Initial Phase of Dendrite Growth: Evidence for the Involvement of High Molecular Weight Microtubule-associated Proteins (HMWP) before the Appearance of Tubulin

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ABSTRACT It has recently been shown that high molecular weight microtubule-associated proteins (HMWP) in the brain are present in dendrites and are absent from axons (Matus et al., 1981, *Proc. Natl. Acad. Sci. U. S. A.* 78:3010–3014). In this study we followed the appearance of both HMWP and tubulin in the neonatal rat cerebellum by immunoperoxidase staining, concentrating particularly on comparing Purkinje cell dendrites with adjacent granule cell axons. In the axons both immunohistochemically demonstrable tubulin and structurally distinct microtubules are present at all stages of development. By contrast the Purkinje cell dendrites contain neither tubulin nor microtubules at early stages of their growth. However, immunoperoxidase staining showed that these developing dendrites are rich in HMWP which are particularly concentrated in the dendritic distal regions. HMWP are also present as patches beneath the surface membrane of the cell body before the emergence of dendrites. Based on this data and the well-documented ability of HMWP to promote microtubule assembly, we propose the hypothesis that during the initial phase of Purkinje neuron differentiation HMWP form part of a specialized cytoskeletal structure which acts as a specifier for the development of dendrites as opposed to axons.

During brain development the postmitotic neurons make two kinds of processes, axons and dendrites, which are strikingly different in morphology and physiology. This structural asymmetry is one of the nerve cell's most distinctive properties and is also a key factor in determining the polarity of neural transmission (17). The cellular mechanisms by which this differentiation of axon and dendrite is achieved are presently unknown. However there is evidence from nerve cells grown in culture which suggests that cytoskeletal structures are involved in the formation of neuronal processes. Microtubules are particularly implicated, because when drugs which cause microtubule depolymerization are applied to cultured neurons, their processes rapidly retract (6, 18, 21, 24).

Recent results have indicated that one class of microtubule-associated proteins, the high molecular weight proteins (HMWP), are associated only with microtubules of neuronal dendrites and are absent from microtubules in axons (4, 11). This led us to ask whether HMWP might play a role in dendrite formation. In support of this idea we show here, by immunohistochemical staining, that in developing Purkinje cells HMWP are intimately associated with the growing tips of dendrites at a time before either tubulin or organized microtubules are present.

MATERIALS AND METHODS

We have used two antisera, one of which reacts only with HMWP (anti-HMWP) while the other reacts selectively with tubulin (antitubulin) as shown by immunoblotting (11). Immunoperoxidase staining was performed as previously described (12) except that the peroxidase reaction was developed with 4-chloro-1-naphthol. This reagent was prepared as a stock solution at 3 mg/ml in methanol which was diluted 1:5 with 50 mM phosphate buffer, pH 6.5, containing 150 mM sodium chloride (PBS) and made 0.01% (vol/vol) in hydrogen peroxide immediately before use. Brains from rats of various ages were fixed by transcardiac perfusion with 4% paraformaldehyde and 0.2% glutaraldehyde (wt/vol) in 50 mM phosphate buffer, pH 7.2, followed by immersion in the same fixative overnight. After transfer to PBS, the tissue blocks were sectioned on a Vibratome at 2°C at a nominal thickness of 40 μm. After immunoperoxidase staining, the sections were mounted in glycerol-PBS (9:1) for light microscopy or postfixed with 1% osmium tetroxide and processed for electron microscopy. Tissue which had not been stained with immunoperoxidase was block-stained with 1% aqueous uranyl acetate before embedding, and the ultrathin sections were stained on the grid with 0.4% lead citrate in 0.1 N sodium hydroxide.

RESULTS

Immunoperoxidase staining with specific antisera showed that tubulin and HMWP have strikingly different patterns of appearance in the developing neonatal rat cerebellar cortex. Both of these proteins had reached their previously described adult distributions by postnatal day 20 (Fig. 1 and reference 11). At

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this time tubulin was present in microtubules of both glial and neuronal cells, and of both axons and dendrites (Fig. 1*B*), whereas HMWP was associated only with microtubules in dendrites (Fig. 1*A*).

