Diminished climbing fiber innervation of Purkinje cells in the cerebellum of myosin Va mutant mice and rats

Running title: Climbing fibers in myosin Va mutants

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Key words: myosin Va; cerebellum; climbing fiber; development; Purkinje cell; axonal transport; Ca^{2+} signaling; mutant mouse

Abstract

Myosin Va is an actin-based molecular motor that is involved in organelle transport and membrane trafficking. Here, we explored the role of myosin Va in the formation of synaptic circuitry by examining climbing fiber (CF) innervation of Purkinje cells (PCs) in the cerebella of *dilute-neurological* (*d-n*) mice and *dilute-opisthotonus (dop)* rats that have mutations in *dilute*–encoded myosin Va. Anterograde labeling of CFs with biotinylated dextran amine (BDA) revealed that they arborized poorly and that their tips extended only half way through the thickness of the molecular layer (ML) in adult *d-n* mice. Using immunohistochemistry specific for vesicular glutamate transporter 2 (VGluT2) to visualize CF synaptic terminals, we found that during development and in adulthood, these terminals did not ascend as far along the proximal shaft dendrites of PCs in *d-n* mice and *dop* rats as they did in normal animals. An irregular distribution of BDA-labeled bulbous varicosities and VGluT2 spots along CF branches were also noted in these animals. Finally, VGluT2-positive CF terminals were occasionally localized on the PC somata of adult *d-n* cerebella. These phenotypes are consistent with our electrophysiological findings that CF-mediated excitatory postsynaptic currents (EPSCs) were significantly smaller in amplitude and faster in decay in adult *d-n* mice, and that the regression of multiple CFs was slightly delayed in developing *d-n* mice. Taken together, our results suggest that myosin Va is essential for terminal CF extension and for the establishment of CF synapses within the proper dendritic territories of PCs.

Introduction

Myosin Va is an actin-based molecular motor protein that is involved in membrane trafficking and organelle transport (Langford and Molyneaux, 1998; Bridgman and Elkin, 2000; Reck-Peterson et al., 2000; Bridgman, 2004). Since myosin Va is abundant and ubiquitous in the brain (Futaki et al., 2000a; Tilelli et al., 2003), elucidation of its function has been an intriguing issue in neuroscience (Langford, 2002; Goldstein, 2003). To date, insights into its potential roles have come from studies in animals that had altered myosin Va function due to a spontaneous genetic mutation (Dekker-Ohno et al., 1996; Takagishi et al., 1996; Wang et al., 1996; Bridgman, 1999; Miyata et al., 2000; Lalli et al., 2003). Others have explored the interactions between myosin Va and cellular organelles and proteins (Prekeris and Terrian, 1997; Tabb et al., 1998; Naisbitt et al., 2000; Walikonis et al., 2000; Rao et al., 2002; Watanabe et al., 2005).

Myosin Va is encoded by the mouse and rat *dilute* locus. *Dilute-lethal* mice (Strobel et al., 1990; Mercer et al., 1991) and *dilute-opisthotonus* (*dop*) rats (Dekker-Ohno et al., 1993; Futaki et al., 2000b), which carry null mutations at this locus, exhibit a diluted coat color due to a defect in the transport of melanosome granules in melanocytes (Reck-Peterson et al., 2000; Wu et al., 2002). Furthermore, they display ataxia and opisthotonic seizures beginning 2 weeks after birth and generally die within 3 weeks. The cellular bases of these neurological deficits have not been elucidated. We previously reported that the dendritic spines of Purkinje cells (PCs) from the cerebella of *dilute-lethal* mice and *dop* rats lack a smooth endoplasmic reticulum (SER: intracellular Ca^{2+} store) (Dekker-Ohno et al., 1996; Takagishi et al., 1996). This

abnormality may affect long-term depression (LTD) at parallel fiber (PF) to PC synapses in these animals (Miyata et al., 2000). However, synaptic transmission at PF-PC synapses is normal (Miyata et al., 2000) and glutamate receptors and their associated proteins are normally retained on PC spines (Miyata et al., 2000; Petralia et al., 2001) in myosin Va null mutants. Thus, whether a loss of myosin Va function affects the development of synaptic circuit remains unknown.

In this study, we determined whether mutated myosin Va influences the formation of proper synaptic wiring in the cerebellum in mice and rats. To this end, we utilized a new mouse model, *dilute-neurological* (*d-n*). Genomic sequence analysis of *d-n* mice has shown that a missense mutation of the myosin Va gene introduces a stop codon that results in the production of a truncated protein (Huang et al., 1998). Although myosin Va mRNA levels are not significantly affected, the protein levels are dramatically reduced (Huang et al., 1998). The phenotype of the *d-n* mice is similar to that of *dilute-lethal* mice and *dop* rats with respect to coat color and neurological abnormalities. However, *d-n* mice are neurologically impaired only between 2 and 3 weeks after birth but as they age they recover progressively. Adult *d-n* mice exhibit apparently normal behavior, although behavior analysis has not been reported. By analyzing *d-n* mice and *dop* rats, we discovered that myosin Va plays a role in the extension of climbing fiber (CF) arbors and in the establishment of CF innervation territories on PC dendrites.

Methods

Animals

Myosin Va mutant *dilute-neurological (d-n) (*C57BL/6J; d^n/d^n*)* mice and *dilute-opisthotonus (dop*) (DOP; *dop/dop*) rats were used in this study. The *d-n* mice were provided by Dr. N.A. Jenkins (National Cancer Institute Frederick Cancer Research & Development Center, MD) (Huang et al., 1998). Homozygous mice were obtained from the mating of homozygous males with heterozygous females. The *dop* rats were discovered and propagated in our laboratory (Dekker-Ohno et al., 1993). Homozygous *d-n* mice and *dop* rats can be distinguished from normal animals 4 - 5 days after birth by their diluted coat color. All animals were cared in accordance with the guidelines of the Committee for Animal Experiment of Nagoya University School of Medicine and Research Institute of Environmental Medicine.

