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Myosin Va is developmentally regulated and expressed in the human cerebellum from birth to old age

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Abstract

Myosin Va functions as a processive, actin-based motor molecule highly enriched in the nervous system, which transports and/or tethers organelles, vesicles, and mRNA and protein translation machinery. Mutation of myosin Va leads to Griscelli disease that is associated with severe neurological deficits and a short life span. Despite playing a critical role in development, the expression of myosin Va in the central nervous system throughout the human life span has not been reported. To address this issue, the cerebellar expression of myosin Va from newborns to elderly humans was studied by immunohistochemistry using an affinity-purified anti-myosin Va antibody. Myosin Va was expressed at all ages from the 10th postnatal day to the 98th year of life, in molecular, Purkinje and granular cerebellar layers. Cerebellar myosin Va expression did not differ essentially in localization or intensity from childhood to old age, except during the postnatal developmental period. Structures resembling granules and climbing fibers in Purkinje cells were deeply stained. In dentate neurons, long processes were deeply stained by anti-myosin Va, as were punctate nuclear structures. During the first postnatal year, myosin Va was differentially expressed in the external granular layer (EGL). In the EGL, proliferating prospective granule cells were not stained by anti-myosin Va antibody. In contrast, premigratory granule cells in the EGL stained moderately. Granule cells exhibiting a migratory profile in the molecular layer were also moderately stained. In conclusion, neuronal myosin Va is developmentally regulated, and appears to be required for cerebellar function from early postnatal life to senescence.

Key words: Myosin Va; Human cerebellum; Postnatal development; Aging; Immunohistochemical expression

Introduction

The myosin superfamily is composed of more than 20 structurally distinct classes of myosins, which are widely expressed in eukaryotes (1). Vertebrate class V myosins are two-headed, actin-based processive motors. They convert the chemical energy of ATP hydrolysis to generate force and movement along actin filaments. Class V myosins are involved in intracellular organelle and mRNA transport and signaling (2,3). Three myosin V genes, encoding myosins Va, Vb and Vc that differ mostly in their C-terminal domains, have been described in mammals and display different expression patterns (4). Myosin Va (2,5,6) is expressed mainly in neurons, neuroendocrine cells and melanocytes, whereas myosins Vb and Vc are expressed in epithelial cells (7). In neurons and neuroendocrine cells, myosin Va has been associated with distribution, docking and release of secretory granules

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(7), with transport of the endoplasmic reticulum to dendritic spines of Purkinje cells (8), with axonal transport (9), with transport of mRNA and protein translation machinery in neurons (10), and with extension of growth cones (11), and has been localized to synaptic vesicles (12).

The movement of melanosomes, the best studied cargo of mammalian myosin Va, depends on the formation of an organelle-specific transport complex, composed of myosin Va, Rab-27a and melanophilin (for a review, see Ref. 7). Mutations in the human myosin Va gene (*MyoVa*) result in the Griscelli syndrome type I, a recessive disease characterized by partial albinism, hypotonia, mental retardation, epilepsy, and ataxia (13). A similar phenotype has also been observed in the *dilute MyoVa* mouse mutant (14). However, mutations in the other components of the human transport complex have not been associated with primary neurological defects (15,16). Thus, myosin Va clearly plays an important role in both normal and pathological CNS physiology. However, very little is known about the expression of myosin Va in the human nervous system from development to senescence.

The cerebellum is a useful model for the study of myosin Va expression because it is a relatively simple adult trilaminar structure that contains only a few neuronal cell types. During the first postnatal year there is a fourth layer, which is a secondary cerebellar proliferative matrix, the external germinative layer (EGL). The EGL generates new prospective granule cells that migrate on Bergman glia processes towards their final destination, the granule cell layer. Therefore, we have studied the expression of immunoreactive myosin Va in the postnatal developing, adult and aging human cerebellum.

