Putative 51,000-M_r Protein Marker for Postsynaptic Densities is Virtually Absent in Cerebellum

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ABSTRACT Cerebrum and cerebellum contain numerous asymmetric synapses characterized by the presence of a postsynaptic thickening prominently stained by phosphotungstic acid and other electron-dense stains suitable for electron microscopy. A 51,000-M_r protein, copurified in postsynaptic density-enriched fractions from cerebrum, is considered to be a well established marker for the postsynaptic density. On the basis of two criteria, our studies demonstrate that the 51,000-M_r protein marker for postsynaptic densities is virtually absent in cerebellum. First, it is present in negligible amounts in deoxycholate-insoluble fractions from cerebellum but abundant in parallel fractions from cerebrum. Secondly, the 51,000-M_r protein, which binds ^{125}I-calmodulin after SDS PAGE, is readily visualized in membrane samples from cerebrum but is virtually undetectable in cerebellar samples. It is apparent that these results require reexamination of the role of the 51,000-M_r protein in postsynaptic density structures.

Synapses in mammalian brain may be broadly classified into two general types based primarily upon morphological parameters of their postsynaptic specializations. One type, termed Gray's Type I or asymmetric synapses, are characterized by a 30-nm separation of pre- and postsynaptic membranes and a pronounced accumulation of material on the cytoplasmic face of the postsynaptic membrane. This prominently stained structure is termed the postsynaptic density (PSD) and is only faintly visible in Type II or symmetrical synapses. Type II synapses are identified in the electron microscope, not by evident postsynaptic densities, but by virtue of a uniform 20-nm separation of membranes together with an association of synaptic vesicles on one side (16). Quite often, Type II synaptic vesicles are somewhat elongated and flattened (32). Such a classification scheme is too rigid to accommodate all synapses, and it is apparent that these characteristics represent major classes at either extreme of a continuum containing intermediate PSD structures (10, 15). It is probable that any classification scheme based upon conventional morphological parameters is likely to underestimate the variety of synaptic structures containing specific biochemical or functional attributes. The identification of protein markers for various synaptic structures would greatly facilitate further classification of synapses.

A widely accepted marker for the PSD structure is a protein with a M_r of 51,000–52,000, which is the most prominent protein in fractions enriched in PSDs (11, 20). This marker protein is generally assumed to reflect quantitatively the number of PSD structures in isolated preparations. As a point of reference, we will adopt the terminology of Kelly and Montgomery (19) in our discussions of the presence of this protein and refer to this protein as the major PSD protein (mPSDp). The PSD morphology is remarkably resistant to detergent action; Triton X-100, a nonionic detergent widely used during the preparation of PSD-enriched fractions, largely solubilizes membrane-bound proteins while leaving the PSD morphology apparently unaffected (9, 23). PSDs may be rendered resistant to stronger detergent treatment by the oxidizing agent, *p*-iodonitrotetrazolium violet, which mediates the formation of disulfide bonds between the mPSDp molecules (19, 21). Cross-linking the PSD, followed by extraction with the ionic detergent, *N*-lauroyl-sarcosinate (which removes the majority of proteins), yields a crescent-shaped structure with PSD-like morphology containing principally the mPSDp (11, 20). Simultaneous purification of PSD-like structures and the mPSDp provides the major evidence that this protein is a constituent of the PSD, at least in cerebral tissue. This correlation has been further verified by assessing the relative content of mPSDp in various cerebrum subcellular fractions. Subcellular fractions, known to contain synaptic structures, also contain the mPSDp; fractions enriched in myelin and mitochondria contain little of the mPSDp (19).

An indication that the mPSDp may not be a universal PSD marker comes from the studies by Carlin et al. (5) of a PSD-enriched fraction derived from canine cerebellum. Carlin et al. (5) used multistep velocity and gradient centrifugation procedures to prepare synaptosome-enriched fractions, followed by treatment with Triton X-100 and gradient centrifugation steps to prepare a PSD-enriched fraction. This procedure, originally designed to purify cerebral PSDs (9), yielded a cerebellar PSD-enriched fraction that contained little mPSDp. The PSDs

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prepared from cerebellum were delineated from the fraction derived from cerebrum by several morphological parameters. Cerebral, but not cerebellar, PSDs contain a central perforation. Furthermore, instead of 20- to 30-nm aggregates observed in cerebral PSDs, cerebellar PSDs contain a latticelike structure. The average cross-sectional thickness of cerebellar PSDs (33 nm) is thinner than that of cerebral PSDs (58 nm). On the basis of the above morphological criteria, these investigators describe the PSD-enriched fraction from cerebellum as derived from Type II synapses, and PSDs from cerebrum, from Type I synapses (5).

