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Microheterogeneity ("neurotypy") of neurofilament proteins

(monoclonal antibodies/immunocytochemistry/monoclonal peroxidase-antiperoxidase/electroblot/neuronal diversity)

MARGI E. GOLDSTEIN, LUDWIG A. STERNBERGER, AND NANCY H. STERNBERGER

Center for Brain Research, University of Rochester School of Medicine, Rochester, New York 14642

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ABSTRACT **Neurofilaments purified from adult rat brainstem by two methods were electrophoresed on $NaDodSO_4$/polyacrylamide gels to separate the triplet proteins (approximate M_rs of 200,000, 155,000, and 68,000) which, in turn, were electroblotted onto nitrocellulose paper. On Coomassie blue-stained gels that were not electroblotted, the same banding pattern was seen with both methods of preparation. Immunocytochemical staining of the electroblots with each of five monoclonal antibodies revealed that three of the monoclonal antibodies were specific for the M_r 200,000 neurofilament protein and two, for both the M_rs 200,000 and 155,000 neurofilament proteins. None of the antibodies reacted with the M_r 68,000 band. The M_r 200,000 band could be resolved into doublet bands. Individual monoclonal antibodies reacted with either one or both of the M_r 200,000 doublets. The immunocytochemical staining of the neurofilament triplets on electroblots was compared to that of adult rat cerebellar paraffin sections. Each monoclonal antibody had a unique pattern of staining, reacting only with certain subpopulations of neurons or their processes. Correlation of the staining patterns in cerebellar tissue sections with those of neurofilament polypeptides on electroblots suggested that different neurofilament polypeptides can be localized to different structures and subpopulations of neurons and that molecular heterogeneity ("neurotypy") may be revealed within the M_rs 200,000 and 155,000 neurofilament polypeptides.**

Neurofilaments are neuron-specific intermediate filaments with a diameter of 10 nm and consist of the neurofilament triplet, three major polypeptides with M_rs of 200,000, 150,000, and 68,000 (1–5). The origin of the triplet is uncertain. Immunological crossreactivity between the neurofilament polypeptides with polyclonal antibodies suggests common antigenic sites (1, 5–14) and implies that the neurofilament proteins are derived from a common precursor (15). On the other hand, peptide maps reveal little homology among the neurofilament polypeptides (14–20) and point to an independent origin for each.

Monoclonal antibodies to a single neurofilament polypeptide would be useful to explore the origin, structure, and organization of neurofilaments and their polypeptides. Monoclonal antibodies that recognize unshared (21) and shared (21, 22) determinants on the three polypeptides have been reported.

In the present study, we have produced five monoclonal antibodies, each of which recognizes one or more of the neurofilament polypeptides. The staining, by each monoclonal antibody, of neurofilament proteins separated by gel electrophoresis and electroblot transfer onto nitrocellulose paper has provided additional evidence for the existence of one or more common antigenic sites. Comparison of these electroblots to the staining of rat cerebellar tissue sections has allowed for closer examination of the structure and distribution of neurofilaments and their polypeptides in the brain. The different staining patterns seen with each of the monoclonal antibodies has suggested that the neurofilament polypeptides are a heterogeneous group of proteins and that neurofilaments in different locations in the brain possess differences in molecular structure.

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MATERIALS AND METHODS

Monoclonal Antibody Production. Monoclonal antibodies were produced as described (23–25) by using the procedure of Kohler and Milstein (26, 27). Five monoclonal antibodies were chosen as primary antibodies for the present study.

