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A Novel Marker for Purkinje Cells, Ribosomal Protein MPS1/S27: Expression of MPS1 in Human Cerebellum

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Abstract. *Background: The ribosomal protein metallopanstimulin-1 (MPS1/S27) serves critical survival purposes in cell division, in normal and cancerous cells; for this reason, selective pressures of evolution have conserved the DNA sequences encoding MPS1/S27 in Archaea and Eukariotic cells. The expression of MPS1/S27 protein in human adult cerebellum has not been established. Material and Methods: The presence of MPS1/S27, was screened in paraffin-embedded human adult brain specimens processed for tissue inmunohistochemistry. Affinity-purified specific antibodies were directed against the N-terminus of MPS1. Results: The antibodies to MPS1 detected Purkinje cells (PC) and their dendrites. In PC, MPS1 antigen-positive staining was found in: the nucleolus, which was strongly stained; ribosomes attached to the external nuclear membrane; cytoplasm of PC, with strong staining in a punctuate fashion; the soma-attached large dendrite trunks of PC, which were MPS1 antigen-positive; and the granular cell layer, where cellular staining in a few cells that appeared to resemble smaller PC was observed. Conclusion: Since MPS1 is involved in cell division, DNA repair, and ribosomal biogenesis, it may be a useful antigen for studying processes such as protein synthesis, oncogenesis, regeneration, aging, and perhaps diseases of the human cerebellum.*

In the human cerebellum, neuroblasts migrate radially and outwards from the germinal matrix layer present in the walls of the fourth ventricle and finally populate in the deep cerebellar nuclei and the Purkinje cell (PC) layer during the embryonic period (1, 2).

Numerous markers for PCs have been found, such as, microtubule-associated protein 2 MAP2 (3), calbindin-D28K (4, 5), inositol 1,4,5, triphosphate type 1 receptor (6, 7), spot 35 protein (8), and KIAA0864 protein (9). Antibodies against these proteins have been used for detection by immunochemistry in analyzing formalin-fixed and paraffin-embedded brain samples from normal and diseased cerebellum.

The high complexity and molecular functions of the cerebellum have baffled scientists for more than 200 years (10-12). Numerous attempts have been made to understand the evolutionary steps of the cerebellum and its behavior as a complex biological machine (11, 12). The techniques used range from simple morphological techniques, to computer-generated models, and sophisticated immunological techniques (11, 12).

When the functions of a protein, as in the case of MPS1/S27, serve a critical and unique survival purpose, the selective pressures of evolutionary laws of nature conserve the DNA sequences encoding such proteins (13-16).

In recent times, the functions of MPS1 have been studied in various normal cells, virus-infected cells, cancerous cells, aging cells, normal and pathological tissues (13-15, 17-22). MPS1 is a ribosomal protein with extra-ribosomal functions such as DNA repair and transcription (16, 19). Berthon *et al*. obtained impressive results with *Archaea* on the functions of MPS1 and other ribosomal proteins by genomic context analysis that indicated a previously unrecognized regulatory network coupling DNA replication, DNA repair, DNA transcription, translation and biogenesis of ribosomes that exists in both *Archaea* and *Eukarya* (16).

We herein describe MPS1 as a new marker of PCs its use in immunohistochemistry in adult human cerebellum, employing specific affinity-purified polyclonal antibodies directed against the *N*-terminuus synthetic peptide corresponding to amino acids 2 to 17 of MPS1/S27 ribosomal protein with extra-ribosomal functions. This marker may be useful in further understanding the structure and function of MPS1/S27 in the cerebellum and perhaps in the pathology encountered in this complex biological organ.

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Materials and Methods

Materials. Brain samples for the immunohistochemical study were obtained at autopsy from 41 men ranging from 62 to 73 years of age. All samples were obtained at autopsy within 12 h after death and were fixed in 10% buffered formalin. In the cerebellum,

transverse, tangential, and frontal sections were obtained from the cerebellar hemispheres and vermis.

Analysis of the patients included both computerized clinical and histopathological data. The patients had no evidence of clinical or pathological cerebellar disease. Hematoxylin and eosin-stained sections were examined in all specimens. The tissue type and diagnosis corresponding to the tissues tested for immunohistochemistry of MPS1 were obtained from a brain bank (Chang Pathological Labs., St. Louis, MO, USA). This tissues were used in this study in accordance with the Helsinki Accord and was approved by the Ethics Committee of each Institution (no. CPL-C-1998b).