Adult *d-n* mice (2-3 months of age) were used for anterograde labeling and immunohistochemistry. For our developmental study, postnatal day (P) 10, 14, and 17 *d-n* mice and P10, 14 and 21 *dop* rats were used. Electrophysiological studies were carried out on young (P11~19) and adult (2 months of age) *d-n* mice, the latter of which were also used for conventional and immunoelectron microscopy. Littermate heterozygous mice or wild-type (C57BL/6J) mice were used as controls.

Anterograde labeling

Animals were anesthetized with 4% chloral hydrate (250 μL injected intraperitoneally/25g body weight), after which their inferior olivary nucleus was

stereotaxically injected with 0.025 μL of 5% biotinylated dextran amine （BDA, 10000MW, Molecular Probes INC, Eugene, OR） in physiological saline using a glass micropipette（inner diameter of the tip was 30-50μm）with a pneumatic picopump (Picospritzer Model PV800). Five to seven days later, the animals were transcardially perfused with 2% paraformaldehyde (PFA) in 0.1*M* phosphate buffer (PB, pH 7.4) (100 ml/25g body weight). Their cerebella were removed and rinsed in PB containing 30% sucrose at 4℃. Serial sagittal sections (50μm) of the cerebellum were cut using a freezing microtome, after which they were rinsed in PBS and incubated overnight in a 15μL solution of streptavidin, 1 mL of 2.5% Triton X-100, and 4 mL of PBS. After being rinsed five times with PBS, the sections were incubated with diaminobenzidine solution containing 50 mL of 0.05*M* Tris-HCl buffer (pH 7.4), 10 mg of diaminobenzidine and 125 mg of nickel ammonium for 5-10 minutes, after which the reaction was stopped by adding 50-80 μL of 0.3% H_2O_2. Finally, the sections were mounted onto the gelatinized slides, counterstained with 0.1% Neutral red and examined by light microscopy.

Immunohistochemistry

Animals were perfused intracardially with 4% PFA in PBS for 5-10 minutes, after which their cerebella were removed and dissected in the sagittal plane. They were immersed immediately and incubated in the same fixative at 4°C overnight. After cryoprotection with PBS containing graded concentrations of sucrose, the tissues were frozen in LN_2; frozen sections (16-20 μm) were cut with a cryostat. Affinity-purified polyclonal guinea

pig antibodies against vesicular glutamate transporter type 2 (VGluT2) were used to visualize CF terminals (Ichikawa et al., 2002; Hioki et al., 2003; Miyazaki et al., 2003). Double immunofluorescence for VGluT2 (1 μg/mL) and calbindin D-28K (CD-28k) (Chemicon Int. Inc, Temecula, CA; diluted to 1:500) was carried out using these antibodies. Sections were incubated with the antibodies overnight, after which they were treated with a mixture of FITC- or Cy3-labeled species-specific secondary antibodies (diluted to 1:250-500, Jackson ImmunoResearch Lab Inc., West Grove, PA) for 1 hour. The immunolabeled sections were examined with a confocal laser scanning microscope (Zeiss LSM 510).

Electron microscopy

Animals were perfused intracardially with 2% PFA and 2.5% glutaraldehyde (GLA) in 0.1*M* PB, after which their cerebella were removed and cut into small pieces. The tissues were postfixed with 1% OsO_4 in PB for 1hr. After dehydration with graded alcohols, the tissues were embedded in epoxy resin. Semithin sections were cut and stained with toluidine blue for light microscopy and ultrathin sections were prepared for electron microscopy. For immunoelectron microscopy, the cerebella were fixed with 0.1% GLA and 2% PFA in 0.1*M* PB. The cryostat sections were prepared as the same procedure described above. The sections were incubated with anti-VGluT2 or anti-vesicular glutamate transporter type 1(VGluT1) antibodies overnight, followed by HRP-labeled secondary antibodies for 1 hour. The anti-VGluT1 antibodies were used to visualize PF terminals (Ichikawa et al., 2002; Hioki et al., 2003; Miyazaki et al., 2003).

They were then visualized with DAB and post-fixed with 1% OsO_4 in PB for 1hr. After being dehydrated, the tissues were embedded in epoxy resin.

Electrophysiology

Parasagittal, cerebellar slices (250 μm) were prepared from *d-n* and normal mice as previously described (Kano et al., 1995, 1997). Whole-cell recordings were made from visually identified PCs using an upright microscope (BX50WI; Olympus Optical) at 31°C. The resistance of our patch pipettes was 3-6 MΩ when filled with the following solution (in m*M*): 60 CsCl, 10 Cs D-gluconate, 20 TEA-Cl, 20 BAPTA, 4 $MgCl_2$, 4 ATP, 0.4 GTP, and 30 HEPES, pH 7.3, adjusted with CsOH. The pipette access resistance was compensated by 70-80%. The constituents of the standard bathing solution were (in m*M*): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 20 glucose, and the solution was bubbled with 95% O_2 and 5% CO_2. Bicuculline (10μ*M*) was always present to block inhibitory synaptic transmission. Ionic currents were recorded with an Axopatch 1D (Axon Instruments, Foster City, CA) patch-clamp amplifier; the signals were filtered at 2 kHz and digitized at 20 kHz. Online data acquisition and offline data analysis were performed using PULSE software (Heka Elektronik, Lambrecht/Pfalz, Germany). Stimulation pipettes (5-10 μm tip diameter), filled with standard saline, were used to apply square pulses of focal stimulation (duration, 0.1 msec; amplitude, 0-90 V). CFs were stimulated in the granule cell layer at a distance of 50-100 μm from the Purkinje cell somata.

