A Chondroitin Sulfate Proteoglycan PTP ζ /RPTP β Regulates the Morphogenesis of Purkinje Cell Dendrites in the Developing Cerebellum

Masahiko Tanaka,¹ Nobuaki Maeda,^{2,3} Masaharu Noda,² and Tohru Marunouchi¹

¹Division of Cell Biology, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan, ²National Institute for Basic Biology, Graduate University for Advanced Studies, Okazaki, Aichi 444-8585, Japan, and ³Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo 183-8526, Japan

PTP ζ /RPTP β , a receptor-type protein tyrosine phosphatase synthesized as a chondroitin sulfate (CS) proteoglycan, uses a heparin-binding growth factor pleiotrophin (PTN) as a ligand, in which the CS portion plays an essential role in ligand binding. Using an organotypic slice culture system, we tested the hypothesis that PTN-PTP ζ signaling is involved in the morphogenesis of Purkinje cell dendrites. An aberrant morphology of Purkinje cell dendrites such as multiple and disoriented primary dendrites was induced in slice cultures by (1) addition of a polyclonal antibody against the extracellular domain of PTP ζ , (2) inhibition of protein tyrosine phosphatase activity, (3) enzymatic removal of the CS chains, (4) addition of exogenous CS chains, and (5) addition of exogenous PTN, all of which disturb PTN-PTP ζ signaling. These treatments also reduced the immunoreactivity to GLAST, a glial glutamate transporter, on Bergmann glial processes. Furthermore, a glutamate transporter inhibitor also induced the abnormal morphogenesis of Purkinje cell dendrites. Altogether, these findings suggest that PTN-PTP ζ signaling regulates the morphogenesis of Purkinje cell dendrites and that the mechanisms underlying that regulation involve the GLAST activity in Bergmann glial processes.

Key words: PTP ζ /RPTP β ; pleiotrophin; GLAST; Purkinje cell; dendritic morphogenesis; cerebellum; organotypic slice culture

Introduction

Neurons are characterized by the specific morphology of dendritic trees and axons, which are essential for information processing. Although the molecular mechanisms of directed axonal outgrowth are beginning to be elucidated, those underlying the morphogenesis of dendritic trees are poorly understood. Among the neurons of the CNS, the cerebellar Purkinje cells have the most elaborate dendritic trees. Mature Purkinje cells have a single primary dendrite, which extends toward the pial surface, branches extensively in the molecular layer (ML), and makes synaptic contacts with parallel fibers, the axons of granule cells. The presence and differentiation of granule cells are necessary for normal development of Purkinje cell dendrites, as shown in agranular cerebella of x-irradiated and mutant animals (Altman and Anderson, 1972; Rakic and Sidman, 1973; Sotelo, 1975; Berry et al., 1978). Coculture experiments using dissociated Purkinje cells and granule cells clearly indicated that the granule–Purkinje cell interaction plays a crucial role in the branching and thickening of the Purkinje cell dendrites (Baptista et al., 1994; Hirai and Launey, 2000). It has been suggested that granule cells exert trophic effects on Purkinje cells by providing neurotrophic substances and electrical activity (Schwartz et al., 1997; Hirai and Launey, 2000). Although Purkinje cells displayed stimulated growth of dendrites in such coculture systems, most of them had

multiple primary dendrites extending in various directions in contrast to Purkinje cells *in vivo* having a single primary dendrite extending in only one direction. This suggests that the polarity of Purkinje cells is determined by alternative mechanisms. Recently, Yamada et al. (2000) found that the lamellate processes of Bergmann glia surrounded the differentiating dendritic trees of Purkinje cells, and more importantly, the growing tips of Purkinje cell dendrites entered the external granular layer (EGL) by contacting the rod-like processes of Bergmann glia. These observations suggest that the Bergmann glia–Purkinje cell interaction is involved in the directed growth and determination of polarity of Purkinje cell dendrites.

Phosphacan/6B4 proteoglycan, a chondroitin sulfate (CS) proteoglycan expressed predominantly in the CNS, is distributed around the cell surface of Purkinje cells during dendritic outgrowth (Maeda et al., 1992). Phosphacan corresponds to the extracellular domain of PTP ζ /RPTP β , a receptor-type protein tyrosine phosphatase composed of an N-terminal carbonic anhydrase-like domain, a fibronectin type III domain, a serine-, glycine-rich domain, a transmembrane segment, and two intracellular tyrosine phosphatase domains (Maurel et al., 1994; Peles et al., 1998). Phosphacan and the transmembrane-type molecules are generated by alternative splicing, and all of the splice variants are synthesized as CS proteoglycans (Maurel et al., 1994; Nishiwaki et al., 1998; Peles et al., 1998). Pleiotrophin (PTN) and midkine (MK), closely related heparin-binding growth factors, bind to PTP ζ /phosphacan with high affinity and trigger signal transduction of this receptor (Maeda et al., 1996, 1999). The CS portion of PTP ζ /phosphacan plays an essential role in binding to PTN and MK, and the removal of CS chains from PTP ζ /phos-

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Correspondence should be addressed to Masahiko Tanaka, Division of Cell Biology, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan. E-mail: mtanaka@fujita-hu.ac.jp.

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phacan resulted in a marked decrease of the binding affinity to PTN and MK and in the loss of signal transduction (Maeda et al., 1996, 1999; Qi et al., 2001). Although Purkinje cells and Bergmann glia express PTP ζ /phosphacan (Canoll et al., 1993; Snyder et al., 1996), PTN and MK distribute along Bergmann glial fibers in postnatally developing cerebellum (Matsumoto et al., 1994; Wewetzer et al., 1995). These expression patterns suggest that PTN/MK secreted by Bergmann glia binds with PTP ζ on Purkinje cells or Bergmann glia, or both. Thus, the signaling of PTP ζ /phosphacan and PTN/MK could be involved in cell–cell interaction between Purkinje cells and Bergmann glia.

In this study, we hypothesized that PTN-PTP ζ signaling is involved in the Bergmann glia–Purkinje cell interaction required for the morphogenesis of Purkinje cell dendrites. To test this hypothesis, we used organotypic slice cultures of postnatal rat cerebellum, which preserve the cytoarchitecture of the cerebellar cortex and reproduce the series of processes in cerebellar cortical development (Tanaka et al., 1994). Using this system, we found that the perturbation of PTN-PTP ζ signaling resulted in a marked increase in the number of Purkinje cells with abnormal dendrites, such as multiple and disoriented primary dendrites, showing that PTN-PTP ζ signaling is involved in the morphogenesis of Purkinje cell dendrites. Furthermore, we obtained evidence suggesting that Bergmann glia play important roles in these mechanisms.

