergic synapses in the cerebellar cortex

GABAergic neurons and synapses

The cerebellar cortex consists of three layers, molecular, Purkinje cell and granular layers (Fig. 1A) [7,8,10]. Each layer contains distinct types of neurons. Stellate cells are scattered in the molecular layer and basket cells are localized in the deep part of the molecular layer. Cell bodies of Purkinje cells are arranged in a single layer between the molecular and granular layers. Numerous granule cells occupy the granular layer and Golgi cells are localized mainly in the upper half of the granular layer. Among the five types of main neurons, four of them, Purkinje, stellate, basket and Golgi cells, release GABA as a neurotransmitter.

The Purkinje cell is a pivotal neuron in the cortex. Each dendrite spreads out in a single vertical parasagittal plane in the molecular layer, receiving excitatory inputs from climbing and parallel fibers at the spines and inhibitory inputs from stellate cells at the shafts. Cell bodies form GABAergic synapses with the collaterals of basket cell axons. The Purkinje cell axons traverse the granular layer into the white matter, and innervate the deep cerebellar and vestibular nuclei. In addition, recurrent collateral branches arise from the third or fourth nodes of Ranvier [11], ramify in the upper granular layer, and give rise to plexuses beneath the Purkinje cell bodies. In the plexus, axon collateral varicosities form GABAergic synapses with Purkinje and Golgi cells at the cell bodies and dendrites.

Each stellate and basket cell extends its dendrite only in a parasagittal plane parallel to the fan of Purkinje cell dendrites within the molecular layer. They receive excitatory inputs from parallel and climbing fibers and inhibitory inputs from stellate cells. The stellate cell axons arborize within the molecular layer, and their varicosities make many GABAergic synaptic contacts with the dendritic shafts of Purkinje cells and other GABAergic neurons, including stellate, basket and Golgi cells. Basket cell axons run deep in the molecular layer just above the cell bodies of Purkinje cells. Axon collaterals descend along the Purkinje cell dendrites, surround the Purkinje cell bodies, give rise to pericellular baskets, forming periaxonal plexuses, '*pinceau*', around the initial segment of the Purkinje cell axon. At the pericellular basket, axo-somatic synapses are formed with Purkinje cell bodies. At the *pinceau*, axo-axonic synapses are formed with the initial segments of Purkinje cells. Both synapses are GABAergic. One basket cell axon innervates about 10 Purkinje cells.

Golgi cells extend their dendrites into the molecular layer. Each Golgi cell dendrite is not confined to a single plane, but opens out into a three-dimentional ungulated field. They receive excitatory input from parallel and climbing fibers in the molecular layer and mossy fibers in the granular layer. Inhibitory inputs come from stellate cells in the molecular layer and Purkinje cell axon collaterals in the granular layer. One to three axons

arise from the cell body and main dendrite, divide repeatedly and give rise to plexuses. At the plexuses, varicosities of Golgi cell axons form GABAergic synapses with granule cell dendrites at the peripheral part of the glomeruli in the granular layer.

GABA, GABA receptors and GABA transporters

GABA is synthesized from glutamate by two isoforms of glutamic acid decarboxylase (GAD65 and GAD67) [12-14], and is loaded into vesicles by the vesicular GABA transporter (VGAT) (Fig.1B) [15-17]. In response to the influx of calcium ions via a voltage-dependent calcium channel, GABA is released by the fusion of vesicles with the presynaptic membrane at the nerve terminals, activating GABA receptors at the postsynaptic membrane. GABAergic signals are terminated by reuptake of the neurotransmitter into nerve terminals or uptake into surrounding glia by plasma membrane GABA transporters (GATs) [18].

GABA receptors are classified into three groups on the basis of pharmacology and biochemistry; $GABA_A$, $GABA_B$ and $GABA_C$. Among them, fast synaptic transmission is mainly mediated by $GABA_A$ receptors [2,19-21]. The $GABA_A$ receptor is a member of the ligand-gated ion channel receptor family, and is thought to be composed of five heteromeric subunits belonging to seven different subunit families; α1-6, β1-3, γ1-3, δ, ε , π, and θ[2,20,22]. Native $GABA_A$ receptors contain at least one α-, one β-, and one γ-subunit [22-25]. In the

cerebellar cortex, the main composition of the $GABA_A$ receptors is $\alpha1\beta2\gamma2$ [26-28]. In addition, Purkinje cells abundantly express the β3 subunit ($\alpha1\beta2/3\gamma2$), while granule cells abundantly express the α6, β3 and δ subunits ($\alpha1/6\beta2/3\gamma2/\delta$). GABA binding opens the pore of $GABA_A$ receptors and induces inhibitory postsynaptic potential by the influx of chloride ions (Fig. 1B).