To assess the possibility that a subpopulation of Type I synapses containing the mPSDp does exist in the cerebellum, it is necessary to use approaches that are not prone to the vagaries of a multistep fractionation scheme, where the distribution of synaptic structures must be assessed in each subfraction. We used two unique properties of the mPSDp to assess its presence in various subcellular fractions: its insolubility in deoxycholate (DOC) and its calmodulin-binding properties. Using these two criteria, we have determined that the mPSDp is virtually absent in cerebellum. These observations require reconsideration of the role of the mPSDp in Type I synapses.

MATERIALS AND METHODS

Preparation and Analysis of Subcellular Fractions

The cerebrum and cerebellum were dissected taking care not to include brain stem tissue. Subcellular fractions were prepared in parallel from cerebellum, from a mixture of cerebellum and cerebrum combined before homogenization, and from cerebrum. The three starting samples (from 150- to 200-g rats) were 16 cerebellums (3.88 g wet weight), eight cerebellums and two cerebrums (1.91 and 1.71 g, respectively), and four cerebrums (3.71 g). The synaptic plasma membrane (SPM) fractions were prepared from each starting sample as described by Jones and Matus (18) with the following modifications: all sucrose solutions (expressed as wt/wt) were buffered with 5 mM HEPES, pH 7.4, and contained 50 μM $CaCl_2$ (36). To increase yields, the initial nuclear pellets (P_1) were rehomogenized and first and second supernatants (S_1) combined (9). Each crude mitochondrial (P_2) pellet (11,000 rpm for 20 min in Sorvall SS34; DuPont Instruments–Sorvall Biomedical Div., DuPont Co., Newtown, CT) was lysed by suspension in 9 ml of 5 mM Tris-HCl, pH 8.1, containing 50 μM $CaCl_2$ (36). After a 45 min incubation at 0°C, the sucrose concentration of each P_2 was adjusted to 34% as described (14). SW 25.2 nitrocellulose tubes (Beckman Instruments Inc., Spinco Div., Palo Alto, CA) were loaded with 5 ml of 10% sucrose, 20 ml of 28.5% sucrose and 35 ml of 34% sucrose containing the lysed P_2 fractions and centrifuged for 18 h at 20,000 rpm. The SPM fraction was collected from the 28.5%/34% sucrose interface and diluted three-fold with H_2O and pelleted. Crude homogenate and S_1 fractions (4-ml aliquots) were each diluted with 12 ml H_2O plus 2 ml 10% sucrose and pelleted (Beckman Ti60 for 45 min at 45,000 rpm) to yield respectively, the crude membrane and crude membrane without nuclei (P_{2+3}) fractions. For preparation of the DOC-insoluble fractions, the three pellets from each starting sample were resuspended in H_2O, adjusted to 1 mg/ml and diluted twofold to a final composition of 1% (wt/vol) sodium deoxycholate (Schwarz/Mann Research Lab. (Spring Valley, NY) Cat No. 1346 Lot No. T1070), 10 mM Tris-HCl, pH 7.6, 50 μM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride (freshly prepared), and 0.5 mg/ml protein in a total volume of 2 ml in Beckman No. 338819 polyallomer tubes. After incubation at 37°C for 1 h, the sample was diluted by addition of 1 ml H_2O, underlayered with 0.1 ml 56% sucrose, overlayered with H_2O to the 3.5 ml total tube capacity and centrifuged in Beckman No. 350575 adapters at 30,000 rpm in the Ty35 rotor. The supernatant was aspirated to 0.8 ml and mixed with 2.4 ml H_2O and centrifuged again as above but without the sucrose underlayer. The pellet was resuspended in a twofold concentrate of modified Laemmli sample buffer (17, 22) and electrophoresis performed as described previously (17). Assessment of the relative proportion of protein bands was performed using an ACD-18 automated microdensitometer (Gelman Instrument Co., Ann Arbor, MI). Peptide analysis of selected bands from the SDS polyacrylamide gels was performed as described previously (13, 17).