Materials and Methods

Slice culture. The methods for slice culture have been described previously (Tanaka et al., 1994). In brief, cerebella were dissected from 9-d-old Wistar rats. The vermes of the cerebella were cut parasagittally into ~600- μ m-thick slices in calcium- and magnesium-free PBS (CMF-PBS). The slices were mounted on a collagen-coated, porous (2.0 μ m) polycarbonate membrane (Nuclepore; Whatman, Clifton, NJ) that was floated at the interface between the air and culture medium in a Petri dish ("interface" culture technique) (Freshney, 1987; Yamamoto et al., 1989; Stoppini et al., 1991; Tanaka et al., 1994). The culture medium consisted of 15% heat-inactivated horse serum (Invitrogen, Grand Island, NY), 25% Earle's balanced salt solution, 60% Eagle's basal medium, 5.6 gm/l glucose, 3 mM L-glutamine, 5 μ g/ml bovine insulin, 5 μ g/ml human transferrin, 30 nM sodium selenite, 20 nM progesterone, 1 mM sodium pyruvate, 50 U/ml penicillin G potassium, and 100 μ g/ml streptomycin sulfate. The rabbit polyclonal antibody (α B4PG) against the extracellular domain of PTP ζ (PTP ζ -ECD) has been described previously (Maeda et al., 1996). The other reagents added to the culture medium were purchased as follows: rabbit IgG, from Chemicon (Temecula, CA); sodium orthovanadate, from Wako Pure Chemicals (Osaka, Japan); chondroitinase ABC (Chase ABC; protease free), CS-C and CS-D from shark cartilage, CS-E from squid cartilage, and CS-A from whale cartilage, from Seikagaku (Tokyo, Japan); recombinant human PTN, from Sigma (St. Louis, MO); and DL-threo- β -benzyloxyaspartate (DL-TBOA), from Tocris (Bristol, UK). Chase ABC was dissolved (6 U/ml) in 60 mM sodium acetate, pH 7.5, 80 mM sodium chloride, and bovine serum albumin (1 mg/ml), and stored as frozen aliquots. These reagents were added at 1 d *in vitro* (DIV). The cultures were incubated at 33°C in 5% CO₂/95% air. Because a large number of cells degenerated in the bottom part (medium side) of the slice cultures, we analyzed the top half (air side) to obtain data in the present study.

Analysis of the Purkinje cell morphology. For immunohistochemistry of inositol 1,4,5-trisphosphate receptor (IP₃R), cerebellar slice cultures were fixed with 4% paraformaldehyde in CMF-PBS for 20 min at room temperature. For analysis of the cerebellum *in vivo*, 250- μ m-thick slices were prepared from the vermes of the cerebella of 9- or 15-d-old rats and fixed as mentioned above. The slices were preincubated for 30 min in 10% normal goat serum and 0.3% Triton X-100 in CMF-PBS and then incubated overnight at 4°C in CMF-PBS containing a rat anti-mouse IP₃R monoclonal antibody (4C11; 1:20) (Maeda et al., 1989). The immu-

noreactivity was visualized using a Cy3-conjugated goat anti-rat IgG antibody and examined under a confocal laser scanning microscope (LSM510; Zeiss, Oberkochen, Germany). For imaging of Purkinje cells, we used a 63 $\times$ water-immersion objective (numerical aperture = 0.9; Achroplan Water; Zeiss) and projected five to eight optical sections of 3 μ m thickness to make one stacked image. For analysis of the morphology of primary dendrites, we used three to four slices from three independent experiments in each experimental condition and randomly selected 222–300 (*in vivo*) and 32–63 (slice cultures) Purkinje cells per slice [total 707–729 (*in vivo*) and 126–205 (slice cultures) cells per condition]. For statistical analysis of the ratios of the three types of Purkinje cells (see Results), the repeated measures ANOVA was used.

Immunohistochemistry of cryosections. For immunohistochemical analysis of IP₃R, PTP ζ , GLAST, PTN, CS, neuronal nuclei (NeuN), vesicular glutamate transporter 1 (VGLUT1), and glial fibrillary acidic protein (GFAP), cerebellar slices before or after culture were fixed with 4% paraformaldehyde in CMF-PBS for 20 min at room temperature, frozen in liquid nitrogen, and sectioned at 12 μ m on a cryostat. The sections were preincubated for 30 min at room temperature in 10% normal goat serum in CMF-PBS with or without 0.3% Triton X-100, and then incubated overnight at 4°C in CMF-PBS containing a rat anti-IP₃R monoclonal antibody (4C11; 1:20), a rabbit anti-PTP ζ -ECD antibody (α B4PG; 20 μ g/ml), a mouse monoclonal antibody against the intracellular domain of PTP ζ (PTP ζ -ICD) (Transduction Laboratories, Lexington, KY; 1:100), a rabbit anti-GLAST antibody (Abcam, Cambridge, MA; 1:600), a guinea pig anti-GLAST antibody (Chemicon; 1:8000), a goat anti-PTN antibody (N-15; Santa Cruz Biotechnology, Santa Cruz, CA; 1:400), a mouse anti-CS monoclonal antibody (CS-56; Sigma; 1:800), a mouse anti-NeuN monoclonal antibody (Chemicon; 1:600), a guinea pig anti-VGLUT1 antibody (Chemicon; 1:10,000), or a rabbit anti-GFAP antibody (Immunon, Shandon, Pittsburgh, PA; 1:1000). For pretreatment with Chase ABC before PTN immunohistochemistry, the sections were incubated for 40 min at 37°C in CMF-PBS containing 20 mU/ml Chase ABC. The immunoreactivities to IP₃R, PTP ζ -ECD, GLAST (rabbit antibody), NeuN, and GFAP were visualized using Cy3-, Cy2-, or Cy5-conjugated secondary antibodies. The immunoreactivities to PTP ζ -ICD, GLAST (guinea pig antibody), and PTN were visualized with a tyramide signal amplification system (TSA-Indirect; NEN Life Science Products, Boston, MA) and Cy2-conjugated streptavidin. Images for fluorescent microscopy were acquired with a confocal laser scanning microscope (LSM510; Zeiss). In some cases of PTN immunohistochemistry, the signals were chromogenically visualized with 3-amino-9-ethylcarbazole (AEC). The immunoreactivities to CS and VGLUT1 were visualized by the streptavidin–biotin affinity method and development with AEC.