Neurofilament Preparation. Neurofilaments were prepared by two methods. In the first method (28, 29), adult rats were killed by an overdose of sodium pentobarbital and their brains were removed, stripped of meninges, and frozen at −80°C. Brainstems and hippocampi were homogenized for 15 min (glass homogenizer with Teflon pestle) in 20 vol of 50 mM Tris·HCl (pH 6.8) containing 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 2 mM EDTA. The homogenate was centrifuged for 10 min at 11,400 rpm (12,000 × *g*) at 1°C in a Sorvall SS34 rotor. The supernatant was decanted and was termed the "soluble fraction." The pellet was rehomogenized in 20 vol of the above buffer with 30% sucrose for 15 min and again was centrifuged at 11,400 rpm at 1°C for 10 min. The floating layer of myelin was removed and the supernatant was decanted and pooled with the soluble fraction. The pellet was resuspended in a small volume of 50 mM phosphate buffer at pH 8. Diethyl ether/ethanol, 3:2 (vol/vol), was added to a total of 20 times the original volume, mixed well, and centrifuged at 11,400 rpm for 10 min. The pellet was washed once with acetone, dried under nitrogen gas, and desiccated overnight. The dried residue was extracted in a Broeck grinder (Thomas) in a small volume of 25 mM phosphate buffer with 8 M urea and 10 mM Cleland's reagent (Sigma) and was centrifuged at 5,750 rpm (3,000 × *g*) for 10 min. The supernatant, containing the neurofilament triplet and glial fibrillary acidic protein as well as other cytoskeletal proteins, such as vimentin, α- and β-tubulins, and actin, was termed the "insoluble fraction."

In the second method, neurofilaments were purified by axonal flotation (1, 16, 30). Brainstems were obtained from adult rats killed by an overdose of sodium pentobarbital or by cervical dislocation and were frozen as before. Two grams of tissue was homogenized in 40 ml of 10 mM KH_2PO_4, pH 6.5/100 mM KCl/5 mM EGTA (buffer A) with 0.85 M sucrose. The homogenate was centrifuged in a Beckman SW 25.1 rotor at 11,000 rpm (13,000 × *g*) for 15 min. The axonal pad floating to the surface was washed three times by homogenization in 40 ml of buffer A with 0.85 M sucrose and centrifugation at 11,000 rpm for 15 min. The axons were homogenized in 10 ml of buffer A with 1% Triton X-100 and centrifuged on 30 ml of buffer A with 0.85 M sucrose at 22,000 rpm (52,000 × *g*) for 1 hr. The pellet was homogenized in 8 ml of buffer A with 1% Triton X-100, layered onto a discontinuous sucrose gradient (8 ml of 1.0, 1.5, and 2.0 M sucrose, each made with buffer A), and centrifuged at 22,000 rpm for 2 hr. Neurofilaments at the interfaces of the 1.5 and

2.0 M sucrose and the 1.0 and 1.5 M sucrose were pooled, diluted with 34 ml of buffer A, and centrifuged for 15 min at 22,000 rpm. The pellet was stored at −80°C. For use, the pellet was resuspended in a small volume of 25 mM phosphate buffer with 8 M urea/2 mM EDTA.

Protein determination for both neurofilament preparations was done by the method of Lowry *et al.* (31).

Gel Electrophoresis. One-dimensional discontinuous gels were run by the method of Laemmli (32) in a vertical slab gel apparatus (Hoeffer Scientific Instruments, San Francisco). The stacking gel contained 125 mM Tris·HCl, pH 6.8/0.1% $NaDodSO_4$/4% acrylamide. The separating gel contained 375 mM Tris·HCl, pH 8.8/0.1% $NaDodSO_4$/8% acrylamide. Samples were incubated for 3 min at 100°C in 63 mM Tris·HCl, pH 6.8/2% $NaDodSO_4$/10% glycerol/5% 2-mercaptoethanol/0.001% bromophenol blue and were frozen at −80°C or used immediately. The reservoir buffer was 25 mM Tris·HCl/0.192 mM glycine/0.5% $NaDodSO_4$, pH 8.3. Electrophoresis was carried out at constant voltage (150 V) for 4.5–5 hr with cooling. The gels were either stained in 0.125% Coomassie blue R-250 in 50% methanol/10% acetic acid and destained in 5% methanol/7% acetic acid or were used directly in electroblot.