Material and Methods

Tissue characterization and processing

Human nervous tissue was obtained from autopsies performed in the Departamento de Patologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, according to protocols approved by the local Ethics Committee. The brains did not show any evidence of disease, as demonstrated by systematic neuropathologic examination. Twenty-nine autopsy cases ranging from the first postnatal day to the 98th year of life were studied. For each case the age, *causa mortis*, and postmortem interval are given in Table 1. Cerebella were fixed in 10% (v/v) formalin for 1 to 4 weeks. Fixed tissues were dehydrated in ethanol, cleared in xylene and embedded in paraffin. Five-micrometer sections were cut, mounted on gelatin-chrome alum-coated microscope slides, and dried at 58˚C for 2 h.

Preparation of anti-myosin Va antibody

Polyclonal antibodies against the head domain of chicken myosin Va were raised in rabbits by inoculation with a recombinant protein corresponding to amino acid residues 5-572 of chicken myosin Va (6,17). These antibodies were affinity-purified using purified chicken brain myosin Va (18) immobilized on polyvinylidene difluoride membranes. The membranes were washed with 50 mM Tris-HCl buffer, pH 7.5, containing 0.9% (w/v) NaCl (TBS), and the antibody was then eluted with 100 mM triethylamine, pH 12.2. The pH of the eluate was adjusted to 8 with 1.0 M Tris-HCl buffer, pH 8.0. The eluate was dialyzed against TBS, and concentrated to 1 mL, at 4˚C. The final concentration of affinity-purified anti-myosin Va antibody was 0.5 mg/mL.

Immunohistochemistry

The immunohistochemical protocol used here has been described in detail by Martins et al. (19,20). Briefly, myosin Va antigenicity in dewaxed and hydrated human cerebellar tissue sections was microwave-retrieved in 10 mM sodium citrate buffer, pH 6, for 20 min at 900 W. Myosin Va detection in cerebellar sections was carried out by incubating sections overnight with an affinity-purified rabbit anti-myosin Va antibody diluted 1:50 (v/v) in 20 mM sodium phosphate buffer, pH 7.4, containing 0.45 M NaCl, 0.3% (w/v) Triton X-100, 5% (w/v) defatted dry milk, and 15% (v/v) normal donkey serum (blocking buffer). Endogenous biotin in tissue sections was blocked using a biotin blocking system (Vector, USA). Anti-myosin Va antibody was detected using a biotinylated swine anti-rabbit IgG (Dako, USA) diluted 1:100 (v/v) in blocking buffer, and the Elite ABC kit (Vector). Peroxidase was detected using a 2000-fold dilution of a 30 volumes hydrogen peroxide solution (Merck, Germany) and 0.66 mg/mL 3,3′-diaminebenzidine tetrahydrochloride as the chromogen, for 12 min. The reaction was stopped with water. All incubations were carried out at room temperature. Immunohistochemical controls were obtained by omitting the primary antibody and by preadsorbing the primary antibody with polyvinylidene difluoride membranes to which recombinant myosin Va was blotted. Tissue sections were examined and photographed using an Olympus light microscope, model BX-60 (Japan). All objectives (4X/0.16, 10X/0.40, 20X/0.70, 40X/0.85, 100X/1.35) were UPlanApo (Olympus).

Gel electrophoresis and immunoblotting

A fragment of human left temporal lobe from a 20-year-old female patient was resected to provide access for the resection of a cavernoma during a temporal craniotomy. We have used a surgical biopsy sample of cerebral cortex instead of a cerebellar one because both cortices express myosin Va, and because of difficulties in obtaining appropriate cerebellar tissue at autopsy. The procedures below were carried out according to protocols approved by the local Ethics Committee. The tissue was frozen and stored in liquid nitrogen until use. All subsequent procedures were performed on ice. The tissue was homogenized in 20 mM Tris-HCl buffer, pH

Table 1. Clinical data of 29 human necropsies whose cerebella were used to evaluate myosin Va expression.