Quantitative analysis of Purkinje and granule cells and Bergmann fibers. For measurement of the length of the longest dendrite per cell and counting the number of dendritic branching points per cell, the stacked confocal microscopic images of Purkinje cells obtained as described above were analyzed using the LSM Ver. 2.8 software (Zeiss). For evaluation of the density of Purkinje and granule cells, cryosections of slice cultures were stained by immunohistochemistry using antibodies against IP₃R and NeuN as described above. The IP₃R- and NeuN-positive cells within 150 μ m of the Purkinje cell layer (PL) and internal granular layer (IGL) were enumerated to determine the density of Purkinje and granule cells, respectively, in confocal microscopic images of 2- μ m-thick optical sections. As another type of evaluation of granule cell density, the number of NeuN-positive cells within square areas of 100 $\times$ 100 μ m² of the IGL was counted. We did these two types of evaluation of granule cell density because migration of these cells from the EGL to the IGL might result in an increase of only one of the number of granule cells per unit length of the IGL and that per unit area of the IGL in slice cultures. When counting the NeuN-positive cells, the cells >10 μ m in diameter (<0.1% of total NeuN-positive cells in the IGL) were not included because they might be Golgi cells. For evaluation of Bergmann fibers, GFAP-positive fibers >30 μ m within 150 μ m of the ML were enumerated in confocal microscopic images of 3- μ m-thick optical sections. Statistical analysis was done using Student's *t* test.

Western blotting. Slice culture and treatment with Chase ABC or CS chains were done as described above. Three cultured slices at 3 DIV were combined and rapidly frozen on dry ice. The frozen slices were homog-

enized in 200 μ l of 1% NP-40, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 1.5 μ M aprotinin, 30 μ M E64, 40 μ M leupeptin, 100 μ M bestatin, and 20 μ M pepstatin A. After centrifugation at $15,000 \times g$ for 15 min at 4°C, the supernatants (15 μ g protein) were applied to 12.5% SDS-PAGE and Western blotting using a goat anti-PTN antibody (R&D systems, Minneapolis, MN; 1:1,000). The density of the bands was quantified by an Epson desktop scanner (GT-9700F) using NIH image software.

Results

Comparison of the morphogenesis of Purkinje cell dendrites *in vivo* and in slice cultures

In the present study, we examined the morphogenesis of Purkinje cell dendrites in postnatal cerebellar development using an organotypic slice culture system of cerebellum from 9-d-old rats (Tanaka et al., 1994). Purkinje cells were visualized by immunohistochemical staining using a monoclonal antibody against IP₃R (Maeda et al., 1989). This antibody stains clearly the overall structure of Purkinje cells, including dendrites, dendritic spines, axons, and cell bodies (Fig. 1).

The morphology of Purkinje cell dendrites changes dramatically during postnatal cerebellar development (Hendelman and Aggerwal, 1980; Armengol and Sotelo, 1991). In the first postnatal week *in vivo*, Purkinje cells have several primary dendrites. During the second postnatal week, most Purkinje cells lose all of their primary dendrites except one, which extends toward the pial surface, branches extensively in the ML, and forms numerous synapses with parallel fibers (Fig. 1A–C).

Our slice culture system preserves the overall structure of cerebellar slices (Fig. 1D) and the cytoarchitecture of the cerebellar cortex and reproduces the serial process of granule cell development, including proliferation, migration, and extension of parallel fibers within 6 DIV, as described previously (Tanaka et al., 1994). Purkinje cells also survive well, are arranged in a row at the PL as *in vivo* (Fig. 1E), extend arborized dendritic trees toward the pial surface (Fig. 1F), and form synapses with parallel fibers (Tanaka et al., 1994).

As the first step in the present study, we carefully observed the morphological changes of Purkinje cell dendrites *in vivo* and in slice cultures under control conditions. The immunohistochemical analysis was done using slices, not sections (cryosections or paraffin sections) of slices, which enabled us to reliably distinguish the morphology of Purkinje cell dendrites. The morphology of Purkinje cells was classified into three types: single primary dendrite (SPD), multiple primary dendrite (MPD), and disoriented primary dendrite (DOPD). The MPD type was defined as Purkinje cells with multiple primary dendrites, all of which extended toward the pial surface. The DOPD type was defined as Purkinje cells with multiple primary dendrites, at least some of which had an abnormal orientation, for example, extending horizontally in the PL or downward into the IGL. No Purkinje cells with a single primary dendrite extending abnormally were observed in the present study.

In the rat cerebellum on postnatal day (P) 9, approximately half (47.9%) of the Purkinje cells were of the SPD type and half (49.6%) were of the MPD type (Fig. 1B, G). Few (2.5%) Purkinje cells were of the DOPD type. On P15, most Purkinje cells showed the SPD-type morphology (SPD/MPD/DOPD = 88.3:11.7:0.0%) (Fig. 1C, G). Also in our slice culture system, the ratio of SPD-type Purkinje cells significantly increased at 6 DIV compared with that on P9 (0 DIV), although somewhat fewer SPD-type and more MPD-type cells were observed under the culture conditions than in the P15 cerebellum (SPD/MPD/DOPD = 62.7:34.5:2.8%) (Fig. 1F, G). These results indicated that nearly normal morphological changes of Purkinje cell dendrites occur in our slice cul-

Figure 1. Morphogenesis of Purkinje cell dendrites *in vivo* and in slice cultures. *A–C*, Fluorescent immunohistochemistry using a monoclonal antibody against IP₃R (4C11) showing the morphology of Purkinje cells in the cerebellum from P9 (*A*, *B*) and P15 (*C*) rats. *A*, Low-power view of an immunostained cryosection. *B*, *C*, Confocal microscopic images of immunostained slices. The multiple primary dendrite (MPD)-type (arrows) and single primary dendrite (SPD)-type Purkinje cells coexist on P9 (*B*), whereas most Purkinje cells are of the SPD type on P15 (*C*). *D–F*, Overview (*D*) and fluorescent immunohistochemistry using 4C11 (*E*, *F*) of cerebellar slices derived from P9 rats and cultured for 6 d under control conditions. *E*, Low-power view of an immunostained cryosection. *F*, A confocal microscopic image of an immunostained slice culture. Scale bars: (in *A*, *E*, 100 μ m; in *B*, *C*, 25 μ m; *D*, 1 mm. *G*, Quantitative representation of the ratios of the three types of Purkinje cells *in vivo* (P9 and P15) and in slice cultures at 6 DIV under control conditions. The ratio of SPD-type Purkinje cells significantly increased in slice cultures at 6 DIV compared with that on P9, although the increase was not as marked as *in vivo*. Cell counts were made in slices, not in cryosections, which enabled us to reliably distinguish the morphology of Purkinje cell dendrites. DOPD, Disoriented primary dendrite. $n = 4$. Error bars represent SEM. * $p < 0.05$, ** $p < 0.001$ versus P9.

tures under control conditions, although these changes appeared to proceed insufficiently *in vitro*.

