

Brain Postsynaptic Densities: Their Relationship to Glial and Neuronal Filaments

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ABSTRACT Preparations of isolated brain postsynaptic densities (PSDs) contain a characteristic set of proteins among which the most prominent has a molecular weight of ~50,000. Following the suggestion that this major PSD protein might be related to a similarly sized component of neurofilaments (F. Blomberg et al., 1977, *J. Cell Biol.*, 74:214–225), we searched for evidence of neurofilament proteins among the PSD polypeptides. This was done with a novel technique for detecting protein antigens in SDS-polyacrylamide gels (immunoblotting) and an antiserum that was selective for neurofilaments in immunohistochemical tests. As a control, an antiserum against glial filament protein (GFAP) was used because antisera against GFAP stain only glial cells in immunohistochemical tests. They would, therefore, not be expected to react with PSDs that occur only in neurons. The results of these experiments suggested that PSDs contain both neuronal and also glial filament proteins at higher concentrations than either synaptic plasma membranes, myelin, or myelinated axons.

However, immunoperoxidase staining of histological sections with the same two antisera gave contradictory results, indicating that PSDs in intact brain tissue contain neither neuronal or glial filament proteins. This suggested that the intermediate filament proteins present in isolated PSD preparations were contaminants. To test this possibility, the proteins of isolated brain intermediate filaments were labeled with ^{125}I and added to brain tissue at the start of a subcellular fractionation schedule. The results of this experiment confirmed that both neuronal and glial filament proteins stick selectively to PSDs during the isolation procedure. The stickiness of PSDs for brain cytoplasmic proteins indicates that biochemical analysis of subcellular fractions is insufficient to establish a given protein as a synaptic junctional component. An immunohistochemical localization at PSDs in intact tissue, which has now been achieved for tubulin, phosphoprotein I, and calmodulin, appears to be an essential accessory item of evidence.

Our findings also corroborate recent evidence which suggests that isolated preparations of brain intermediate filaments contain both neuronal and glial filaments.

The postsynaptic density (PSD) is a disk-shaped proteinaceous structure that is attached to the postsynaptic junctional membrane at synapses in mammalian brain (17, 31–33). In several recent studies, it has been shown that subcellular fractions highly enriched in PSDs can be made by treating isolated synaptosomal plasma membranes with detergents (7, 8, 28, 29, 42, 49).

Analysis by SDS-gel electrophoresis has shown that isolated PSDs contain a characteristic set of polypeptides, among which is a major component of ~50,000 mol wt. Initially, it was thought that this 50,000 PSD protein might be tubulin because the two comigrated in SDS gels and showed apparently similar

tryptic digest peptide maps (13, 48). The presence of tubulin antigen at PSDs in intact brain tissue has been confirmed by immunohistochemical staining (30, 48), but it has become clear that tubulin is a minor PSD component distinct from the major ~50,000 band (3, 22, 28).

Another possible affiliation suggested for the ~50,000 PSD protein was to a polypeptide of approximately the same size that occurs in preparations of isolated brain intermediate filaments (3, 54). This 50,000-dalton intermediate filament (IF) protein was, until recently, thought to be specifically associated with neurofilaments (11, 12, 53). The proposed relationship between neurofilaments and the PSD was also based on the

observation that isolated PSDs examined in the electron microscope appear to contain intermediate-sized filaments (7). In addition, neurofilaments are found in a variety of postsynaptic structures in the nervous system (21, 34, 35, 51). From the functional viewpoint, the known involvement of neurofilaments in slow axoplasmic flow (19) could indicate a relationship between the PSD and one of the major mechanisms for transporting materials within neuronal processes.

The possible significance of neurofilaments for PSD structure and function suggested by these ideas lead us to begin investigating the relationship between brain intermediate filaments and PSDs in more detail. During the course of our experiments, several relevant studies appeared reporting results that correlate with those we have obtained. First, it has become clear that the 50,000-dalton protein of brain IFs does not belong to neurofilaments but to glial filaments (10, 15, 24). Neurofilaments, it is now agreed, contain instead three characteristic polypeptides of molecular weights 210,000, 160,000, and 68,000 as their major components (1, 19, 25, 40, 44). Second, detailed electrophoretic analysis and peptide mapping have confirmed our earlier findings (28) in showing that the 50,000 mol wt PSD protein is distinct from any independently recognized protein species and seems to be specifically associated with brain synapses (22).

These recent developments leave unanswered the question of what relationship, if any, exists between brain IFs (either neuronal or glial) and PSDs. It is this issue that is dealt with here.

MATERIALS AND METHODS

Subcellular Fractionation

Synaptosomes, synaptosomal plasma membranes, myelin, and mitochondria were isolated from a crude mitochondrial fraction (P_2) of rat whole brain made according to Gray and Whittaker (18). From the P_2 synaptosomes were isolated on a discontinuous gradient of 8 and 14% (wt/vol) Ficoll (Pharmacia AG, Zurich, Switzerland) in isotonic (10% wt/wt) sucrose (9). In other cases, the P_2 fraction was lysed by hypotonic shock in 5 mM Tris, pH 7.4, and then made up to a sample layer of ~32% (wt/wt) sucrose by the addition of 2 vol 48% (wt/vol) sucrose. This lysate was fractionated by the sedimentation-flotation sucrose density gradient method of Jones and Matus (20), adapted for the Sorvall SS-90 vertical rotor (Digitana AG, Zurich, Switzerland). The gradients consisted of the following sucrose layers (all percentages wt/wt): 5 ml of 48%, 16 ml of sample, 11 ml of 28.5%, and 4.5 ml of 10%. The gradients were centrifuged for 90 min at 18,000 rpm (28,000 avg) in a Sorvall RC-5 centrifuge at 4°C after which enriched mitochondria were collected from the lowest interface, synaptosomal plasma membranes (SPM) from the center, and myelin from the uppermost. Unless otherwise stated, all solutions contained 10 mM HEPES buffer, pH 7.4, and 50 μ M Ca^{++} . Each fraction was diluted with 2 vol of this buffer (containing 10% wt/wt sucrose in the case of synaptosomes) and pelleted by centrifugation at 18,000 rpm (30,000 avg) for 30 min in the 8 \times 50 ml rotor of the Sorvall RC-5. PSDs were prepared from either synaptosomes or SPM by extraction with 0.5% Triton X-100 and washing with 75 mM KCl, as described by Cohen et al. (7). In making PSDs from synaptosomes, 10% (wt/wt) sucrose was present up to and during the Triton X-100 treatment to avoid lysis.

Brain intermediate filaments were prepared essentially by the method of Yen et al. (53), modified in detail to produce optimal morphological preservation. To minimize proteolytic degradation that occurs in postmortem tissue (A. I. Matus, D. H. Jones, and B. H. Anderton, unpublished observations), all solutions contained EDTA, phenylmethylsulfonyl fluoride, and *p*-hydroxymercuribenzoate at 1 mM concentration. These inhibitors, together with 50 mM 2-*N*-morpholinoethane sulfonic acid buffer, pH 6.5, and 30 mM KCl, constituted the isolation buffer, referred to as MKI. Each preparation used 4 g rat brain stems which were individually dispersed immediately after dissection in 80 ml of ice-cold 0.85 M sucrose in MKI by eight strokes in a tight-fitting Teflon-glass homogenizer, motor driven at 800 rpm. When all the tissue had been homogenized, a further 80 ml of sucrose-MKI was added and the suspension was centrifuged at 10,000 rpm (15,000 avg) for 30 min in a Sorvall HB-4 rotor at 4°C. The pellicle of myelinated axons, which floats to the surface, was collected with a spoon and washed twice more by homogenization in sucrose-MKI and centrifugation. During this proce-

dures, the volume of suspension was halved at each successive step. The material obtained at this stage was the myelinated axon preparation used in some of the experiments. Most of the material was resuspended in MKI by homogenization, the total volume made up to 80 ml with further MKI, and this suspension was vigorously stirred (by magnetic stirrer) at 6°C for 90 min. An equal volume (80 ml) of 1.7 M sucrose in MKI was added and the suspension was centrifuged at 11,500 rpm (20,000 avg) in the HB-4 rotor for 30 min. The pellet was washed twice with 0.85 M sucrose in MKI and finally resuspended in 2 ml MKI. After the lysis step, a loose-fitting, hand-operated homogenizer was used. The yield is usually between 4 and 5 mg protein. Every preparation was checked by electron microscopy for purity and filament integrity because preparations occasionally show, for no obvious operational reason, either poor preservation of filament structure or gross myelin contamination. If either was the case, the preparation was rejected.

Gel Electrophoresis

Electrophoresis in SDS-containing polyacrylamide gels was performed using the same buffer system as Laemmli (25). Samples were solubilized in 2.5 mg SDS per milligram protein and 2% 2-mercaptoethanol in stack buffer diluted 1:4. Samples were immediately heated at 90°C for 10 min, cooled, and glycerol (10–15%) and trace amounts of bromophenol blue tracking dye were added. The gels were slabs 1.5-mm thick containing a 7.5–15% (wt/vol) gradient of polyacrylamide as separating gel, topped with a 5% stacking gel.

