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Postsynaptic Density Antigens: Preparation and Characterization of an Antiserum against Postsynaptic Densities

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ABSTRACT Long-term immunization of rabbits with postsynaptic densities (PSD) from bovine brain produced an antiserum specific for PSD as judged by binding to subcellular fractions and immunohistochemical location at the light and electron microscope levels. (*a*) The major antigens of bovine PSD preparations were three polypeptides of molecular weight 95,000 (PSD-95), 82,000 (PSD-82), and 72,000 (PSD-72), respectively. Antigen PSD-95, also present in mouse and rat PSDs, was virtually absent from cytoplasm, myelin, mitochondria, and microsomes from rodent or bovine brain. Antigens PSD-82 and PSD-72 were present in all subcellular fractions from bovine brain, especially in mitochondria, but were almost absent from rodent brain. The antiserum also contained low-affinity antibodies against tubulin. (*b*) Immunohistochemical studies were performed in mouse and rat brain, where antigen PSD-95 accounted for 90% of the antiserum binding after adsorption with purified brain tubulin. At the light microscope level, antibody binding was observed only in those regions of the brain where synapses are known to be present. No reaction was observed in myelinated tracts, in the neuronal cytoplasm, or in nonneuronal cells. Strong reactivity was observed in the molecular layer of the dentate gyrus, stratum oriens and stratum radiatum of the hippocampus, and the molecular layer of the cerebellum. Experimental lesions, such as ablation of the rat entorhinal cortex or intraventricular injection of kainic acid, which led to a major loss of PSD in well-defined areas of the hippocampal formation, caused a correlative decrease in immunoreactivity in these areas. Abnormal patterns of immunohistochemical staining correlated with abnormal synaptic patterns in the cerebella of *reeler* and *staggerer* mouse mutants. (*c*) At the electron microscopic level, immunoreactivity was detectable only in PSD. The antibody did not bind to myelin, mitochondria or plasma membranes. (*d*) The results indicate that antigen PSD-95 is located predominantly or exclusively in PSD and can be used as a marker during subcellular fractionation. Other potential uses include the study of synaptogenesis, and the detection of changes in synapse number after experimental perturbations of the nervous system.

The appearance of a submembranous specialization, the postsynaptic density (PSD), seems to mark the commitment of two neurons to engage in synaptic contact (10, 43) both in the course of development (developmental synaptogenesis; 21, 31) and after damage of the nervous system (reactive synaptogenesis; 10, 13). However, the precise functional role of these specializations and the mechanisms that control their biosynthesis and removal are not known. Antibodies directed against molecules that occur in the PSD and nowhere else in the brain would be extremely valuable tools to approach these problems and would also provide much needed molecular markers to help in ascertaining the purity of subcellular fractions. Therefore, it was decided to prepare PSD on a comparatively large scale and immunize rabbits with high concentrations of these PSD over prolonged periods of time. This report describes the properties of the antisera thus obtained and shows that at least one antigenic protein, denominated antigen PSD-95, appears to be specifically enriched in PSD.

MATERIALS AND METHODS

Subcellular Fractionation of Rat Brain

Adult Sprague-Dawley rats were either purchased from Simonsen Laboratories (Gilroy, Calif.) or bred in the University's animal facility. Subcellular

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fractions were prepared from forebrains obtained after decapitation of the animals and free-hand dissection of brains rostral to the superior colliculi. Purified synaptic plasma membranes (SPM), mitochondria, and myelin were prepared by sucrose density gradient centrifugation of a lysed synaptosomal fraction treated with *p*-iodonitrotetrazolium (INT) and succinate according to the method of Cotman and Taylor (14). Similar fractions were also obtained by an identical method but without the use of INT. Synaptic junctions (SJ) were prepared by Triton X-100 treatment from the SPM fraction, as previously described (14, 23). PSD were prepared from SPM or SJ by treatment with sodium *N*-lauroyl sarcosinate, as described by Cotman et al. (11). Microsomes were obtained from the supernate of a synaptosomal-mitochondrial P_2 pellet by centrifugation at 65,000 *g* for 2 h. The clear supernate was the soluble cytoplasmic fraction. The yield of SPM in eight different instances was 2.9 ± 1.3 (SD) mg of protein/g wet weight of brain tissue. The average yield of SJ was 0.16 ± 0.06 mg of protein/mg of SPM protein and that of PSD was 0.054 ± 0.04 mg of protein/mg of SPM protein. Protein was estimated by a modification (4) of the method of Lowry et al. (30), correcting for the presence of formazan as described previously (11). In three different occasions, we used frozen tissue as starting material. Forebrains were rinsed in ice-cold 0.32 M sucrose, frozen whole at −80°C, and stored at that temperature until required. Thawing was carried out at 4°C in 0.32 M sucrose. The yield of SPM and SJ, the polypeptide pattern of these fractions in SDS PAGE and their ultrastructural appearance were indistinguishable from the equivalent fractions prepared from fresh tissue.

Subcellular Fractionation of Bovine Brain

Bovine brains were obtained from a local slaughterhouse within 30 min after sacrifice of the animal. They were kept in ice-cold 0.32 M sucrose for 1.5–2 h before processing. Neocortex (400 g) was dissected free of pial membranes and blood vessels, finely minced, and processed using the methods described for rat brain, adapted to process larger amounts of tissue using the Sorvall RC2-B centrifuge (Du Pont Co., Sorvall Biomedical Div., Newtown, Conn.) and large capacity rotors in the initial stages of the preparation. Thus, the nuclear pellet (P_1) was obtained by centrifugation for 5 min at 3,000 rpm (1,500 *g*) in the Sorvall GS-3 rotor. The synaptosomal-mitochondrial pellet (P_2) was obtained by centrifugation at 12,000 rpm (16,000 *g*) for 20 min in the Sorvall GSA rotor. SPM were prepared from the P_2 as for rat brain, with slight modifications. The concentration of INT/succinate used was 1.4 mg of INT and 36 mg of disodium succinate hexahydrate per gram of wet brain tissue. This amount of INT, only one third of that used by Cotman and Taylor (14), was found to give SPM of adequate purity and in higher yield than the standard concentration prepared from rat brain (39). The discontinuous gradients of sucrose used in the purification of SPM were made up, from bottom to top, as follows: (*a*) Beckman SW27 nitrocellulose tube, 1.3 M sucrose, 10 ml; 1.0 M sucrose, 9 ml; 0.8 M sucrose, 8 ml; and sample, 10–12 ml; (*b*) Beckman SW25.2 nitrocellulose tube, 14 ml each of 1.3 M, 1.0 M, and 0.8 M sucrose and 16 ml of sample. The use of 1.3 M sucrose in the bottom layer made the SPM band (that floats over that density) more compact and less likely to be contaminated by mitochondria when the gradients were slightly overloaded. The yield of SPM obtained in seven different preparations starting with amounts of tissue ranging from 50 to 600 g of wet weight was 2.4 ± 1.7 mg of protein/g of tissue. SJ and PSD were prepared from the SPM fraction exactly as described for rat brain (11, 14, 23). The yield of SJ from SPM was 40 ± 6% by weight of protein, while that of PSD was 6 ± 4% of SPM protein.

Particulate Subfractions from Other Tissues

Membranes from rat erythrocytes were prepared as described by Dodge et al. (16). To prepare particulate material from rat liver, kidney, and heart, the tissue was finely minced, suspended in 10 mM sodium phosphate buffer, pH 7.0 (5 ml/g of tissue) and dispersed with the help of a Brinkmann Polytron (setting 4, three blasts of 30-s duration; Brinkman Instruments, Inc., Westbury N. Y.). The homogenates were then filtered through two layers of cheesecloth, diluted to 10% (wt/vol) in the same buffer as described above, and centrifuged at 20,000 *g* for 30 min. The pellets were used in the absorption of antiserum after washing with the same buffer four times by centrifugation.

Iodination of Protein A and Goat IgG

These proteins were iodinated by the chloramine-T method essentially as described by Dorval et al. (17). The binding capacity of the iodinated proteins was tested by measuring their ability to bind to glutaraldehyde-cross-linked rabbit IgG, prepared as described by Avrameas and Ternynck (2). For protein A, half-saturation of this IgG preparation occurred at ~20 ng of ^{125}I-protein A/μg of insolubilized IgG. Either iodinated protein could be displaced completely by its nonradiolabeled homologue.

Immunization Procedure

Six female white, New Zealand rabbits (Ray Simunek Co., Vista, Calif.), 4 mo old, were immunized by subcutaneous injection in four different sites on the back with 1.2 mg of bovine PSD emulsified in complete Freund's adjuvant. Successive similar injections of 1 mg of PSD protein per rabbit were given in incomplete Freund's every 15 d for the first 2 mo, then every 25 d, up to a total of eight injections. Samples of blood were obtained 10–12 d after each injection, and their titer was examined using the test-tube binding assay described below. Maximum titer was achieved after the fourth injection in three animals, declining thereafter. In the other three rabbits the titer increased continuously during the next 4 mo; 13 d after the last injection, the animals were exsanguinated by heart puncture. The sera were aliquoted and stored frozen at −80°C.