Preparation of antibodies to MPS1 using a synthetic peptide containing the N-terminus region of the MPS1 protein and affinity purification of antibodies. Peptide antisera against the amino terminus of the MPS1 protein were prepared as follows (19). One sequence, PLAKDLLHPSPEEEKR, corresponding to MPS1 amino acid residues 2 to 17, was designated as the *N*-terminal region of the MPS1 protein, located between the *N*-terminus and the zinc-finger domain (19). The *N*-terminal peptide was selected so that it would not contain any portion of the zinc-finger domain of the MPS1 protein, since the zinc-finger domain is a highly conserved structure and antisera against it may cross-react with other zinc-finger proteins (17, 20). The production of anti-peptide antibodies is described elsewhere (19). IgG antibodies were purified by affinity chromatography (19). Specificity of the IgG antibodies was determined by peptide antibody neutralization studies using western blot analysis of human recombinant MPS1 protein (19) and neutralization of antibodies directed against the synthetic peptide 2-17 of MPS1 (19, 22).

Light microscopy immunohistochemistry. Immunohistochemical assays to detect the localization of MPS1 protein were performed on routinely processed formalin-fixed paraffin-embedded cerebellum tissue specimens. The method used to detect the localization of the MPS1 antigen using anti-MPS1-*N*-terminus peptide was the biotin-streptavidin amplified system (StrAviGen™ ; BioBenex, San Ramon, CA, USA). In this system, the second antibody link is biotinylated and streptavidin is conjugated to alkaline phosphatase, which generates a chromogenic reaction with the appropriate reagents (22).

Formalin-fixed tissues were embedded in paraffin, cut, and placed on Vectabond-treated microscope slides and baked for 1 h at 56°C. The tissues were then de-paraffinized with xylene, rinsed with 100% ethanol, and rehydrated in a graded alcohol series. The tissues were then microwaved according to standard procedures (energy unit per mm^2: 2.506×10^{-12} Hz).

To eliminate non-specific binding, the tissues were treated with avidin/biotin blocking solutions containing 10% normal goat serum (Vector Labs, Inc; purchased from Sigma-Aldrich, St. Louis, MO, USA). The rabbit anti-MPS1-*N*-terminus peptide IgG was diluted (1:500) in 1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS), and incubated with the tissue slides. Slides were covered with a coverslip and incubated at room temperature for 60 min in a humidified chamber.

Each tissue section was analyzed for the presence of MPS1-*N*-terminus antigen and a contiguous tissue section was analyzed in parallel, as a control, in which the primary antibody was omitted from the preparation. An additional control for each contiguous section from the same tissue was prepared as follows. The rabbit antibodies to MPS1-*N*-terminus were neutralized by incubation with a synthetic *N*-terminus peptide corresponding to region 2-17 of MPS1 protein, at 100-fold higher concentration than that of the antibodies to MPS1-*N*-terminus, and incubated in a test tube for 30 min at room temperature prior to addition to the tissue slides. The tissues were then incubated in PBS containing 2% human serum, 1% BSA and biotinylated goat anti-rabbit antibodies for 20 min at room temperature. Streptavidin alkaline phosphatase (BioGenex, San Ramon, CA, USA) in 1% BSA/PBS was added for 20 min. The tissues were then labeled using stable Fast Red/Stable Naphthol Phosphate as a chromogen (Research Genetics, Inc., Huntsville, AL, USA). Slides were rinsed with deionized water and were then counterstained in hematoxylin (22).

Immunohistochemistry of glial fibrillary acid protein (GFP). Detection of GFP [Homo sapiens, NCBI, sequence: NP-002046 (23)] was performed with goat IgG affinity-purified polyclonal antibodies to GFP. The antibodies were purchased from Sigma-Aldrich. They were used to detect GFP in the cerebellum, directly or as a counterstain followed immunohistochemistry with MPS1-*N*-terminus antibodies to detect MPS1 antigen.