Molecular Weight Calculation

Molecular weights were based on a series of gels in which subcellular fractions were run in slots adjacent to molecular weight marker proteins. The markers were: myosin (200,000), β -galactosidase (130,000), phosphorylase B (94,000), bovine serum albumin (BSA) (68,000), ovalbumin (43,000) (Kit I, Bio-Rad, Munich, W. Germany); phosphorylase B, BSA, ovalbumin, carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), lysozyme (14,300) (Kit II, Bio-Rad); microtubule-derived tubulin (unresolved, 55,000). In a single slab gel were run: brain IFs (slots 1 and 2), kit I (slot 3), kit I + kit II (slot 4), kit II (slot 5), microtubules (slots 7–9). A plot of log molecular weight vs. relative mobility was prepared for these standards. From our gradient gels, this plot was slightly concave upward and was, therefore, fitted by third-order polynomial regression with an HP 9845T and manufacturer's software (Hewlett-Packard Instruments, Zurich, Switzerland). From the computed curve, molecular weights for each of the IF polypeptide bands were separately calculated for each of the two samples. A second identical gel was run and the process repeated so that four sets of calculated molecular weights for the IF polypeptides were generated. These were averaged and rounded to the nearest 500 dalton. A similar process was used to arrive at molecular weights for polypeptides of other subcellular fractions using gels in which a variety of standardized samples and unknowns were coelectrophoresed in adjacent slots. A typical gel contained myelin, SPM, kit I, PSD, kit II, microtubules, and IFs.

Immunochemical Staining of SDS Gel Blots

The separated proteins from SDS gels were blotted onto cellulose nitrate membranes (Sartorius Balances, Brinkman Instruments, Inc., Westbury, N. Y. and Millipore Corp., Bedford, Mass.) by transverse electrophoresis (45). The electrophoresis medium was the Laemmli (23) run buffer with the SDS omitted and 20% (vol/vol) methanol added. Electrophoresis was performed for 2 h at 38 V. In each experiment, several blots of the same combination of samples, run in the same slab gel if possible, were made; one blot from each set was stained for protein with 0.55% amido black in methanol:acetic acid:water (5:1:5) for 3 min, washed in water for 2 min, and destained in methanol:acetic acid:water (90:2:100). Once the fidelity of the transfer had been established, other blots were stained with specific antisera. The steps below utilize a basic buffer of 50 mM Tris, pH 7.4, 150 mM sodium chloride (TBS), or TBS containing 3% BSA (TBSA). The blots are incubated for 40 min with TBSA in a shaking water bath at 40°C and then rinsed with TBS for 1 min. They are then transferred to shallow boats constructed from folded parafilm in which they are laid "good side" (i.e., the side against the gel during the transfer procedure) up. They are overlaid with 1 ml of TBSA containing 100 μ l normal goat serum and 50 μ l specific antiserum. 1 ml of this antiserum solution is sufficient for one or two samples. The specific antiserum can be diluted or undiluted antiserum can be used in making up the formulation. Undiluted antiserum is used repeatedly to stain five or six sets of blots. In either case, the incubation proceeds for 1.5 h at ambient temperature with gentle agitation, after which the blots are washed with four changes of 50 ml of TBS, 40 min for each change. They are next incubated with peroxidase-coupled goat IgG (3 μ l in 5 ml BSA) for 1 h at ambient temperature and then washed four times with 50 ml TBS (20 min per wash). After rinsing in 50 ml of

10 mM Tris, pH 7.4, for 5 min, the peroxidase stain is developed with 5 ml of 10 mM Tris, pH 7.4, 125 μ l of 1% *o*-dianisidine (made up in methanol), and 125 μ l of 0.3% hydrogen peroxide. When sufficiently developed, the blots are transferred into a large volume of 10 mM Tris, pH 7.4, to stop the reaction and then air-dried. They are stored in the dark in heat-sealed plastic bags.

Antisera

Neurofilament-selective antiserum was obtained by separating the proteins of isolated brain IF preparations as a continuous band in slab SDS gels. Strips taken from the edges of the gels were stained to reveal proteins and, using these strips as a guide, the main part of the gel was cut across to isolate all material with an apparent mol wt >60,000. Either these proteins were recovered from the gel or the gel that contained them was homogenized in a small volume of TBS. In either case, the material was further homogenized with Freund's complete adjuvant (1:1 vol/vol) to form a homogeneous emulsion. 2 ml of this emulsion containing ~1 mg of protein was injected subcutaneously at eight sites into the backs of albino rabbits. The injection schedule began with three inoculations at 14-d intervals followed by further injections at irregular intervals until nine such inoculations had been given over a period of 9 mo. At this time, an antiserum that selectively stained brain neurofilaments in histochemical tests was obtained from one rabbit (27). Anti-GFAP was raised in rabbit against GFAP purified by column chromatography (36). The antitubulin serum was raised against vinblastine-precipitated tubulin from sea urchin eggs (46).

Protein Iodination

A sample of isolated brain IFs containing 2.2 mg of protein was iodinated with carrier-free [¹²⁵I]iodide using the method of Markwell and Fox (26), which gave the best preservation of the SDS-gel profile of the IFs. 2 mg of this preparation was added to 5 g of brain tissue at the start of a subcellular fractionation procedure, immediately after two strokes of the homogenizer.

Electron Microscopy

Intermediate filament fractions were prepared for electron microscopy by the "sandwich" negative-staining procedure of Boublik et al. (5). 5% uranyl acetate was used.

Other subcellular fractions were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, postfixed in 1% osmium tetroxide in the same buffer, block-stained with 2% uranyl acetate, dehydrated via graded alcohols, embedded in Spurr resin (Serva Chemicals, Heidelberg, W. Germany) sectioned, and grid stained with 0.4% lead citrate in 0.1 N NaOH. Block faces were prepared to give a section through the entire pellet depth. Morphometric measurements were made on prints of $\times 100,000$ final magnification with a MOP-Digiplan electronic planimeter (Kontron Instruments, Zurich, Switzerland).

Immunohistochemistry

The procedures were performed as previously described (27); however, to lessen the intrinsic electron-dense staining of the synaptic junctional structures, tissue samples were not stained with uranyl acetate. They were, however, stained on the grid with lead citrate (see above).

RESULTS

Morphology and Protein Composition of Isolated SPM and PSD Fractions

Fig. 1 compares the essential morphological features of isolated SPMs and PSDs that have been established in several previous studies (7, 8, 28, 29, 49). The apparent morphological purity of the PSD subfraction prepared by Triton X-100 treatment and mild salt washing (7) can be judged from Fig. 2. The polypeptide content of the SPM and PSD fractions is shown in Fig. 3. The PSD fraction contains the characteristic ~50,000-dalton "major" PSD protein, a band at 46,000 daltons and the doublet 280,000-, 270,000-dalton proteins that also occur in SPM and myelinated axons and more faintly in brain IFs and purified myelin (Fig. 5, slots 7-9). Also enriched in PSD, as compared with SPMs, were bands with apparent molecular weights of 180,000, 143,000, 136,000, 113,000, 62,000, and

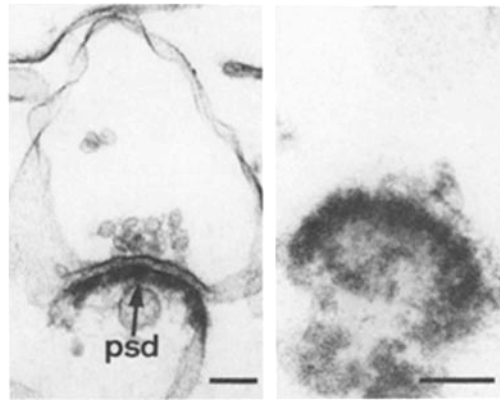


FIGURE 1 Morphological features of isolated SPM (left) and PSDs (right). The plasma membranes contain a PSD (*psd*) as an intrinsic structural element attached to the postsynaptic junctional membrane. Bars, 100 nm.

59,000. On the other hand, the PSD preparations were depleted compared with SPMs in bands of 170,000, 65,000, 52,000 (doublet), 43,000, 41,000, 39,000, and 36,000 daltons.

One of the problems with biochemical studies of the PSD has been the lack of uniformity in the methods used for their isolation. A variety of detergents have been used (7, 8, 29), and the one detergent used by all authors, Triton X-100, has been applied inconsistently, sometimes being used in concentrations that isolate PSDs (7, 28) and sometimes in concentrations deliberately chosen to leave the junctional plasma membrane undissolved, yielding the so-called synaptic junctional complex (SJC) preparation (22, 42, 49). Despite this, a consensus is emerging regarding the identity of characteristic synaptic junctional proteins. Table I compares the molecular weights of PSD and SJC proteins found in this study and by various other authors. The features common to all reports are the major ~50,000 band, a ~45,000 band, which is almost certainly actin (3, 22), and material migrating with an apparent molecular weight of ~100,000. Bands with mol wts of 55,000 (probably tubulin, see references 22 and 28) and a prominent high mol wt doublet, variously reported with mol wts from 185,000 to 280,000, are also usually found. Another common feature is the absence from the PSD of several prominent SPM proteins with molecular weights between 30,000 and 46,000, which is also noticeable in our preparations (Fig. 3).

Fig. 3 also shows that the various SPM and PSD proteins could be blotted onto cellulose nitrate. Most of the bands transferred with little loss of resolution but there was a small proportion which failed to appear in the blot. The most notable absentees were the 143,000 and 136,000 components of the PSD fraction (marked with a triangle in Fig. 3) and the more slowly migrating member of the 52,000-dalton doublet from the SPM fraction. There are several plausible explanations for the absence of these components. They may not stick well to cellulose nitrate, or they may be poorly stained by amido black compared with Coomassie Blue, or they may not migrate out of the polyacrylamide gel efficiently. Whatever the reasons, none of the polypeptides involved in the present study appeared to be affected by this difficulty.