Test-tube Assay of Antiserum Binding

Particulate subcellular fractions were incubated with 100 μl of the appropriate serum dilution in 25 mM sodium phosphate/0.15 M sodium chloride (PBS), pH 7.4, at 0°C for 90 min. Control incubations contained either the same dilution of preimmune serum or the serum exhaustively adsorbed with the immunogen. After centrifugation in a Beckman microfuge for 3–10 min, the supernate was aspirated off and the pellet was washed three times with PBS containing 1 mg/ml ovalbumin (PBS-ovalbumin; 600 μl each time). The pellets were then treated with ^{125}I-labeled protein A (10 ng in 20 μl of PBS-ovalbumin) and incubated at 0°C for 30 min. After three washes with PBS-ovalbumin as before, the pellets were counted in a Beckman radioactivity counter model Gamma 8000. The amount of particulate protein used per assay depended on the subcellular fraction tested: binding to bovine or rat SJ and PSD could be easily determined on 15 μg of protein of these fractions. Comparable amounts of binding to SPM required ~50–100 μg of protein, whereas 100–200 μg of myelin protein showed little binding above controls. The radioactivity bound specifically was the difference between that bound by immune serum and the corresponding control. Binding by controls was ~30–40% of the specific binding to the immunogen; this amount was not decreased by coating the microfuge tubes with ovalbumin, as recommended by Dorval et al. (17). Radioactive protein A alone showed <2% of the binding observed in the presence of nonimmune serum. The same binding method was occasionally applied, with similar results, using as secondary reagent ^{125}I-labeled goat IgG anti-rabbit IgG.

PAGE

One-dimensional slab gel electrophoresis was performed using the discontinuous SDS-buffer system of Laemmli (28) and linear-exponential acrylamide gradients cast as described by Kelly and Luttges (26). Membrane fractions were solubilized in SDS-mercaptoethanol-Tris buffer, and gels were fixed and stained as described (26). Protein standards (molecular weight in parentheses) were: microtubule-associated proteins (350,000; 300,000); sketetal muscle myosin (200,000); phosphorylase *a* (96,000); lactoperoxidase (82,000); bovine serum albumin (68,000); α- and β-tubulins (56,000; 54,000); actin (45,000); soybean trypsin inhibitor (21,500); and cytochrome *c* (11,700).

Localization of Antigens in Polyacrylamide Gels

Antibody binding to SDS polyacrylamide gel electropherograms of subcellular fractions (10–30 μg of protein) was carried out as described by Adair et al. (1). One whole gel, of area 15 cm^2, was usually incubated with 12–15 ml of serum (1:50 dilution in 50 mM Tris-HCl-0.5 M NaCl, pH 7.4), and 15–20 ml of radiolabeled protein A solution (0.3 μg/ml) containing ~20 μCi/μg of protein.

Adsorption of Antisera

Antisera were adsorbed with nonbrain particulate fractions prepared as described above. Antiserum diluted 1:10 in PBS, pH 7.2 (10 ml), was treated with 150 mg of particulate protein from rat liver, kidney, heart, or erythrocytes, at 4°C for 2 h, with occasional shaking. After this period the suspension was homogenized in a Dounce homogenizer and incubated a further 2 h at 4°C. The mixture was centrifuged at 27,000 rpm in a Beckman type 30 rotor for 30 min and the treatment repeated; the supernate was the adsorbed serum. A similar treatment was used to adsorb the serum with bovine PSD (38 mg of PSD protein/ml of serum), myelin purified from rat brain (45 mg of protein/ml of serum diluted 1:10), and with a mixture of denatured and native insolubilized tubulin from rat brain. The latter mixture was prepared as follows: tubulin from rat brain was prepared by three successive cycles of polymerization/depolymerization (5), and insolubilized by glutaraldehyde cross-linkage (2). Cross-linked tubulin (40 mg) was denatured by treatment at 85°C for 10 min with 2.5% SDS, 5% 2-mercaptoethanol in 0.0625 M Tris buffer, pH 6.8. The cross-linked tubulins, denatured but still insoluble, were recovered by centrifugation in the Beckman microfuge for 5 min, resuspended in PBS, pH 7.2 (1 ml), and centrifuged as before. This washing procedure was repeated five times. Nondenatured, cross-linked tubulin (10 mg) was added to the washed pellet of denatured protein and centrifuged again, and the pellet was resuspended in 1 ml of undiluted serum. The mixture was incubated for 16 h at 4°C and the adsorbed serum separated by high-speed centrifugation.

IgG was purified from immune and nonimmune sera by ammonium sulfate (40% wt/vol) precipitation, followed by ion-exchange chromatography on a DEAE-cellulose column (20).

Immunohistochemical Location of Antigens

Rats were anesthesized with an overdose of Nembutal (40 mg) and perfused through the heart with 200 ml of Tyrode's buffer, pH 7.0, at 4°C, followed by 300 ml of a filtered solution of fixative at the same temperature. Preliminary observations indicated that the conditions of fixation of the tissue were rather critical. Antigenicity was well-conserved by perfusing the animals with freshly prepared 1–4% paraformaldehyde, 0.05% glutaraldehyde in Sorensen's buffer (0.212 M sodium, potassium phosphate), pH 7.2, followed by postfixation for 90 min in the same fixative. After postfixation, the brains were washed for a minimum of 24 h at 4°C in Sorensen's buffer containing 10% sucrose (wt/vol). When slices of frozen, fresh tissue (20–40 μm) were cut and subsequently fixed, then optimal fixation was achieved by immersion of the slices in the above fixative for 7–15 min at 21°C. Longer periods of fixation diminished tissue antigenicity, and after 90 min this was virtually abolished. Fixation times shorter than 5 min did not improve staining, and tissue structure was very poorly preserved. Concentrations of glutaraldehyde in the fixative higher than 0.25% (wt/vol) greatly diminished or abolished antigenicity while increasing nonspecific staining.

Detergent (0.1%, wt/vol, Triton X-100 or 0.02%, wt/vol, saponin) was required either during fixation or during incubation of fixed tissue with primary antiserum. Omission of detergent led to very little or no reaction, presumably because of lack of access of antibody molecules to the interior of the cells.

Brain sections for light microscopy (20–25 μm thick) were cut with a cryostat and for electron microscopy (40–60 μm thick) with an Oxford Vibratome model G (Foster City, Calif.). For either purpose, individual free-floating sections of tissue were washed for at least 30 min in PBS, pH 7.3 (10 ml), and stained by the unlabeled three-layer peroxidase-antiperoxidase (PAP) method (45), using as the primary reagent immune rabbit serum adsorbed with cross-linked native and denatured tubulin. Immune serum exhaustively adsorbed with bovine PSD, preimmune serum from the same animal, or nonimmune serum from other animals was used as the control for nonspecific staining. The staining protocol was as follows. Individual free-floating sections of tissue were washed in PBS for 30–60 min at 21°C and then incubated overnight at 4°C with rabbit antiserum (1 ml) at dilutions of 1:500 or 1:1,000 in PBS containing 1% normal goat serum and 0.1% Triton X-100 (wt/vol), pH 7.3 (buffer A). After the sections were washed twice with PBS, 30 min each time, they were incubated for 1 h at 21°C with goat IgG anti-rabbit IgG at a dilution of 1:100 in buffer A. After two washes in PBS as before, the brain slices were incubated for 1 h at 21°C with horseradish peroxidase-rabbit antiperoxidase complex (rabbit PAP) diluted 1:250 or 1:500 with buffer A, washed twice again in PBS, and incubated for 10–20 min at 21°C with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (0.5 mg/ml) and hydrogen peroxide (0.03% wt/vol) in PBS. The stained slices were washed for at least 1 h in PBS and mounted on glass slides for light microscopy. Tissue to be processed for electron microscopy was initially fixed in paraformaldehyde containing 0.05–0.25% glutaraldehyde. After immunostaining, the tissue was treated with OsO_4 and embedded as described by Wood et al. (48). Ultrathin sections, cut parallel to the surface of the stained tissue, were examined in a JEOL 100C electron microscope without prior staining in either uranyl acetate or lead citrate.

Subcellular fractions from rat brain, purified as described under subcellular fractionation, were immunostained for electron microscopic examination using a sequence of steps essentially identical to that described for intact tissue. The process was carried out in microfuge tubes and washes, and changes of reagents were performed by centrifugation at 10,000 *g*. After being incubated with DAB/H_2O_2 and then washed, the pellets were fixed and processed for electron microscopy with or without staining with uranyl acetate, as previously described (47).