Visualization of Calmodulin-binding Proteins

Calmodulin was obtained from Calbiochem (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) and iodinated by a lactoperoxidase method (7) to a specific activity of 50 to 125 μCi/mol. SDS PAGE and ^{125}I-calmodulin (^{125}I-CaM) binding to the separated protein were performed exactly as described previously (7). Control experiments with 10 mM EDTA added to the ^{125}I-CaM incubation mixture were performed in parallel. After drying, the extent of ^{125}I-CaM binding was visualized by autoradiography. To quantify the results, slices were cut from the dried gels and counted in a Beckman Biogamma II scintillation counter (Beckman Instruments, Inc., Scientific Instruments Div., Irvine CA).

RESULTS

Electrophoretic Analysis of DOC-insoluble Fractions

In confirmation of a previous report (36), the DOC-insoluble fraction from cerebrum contains high quantities of tubulin (Fig. 1). Based upon densitometry, the Coomassie-Blue-staining band ascribed to tubulin accounted for at least 20%–30% of

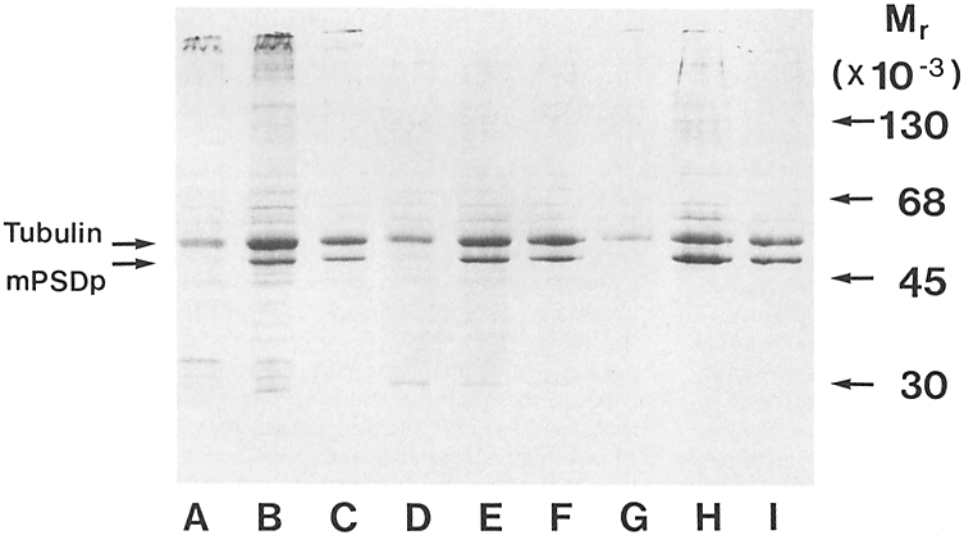

FIGURE 1 SDS PAGE of protein residue after solubilization in 1% sodium deoxycholate. Crude membranes (*A*, *B*, and *C*), combined P_{2+3} (*D*, *E*, and *F*) and SPM fractions (*G*, *H*, and *I*) were collected during successive stages of the subcellular fractionation described in Materials and Methods, using cerebellum (*A*, *D*, and *G*), combined cerebellum and cerebrum (*B*, *E*, and *H*) or cerebrum (*C*, *F*, and *I*) starting material. The tissue sample was extracted with deoxycholate as described in Materials and Methods, and the equivalent of 150 μg of protein (measured before extraction) subjected to electrophoresis and stained by Coomassie Blue; in the case of *B*, *E*, and *H*, an equivalent of 150 μg of cerebral protein was electrophoresed along with the cerebellar protein. With the gel system employed in these studies, α-tubulin is not resolved from β-tubulin.

the total staining pattern. A second feature of the DOC-insoluble fraction from cerebrum is the presence of a 51,000-M_r protein, migrating in a manner similar to the previously identified prominent macromolecular component of PSD preparations from cerebrum (9, 20). Earlier analysis of the DOC-insoluble fraction from cerebrum (36) had failed to identify this protein because of its electrophoretic mobility is almost identical to that of tubulin (54,000 M_r).

Cerebellar DOC-insoluble fractions contained similar amounts of tubulin but considerably lower amounts of the 51,000-M_r protein than observed in cerebral fractions. Densitometry measurements indicate that 3%, 4%, and 6.5% of the Coomassie Blue stain is present in the 51,000-M_r region of DOC-insoluble material of, respectively the crude membrane, P_{2+3}, and SPM fractions from cerebellum. For cerebrum, 16%, 16% and 15% of the Coomassie Blue staining was ascribed to same respective fractions.