Morphology and Protein Composition of Brain Intermediate Filament Preparations

The brain IF preparations contained intermediate-sized fil-

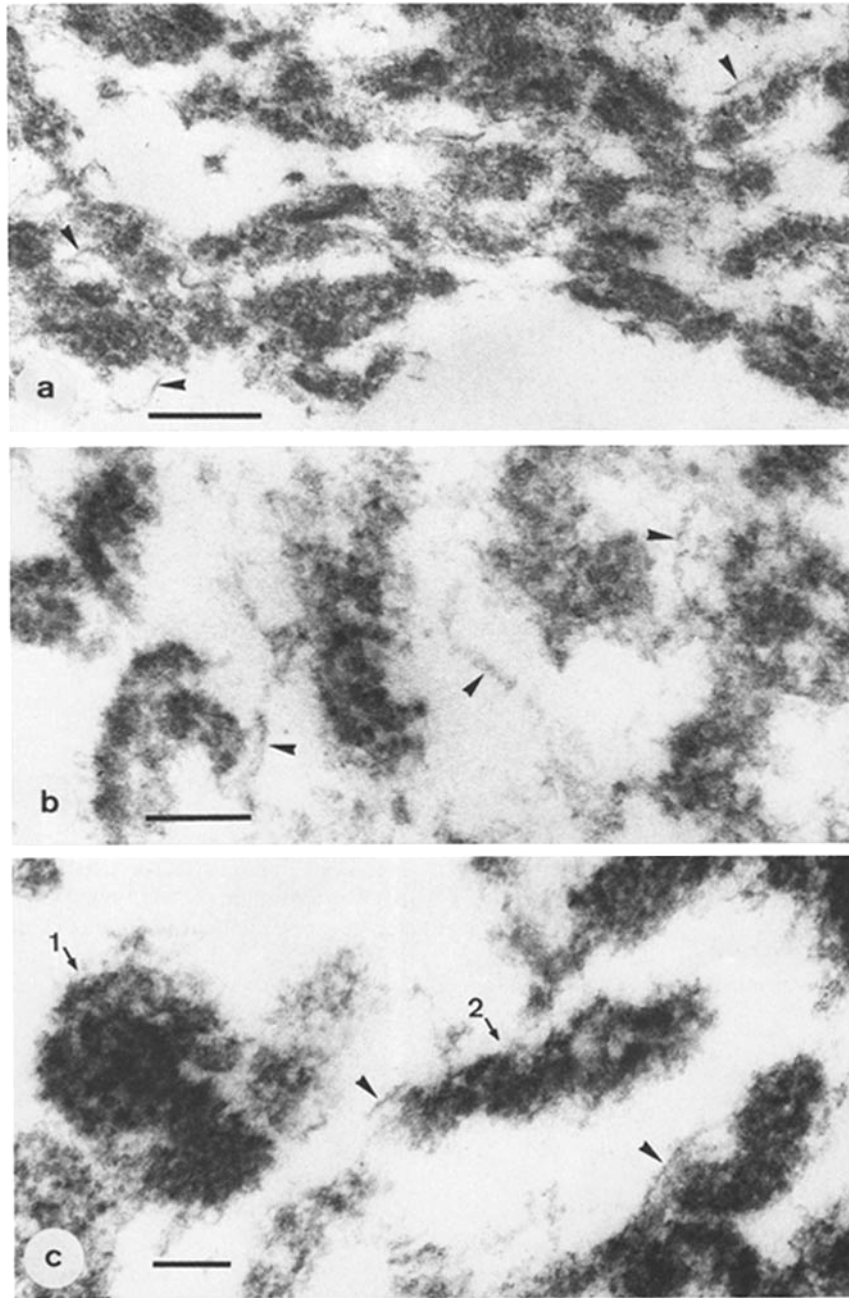


FIGURE 2 Isolated PSDs from some of the preparations used in this study. In each micrograph, intermediate-sized filament fragments stuck to PSDs are indicated by arrow heads. In *c*, PSDs cut en face (1) and in transverse section (2) are displayed. Bars, *a*, 500 nm, *b* and *c*, 100 nm.

aments both in bundles (Fig. 4*a*) and in loose aggregates (Fig. 4*b*). The filaments in our preparations had the unbranching flexible morphology which is characteristic of both glial and neuronal filaments in whole tissue (34, 35, 50, 51) and which has been previously remarked upon in isolated brain IF preparations (11, 40, 53).

Electrophoretic analysis revealed prominent IF proteins with apparent molecular weights of 210,000, 160,000, 68,000, and 53,000 (Fig. 5, slot 9). Additional faint bands had calculated molecular weights of 280,000, 270,000, 64,000, and 48,000. The 48,000 band is probably a myelin contaminant because one of the major polypeptides of purified rat brain myelin migrates with this apparent molecular weights in our electrophoresis

system (Fig. 5, slot 7). The high molecular weights doublet (280,000 and 270,000 daltons) components also probably do not belong specifically to the filaments because they are prominent in unlysed myelinated axon (Fig. 4, slot 8) where the characteristic filament components are weak.

Antiserum Staining of SDS-Polyacrylamide Gels

The staining of brain IF proteins by anti-NF and anti-GFAP sera is shown in Fig. 5. We also tested, in adjacent channels of the same polyacrylamide gel, samples of unlysed myelinated axons and of purified myelin. We thus had sets composed of these three samples. Three such sets were run in a single slab

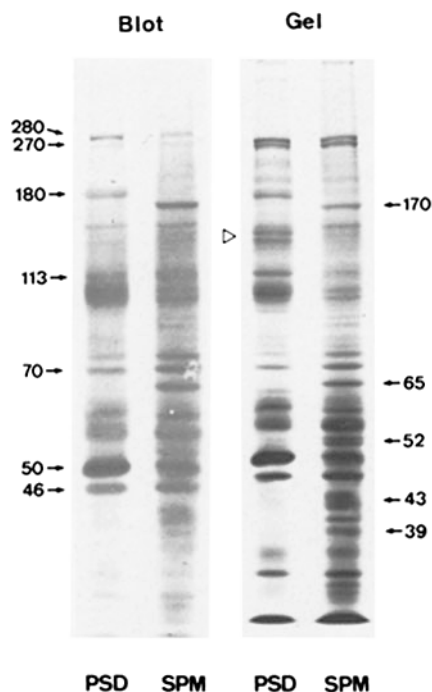


FIGURE 3 Protein components of PSD and SPM fractions separated in 7.5–15% SDS-polyacrylamide gels (25). The pattern is shown twice. On the right-hand side is the result obtained by Coomassie Brilliant Blue staining of a gel (under the heading Gel), whereas on the left is shown a second pair of samples that were electrophoretically transferred onto a nitrocellulose sheet and then stained with amido black (under the heading Blot). The calculated molecular weights of some prominent PSD bands (daltons $\times 10^{-3}$) are indicated on the left and those for some prominent SPM bands on the right.

TABLE I
Reported Molecular Weights of PSD Proteins *

Matus et al. †, §	Cohen et al. (7) §	Kelly and Cotman (22) , ¶	Wang and Mahler (49) , **	Therien and Muyschinski (42)
<280,000	185,000 ‡‡	≥200,000 ‡‡	225,000 ‡‡	
270,000				
180,000			175,000	
143,000	150,000		140,000	
136,000			135,000	
113,000			110,000	
110,000	100,000	95,000	105,000	97,000
70,000			68,000	
59,000	59,000		61,000	
55,000	55,000	56,000		55,000
50,000	51,000	52,000	54,000	50,000
46,000	45,000	45,000	47,000	45,000
36,000	§§		33,000	
30,000	31,000	30,000	28,000	

* Probable equivalent bands are presented on the same line with the calculated molecular weights reported by each set of authors.

† Results from this study. These are the calculated molecular weights of bands found reproduced in 11 separate PSD preparations taken from 40 SDS gels.

§ Triton X-100 PSDs used.

|| Triton X-100 SPCs used.

¶ Sarcosinate PSDs used.

** Deoxycholate PSDs used.

‡‡ Closely spaced doublet.

§§ Cohen et al. (7) also report PSD components with molecular weights of 43,000 and 40,000.

gel which was afterward cut so that each set could be handled independently. One set was conventionally stained with Coomassie Brilliant Blue (Fig. 5, headed CBB), whereas the other two sets were transferred onto cellulose nitrate membranes to give two electrophoretic blots, one of which was stained with anti-NF and the other with anti-GFAP. The results reported here are drawn from 34 Coomassie Blue-stained gels, 9 amido black-stained blots, 11 anti-NF-stained blots, and 3 anti-GFAP-stained blots.

Fig. 5 shows that the two antisera stained different, and apparently mutually exclusive, subsets of the brain IF proteins. Anti-GFAP stained most strongly the 53,000-dalton band and two further bands with apparent molecular weights of 45,000 and 43,000 (Fig. 5, slot 3). Bands comigrating with these molecular weights were also faintly stained in myelinated axons (Fig. 5, slot 2). There was no distinct staining of the purified myelin sample (Fig. 5, slot 1). In myelinated axons, but not in brain IFs or purified myelin, anti-GFAP stained faintly two bands with apparent molecular weights of 69,500 and 67,000.

Our anti-NF serum stained a number of proteins in brain IF preparations, among which the most prominent have molecular weights of 210,000, 185,000, 170,000, 160,000, and 68,000 (Fig. 5, slot 6). The same antiserum produced faint staining of several bands in myelinated axon samples and faintly stained one band, at 68,000 daltons, in preparations of purified myelin (Fig. 5, slots 4 and 5).

Antiserum Staining of Brain Subcellular Fractions

The neurofilament-selective antiserum was next tested against brain SPM or PSD preparations. For this purpose, samples of IF, SPM, and PSD preparations were run in adjacent channels of a slab gel. The same set of three samples was repeated three times in the same gel to give one set for Coomassie Blue staining, one set for staining with anti-NF (Fig. 6), and one set for staining with amido black, to check the blotting efficiency (not shown). The results are drawn from experiments with eight separate SPM and PSD preparations and four anti-NF stained SDS gel blots.