We also studied the location of the antigens in crude particulate material from rat brain (25), using ferritin coupled to goat IgG anti-rabbit IgG. All procedures were carried out at 0°–4°C in microfuge tubes; separation of reagents and membranes and washes was carried out by centrifugation in the Beckman microfuge for 2–5 min. Membrane fractions (200 μl; 0.2–0.4 mg of protein) were preincubated for 30 min with 0.1 M sodium phosphate buffer, pH 7.1 (buffer B), containing 10% normal goat serum (600 μl), washed once with the same buffer, and incubated for 4 h with immune rabbit serum diluted 1:500 in buffer B. After two washes with 5% normal goat serum in buffer B, the membranes were incubated for 1 h with the same buffer containing in addition 5 mg/ml apoferritin. They were then treated for 1 h with ferritin coupled to goat IgG anti-rabbit IgG (400 μl of a 0.12 mg/ml solution in buffer B), washed three times with buffer B, fixed, and processed for electron microscopy, as described below.

Electron Microscopy

Unless otherwise indicated, subcellular fractions were fixed in 4% glutaraldehyde, postfixed in 1% osmium tetroxide, stained in uranyl acetate, and, if required, counterstained with lead citrate as previously described (47). Thin sections were cut with an LKB III ultramicrotome and examined in a JEOL electron microscope (model 100C).

Surgical Procedures

Stereotaxic ablation of the rate entorhinal cortex and intraventricular injection of kainic acid were performed as described elsewhere (29, 36).

Reagents

Lactoperoxidase, horseradish peroxidase, soybean trypsin inhibitor, ovalbumin, bovine serum albumin, *p*-iodonitrotetrazolium violet, DAB, and acrylamide were obtained from Sigma Chemical Co. (St. Louis, Mo.). Triton X-100, ScintillAR grade, was purchased from Mallinckrodt Inc. (St. Louis, Mo.). Sodium *N*-lauroyl sarcosinate was obtained through ICN Pharmaceuticals (Plainview, N. Y.). Freund's complete and incomplete adjuvants were purchased from Difco Laboratories (Detroit, Mich.). Paraformaldehyde, osmium tetroxide, and glutaraldehyde were products of Polysciences, Inc (Warrington, PA). Chloramine-T and *N,N,N′,N′*-tetramethylethylenediamine were purchased from Eastman Organic Chemicals Div. (Rochester, N. Y.). Protein A was supplied by Pharmacia Fine Chemicals (Piscataway, N. J.), and phosphorylase *a* was provided by Worthington Biochemical Co. (Freehold, N. J.). Sodium iodide (^{125}I) was purchased from Amersham Corp. (Arlington Heights, Ill.). Normal goat serum and IgG fraction of goat anti-rabbit IgG coupled to ferritin were products of Miles Laboratories, Inc. (Elkhart, Ind.). IgG fraction of goat anti-rabbit IgG, and rabbit PAP complex were obtained from Cappel Laboratories Inc. (Cochranville, Pa.). A preparation of the soluble PAP complex was also purchased from DAKO Corp. (Santa Barbara, Calif.). *Bis*-acrylamide and ammonium persulfate were obtained from Bio-Rad Laboratories (Richmond, Calif.). Kodak X-Omat R film (XR-2 Ready pack) was purchased from various local distributors. All other chemicals were analytical reagent grade or the best grade commercially available.

RESULTS

Large-scale Preparation of PSD

The preparation of synaptic fractions (SPM, SJ) and PSD by the methods of Cotman and Taylor (14) and Cotman et al. (11), respectively, was scaled up to 30-fold with only a slight decrease in the yield of the subcellular fractions. The average yield of SPM from bovine brain was 2.6 mg of protein/g wet tissue, a value similar to that obtained in preparations from rat brain. The major difference between the preparations from both species was the higher yield of SJ obtained from bovine SPM: 40% of the protein in SPM was recovered in the SJ fraction, as compared with 16% in the corresponding preparation from rat. That the yield of PSD was, however, similar in preparations from both species (~6% of the SPM protein) suggests that the bovine SJ fraction contained a greater proportion of nonjuctional membranes. The purity of the bovine PSD fraction used as immunogen, as judged by its ultrastructural appearance and SDS gel electrophoretic pattern (Fig. 1), compared favorably with that reported for similar fractions from rat brain (11, 12).

Types of Antisera and Their Titers

The binding of immune rabbit IgG to particulate subcellular fractions or to their SDS gel electropherograms was detected with the help of ^{125}I-labeled protein A, as described in Materials and Methods. The sera from six immunized rabbits (R1 to R6) could be grouped into three classes, according to their titer and antigenic specificity. The titer, determined by testing the binding to bovine PSD of sequential dilutions of the sera in PBS, pH 7.1, increased steadily in three animals (R4, R5, and R6) after the second injection of immunogen. After the eighth immunization the sera of these rabbits had titers that ranged from 1:2,000 to 1:5,000. The sera of the remaining three animals (R1, R2, and R3) had a maximum titer of 1:300 after the fourth immunization, decreasing thereafter.

To study the antigenic specificity of the sera, SDS gel electropherograms of the immunogen were treated sequentially with antiserum and ^{125}I-labeled protein A (1). The gels were then stained with Coomassie Blue, destained, dried, and autoradiographed. The pattern of binding observed is shown in Fig. 2. Radioactive protein A alone, or after incubation with nonimmune rabbit serum (1:20 dilution), did not bind to the gels (Fig. 2). The immune sera, on the other hand, showed three different types of binding pattern. Sera R1 and R2, which had titers below 1:300, showed weak reaction restricted to components of low electrophoretic mobility (high molecular weight), as seen in Fig. 2. Sera R3 and R4 showed reaction with two well-defined bands of molecular weight 82,000 ± 3,000 and 72,000 ± 2,000, denominated antigens PSD-82 and

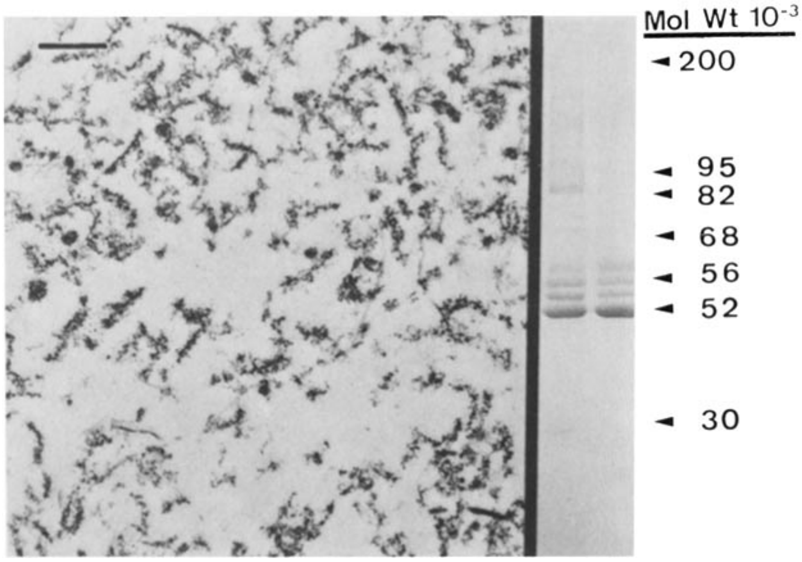

FIGURE 1 Bovine PSD, purified on a large scale. Bovine PSD were stained with uranyl acetate and counterstained with lead citrate (47). More than 85% of the profiles observed in electron micrographs were PSD. Bar, 0.5 μm. × 18,200. On the right-hand side, SDS polyacrylamide gel electropherograms of the two PSD preparations used as immunogens.