At earlier times the distribution of both tubulin and HMWP differed markedly from this adult pattern in a fashion which was most clearly demonstrated in Purkinje cell dendrites (Fig. 2). In sections taken from 10-d-old cerebellum, the growing dendrites of the Purkinje cells, and particularly their distal portions, are rich in HMWP (Fig. 2*A*). Furthermore, as expected from the distribution of HMWP in adult brain, there is no anti-HMWP staining in the parallel fiber axons which surround the Purkinje cell dendrites (Fig. 2*A*). Staining an adjacent section from the same tissue block with antitubulin produces an inversion of this pattern; the parallel fiber axons are strongly stained and they effectively outline the Purkinje cell dendrites which are not stained by antitubulin (Fig. 2*B*).

In still younger tissue (3 d postnatal), strong anti-HMWP

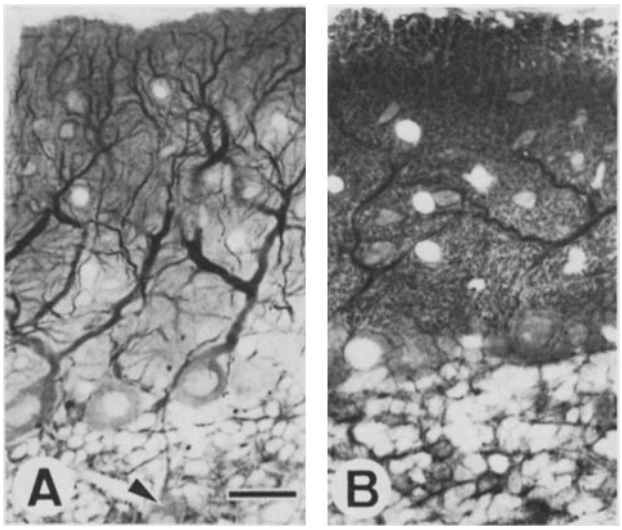

FIGURE 1 Two sections taken from the cerebellum of a 21-d-old rat stained with anti-HMWP (*A*) and antitubulin (*B*). Anti-HMWP staining appears in Purkinje cell bodies and dendrites. Also visible is the stained cell body and an apical dendrite of a Golgi neuron (arrowhead) as well as stained dendrites of granule and other neurons in the granular layer. Antitubulin (*B*) stains cell bodies and dendrites of Purkinje cells and other types of neurons and in addition axons, of which the most prominent are the parallel fibers which are cut in cross section and appear as stained dots filling the space between the Purkinje cell dendrites. Bar, 30 μm. × 300.