Plasma membrane GABA transporters (GAT) are high-affinity transporters, and co-transport GABA, sodium ion (Na^+) and chloride ions (Cl^-) [29,30]. Molecular cloning has isolated four GATs, GAT-1, GAT-2, GAT-3 and BGT-1. (Mouse GAT2, GAT3 and GAT4 are species homologs of rat BGT-1, GAT-2 and GAT-3, respectively.) In the cerebellar cortex, GAT-1 is mainly localized at the axon terminals containing GABAergic vesicles[31,32]. In contrast, GAT-3 is localized at the astrocyte processes in the granular layer. GAT-2 is only localized at the leptomeningeal, ependymal cells and choroids plexus [33]. GAT-1 and GAT-3 remove GABA from the synaptic cleft into the presynapse and surrounding glia, respectively.

Development of GABAergic synapses in the cerebellar cortex

GABA, GABA receptors and GABA transporters

In the developing cerebellum, GABA is distributed throughout the GABAergic neurons, including cell bodies, dendrites, axons, axon varicosities, and growth cones [34,35].

During synapse formation, GABA becomes confined to the axon terminals (Table). After finishing synapse formation, GABA is almost completely co-localized with VGAT at the synaptic sites, indicating that most GABA is exclusively localized in the synaptic vesicles within the axon terminals.

In the immature cerebellar cortex, VGAT is localized at the axon varicosities and growth cones and may be involved in extrasynaptic exocytosis (Table) [34]. Subsequently, the axon varicosities and growth cones may give rise to terminals, and VGAT is confined to these terminals.

The subunit compositions of $GABA_A$ receptors also drastically change during cerebellar development[36,37]. Cerebellar neurons start to express the functional $GABA_A$ receptors after finishing cell proliferation. Differentiating Purkinje cells express the α3 subunit, migrating and maturating granule cells express the α2 subunit, and both subunits disappear from the cerebellar cortex after finishing synapse formation. In addition, the β3, γ1 and γ3 subunits are also abundantly expressed in the developing cerebellum. During synapse formation, the subunit composition shifts from α2/3βγ1/3 to α1/6β2/3γ2 (Table).

In the developing cerebellum, plasma membrane GABA transporters appear during synapse formation [38]. GAT-1 appears at postnatal day 5 (P5), and is localized at axons, varicosities, and terminals, and GAT-3 appears at postnatal day 10 (P10) in the granular

layer, and is localized in the processes of astrocytes.

GABAergic synapses

In the molecular and Purkinje cell layers, symmetric synapses between VGAT or GAD-positive terminals and dendrites appear at postnatal day 5 (P5) (Fig. 2A), and are often observed at postnatal day 7 (P7) (Fig. 2B-D). In the granular layer, symmetrical synapses between VGAT- or GAD-positive terminals and granule cell dendrites appear in the deep part at P7 (Fig. 2E) and are often detected in all layers at P10 (Fig. 2F) [35]. The $GABA_A$ receptor α1 subunit protein, which is an essential subunit for mature $GABA_A$ receptors in the cerebellar neurons[27,28], appears on the same postnatal days in the cerebellar cortex [35]. The number of GABAergic synapses massively increases in all layers during the second and third post natal weeks [35,39-41], and the expression and localization of the $GABA_A$ receptor α1 subunit increase simultaneously. These results indicate that GABAergic synapses are massively formed during the second and third postnatal week.

The cerebellum is deeply involved in learning motor skills [7,8,42]. 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. During the same developmental period, GABAergic inhibition starts in the cerebellar cortex. These results suggest that the start of

GABAergic inhibition is crucial for acquisition of motor learning.