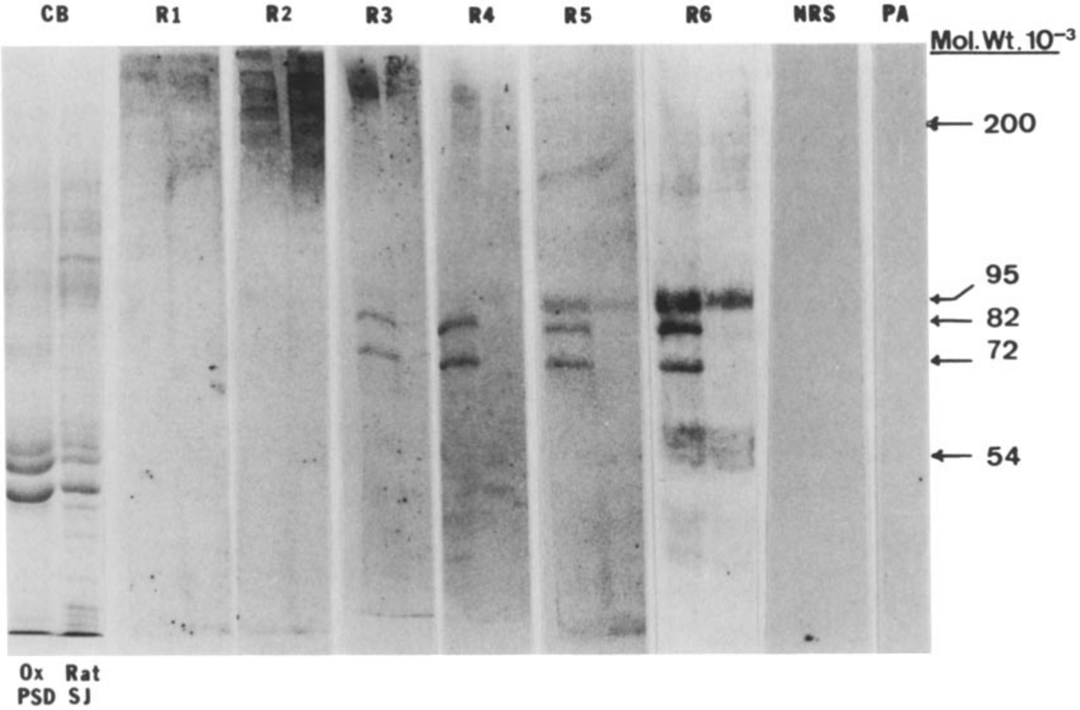

FIGURE 2 Antigenic specificity of rabbit anti-PSD antisera. Bovine postsynaptic densities (*Ox PSD*) and rat synaptic junctions (*Rat SJ*) were subjected to electrophoresis in the presence of SDS on 8% polyacrylamide gel slabs. From left to right, their Coomassie Blue staining pattern (*CB*) and autoradiograms after treatment of the gels with antisera R1 to R6 and ^{125}I-labeled protein A according to Adair et al. (1). Nonimmune rabbit serum (*NRS*) and ^{125}I-labeled protein A alone (*PA*) did not bind to any junctional component.

PSD-72, respectively, because of their apparent molecular weight (Fig. 2). The third group of sera, R5 and R6, also recognized these two antigens, but in addition bound to a component of molecular weight 95,000 (antigen PSD-95), and more weakly to polypeptides of molecular weight 56,000 and 54,000 (Fig. 2). Similar results were obtained if ^{125}I-labeled protein A was substituted by ^{125}I-labeled goat anti-rabbit IgG as secondary reagent, indicating that reactive IgG allotypes were those recognized by protein A. The maximum dilutions at which antisera R5 and R6 bound to bovine PSD in the test tube assay were 1:3,000 and 1:5,000, respectively. Serum R6 had the highest titer and was chosen for detailed characterization. Unless otherwise specified, R6 will be the only antiserum considered in the rest of this report.

Purified IgG from all the immune sera conserved the binding properties described for whole serum. For serum R6, a working dilution of 1:1,000 was frequently used; it corresponded to a total IgG concentration of 2.6 μg/ml.

Identification of Antitubulin Antibodies

Serum R6 contained antibodies that recognized two PSD polypeptides of molecular weight 56,000 and 54,000, respectively (Fig. 2). These polypeptides have been previously identified by their electrophoretic mobility and peptide maps as brain α- and β-tubulin (24). To confirm the identity of these antigens, we purified tubulin from bovine or rat brain by successive cycles of polymerization and depolymerization (5) and tested its reaction with anti-PSD antiserum on SDS gel electropherograms and in the test tube assay. For the latter assay, tubulin was insolubilized by glutaraldehyde cross-linkage (2) and, when required, denatured by treatment with SDS-2-mercaptoethanol, as described in Materials and Methods. Antibodies in serum R6 combined specifically with purified brain tubulin in both types of assay (Fig. 3). The average value of the dissociation constant for the binding of antitubulin antibodies in R6 to native or denatured tubulin was $K_d = 1.1 \times 10^{-5}$ M, estimated from the binding data obtained by test-tube assay. This K_d value is characteristic of low-affinity antibodies. Adsorption of the serum with a mixture of native and denatured, cross-linked tubulins abolished its reactivity towards both purified brain tubulin and the polypeptides of 56,000 and 54,000 mol wt in the PSD fraction (Fig. 3, *inset*).

Specificity of Tubulin-adsorbed Antiserum

The nature of the antigenic molecules of the PSD recognized by the antiserum, the organ specificity of these antigens, and the content of the antigens in purified brain subcellular fractions will be described separately.

ANTIGENS OF THE PSD: The binding of excess antiserum R6 to SDS gel electropherograms of bovine PSD and rat SJ is shown in Fig. 2. Mouse junctions behaved as those from rat. Binding to antigens PSD-82 and PSD-95 and to tubulins was observed in all three animal species. Antigen PSD-72, on the other hand, was present in bovine PSD but absent from rodent PSD. An estimate of the distribution of antibody binding among the antigenic polypeptides is shown in Table I. Binding to tubulins, that accounted for 15–30% of the total, was excluded from the calculations because subsequent studies were carried out with tubulin-adsorbed serum. Antigen PSD-95 was responsible for 59% of the antibody reaction with bovine PSD and represented as much as 89% of the binding to rodent fractions.

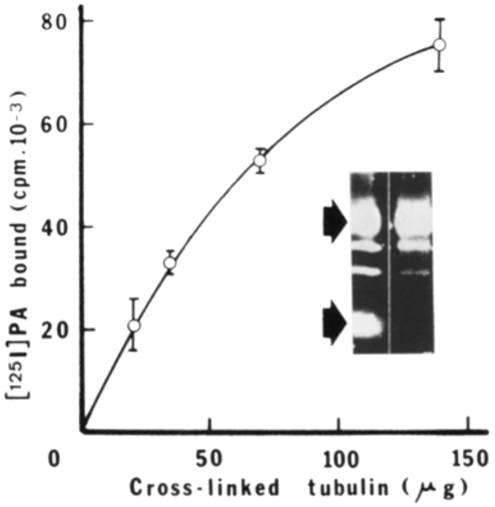

FIGURE 3 Binding of antiserum R6 to insolubilized tubulin. Antiserum R6 (220 μl, 1:20 dilution in PBS, pH 7.1) was titrated at 21°C with increasing amounts of glutaraldehyde-cross-linked tubulin, followed by saturating concentrations of ^{125}I-protein A ([^{125}I]PA). Both native and denatured, cross-linked tubulins gave essentially the same binding curves. The curve shown was obtained with a mixture of equal parts of both species, and incubations were carried out in quadruplicate. In the inset, an autoradiogram of the binding of the serum to SDS gel electropherograms of bovine PSD before (*left*) and after (*right*) exhaustive adsorption with a mixture of native and denatured, cross-linked tubulins. The arrows point to molecular weights 95,000 and 55,000. The radioactive protein A used in these experiments had a specific activity of 1.44×10^7 cpm/μg (efficiency, 70%).

TABLE I

Relative Content of Antigenic Polypeptides in Bovine and Rodent PSD

Species	^{125}I Labeled protein A bound, % of *total*		
	PSD-95	PSD-82	PSD-72
Bovine (11)	59 ± 9	22 ± 5	19 ± 6
Rat (9)	89 ± 7	11 ± 6	0
Mouse (3)	88 ± 6	12 ± 6	0

Reaction of excess serum R6 (tubulin-adsorbed) and radioiodinated protein A with PSD polypeptides on polyacrylamide gels was performed as described in Materials and Methods. The distribution of radioactivity among the three antigen bands was estimated by densitometry of the autoradiograms, or by direct gamma counting of the gel bands. Both methods agreed within experimental error. The number in parentheses after the name of the animal species is the number of independent determinations from which the values given ± SD were calculated. Typical values of total protein A radioactivity bound per μg of PSD protein were 750–2,000 cpm (counting efficiency 77%) in gels, compared with 2,000–4,000 cpm/μg in test-tube assays.

The species selectivity of antigens PSD-82 and PSD-72 observed in the reaction on gels was confirmed by use of the test-tube binding assay. Antisera R3 and R4, that recognized only these antigens, did not show specific binding in test-tube or gel assays to rat PSD (Fig. 2). Furthermore, binding of rat PSD to tubulin-adsorbed serum R6 was 72% of the binding exhibited by bovine PSD, whereas a parallel experiment using serum R5 showed only 15% of the binding to bovine PSD. In serum R5, antibodies to PSD-95 were present but those directed against PSD-82 and PSD-72 predominated.

ORGAN SPECIFICITY: Tubulin-adsorbed antiserum did not bind to particulate fractions from rat liver, kidney, heart, or erythrocytes, either in gels or in the test-tube assay. Conversely, exhaustive adsorption with these fractions did not reduce appreciably the ability of the serum to bind to bovine PSD or rat SJ (Table II). The observed 10–30% decrease in binding to bovine PSD after adsorption was probably attributable to nonselective removal of serum IgG by the large volume of adsorbent repeatedly used. The variations observed in the gel assay (Table II) were attributable to the semiquantitative nature of the method. Within this limitation, the results were in agreement with those obtained by test-tube assay. Adsorption did not cause qualitative changes in the pattern of antigen recognition on SDS gels.