Results

In all 41 cases, positive immunostaining appeared in PCs in three to five layers. Figures 1 to 3 illustrate some of these cases. Below the PC layer, in the granular cell layer (GCL), a few cells were positive for the MPS1 antigen (Figure 2A and B). Positive immunoreactivity with antibodies to *N*-terminus of MPS1 was observed in the soma of PCs and the large dendritic trunks emanating from these cells (Figure 1 and 3). The short and long processes originating from these large dendrites extended into the molecular layer (ML) and exhibited various degrees of positivity for the MPS1 antigen (Figures 1 and 2). MPS1-positive dendrites of PCs were prominent as a single layer in some of the specimens (Figure 1A-D). MPS1 immunoreactive dendrites elongated into the ML but they were much less stained (Figure 1D). In all specimens studied, dendrites of the PCs penetrated the ML and reached the pial surface of the cerebellum, but were faintly stained for MPS1 (Figure 3B). Thus, MPS1 immunoreactivity was confined to PC-type neurons.

In the white matter of the cerebellum, some nerve fibers were weakly stained for MPS1 antigen. The deep cerebellar cell nuclei identified as the embolous, dentate, roof, and spheric, were positive for the MPS1 antigen (data not shown).

No positive immunoreactivity was found when omitting primary antibodies or antibodies pre-reacted with MPS1-*N*-terminus peptide (data not shown).

GFP was detected by anti-GFP antibodies to compare the patterns of stain of MPS1 and GFP (Figure 3). GFP is the major protein of glial intermediate filaments in differentiated fibrous and protoplasmatic astrocytes of the central nervous system (23). A function of glial filaments is as a component of the cytoskeleton in defining and maintaining the shape of the astrocytes. Neither PCs nor their dendrites were labeled for GFP antigen (Figure 3A). In the ML of the cerebellum in all