Case	Gender	PMI (h)	Age	Diagnosis
1	Female	8.5	1 day	Hyaline membrane disease
2	Female	3.3	1 day	Sepsis
3	Male	7.3	3 days	Aortic stenosis/congestive heart failure
4	Female	10	10 days	Postoperative status/complex congenital heart disease
5	Male	3	21 days	Postoperative status/patent ductus arteriosus
6	Male	6	3.5 months	Bilateral acute pneumonia/congenital heart disease
7	Male	17.3	4 months	Aspiration bronchopneumonia/glossoptosis/ retrognathia
8	Female	5.5	4.5 months	Bronchopneumonia/diffuse alveolar damage
9	Female	6.75	7 months	Bronchopneumonia/lung abscess
10	Female	4.25	10 months	Acute enterocolitis/dehydration
11	Male	3.5	1 year 3 months	Acute anemia/lower intestinal bleeding
12	Male	6	1 year 5 months	Bilateral bronchopneumonia
13	Female	3	1 year 6 months	Diffuse alveolar damage/enterocolitis
14	Male	4	1 year 6 months	Postoperative status/ventricular septal defect
15	Female	4	1 year 8 months	Pulmonary hemorrhage/chronic Epstein-Barr virus infection
16	Male	5.5	10 years	Congestive heart failure
17	Female	8	21 years	Complex congenital heart disease/congestive heart failure
18	Male	4	26 years	Sepsis
19	Male	4	29 years	AIDS
20	Male	3	33 years	AIDS
21	Male	5.5	37 years	Acute respiratory distress syndrome/cirrhosis
22	Male	4.45	37 years	Bilateral aspiration pneumonia
23	Male	4	43 years	Chronic renal failure
24	Male	3.5	53 years	Acute pulmonary edema
25	Female	3.45	63 years	Pneumonia
26	Female	5	71 years	Acute respiratory insufficiency
27	Female	7	78 years	Cardiogenic shock/multiple peptic ulcerations
28	Male	7.3	90 years	Lower digestive bleeding
29	Male	8	98 years	Sepsis/bilateral pneumonia

PMI = postmortem interval.

7.5, containing 10 mM EDTA, 0.3 mM PMSF, 1 mM benzamidine and 3 μM aprotinin. The total homogenate (10 mg protein/mL) was diluted 4-fold with 0.26 M Tris-HCl buffer, pH 6.8, containing 7.3% (w/v) sodium dodecyl sulfate, 16.6% (w/v) sucrose, 3.5 M β-mercaptoethanol and 0.005% (w/v) bromophenol blue, and boiled for 4 min. The material was frozen and stored at -80˚C until the time for SDS-PAGE and Western blotting.

SDS-PAGE was carried out on 8% gradient minislab gels using a discontinuous system (21). Western blotting to nitrocellulose membranes (Hybond-C extra, Amersham, USA) was performed according to Towbin et al. (22). Molecular mass standards used were: 205-kDa rabbit muscle myosin, 116-kDa *E. coli* β-galactosidase, 97.4-kDa rabbit muscle phosphorylase b, 66-kDa bovine serum albumin, and 45-kDa egg albumin (Sigma, USA).

Protein determination

Protein was measured by the method of Lowry et al. (23) using bovine serum albumin as the standard.

Results

The detection of myosin Va was specific since the affinity-purified rabbit anti-myosin Va antibody used here labeled a single-intense band corresponding to 200 kDa in Western blots of a human cerebral cortex (Figure 1A). As immunohistochemical specificity controls, anti-myosin Va antibody was diluted 1:25 (v/v) in blocking buffer and incubated with purified myosin Va bound to polyvinylidene membranes for up to 250 min, at room temperature. Aliquots of non-adsorbed material were collected at 0, 50, and 250 min, and used to immunostain adult (data not shown) and developing (Figure 1) adjacent cerebellar sections. The 0-min aliquot (non-adsorbed antibody) stained all three cerebellar layers, i.e., molecular, Purkinje and granular layers (Figure 1B), but the 50- (Figure 1C) or 250-min (Figure 1D) aliquots showed a decrease or a complete loss of the immunostaining pattern, respectively.