Expression patterns of PTP ζ and PTN in postnatally developing cerebellum

The expression pattern of PTP ζ in P9 rat cerebellum was examined by double-fluorescent immunohistochemistry using antibodies against PTP ζ -ECD or -ICD and IP₃R (Fig. 2*A*, *B*). Signals obtained by a polyclonal antibody against PTP ζ -ECD were abundant around Purkinje cells in the PL and ML (Fig. 2*A*). On the other hand, the immunoreactivity to the monoclonal antibody against PTP ζ -ICD was detected in the cytoplasm and surroundings of Purkinje cells (Fig. 2*B*). Western blotting showed that the

Figure 2. Expression patterns of PTP ζ and PTN in postnatally developing cerebellum. *A–C*, Confocal microscopic images of cerebellar cryosections derived from P9 rats and double stained by fluorescent immunohistochemistry using antibodies against the extracellular domain (ECD) (*A1*) or the intracellular domain (ICD) (*B1*, *C1*) of PTP ζ (Cy2) and IP₃R (*A2*, *B2*) or GLAST (*C2*) (Cy3). *A3*, *B3*, and *C3* are the merged images. The immunoreactivities to PTP ζ -ICD and GLAST partially overlapped each other, especially around Purkinje cells. EGL, External granular layer; ML, molecular layer; PL, Purkinje cell layer; IGL, internal granular layer. Scale bar, 25 μ m. *D*, *E*, A cerebellar cryosection derived from a P9 rat and stained by immunohistochemistry using an antibody against PTN. *E* is the high-magnification image of the enclosed area in *D*. PTN distributes abundantly in the ML. Scale bars: *D*, 50 μ m; *E*, 25 μ m. *F*, An adjacent section stained with toluidine blue. Scale bar, 25 μ m.

amount of the transmembrane forms of PTP ζ is very low compared with that of the secreted form (data not shown), which suggests that the signals of PTP ζ -ECD mainly correspond to the presence of the secreted form, phosphacan. In addition, the signals of both PTP ζ -ECD and -ICD were detected in the IGL and white matter.

During postnatal development of the cerebellum *in vivo*, differentiating dendrites of Purkinje cells are surrounded by the lamellate processes of Bergmann glia, which express GLAST, a glial glutamate transporter (Yamada et al., 2000). Double-fluorescent immunohistochemistry using antibodies against PTP ζ -ICD and GLAST showed that the immunoreactivities to

these proteins partially overlapped each other, especially around Purkinje cells. This suggests that the PTP ζ -ICD-positive structures surrounding Purkinje cells were the GLAST-positive lamellate processes of Bergmann glia (Fig. 2*C*).

Immunohistochemical analysis using an antibody against PTN revealed that this growth factor shows a characteristic localization in the developing cerebellum (Fig. 2*D–F*). PTN was distributed abundantly in the ML and moderately in the IGL (Fig. 2*E*). Thus, signal transduction of PTP ζ by PTN could occur most strongly in the ML in the developing cerebellum. In addition, abundant signals of PTN were also observed in the white matter.

Involvement of PTP ζ in the morphogenesis of Purkinje cell dendrites

From the abundant expression of PTP ζ and PTN around developing Purkinje cells, we hypothesized that PTN-PTP ζ signaling is involved in the morphogenesis of Purkinje cell dendrites. To test this possibility, we examined the effects of the polyclonal antibody against PTP ζ -ECD, α 6B4PG, on the morphogenesis of Purkinje cell dendrites in slice cultures. This antibody disturbs PTP ζ signaling activated by PTN and MK (Maeda et al., 1996; Maeda and Noda, 1998; Qi et al., 2001). On addition of this antibody (200 μ g/ml) into the medium of slice cultures, the MPD (Fig. 3*A*) and DOPD (Fig. 3*B*) types of Purkinje cells significantly increased at 6 DIV (SPD/MPD/DOPD = 32.4:48.4:19.2%) (Fig. 3*F*). Notably, the DOPD type of Purkinje cells increased remarkably. Addition of the control rabbit IgG (200 μ g/ml) did not influence the morphogenesis of Purkinje cells (SPD/MPD/DOPD = 62.0:34.1:3.9%) (Fig. 3*C,F*). These findings suggest that PTP ζ regulates the morphogenesis of Purkinje cell dendrites during cerebellar development. The length of the longest dendrites and the number of branching points per cell were not significantly different between the α 6B4PG- and control IgG-added conditions, indicating that the growth of Purkinje cell dendrites itself was not influenced by this treatment (Table 1). Thus, PTP ζ does not simply promote growth of Purkinje cell dendrites but is involved in the morphological change from the MPD- to the SPD-type Purkinje cells and the directed growth of Purkinje cell dendrites.

Consistently, sodium vanadate and phenylarsine oxide, protein tyrosine phosphatase inhibitors, also increased the MPD and DOPD types of Purkinje cells at 6 DIV [SPD/MPD/DOPD = 25.4:63.3:11.4% (sodium vanadate; 10 μ M); 25.1:64.3:10.7% (phenylarsine oxide; 0.1 μ M)] (Fig. 3*D–F*). We cannot exclude the possibility that these treatments retarded Purkinje cell development, however, because they concomitantly resulted in re-

Figure 3. Involvement of PTP ζ in the morphogenesis of Purkinje cell dendrites. *A–E*, Effects of the polyclonal antibody against PTP ζ -ECD ($\alpha 6B4PG$) (*A*, *B*), control IgG (*C*), and sodium vanadate (*D*, *E*) on the morphogenesis of Purkinje cell dendrites. Shown are confocal microscopic images of cerebellar slices derived from P9 rats, cultured for 6 d with culture medium containing $\alpha 6B4PG$ (200 $\mu g/ml$) (*A*, *B*), control IgG (200 $\mu g/ml$) (*C*), and sodium vanadate (10 μM) (*D*, *E*) and stained by fluorescent immunohistochemistry using 4C11. On addition of $\alpha 6B4PG$ and sodium vanadate, the MPD (*A*, *D*) and DOPD (*B*, *E*) types of Purkinje cells markedly increased. Control IgG did not influence the morphogenesis of Purkinje cell dendrites (*C*). Scale bar, 25 μm . *F*, Quantitative representation of the effects of $\alpha 6B4PG$, control IgG, sodium vanadate, and phenylarsine oxide (PAO) on the morphogenesis of Purkinje cell dendrites in slice cultures at 6 DIV. $n = 3$ (Vanadate, PAO) or 4 ($\alpha 6B4PG$, Control IgG). Error bars represent SEM. * $p < 0.05$ versus control IgG (200 $\mu g/ml$); ** $p < 0.005$ versus control (data in Fig. 1*G*).

duced extension and branching of Purkinje cell dendrites [longest dendrite per cell = $64.8 \pm 3.1 \mu m$ ($n = 41$), branching points per cell = 40.8 ± 6.0 ($n = 16$) under vanadate-added (compare Table 1)].