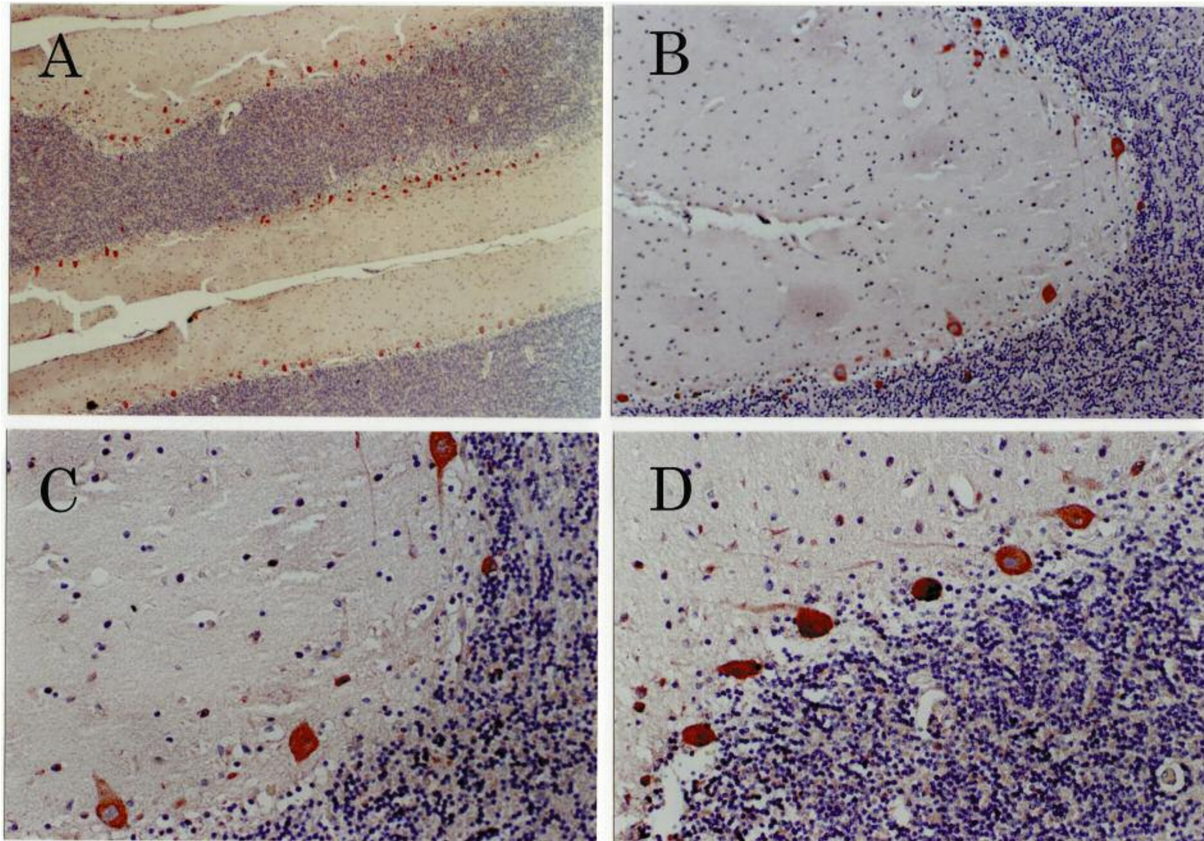

Figure 1. *Comparison in different sections showing immunohistochemical distribution in the cerebellum of metallopanstimulin-1 (MPS1) protein in a 71-year-old man. A: MSP1-immunopositive Purkinje cells are clearly visible along the Purkinje cell layer. Detailed observation of the Purkinje cells showed intense stain of the Purkinje cells soma. The granular cell layer is not stained. Original magnification, ×40. B: Purkinje cell immunoreactivity in the boundary between the Purkinje cell layer and the molecular layer. Purkinje cell soma and dendritic trunks were positive for MPS1 antigen. Original magnification, ×100. C: Detailed view of Purkinje cells showing intense staining of soma in a punctuated fashion in the cytoplasm, which extends to their large dendrites which penetrate the molecular layer. Original magnification, ×200. D: In the dendrites, the material strongly stained punctately for MPS1 antigen which appears to be predominantly bound to the dendrite's membrane. Original magnification, ×400.*

patient samples studied, we found GFP immunostaining in axons and dendrites of astrocytes (Figure 3A). In double staining, first with antibodies to MPS1 and then with antibodies to GFP (Figure 3B and C), the MPS1 antigen was visualized in numerous PC soma as positive staining, and in the large dendritic branches of PCs, and in the smaller branches which penetrated the ML (Figure 3B and C). The long dendrites in the ML, ordered in a parallel pattern, terminated in the pial surface of the cerebellum (Figure 3B and C).

Discussion

Whitney *et al*. compared calbindin-D28K staining with Nissl's stain in the cerebellum. With calbindin D28K staining, the PC nucleus and nucleolus were essentially impossible to visualize, as they were most often obscured by the dense and diffused inmunoreaction products filling the soma of PCs (5). In contrast, this was not the case when using anti-MPS1-*N*-terminus peptide detection for visualization of PCs (Figures 1-3).

The PC soma in our samples measured an average of 35 μm ×26 μm, height-width). Thus, with the ×40 objective lens we were able to see the soma of all PCs in the field of view.

It is worth noting that Nissl stain strongly labels proteins (5), including ribosomal proteins, and thus some overlapping of staining must occur in PCs when one uses the MPS1 antibodies to detect MPS1 antigen in combination with Nissl stain. However, the main component in Nissl stain, thionin, labels any protein containing a cysteine group, while antibodies to MPS1-N-terminus only label the very specific N-terminal region of MPS1/S27, in the following discrete intracellular areas of PCs: Prominently, the nucleolus and the ribosomes

