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LETTER TO NEUROSCIENCE

DIFFERENTIAL DISTRIBUTION OF VESICULAR GLUTAMATE TRANSPORTERS IN THE RAT CEREBELLAR CORTEX

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Abstract—The chemical organization of excitatory axon terminals in the rat cerebellar cortex was examined by immunocytochemistry and *in situ* hybridization histochemistry of vesicular glutamate transporters 1 and 2 (VGluT1 and VGluT2). Chemical depletion of the inferior olivary complex neurons by 3-acetylpyridine treatment almost completely removed VGluT2 immunoreactivity from the molecular layer, leaving VGluT1 immunoreactivity apparently intact. On the other hand, neuronal deprivation of the cerebellar cortex by kainic acid injection induced a large loss of VGluT1 immunoreactivity in the molecular layer. In the cerebellar granular layer, both VGluT1 and VGluT2 immunoreactivities were found in mossy fiber terminals, and the two immunoreactivities were mostly colocalized in single-axon terminals. Signals for mRNA encoding VGluT2 were found in the inferior olivary complex, and those for VGluT1 and VGluT2 mRNAs were observed in most brainstem precerebellar nuclei sending mossy fibers, such as the pontine, pontine tegmental reticular, lateral reticular and external cuneate nuclei.

These results indicate that climbing and parallel fibers selectively use VGluT2 and VGluT1, respectively, whereas mossy fibers apply both VGluT1 and VGluT2 together to accumulate glutamate into synaptic vesicles. Since climbing-fiber and parallel-fiber terminals are known to make depressing and facilitating synapses, respectively, VGluT1 and VGluT2 might have distinct properties associated with those synaptic characteristics. Thus, it would be the next interesting issue to determine whether mossy-fiber terminals co-expressing VGluT1 and VGluT2 show synaptic facilitation or depression.

Key words: glutamatergic neurons, climbing fibers, mossy fibers, parallel fibers, precerebellar nuclei, cerebellum.

It has been established that mammalian cerebellar cortex has three kinds of excitatory nerve terminals: axon terminals of climbing fibers, parallel fibers and mossy fibers (for review, Llinás and Walton, 1998). The parallel fibers that are derived from cerebellar granule cells are well known to use L-glutamate for their excitatory transmission (Llinás and Walton, 1998). Recently, mossy fibers have also been supposed to employ L-glutamate as the transmitter (for review, Laake et al., 1999). On the other hand, climbing fibers, which are derived from the inferior olivary complex neurons in the medulla oblongata, have been suggested to use L-aspartate for the transmission by a few lines of evidence. Inferior olivary complex neurons were retrogradely labeled by injection of D-[^{3}H] aspartate into the cerebellar cortex (Wiklund et al., 1984), and L-aspartate-like immunoreactivity was reported in inferior olivary complex neurons and climbing fibers (Aoki et al., 1987; Madl et al., 1987).

Two vesicular glutamate transporters, named VGluT1 and VGluT2, have been identified recently (Bellocchio et al., 2000; Takamori et al., 2000; Bai et al., 2001; Fremeau et al., 2001; Herzog et al., 2001; Takamori et al., 2001; Varoqui et al., 2002). As far as they reported, no significant pharmacological differences were detected between VGluT1 and VGluT2; in particular, the two transporters could accumulate L-glutamate and D-glutamate, but not L-aspartate or D-aspartate into vesicles. VGluT1 immunoreactivity (Bellocchio et al., 1998) and VGluT2 immunoreactivity (Fremeau et al., 2001; Fujiyama et al., 2001; Herzog et al., 2001; Kaneko and Fujiyama, 2002) were electron-microscopically located on synaptic vesicles of the axon terminals which made asymmetric, putatively excitatory synapses in some brain regions, including the cerebellar cortex. VGluT1 and VGluT2 were suggested to be located in parallel-fiber and climbing-fiber terminals, respectively, and both transporters were found in the mossy-fiber terminals. More recently, the third vesicular glutamate transporter (VGluT3) was reported (Gras et al., 2002). Although VGluT3 has the molecular and pharmacological similarity to VGluT1 and VGluT2, its expression pattern is considerably restricted and was not detected in the cerebellum.