The polypeptide composition of each of the three subcellular fractions (IFs, SPMs, and PSDs) was consistent throughout the various preparations used in our studies (cf. the protein-stained images in Figs. 6 and 7 which use separately prepared subcellular fractions). As before, staining of IF preparations with anti-NF serum produced distinct staining of bands with apparent mol wts of 210,000, 185,000, 170,000, 160,000, and 68,000 daltons. In this case, the 170,000 component was resolved into a narrowly spaced doublet of equal anti-NF staining intensity (Fig. 6, slot labeled IF, left-hand side). There was little anti-NF staining of SPM proteins at the gel loadings used here, a reaction at the threshold of detectability occurring in the 210,000- and 68,000-dalton band positions. By contrast, the PSD sample, loaded with the same amount of protein as the SPM, showed strong anti-NF staining of the 210,000, 185,000, 160,000, and 68,000 bands (Fig. 6, slot labeled PSD, left-hand side). It is noticeable that the 170,000 mol wt band stained by anti-NF in IF preparations does not appear in the PSD sample. This result was consistently obtained with all the PSD preparations tested.

As a control, we used antiserum against glial filament protein (anti-GFAP) with the expectation that if PSDs were present only in neurons and if glial filaments were limited to glial cells,

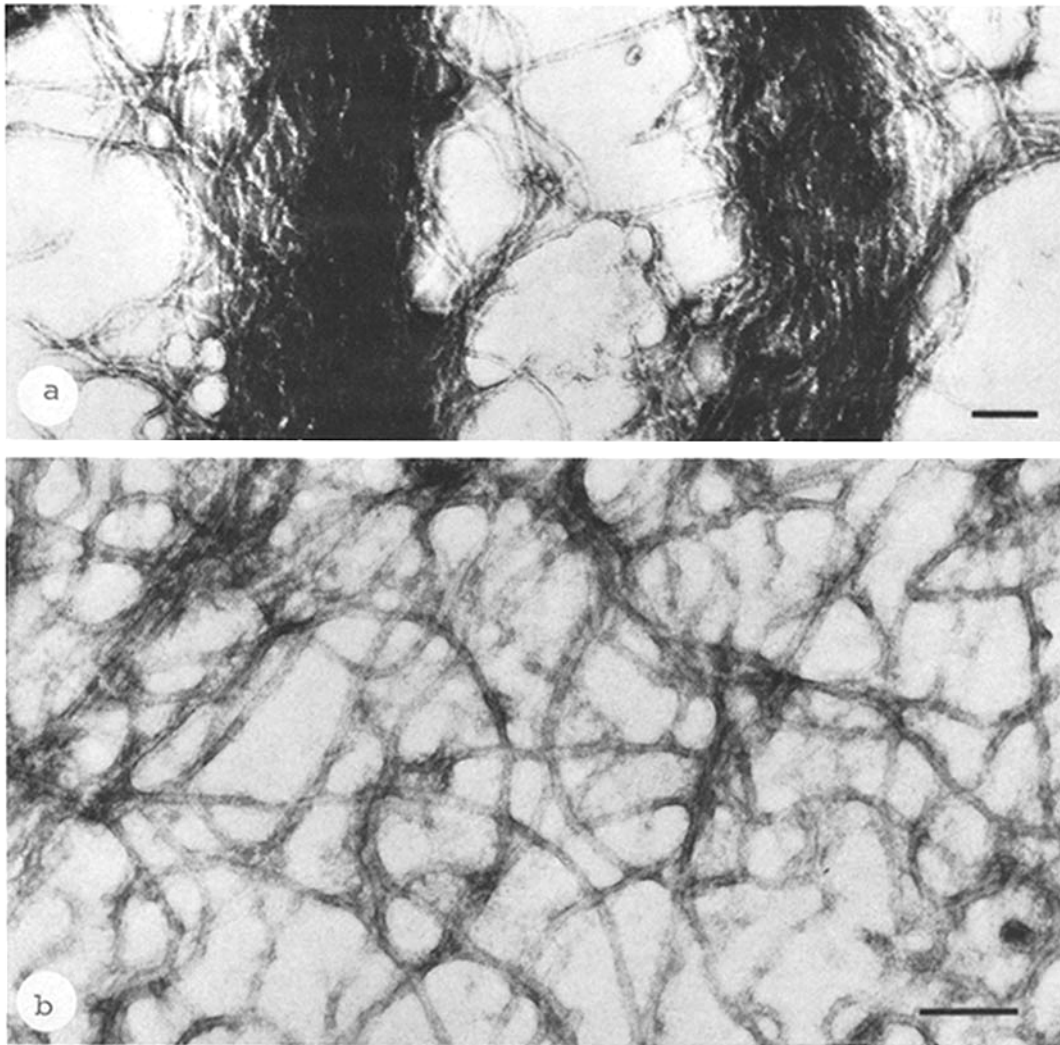


FIGURE 4 Intermediate-sized filaments isolated from rat brain stems as revealed by electron microscopy after negative staining with uranyl acetate. The preparations contain filaments in tight bundles (a) and in loose aggregates (b). In a planimeter analysis of five 400×600 nm randomly selected fields, filaments accounted for $88 \pm 4\%$ of the particulate material. Bars, 100 nm.

then PSDs should not contain glial filament protein. As Fig. 7 shows, that expectation was confounded. Each of the protein bands stained by anti-GFAP in brain IFs was also stained by this antiserum in isolated brain PSDs. The 53,000 band (GFAP itself) was also detectable in SPM preparations. SPM proteins of 72,000, 70,000, and 65,000 mol wts also stained faintly with anti-GFAP.

Immunohistochemical Staining of Histological Brain Sections

Taken at face value, the results presented so far suggest that brain PSDs contain polypeptides characteristic of both brain neuronal and glial cytoplasmic filaments. This, however, contradicts previous indications that antisera against GFAP and against neurofilament proteins do not react with brain PSDs in whole tissue when tested by immunohistochemical staining (27, 39). Because of this conflict, we reexamined in detail the immunohistochemical staining of PSDs in intact brain tissue by both antisera.

The anti-GFAP staining of histological sections of perfusion-fixed brain is shown in Fig. 8. Examination by light microscopy

shows staining of glial cell bodies (arrowheads) and of an envelope around blood vessels, which is consistent with the staining of the astroglial endfeet that are known to occur with this distribution (35). The latter proposition was confirmed by subsequently processing the same material for electron microscopy. Fig. 8b shows an anti-GFAP-stained astroglial process in contact with the endothelial lining of a blood vessel. This field was selected because the stained astroglial process is in close proximity to a synapse. None of the elements of this synapse, including the PSD, is decorated with anti-GFAP-induced peroxidase reaction product. Anti-GFAP-stained tissue from cerebral cortex, caudate nucleus, and cerebellum was surveyed. Two well-stained vibratome sections from each area were processed for electron microscopy and material from them was examined. In all, 37 ultrathin sections of immunoperoxidase-stained brain tissue were searched for evidence of anti-GFAP-stained PSDs, but none was found.

We next tested the anti-NF serum by immunohistochemistry. In the cerebellum, this antiserum strongly stains the neurofilament-rich basket cell axons around Purkinje cells (Fig. 9a). When such areas are found in the electron microscope, the identity of the stained elements, such as basket cell axons, is

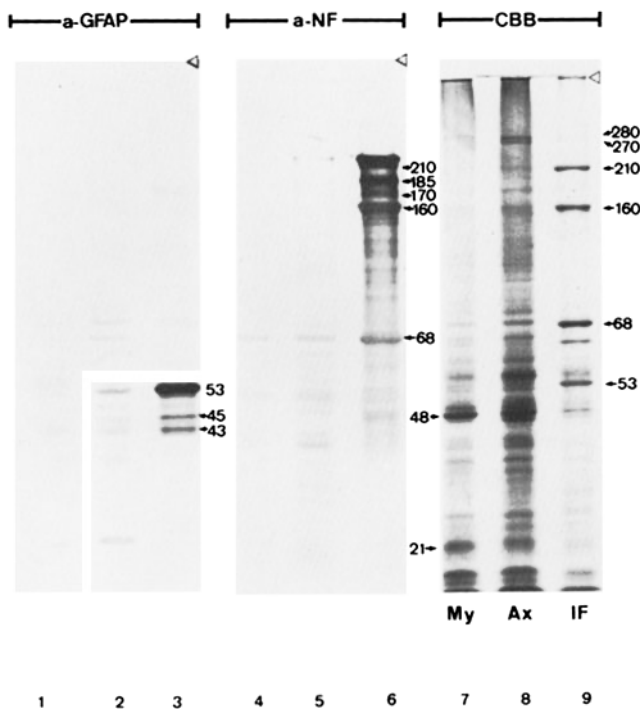


FIGURE 5 Immunoblot staining of brain intermediate filament proteins by two antisera, one against glial fibrillary acidic protein (a-GFAP) and the other against neurofilaments (a-NF). Each antiserum was tested against three different brain subcellular fractions; myelin (My), intermediate filaments (IF), and myelinated axons (Ax) before their separation into My and IF. Samples of each fraction were separated in adjacent channels of an SDS-polyacrylamide gel. The protein patterns after staining with Coomassie Brilliant Blue (CBB) are shown on the right-hand side for myelin (slot 7), myelinated axons (slot 8) and IF (slot 9). Four further sets of these three samples were prepared and blotted onto cellulose nitrate sheets by transverse electrophoresis. Two sets were stained with a-NF and two by a-GFAP using the immunoperoxidase procedure. The a-GFAP-stained data (slots 1-3) is a composite of the two sets. The a-NF-stained data appears in slots 4, 5, and 6. The data for each antiserum were congruent in each of the two sets and in further immunoblotting tests of intermediate filament proteins by a-NF (11 blots) and a-GFAP (3 blots). Note the different staining of IFs by a-GFAP (slot 3) and a-NF (slot 6). Molecular weights of prominent bands are given in daltons $\times 10^{-3}$. The origins of the electrophoretic gels in each case are indicated by triangles. Loadings: My, 30 μ g; Ax, 30 μ g; IF, 10 μ g.

readily apparent, as is the fibrous nature of their stained contents (Fig. 9 *b*, and reference 27). Fig. 9 *c* shows an example of a basket cell axon with anti-NF-stained cytoplasmic contents that lies in the vicinity of synapses that are not stained by the antiserum. In the same field, astrocytic processes occur that are unstained by the anti-NF serum, which is thus truly selective for neuronal cytoplasm filaments.