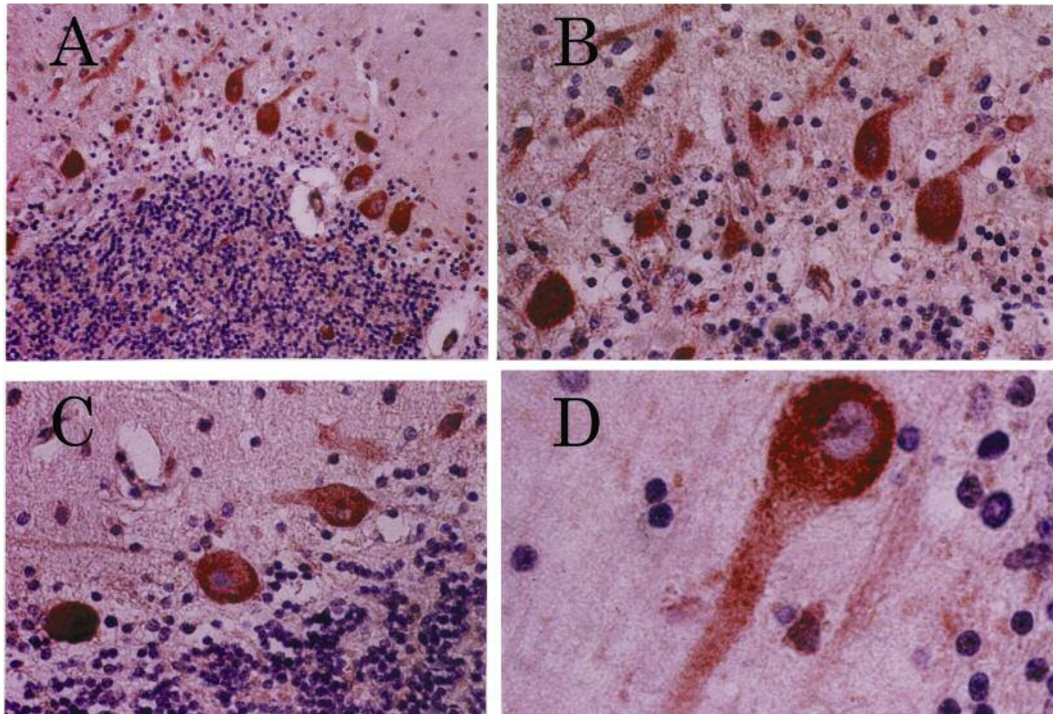

Figure 2. *Detection of metallopanstimulin-1 (MPS1) in various sections corresponding to specimens obtained from a 67-year-old man. A: The large dendrites originating from Purkinje cells and penetrating the molecular layer can be distinctly traced from the MPS1-positive staining. Punctate MPS1-positive staining can also be seen in the dendrites. Original magnification, ×100. B: MPS1 antigen-immunopositive Purkinje cells are clearly visible along the Purkinje cell layer. Detailed observation of the Purkinje cells showed intense staining of their soma and curved dendrites penetrating the molecular layer. The punctuately stained material in all dendrites appears to be bound to the dendritic membranes. Original magnification, ×200. C: The nucleoli of Purkinje cells, the external nuclear membrane and the dendrites stained for MPS1 antigen. In addition, the cytoplasmic cell membrane exhibited intense punctated positive staining for MPS1 antigen. Original magnification, ×400. D: Immunohistochemical distribution of MPS1 antigen in Purkinje cells. High magnification using an oil immersion objective. Microscopic examination at ×1000 showed the following: Strong punctated MPS1-positive staining of the nucleolus, surrounded by unstained nucleolar areas; MPS1 staining of the external nuclear membrane was also punctate; Purkinje cell cytoplasm strongly stained for MPS1 in larger granules, resembling ribosomal protein staining containing MPS1 antigen; most interestingly, the strong punctate staining for MPS1 extended to the large dendritic trunks of Purkinje cells, but areas of punctately stained material in the dendrites are much more distinctly separated from each other than in similar areas in the Purkinje cell cytoplasm (soma). The nucleoli of granular cells are seen to be negative for MPS1 antigen staining. Original magnification, ×1000.*

attached to the external nuclear membrane; conspicuously, the large trunks of a few dendrites attached to the soma of PCs. The MPS1 antigen in many instances can be observed attached to the dendrites. A large number of dendrites faintly stained in a graded fashion, as a function of their distance from the soma of these cells (Figure 3B and C). Some PC axons in some cells faintly stained for MPS1 antigen. The staining for MPS1 is specific, as omission of antibodies to MPS1, or blocking MPS1 antibodies with the synthetic MPS1-*N*-terminus peptide eliminated the visualization of PCs. Antibodies against MPS1 C-terminus are unable to detect MPS1 antigen in PCs (unpublished data). Thus, the detection of MPS1 antigen in such a specific fashion could be useful for studying the ribosomal and extra-ribosomal function of this protein in PCs of the cerebellum of humans and perhaps of other species.