SPECIFICITY OF BINDING TO PURIFIED BRAIN SUBCELLULAR FRACTIONS: The binding of antiserum R6 to purified brain subcellular fractions was also examined by means of test-tube and polyacrylamide gel assays with the help of ^{125}I-labeled protein A. Both methods of measuring antibody binding gave similar values, within experimental error (Table III), suggesting that the more native antigens observed in the test-tube assay were also those observed in SDS gel electropherograms. The variable accessibility of the antibodies and protein A to the antigen molecules in different gel slabs, or in different lanes of the same gel (7), caused large standard deviations in the gel assay.

The relative content of the three main antigens in different subcellular fractions, as revealed by binding of saturating antibody concentrations to SDS polyacrylamide gels, is included in Table III. In bovine brain, antigens PSD-82 and PSD-72 predominated over PSD-95 in microsomes, mitochondria, and myelin. On the other hand, antigen PSD-95 became more enriched in those fractions that contained more PSD, in the order: microsomes < SPM < SJ < PSD. The fraction of SPM that floated over 1.0 M sucrose (*SPM 1.0 M*) had a lower content of junctional complexes than the SPM fraction over 1.3 M sucrose (*SPM 1.3 M*) (11, 14). It also showed a lower content of antigen PSD-95 (Table III). In rodent brain, antigen PSD-95 predominated in all subcellular fractions, constituting ~80–100% of the total binding. Antigen PSD-72 could not be detected in any fraction, and PSD-82 was also absent from some of them (Table III, mouse myelin and microsomes), probably undetectable under the conditions used. Brain soluble cytoplasmic components did not bind R6 antibodies. Repeated adsorption of the serum with purified myelin decreased binding to immunogen by 50%, probably attributable in part to contamination of this fraction with synaptic plasma membranes.

TABLE II

Organ Specificity of Tubulin-adsorbed Antiserum R6: Binding to Bovine PSD and Rat SJ after Additional Adsorption with Nonbrain Particulate Fractions

	Binding to bovine PSD		Binding to rat SJ	
Antiserum adsorption	Test tube	Gel	Test tube	Gel
	%		%	
None	100	100	100	100
Liver	81	82	70	130
Kidney	82	120	—	86
Heart	71	73	—	86
Erythrocytes	93	85	86	102
Liver + kidney	72	95	75	86
Liver + kidney + heart	76	107	65	92

The preparation of nonbrain particulate fractions and their use in the exhaustive adsorption of serum has been described in Materials and Methods. When more than one appears on the table, adsorption was performed sequentially, in the order written. Bovine PSD and rat SJ, both 20 μg of protein, were tested for antibody binding by a microcentrifugation assay (100 μl of a 1:500 dilution of antiserum) or on SDS polyacrylamide gel electropherograms (serum dilution, 1:50). After treatment with excess ^{125}I-labeled protein A, the bound radioactivity was measured in a gamma counter (test-tube assay) or by densitometry of autoradiograms (gel assay). The amount of antibody binding is expressed as a percentage of that for unadsorbed antiserum. The values reported are the average of two independent experiments.

Antigen Content of SJ and PSD

The antigens recognized by serum R6 were undetectable by Coomassie Blue staining of SDS gel electropherograms of bovine or rodent PSD. Their content in these fractions was determined by test-tube titration with tubulin-adsorbed antiserum and radioactive protein A. Titration curves are shown in Fig. 4. Assuming that the antigens were fully accessible to the antibodies, that only one molecule of antibody reacted per molecule of antigen and that, in turn, only one molecule of protein A was bound per molecule of bound IgG, we estimated that bovine PSD contained ~4.0 pmol of reactive antigens/mg of protein, and rat SJ 2.1 pmol/mg. In rat PSD the total amount of antigen binding sites was 3.4 pmol/mg protein (Fig. 4), of which 2.99 pmol (88%) was contributed by antigen PSD-95 (Table III). This value corresponds to ~0.28 μg of PSD-95 antigen/mg of rat PSD protein, a quantity undetectable by conventional staining of gel electropherograms. This content is probably underestimated, because not all the antigen molecules are likely to be fully accessible to the antibodies.

Lack of Identity between PSD-95 and Concanavalin A Binding Components

Purified SJ contained four main bands, previously termed Con A I, II, III, and IV, capable of binding concanavalin A (19, 23). A small proportion of these components persisted in the preparation of bovine PSD. One of them, Con A IV, had a molecular weight similar to that of the antigen PSD-95 (23), suggesting that both could be identical. However, saturation of isolated bovine PSD or rat SJ with antiserum did not prevent the subsequent binding of ^{125}I-labeled concanavalin A. Conversely, concanavalin A saturation of its binding sites in SJ did not affect the binding of serum either in gels or in the test tube. Furthermore, bovine or rat SJ contained both Con A IV and PSD-95. However, rat PSD prepared from SJ by extraction with sodium *N*-lauroyl sarcosinate lacked Con A IV, whereas its content of PSD-95 per total weight of protein was increased. Finally, concanavalin A–binding components, purified by affinity chromatography from the fraction of SJ soluble in sodium *N*-lauroyl sarcosinate (E. E. Mena et al., unpublished observations), did not bind to the serum on a gel where Con A IV was readily detectable by its Coomassie Blue staining. Therefore, PSD-95 and Con A IV are different junctional proteins.

Electron Microscope Observation of Binding of Serum R6 to Subcellular Structures

Crude particulate material from rat brain (25) was treated with antiserum adsorbed with liver, heart, and kidney (Table II), at a dilution of 1:500, followed by ferritin conjugated to the IgG fraction of goat serum anti–rabbit IgG. The treated particles were fixed and stained for electron microscope examination. Incubations with similar dilutions of preimmune serum from the same rabbit, immune serum adsorbed with

TABLE III

Antigen Content of Purified Brain Subcellular Fractions

		Maximum antibody binding per mg of protein, % *of immunogen*		Distribution of binding among antigens, % *of total* ± *SD*		
Animal species	Subcellular fraction	Test tube	Gel	PSD-95	PSD-82	PSD-72
Ox	PSD (11)	100	100	59 ± 9	22 ± 5	19 ± 6
	Microsomes (2)	—	11 ± 4	10 ± 15	52 ± 11	43 ± 5
	Myelin (3)	3	10 ± 7	6 ± 10	50 ± 15	45 ± 11
	Mitochondria (3)	12	57 ± 16	27 ± 2	39 ± 3	34 ± 4
	SPM 1.0 M (2)	3	14 ± 10	44 ± 6	28 ± 7	28 ± 1
	SPM 1.3 M (4)	10	46 ± 28	49 ± 3	28 ± 2	24 ± 2
	SJ (3)	36	56 ± 10	56 ± 6	21 ± 2	24 ± 7
Rat	Microsomes (2)	5	12 ± 5	90–100	0–10	0
	Myelin (2)	3	5 ± 3	72 ± 5	28 ± 5	0
	Mitochondria (4)	13	5 ± 3	67 ± 3	33 ± 3	0
	SPM 1.0 M (1)	3	12	74	26	0
	SPM 1.3 M (2)	8	36 ± 15	85 ± 10	15 ± 12	0
	SJ (9)	51	58 ± 13	88 ± 6	12 ± 5	0
	PSD (9)	85	72 ± 12	89 ± 7	11 ± 6	0
Mouse	Microsomes (2)	6	9 ± 0	100	ND	ND
	Myelin (1)	5	9	100	ND	ND
	SPM 1.0 M (1)	7	33	79	21	0
	SPM 1.3 M (1)	9	44	87	13	0
	SJ (3)	49	75 ± 16	88 ± 6	12 ± 6	0

Abbreviations: *SPM 1.3 M*, synaptic plasma membrane fraction that floats over 1.3 M sucrose (used in the preparation of SJ and PSD); *SPM 1.0 M*, synaptic plasma membrane fraction that floats over 1.0 M sucrose and contains fewer junctional complexes than SPM 1.3 M (39). Test-tube assay of antibody binding (see Materials and Methods) was performed on 15–20 μg of SJ or PSD protein per assay, 50 μg of SPM protein and 100 μg of protein in the case of mitochondria, myelin, and microsomes. The fractions were incubated with excess of tubulin-adsorbed antiserum (100 μl of a 1:500 dilution) followed by excess ^{125}I-labeled protein A (~2 × 10^5 cpm per incubation; specific activity, 1.78 × 10^7 cpm/μg of protein A). The efficiency of counting was 70%. Under these conditions, binding to the immunogen, bovine PSD, was 3.36 × 10^6 cpm/mg of protein. Binding to gel electropherograms was also detected using ^{125}I-labeled protein A as a secondary reagent, and was carried out on gel slabs (0.5 mm thick) containing 20–40 μg of protein per sample well. The distribution of binding among the three major antigens was estimated as indicated in Table I. The total binding of radioactive protein A to 20 μg of bovine PSD on gels ranged from 15,000 to 40,000 cpm in different experiments. No binding to soluble cytoplasmic components was observed in any of the three animal species examined. In parentheses, after the name of the fraction, is given the number of experiments from which the value ± SD was calculated. *ND*, not detectable.