40 anti-NF-stained ultrathin sections of cerebellar cortex were examined for evidence of reactive PSDs. A further 40 ultrathin sections of two separate, well-stained samples of cerebral cortex and 40 ultrathin sections from two samples of caudate nucleus were examined. Fig. 10 shows representative results obtained with the cerebral cortex. In all this material, no anti-NF-stained PSDs were found. Throughout, the anti-NF serum consistently stained neurofilaments and not glial filaments.

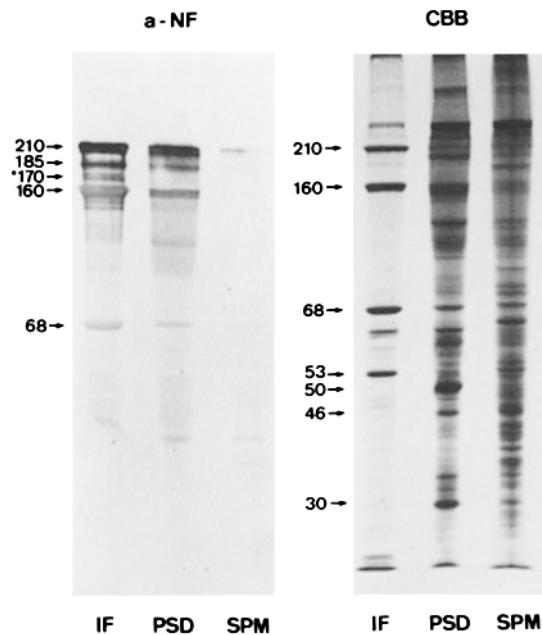


FIGURE 6 Immunoblot demonstration of neurofilament proteins in the PSD. Samples of IF, SPM, and PSD were run in adjacent slots of a slab gel. One set was stained with CBB. A further set was blotted onto cellulose nitrate and stained with a-NF. Each of the a-NF reactive bands occurs in the PSD fraction, except the 170,000 doublet (*). Reaction of the SPM proteins is barely detectable. Congruent results were obtained with five separate PSD/SPM preparations. Loadings: IF, 10 μ g; PSD, 35 μ g; SPM, 35 μ g.

Evidence that PSDs are Immunohistochemically Stainable

This succession of negative results might suggest that the PSD is unstainable by immunoperoxidase procedures were it not that it has been convincingly stained in past studies with antiserum against tubulin (30, 48) and more recently with antisera against the synaptic phosphoprotein I (4) and calmodulin (52). To demonstrate the feasibility of immunoperoxidase staining the PSD with our present techniques, we repeated our former experiments with antiserum against tubulin. The anti-tubulin serum was applied to a section of cerebellum (Fig. 11) and gave results very different from those obtained with anti-NF. Instead, the antitubulin serum most strongly stained the microtubule-rich dendrites of Purkinje cells (Fig. 11 *a*). In the electron microscope, dendrites cut in transverse section showed staining of round profiles of approximately microtubular dimensions (Fig. 11 *b*), whereas examples cut in longitudinal sections showed clearly the microtubular nature of the stained elements (Fig. 11 *c*). In both these examples adjacent myelinated axons are present whose neurofilament contents are unstained by the antitubulin serum (compare with the anti-NF stained example in Fig. 10 *c* where the neurofilaments are stained). In both examples, synapses are present whose PSDs are distinctly and selectively stained compared with other parts of the synapse, such as the synaptic cleft (see particularly Fig. 11 *b*). Compare the way in which the stained PSDs shown here are far more electron dense than the contents of the synaptic cleft, whereas in examples shown in Figs. 8-10, where antisera are used that do not stain the PSD, the level of electron density of the PSD and synaptic cleft material is comparable (see particularly Figs. 8 *b* and 10 *c*).

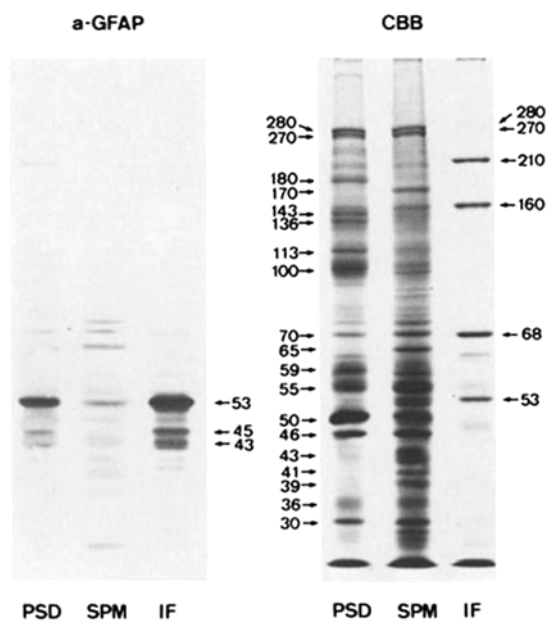


FIGURE 7 Immunoblot demonstration of α -GFAP reactive proteins in PSD, SPM, and IF preparations. The experiment is equivalent to the one shown in Fig. 6, except that α -GFAP was used instead of α -NF to develop the immunoblot. GFAP (53,000 daltons) appears in both SPM and PSD, more strongly in the latter. This experiment was performed on three separate PSD/SPM preparations, which gave congruent results. Loadings: PSD, 35 μ g; SPM, 35 μ g; IF, 10 μ g.

Contamination of Isolated PSDs by Intermediate Filaments

Our observations now were that antisera against neurofilament and glial filament proteins reacted with protein components of isolated PSDs, as shown by immunochemical staining of gel blots, but did not react with PSDs in histological sections of intact brain tissue. There were two possible explanations for this apparent contradiction. Either (a) the antigens stained in the SDS gel blots are inaccessible to the antiserum in tissue sections or (b) the neuronal and glial filament antigens are indeed absent from PSDs in intact tissue and become artifactually associated with isolated PSDs during subcellular fractionation. To test the latter possibility, we prepared brain IFs and iodinated them with 125 I. This preparation was separated in an SDS gel and stained to confirm that it contained the usual neurofilament and glial filament proteins (Fig. 12, right-hand slot). Other samples of the same preparation were run in gels that were dried and autoradiographed. The autoradiograms showed that each of the prominent neurofilament and glial filament bands was iodinated and had apparently survived the labeling process without suffering extensive degradation (Fig. 12, slot labeled 125 I and IF). A considerable quantity of radioactivity ran near the gel front.

Part of this brain IF preparation was added at the beginning of a PSD isolation schedule. From the ensuing procedure, aliquots of various subcellular fractions were collected. Fig. 12 shows an SDS slab gel in which four of them, myelin, mito-

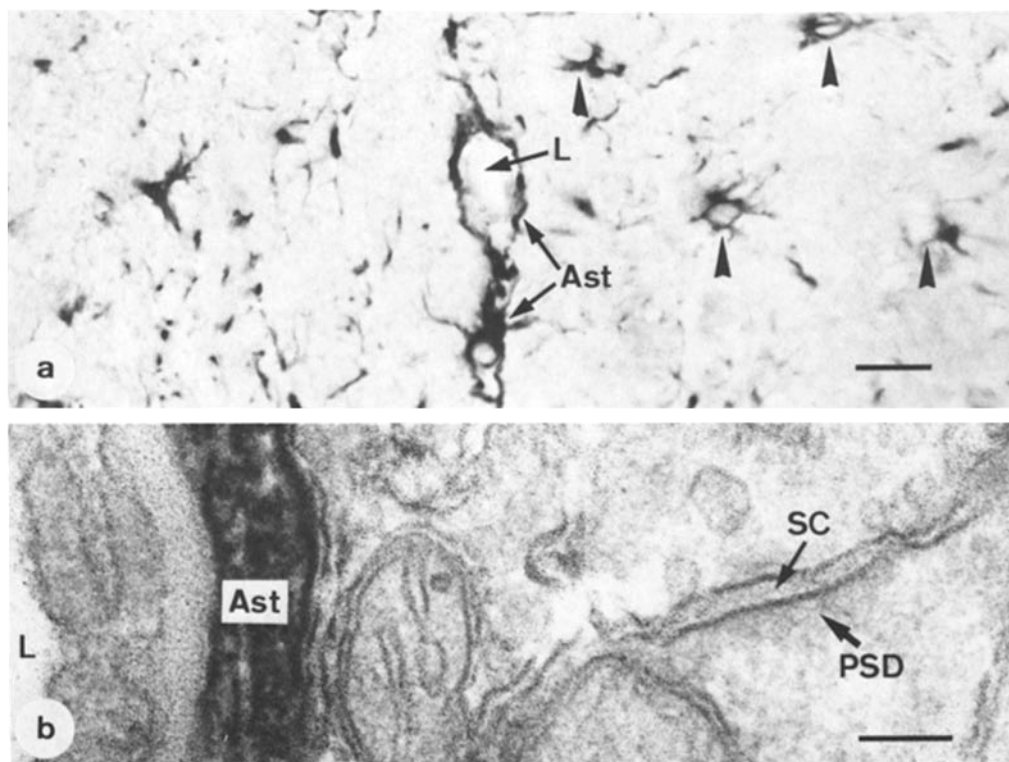


FIGURE 8 Immunohistochemical staining of rat cerebral cortex with α -GFAP. (a) A 40- μ m vibratome section viewed in the light microscope. The section is coronal with the upper border of the corpus callosum showing at the left, pia to the right. Astrocytic cell bodies (arrow heads) and their proximal processes are stained, as are astrocytic endfeet (Ast) surrounding blood vessels (L, lumen of blood vessel). Bar, 10 μ m. (b) An ultrathin section from the same vibratome slice. A stained astrocytic endfoot (Ast) is applied to a blood vessel (L, lumen). At an adjacent synapse, none of the junctional structures, including the PSD, are stained (SC, synaptic cleft). Bar, 100 nm.