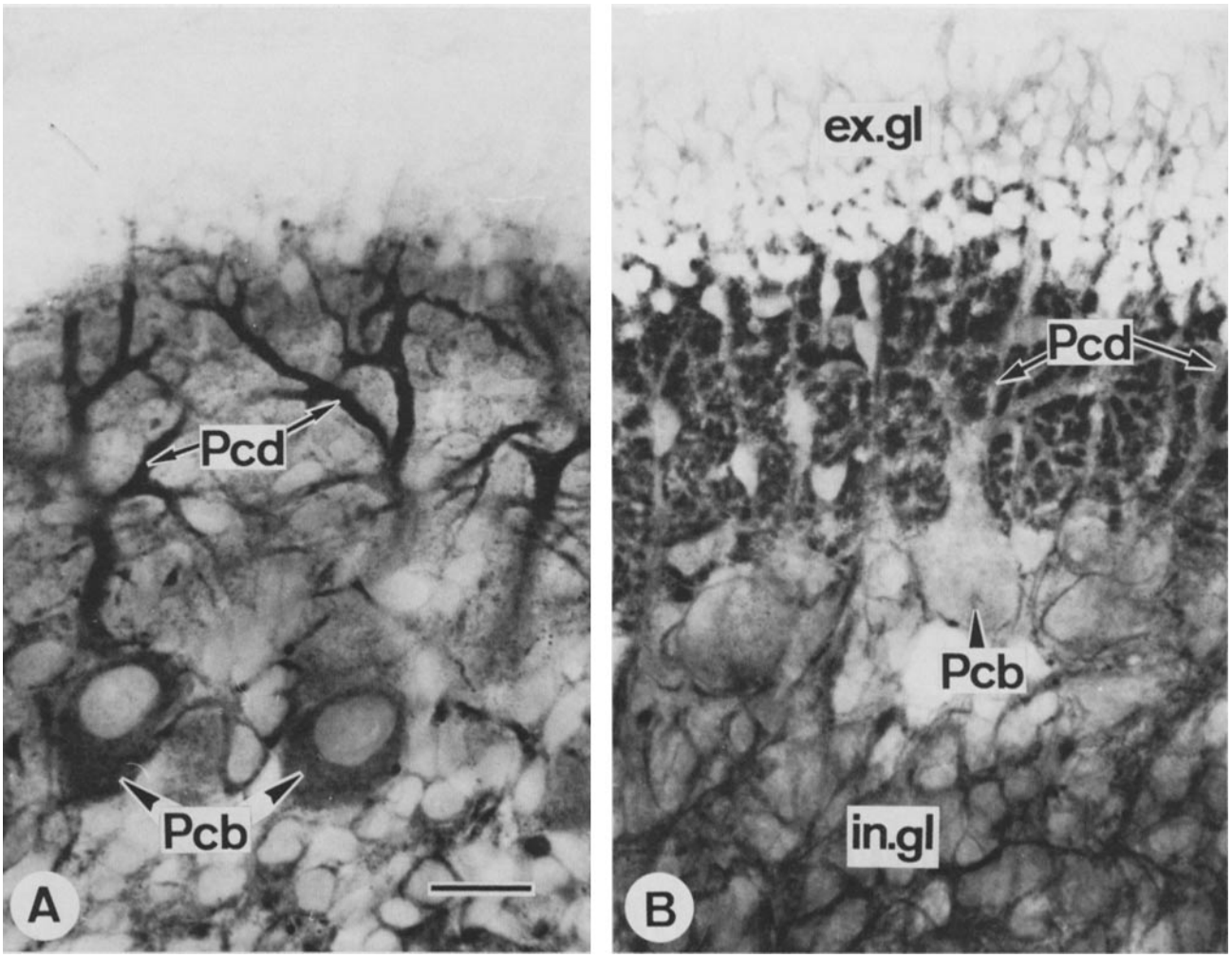

FIGURE 2 A pair of sections from 10-d-old rat cerebellum. When stained with anti-HMWP (*A*) Purkinje cell bodies (*Pcb*) and dendrites (*Pcd*) are the most prominent feature. There is no staining of the parallel fiber axons. Staining with antitubulin (*B*) shows a quite different pattern, in which Purkinje cell bodies and dendrites are unstained but the parallel fiber axons are strongly stained. The dividing granule cells in the external granular layer (*ex.gl*) are faintly stained. *In.gl*, inner granular layer. Bar, 30 μm. × 500.