Protein Electroblot Transfer to Nitrocellulose Paper. Separated proteins were transferred to nitrocellulose paper as described by Towbin *et al.* (33) in a Hoeffer electroblot unit with 50 mM sodium phosphate buffer (pH 7.4) at 100 mA overnight. Molecular weight standards were cut from the blot, stained with 0.125% Coomassie blue R-250 in 50% methanol/10% acetic acid, and destained with several changes of 50% methanol/10% acetic acid. The remainder of the blots that were to be stained immunocytochemically were fixed in 25% isopropanol/10% acetic acid for 30 min, rinsed carefully with 0.05 M Tris·HCl, pH 7.6/1.5% sodium chloride (1.5 T buffer) for 5 min, and incubated in 3% bovine serum albumin and 1% normal sheep serum in 1.5 T buffer for 30 min while covered with aluminum foil to avoid exposure to light. The blots were rinsed carefully for 15 min in 1.5 T buffer and cut into strips corresponding to separate lanes on the original gel.

Immunocytochemistry. Strips of nitrocellulose paper were stained immunocytochemically (34, 35) by incubating with (*i*) one of the five mouse monoclonal antibodies as ascites fluids, diluted 1:2,000, for 1 hr; (*ii*) goat anti-mouse IgG, diluted 1:20, for 30 min; (*iii*) mouse peroxidase-antiperoxidase prepared from monoclonal antibody (24, 25), diluted 1:200, for 30 min; and (*iv*) 0.05% diaminobenzidine tetrahydrochloride/0.01% hydrogen peroxide, for 8 min. Each step was followed by careful rinsing for 15 min with several changes of 1.5 T buffer. All dilutions were made with 1.5 T buffer containing 1% normal sheep serum. Antibodies 02-135, 06-32, 02-40, 03-44, and 06-17 were chosen as primary antibodies for this study because their staining of neuronal cell somata or fibers (or both) (23–25) suggested that they reacted with neurofilament proteins.

Immunocytochemical staining of paraffin sections of rat cerebella was done as described (23, 24).

RESULTS

Coomassie blue staining of neurofilaments prepared by two methods on polyacrylamide gels showed that the M_r 200,000 neurofilament protein could be resolved into two bands (Fig. 1) corresponding to a M_r 200,000 and a M_r 185,000 component. Cytoskeletal proteins other than the neurofilaments (glial fibrillary acidic protein, vimentin, α- and β-tubulins, and actin, for example) were found in larger amounts in preparations of neurofilaments isolated by insolubility in Triton X-100 rather than by axonal flotation.

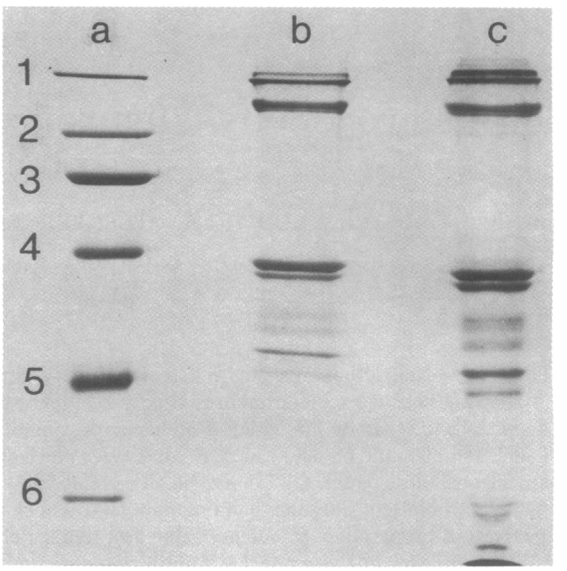

FIG. 1. Neurofilament polypeptides separated by $NaDodSO_4$/8% polyacrylamide gel electrophoresis and stained by Coomassie blue. Lane a, molecular weight standards: 1, M_r 200,000; 2, M_r 116,250; 3, M_r 92,500; 4, M_r 66,200; 5, M_r 45,000; and 6, M_r 31,000. Lane b, neurofilament triplet proteins prepared by axonal flotation. Lane c, neurofilament proteins insoluble in Triton X-100.