Involvement of CS and PTN in the morphogenesis of Purkinje cell dendrites

CS plays essential roles in the signal transduction of PTP ζ , especially for the signaling of PTN and MK. As revealed by immunohistochemistry using a monoclonal antibody against CS, CS-56, this glycosaminoglycan is present in the ML, IGL, and white matter *in vivo* (data not shown) and in slice cultures (Fig. 4*A*). Addition of Chase ABC (200 mU/ml), an enzyme that hydrolyzes CS chains, into the medium of slice cultures markedly decreased the immunoreactivity to CS-56 (Fig. 4*B*). This treatment also resulted in a significant increase in the MPD and DOPD types of Purkinje cells (SPD/MPD/

Table 1. Quantitative evaluation of the growth of Purkinje cell dendrites and the density of Purkinje cells

Culture conditions	Longest dendrite (μm)	Branching points (number/cell)	Cell density (cells/150 μm PL)
Control	81.3 ± 3.1 (48)	56.2 ± 5.1 (20)	4.4 ± 0.3 (28)
CS-D	75.1 ± 4.5 (22)	54.2 ± 5.1 (13)	4.4 ± 0.2 (17)
Control IgG	82.1 ± 5.2 (24)	56.3 ± 5.6 (17)	4.2 ± 0.2 (27)
$\alpha 6B4PG$	71.4 ± 3.0 (36)	52.8 ± 7.1 (15)	4.0 ± 0.3 (25)

Cerebellar slices derived from P9 rats were cultured for 6 d with or without CS-D (50 $\mu g/ml$), control IgG (200 $\mu g/ml$), and $\alpha 6B4PG$ (200 $\mu g/ml$). For measurement of the length of the longest dendrite per cell and counting the number of dendritic branching points per cell, confocal microscopic images of slice cultures stained by immunohistochemistry using an antibody against IP $_3$ R were analyzed. For analysis of the density of Purkinje cells, cryosections of slice cultures were stained by immunohistochemistry using an antibody against IP $_3$ R, and the IP $_3$ R-positive cells within 150 μm of the PL were enumerated in confocal microscopic images of 2- μm -thick optical sections. Values are expressed as the mean $\pm$ SEM (n). Statistical analysis using Student's *t* test detected no significant difference between any set of conditions.

DOPD = 36.5:50.2:13.3%; compare SPD/MPD/DOPD = 63.7:31.6:4.6% under control conditions) (Fig. 4*C*, *D*, *M*), as in the case of $\alpha 6B4PG$. These results indicated that endogenous CS is involved in the morphogenesis of Purkinje cell dendrites.

Next we examined the effects of exogenously added CS chains. We indicated previously that various CS samples differentially inhibit the signaling of the PTN-PTP ζ pathway (Maeda et al., 1996, 1999). Although CS-C, -D, and -E markedly inhibited the binding of PTN to PTP ζ , CS-A scarcely influenced the binding. A similar selectivity was observed in the effects on the morphogenesis of Purkinje cell dendrites. CS-C, -D, and -E (50 $\mu g/ml$) increased the MPD and DOPD types of Purkinje cells when added to the medium of slice cultures [SPD/MPD/DOPD = 36.2:57.9:5.9% (CS-C); 25.1:64.4:10.5% (CS-D); 42.6:46.1:11.2% (CS-E)] (Fig. 4*E*, *F*, *M*). In contrast, the morphology of Purkinje cell dendrites was not influenced by addition of 50 $\mu g/ml$ CS-A (SPD/MPD/DOPD = 64.0:30.1:5.9%) (Fig. 4*G*, *H*, *M*). These observations suggested that PTN signaling is involved in the morphogenesis of Purkinje cell dendrites.

In our slice culture system, exogenously applied PTN also induced an abnormal morphology in Purkinje cell dendrites (SPD/MPD/DOPD = 37.4:55.2:7.4%) (Fig. 4*I*, *J*, *M*). This may be caused by perturbation of normal distribution of the endogenous PTN by the exogenous PTN.

The morphological change of Purkinje cells from the MPD to the SPD type is accompanied by the formation of many dendritic spines *in vivo*. This later aspect of maturation appeared not to be influenced by treatment with the reagents affecting PTN-PTP ζ signaling in slice cultures. Spine formation was apparently normal in the MPD and DOPD types of Purkinje cells in the slice cultures treated with Chase ABC and CS chains (Fig. 4*K*, *L*).

In contrast to the dendrite formation of Purkinje cells, development of granule cells proceeded normally even in the presence of the reagents affecting PTN-PTP ζ signaling. As under control conditions, migration of granule cells from the EGL to the IGL was nearly completed by 6 DIV in the treated cultures, as revealed by the almost complete disappearance of the EGL (Fig. 5*A*, *B*). The density of granule cells in the IGL at 6 DIV was not significantly different between the treated and control conditions (Fig. 5*C*, *D*, Table 2). No abnormalities such as increased pyknosis were observed in granule cells in sections of the treated cultures processed for Nissl staining (Fig. 5*A*, *B*) and immunohistochemistry against NeuN (Fig. 5*C*, *D*). Furthermore, VGLUT1, a vesicular glutamate transporter that is localized to the synaptic vesicles (Bellocchio et al., 1998), was expressed in the ML under the treated as well as control conditions, suggesting that normal pre-synaptic structures of parallel fiber terminals were formed under both conditions (Fig. 5*E*, *F*).

Figure 4. Involvement of chondroitin sulfate (CS) and PTN in the morphogenesis of Purkinje cell dendrites. *A, B*, Distribution of CS in the control (*A*) and chondroitinase ABC (*Chase ABC*)-treated (*B*) slice cultures. Cryosections of cerebellar slices derived from P9 rats, cultured for 6 d without (*A*) or with (*B*) *Chase ABC* (200 mU/ml), and stained by immunohistochemistry using a monoclonal antibody against CS (CS-56). Scale bar, 25 μ m. *C–J*, Effects of *Chase ABC*, CS, and PTN on the morphogenesis of Purkinje cell dendrites. Shown are confocal microscopic images of cerebellar slices cultured for 6 d with culture medium containing *Chase ABC* (200 mU/ml) (*C, D*), CS-C (50 μ g/ml) (*E, F*), CS-A (50 μ g/ml) (*G, H*), and PTN (1 μ g/ml) (*I, J*), and stained by fluorescent immunohistochemistry using 4C11. On addition of *Chase ABC*, CS-C, and PTN, the MPD (*C, E, I*) and DOPD (*D, F, J*) types of Purkinje cells increased. In contrast, many Purkinje cells had an SPD under the CS-A-added conditions (*G, H*). Scale bar, 25 μ m. *K, L*, High-power views of the enclosed areas in *D, H*. Spine formation on Purkinje cell dendrites did not differ between the *Chase ABC* (*K*)- and CS-A (*L*)-added conditions. Scale bar, 3 μ m. *M*, Quantitative representation of the effects of *Chase ABC*, CS, and PTN on the morphogenesis of Purkinje cell dendrites in slice cultures at 6 DIV. $n = 3$ (CS chains) or 4 (Control, *Chase ABC*, PTN). Error bars represent SEM. * $p < 0.05$, ** $p < 0.005$ versus control.