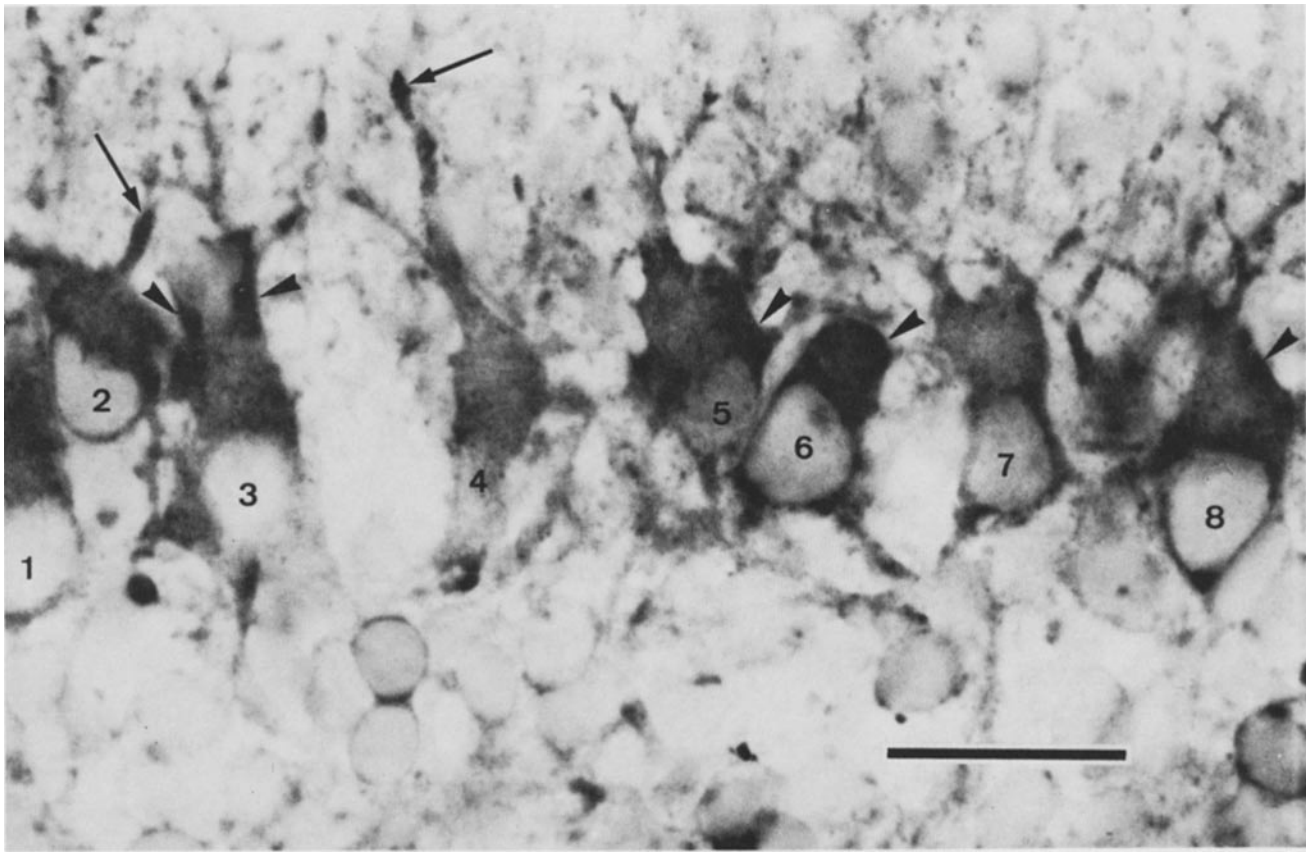

FIGURE 3 Purkinje cells in a section from 3-d-old cerebellum stained with anti-HMWP. Staining occurs throughout the cell body except for the nucleus (where the cells are numbered). The staining is weakest in the center of the cell and the stained material tends to form patches under the surface membrane (at points marked by arrowheads in cells *3, 5, 6,* and *8*). The section is focused to optimally display the two HMWP patches in cell *3*. Staining is also particularly strong in dendritic tips (indicated by arrows on cells *2* and *4*). The tip on cell *4* can be traced back to the cell body and shows the stronger staining of the distal dendrite as compared to the proximal dendrite and cell body. Bar, 40 μm. × 800.

staining can be seen in the advancing tips of the growing Purkinje cell dendrites as they first emerge from the cell body (cells *2* and *4* in Fig. 3). In cells where the first dendritic protrusions are just beginning to form, concentrated patches of HMWP occur beneath the cell surface (arrowheads in Fig. 3). These patches are not limited to the apical surface of the cells because high concentrations of HMWP are also present in the somatic spines which form transitorily during Purkinje cell development (some can be seen in Fig. 3). These somatic spines are dendritelike, being postsynaptic to the developing climbing fiber axons (9, 17). As the apical dendrites grow, the strongest anti-HMWP staining remains associated with the advancing dendrite tip. In places where the cell body and all of the growing dendrite are within the plane of section the appearance of the stained material suggests that there is a decreasing gradient of HMWP from tip to cell body (cell *4* in Fig. 3).

Thus, at this early stage of dendrite growth, immunohistochemical staining indicates that the dendrites contain HMWP but lack tubulin, whereas the surrounding axons do contain tubulin. Since tubulin is the major structural constituent of microtubules, we examined samples of postnatal cerebellar cortex by electron microscopy to see whether there was a corresponding difference in microtubular organization between axons and dendrites. Fig. 4*A* shows the apical portion of a Purkinje cell, in 10-d-old rat cerebellum, from which dendrites (numbered *1–4*) are growing in between developing parallel fiber axons. Examining areas of such neuropil at higher magnification (Fig. 4*B*) shows that whereas microtubules are abundant in axons (arrowheads), they are very sparse in Purkinje cell dendrites, which instead contain smooth endoplasmic reticulum as their most prominent feature. These growing dendrites also possess a dense filamentous network filling the cytoplasm between the subcellular organelles (Fig. 4*C*).