Quantitative measurement and data analyses

In BDA labeling and immunofluorescence, to evaluate quantitative differences in CF extensions between normal and mutant cerebella, we measured the distance from the base of the ML to the tips of BDA-labeled CFs or VGluT2 positive CFs relative to the vertical length of the entire ML in the more than 3 (the BDA labeling) or 5 (immunofluorescence) images randomly collected from each animal. Furthermore, to estimate the translocation of CFs from the soma to dendrites of PCs, PCs were counted in double immunofluorescence sections and divided into two categories; PCs with and without several VGluT2-positive puncta on the somatic surface. Data are expressed as the mean $\pm$ S.E.M. The *p* value was calculated using Student's or Welch's unpaired *t* test (two-tailed).

Results

The cerebellum of the *d-n* mouse was found to be slightly smaller in size than that of the normal mouse, although the organization of the cerebellar cortex such as foliation, layer formation, and monolayer alignment of PCs was normal in the *d-n* mouse (Fig. 1). The anterior lobules (lobules I-V) were selected for the following histological analyses.

Reduced terminal extension and branching of CFs in adult *d-n* mice

CF innervation in the cerebellar cortex of adult *d-n* mice was assessed by anterograde labeling of CFs with BDA, which was injected into the inferior olivary nucleus in the medulla. Labeled olivocerebellar axons were traced into the cerebellar cortex and their

distal extensions, designated as CFs, were found to pass through the granular and PC layers, and terminated in the ML. Once the CFs reached the base of the ML, they gave off numerous branches in a fan-like fashion. The branches possessed fine tendrils and small, presynaptic varicosities (Strata et al., 1997; Strata and Rossi, 1998). The CFs of normal mice had small, bulbous varicosities of similar size and shape that were regularly placed along their branches (Fig. 2*A* and *C*) and their tips reached close to the pial surface (Fig. 2*A*). In contrast, the labeled CFs of *d-n* mice only extended about half way through the ML (Fig. 2*B)*. The mean relative height of CFs in the molecular layer was 86.2 ± 0.2% of its thickness in normal mice (n=10 images from three mice) and 58.9 ± 0.3% in *d-n* mice (n=11 images from three mice, $p < 0.0001$, Welch's *t* test). CFs in *d-n* mice were noted to arborize poorly and to have an altered branching pattern (Fig. 2*B*, *D-H*), indicating that CF extension are affected in both vertical and lateral dimension in *d-n* mice. The CF arbors in normal mice were found, under higher magnification, to consist of short and thick proximal branches and numerous distal varicose tendrils that branched regularly and organized themselves into several "orders" (Fig. *2C*). In contrast, mutant CFs branched less frequently (Fig. *2D-H*) and displayed irregular proximal and distal contours that included distorted, rugged, and complex, ramified branches (arrowheads in Fig. *2D, E, G* and *H*). Bulbous varicosities were irregular in size (arrows in Fig. *2F* and *G*) and sometimes formed clusters (white arrows in Fig. *2D* and *E*). Their distribution along tendrils was also irregular (asterisks in *H*). Beaded CF tendrils were also present around the somata of PCs in *d-n* mice (arrows in Fig. *2B*), although they were rarely encountered in normal mice.

VGluT2-positive CFs were found to extend up approximately four-fifth of the ML in normal mice (Fig. 3*A* and *a*), whereas they were confined to the inner half of the ML in *d-n* mice (Fig. 3*B* and *b*). The mean relative height of CFs in the ML was 83.0 ± 0.9% in normal mice (n=17 images from three mice) and 57.4 ± 1.2% in *d-n* mice (n=24 images from three mice, $p<0.0001$, Welch's *t* test). The spot-like distribution of VGluT2-positive CFs was evenly diffused in the lower and upper ML in normal mice (Fig. 3*a*), whereas they were more distributed in the lower ML relative to the upper layer in the *d-n* mice (Fig. 3*b*). These findings were consistent with those obtained by BDA anterograde labeling.

Using double immunofluorescence with calbindin D-28k (CD-28k), we found that PC dendrites of *d-n* mice displayed their characteristic branching pattern similar to that of normal mice and that punctate, VGluT2–positive CF terminals were distributed along shaft dendrites in both types of mice (Fig. 3*C* and *D*). However, the VGluT2 labeling was localized along more basal portions of the proximal dendrites and the distal shaft dendrites were free of VGluT2 imunofluorescence in *d-n* mice (Fig. 3*D* and *d*), while they were distributed throughout proximal and distal shaft dendrites in normal mice (Fig. 3*C* and *c*). The VGluT2-labeled spots were more varied in size in *d-n* mice than in normal mice (Fig. 3*c* and *d*). In addition, VGluT2 fluorescence was occasionally localized around PC somata (arrow head in Fig. 3*d*). By counting PCs in which the soma were associated with more than several VGluT2-positive punctate spots, 16.7 ± 2.2% of PCs retained somatic CFs in *d-n* mice (n=27 sections from four mice), whereas 6.6 ± 2.2% of PCs retained somatic CFs in normal mice (n=25 sections from three mice,

$p<0.005$, Student's *t* test). Taken together, the results of our BDA anterograde labeling and VGluT2 immunohistochemical studies suggest that CF terminal arborization is reduced and its innervation of PC dendrites is diminished in myosin Va mutant cerebella.