Interestingly, *Chase ABC* digestion of the cerebellar sections markedly reduced the PTN immunoreactivity in the ML (Fig. 6*A, B*), indicating that most of the endogenous PTN is present in a CS-bound form in this tissue. This also suggests that the effects of *Chase ABC* and CS chains on the morphogenesis of Purkinje cell dendrites were caused by the displacement of PTN from the ML. In fact, Western blotting showed that the addition of CS-D but not CS-A into the culture medium markedly decreased the PTN contents in the cerebellar slices [control/CS-D/CS-A = 100; 68 ± 8 ($p < 0.01$ vs control; t test); $100 \pm 4\%$ ($n = 3$)] (Fig. 6*C*).

Involvement of GLAST on Bergmann glial processes

Because the GLAST-positive lamellate processes of Bergmann glia expressed PTP ζ , we next examined whether the reagents affecting PTN-PTP ζ signaling influence these processes of Bergmann glia. For this purpose, cryosections of cerebellar slice cultures were double stained by immunohistochemistry using the antibodies against IP $_3$ R and GLAST. Although the cell bodies and dendrites of Purkinje cells were surrounded by the GLAST-positive lamellate processes of Bergmann glia in slice cultures under control conditions (Fig. 7*A, B*), the GLAST immunoreactivity was markedly reduced in the slice cultures treated with α B4PG, *Chase ABC*, and CS-C, -D, and -E (Fig. 7*C, D*). In contrast, the same treatments did not reduce the number of Bergmann fibers, the shaft processes of Bergmann glia that express GFAP, a glial intermediate filament protein (Fig. 7*E, F*, Table 3). This indicated that the reduction in GLAST immunoreactivity was not caused by the nonspecific degeneration of Bergmann glia. Western blotting also showed that the amount of GLAST but not GFAP was reduced by these treatments (data not shown). These observations strongly suggest that the morphogenesis of Purkinje cell dendrites involves their interaction with the GLAST-positive lamellate processes of Bergmann glia.

The reduction in GLAST immunoreactivity by the reagents affecting PTN-PTP ζ signaling suggests that the glutamate-transporting activity of GLAST is involved in the morphogenesis of Purkinje cell dendrites presumably downstream of PTN-PTP ζ signaling. To test this hypothesis, slice cultures were treated with DL-TBOA, an inhibitor of glutamate transporters (Shimamoto et al., 1998). DL-TBOA (10 μ M) actually increased the MPD and DOPD types of Purkinje cells (SPD/MPD/DOPD = 36.0:49.3:14.7%; compare SPD/MPD/DOPD = 59.3:36.5:4.1% under control conditions) (Fig. 8). This treatment reduced neither the survival of Purkinje cells [IP $_3$ R-positive cells per 150 μ m of the PL under DL-TBOA-added conditions = 4.4 ± 0.3 ($n = 28$) (compare Table 1)] nor the extension or

branching of Purkinje cell dendrites (Fig. 8*A, B*). Thus, the morphogenesis of Purkinje cell dendrites may be regulated by the glutamate-transporting activity of GLAST on Bergmann glial processes.

Discussion

The postnatal development of Purkinje cells is characterized by a specific pattern of dendritic morphogenesis. The change of Purkinje cells from the MPD or DOPD type to the SPD type strictly

Figure 5. Development of granule cells in slice cultures under control and CS-D-added conditions. Cerebellar slices cultured for 6 d without (*A, C, E*) or with (*B, D, F*) CS-D were processed for several histological analyses. *A, B*, Nissl staining with toluidine blue. Migration of granule cells from the EGL to the IGL was nearly completed even in the CS-D-treated cultures. Scale bar, 50 μ m. *C, D*, Confocal microscopic images of cryosections double stained by fluorescent immunohistochemistry using antibodies against NeuN (Cy2, green) and IP₃R (Cy3, red). The density of granule cells in the IGL was not significantly different between these two conditions (Table 2). Scale bar, 25 μ m. *E, F*, Cryosections stained by immunohistochemistry using an antibody against vesicular glutamate transporter 1 (VGLUT1). VGLUT1 was expressed in the ML under both conditions. Scale bar, 50 μ m.

Table 2. Evaluation of the density of granule cells

Culture conditions	Density of granule cells	
	(Cells/150 μ m IGL)	(Cells/100 $\times$ 100 μ m ²)
Control	189.6 $\pm$ 7.1 (28)	95.5 $\pm$ 1.9 (28)
CS-D	189.3 $\pm$ 8.9 (17)	97.8 $\pm$ 2.6 (17)
Control IgG	186.6 $\pm$ 6.1 (27)	97.7 $\pm$ 2.2 (27)
α 6B4PG	189.0 $\pm$ 5.5 (25)	91.7 $\pm$ 2.1 (25)

Cerebellar slices derived from P9 rats were cultured for 6 d with or without CS-D (50 μ g/ml), control IgG (200 μ g/ml), and α 6B4PG (200 μ g/ml). Cryosections of slice cultures were stained by immunohistochemistry using an antibody against NeuN, and the NeuN-positive cells within 150 μ m or 100 $\times$ 100 μ m² of the IGL were enumerated in confocal microscopic images of 2- μ m-thick optical sections. Values are expressed as the mean $\pm$ SEM (*n*). Statistical analysis using Student's *t* test detected no significant difference between any set of conditions.