DISCUSSION

Previous work has indicated that in various types of brain neurons the HMWP are selectively associated with microtubules in dendrites (4, 11). Here we used the cerebellar Purkinje cell as a model to discover what role the HMWP might play in dendrite formation during neuronal differentiation. The cerebellar cortex is particularly suitable for such a study because the molecular layer in which the developing Purkinje cell dendrites are situated consists mainly of them and of developing parallel fiber axons which provide a convenient control.

The results demonstrate a striking difference between the patterns of microtubular development in these axons and dendrites. In the Purkinje cell dendrites, the HMWP appear several days before either tubulin or microtubular structures. In contrast, the adjacent parallel fiber axons contain both tubulin and structurally distinct microtubules from the earliest stage of their extrusion from the granule cell body.

This is in general agreement with previous ultrastructural observations of the cerebellar cortex during postnatal devel-

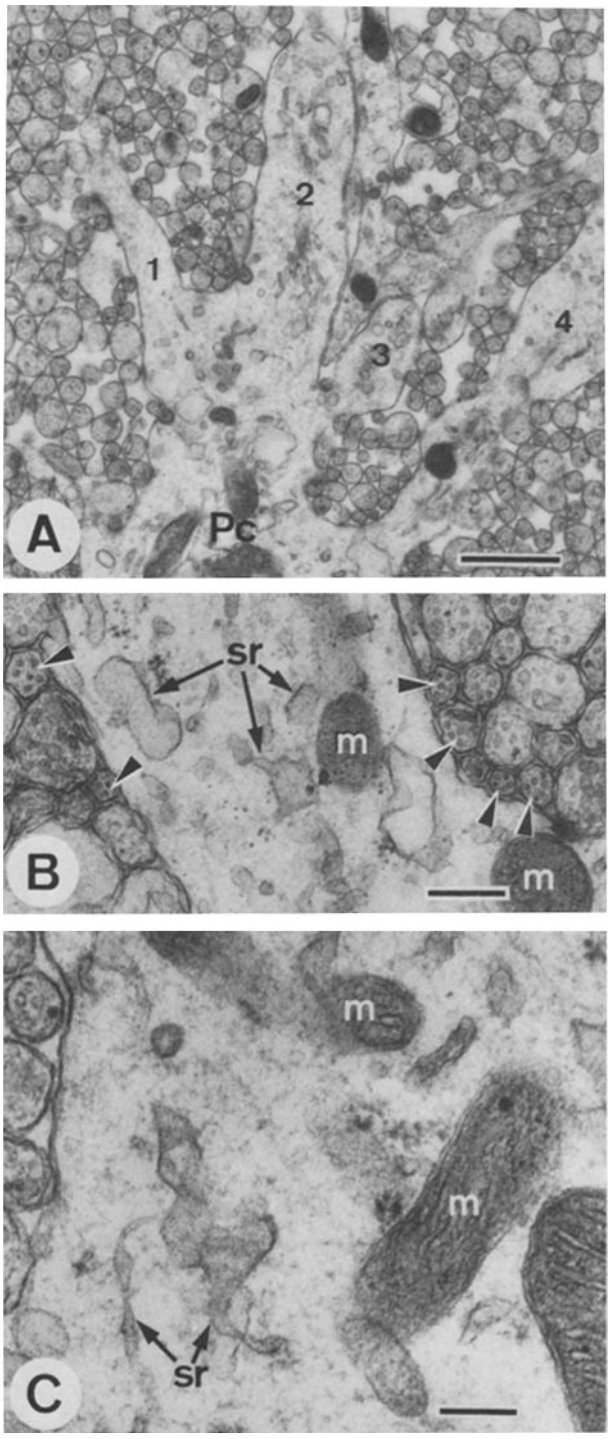

FIGURE 4 Electron micrographs of cerebellar cortex from 10-d-old rat brain. (*A*) The apical expansion of a growing Purkinje cell (*Pc*) from which emerge four dendritic branches (numbered *1–4*). Surrounding it is a mixture of growing parallel fiber axons and some smaller dendrite branches cut in transverse section. (*B*) Part of a growing dendrite and surrounding processes. The parallel fiber axons (arrows) each contain several microtubules in contrast to the Purkinje cell dendrite (*Pd*) which contains smooth endoplasmic reticulum (*sr*) and mitochondria (*m*) but no visible microtubules. (*C*) Part of a Purkinje cell dendrite showing the filamentous network which fills the cytoplasm between the mitochondria and smooth endoplasmic reticulum. Bars: *A*, 1 μm; *B*, 0.4 μm; *C*, 0.2 μm. *A*, × 13,000; *B*, × 25,000; *C*, × 50,000.

opment. While such studies have tended to concentrate on the growth cones of developing dendrites and axons, it has been noticed that dendrites contain few, if any, microtubules at early phases of their development (1, 7, 19). Literature surveys have emphasized that growing dendrites contain networks of 5-nm filaments and smooth endoplasmic reticulum but exclude microtubules and other subcellular organelles (8, 15). 5-nm filaments have also been recognized as the predominant cytoplasmic feature in the growth cones of neuronal processes in culture (5). Previous studies have also demonstrated that granule cell axons contain microtubules during development, in even greater abundance than they do in adult brain (2, 13, 23).

The most straightforward explanation of our observations is that tubulin is present in axons but absent from dendrites at early stages of neuronal differentiation. However, we cannot yet exclude the possibility that tubulin is present in both structures but in different forms of organization such that it is fixed and detected in axons but not in dendrites. On the other hand the absence of microtubular structures from developing dendrites and the contrasting presence of microtubules in nascent axons correlates well with the absence of tubulin from the former and its presence in the latter.