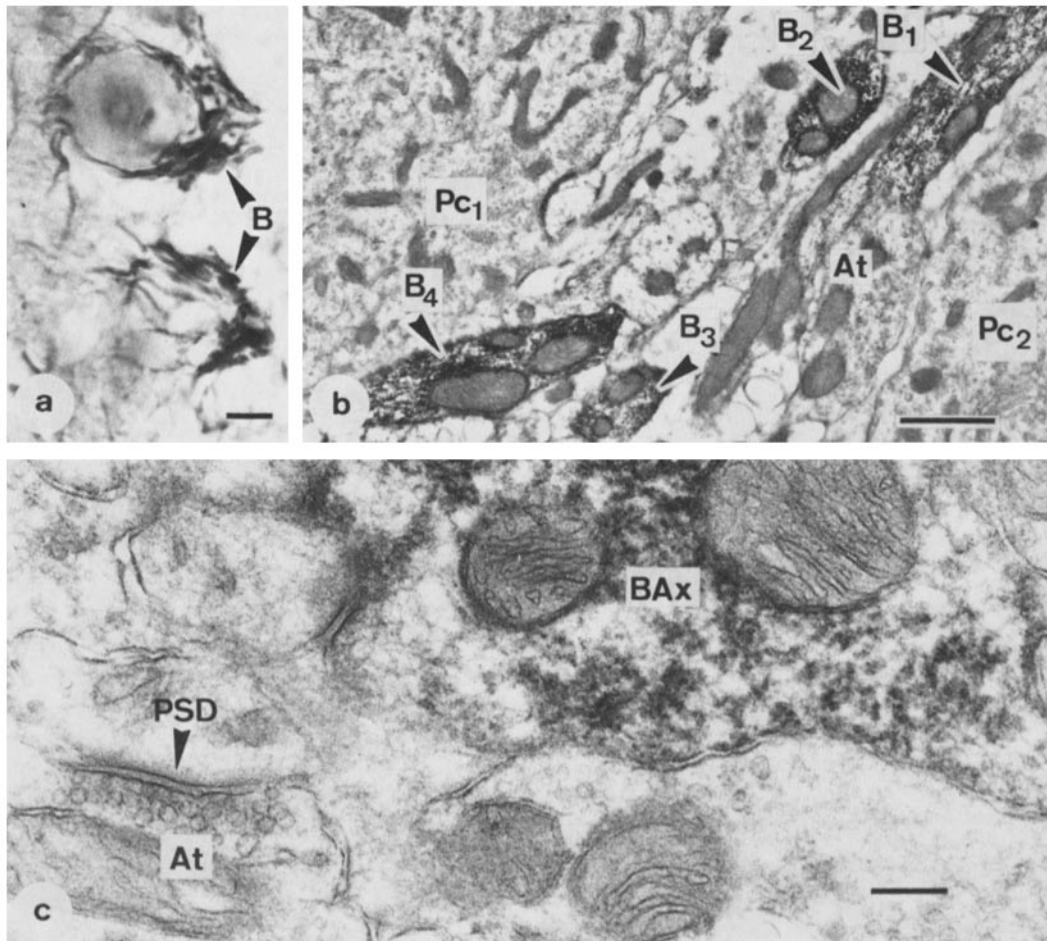


FIGURE 9 Immunohistochemical staining of cerebellar cortex with a-NF. (a) Light micrograph including two Purkinje cells with a-NF-stained basket cell axons (B). The lower example is a glancing section which shows the perikaryal basket rather than the cell body itself. Bar, 9 μm . (b) Electron micrograph of the area between two Purkinje cells (Pc_1 and Pc_2) showing basket cell axons (B_1 – B_4) containing stained neurofilaments. B_1 has an axon terminal (At) within the plane of section, verifying that it is a neuronal and not a glial process. Bar, 1 μm . (c) A basket cell axon (BAx) contains a-NF-stained material. At an adjacent synapse (axon terminal, At) the PSD is not peroxidase labeled. Bar, 200 nm.

chondria, SPM, and PSD, were separated, yielding their usual characteristic SDS gel polypeptide patterns (Fig. 12, CBB, left-hand side). This gel was then dried and autoradiographed (Fig. 12, ^{125}I , slots 1–4). Labeling was barely detectable in the myelin, mitochondrial, and SPM fractions, but the PSD fraction was strongly labeled. However, the ^{125}I -labeling pattern in the PSD was not identical to that found in the original labeled IF preparation. In the PSDs, the radioactive material near the electrophoresis front was less heavily represented. Also, the glial filament bands (53,000 and the 46,000, 43,000 pair) were much more strongly represented in the isolated PSDs than in the original exogenous labeled IF preparation.

Comparison of PSDs from SPM and Synaptosomes

The results obtained with ^{125}I -labeled brain IFs suggest that both neuronal and glial filaments stick selectively to PSDs in broken cell preparations. One possibility we considered was that the filament contamination in PSD, revealed by immunohistochemical staining of SDS gel blots, might arise by the release of filament material from vesicularized glial and neuronal fragments present in the P_2 fraction during the lysis step, which

frees the plasma membranes from their cytoplasmic contents. If this were true, it might be possible to avoid the filament contamination by preparing PSDs directly from unlysed synaptosomes (7). To test this possibility, we fractionated 12 g of rat brain up to the P_2 fraction, then processed one portion by our usual method to provide SPM and the other portion without lysis to obtain synaptosomes. From both SPM and synaptosomes, we then made PSDs by the methods of Cohen et al. (7). Each of the four samples was separated in adjacent slots of an SDS slab gel and three such gels were prepared. One was stained with Coomassie Blue (CBB, Fig. 13). Comparing SPM with synaptosomes (slots 1 and 2) shows them to contain similar populations of proteins. Bands with apparent molecular weights of 88,000, 55,000, and 30,000 are proportionately more greatly represented in synaptosomes than in SPM. Comparing PSDs made from synaptosomes (Fig. 13, CBB, slot 4) with PSDs made from SPM (slot 3) shows that the former contain a substantially higher proportion of material in the 55,000- and 30,000-dalton bands. There are no components that are eliminated from isolated PSD as a result of making them from synaptosomes rather than SPM.

Staining this combination of samples with anti-GFAP shows that substantially more GFAP is present in synaptosome prep-