Peptide Mapping of Major DOC-insoluble Proteins

Peptide maps of the 54,000-M_r regions of the gels allowed verification that the proteins comigrating with tubulin standards are indeed identical to tubulin as purified from cytosol (Figs. 2*A*, *B*, and *C*). The 51,000-M_r protein prominent in DOC-insoluble fraction purified from cerebrum yielded eight major tryptic ^{125}I-labeled-peptides (Fig. 2*E*); of these, seven were identical to published maps of the mPSDp (19). Therefore, we conclude that the 51,000-M_r protein present in cerebral DOC-insoluble fractions is identical to the previously described mPSDp. In contrast, the peptide map of the 51,000-M_r region of the cerebellar sample does not contain significant amounts of the peptides characteristic of the mPSDp. In particular, the two peptides indicated by two arrows in the cerebellar map (Fig. 2*D*) are only faintly apparent—although autoradiographic exposure time was increased twofold to enhance the detection of any minor mPSDp peptides present. The complex peptide mapping pattern of the cerebellar sample is indicative of a mixture of 51,000-M_r polypeptides. Control maps indicate little label incorporated into slices from blank polyacrylamide gel (Fig. 2*F*).

These results strongly indicate that the mPSDp associated with cerebrum PSDs is essentially absent from the cerebellum. However, another explanation is that the mPSDp exists in two forms. One form would be insoluble in DOC, while a postulated second form would be soluble in the presence of detergents. If we further postulate that enzymatic activities present in cerebellum (e.g., specific protease or protein phosphatase activities) mediate the transformation of detergent-insoluble into detergent-soluble forms, then mixing of cerebellum and cerebrum tissues before homogenization would be expected to decrease the content of mPSDp present in the DOC-insoluble samples. In contradiction to this hypothesis, all three subcellular fractions derived from mixtures of cerebellum and cerebrum tissue, whether crude or enriched in SPM structures, contain virtually identical amounts of the mPSDp as the equivalent fractions from cerebrum (Fig. 1*B*, *C*, *E*, *F*, *H*, and *I*).

Detection of mPSDp by ^{125}I-CaM Binding

A protein of 51,000 M_r has been reported to be a prominent calmodulin-binding protein (7). Remarkably, the calmodulin-binding property of this protein is at least partially retained or renatured by removing SDS from gels. Visualization is accomplished by incubating the gel with ^{125}I-CaM, washing away unbound ^{125}I-CaM, and autoradiography of the dried gel. This procedure provided visualization of ^{125}I-CaM-binding proteins in a complex mixture and is highly selective for a fraction of the protein species observed by Coomassie blue staining (Fig. 3). ^{125}I-CaM binds to an identical pattern of cerebral SPM proteins as described by Carlin et al. (7). Since other proteins derived from mitochondria or glia have an electrophoretic mobility near 51,000 M_r, it was necessary to verify that the ^{125}I-CaM-binding protein observed in the cerebral DOC-insoluble fraction is identical to the mPSDp. Readily detectable ^{125}I-CaM binding to the mPSDp was observed even when <1 μg of the DOC-insoluble residue was loaded on the gels. Precise alignment of the ^{125}I-CaM-labeled band and the mPSDp Coomassie-Blue-staining band was observed. The mPSDp was the