Myosin Va was expressed in molecular, Purkinje and granular cell layers in human cerebella from the 10th

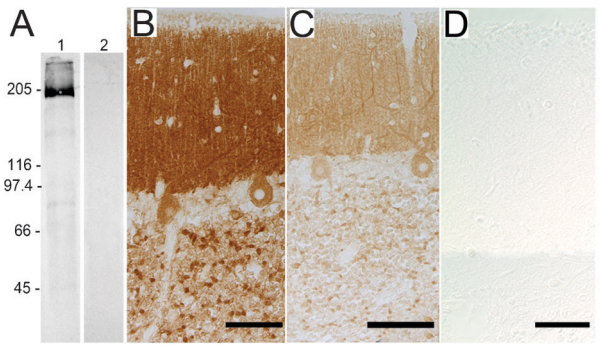

Figure 1. Specificity of anti-myosin Va antibody and immunohistochemical controls. *A*, Western blotting of a homogenate (20 µg per lane) of human cerebral cortex shows intense staining of a 200-kDa region, corresponding to myosin Va heavy chain (*lane 1*). The control lane (*lane 2*) was probed only with the secondary antibody. Immunohistochemical controls were obtained by pre-adsorbing anti-myosin Va antibody with myosin Va for 0 (*B*), 50 (*C*) and 250 (*D*) min before performing the assay on adjacent cerebellar sections. Nomarski optics. Magnification bars: 100 µm.

postnatal day to 98 years (Figure 2A, K, N). The molecular layer (MOL) exhibited a radial-like staining at all ages (Figure 2B, K, N) that was more pronounced during the first postnatal year (Figure 2N). Stellate cells in the outer MOL and interneurons in the inner MOL were also stained at all ages (Figure 2C, K, M). Anti-myosin Va antibody strongly decorated dendrites, and structures resembling cytoplasmic vesicles and the subcortical region in Purkinje cells [Figure 2A, D, K, L, M, N (inset)]. Structures resembling climbing fibers were stained along Purkinje cell dendrites of adult and elderly specimens (Figure 2M). A thin rim of granule cell cytoplasm was strongly stained at all ages (Figure 2E). In the dentate nucleus, myosin Va showed a strong punctate expression in the neuropile, perikarya (Figure 2G, H), nucleolus (Figure 2H, inset) and neuronal processes (Figure 2I, J), some of which were extended for hundreds of micrometers (Figure 2I). Omitting the anti-myosin Va antibody from the tissue section incubation led to no staining (Figure 2F).

The EGL, a germinative cerebellar matrix that persists throughout the first postnatal year (24), was lightly stained (Figure 2N). The prospective granule cells in the EGL proliferative zone (PZ) were not stained, but those in the premigratory zone (MZ) were well stained by the anti-myosin Va antibody (Figure 2O). Fibers exhibiting a radial-like pattern were also stained (Figure 2O, arrowheads). Granule cells exhibiting a migratory profile in the MOL expressed myosin Va in their subcortical region and processes (Figure 2P, arrow and inset).

Discussion

Using an affinity purified antibody against myosin Va to determine its expression in the human cerebellum, we showed that i) myosin Va was expressed in the cytoplasm of interneurons in the MOL, Purkinje, granule and dentate nucleus neurons from birth to advanced age; ii) myosin Va was expressed in structures resembling cytoplasmic granules and perinuclear and subcortical regions of Purkinje and dentate neurons; iii) no significant differences in this pattern of expression of cerebellar myosin Va described above were observed in this age series; iv) prospective granule cells in the MZ of the external granular layer of infants moderately expressed myosin Va, whereas those in the PZ were lightly or not detectably stained; v) the subcortical region of cells with a migratory profile in the molecular layer were moderately stained, as were fibers in the molecular layer that penetrated into the EGL.

Myosin Va is highly expressed in the human brain (19), as well as in neurons of most other eukaryotes such as rats (25), chickens (26) and squid (27). Taken together, these demonstrations of the ubiquitous localization of myosin Va to neurons and nervous tissue over a broad phylogenetic range underscore the importance of this molecular motor to fundamental neuronal processes. In the mouse, myosin Va is encoded by the *dilute* gene (14), where it has been shown to function in the transport and/or tethering of organelles, such as melanosomes within the dendritic processes of melanocytes (28,29), and synaptic (12) and secretory pancreatic acinar vesicles (30). Also, the transport and/or positioning of smooth endoplasmic reticulum within the dendritic spines of Purkinje cells (8), as well as the insertion of AMPA receptors in spines during synaptic plasticity (31), require myosin Va. The immunolocalization of myosin Va in cerebellar neurons and neuronal processes shown here can be related to activities in Purkinje cells and dentate neurons.