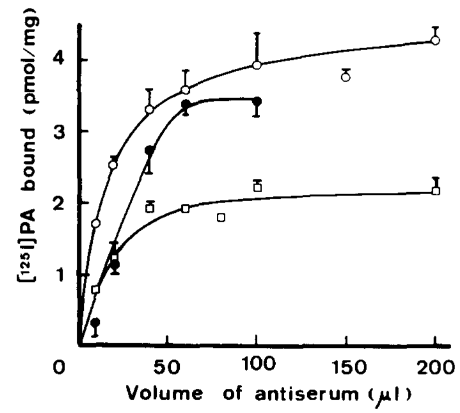

FIGURE 4 Titration of PSD and SJ with anti-PSD antiserum R6. Suspensions of bovine PSD (○), rat PSD (●), and rat SJ (□) (each:20 μg of protein) were treated for 1 h at 21°C with the indicated volumes of serum R6 (dilution, 1:500) in a final volume of 500 μl of PBS, pH 7.2, followed by saturation with ^{125}I-labeled protein A ([^{125}I]PA; specific activity, 1.0–1.8 × 10^7 cpm/μg). Experiments were performed in triplicate, and the standard deviation is indicated in those cases in which it was larger than the size of the symbol.

bovine PSD, and ferritin conjugate alone were used as controls. Ferritin particles were observed on 35% of the structures recognizable as PSD and, occasionally, on amorphous particulate material of unknown origin. No binding was observed to myelin, plasma membranes, mitochondria, or presynaptic elements, although these structures had accessibility to reagents similar to that of the PSD. Preimmune serum from the same or other animals also showed binding to 22% of the observed PSD (100 profiles counted), and ~15% binding was observed even in the absence of any rabbit serum.

The above experiments demonstrated that serum R6 did not react with non-PSD structures. However, the presence of ferritin particles attached to PSD in the absence of immune serum showed that conclusive results could not be obtained using this method. Therefore, binding of tubulin-adsorbed immune and nonimmune serum (1:1,000 dilution) to purified SJ was also tested using the PAP method. As with the immunoferritin method, strong reaction was restricted to the postsynaptic elements. However, in this case, binding to nonimmune serum was either very weak or absent (Fig. 5).

The subcellular location of the DAB-peroxidase reaction product was examined in intact tissue after treatment of stained slices with osmium tetroxide, without any subsequent staining. The results obtained were influenced by the conditions of fixation of the brain tissue. Fixation under conditions that led to good tissue preservation (perfusion with 4% paraformaldehyde, 0.25% glutaraldehyde, followed by 3 h postfixation in the same fixative) led to partial loss of antigenicity. High electron density was restricted to PSD (Fig. 6*a*) but only ~50–60% of the observed PSD were stained. No staining was observed in control samples treated with nonimmune serum or immune serum adsorbed with bovine PSD (Fig. 6*b*).

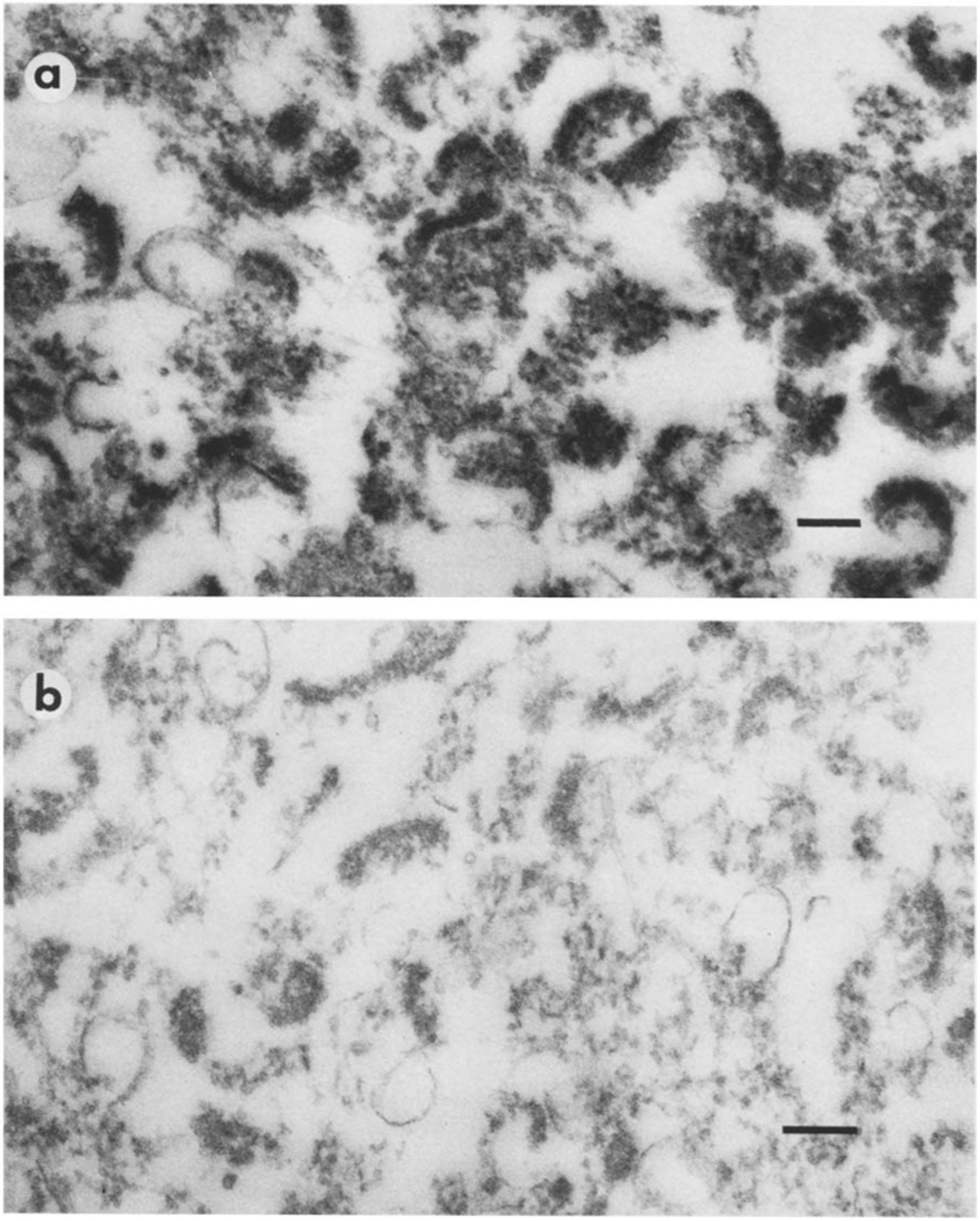

FIGURE 5 Antiserum R6 binding to SJ from rat brain. Purified SJ from rat brain (14) were treated either with tubulin-adsorbed antiserum R6 (*a*) or with the same antiserum adsorbed in addition with bovine PSD (*b*), both at a dilution of 1:1000 in PBS. Samples were stained by the three-layer PAP method (45), treated with osmium tetroxide, and block-stained with uranyl acetate. Bars, 0.2 μm. *a*, × 50,000; *b*, × 59,000.

Milder fixation conditions (perfusion with 1% paraformaldehyde, 0.05% glutaraldehyde in Sorensen's buffer, followed by 30 min postfixation in the same mixture; or, a 15-min fixation of 60-μm Vibratome sections of fresh tissue) led to a much stronger reaction in virtually every structure recognizable as a PSD. However, fixation was poor and the tissue became extremely disorganized during staining. Regardless of the mildness of fixation, no reaction was observed after the antiserum had been adsorbed with bovine PSD.