Impaired terminal growth of CFs in myosin Va mutants during development

To clarify the roles of myosin Va in terminal growth of CFs, we examined CF innervation of PC dendrites in developing cerebella. At P10, the punctate, fluorescence-labeled VGluT2 was predominantly localized to CFs around somata of PCs in both normal and *d-n* mice (Fig. 4*A*, *D*). These transitory, pericellular CF nests were referred to as "nid" formations. VGluT2 was reportedly expressed in both CF and PF terminals during early postnatal mouse development (Miyazaki et al., 2003). We also observed VGluT2 immuofluorescence in the ML as well as around somata of PCs (Fig. 4*A*, *D*), which makes it difficult to distinguish between CF and PF terminals (Fig. 4*A*, *D*). Even so, the perisomaticVGluT2 immuofluorescence appeared as coarse puncta (arrowheads in Fig. 4*A*, *D*), whereas the VGluT2 spots in the ML were tiny and densely packed, indicating that they belong to PF terminals. VGluT2 labeling was also occasionally found at the base of the stem dendrites in normal and mutants (dwata not shown). They might belong to CF terminals.

At P14, VGluT2-positive labeling was associated with relatively branched dendrites of PCs in normal mice (arrowheads in Fig. 4*B*), although VGluT2-labeled CFs and PFs were still indistinguishable at this developmental stage. In mutants,

VGluT2-labeled CFs were similarly located on PC dendrites, but they did not ascend as distally along these dendrites as did the CFs of normal mice (Fig. 4*E*). Hence, the mean relative height of CFs to the molecular layer was 53.0 ± 2.1% of its thickness for normal mice (n=16 images from two mice) and 30.8 ± 2.3% of its thickness for *d-n* mice (n=16 images from two mice, $p < 0.0001$, Student's *t* test). Furthermore, CF immuofluorescence was more often encountered around PC somata in *d-n* mice (48.9 ± 5.9%, n=16 sections from two mice) (arrowheads in Fig. 4*E*) than in normal mice (25.2 ± 4.8%, n=15 sections from two mice), showing a significant difference ($p<0.005$, Student's *t* test).

At P17, the tips of VGluT2 positive CFs extended approximately the two-thirds of the way through the ML in normal mice (the mean height: 64.6 ± 1.4%, n=12 images from two mice) (Fig. 4*C*), with only a few tiny VGluT2 spots present in the upper molecular layer. The punctate immuofluorescence of CFs was located on and along primary to tertiary branches of PC dendrites but rarely surrounding the somata of PCs in normal mice (Fig. 4*C*). In contrast, VGluT2-positive CFs in mutant animals were restricted to the inner regions of the ML (the mean height: 39.8 ± 1.4%, n=12 images from two mice, $p < 0.0001$, Student's *t* test) and were only found on more proximal portions of PC dendrites than the CFs of normal mice (Fig. 4*F*). In addition, perisomatic CFs were undiminished in mutant cerebella at this developmental stage. The VGluT2-positive punctate fluorescence remained on the somata of 46.0 ± 5.0% of PCs in mutant mice (n=10 sections from two mice), whereas they were observed on 13.9 ±5.1 % of PCs in the normal mice (n=11 sections from two mice, $P<0.0005$,

Student's *t* test).

We also investigated the CF innervation in postnatal *dop* rats that carry a myosin Va null mutation and found that they displayed phenotypes that were similar to those seen in *d-n* mice (Fig. 5). The perisomatic nests of CF terminals were observed at P10 in both normal and *dop* rats (Fig. 5*A* and *D*). Thereafter, CFs were found to climb along the PC dendrites in both rats, although VGluT2 labeled CF terminals were consistently lower in height [at P14, 32.0 ± 2.2% for *dop* rats (n=13 images from two rats) and 53. 8 ± 1.8% for normal rats (n=12 images from two rats, $p< 0.0001$, Student's *t* test) and at P21, 49.5 ± 1.3% for *dop* rats (n=23 images from three rats) and 72. 6 ± 1.3% for normal rats (n=12 images from two rats, P< 0.0001, Student's *t* test)]. The CFs also persisted in greater numbers around PC somata in *dop* rats [at P14, 71.6 ± 4.7% (n=12 sections from two rats) and at P21, 55.5 ± 6.8% (n=17 sections from three rats)] than in normal rats [at P14, 38.0 ± 5.1% (n=10 sections from two rats) and at P21, 16.8 ± 3.8% (n=12 sections from two rats)]. These differences were significant ($p<0.0001$ at P14, Student's *t* test; $p<0.0001$ at P21, Welch's *t* test) (Fig. *5B, C, E*, and *F*). All of the above data suggest that the translocation of CFs from the somata to the dendrites of PCs is delayed and that the CF innervation of PC dendrites is diminished in the developing cerebella of myosin Va mutant mice and rats. It is conspicuous that the VGluT2 immuofluorescence was very weak in the ML during development in both normal and *dop* rat cerebella at all stages examined in the present study (Fig. 5*A*-*F*). This result indicates that VGluT2 expression level is very low in rat PF terminals during development and could not be detected by immunofluorescence.

This VGluT2 expression profile is in contrast to that of mice (Fig. 4) and enabled us to demonstrate impaired growth of CFs clearly in *dop* mutants.