Immunocytochemical staining of neurofilament proteins separated by electrophoresis and transferred to nitrocellulose paper showed that all five monoclonal antibodies used as primary antibodies stained the M_r 200,000 neurofilament protein (Fig. 2*A*). Two antibodies (02-40 and 06-17) reacted with the M_r 155,000 neurofilament component as well. Only antibodies 02-135, 06-32, and 02-40 stained both bands of the doublet M_r 200,000 neurofilament protein. Antibodies 03-44 and 06-17 stained only the higher molecular weight (M_r 200,000) band of the doublet. Larger amounts of protein were needed to obtain staining of the M_r 155,000 band with 06-17 (Fig. 2*B*) than with 02-40. Increases in the amount of protein loaded on the gel did not reveal additional bands with monoclonal antibodies 02-135, 06-132, and 02-40. Some faint bands became apparent with antibody 03-44 (Fig. 2*B*).

Correlation of immunocytochemical staining in cerebellar tissue sections with electroblot patterns for each of the monoclonal antibodies is given in Fig. 3. There was no difference in staining intensities of any structures when antibody dilutions of 1:500 or 1:2,000 were used. With dilutions greater than 1:2,000, intensities of more weakly stained structures diminished progressively, but those of more strongly stained structures (perikaya of selected brainstem nuclei for 02-135 and 06-32 or basket cell fibers for 02-40, 03-44, and 06-17) remained undiminished until dilutions greater than 1:6,000. Antibodies of 02-135, 06-32, 03-44, and 06-17 still stained at dilutions of 1:80,000.

DISCUSSION

The origin of the neurofilament polypeptides, whether they are synthesized separately or are products of a common precursor, has been a source of debate. Immunological crossreactivity of polyclonal antibodies against the neurofilament proteins supports the view that there are common antigenic sites (1, 5–14) and suggests that they may be derived from a common precursor. Axoplasmic transport studies show the triplet to be present *in vivo* (36). Therefore, if there is a common precursor, processing must be done in the cell body (5). Peptide mapping