Formation of GABAergic synapses

Assembly of GABAergic synapses in the cerebellar cortex is considered to be a multi-step process [18,43,44], including target determination of GABAergic axons, maturation of postsynapses such as GABA receptor-expression and localization at synaptic sites, expression of plasma membrane transporters and so on, as mentiond in the last paragraph (Table).

Target determination

The mechanism underlying the target determination of GABAergic axons is almost unknown. Only the specificity of neuron-to-neuron connection was investigated in the mutant cerebellum[45-47]. In the normal cerebellar cortex, five major types of neurons innervate distinct types of target neurons (Fig. 1) [7,8]. The specific innervation patterns, however, are not preserved in the abnormal environment of the reeler and weaver cerebellum, as shown in Figure3. Golgi cells directly innervate Purkinje cells in the central mass of the reeler cerebellum and weaver cerebellum. In both regions, there are few granule cells or they are absent. Thus, Golgi cell axons form synapses with neighboring neurons instead of granule cells. This result indicates that the targets of Golgi cells are not

genetically and strictly determined, but are influenced by the environment.

Changes in subunit compositions

In the cerebellar cortex, expression of the $GABA_A$ receptor subunits, in particular α subunits, changes during GABAergic synapse formation. The α subunits in the $GABA_A$ receptors shift from the α2 and α3 subunits to the α1 and α6 subunits [36,37,48] (Table). This result suggests that disappearance of subunits which are involved in morphogenesis, and the appearance of subunits which mediate inhibitory synaptic transmission are crucial for GABAergic synapse formation.

To test the mechanism underlying the change in subunit compositions, we investigated its relationship with aspects of 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 is assumed to be arrested in terms of the synaptic architecture and dendritic arborization [45-47]. 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 form synapses directly with mossy fibers and Golgi cell axons. Dendrites of Purkinje cells are poorly developed and extend almost randomly. The α3 subunit, however, is negative as in normal mature Purkinje cells [49], and

malpositioned Purkinje cells abundantly express the α1 subunit (Fig. 2) [49,50]. These results indicate that developmental changes in subunit composition are independent of neuronal maturation, such as settling in the normal neuronal position, and maturation of excitatory networks. Absence of normal inhibitory synapses with stellate and basket cell axons and heterologous input from Golgi cells do not affect the developmental change in subunit composition. Previous in vitro studies have indicated that GABAergic stimulation induces low-affinity type GABA receptor expression, which is involved in inhibitory synaptic transmission [51-53]. These results as a whole suggest that innervation of GABAergic fibers may be important for the changes in subunit composition, even if the synapses are heterologous and ectopic; GABAergic innervation may initiate and/or accelerate the changes in subunit composition.

Specific subunit expression

In the CNS, distinct types of subunits are expressed at distinct synapses as shown in Figure1 [27,54]. In the normal cerebellum, GABAergic transmission between stellate cell axons and Purkinje cell dendrites is mediated by $GABA_A$ receptors containing only the α1 subunit, but not the remaining five α subunits. In contrast, inhibitory transmission between Golgi cell axons and granule cell dendrites is mediated by $GABA_A$ receptors containing both the α1 and α6 subunits [27,54].

To test the relationship between types of presynapse and subunits in the postsynapse, we examined the expression of $GABA_A$ receptor subunits in the reeler cerebellum. In the central cerebellar mass of the reeler cerebellum, Purkinje cells directly form synapses with Golgi cell axons [45-47]. If presynaptic neurons determine the type of receptor subunits in the postsynaptic neurons, GABAergic innervation from Golgi cells should induce Purkinje cells to express the α6 subunit in the central cerebellar mass. Nevertheless, Purkinje cells in the central cerebellar mass did not express the α6 nor α2 subunits (Fig. 2) [49]. This result indicates that Golgi cell innervation does not induce expression of the α6 subunit in Purkinje cells, and suggests postsynaptic self-autonomous process, but not presynaptic neurons, determines the types of subunits.

Activity-dependent synaptic remodeling

Recent investigations revealed that GABAergic synapses are remodeled by the change in GABAergic input in the auditory systems during the critical period [55-57]. Auditory experience guides sub-cellular localization of the receptor proteins [57], induces functional and structural elimination of inhibitory synapses during the establishment of precise topography in the GABAergic/glycinergic pathway [56], and mediates aural dominance bands in the inferior colliculus [58]. In the cerebellum, the above activity-dependent remodeling of GABAergic synapses has not yet been revealed, but could play roles in the formation and

maturation of GABAergic synapses.