In the present study, we produced rabbit antibodies against VGluT1 and VGluT2, and studied colocalization of VGluT1 and VGluT2 immunoreactivities in cerebellar nerve terminals by the double-immunofluorescence method with the rabbit antibodies and the previously produced guinea-pig antibodies to VGluT1 and VGluT2. Furthermore, we observed changes in VGluT1 and VGluT2 immunoreactivities after depletion of inferior olivary com-

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Abbreviations: 3-AP, 3-acetylpyridine; KA, kainic acid; VGluT1, vesicular glutamate transporter 1; VGluT2, vesicular glutamate transporter 2; VGluT3, vesicular glutamate transporter 3.

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plex neurons or cerebellar granule cells, and examined mRNA signals for VGluT1 and VGluT2 in the brainstem precerebellar nuclei.

EXPERIMENTAL PROCEDURES

The experiments were conducted in accordance with the rules of animal care by Institute of Laboratory Animals, Faculty of Medicine, Kyoto University. Twenty-six adult male Wistar rats (200–300-g body weight; Japan SLC, Shizuoka, Japan) and four female white rabbits (2-kg body weight; Japan SLC) were used in the present study. All efforts were made to minimize animal suffering and the number of animals used.

Production and characterization of rabbit antibodies against C-terminals of VGluT1 and VGluT2

The rabbit antibodies that recognize VGluT1 and VGluT2 were produced as described in the previous report on the production of guinea-pig antibodies (Fujiyama et al., 2001). Briefly, C-terminal 19 amino acids (residues 552–560) of rat VGluT1 and C-terminal 29 amino acids (residues 554–582) of rat VGluT2 were synthesized. Four female white rabbits were injected intracutaneously with these antigens. The rabbit antibodies were affinity-purified and characterized by Western blotting as described in the previous report (Fujiyama et al., 2001).

Chemical depletion of neurons and immunoperoxidase staining

Under deep anesthesia with chloral hydrate (35 mg/100-g body weight), 10 rats were injected intraperitoneally with 3-acetylpyridine (3-AP, 65 mg/kg body weight) in saline to deplete the inferior olivary complex neurons (Desclin and Escubi, 1974). The other six rats were injected with 0.5 μl of 0.2% (w/v) kainic acid into the left cerebellar cortex or vermis by pressure through a glass micropipette attached to Picospritzer II (General Valve Corporation, East Hanover, NJ, USA). The treated rats, which were allowed to survive for 7–14 days, and four normal rats were fixed and immunostained for VGluT1 or VGluT2 by the avidin-biotinylated peroxidase complex method with diaminobenzidine as described in the previous report (Fujiyama et al., 2001).

Double immunofluorescence study

The rat sections were incubated overnight with a mixture of 1 μg/ml anti-VGluT1 rabbit IgG and 1 μg/ml anti-VGluT2 guinea-pig IgG. After a wash, the sections were incubated for 1 h with biotinylated anti-guinea-pig IgG donkey antibody (Jackson, West Grove, PA, USA), and then for 1 h with fluorescein isothiocyanate-conjugated anti-rabbit IgG donkey antibody (Chemicon, Temecula, CA, USA) and Alexa594-conjugated streptavidin (Molecular Probes, Eugene, OR, USA) in the presence of 10% (v/v) normal guinea-pig serum. The sections were observed under epifluorescence microscope Axiophot and under con-focal laser scanning microscope LSM 410 (Zeiss, Oberkochen, Germany) with a con-focal depth of 0.5 μm.