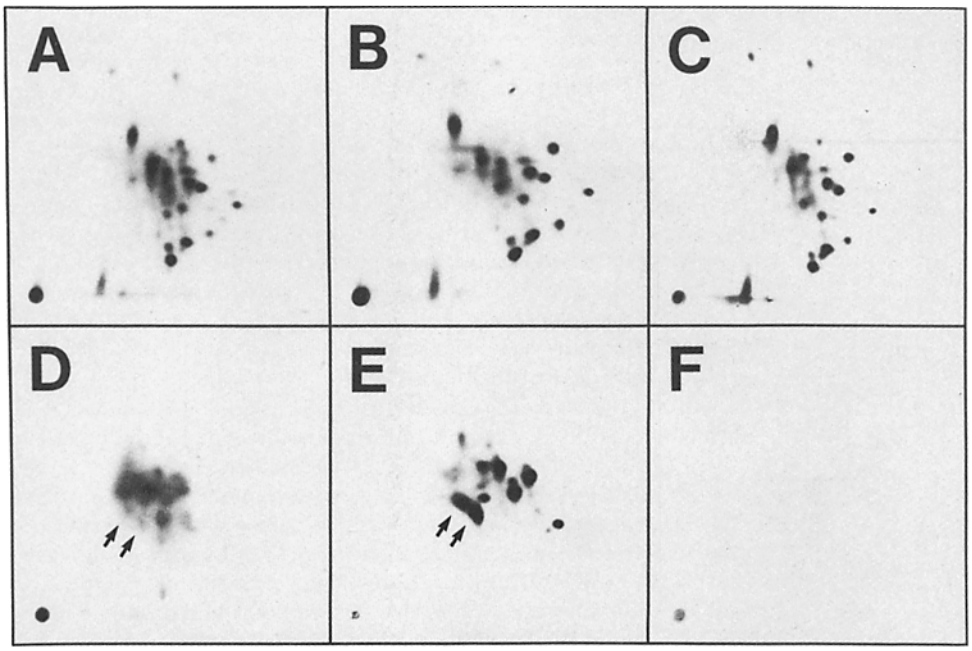

FIGURE 2 Autoradiographs of ^{125}I-labeled tryptic peptides from 54,000-M_r and 51,000-M_r regions of SDS polyacrylamide gels. Gel slices were obtained from the 54,000-M_r region of gels: peptide map A is from gel Fig. 1 *D*; peptide map *B* is from Fig. 1 *F*; peptide map *C* is from a gel of tubulin standard prepared from rat brain cytosol. The following peptide maps were from the 51,000-M_r region of the following gels: peptide map *D* from gel 1 *D*; peptide map *E* is from Fig. 1 *F*. Peptide map *F* is from a blank region of the gel. Peptide maps were exposed for 6 h, except *D*, 20 h. Electrophoresis was performed from left to right, and chromatography from bottom to top.

most prominent ^{125}I-CaM-binding protein in the cerebral DOC-insoluble fraction; also detected were significant ^{125}I-CaM-binding proteins of 61,000 and 224,000 M_r; and fainter ^{125}I-CaM-binding proteins at 32,000, 30,000, and 143,000 M_r. On the basis of estimates of the mPSDp content of the cerebral DOC-insoluble fraction and measurement of the amount of ^{125}I-CaM bound, we estimate that ^{125}I-CaM binds at a level less than one-tenth that calculated assuming a stoichiometry of one calmodulin bound to each mPSDp molecule. This is probably due to the limited access of calmodulin to the protein retained in the gel (7), but it could also be due to incomplete renaturation of the mPSDp calmodulin-binding site. The cerebral SPM fraction also contained a 75,000-M_r ^{125}I-CaM-binding protein not observed in the DOC-insoluble fraction. ^{125}I-CaM labeling of the 30,000- and 143,000-M_r proteins was more pronounced in cerebral SPM than in cerebral DOC-insoluble fraction. The total homogenate fraction from cerebrum contained in addition, a 24,000-M_r ^{125}I-CaM-binding protein (Fig. 3). Most of the ^{125}I-CaM-binding proteins observed in cerebrum were also observed in cerebellum with the notable exceptions of the 75,000- and 51,000-M_r proteins, indicating that little mPSDp is found in the cerebellum. ^{125}I-CaM binding to crude homogenates from cerebellum and cerebrum yielded essentially the same results as when SPM fractions were analyzed.

We investigated the binding profile of ^{125}I-CaM binding to the mPSDp fraction in cerebral and cerebellar SPM fractions as well as the DOC-insoluble fraction derived from cerebral SPM. As previously observed (7), ^{125}I-CaM binding to gel bands is not linear over a wide range of applied protein. However, when the gel slices from gels loaded with <75 μg cerebellar or cerebrum protein or <5 μg of DOC-insoluble protein are analyzed, the binding is approximately linear (Fig. 4). The linear interval of the isotherm corresponds to conditions where <20,000 cpm of ^{125}I-CaM is bound per 51,000-M_r gel slice. Quantification of the level of mPSDp indicates the following calculated slopes: cerebral DOC-insoluble fraction, 4,600 cpm/μg protein; cerebrum SPM, 220 cpm/μg; cerebellum SPM, 10 cpm/μg. When cerebral DOC-insoluble sample was diluted with 50 or 75 μg of cerebellar SPM protein in the Laemmli sample buffer, the slope was increased almost twofold to 8,500 cpm/μg (data not shown). This enhancement of the binding of ^{125}I-CaM in the presence of SDS denatured cerebellar proteins could be due to a carrier effect during dilution