The appearance of HMWP in the developing Purkinje cells follows a pattern which suggests that they play a significant role in the initiation and subsequent growth of the developing dendrites. First, before the appearance of the dendritic protrusion from the cell body, concentrated patches of HMWP form under the surface membrane. Once the dendrite has emerged, high concentrations of HMWP are present in their distal tips. As the dendrite elongates, the highest concentration of HMWP remains in the advancing tip. This sequence of events suggests that the HMWP in the cell body may be involved in forming a local evagination of the surface membrane which initiates dendrite formation and that the continuation of this process in the advancing dendrite promotes its further elongation. We are now examining ultrathin sections stained with anti-HMWP to determine whether the stained material at the tip of the growing dendrite is indeed associated with the growth cone itself.

The process described above does not by itself seem to be sufficient to produce a mature Purkinje cell dendrite. HMWP are also concentrated in the short protrusions which transitorily appear on the perikaryon of developing Purkinje cells and which are contacted by growing climbing fibers (9, 17). However, the climbing fibers move onwards to the apical dendrites and abandon these somatic spines, which subsequently disappear. In addition to this phenomenon there are a number of examples of developmental abnormalities which suggest that innervation is involved in the final modeling and consolidation of the form of these dendrites (10, 16, 22). Thus, the HMWP seem to be involved in a flexible early growth phase when the ultimate shape or even the survival of a particular dendritic branch depends upon a secondary process of endorsement which involves factors external to the Purkinje cell.

Historically, HMWP have been recognized as integral microtubule components which facilitate the assembly of tubulin (3, 14, 20). Here we have shown that they appear in growing Purkinje cell dendrites before tubulin itself is present. This suggests that the HMWP act as a framework upon which the microtubules are subsequently built. It may be this maturation of the cytoskeleton which consolidates the form of the dendrites after the initial flexible growth phase.

As we have suggested (11), the differences in molecular structure of microtubules in dendrites and axons might serve

as a means of specifying export routes from the cell body for molecules destined to participate in either dendritic or axonal structure and function. In this way, the specialization of the dendritic cytoskeleton, of which the HMWP are part, may account for both the initiation and the maintenence of a differentiated dendritic structure.