In situ hybridization histochemistry

Complementary DNA fragments corresponding to the regions of the rat VGluT1 cDNA (nucleotides 1665-1980 of gb:RNU07609, GenBank) and VGluT2 cDNA (nucleotides 166-500 of gb: AF271235) were cloned into pBluescript II SK(+) (Stratagene, La Jolla, CA, USA). Using these plasmids as templates, sense and antisense RNA probes were synthesized with a digoxigenin labeling kit (Roche Diagnostics, Tokyo, Japan). The six rat-brain tissues fixed by perfusion with 4% paraformaldehyde in 0.1-M phosphate buffer, pH 7.4, were cut into 30-μm-thick frontal sections. The free-floating sections were hybridized with 500 ng/ml digoxigenin-labeled sense and antisense RNA probes for VGluT1 or VGluT2 in a mixture of 50% (v/v) formamide, 5× SSC, 5× Denhardt's solution, 250 μg/ml yeast tRNA, and 500 μg/ml salmon sperm. Then, the sections were incubated with 1/2000-diluted alkaline phosphatase-conjugated anti-digoxigenin antibody Fab fragment (Roche). The bound phosphatase was visualized by reaction for 24–36 h with 0.375 mg/ml nitroblue tetrazolium and 0.188 mg/ml 5-bromo-4-chloro-3-indolyl phosphate.

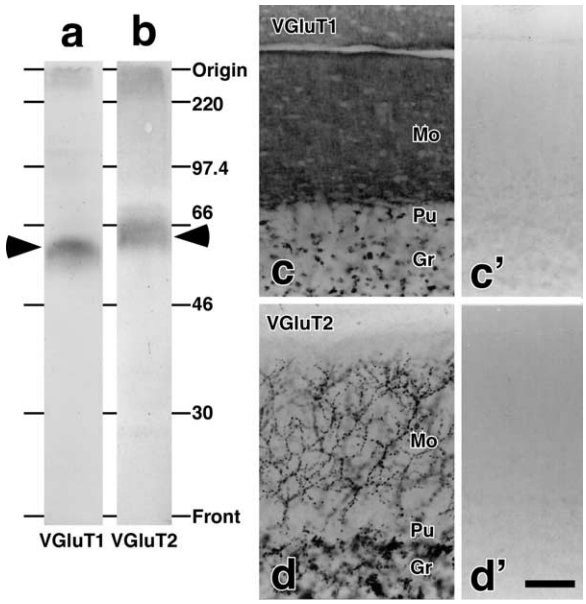

Fig. 1. Characterization of rabbit anti-vesicular glutamate transporter 1 (VGluT1) and anti-VGluT2 antibodies. Rat-brain extract with 1% sodium dodecyl sulfate (SDS) was electrophoresed in 10% polyacrylamide gel in the presence of SDS (Fujiyama et al., 2001). The proteins were blotted onto the polyvinylidene difluoride membrane, and immunostained with the affinity-purified anti-VGluT1 (a) or anti-VGluT2 rabbit antibody (b). Arrowheads indicate the positive bands of about 58,000 or 62,000 Da, respectively. The sagittal sections of the cerebellar cortex were immunostained with the anti-VGluT1 and anti-VGluT2 antibodies (c, d). For control experiments, when the sections were incubated with the primary antibody in the presence of 10,000-fold (in mol) excess amount of the antigen peptide, no immunoreactivity was observed in the cerebellar cortex (c', d'). Scale bar=40 μm (c–d').

RESULTS

Characterization of antibodies

Antibodies to VGluT1 and VGluT2 were raised in rabbits, and affinity-purified on the antigen columns. In the immunoblotting tests with 1% sodium dodecyl sulfate extract of rat brain, the anti-VGluT1 and anti-VGluT2 antibodies recognized protein bands of 58,000 and 62,000 Da, respectively (Fig. 1a, b). The molecular weights of the positive bands were the same as those reported previously (Fujiyama et al., 2001).