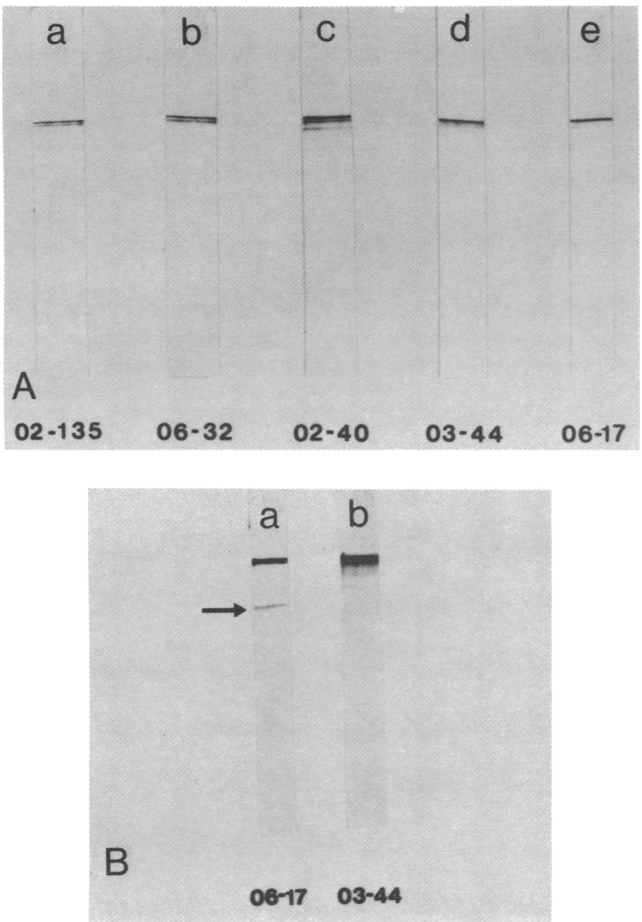

FIG. 2. (*A*) Neurofilaments separated by NaDodSO$_4$/8% polyacrylamide gel electrophoresis with 10 μg of protein per lane, transferred to nitrocellulose, and stained with each of five monoclonal antibodies. Lanes: a, antibody 02-135; b, antibody 06-32; c, antibody 02-40; d, antibody 03-44; and e, antibody 06-17. (*B*) Neurofilaments separated by NaDodSO$_4$/6% polyacrylamide gel electrophoresis with 30 μg of protein per lane, transferred to nitrocellulose, and stained with each of two monoclonal antibodies. Lanes: a, antibody 06-17, and b, antibody 03-44. Note the staining of the M_r 155,000 neurofilament polypeptide by antibody 06-17 when 30 μg of protein was loaded on the gel (arrow).

has revealed no homology among the neurofilament polypeptides (14–20), implying an independent origin for each. However, Julien and Mushynski (37) isolated a segment with a common amino acid sequence. It is possible that the polypeptides may be synthesized separately (3, 5, 7) yet still share common antigenic sites (8) and that the genes coding for the neurofilament polypeptides have evolved from the same ancestral gene (7). Immunological crossreactivity on electroblots may also be explained by nonhomogeneous bands (5). The M_r 155,000 protein may consist of a proteolytic product of the M_r 200,000 protein in addition to a unique M_r 155,000 component, or each polypeptide band may consist of more than one molecular form. Although the neurofilaments are sensitive to proteolysis (8), Thorpe (20) has shown by two-dimensional analysis that the M_r 155,000 band is homogeneous. However, different molecular forms of the M_r 155,000 polypeptide may be present in quantities insufficient to be detected by Coomassie blue but capable of being resolved by a sensitive immunocytochemical method.

In the present study, we have stained neurofilaments separated by gel electrophoresis and transferred onto nitrocellulose paper and found that all five antibodies reacted with the M_r 200,000 polypeptide. The M_r 200,000 band was a doublet, consisting of bands corresponding to M_rs 200,000 and 185,000. All five of the monoclonal antibodies reacted with the upper band of the M_r 200,000 polypeptide, but only antibodies 02-135, 06-32, and 02-40 stained the lower band (M_r 185,000). Two antibodies (02-40 and 06-17) also stained the M_r 155,000 polypeptide, although decreasing the amount of protein on the gel eliminated this staining with 06-17.

Comparison of the staining of these electroblots with the staining of rat cerebella leads to interesting conclusions. Antibodies 02-135 and 06-32, each of which stained the two bands of the doublet M_r 200,000 protein, have similar staining patterns, although staining was more selective with antibody 06-32. In the cerebellum, Purkinje cell bodies with their axons and dendrites, cells tentatively identified as Lugaro cells, processes in the granular layer, and inner medullary fibers of the white matter layer were stained by both monoclonal antibodies. Similar staining reactivities were seen in cerebella stained with polyclonal antisera against the neurofilament proteins by other groups (38–40), although none of these patterns was faithfully reproduced by our two antibodies. The electroblot of neurofilaments stained with antibody 02-40 also showed the doublet M_r 200,000 band and, in addition, the M_r 155,000 band was stained. The electroblot correlates with the more widely distributed staining pattern seen with this antibody (24). The Purkinje cell pattern was similar to that of antibodies 02-135 and 06-32. Differences were that 02-40 stained (*i*) basket cell fibers in the molecular layer of the cerebellum and (*ii*) a larger number of axons and neurons throughout the brain. At this point, it would seem logical to conclude that antibodies 02-40, 02-135, and 06-32 are reacting with an antigen localized in or near the cell body of a subpopulation of neurons, specifically a component of the M_r 200,000 neurofilament doublet.