EM structure of CF-PC synapse and PF-PC synapse

Then, we observed the ultrastructure of CF to PC synapses by using conventional and immunoelectron microscopic techniques (Fig. 6). The CF synapses in normal animals were easily identified by their large size, abundance of synaptic vesicles (including dense-cored vesicles), proximity to or apposition with thick PC dendrites, and mitochondria in their cytoplasm, as previously reported (Palay and Chan-Palay, 1974). CF terminals were found to make synaptic contacts with typically several PC spines and to form asymmetrical synaptic junctions (Fig. 6*A*). Immunoelectron microscopy for VGluT2 confirmed that these synapses were indeed CF synaptic terminals (Fig. 6*a*). The CF synapses of *d-n* mice appeared essentially normal i.e., they had large terminals containing densely packed vesicles and formed asymmetric junctions with multiple PC spines (Fig. 6*B* and *b*). On the contrary, atypical PF-PC synapses were occasionally observed in *d-n* mice. The terminals were enlarged, contained many SVs and made synaptic contacts with more than two PC spines (Fig. 6*D*), while normal PF terminals were small in size, contained a small number of SVs and made synaptic contacts with mostly one or occasionally two PC spines (Fig. 6*C and c*). Table 1 shows the frequency of multiple synaptic contacts in 1000 PF terminals randomly collected from the entire ML of *d-n* and normal mice (n=3 for each). The percentage of multiple synaptic contacts was 13.0% for *d-n* mice and 4.3% for normal mice. Importantly, no PF

boutons contacting with three or more PC spines were present in normal mice, but they were observed in *d-n* mice (Table 1) (Fig. 6*D* and *d*). The VGluT1 immunoelectron microscopy confirmed that these atypical terminals belonged to PF synaptic terminals and not to CF terminals (Fig. 6*d*). These findings suggest that PF-PC synapses in adult *d-n* mouse cerebella undergo morphological rearrangement presumably because of reduced number of CF-PC synapses.

CF to PC synaptic transmission is altered in *d-n* mice

To determine whether a myosin Va mutation results in changes in synaptic transmission, we measured CF-mediated excitatory postsynaptic currents (CF-EPSCs) in PCs of adult (2 months of age) *d-n* mice. In parasagittal cerebellar slices, PCs were recorded in the whole-cell configuration and CFs were stimulated with a glass pipette that was placed in the granular layer near the recorded PCs. To search for PCs that were innervated by multiple CFs, we moved the stimulation pipette systematically and gradually increased the stimulus intensity at each stimulation site. In most adult *d-n* and normal mice, a clearly discernible CF-EPSC was elicited in an all-or-none fashion (Fig. 7*A*), indicating that these cells were innervated by single CFs. The frequency distribution of PCs in terms of their number of CF-EPSCs steps (Fig. 7*B*) showed no significant difference between normal and *d-n* mice ($p>0.05$ by χ^2-test), suggesting that most PCs in *d-n* mice were innervated by single CFs in a manner similar to that seen in normal PCs at 2 months of age. However, the amplitudes of the CF-EPSCs in *d-n* mice were significantly smaller than those in normal mice (Fig. 7*A*). As summarized in Table 2, the

CF-EPSCs of *d-n* mice had a smaller chord conductance and shorter decay time constant than that observed in normal mice. Furthermore, the magnitude of the paired-pulse depression of the CF-EPSCs was significantly greater in *d-n* mice than in normal mice (Fig. 7*E*). These results suggest that CF-PC synaptic transmission in *d-n* mice is functionally weaker than in normal mice. These electrophysiological data are consistent with our BDA labeling and immuohistochemical findings of reduced CF innervation in *d-n* mice (Figs. 2-5). In contrast to CF-PC synapses, we did not detect any significant differences in paired-pulse facilitation of PF-EPSCs, as summarized in Figure 7*F*.

The CF-PC synapse was also examined in developing (P11-15) mice. In early postnatal days of the rodent cerebellum, PCs are innervated by multiple CFs (Crepel, 1982). Massive elimination of supernumerary CFs occurs subsequently until the adult type mono innervation pattern is established during the initial three postnatal week in mice (Hashimoto and Kano, 2003). In developing normal and *d-n* mice, PCs with multiple CF-EPSC steps were sampled more frequently than in the adult mice (Fig. 7*C*). The summary graph in Fig. 7*D* shows that about 50% of the PCs were found to be innervated by multiple CFs in normal mice. In contrast, more than 70% of the PCs remained innervated by multiple CFs in *d-n* mice (Fig. 7*D*). This result suggests that the elimination of surplus CFs is somewhat delayed in the developing cerebella of *d-n* mice. This finding was consistent with our demonstration that the disappearance of VGluT2-positive spots around PC somata was delayed in *d-n* mice (Fig. 4).

Discussion

We have shown that the terminal extension of CFs and their synaptic territories on PC dendrites were diminished in the developing and adult cerebella of myosin Va mutant mice and rats. Our anterograde labeling of CFs with BDA and immunohistochemical staining of VGluT2 demonstrated a reduction of CF extension in both vertical and lateral dimension and a diminished CF innervation on more proximal portions of PC dendrites. Consistent with these morphological phenotypes, CF-mediated EPSCs were reduced in amplitude in adult *d-n* mice and the regression of multiple CFs was delayed in developing *d-n* mice.

Diminished CF innervation in myosin Va mutants

A characteristic feature of developing CFs is an extensive terminal arborization (Palay and Chan-Palay, 1974; Strata et al., 1997). Each CF arbor ramifies parasagittally into over 100 branches that are organized in four orders of ramifications in rats (Strata et al., 1997). Our data confirmed that there were extensive terminal arborizations of CFs, as demonstrated by BDA labeling and VGluT2 immunofluorescence, in normal mouse adult cerebella (Figs. 2 and 3) (Ichikawa et al., 2002; Miyazaki et al., 2003). Similarly, diminished CF extension and PC innervation in mutant cerebella was also well characterized by these methods (Figs. 2 and 3). The diminished CF innervation first became apparent during postnatal development, which is a time when CFs climb along the dendrites of PCs. Our findings suggest that CFs grow and innervate PC somata

normally during early development in myosin Va mutant animals, but that they lag behind and never fully attain the innervation pattern of normal animals at later developmental periods.