VGluT1 and VGluT2 immunoreactivities in the cerebellar cortex (Fig. 1c, d) were almost the same as those reported in our recent work using the guinea-pig antibodies (Kaneko et al., 2002; Kaneko and Fujiyama, 2002). Fur-

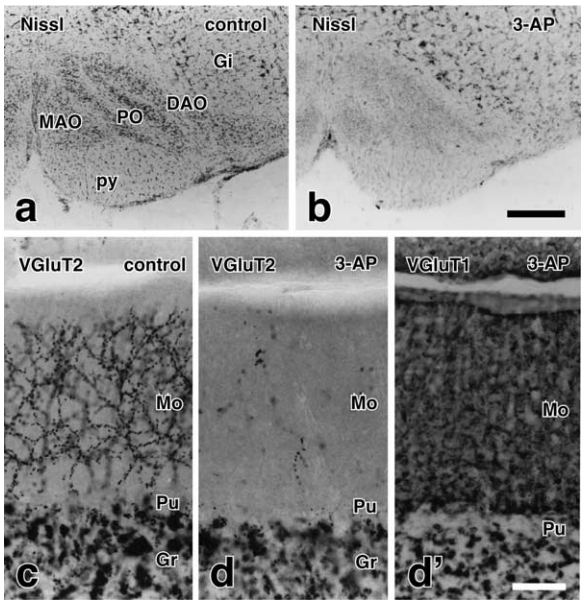

Fig. 2. Chemical lesion of inferior olivary complex neurons with 3-acetylpyridine (3-AP). By i.p. injection of 3-acetylpyridine, almost all neurons were removed from the inferior olivary complex (b), resulting in almost complete loss of vesicular glutamate transporter 2 (VGluT2)-immunoreactive axon terminals in the cerebellar molecular layer (d). However, VGluT1 immunoreactivity was mostly preserved in the molecular layer (d'). (a) Control inferior olivary complex. (c) VGluT2-immunoreactive axon terminals in the molecular layer in the control cerebellar cortex. DAO, dorsal accessory olivary nucleus; Gi, gigantocellular reticular nucleus; MAO, medial accessory olivary nucleus; PO, principal inferior olivary nucleus; py, pyramidal tract. Scale bar=50 μm (c, d, d'); 400 μm (a, b).

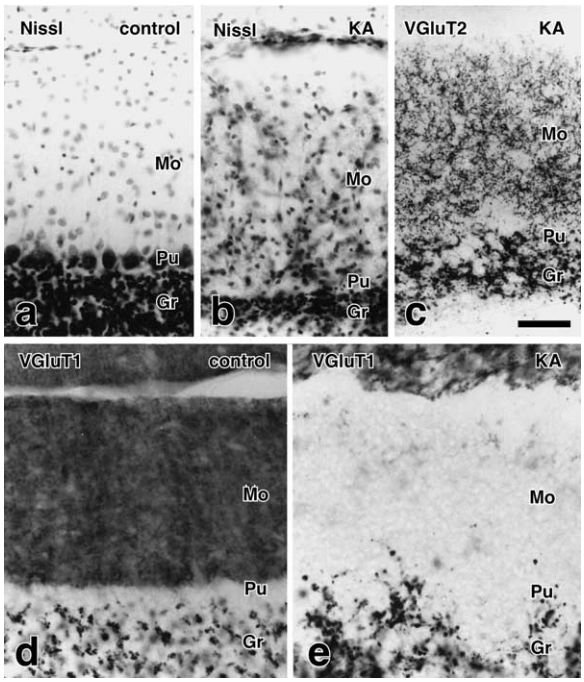

Fig. 3. Chemical lesion of the cerebellar cortex with kainic acid (KA). Local application of KA induced a severe neuronal loss in the affected cerebellar cortical region (b), where only a very thin granular layer or no Purkinje cell was found. In the molecular layer within and near the chemical lesion, vesicular glutamate transporter 1 (VGluT1) immunoreactivity was almost completely abolished (e). In contrast, VGluT2 immunoreactivity was preserved well in the injured molecular layer, although VGluT2-immnoreactive axon terminals appeared distorted (c). (a, d) Control cerebellar cortex far from the chemically injured region. Scale bar=50 μm (a–e).

thermore, VGluT1 or VGluT2 immunoreactivity was completely abolished by preincubation of the anti-VGluT1 or anti-VGluT2 antibody with an excess amount of the C-terminal peptide of VGluT1 or VGluT2, respectively (Fig. 1c', d').