Antibodies 03-44 and 06-17 had reactivities different from antibodies 02-135, 06-32, and 02-40 in both the electroblots and the rat cerebellum. The staining patterns were more like those seen in most reports of staining with both polyclonal and monoclonal antibodies against the neurofilament proteins (5, 9, 10, 39, 40). In the cerebellum, basket cell fibers surrounding Purkinje cell bodies and inner medullary fibers in the white matter layer were stained. No neuronal cell bodies were stained. Elsewhere in the brain, staining was restricted to axons and dendrites. On electroblots, both 03-44 and 06-17 stained only the upper band of the M_r 200,000 doublet, and 06-17 stained the M_r 155,000 band as well. It is possible that absence of staining of the lower bands of the M_r 200,000 doublet with antibodies 03-44 and 06-17 accounts for the lack of neuronal cell body staining by these two antibodies. The M_r 155,000 band is not consistently stained by antibody 06-17 and it seems that this antigen may be present in much smaller amounts in the brain so that decreasing the amount of the total protein on the electroblot decreases the antigen beyond a detectable level. The requirement of higher concentration of neurofilament preparation for staining of the M_r 155,000 band by 06-17 may suggest that this antibody reacts with an epitope expressed by only some of the polypeptides banding in the M_r 155,000 region.

All of the five antibodies stain the upper M_r 200,000 band. Because antibodies 02-135 and 06-32 stain Purkinje cell bodies and their projections but not basket cell fibers, whereas antibodies 03-44 and 06-17 stain basket cell fibers but not Purkinje cell bodies or projections, the antigen represented by the upper M_r 200,000 band appears to possess a heterogeneity not resolved by the electroblot. The lower M_r 200,000 band is only stained by antibodies that reveal Purkinje and other cell bodies. Because not all of these antibodies stain basket cell fibers, the lower M_r 200,000 band may indicate a neurofilament hetero-