The human cerebellum continues its development, produces granule cells in the EGL, and prospective granule cells continue to migrate from the EGL to the granular layer during the first 12-18 postnatal months (32). These are among the reasons that make the postnatal cerebellum a classical choice for developmental studies. Particularly for human studies, the prenatal cerebellum is obtained after abortion, and its morphology is usually not well preserved. Using the postnatal human cerebellum, we have demonstrated a differential expression of myosin Va within the EGL, where prospective granule cells in the MZ expressed myosin Va, whereas those cells in the PZ showed faint or no staining by anti-myosin Va antibody. The prospective granule cells in the MZ are indeed in a migratory state, moving in the direction of the long axis of the cerebellar folia (33,34). Moreover, cells exhibiting a migratory profile in the MOL (34,35) clearly expressed myosin Va in the subcortical region, leading and trailing processes, and were apposed to radial glial fibers. Granule cell migration in the developing cerebellum occurs within a crowded terrain, and requires the ability

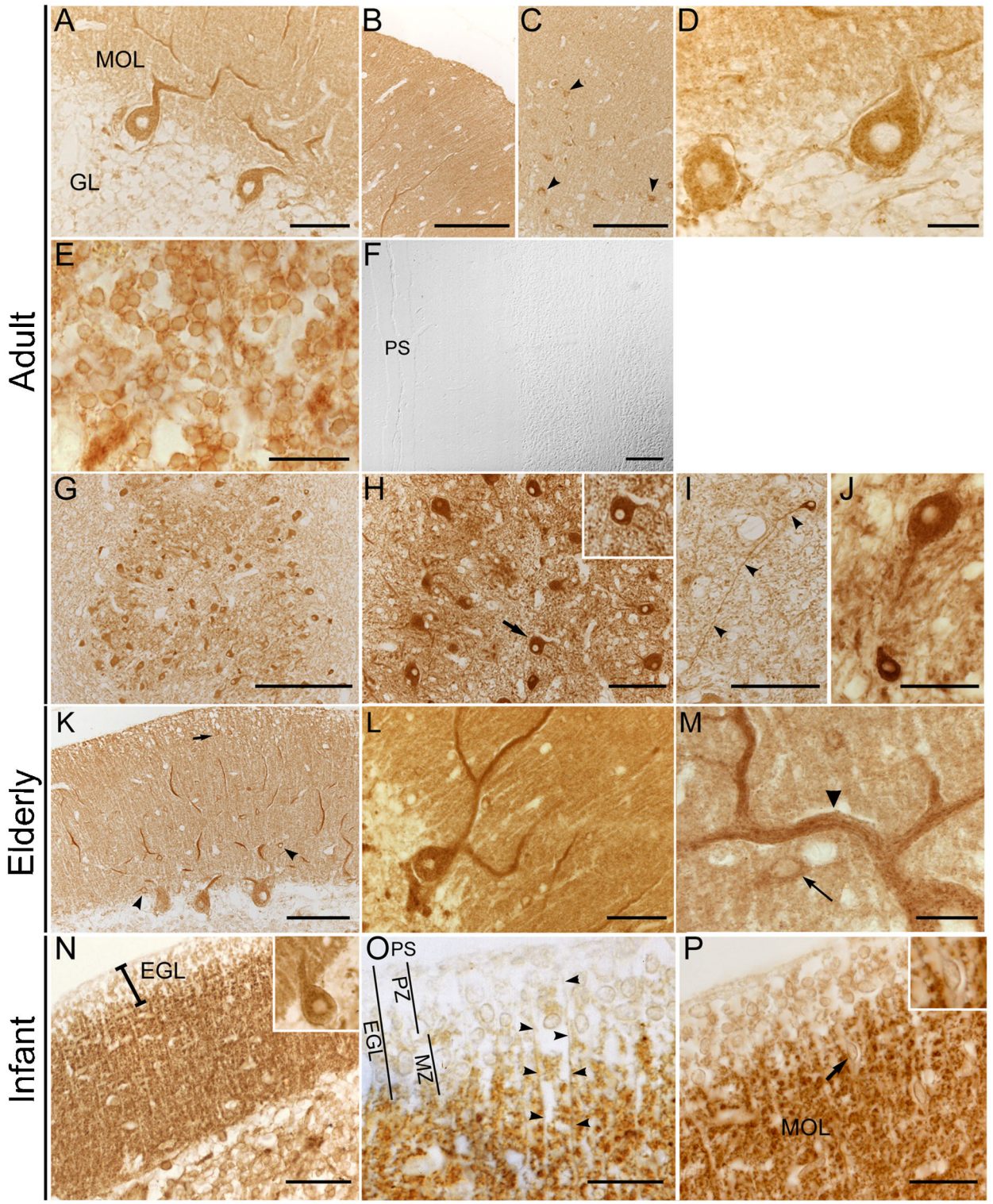

Figure 2. See legend on next page.

Figure 2. Expression of myosin Va in the human cerebellum from birth to old age. *Panels A-J*, adult; *panels K-M*, elderly; *panels N-P*, postnatal development. *A*, Myosin Va was expressed in all three adult cerebellar layers, the molecular layer (MOL), Purkinje and granule cell layers (GL). *B*, The MOL exhibited a radial-like pattern upon staining by anti-myosin Va antibody. *C*, Arrowheads indicate stained interneurons in the MOL. *D*, A coarse punctate expression of myosin Va is evident in the cytoplasm and primary dendrites of Purkinje cells. *E*, Myosin Va is seen in the thin rim of cytoplasm of granule cells. *F*, Control section obtained by omitting the anti-Myo Va antibody during staining. *G*, Low magnification shows strong staining of neuronal soma and fibers throughout a cerebellar dentate nucleus. *H*, Dentate neurons and neuropile are strongly stained; the arrow indicates a neuron with a stained nucleolus, shown in the inset. *I*, A long dentate neuronal process is strongly stained (arrowheads). *J*, High magnification shows strong staining in the cytoplasm and processes of a dentate neuron. *K*, The staining pattern of the cerebellar cortex of an aged person is similar to that of an adult; arrow indicates outer MOL-situated