Lesion experiments

When 3-AP was injected intraperitoneally, almost all neurons in the inferior olivary complex were abolished (Fig. 2b). By this treatment, VGluT2-immunoreactive axon fibers and terminals in the molecular layer were drastically decreased (Fig. 2d), although VGluT1 immunoreactivity in the molecular layer (Fig. 2d') and VGluT2 immunoreactivity in the granular layer (Fig. 2d) were well preserved. This clearly indicates that VGluT2-immunoractive axon fibers in the molecular layer are climbing fibers derived from the inferior olivary complex.

When kainic acid was injected locally into the cerebellar cortex, the chemically affected region appeared deprived of a large population of granule cells, Purkinje cells and the other neurons (Fig. 3b). Within and near the site of chemical lesion, the molecular layer lost most of VGluT1 immunoreactivity (Fig. 3e). In contrast, VGluT2-immunoreactive axon fibers were preserved considerably, although the running pattern of the axon fibers was severely distorted probably owing to the loss of their targets, i.e. Purkinje-cell dendrites (Fig. 3c). Since granule cells are the only source of glutamatergic axon fibers in the cerebellar cortex, it is likely that this large reduction of VGluT1 immunoreactivity in the molecular layer was caused by the depletion of granule cells.

Double-immunofluorescence study

Double-immunofluorescence study revealed no colocalization of VGluT1 and VGluT2 immunoreactivities in the molecular layer (Fig. 4a, a'), but showed extensive colocalization of those immunoreactivities in the granular layer of the cerebellar cortex (Fig. 4b, c). Although a few structures in the granular layer exclusively showed either VGluT1 (2 in Fig. 4d, d') or VGluT2 immunoreactivity (1 in Fig. 4d', d″), more than 80% of large terminal-like structures showed immunoreactivities for both transporters; in the vermian region (Fig. 4b), 244 of 246 VGluT1-immunoreactive terminal-like structures showed VGluT2 immunoreactivity, and 244 of 290 VGluT2-immunoreactive structures displayed VGluT1 immunoreactivity. In the hemispheric region (Fig. 4c), 270 of 271 VGluT2-immunoreactive structures were immunopositive for VGluT1, and 270 of 297 VGluT1-immunoreactive structures were positive for VGluT2.