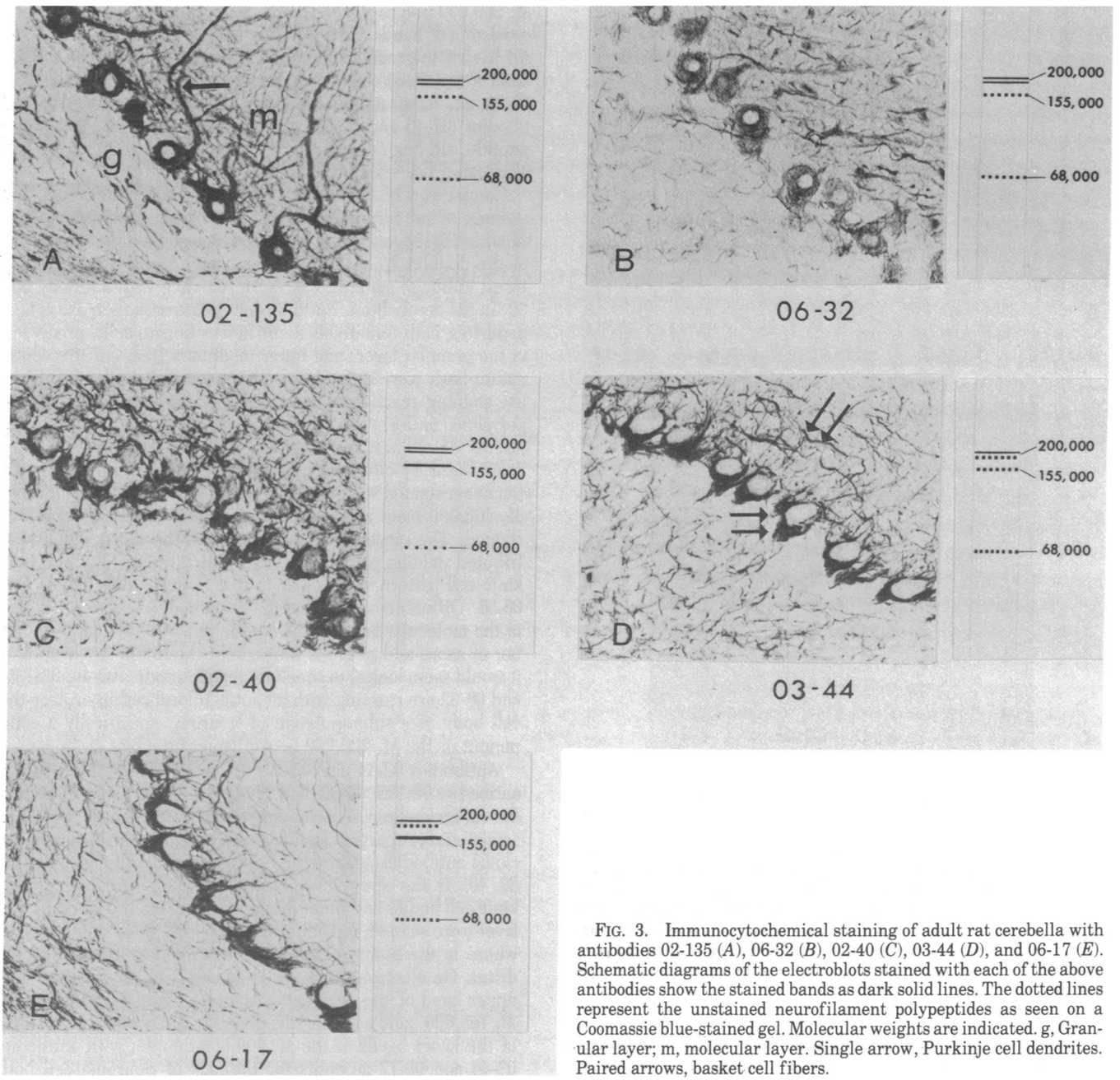

FIG. 3. Immunocytochemical staining of adult rat cerebella with antibodies 02-135 (*A*), 06-32 (*B*), 02-40 (*C*), 03-44 (*D*), and 06-17 (*E*). Schematic diagrams of the electroblots stained with each of the above antibodies show the stained bands as dark solid lines. The dotted lines represent the unstained neurofilament polypeptides as seen on a Coomassie blue-stained gel. Molecular weights are indicated. g, Granular layer; m, molecular layer. Single arrow, Purkinje cell dendrites. Paired arrows, basket cell fibers.

geneity present in Purkinje cells and absent from basket fibers.

Only antibody 02-40 stained all of the three bands (both of the M_r 200,000 doublet bands and the M_r 155,000 band). Interestingly, it is this antibody that revealed the broadest staining distribution among all of the antibodies investigated (25). The broad histologic staining distribution by this antibody and the elucidation of three bands resolved by electroblot suggests that this antibody reacts with a common antigen in a relatively large number of heterogeneous neurofilament polypeptides ("neurotypes").

The electroblot patterns alone could be interpreted as recognition of different antigenic determinants by each monoclonal antibody in a single neurofilament polypeptide. However, the correlation of these different reactivities with the characteristic, yet varied, anatomical distributions in different cells and fibers suggests microheterogeneity either in the primary structure of neurofilament peptides or in their functional interaction with characteristic, variable environmental factors. The latter explanation would imply the existence of heterogeneous factors, other than neurofilaments, that mask specific neurofilament epitopes in some neurons or fibers and not in others. It would also imply the existence of many different masking factors specifying as many neurofilament epitopes as we have antibodies that reveal different immunocytochemical distributions of neurofilaments. This explanation, although devoid of any experimental basis, must be considered until disproven. However, a more likely explanation of the observed phenomenon, not only in the cerebellum but also in the rest of the brain and selected peripheral sites in adult and developing animals (23–25), lies in an intrinsic microheterogeneity of the neurofilament peptides themselves (41), thus suggesting that different and unshared determinants are characteristic of different types (neurotypes) of the neurofilament peptides expressed in different regions of the brain. These neurotypes may have different functions in axoplasmic transport and cytoskeletal integrity.

Neurofilaments may not be the only brain- or neural crest-specific proteins that exhibit microheterogeneity ("neurotypy"). Antigens of the synapse-associated group (23, 24) and precursors of neuropeptides (42) provide other examples. Heterogeneity of the latter is, indeed, an essential corollary of the fundamental concept of the Scharrers (43–45) that has established neuropeptides as an essential principle of neuronal, vascular, and private (paracrine) intercellular communication. Heterogeneity seems to be important to give neurons their specificity in communication.

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