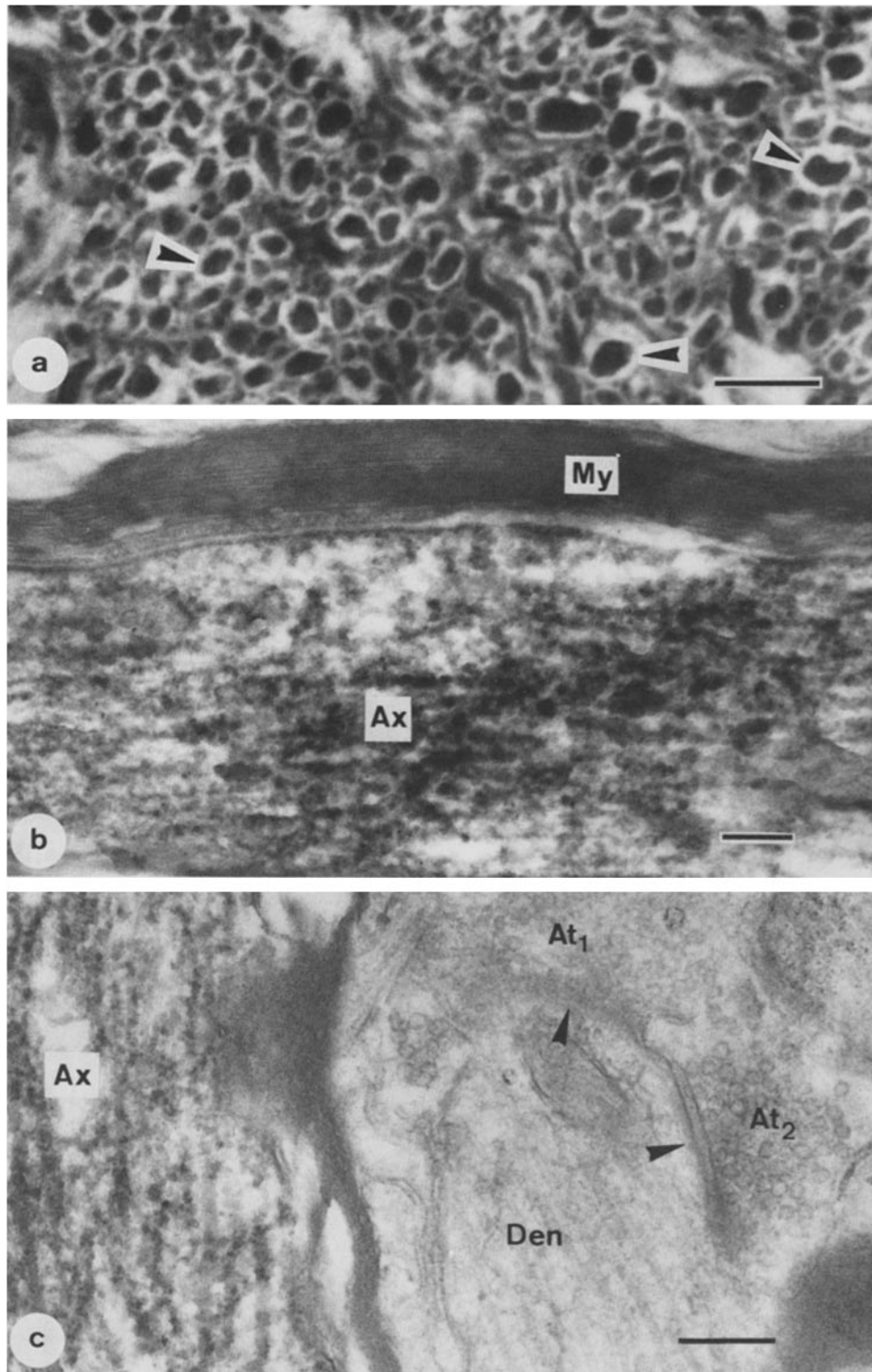


FIGURE 10 Immunohistochemical staining of rat cerebral cortex with a-NF. (a) Transverse section of the corpus callosum. The neurofilament-rich cores of myelinated axons are stained, whereas the myelin sheaths (arrow heads) are not. Bar, 5 μ m. (b) A single myelinated axon in the electron microscope. Filamentous material in the axoplasm (Ax) is labeled with a-NF-induced peroxidase reaction product. The myelin itself (My) shows only osmium/lead staining. Bar, 200 nm. (c) A myelinated axon (left) shows a-NF staining of filamentous material in its axoplasm (Ax). An adjacent dendrite (Den) contains microtubules that are unstained. Two axon terminals (At₁ and At₂) make synaptic contacts with this dendrite. In neither case are elements of the synapse a-NF stained. Bar, 200 nm.

arations (Fig. 13, a-GFAP, slot 2) than in SPM (slot 1). Similarly, the GFAP content of synaptosome-derived PSDs (slot 4) is higher than that of those prepared from SPM (slot 3). A similar result was found when this combination sample

was stained with anti-NF (a-NF, Fig. 13). Anti-NF staining was undetectable in SPM but clearly present in synaptosome preparations. Anti-NF staining was also stronger in synaptosome-derived PSDs than those made from SPM.

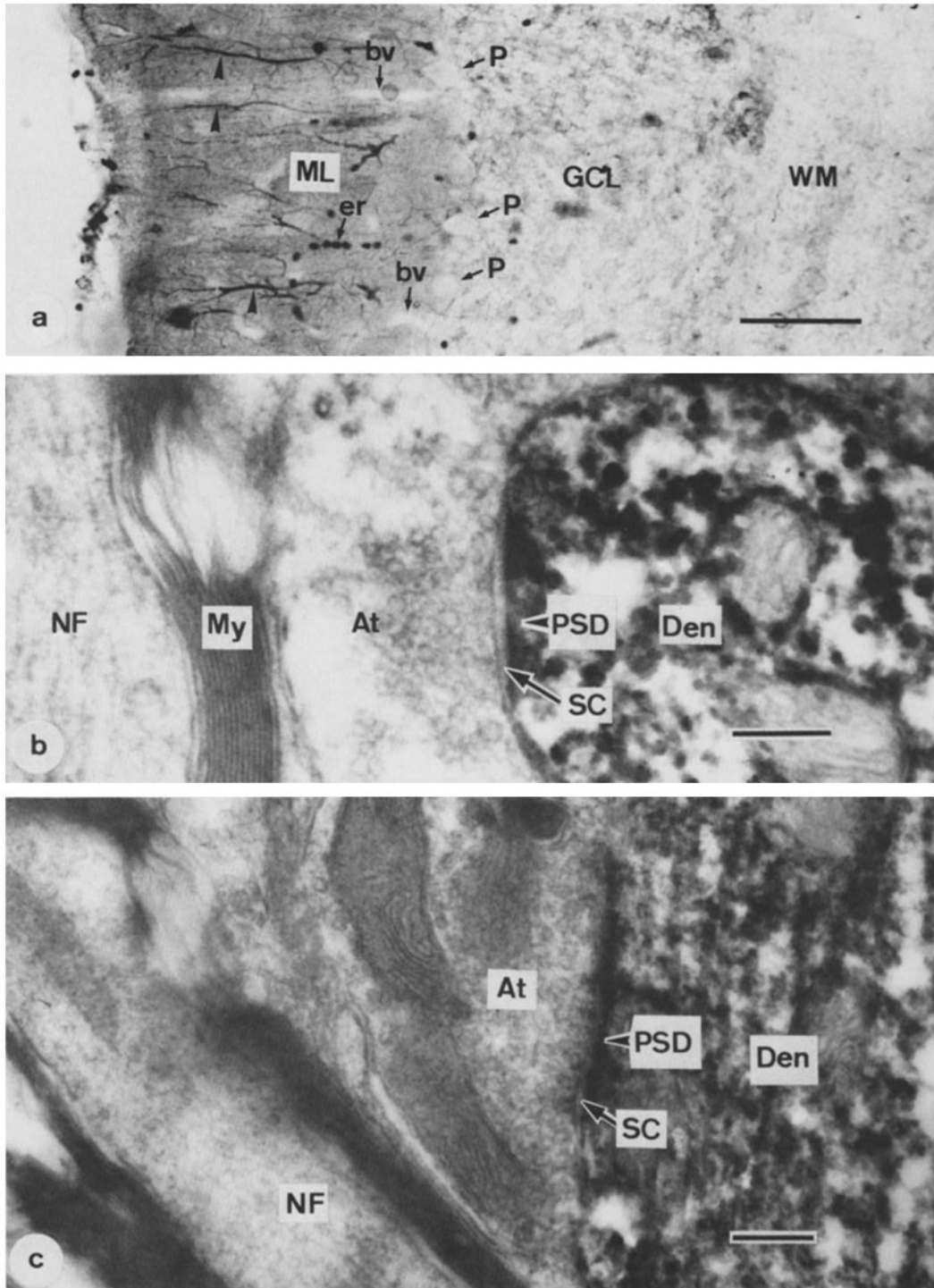


FIGURE 11 Immunohistochemical staining of cerebellar cortex with antitubulin. (a) Viewed in the light microscope there is little staining of white matter (WM) or granule cell layer (GCL). In the molecular layer (ML), Purkinje cell dendrites are stained (arrow heads), whereas Purkinje cell bodies (P) are not. Some erythrocytes (er) in blood vessels (bv) show endogenous (i.e., nonimmune) peroxidase reaction. Bar, 100 μ m. (b) An axon terminal (At) synapsing with a Purkinje cell dendrite (Den) that contains antitubulin stained microtubules cut in transverse sections. Mitochondria are unstained. Staining is evident in the PSD right up to the postsynaptic plasma membrane but is absent from the postsynaptic surface in the synaptic cleft (SC). Note the absence of antitubulin staining on the neurofilaments (NF) in an adjacent myelinated (myelin, My) axon. Bar, 200 nm. (c) A further example of a Purkinje cell dendrite, this time cut longitudinally, which contains stained microtubules. An axon terminal (At) makes a synapse whose PSD is stained. The synaptic cleft (SC) twists out of the plane of section. It was examined in detail with goniometer tilting and found not to contain peroxidase label. Note again the lack of neurofilament staining (NF). Bar, 200 nm.

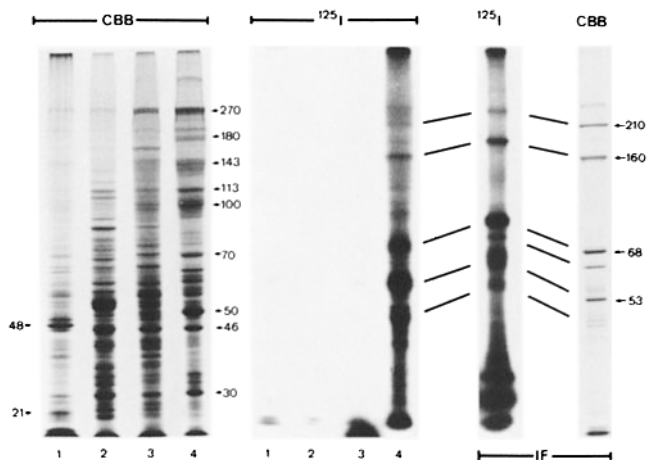


FIGURE 12 The selective contamination of isolated PSDs by brain IF proteins. An isolated brain IF preparation was labeled with ^{125}I . A gel autoradiogram (labeled ^{125}I and IF, right-hand side) shows that each of the neurofilament triplet proteins (210,000, 160,000, and 68,000 daltons) and GFAP (53,000 daltons) and its subjacent degradation products are labeled. Note the low molecular weight label running at the dye front. On the left-hand side is shown an SDS slab containing samples of brain subcellular fractions made in the presence of ^{125}I -labeled IFs. Protein staining (CBB) shows characteristic band patterns for myelin (slot 1), mitochondria (slot 2), SPM (slot 3), and PSD (slot 4). This gel was then dried and autoradiographed (under the heading ^{125}I). Labeling is barely detectable in slots 1–3, whereas each of the ^{125}I neuronal and glial filament proteins is represented in the PSD fraction (slot 4). Note the reduction in ^{125}I migrating at the dye front. Loadings: all slots 40 μg .