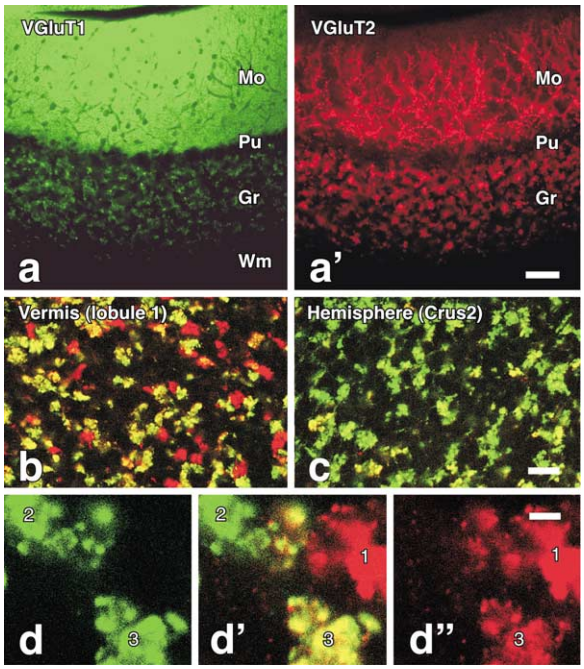

Fig. 4. Double-immunofluorescence staining of the cerebellar cortex. Vesicular glutamate transporter 1 (VGluT1) and VGluT2 were visualized green and red with rabbit anti-VGluT1 antibodies and guinea-pig anti-VGluT2, respectively. The photoimages of a and a' are taken in the same site of the cerebellar cortex (sagittal section) under different excitations. In the granular layer (b, c, d–d'; frontal section), many mossy-fiber terminal-like structures showed both VGluT1 and VGluT2 immunoreactivities and the colocalization was indicated by merged yellow image, although some profiles only showed either VGluT1 (2 in d) or VGluT2 immunoreactivity (1 in d'). The images in d–d' were taken in the same site with a con-focal laser scanning microscope. Scale bar=50 μm (a, a'); 20 μm (b, c); 2.5 μm (d–d').

In situ hybridization histochemistry

Inferior olivary complex neurons showed moderate signals for VGluT2 but not for VGluT1 (Fig. 5a, a'), and the other precerebellar nuclei displayed signals for both transporters. However, the pontine nuclei and pontine tegmental reticular nucleus of Bechterew, expression of VGluT1 mRNA was dominant compared with that of VGluT2 mRNA (Fig. 5b, b'). In contrast, the external cuneate nucleus and lateral reticular nucleus showed moderate to intense signals for both VGluT1 and VGluT2 (Fig. 5c, c', d, d'). Signals for VGluT1 mRNA were observed in the granular layer of the cerebellar cortex as reported previously (Ni et al., 1995; Fremeau et al., 2001; Herzog et al., 2001), but no signal for VGluT2 mRNA was found in the granular layer (not shown).

VGluT1 and VGluT2 in the deep cerebellar nuclei

The neuropil of the deep cerebellar nuclei has been reported to show weak to moderate VGluT1 immunoreactivity and moderate VGluT2 immunoreactivity (Kaneko et al., 2002). Since both mossy fibers and climbing fibers have collateral inputs to the deep cerebellar nuclei (for review, cf. Shinoda et al., 2000), VGluT1 and VGluT2 immunoreactivities are expected to be located in excitatory axon

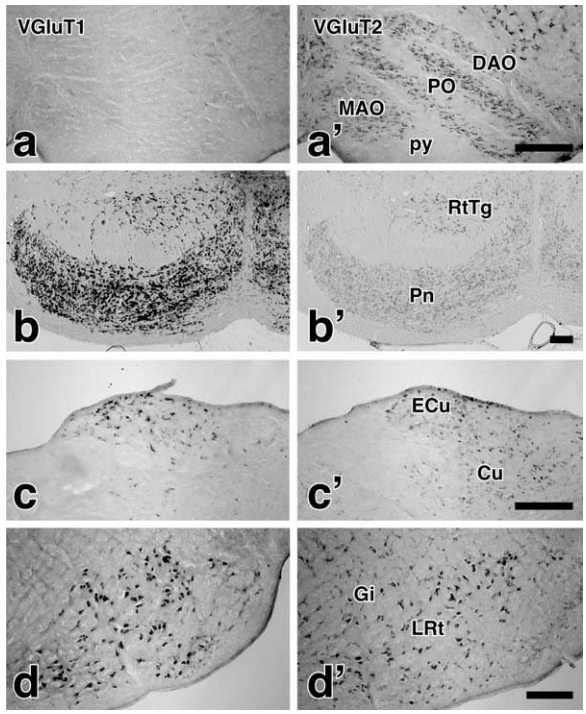

Fig. 5. *In situ* hybridization histochemistry for vesicular glutamate transporter 1 (VGluT1) and VGluT2 mRNAs in the precerebellar nuclei and deep cerebellar nuclei. In the inferior olivary complex, moderate signals for VGluT2 mRNA (a') but no signals for VGluT1 (a) were observed. In contrast, intense VGluT1 signals (b) and very weak VGluT2 signals (b') were seen in the pontine nuclei (Pn) and pontine tegmental reticular nucleus (RtTg). There were moderate to intense signals for both VGluT1 and VGluT2 distributed in the external cuneate nucleus (ECu; c, c') and lateral reticular nucleus (LRt; d, d'). Cu, cuneate nucleus; DAO, dorsal accessory olivary nucleus; Gi, gigantocellular reticular nucleus; MAO, medial accessory olivary nucleus; PO, principal inferior olivary nucleus; py, pyramidal tract. All scale bars=200 μm.