Myosin Va is highly expressed in the inferior olivary neurons of normal rats (Futaki et al., 2000a) and mice (Takagishi, unpublished observation). Although we did not trace the projection of olivocerebellar axons from the inferior olive to the cerebellum, the olivocerebellar axons were expected to project normally into the cerebellar cortex because CFs innervate PC somata normally during early developmental stage in myosin Va mutant animals. *d-n* mice are not null myosin Va gene mutants, but the expression of this myosin form was reported to be quite low in these animals during early development (Huang et al., 1998; Takagishi, unpublished observation). Therefore, the above data suggest that the lack of myosin Va function does not affect the growth of olivocerebellar axons but rather impairs their terminal extension and synapse formation on the proper PC dendritic territories.

Possible roles of myosin Va in organelle/vesicle transport in CF axon terminals

Long-range transport in axons requires intact microtubules (MTs) and MT-based motors, whereas the local movement of organelles is mediated by actin that is driven by myosin motors in areas that are devoid of MTs such as nerve terminals and growth cones (Langford and Molyneaux, 1998; Bridgman and Elkin, 2000; Goldstein, 2003; Vale, 2003; Bridgman, 2004; Brown and Bridgman, 2004). However, recent reports suggest that the actin-myosin motor system may also be involved in long-range axonal transport

(Rao et al., 2002; Bridgman, 2004; Cao et al., 2004; Hasaka et al., 2004; Jung et al., 2004). The present findings that somatic CF innervation was normal during early development suggest that impairment of long-range axonal transport, if any, does not affect olivocerebellar axonal growth in myosin mutant animals.

A role for myosin Va in local organelle/vesicle transport has been proposed (Bridgman and Elkin, 2000; Langford, 2002; Bridgman, 2004; Brown et al., 2004). Myosin Va is known to be distributed in the growth cone and is associated with vesicular and cytoskeletal structures (Evans et al., 1997; Suter et al., 2000). Furthermore, it was reported to be associated with synaptic vesicle proteins such as SV2 (Evans et al., 1998) and synaptobrevin-synaptophysion complexes (Prekeris and Terrian, 1997). These results suggest the possibility that myosin Va plays a role in organelle/vesicle transport in axon terminals and contributes to their growth. Our present studies support this hypothesis, as does our unpublished observation of reduced thalamocortical projections in layer IV of the neocortex in *d-n* mice and *dop* rats.

Our BDA labeling study and VGluT2 immunohistochemical analysis also demonstrated various kinds of irregular profiles of CF terminals (Figs. 2 and 3). While these findings may reflect an altered distribution of synaptic vesicles along CF axons, supporting the notion that myosin Va acts as a synaptic vesicle motor (Prekeris and Terrian, 1997; Langford and Molyneaux, 1998; Bridgman, 1999; Bridgman, 2004; Brown et al., 2004), we did not detect any altered profiles of individual CF synaptic terminals in *d-n* mice by EM analysis (Fig. 6). Morphological features of CF synaptic terminals, such as the density of synaptic vesicles and the size of synaptic boutons vary

among individual terminals even in normal animals (Xu-Friedman et al., 2001). It is possible that plane-of-section limitations may have accounted for our inability to detect any change in synaptic vesicles transport along CF axons. Alternatively, we found that PF-PC synapses appeared somehow altered morphologically in *d-n* mice such that enlarged PF terminals with multiple PC spines were occasionally encountered (Fig. 6*D* and *d*). Similar morphological changes are also found in myosin Va null cerebella (Bridgman, 1999) as well as in several other neurological mutant mice such as *hyperspiney* (Guenet et al., 1983), *nodding* (Sotelo et al., 1983) and the P/Q-type Ca^{2+} channel α1A subunit ($Ca_V2.1$) mutants; *tottering, leaner, rolling Nagoya* (Rhyu et al., 1999a, b) and $Ca_V2.1^{-/-}$ mice (Miyazaki et al., 2004). The presence of multiple spine synapses with PFs was explained by a compensatory response of PF-PC synapses to a loss of granule cells in $Ca_V2.1$ mutants (Rhyu et al., 1999a, b). In *dilute-lethal* mice, a loss of PCs was reported, with TUNEL-positive cells detected on embryonic day 12 (Sawada et al., 2004). However, we have not observed cell death of either PCs or granule cells during postnatal development in myosin Va null mutant cerebella (Dekker-Ohno et al., 1996; Takagishi et al., 1996; Miyata et al., 2000). Thus, we assume that the large PF terminal with multiple PC spines might be caused by the loss of CF-PC synapses. This rearrangement of PF-PC synapses may not significantly alter synaptic transmission because there was no difference in paired-pulse facilitation of PF-EPSCs between *d-n* and normal mice (Fig. 7*B*).