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REFERENCES

1. Altmann, J. 1972. Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. *J. Comp. Neurol.* 145:335–398.
2. Altmann, J. 1972. Postnatal development of the cerebellar cortex in the rat. II. Phases of the maturation of Purkinje cells and the molecular layer. *J. Comp. Neurol.* 145:399–464.
3. Berkowitz, S. A., J. Katagiri, H. K. Binder, and R. C. Williams. 1977. Separation and characterization of microtubule protein from calf brain. *Biochemistry.* 16:5610–5617.
4. Black, M. M., and R. J. Lasek. 1980. Slow components of axonal transport: two cytoskeletal networks. *J. Cell Biol.* 86:616–623.
5. Bunge, M. B. 1973. Fine structure of nerve and growth cones of isolated sympathetic neurons in cultures. *J. Cell Biol.* 56:713–735.
6. Daniels, M. P. 1972. Colchicine inhibition of nerve fiber formation in vitro. *J. Cell Biol.* 53:164–176.
7. Hinds, J. W., and P. L. Hinds. 1972. Reconstruction of dendritic growth cones in neonatal mouse olfactory bulb. *J. Neurocytol.* 1:169–181.
8. Jacobson, M. 1978. Developmental Neurobiology. Plenum Press, New York. 183–188.
9. Larramendi, L. M. H. 1969. Analysis of synaptogenesis in the cerebellum of the rat. *In* Neurobiology of Cerebellar Evolution and Development. R. Llinas, editor. American Medical Association Press, Chicago. 808–843.
10. Lewis, P. D., A. J. Patel, A. L. Johnson, and R. Balazs. 1976. Effect of thyroid deficiency on cell acquisition in the postnatal rat brain: a quantitative histological study. *Brain Res.* 104:49–62.
11. Matus, A., R. Bernhardt, and T. Hugh-Jones. 1981. HMWP proteins are preferentially associated with dendritic microtubules in brain. *Proc. Natl. Acad. Sci. U. S. A.* 78:3010–3014.
12. Matus, A., G. Pehling, M. Ackermann, and J. Maeder. 1980. Brain postsynaptic densities: their relationship to glial and neuronal filaments. *J. Cell Biol.* 87:346–359.
13. Mugnaini, E. 1969. Ultrastructural studies on cerebellar histogenesis. *In* Neurobiology of Cerebellar Evolution and Development. R. Llinas, editor. American Medical Association Press, Chicago. 749–782.
14. Murphy, D. B., and G. G. Borisy. 1975. Association of high molecular weight proteins with microtubules and their role in microtubule assembly in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 72:2696–2700.
15. Peters, A., S. L. Palay, and H. deF. Webster. 1976. The Fine Structure of the Nervous System. Saunders. Philadelphia. 86–89.
16. Purpura, D. P., R. J. Shofer, E. M. Housepian, and C. R. Noback. 1964. Comparative ontogenesis of structure-function relations in cerebral and cerebellar cortex. *Prog. Brain Res.* 4:187–221.
17. Ramon y Cajal, S. 1911. Histologie du Système Nerveux de l'Homme et des Vertèbres. Maloine, Paris.
18. Seeds, N. W., A. G. Gilman, Tamano, and M. W. Nirenberg. 1970. Regulation of axon formation by clonal lines of a neural tumour. *Proc. Natl. Acad. Sci. U. S. A.* 66:160–167.
19. Skoff, R. P., and V. Hamburger. 1974. Fine structure of dendritic and axonal growth cones in embryonic chick spinal cord. *J. Comp. Neurol.* 153:107–148.
20. Sloboda, R. D., S. A. Rudolph, J. L. Rosenbaum, and P. Greengard. 1975. Cyclic-AMP-dependent endogenous phosphorylation of a microtubule-associated protein. *Proc. Natl. Acad. Sci. U. S. A.* 72:177–181.
21. Solomon, F., and M. Magendantz. 1981. Cytochalasin separates microtubule disassembly from loss of asymetric morphology. *J. Cell Biol.* 89:157–161.
22. Sotelo, C. 1978. Purkinje cell morphology: formation and maintainence of spines. *Prog. Brain Res.* 48:149–170.
23. Woodward, D. J., B. J. Hoffer, G. R. Siggins, and F. E. Bloom. 1971. The ontogenetic development of synaptic junctions. Synaptic activation and responsiveness to neurotransmitter substances in rat cerebellar Purkinje cells. *Brain Res.* 34:73–97.
24. Yamada, K. M., B. S. Spooner, and N. K. Wessels. 1971. Ultrastructure and function of growth cones and axons in cultured nerve cells. *J. Cell Biol.* 49:614–635.