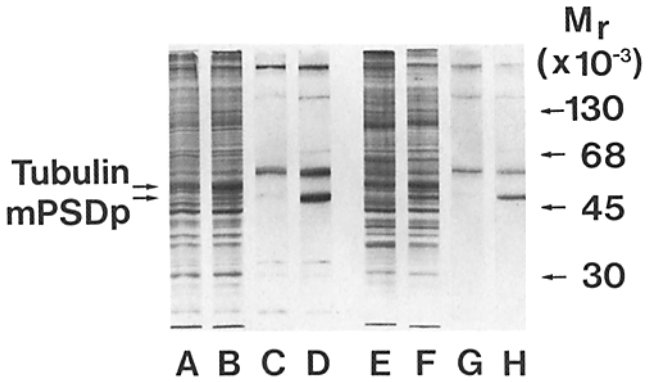

FIGURE 3 ^{125}I-CaM overlay of SDS PAGE of crude membrane and synaptic plasma membrane fractions. Gels *A–D*, crude homogenates; gels *E–H*, synaptic plasma membrane fractions from cerebellum *A, C, E, G*, and cerebrum *B, D, F, H*. After binding ^{125}I-CaM and washing, the gels were stained with Coomassie Blue *A, B, E, F* or autoradiographed *C* and *D* (38 μg, 20-h exposure); *G* and *H* (50 μg, 6-h exposure).

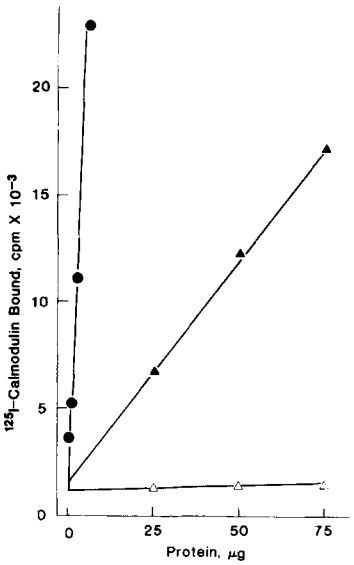

FIGURE 4 Scintillation counting of 51,000-M_r region from gels loaded with various amounts of DOC-insoluble and SPM fractions. The indicated amounts of protein were loaded onto gels and electrophoresis and ^{125}I-CaM overlay as described in Materials and Methods. Gel slices from the 51,000-M_r region were counted at an efficiency of 75%. (●) DOC-insoluble fraction from cerebrum SPM. (▲) Cerebrum SPM. (△) Cerebellar SPM.

or to an increase in the gel band width which has been correlated with the degree of ^{125}I-CaM binding (7). Whatever the cause for the enhanced ^{125}I-CaM binding, it serves to strengthen the conclusion that little mPSDp is present in the cerebellum.

DISCUSSION

To relate differences in the cerebellar and cerebral content of the mPSDp to its structural role in Type I and Type II synapses, it would be of value to estimate the relative proportions of the synaptic classes in the two tissues. There are numerous reports referring to Type I synapses in both the cerebrum and cerebellum, (cf. references 15, 35). In some cases the relative proportions of Type I and Type II synapses in the various identified regions have been reported. For example, 95% of synapses in the cat cerebellar molecular layer have morphological characteristics similar to those of Type I synapses (33), consistent with the observation that a large number of synaptic contacts with the Purkinje cell spines are almost exclusively Type I. On the other hand, Type II synapses are observed on the dendritic shafts of the Purkinje cell and on the bulbous enlargements of stellar cell dendrites and basket cell bodies. Overall, these observations convey the impression that Type I synapses are very common in the cerebellum. Ideally, an estimate of the relative protein content of Type I synapses in the two tissues would be most useful, so that the specific content of mPSDp could be related directly to the content of Type I synaptic protein in the two tissues. Such quantitative data are, unfortunately, not available. However, if we assume that the content of mPSDp is directly proportional to the counts of Type I synapses in the two tissues, then our data would imply that there is one-twentieth the number of Type I synapses in cerebellum as in cerebrum. This is inconsistent with the large body of cerebellar ultrastructural observations.