terminals in the cerebellar nuclei. VGluT1 and VGluT2 immunoreactivities were colocalized in some axon terminals (3 in Fig. 6a–a″). Since the collaterals of climbing

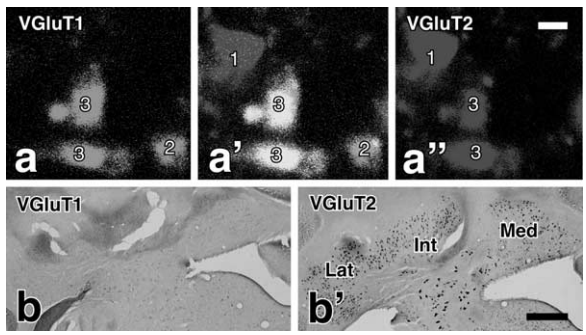

Fig. 6. Immunoreactivity and *in situ* hybridization signals for vesicular glutamate transporter 2 (VGluT2) and VGluT1 in the deep cerebellar nuclei. By confocal laser-scanning microscopy, some axon terminals were shown to be immunoreactive for both VGluT1 and VGluT2 (3 in a–a'); a' is the merged image of a and a'. mRNA signals for VGluT2 (b') but not for VGluT1 (b) were found in the deep cerebellar nuclei. Int, interpositus cerebellar nucleus; Lat, lateral cerebellar nucleus; Med, medial cerebellar nucleus. Scale bar=5 μm (a–a'); 500 μm (b, b').

fibers were considered to show VGluT2 immunoreactivity alone, these axon terminals immunoreactive for both VGluT1 and VGluT2 were probably the collaterals of mossy fibers.

The deep cerebellar nuclei showed intense mRNA signals for VGluT2 but not for VGluT1 (Fig. 6b, b'). Thus, the excitatory output of the cerebellum appeared to selectively use VGluT2 for vesicular accumulation of transmitter glutamate. Furthermore, axon terminals immunoreactive for VGluT2 alone in the deep cerebellar nuclei might contain the local axon collaterals of these output neurons (Matsushita and Iwahori, 1971).

DISCUSSION

The present morphological results together with the previous findings (Fremeau et al., 2001; Kaneko et al., 2002; Kaneko and Fujiyama, 2002; Varoqui et al., 2002) have clearly proven that climbing fibers exclusively use VGluT2, and that parallel fibers only employ VGluT1 for vesicular glutamate uptake in the molecular layer of the cerebellar cortex. In the granular layer of the cerebellar cortex, coarsely granular immunodeposits were observed with anti-VGluT1 and anti-VGluT2 antibodies (Bellocchio et al., 1998; Fremeau et al., 2001; Kaneko et al., 2002; Varoqui et al., 2002). Since VGluT1- and VGluT2-immunoreactive axonal profiles in the granular layer were very large, contained numerous mitochondria and made asymmetric contacts with small dendritic profiles (Fremeau et al., 2001; Kaneko et al., 2002), the axonal profiles were considered to be mossy fiber terminals. The present study further revealed that VGluT1 and VGluT2 were colocalized in many single mossy-fiber terminals. Thus, excitatory nerve terminals in the cerebellar cortex are characterized by expressions of vesicular glutamate transporters as summarized in Fig. 7.