interneurons, probably stellate cells; arrowheads indicate unidentified interneurons in the inner MOL. *L*, A higher magnification of the cerebellar cortex of an elderly person; staining intensity of adult and elderly cerebellum is about the same; *M*, High magnification of the Purkinje cell dendrites of a 98-year-old person shows strong labeling of discrete points along the dendrite, and also apparent overlaying structures that resemble climbing fibers (arrowhead); the arrow indicates an interneuron. *N*, anti-myosin Va weakly stains cells in the external granular layer (EGL), but strongly stains the MOL of a 10-day-old infant. The inset shows granular staining of the subcortical, perinuclear and dendritic regions of a Purkinje cell. *O*, High magnification of a 10-day-old infant cerebellum shows more intense staining of prospective granule cells in the premigratory zone (MZ) of EGL than those in the proliferative zone (PZ). Also note the staining of radial-like fibers (arrowheads) that penetrate the EGL up to the pial surface (PS). *P*, Cells in the MOL exhibiting a migratory profile (arrow) moderately expressed myosin Va (see inset). Magnification bars: A, H, L, N 50 μm; B, C, F, I, J, K, 100 μm; D, E, M, O, P, 20 μm; G, 200 μm.

of exerting forces to perform the calcium-dependent (36), saltatory (34) movement towards their final destination. It is noteworthy that the structure and enzymatic activity of myosin Va also depends on calcium (for a review, see Ref. 37). Moreover, the movement of the leading process is similar to that of a growth cone, for which a role for myosin Va has been proposed (11). Therefore, the results presented here suggest that myosin Va expression is developmentally regulated, and can play a role in cerebellar granule cell migration.

Myosin Va was differentially expressed during the proliferation, differentiation and migration of the granule cell, and can thus be considered to be developmentally regulated during this period. In addition, it was expressed in most neuron types of the human cerebellum, both within their soma and processes. The striking similarity of the myosin Va localization pattern shown here for children, adults and elderly individuals, together with the association of mutations in the *dilute* gene with severe neurological disturbances and lethality in both mice and humans (13,38), suggest that myosin Va expression is required for cerebellar function from early postnatal life to senescence.

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