This confusing situation could be remedied by assuming that the mPSDp is an authentic marker for Type I PSD structures present in the cerebrum but rare in the cerebellum. If the mPSDp is, indeed, a component of cerebral Type I synapses, then the fact that the mPSDp is virtually absent in cerebellum implies that cerebellar and cerebral Type I synapses must now be further delineated. However, the evidence that the mPSDp is an authentic component of the PSD is based entirely upon the results of subcellular distribution studies (19, 20). Progress

in determining the precise localization of the mPSDp has been hindered by the fact that the mPSDp is nonantigenic; thus immunocytochemical localization studies have not been possible. In addition to containing readily identified PSD profiles, preparations of PSD contain the so-called subsynaptic web structures and interwoven filaments, as well as ~50% nondescript PSD-like material (9). It is not possible to identify which PSD structural feature contains the mPSDp, nor is it possible to state that all of the PSD structures and PSD-like material contains the mPSDp.

In addition to the mPSDp, other macromolecules have been proposed as PSD constituents (3, 9, 20, 26, 27). These include tubulin, intermediate filament subunits, and actin. Although tubulin has been localized to the PSD by immunocytochemical techniques (25, 26, 36), recent evidence suggests that tubulin accumulates in the PSD during the postmortem period before homogenization, thus weakening the hypothesis that tubulin is an authentic component of the PSD (6). Intermediate filament proteins are generally thought to be contaminants of PSD preparations (25). The presence of actin in the PSD structure has been inferred on the basis of co-electrophoresis of pairs of molecular species upon two-dimensional gels with purified cytoplasmic actins. These comigrating proteins exhibited tryptic peptide maps very similar, if not identical, to those generated from authentic actins (20, 27). Actin is widely distributed in the cytoplasm and appears, as well, in other cell organelles (28, 31) so that it could not be used with any certainty as a marker of PSD structures in isolated fractions. Other functional components such as Thy-1 antigen, neurotransmitter receptors, phosphodiesterases, and other modulatory proteins have also been postulated to be present in the synapse; however, these components may exist in extrasynaptic regions as well (1, 4, 12, 14, 29, 34). Recently, an antigenic determinant of 95,000 M_r has been proposed as a rather general marker for synaptic structures (30). Further delineation of this protein's role in synaptic structures must await development of monospecific antisera or monoclonal antibodies against this antigenic marker. Thus, no macromolecule can be considered, without reservation, to be an exclusive marker for the PSD.

The cerebral DOC-insoluble fraction, is reportedly rich in a postjunctional lattice structure (23, 24, 36) and is remarkably rich in tubulin. This is consistent with tubulin's localization to the postsynaptic density by immunocytochemical techniques (25, 26, 36). It is not possible to determine whether the mPSDp directly interacts with tubulin to form the cerebral DOC-insoluble fraction. However, it is possible to conclude that the mPSDp is not required to obtain significant quantities of DOC-insoluble tubulin, at least from cerebellar tissue. Further studies are required to assess the relationship of the putative underlying postjunctional lattice structure to the native postsynaptic density.

The conclusions drawn from ^{125}I-CaM gel overlay experiments do not depend on any assertion that the binding of this probe to the SDS denatured proteins is of any particular biological significance. However, studies using azido-^{125}I-CaM as a photoaffinity-labeling reagent for calmodulin-binding proteins (2), which can be used under nondenaturing conditions, yield a profile of cerebral calmodulin-binding proteins similar to that described here and earlier (7). The 51,000-M_r protein that is the focus of this communication is apparently identical to the 57,000-M_r protein described by Andreasen et al. (2). The determination of the ^{125}I-CaM-binding protein M_r by cross-linking experiments may be subject to nonsystematic errors. Should the mPSDp prove to be an authentic calmodulin binding protein, then the great difference in mPSDp content in cerebellum and cerebrum should have implications for the role of calmodulin in cerebellar and cerebral synaptic structure and physiology. It should be noted that the brain and muscle specific Ca^{++}-binding protein, parvalbumin, is localized to neurons scattered throughout the nervous system (8). In the cerebellum, Purkinje cells were strongly labeled with an anti-parvalbumin antiserum, whereas no immunostaining was observed in the granular cell layer. Thus, the nonuniform distribution of the 51,000-M_r calmodulin-binding protein and parvalbumin may be indicative of unique Ca^{++} modulated metabolic pathways in various cerebellar and cerebral neurons. The striking degree of segregation of the mPSDp in cerebellum and cerebrum provides an opportunity for the elucidation of the role of calmodulin in synaptic structures, by comparing the neurophysiological and ultrastructural properties of cerebral and cerebellar synapses.

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