proceeds under *in vivo* conditions, and almost all of the Purkinje cells are of the SPD type in the matured cerebellum. In our organotypic slice culture system of postnatal rat cerebellum, the morphological changes of Purkinje cells basically proceeded as *in vivo* (Fig. 1). This suggested that some of the primary dendrites were withdrawn in slice cultures as *in vivo*. However, more MPD-type Purkinje cells were found in slice cultures at 6 DIV than in P15 cerebellum. In addition, a significant population of Purkinje cells was of the DOPD type at 6 DIV, whereas at the corresponding *in vivo* stage (P15), this type of Purkinje cell was not observed. We consider that this insufficient progress of Purkinje cell development in slice cultures is caused by a decrease in signal transduction levels required for the dendrite formation under the culture conditions. However, it seems that this property of the slice culture system makes it a highly sensitive assay system for Purkinje cell development. Using this culture system, we demonstrated that the perturbation of PTN-PTP ζ signaling induced the aberrant morphology of Purkinje cell dendrites such as MPDs and DOPDs (Figs. 3, 4), clearly showing that PTN-PTP ζ signaling is involved in the morphogenesis of Purkinje cell dendrites. Berry and Bradley (1976) reported that Purkinje cells already acquire their polarity on P9. However, our findings that the perturbation of PTN-PTP ζ signaling induced the

DOPD-type Purkinje cells suggest that this signaling needs to function continuously to maintain the polarity of Purkinje cells. If this signaling is lost, new primary dendrites could sprout and extend to an abnormal orientation even after P9.

CS proteoglycans are reported to contribute to the regulation of directed axonal outgrowth of various neurons, including retinal ganglion cells (Brittis and Silver, 1994; Chung et al., 2000) and spinal motor neurons (Bernhardt and Schachner, 2000). These findings suggest that the signal transductions mediated by CS proteoglycans are involved in the directed outgrowth of neurites, although it is not known what kinds of CS proteoglycans contribute to these phenomena. The present study identified PTP ζ as a CS proteoglycan regulating the dendritic morphogenesis of cerebellar Purkinje cells.

The treatment with Chase ABC and CS chains induced an aberrant Purkinje cell morphogenesis in slice cultures (Fig. 4). The same treatments also markedly decreased PTN contents in the cerebellum

(Fig. 6), indicating that most PTN is present in the CS proteoglycan-bound form in this tissue. It is notable that the effects of CS chains were highly dependent on the structure of CS chains. Although CS-C, -D, and -E influenced the morphogenesis of Purkinje cells, CS-A gave no effect on this type of cells. CS-C and -D contain $\sim$ 10–20% GlcUA(2-sulfate) β 1–3GalNAc(6-sulfate) disaccharide units, and CS-E contains 60–65% GlcUA β 1–3GalNAc(4,6-disulfate) disaccharide units (Sakai et al., 2000). In contrast, the contents of these oversulfated disaccharide units are very low in CS-A, suggesting that the oversulfated portion of CS is functionally important for the binding to PTN. Exogenously applied PTN also induced an abnormal mor-

Figure 6. Effects of the Chase ABC and CS treatments on PTN contents in the cerebellum. *A, B*, Confocal microscopic images of adjacent cryosections derived from the cerebellum of a P9 rat and stained by fluorescent immunohistochemistry using an antibody against PTN without (*A*) or with (*B*) pretreatment by Chase ABC (20 mU/ml; 37°C; 40 min). The pretreatment with Chase ABC reduced PTN immunoreactivity. Scale bar, 25 μ m. *C*, Western blotting analysis showing effects of CS-D and -A on PTN contents in cerebellar slice cultures. Addition of CS-D but not CS-A decreased the PTN contents in the slices.

Figure 7. Reduction in GLAST immunoreactivity on Bergmann glial processes by the reagents affecting PTN-PTP ζ signaling. Shown are confocal microscopic images of cryosections of cerebellar slices cultured for 6 d with culture medium containing control IgG (A, B) and α 6B4PG (C, D) (200 μ g/ml) and double stained by fluorescent immunohistochemistry using antibodies against IP ζ R (A1, B1, C1, D1) and GLAST (A2, B2, C2, D2). A3, B3, C3, and D3 are the merged images. Although the cell bodies and dendrites of Purkinje cells were surrounded by the GLAST-positive lamellate processes of Bergmann glia under the control IgG-treated conditions (B, arrowheads), the GLAST immunoreactivity was markedly reduced under the α 6B4PG-treated conditions (C, D). Scale bars: (in A) A, C, 25 μ m; (in B) B, D, 5 μ m. E, F, Distribution of GFAP in cerebellar slice cultures. Confocal microscopic images of cryosections of slices cultured for 6 d without (E) or with (F) CS-C (50 μ g/ml) and stained by fluorescent immunohistochemistry using an antibody against GFAP. The GFAP-positive shaft processes of Bergmann glia did not differ between the two culture conditions. Scale bar, 25 μ m.

phology in Purkinje cell dendrites (Fig. 4), suggesting that normal distribution of PTN is required for the action of PTN-PTP ζ signaling in this phenomenon.

Although PTP ζ is expressed by both Purkinje cells and Berg-

mann glia, PTN is expressed by the latter cells in the postnatal cerebellum (Matsumoto et al., 1994; Wewetzer et al., 1995). These expression patterns suggest two possibilities concerning the action of PTN-PTP ζ signaling, although they are not mutually exclusive. One possibility is that PTN secreted by Bergmann glia binds with PTP ζ on the same cells in an auto-crine or paracrine manner (Fig. 9, arrow 3). The other is that PTN secreted by Bergmann glia binds with PTP ζ on Purkinje cells as one of the glia–neuron interactions (Fig. 9, arrow 3'). Before binding to the transmembrane form of PTP ζ , PTN may be pooled by binding to the secreted form of PTP ζ (phosphacan) in the extracellular matrix of the ML (Figs. 6, 9, arrow 2).

Our study showed that the immunoreactivity to the glutamate transporter GLAST on the lamellate processes of Bergmann glia was reduced in slice cultures treated with the reagents affecting PTN-PTP ζ signaling (Fig. 7). In contrast, the number of GFAP-positive Bergmann fibers was not reduced under the same conditions, indicating that the decrease in GLAST immunoreactivity was not caused by toxic effects of the reagents on Bergmann glia. Moreover, inhibition of glutamate transporter activity by DL-TBOA induced the aberrant morphogenesis of Purkinje cells just as in the case of the perturbation of PTN-PTP ζ signaling (Fig. 8). These findings suggest that PTN-PTP ζ signaling acts on the Bergmann glial processes and controls the formation and maintenance of the GLAST-positive lamellate processes of Bergmann glia, which regulate the morphogenesis of Purkinje cell dendrites (Fig. 9). Further studies are necessary to elucidate how PTN-PTP ζ signaling controls GLAST expression (Fig. 9, arrow 4). On the other hand, we cannot exclude the possibility that PTN directly affects Purkinje cells (Fig. 9, arrow 3').