Acknowledgement

I am grateful to Yoshihiko Ogawa and Hideki Nakamura at the Central Research Department of Hokkaido University School of Medicine for their technical assistance. I also thank Prof. Yoshiro Inoue and Dr. Takayuki Yoshida at the Department of Molecular Neuroanatomy Hokkaido University School of Medicine, and Dr. Hitoshi Komuro at the Cleveland Clinic Foundation for valuable discussions.

Figure Legends

Figure 1 Schematic illustrations of the neural circuit in the cerebellum (A) and GABAergic transmission in the GABAergic synapse (B).

(A) Schematic illustration of the neural circuit in the normal mature cerebellar cortex. The full lines are GABAergic axons and dendrites. Gray circles are cell bodies of GABAergic neurons. Arrows indicate GABAergic synapses. Dotted lines and circles are axons, dendrites and cell bodies of glutamatergic neurons.

Abbreviations; MoL: molecular layer, PuL: Purkinje cell layer, GrL: granular layer, PF: parallel fiber, CF: climbing fiber, MF: mossy fiber, St: stellate cell, Ba: basket cell, Pu: Purkinje cell, Gr: granule cell, Go: Golgi cell, IO: inferior olive nucleus, Nu: deep cerebellar and vestibular nuclei, PN/SC: pontine nucleus/ spinal cord, α1: $GABA_A$ receptor containing the α1 subunit, α1/6:$GABA_A$ receptor containing the α1 and 6 subunits.

(B) Schematic illustration of GABAergic transmission in the mature GABAergic synapse. GABA is synthesized from glutamate by glutamic acid decarboxylase (GAD), and is loaded into vesicles by the vesicular GABA transporter (VGAT). GABA is released by the fusion of vesicles with the presynaptic membrane at the nerve terminals, and activates GABA receptors (GABAR) at the postsynaptic membrane. In the adult synapses, activation of $GABA_A$ receptors mediates hyperpolarization of postsynaptic membrane potential (IPSP) by

the influx of chloride ions (Cl^-). GABAergic signals are terminated by uptake and reuptake of neurotransmitters into nerve terminals and surrounding glia by the plasma membrane GABA transporters (GAT).

Abbreviations GAD: glutamic acid decarboxylase, VGAT: vesicular GABA transporter, GAT: (plasma membrane) GABA transporter, GABAR: GABA receptor, IPSP: inhibitory postsynaptic potential.

Figure 2 Immunohistochemistry for VGAT in the developing cerebellar cortex

(A-D) Immunohistochemical localization of VGAT in the molecular (A, C, D) and Purkinje cell layers (B) at postnatal day 5 (P5) (A) and postnatal day 7 (P7) (B-D). Symmetric synapses are detected at P5 (arrowhead in A) between VGAT-positive terminals containing flattened vesicles and dendrites (den). At P5, excitatory synapses are already detected (asterisks) among parallel fiber bundles (Pf). At P7, GABAergic synapses are more frequently observed between basket cell axon terminals and Purkinje cell bodies (PC) (arrowheads in B), and stellate cell axon terminals and dendrites (den).

(E and F) Immunohistochemical localization of VGAT in the granular layer at P7 (E) and P10 (F). At P7, symmetric synapses (arrowheads) are detected between VGAT-positive Golgi cell terminals and granule cell dendrites (Gd) in the deep part of the granular layer

(E). At P10, GABAergic synapses are more frequently detected in the more mature synaptic glomeruli (F).

Abbreviations and symbols: den: dendrite, Pf: parallel fiber bundle, asterisk: asymmetric synapse, arrowhead: symmetric synapse, BG: sheet of Bergman glial process, Gd: granule cell dendrite, Mf: mossy fiber terminal

Bars: 0.5μm

Figure 3 Schematic illustrations of GABAergic input abnormalities in the reeler cerebellum. In the central cerebellar mass of the reeler cerebellum, Purkinje cells (Pu) directly receive inhibitory inputs (arrows) from Golgi cells (Go) instead of from stellate (st) and basket (ba) cells. $GABA_A$ receptors containing only the α1 subunit (α1), but not the remaining five α subunits, are localized at the GABAergic synapses on the Purkinje cells, although Golgi cells innervate them. In addition, GABAergic input from the Purkinje cell axon collaterals increased markedly.