Possible roles of myosin Va in organelle transport and Ca^{2+} signaling at the

postsynaptic site in PC spines

We previously reported that the SER and IP3 receptors were absent from the dendritic spines of PCs, whereas they were normally distributed in the cytoplasm of PC dendrites and somata in myosin Va null mutants (Dekker-Ohno et al., 1996; Takagishi et al., 1996, Petralia et al., 2001). We also demonstrated that Ca^{2+} elevation was significantly smaller in PC dendritic spines in myosin Va mutants, *dilute-lethal* mice and *dop* rats, following the induction of IP3 mediated Ca^{2+} release from internal stores by repetitive PF stimulation and resultant activation of metabotropic glutamate receptor type 1 (mGluR1) (Miyata et al., 2000). This change in Ca^{2+} signaling was attributed to the lack of Ca^{2+} stores (i.e., SER) within the dendritic spines of *dilute-lethal* mice and *dop* rats (Miyata et al., 2000). The dendritic spines of PCs in adult *d-n* mice contain the SER although the dendritic spines in juvenile *d-n* mice do not (unpublished observations), suggesting that Ca^{2+} signaling in PC dendritic spines may be diminished in the juvenile *d-n* mouse. Since the "critical period" of CF synapse elimination (Kakizawa et al., 2000) corresponds to the period in which dendritic spines of *d-n* PCs lack the SER and IP3 receptors, it is possible that the reduced CF innervation seen in *d-n* mice and *dop* rats may be attributed to altered Ca^{2+} signaling in the dendritic spines of PCs.

Previously we reported that the CF innervation territory was significantly limited to the proximal region of PC dendrites in $Ca_V2.1^{-/-}$ mice (Miyazaki et al., 2004). Altered CF innervation in $Ca_V2.1^{-/-}$ mice resembles that of myosin Va mutant animals, although its abnormalities are more severe presumably because of their greater limitation of Ca^{2+} influx into PCs. Finally, we recently demonstrated that the chronic

blockade of postsynaptic AMPA receptors in PCs caused the retraction of CF innervation of PC dendrites (Kakizawa et al., 2005). These results suggest that neuronal activity and Ca^{2+} signaling in postsynaptic PCs are required for expansion of CF innervation over PC dendrites.

Conclusion

Taken together, our results strongly suggest that myosin Va is required for the terminal arborization of CFs and for the establishment of CF synapses on their proper PC dendritic territories. The mechanism by which myosin Va promotes CF innervation is not entirely clear. However, myosin Va may facilitate organelle/vesicle transport within the CFs, thereby contributing to their growth directly. It is also possible that myosin Va is required for the localization of SER within PC dendritic spines, thereby contributing to proper Ca^{2+} signaling in postsynaptic PC dendrites and maintaining CF innervation through some retrograde signaling mechanism. Thus, the *d-n* mouse is an intriguing model that can be used to elucidate the role of myosin Va in the formation and maintenance of CF synapses in developing and mature cerebella.

Furthermore, our current studies provide a new insight into neuronal phenotypes of *dilute*-encoded myosin Va mutants. In this study, we utilized two alleles of the *dilute*, *dilute-neurological (d-n)* and *dilute-opisthotonus (dop)* and found that both mutants had a similar defect of CF terminal extension and its innervation of PC dendrites (Figs. 4 and 5). The severity of the neurological phenotype, however, was

different between the two mutants since the *d-n* mice are viable as a result of recovery of neurological defect by adulthood, while the *dop* rats are lethal within 3 weeks of age. Thus, it seems that the CF defect is not simply attributable to the neurological phenotypes of these mutants. We previously demonstrated that the absence of SER from the PC spins causes impairment of IP3-mediated Ca^{2+} signaling, resulting in a defect of LTD, a form of synaptic plasticity underlying cerebellar motor learning, at PF-PC synapses in *dop* rats and *dilute-lethal* mice (Miyata et al., 2000). Recently, we have found that PC spines lack the SER in juvenile *d-n* mice, while the SER is observed in adult *d-n* mice and the LTD is also recovered in adult *d-n* mice (unpublished observations). Thus, the defect of PC spine is potently responsible for the neurological phenotype of myosin Va mutants.

Acknowledgments

Authors thank Ms. Akiko Matsumiya for excellent technical assistance in preparation of cryostat and EM sections.

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Legends

Figure 1.

Cerebellar cortical formation in normal (*A*) and *dilute-neurological (d-n*) (*B*) mice. Molecular (ML) and granular (GL) layers are well formed and neurons in each cortical layer and Purkinje cells (PCs) appeared to have developed normally in *d-n* mice. 1.5μm-thick epoxy sections taken from postnatal day 22. Toluidine-blue staining. Scale bars: 50 μm

Figure 2.

CF branching patterns determined by anterograde labeling with biotinylated dextran amine (BDA). *A, C*: Normal mice at 3 months. *B, D-H*: *d-n* mice at 2-3 months. *A*: BDA-labeled CFs can be seen branching throughout the molecular layer (ML), with their tips approaching the pial surface (Pia) in normal mice. *B*: On the other hand, mutant CFs extended only about half way through ML. *C*: A representative image showing a typical CF arbor in normal mice. *D-H*: Representative CF arbor in *d-n* mice showing reduced (*D-H*), irregular branching (arrowheads in *D, E, G* and *H*). The bulbous varicosities along the CFs were irregular in size (arrows in *F* and *G*) and sometimes formed clusters (white arrows in *D* and *E*), and their distribution was irregular along tendrils (* in *H*). Beaded tendrils with bulbous terminals were also found surrounding the soma of PCs (arrows in *B*). Scale bars: *A, B*, 50 μm; *C-H*, 20 μm

Figure 3.