Immunohistochemical Location of the Antigen

Coronal sections of mouse and rat forebrain and saggital sections of cerebellum were incubated with tubulin-adsorbed antiserum at dilutions of 1:500 or 1:1,000 and stained as indicated in Materials and Methods. At the light microscope level, it was observed that there was no binding to nonneuronal cells, to cytoplasmic components of neurons and to myelinated tracts (Fig. 7), in agreement with the results obtained in gel

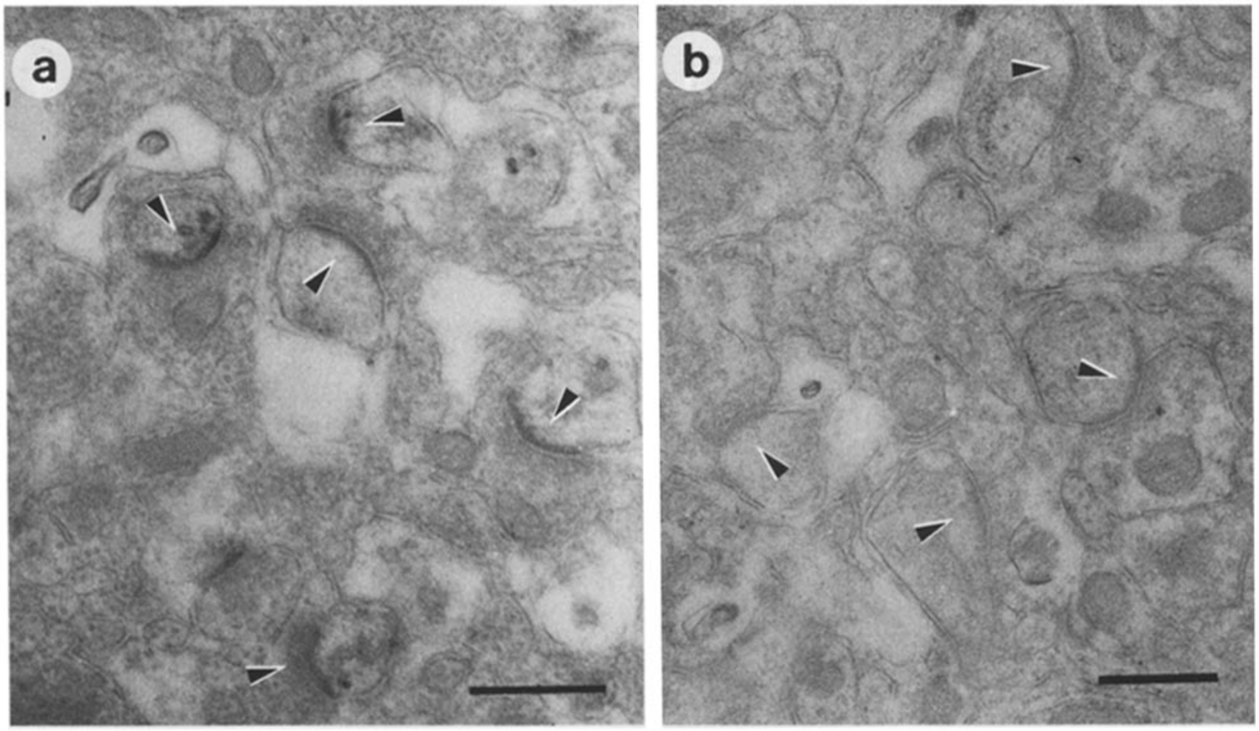

FIGURE 6 Subcellular location of antiserum R6 binding sites in sections of rat cerebellum. Saggital sections of rat cerebellum were stained by the three-layer PAP method (45) either with tubulin adsorbed R6 (*a*) or with the same antiserum adsorbed with bovine PSD (*b*). The tissue was then treated with OsO_4 and embedded, and ultrathin sections were cut parallel to the stained surface. No additional stain was used. Arrowheads point to PSD. Bars, 0.5 μm. *a*, × 35,000; *b*, × 37,000.

and test-tube binding. In cerebellum (Fig. 7 *c* and *d*), most of the reaction occurred in the molecular layer as well as in the dendritic shafts and some loci of the periphery of Purkinje cell bodies. In the granule cell layer of the cerebellum, the overall reaction was weaker and showed a less homogeneous distribution of stain compared with the molecular layer (Fig. 7 *d*). In the forebrain, the strongest staining also occurred in the neuropil. Focusing on the hippocampal formation, we observed strong reaction in the molecular layer of the dentate gyrus, and in the stratum oriens and stratum radiatum of the hippocampus (Fig. 7 *a* and *b*). In the rat spinal cord the strongest reaction took place in the dorsal horn, as well as on the periphery of motor neuron cell bodies, where these large cells are known to receive many synaptic contacts. Immune serum adsorbed with bovine PSD or normal rabbit serum (e.g., from nonimmunized animals), at the same dilution, did not stain nervous tissue appreciably (Fig. 7 *d* and *e*).

Immunohistochemical staining with R6 serum also detected selective changes in number of synapses in discrete regions of the brain after experimental lesions. Unilateral ablation of the rat entorhinal cortex caused a localized loss of synapses in the outer two thirds of the molecular layer of the dentate gyrus ipsilateral to the lesion (10), and in stratum lacunosum-moleculare of the hippocampus (38, 42). These synaptic changes correlated with a selective decrease in immunostaining with respect to the side contralateral to the lesion or to unoperated animals. Elimination of commissural/associational fibers by means of intraventricular injection of the neurotoxin kainic acid (46) denervates the inner one third of the molecular layer of the dentate gyrus (36, 37). This event correlates with a loss of specific immunohistochemical staining in the inner one third of the dentate gyrus molecular layer, the zone of termination of commissural/associational fibers.

A strong correlation between well-characterized synaptic abnormalities (8) and immunohistochemical staining with serum R6 was also shown in the cerebella of the *reeler* and *staggerer* mouse mutants. In the *staggerer*, the two-layer pattern of immunohistochemical staining observed in the cerebellum of normal mice (Fig. 7) was absent. In the cerebellum of the *reeler*, only a very narrow molecular layer was stained; however, specific immunohistochemical reaction was also observed on the periphery of the dendrites and the cell body of large cells, abnormally located in the granule cell layer and within myelinated tracts.

DISCUSSION

Antigenicity of Bovine PSD

Bovine PSD, purified as described in Materials and Methods, contained three major and ~14 minor Coomassie Blue–staining bands in monodimensional SDS gel electropherograms. The most abundant of these, a polypeptide of 52,000 mol wt (PSD-52; 24), seems to be a very poor immunogen, as no antibodies to it were observed in any of the rabbits. Other immunogenic proteins located in the PSD *in situ*, such as the kinase substrate protein I (6), were either lost during the sarcosinate treatment (34) or their antigenicity was destroyed. The three bands that were most immunogenic in our preparation stained either very weakly with Coomassie Blue or not at all. Under our electrophoresis conditions, ~0.1–0.3 μg of protein could be detected by conventional staining methods. Therefore, it can be estimated that the major antigen, PSD-95, accounts for <0.3% of the total PSD protein, in agreement with a content of 0.28 μg of PSD-95/mg of PSD protein, calculated from direct test-tube titration of rat PSD with serum and radioactive protein A (Fig. 4 and Table III). Assuming that PSD are disks of 400 nm