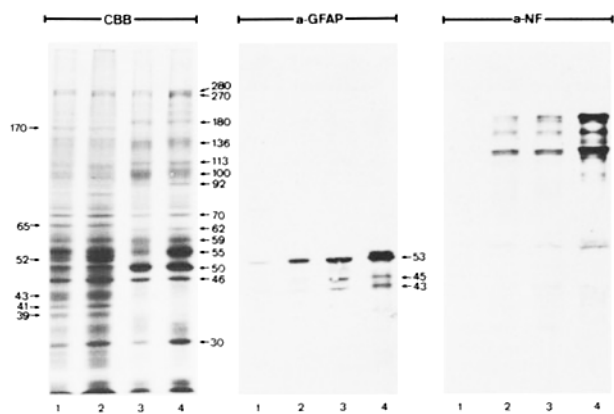


FIGURE 13 SDS-gel electrophoresis of proteins from SPM (slot 1), synaptosomes (slot 2), PSDs made from SPM (slot 3), and PSDs made from synaptosomes (slot 4). Bands with mol wts 92,000, 55,000, and 30,000 are particularly increased in synaptosomal-PSDs as compared with SPM-PSDs (cf. slots 3 and 4 under the heading CBB). Under the headings a-GFAP and a-NF are two immunoblots made from identical samples to those shown protein-stained under CBB. Notice the increase in both a-NF and a-GFAP staining of their respective antigens in synaptosomes compared with SPM (slots 1 and 2) and synaptosomal PSD compared with SPM PSDs (slots 3 and 4). Loadings: all slots 35 μg .

Examination of PSD Preparations for Intermediate Filaments

Because our data suggest that preparations of isolated PSD are contaminated by brain intermediate filaments, we reexamined our PSD preparations for morphological evidence of

intermediate-sized filaments. As Fig. 2 shows, such filaments were readily detectable. They typically appeared as short stretches of 10-nm filament adhering to a PSD (Fig. 2*b* and *c*). Nothing about their total length can be inferred from examining ultrathin sections, but previous studies using replica techniques suggest that they may be of substantial extent (7).

DISCUSSION

Initial attempts to identify the protein components of isolated PSDs produced more confusion than enlightenment. The “major” ~50,000-dalton PSD protein was first thought to be tubulinlike (13, 48), then a neurofilament component (3, 54), and presently lacks any affiliation outside its synaptic context (22, 28). The most recent development is that this 50,000-dalton protein seems to be a major PSD component only in the forebrain; in PSDs isolated from cerebellum, it is far less prominent (6; Pehling and Matus, unpublished observations).

To overcome the lack of uniformity in methods used to isolate PSDs, we recently examined the effect of a variety of detergents upon PSD structure (28). We found, in agreement with Cohen et al. (7), that Triton X-100 gives the best preservation of PSD morphology, whereas more powerful detergents, such as deoxycholate (7, 29) and sarcosinate (8), extract more proteins from the PSD and leave a less electron-dense fibrous structure, which may be a cytoskeletal basis for the PSD. We refer to this as the postsynaptic junctional lattice (28). Using Triton X-100 exactly as described by Cohen et al. (7), we obtain PSD preparations which are highly reproducible with respect to morphology (Fig. 2) and protein composition (Table I). Among the proteins, tubulin (the ~55,000 band) seems to be established as a minor PSD component on the basis of biochemical (22) and immunohistochemical (references 30 and 48 and this study) evidence. The ~45,000 band in Triton X-100-derived PSDs is almost certainly actin (3, 22), but it is not present in the postsynaptic junctional lattice prepared by deoxycholate (3; Pehling and Matus, unpublished observations) or sarcosinate (22) treatment.

A high molecular weight doublet, variously reported from 185,000 (8) up to 280,000 (this study) mol wt, is also commonly found (Table I). Our molecular weight assignment is based on this doublet's rate of migration relative to two high molecular weight neurofilament components (210,000 and 160,000) in our gels. It seems unlikely that this doublet is a PSD-specific feature because it occurs with comparable intensity in gels of SPM proteins and is also prominent in isolated myelinated axons (Fig. 5, slot 8). Because it is much depleted in both myelin and IFs (Fig. 5, slots 7 and 9), it is most probably a cytoplasmic constituent of either axonal or glial origin. Its presence in SPM and PSD then suggests that it may conceivably be yet another SPM/PSD contaminant (see below).

Like Cohen et al. (8), we also prepared PSDs from synaptosomes without first lysing them to extract the cytoplasmic contents of the presynaptic terminal. Unlike them, we did not find that the protein content of synaptosome-PSDs was identical to that of PSDs made from isolated SPM. Compared with PSDs made from SPM, those made from synaptosomes contained appreciably greater proportions of the 280,000, 270,000 doublet and of bands with 92,000, 74,000, 62,000, 55,000 (presumptive tubulin), and 30,000 (Fig. 13, slots 3 and 4) mol wt. The implication is that all of these bands are, at least in part, present as contaminants in the PSD fraction. However, because tubulin can be localized in the PSD by immunoperoxidase histochemistry, it appears to be both an intrinsic component

and a contaminant of isolated PSDs. The same could be true of any of the others, and indicates the value of histochemical methods for substantiating the biochemical analysis of isolated subcellular organelles. Our finding that nonmembrane tubulin sticks to PSDs is also interesting in that, with radioiodinated tubulin, Bhattacharyya and Wolff (2) reported no contamination of isolated brain membranes. It seems, however, that at the PSD level such contamination can occur.

During the course of our experiments, several reports appeared showing that the high molecular weight triplet (210,000, 160,000, and 68,000) in isolated brain IFs are of neurofilament origin (1, 10, 25, 43) and that the ~50,000-dalton component in these preparations is GFAP (10). Our data, based upon the staining of IF proteins by anti-NF and anti-GFAP in the immunoblotting procedure, confirm these results. In addition, we find two further anti-NF reactive bands in isolated IFs with molecular weights of 170,000 and 185,000. Because our anti-NF serum stains these proteins strongly, it seems quite possible that they are also stained in immunohistochemical sections. This would imply that they too are component proteins of neurofilaments. However, it remains possible that although they are accessible to the antiserum after denaturation and separation in SDS gels, they are nevertheless inaccessible for immunohistochemical staining in sections of intact tissue so that we cannot, at present, make a conclusive statement about their origin. The immunoblotting method also shows a variety of faintly reactive IF bands below 60,000 daltons. Because the original inoculum contained only proteins with SDS gel mobilities indicative of ~60,000 mol wt, this cross-reactivity of the antiserum with lower molecular weight material lends further support to the suggestion (14, 41, 44) that the neurofilament proteins are susceptible to degradation.

Intermediate Filament Proteins and Brain PSDs

Our findings indicate that isolated PSDs are contaminated by both neuronal and glial filament proteins. However, the observation of such contamination does not by itself exclude the possibility that they may also be present as intrinsic components of PSDs in vivo. On the other hand, there are several considerations that support the argument that neither filament category is a PSD component. First, glial-type filaments do not occur in neurons (38, 39), so for the PSD to contain their proteins would require the expression in neurons of a filament-forming protein that can self-assemble in vitro (37) but that is somehow inhibited from forming filaments in the neuronal cytoplasm. Second, neuronal filaments occur mainly in presynaptic processes and, whereas they are present in the dendrites of some neurons (21, 34, 51), in other PSD-rich dendrites (such as those of Purkinje cells) they do not occur (35). If PSDs in nonneurofilament-containing processes did contain neurofilament proteins, then in this case too it would be necessary to propose some presently unknown mechanisms for their structural deposition in the PSD without the formation of filamentous structures in the local cytoplasm. Third, PSD-associated proteins (16, 47) can be stained in sections of intact brain tissue with appropriate antisera, such as those against calmodulin (52), the phosphoproteins Ia and Ib (4), or, as we have shown previously (30, 48) and in this study, antisera against tubulin. If glial and neuronal filaments are present in the PSD, then they must be in some special way prevented from exhibiting the immunohistochemical staining that they show in immediately adjacent cytoplasmic situations. Thus, on balance, it seems most likely that brain PSDs do not contain proteins

belonging to either of the categories of brain intermediate filaments. There exists one published report that PSDs in brain tissue sections are stained by an antiserum against ~50,000 "neurofilament" protein (54). This finding has been contradicted by two previous studies (27, 39), and the findings obtained here also weigh against it.

The demonstration that PSDs can become contaminated by exogenous brain cell proteins has obvious implications for the biochemical study of synaptic function in broken cell preparations. Whereas the levels of such contamination may be low in absolute amounts of protein, as suggested in previous studies of PSD contamination by exogenous radioiodine-labeled proteins (7), and whereas PSD contamination by some exogenous enzymes may also be low (7, 8), the possibility must now be faced that any biochemical marker measured in isolated synaptic membranes or their subfractions may have arisen by contamination. Fortunately, a combination of immunohistochemical and immunoblotting techniques together with contamination checks with radioisotopically labeled exogenous proteins should be able to settle such issues.

We thank Dr. D. Dahl for a gift of antiserum against GFAP, Drs. R. W. Tucker and K. Fugiwara for antiserum against tubulin, Dr. D. H. Jones for assistance in raising antiserum against brain neurofilaments, and Dr. Frances Jay for performing the radioiodination of our intermediate filament preparations.

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