Vesicular glutamate transporter type 2 (VGluT2) immunohistochemical labeling of CFs. *A, a* and *C, c*: Normal mice at 2 months of age. *B, b* and *D, d*: *d-n* mice at 2 months of age. VGluT2 and calbindin D-28k (CD-28k) immunostaining appears green and red, respectively, in *A-D* and *a-d*. *a* and *b:* projection images constructed from ~20 serial optical sections through the 20 μm thickness of the tissue section. *c* and *d*: higher magnification images of VGluT2 and CD-28k double labeling VGluT2 labeled CF spots can be seen extending most of the way through the molecular layer in normal mice (*A* and *a*). In contrast, they appeared to be confined to the inner regions of the molecular layer in *d-n* mice (*B* and *b*). VGluT2 CF spots are distributed along shaft dendrites of PCs labeled with CD-28k in both normal and *d-n* mice (*C, c and D, d*). In *d-n* mice, however, the CF spots are located mainly on the proximal shaft and basal portions of PC dendrites (arrows in *D, d*). Furthermore, some CF spots were found on the somata of PCs (arrowhead in *d*). Scale bars: *A-D, a, b*, 50 μm; *c, d*, 10 μm

Figure 4.

CF innervation of PCs during cerebellar development in normal (*A-C*) and *d-n* (*D-F*) mice. Green: VGluT2 immunostaining. Red: CD-28k immunostaining. *A, D*: Postnatal day 10. VGluT2 immunofluorescence appears as coarse puncta (arrowheads) around PC somata and as densely packed tiny spots in the ML. *B, E*: Postnatal day 14. The CFs extended through half of the height of the proximal PC dendrites in normal mice (arrowheads in *B*), whereas they were restricted to the lower dendritic regions in

mutant mice. Arrowheads in *E* indicate perisomatic CFs. *C, F*: Postnatal day 17. The punctate CF spots extended through approximately two-thirds of the height of the PC proximal dendrites (arrowhead in *C*) in normal mice, but only on the more proximal extensions of these dendrites in *d-n* mice (arrowhead in *F*). Scale bars: 50 μm.

Figure 5.

CF innervation of PCs during development in normal (*A-C*) and *dilute-opisthotonus (dop)* (*D-F*) rats. *A, D*: Postnatal day 10. Strong spots of VGluT2 immunofluorescence (green) were found around the somata of PCs in normal and *dop* rats (arrowheads). *B, E*: Postnatal day 14. VGluT2 labeling was localized along primary and secondary branches of PC dendrites in normal rats (arrowheads in B), but was limited to the region around the somata of PCs and lower, primary dendritic extensions in *dop* rats (arrowheads in E). *C, F*: Postnatal day 21. Punctate VGluT2 positive CF staining was apparent along the primary to tertiary PC dendrites in normal rats (arrowheads in C), but only along the more proximal portions PC dendrites in *dop* rats (arrowheads in F). Scale bars: 50 μm

Figure 6

Electron microscopic structure of CF and PF synapses in normal (*A, C* and *a, c*) and *d-n* (*B, D* and *b, d*) mice. *A, B, C* and *D*: Conventional EM. *a, b, c* and *d*: Immuno EM for VGluT2 (*a* and *b*) and VGluT1 (*c* and *d*). *A* and *B* show putative CF synapses; Large terminals (CF) can be seen that contains numerous synaptic vesicles and a few

mitochondria that are located close to the thick PC dendrite (PCD), and which make synaptic contacts with several PC spines (S). Synaptic terminals with these morphological features were VGluT2 positive, confirming that they were indeed CF terminals (*a* and *b*). Typical PF-PC synapses are formed between one or two PC spines and a small PF synaptic bouton (*C* and *c*). In contrast, enlarged terminals that have synaptic contacts with several PC spines (S) are occasionally encountered away from the thick PC dendrite in the mutant ML (*D*). They are VGluT1 positive (*d*), again confirming that they were PF terminals. CF: CF synaptic terminal; PCD: PC dendrite; S: PC spine. PF: PF synaptic terminal. Scale bars: 1 μm.

Figure 7

Electrophysiological analyses of *d-n* mice.

A-D: Climbing fiber (CF)-mediated excitatory postsynaptic currents (EPSCs) in PCs of adult (2 months) (*A, B*) and young (P11-15) (*C, D*) *d-n* mice.

A, C: Examples of CF-EPSCs recorded from single PCs of normal (upper panels) and mutant (lower panels) mice. CFs were stimulated at 0.2 Hz in the granular layer. Two to three traces were superimposed at each threshold stimulus intensity. The number of discrete CF-EPSC steps represents the number of CFs that innervate the recorded PC. Note that the CF-EPSC amplitude of the adult mutant mouse was much smaller than that of the normal (*A*). The holding potential was -20 mV.

B, D: Summary histograms showing the number of discrete steps of CF-EPSCs from normal (open columns) and *d-n* (filled columns) mice. Note that the percentage of

multiple-innervated PCs in young mutant mice tended to be larger than that in young normal mice (*D*).

E–F: Short-term synaptic plasticity of CF and PF synapses in adult (2 months of age) *d-n* mice.

E: Paired-pulse depression of CF-EPSCs in normal (open circles) and mutant (filled circles) PCs. The amplitude of the second response is expressed as a percentage of the first response (mean ± SEM) and is plotted as a function of the inter-pulse intervals. Stimulus pairs were applied at 0.2 Hz. Insets are examples of CF-EPSCs with an inter-pulse interval of 50 ms.

F: Paired-pulse facilitation of PF-EPSCs in normal (open circles) and mutant (filled circles) mice. Stimulus pairs were applied at 0.5 Hz. Insets are examples of PF-EPSCs with an inter-pulse interval of 50 ms. Ten consecutive traces were averaged at a holding potential of -80 mV.

*, $p < 0.05$; **, $p < 0.01$; comparison between mutant and normal mice by Student's *t* test