In the developing cerebellum, glutamate is produced and released by granule cells (Levi et al., 1991; Miranda-Contreras et al., 1999). Studies using dissociated cell cultures have shown that glutamate stimulates the dendritic growth of Purkinje cells (Cohen-Cory et al., 1991; Hirai and Launey, 2000), suggesting that glutamate released in the extracellular space by granule cells influences the morphogenesis of Purkinje cell dendrites *in vivo*. However, it is not plausible that the aberrant morphogenesis of Purkinje cells observed in this

study was caused by the abnormal development of granule cells, because the migration and differentiation of granule cells proceeded normally in our slice cultures even in the presence of the reagents affecting PTN-PTP ζ signaling (Fig. 5, Table 2). Further-

Table 3. Evaluation of the number of Bergmann fibers

Culture conditions	Bergmann fibers (cells/150 μ m ML)
Control	18.1 $\pm$ 1.5 (28)
CS-D	18.8 $\pm$ 1.3 (27)
Control IgG	16.6 $\pm$ 2.3 (20)
α 6B4PG	14.1 $\pm$ 3.1 (7)

Cerebellar slices derived from P9 rats were cultured for 6 d with or without CS-D (50 μ g/ml), control IgG (200 μ g/ml), and α 6B4PG (200 μ g/ml). Cryosections of slice cultures were stained by immunohistochemistry using an antibody against GFAP, and GFAP-positive fibers >30 μ m within 150 μ m of the ML were enumerated in confocal microscopic images of 3- μ m-thick optical sections. Values are expressed as the mean $\pm$ SEM (n). Statistical analysis using Student's t test detected no significant difference between any set of conditions.

Figure 8. Effects of a glutamate transporter inhibitor on the morphogenesis of Purkinje cell dendrites. *A, B*, Confocal microscopic images of cerebellar slices derived from P9 rats, cultured for 6 d with culture medium containing DL-threo- β -benzyloxyaspartate (TBOA) (10 μ M), an inhibitor of glutamate transporters, and stained by fluorescent immunohistochemistry using 4C11. On addition of DL-TBOA, the MPD (*A*) and DOPD (*B*) types of Purkinje cells increased. Scale bar, 25 μ m. *C*, Quantitative representation of the effects of DL-TBOA on the morphogenesis of Purkinje cell dendrites in slice cultures at 6 DIV. $n = 4$. Error bars represent SEM. * $p < 0.05$ versus control.

more, although the growth of Purkinje cell dendrites was stimulated in the dissociated coculture system of Purkinje cells and granule cells, most of the Purkinje cells had multiple primary dendrites extending in various directions (Baptista et al., 1994; Hirai and Launey, 2000), suggesting that granule cells do not function in the determination of polarity in Purkinje cells.

In this context, Bergmann glia settle in a quite important position. The cell bodies of Bergmann glia closely associate with Purkinje cell bodies and extend polarized shaft processes with fine lamellate processes toward the pial surface. Of the two, the latter processes express GLAST and closely surround both the cell bodies and dendrites of Purkinje cells during postnatal development (Furuta et al., 1997; Ullensvang et al., 1997; Yamada et al., 2000) and in adults (Rothstein et al., 1994; Lehre et al., 1995), regulating the extracellular glutamate levels around this type of cell (Barbour et al., 1994). In the present study, we found that the GLAST immunoreactivity was reduced around the Purkinje cells of aberrant morphology and that the inhibition of the glutamate transporter activity induced the abnormal morphogenesis of Purkinje cell dendrites. On the basis of these findings, we suggest that at least one mechanism for Bergmann glia to regulate the morphogenesis of Purkinje cell dendrites is by modulating the extracellular glutamate levels through the glutamate-transporting activity of GLAST (Fig. 9, arrows 5, 6).

Figure 9. A model for regulation of the morphogenesis of Purkinje cell dendrites by PTN-PTP ζ signaling. 1, PTN is produced by Bergmann glia. 2, Before binding to the transmembrane (receptor) form of PTP ζ (rPTP ζ), PTN may be pooled by binding to the secreted form of PTP ζ (Phosphacan) in the extracellular matrix of the ML. 3, PTN is suggested to bind with rPTP ζ on Bergmann glia. 3', The possibility that PTN directly binds with rPTP ζ on Purkinje cells cannot be excluded. 4, PTP ζ signaling controls the formation or maintenance, or both, of the GLAST-positive lamellate processes of Bergmann glia, the mechanism of which is not known at present. 4', The existence of a GLAST-independent mechanism cannot be excluded. 5, 6, GLAST regulates the extracellular levels of glutamate, which can induce growth or retraction of Purkinje cell dendrites. D, Tyrosine phosphatase domain.

Although the directed growth of Purkinje cell dendrites might be regulated simply by the interaction between the leading processes of the dendrites and the Bergmann glial processes (Yamada et al., 2000), the mechanism for the morphological change from the MPD- to the SPD-type Purkinje cells is a profound problem because both the growth and retraction of the primary dendrites have to occur in the same cell. Wilson and Keith (1998) found that glutamate can both facilitate and inhibit the dendritic growth of hippocampal neurons, depending on the exposure time to glutamate in dissociated cell cultures. Fine regulation of the extracellular glutamate levels by GLAST on Bergmann glial processes might produce a spatiotemporal pattern of the glutamate levels around MPDs, and such a pattern might influence the final selection of one primary dendrite. This mechanism may involve voltage-dependent calcium channels activated after glutamate stimulation, because a mutation in the gene encoding the α 2 δ -2 voltage-dependent calcium channel accessory subunit was found to increase the MPD-type Purkinje cells (Brodbeck et al., 2002). In addition, we can propose another mechanism for Bergmann glia to regulate Purkinje cell morphology. The Bergmann glial processes closely surrounding Purkinje cell bodies may prevent the sprouting of new primary dendrites and maintain the SPD-type morphology of Purkinje cells.

It was reported recently that mutant mice deficient in PTP ζ (Shintani et al., 1998; Harroch et al., 2000) and PTN (Amet et al., 2001) showed no gross morphological abnormality, at least in adult animals. This suggests that the morphogenesis of Purkinje cell dendrites is regulated by multiple signaling mechanisms, including that of PTN-PTP ζ , and that the loss of PTN or PTP ζ alone is compensated for by the other molecules. For example, MK might compensate for PTN deficiency, and PTP ζ deficiency might be compensated for by the other proteoglycans such as neurocan and syndecan-3, which bind with PTN (Bandtlow and Zimmermann, 2000). On the other hand, Purkinje cells in the GLAST-deficient mice were abnormal in that they were multiply innervated by the climbing fibers even at the adult stage (Watase et al., 1998). A detailed description of Purkinje cell development in these mutant mice has not been reported, and it will be interesting to analyze the development of the cerebellum in single- and double-mutant mice for these genes.

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