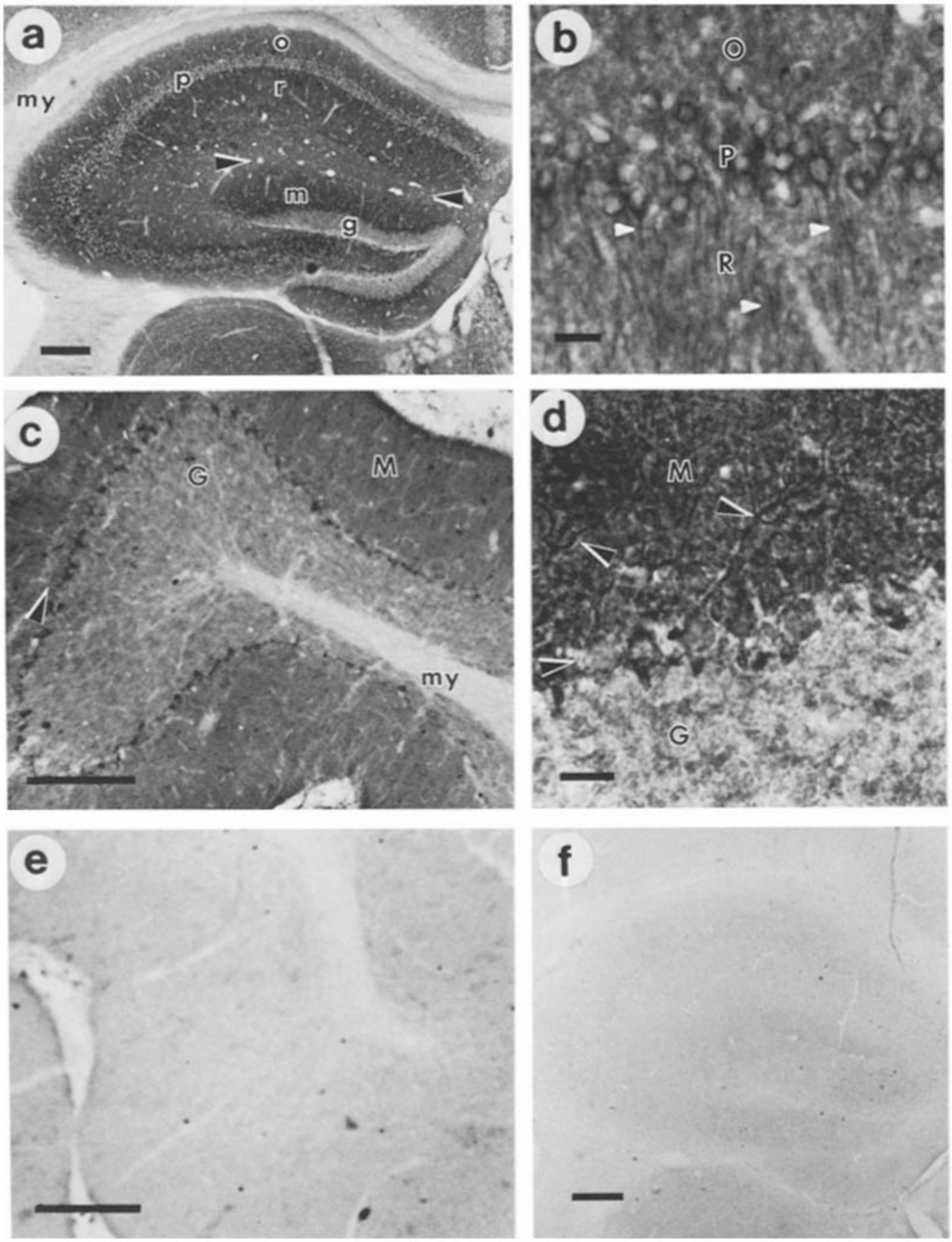

FIGURE 7 Immunohistochemical staining of tissue sections from rodent brain. Sections (25 μm thick) of rat and mouse brain were stained using anti-PSD antiserum R6, adsorbed with tubulin (dilution 1:500 to 1:1,000), and the three-layer PAP method (45). (*a*) Mouse hippocampal formation; *my*, myelinated fiber tracts of the corpus callosum; *o*, stratum oriens; *p*, pyramidal cell layer; and *r*, stratum radiatum of the hippocampus. Black arrowheads point to the hippocampal fissure that separates the hippocampus from the dentate gyrus. In the latter, *m*, molecular layer; *g*; granule cell layer. Bar, 200 μm. × 35. (*b*) Rat hippocampus. Detail of staining in stratum oriens (*O*), on the periphery of the cell bodies of the pyramidal cells (*P*) and their apical dendrites (arrowheads) in stratum radiatum (*R*). Bar, 20 μm. × 300. (*c*) Rat cerebellum. Staining was stronger in the molecular layer (*M*) and on parts of the periphery of Purkinje cell bodies (arrowhead) in the bondary with the granule cell layer (*G*). Bar, 200 μm. × 175. (*d*) Detail of immunohistochemical staining of mouse cerebellum. Arrowheads point to Purkinje cell dendritic shafts and soma. Note the discrete staining in the granule cell layer (*G*). Bar, 20 μm. × 350. Control sections of rat cerebellum (*e*) and mouse hippocampal formation (*f*) were treated with antiserum adsorbed with bovine PSD. Bar, 200 μm. *e*, × 75; *f*, × 35.

diameter, 50 nm thickness, and density 1.24 g/cm^3 (3), it can be estimated that the antigen content is equivalent to ~10–20 molecules/PSD, provided all the antigen molecules are accessible to antibody. Compared with PSD-95, the most abundant PSD polypeptide (PSD-52) has ~18,000 molecules per organelle. The identity of antigen PSD-95 is unknown. It is different from the concanavalin A–binding components of SJ. Other known polypeptides of similar molecular weight, such as alpha-actinin or the catalytic subunit of Na$^+$, K$^+$-ATPase, have different solubility properties and/or subcellular location. Antigen PSD-95 may be similar to the 95-kdalton component of intestinal brush border, a protein probably involved in the interaction between plasma membrane and cytoskeletal components (15). Whatever the identity of PSD-95, it must be quite relevant to the structure and/or function of the PSD because it is also present in fish, reptiles, amphibians, and birds (Nieto Sampedro et al., unpublished observations).

Binding in test-tube to nondenatured subcellular fractions correlates well with binding to SDS gel electropherograms, where the polypeptides have been unfolded. The antigenic sites may be either conserved during the SDS-mercaptoethanol treatment or reformed during the long equilibration of the gel with buffer previous to antibody treatment (1). Whatever the case, it seems likely that the antigenic specificity of serum R6 observed in gels is the same as that shown in test-tube or in brain slices. However, the possibility cannot be ruled out that antigens other than those observed in the SDS gels may be recognized by the antiserum in both the native fractions and the intact brain tissue.

Conventional antisera against purified PSD from dog brain were prepared by Cohen et al. (9). However, the binding properties of the serum were not characterized. Other antisera have been prepared using synaptosomes as immunogens (22, 27, 33, 35, 40, 44). Synaptosomal fractions contain various types of subcellular particles originated in many different types of brain cells. The resulting antibodies were shown to bind to several subcellular sites in neuronal as well as nonneuronal cells. An exception appears to be the antiserum prepared by Rostas and Jeffrey (44) against purified synaptic plasma membranes. The reactivity of this antiserum seems restricted to the axons and presynaptic region of the neuronal plasma membrane (44). Two groups have reported antisynaptosome antisera apparently specific for PSD antigens (33, 40). Both groups used high concentrations of immune rabbit IgG or IgG fragments coupled to ferritin to locate binding sites at the electron microscope level and reported micrographs very similar to those obtained by us. In the absence of quantitative data, the specificity of that binding is uncertain. We found that treatment of disrupted brain tissue with nonimmune IgG at a dilution of 1:50 relative to the original serum, followed by ferritin conjugate (0.1–0.2 mg/ml), or even treatment of tissue with ferritin conjugate alone, showed almost as much binding to PSD as anti-PSD IgG at the same dilution. These results could be explained by nonspecific adhesiveness of ferritin conjugates to the highly adhesive PSD and, perhaps, by the presence of anti-PSD antibodies in nonimmunized animals.

Subcellular Location of PSD-95

The specificity of binding of the antiserum in test-tube and gel electrophoresis suggests that antigen PSD-59 is selectively enriched in PSD. The small amount of binding to PSD-95 observed in microsomes and nonjuctional plasma membranes could be attributable to a combination of the contamination of these fractions with junctional material or to the true presence of the antigen in these fractions. Indeed, PSD-95 has to be synthesized in the endoplasmic reticulum before being transported to its final location on the different postsynaptic sites of the neuron.

Electron microscope observations of serum-treated subcellular fractions and tissue sections (Figs. 5 and 6) show that reactivity occurred selectively in the postsynaptic specialization. This conclusion is sustained by the immunohistochemical observations at the ligh microscope level. In all cases studied, staining was restricted to areas where synapses are known to be present. The distribution and intensity of the stain were also those expected from the known anatomy of the brain area under consideration (Fig. 7). For example, in cerebellum the maximum intensity occurred in the molecular layer, where parallel fibers from the granule cells form numerous synapses with the spiny branchlets on the dendrites of Purkinje cells. The weaker and irregular staining of the granule cell layer corresponds to the presence of discrete clusters, the glomeruli, which are the loci where synaptic contact takes place between the dendrites of the granule cells on one hand and the axons of Golgi cells and mossy fibers on the other (41). When the anatomy is drastically altered, as in the mutant mice (8), so is the immunohistochemical staining reaction. Similar observations apply to other brain regions, such as neocortex, hippocampal formation, or corpus striatum. Selective alterations of the staining pattern have also been observed in response to experimental damage, such as ablation of the entorhinal cortex or intraventricular injection of kainic acid, both lesions that cause extensive loss of PSD in select laminae within the molecular layer of the rat dentate gyrus (10, 32, 36, 37).

In the immunohistochemical studies, both symmetric (type II) and asymmetric (type I) synapses (18) appear to react with the antiserum. For example, in the cerebellum, type I synapses are present on Purkinje cell dendrites, whereas type II synapses are observed on the soma of these cells (41). Both of these cell loci are stained by antiserum (Fig. 7).

Taken together, the reported observations indicate that antigen PSD-95, in common with the major PSD-52 polypeptide (24), is highly enriched in PSD with respect to other subcellular loci. Therefore, PSD-95 can be proposed as another marker for SJ and PSD during the purification of these structures by subcellular fractionation, as well as in the study of their biosynthesis. Other potential uses of antibodies against PSD-95 include the chemical determination of changes in synapse density after various experimental manipulations. The reaction of the antibody with slices of brain tissue, followed by treatment with radioactive protein A, opens the way to make this estimate quantitative.

An advantage of the antiserum described is its reproducibility. In our experiments two of six animals developed high titer antibodies to PSD-95, and the pattern of antigens recognized was conserved. On the other hand, a problem is the heterogeneity of conventional rabbit antisera. Fortunately, antigens PSD-82 and PSD-72 are almost absent from rodent brain. The purification of PSD-95 and the preparation of monospecific antibodies have been hampered so far both by the insolubility of the protein and by its scarcity in nervous tissue.

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