Abbreviations; Nu: cerebellar nucleus

Table Developmental changes in localization and composition of the elements of GABAergic synapses in the cerebellar cortex

	Before synapse formation	After synapse formation
GABA	throughout the neurons	terminals (and cell bodies)
VGAT	varicosities and growth cones	terminals (including varicosities)
$GABA_A$ receptor	α2/3β3γ1/3	α1/6β2/3γ2
GATs	none	GAT-1 (presynapse) GAT-3 (astrocytes)

Abbreviations: VGAT: vesicular GABA transporter, GATs: plasma membrane GABA transporters

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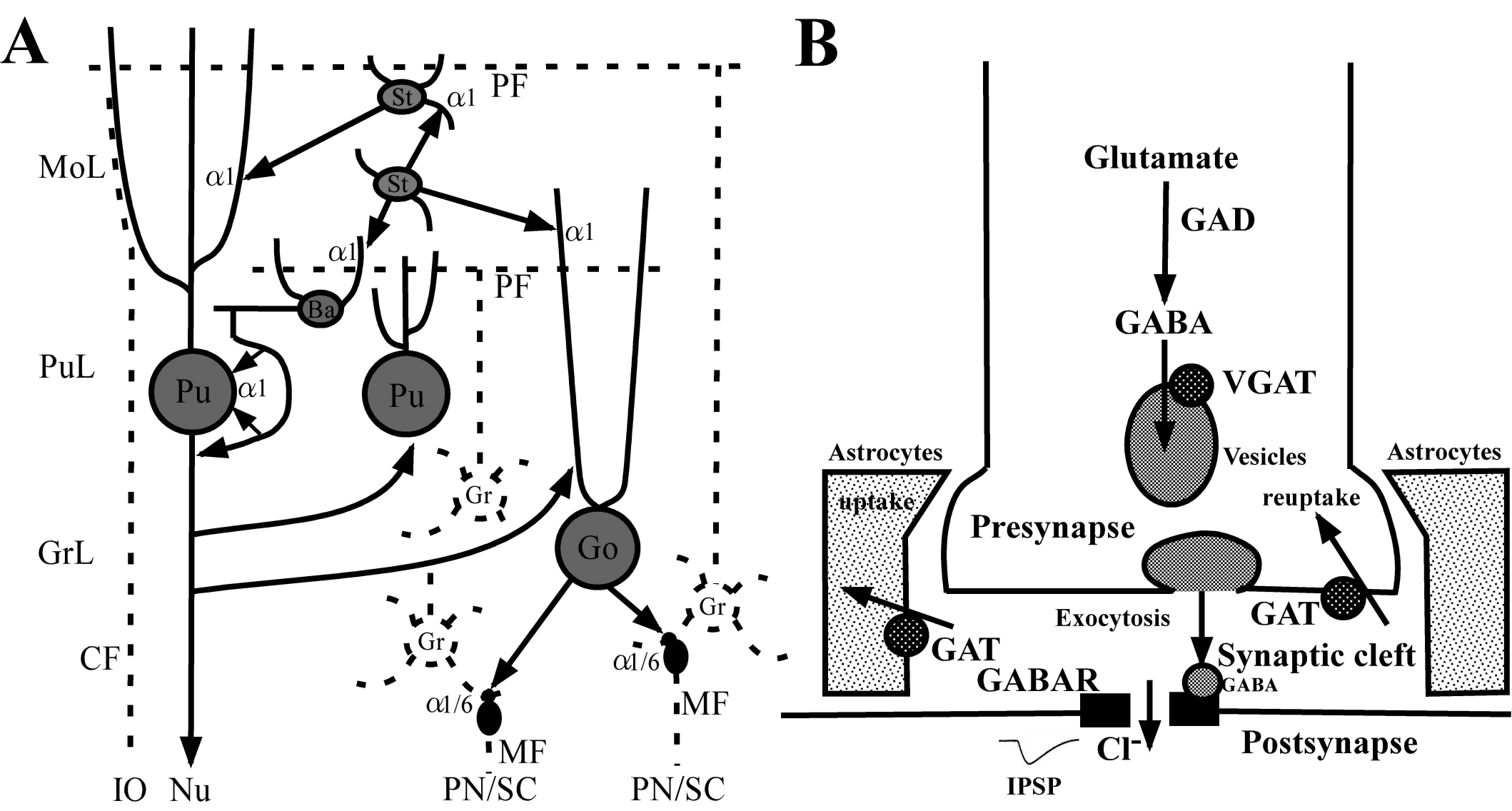
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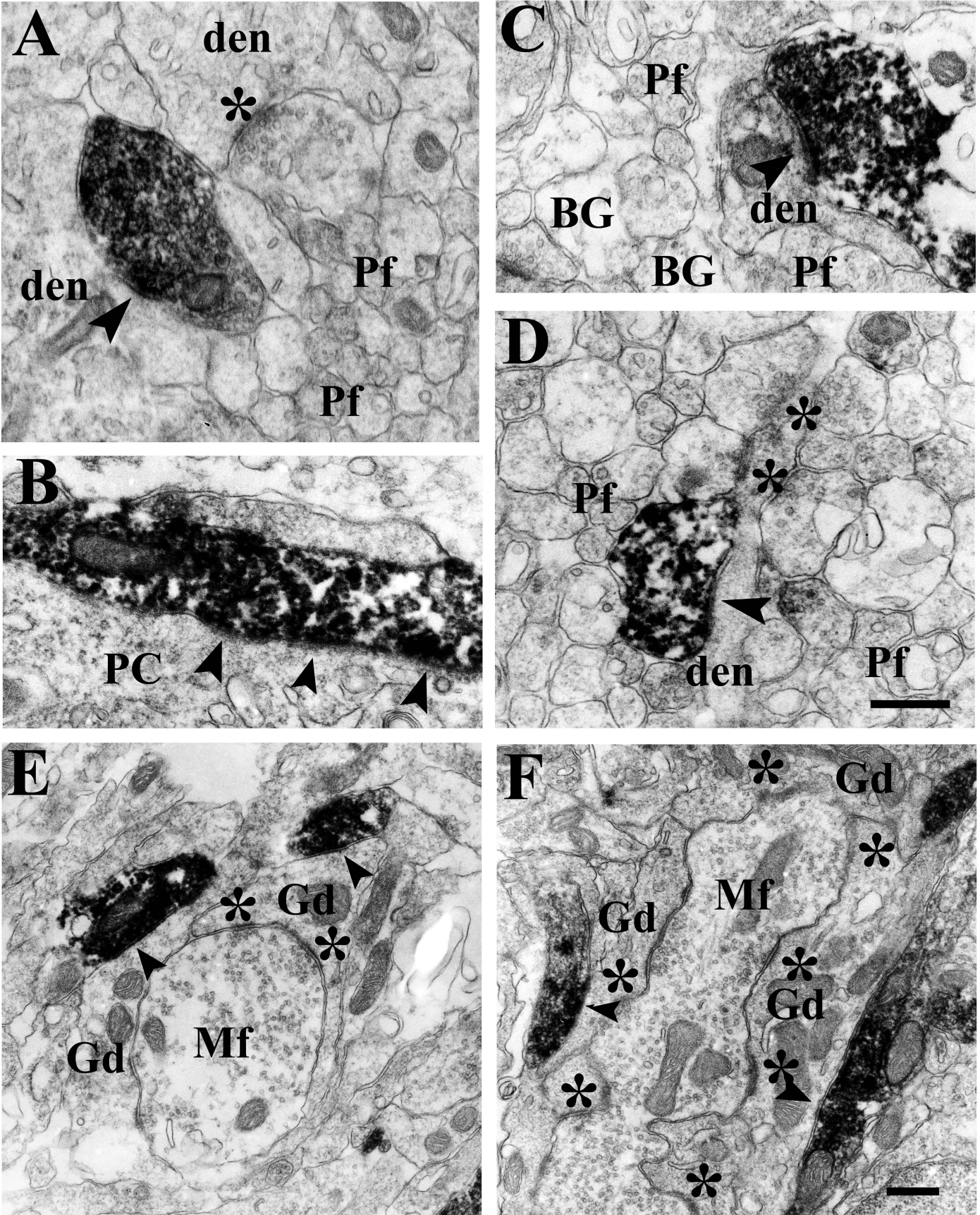