The nucleolus of PCs was easily identified in virtually every PC (Figure 2C and D), and the granular nature of this staining (Figure 2D) is likely to reflect an active nucleolus (24), showing ribosome subunits either in the process of assembly or having already formed ribosomes to be transported to the cytoplasm of PCs (Figure 2D). Thus, the results appear to indicate that the nucleoli of PCs were very active in generating MPS1 ribosomal protein antigen.

The detection of MPS1 antigen may be a valuable tool for examining the histology of the cerebellum at different stages of development and in diseases of the cerebellum. We suggest that the ribosomal proteins, and in particular MPS1 in PCs, are continuously synthesized from the formation of the cerebellum in the embryo to the adult cerebellum. The present study suggests that PCs may have to maintain continuous protein

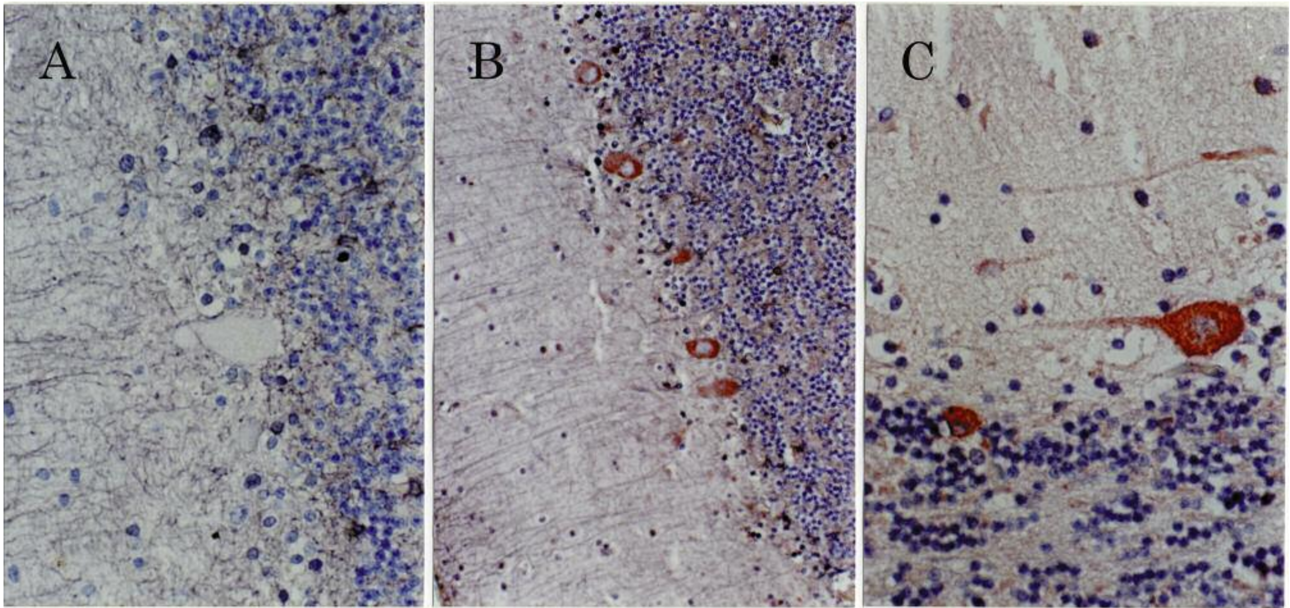

Figure 3. *Immunohistochemical distribution of metallopanstimulin-1 (MPS1) antigen and glial fibrillary acid protein (GFP) antigen in the cerebellum of a 68-year-old man. A: The GFP antibody revealed numerous antigen-positive cells in all the cerebellar layers. A diffuse network of GFP-positive dendrites and axons in the granular cell layer, the Purkinje cell layer and in particular the molecular layer can be seen. The GFP-positive axons and dendrites in the molecular layer appear to have a random staining pattern. Sagittal section; detection of GFP antigen alone. Original magnification, ×200. B: Contiguous section, stained for MPS1 antigen first and then stained for GFP antigen, illustrates the trajectories of dendrites originitating from Purkinje cells, which are positive for MPS1 antigen. The MPS1 antigen is clearly visible in the Purkinje cell soma and its ascending dendrites, which are present in a parallel fashion. Purkinje cell dendrites exhibited faint MPS1 antigen positivity, with their parallel dendrites reaching the pial surface of the cerebellum through the molecular layer. Original magnification, ×200. C: MPS1 antigen detection plus GFP antigen staining clearly showed that the MPS1-positive dendrites did belong to the Purkinje cells, as shown by the intense positive staining of Purkinje cells. The MPS1 antigen detection decreases and is much fainter in dendrites as they penetrate the molecular layer towards the pial cerebellar surface. Higher magnification of the section shown in B. Original magnification, ×400.*