L-aspartate but not L-glutamate has long been assumed to be an excitatory transmitter of climbing fibers by several reasons (Wiklund et al., 1984; Aoki et al., 1987; Madl et al., 1987; Rea et al., 1980; Toggenburger et al., 1983). However, Zhang and Ottersen (1993) reported that L-aspartate immunoreactivity was much less concentrated in climbing fibers than in the neuronal cell bodies of the inferior olivary complex, whereas L-glutamate immunoreactivity in climbing fibers was as intense as that in the cell bodies. They thus discussed that L-aspartate was not a candidate for the excitatory transmitter of climbing fibers. The recent findings (Fremeau et al., 2001; Kaneko et al., 2002; Varoqui et al., 2002) and present results have revealed that climbing fibers use VGluT2, which accumulates L-glutamate but not L-aspartate, indicating that climbing fibers were glutamatergic.

The inferior olive-climbing fiber system and granule cell-parallel fiber system use VGluT2 and VGluT1, respectively, in a mutually exclusive way. Both glutamatergic axon terminals are well known to make excitatory synaptic contacts with dendrites of Purkinje cells. Climbing-fiber terminals have been reported to make depressing synapses; when activated repeatedly by electrical stimulation, the second excitatory postsynaptic potential induced in Purkinje cells is smaller than the first one (Konnerth et al., 1990; Perkel et al., 1990; Hashimoto and Kano, 1998). In contrast, parallel-fiber terminals make facilitating synapses (Konnerth et al., 1990; Perkel et al., 1990). In the cerebellar cortex, no significant differences in synaptic structures, such as the number of docked vesicles per release site and postsynaptic density size, were found between climbing-fiber and parallel-fiber terminals (Xu-Friedman et al., 2001). Thus, some biochemical differences could be assumed as the basis for the distinct synaptic characteristics of the two excitatory inputs to Purkinje cells. It may be interesting that such differences in synaptic characteristics are related to segregated localizations of VGluT1 and VGluT2, although no significant pharmacological differences between VGluT1 and VGluT2 have been reported yet (Bellocchio et al., 2000; Takamori et al., 2000; Bai et al., 2001; Fremeau et al., 2001; Herzog et al., 2001; Takamori et al., 2001; Varoqui et al., 2002). In this view, it may also intriguing to examine whether mossy-fiber terminals, which are loaded with both VGluT1 and VGluT2, show synaptic facilitation or depression.

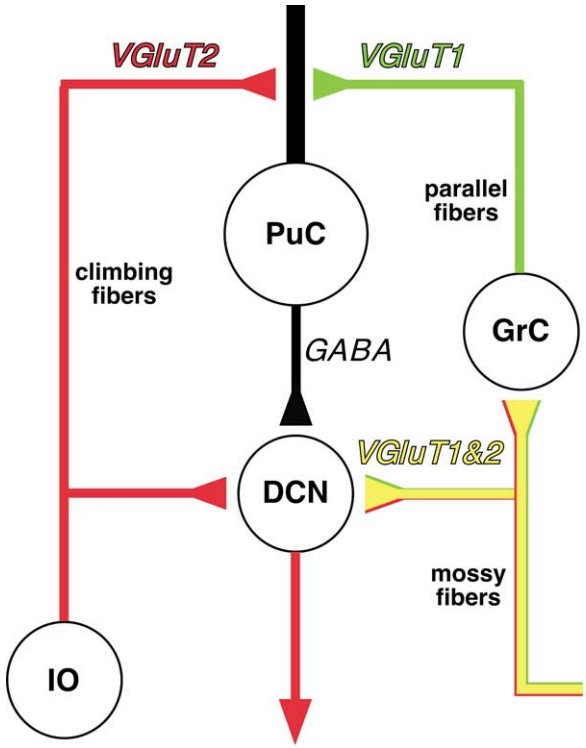

Fig. 7. Summary diagram of cerebellar glutamatergic circuits. Red, vesicular glutamate transporter 2 (VGluT2)-loaded axon fibers; green, VGluT1-laden axon fibers; yellow, axon fibers using VGluT1 and VGluT2 together. DCN, deep cerebellar nucleus neurons; GrC, granule cells; IO, inferior olivary complex neurons; PuC, Purkinje cells.

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