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Gd
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Gd
Mf
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Gd
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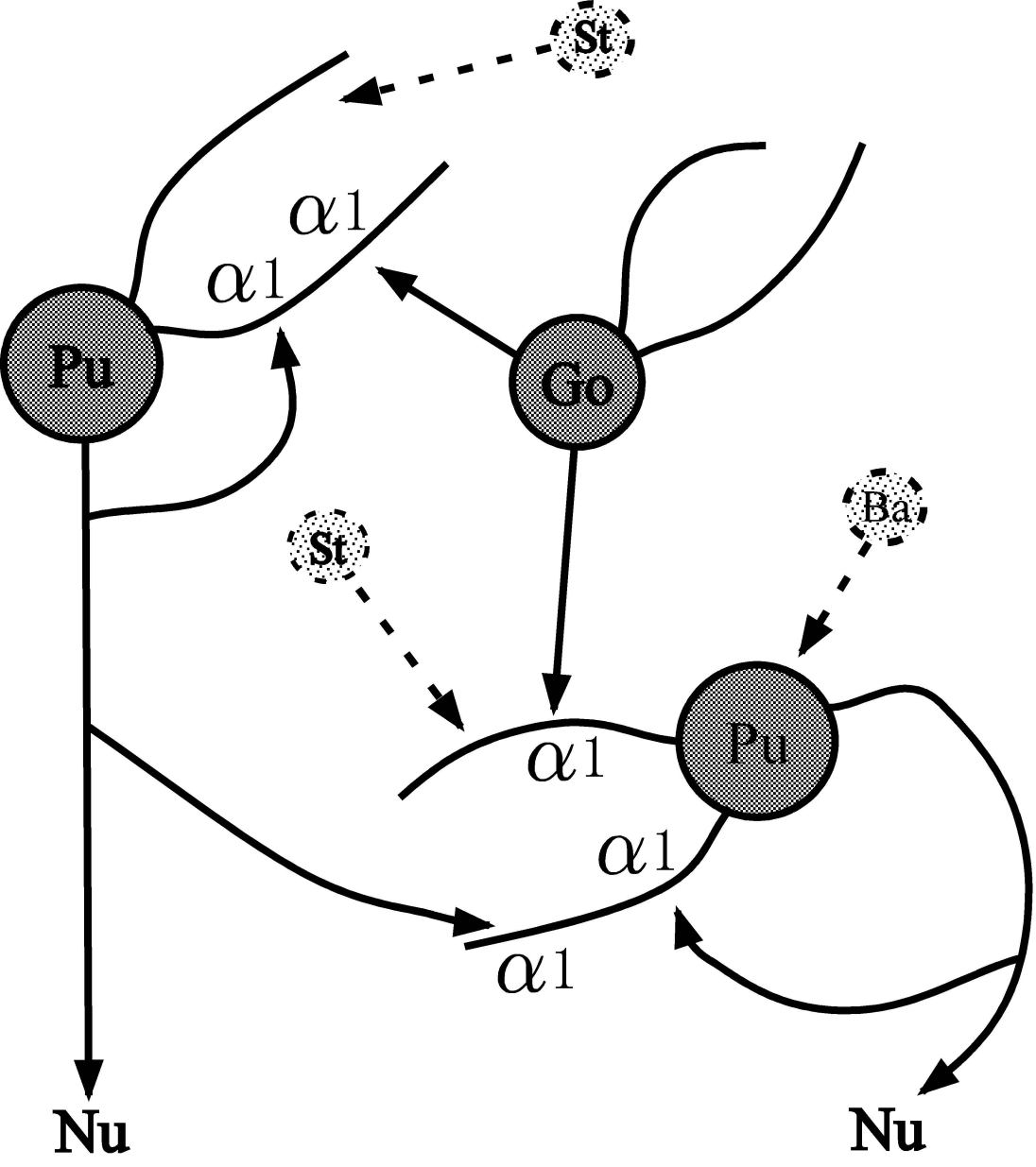
St
α1
α1
Pu
Go
St
Ba
α1
Pu
α1
α1
Nu
Nu