synthesis, perhaps for viability or plasticity of PC dendrites and its axons. In addition, after secondary modification by phosphorylation (20) or other modifications, the MPS1 protein, or specific interactions with survival proteins such as p53 (25), processed MPS1 and MPS1-like proteins (25), may be transported to different compartments of PCs, including the initial portion of the large dendritic trunks of PCs, and from there to the smaller dendritic branches.

After detailed examination of the comprehensive reviews by Sotelo *et al*. (11) and D'Angelo *et al*. (12), essentially there is no significant knowledge of ribosomal or extra-ribosomal MPS1 protein functions in PCs, in normal or pathologically altered cerebellum. The involvement of MPS1 in PC metabolism, nervous cell division, repair of damage DNA, DNA mutation of *MPS1* gene, damage to PC genome or mRNA of *MPS1*, by mutagenic chemical agents, radiation, and commonly used environmental herbicides and pesticides are being study in our laboratory at present.

The finding of MPS1-positive staining in a few sparse cells in the GCL, and in particular at a certain distance below the PC layer in the adult cerebellum appears to be of interest. It is not unconceivable that the GCL in the adult human brain may contain dormant Purkinje stem cells that slowly replace aging PCs, or the stained cells could be Lugarno's cells (11, 12) localized below the PC layer, or young PCs migrating outwards to the PC layer, or they may be involved in changes in the plasticity of PCs in order to adapt to specific neural inputs. Of course, the positive and specific MPS1 staining of cells in the GCL with the appearance of PCs may be an artifact of the preparations, although the author believes that this is highly unlikely.

Plasticity of the cerebellum, requires a precise pattern of formation (11, 12), and it is conceivable that plasticity of PCs and other cerebellar cells cannot be achieved without *de novo* protein synthesis and ribosomal and extra-ribosomal functions of MPS1 and other ribosomal proteins (13,16). This is clearly indicated by studies on the role of MPS1 in cell division in embryonic cells, normal adult cells, aging cells, and cells in carcinogenesis (13, 15, 17). Determining the metabolic activity of MPS1 (13, 15, 17), may be useful in

studying plasticity in the cerebellum at different stages of development, including of adults.

Eccles *et al*. proposed that the most significant property of the cerebellar circuitry would be its plasticity (26, 27), which can participate in motor learning, the acquisition of skills and awareness of balance (11, 12). They proposed that the double innervations of PCs must play a key role in motor skill learning. It is conceivable that ribosomal proteins, including MPS1, may play a role in plasticity of PCs in the cerebellum, whose key role is learning motor skills and relearning them after disease (11, 12). It is conceivable that without *de novo* protein synthesis, plasticity may effectively be lacking.

Perhaps, when adults start recovering from cerebellar diseases, such as stroke, and learn new precise proprioceptive skills or develop specific abilities, new PCs are generated from stem cells, not yet recognized in the cerebellum. It appears unlikely that plasticity in order to acquire new cerebellar skills after cerebellar disease can occur without new ribosomal protein synthesis. Precise proprioceptive patterns of balance may occur by ribosomal protein synthesis and MPS1 may be involved in such processes. Extensive studies are needed to confirm or refute this hypothesis.

In summary, the development of new techniques, in most cases, is the beginning of new discoveries. The study of MPS1 and other ribosomal proteins (16) in PCs may shed some light on the functions of these proteins and their role in plasticity.

Since MPS1 is involved in cell division and DNA repair, it may be useful to study actions of MPS1 in the cerebellum, in processes such as oncogenesis due to environmental chemicals, aging, regeneration, and in maintaining the plasticity of cerebellar cells.

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