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Biochemical and immunochemical evidence that the "major postsynaptic density protein" is a subunit of a calmodulin-dependent protein kinase

(synaptic structure/brain phosphoproteins/synaptic regulation)

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ABSTRACT **By three criteria, two biochemical and one immunochemical, the major postsynaptic density protein (mPSDp) is indistinguishable from the 50-kilodalton (kDa) α subunit of a brain calmodulin-dependent protein kinase. First, the two proteins comigrate on NaDodSO$_4$/polyacrylamide gels. Second, iodinated tryptic peptide maps of the two are identical. Finally, a monoclonal antibody (6G9) that was raised against the protein kinase binds on immunoblots to a single 50 kDa band in crude brain homogenates and to both the α subunit of the purified kinase and the mPSDp from postsynaptic density fractions. The purified kinase holoenzyme also contains a 60-kDa subunit termed β. A comparison of the peptide map of β with the maps of 60-kDa proteins from the postsynaptic density fraction suggests that β is present there but is not the only protein present in this molecular weight range. These results indicate that the calmodulin-dependent protein kinase is a major constituent of the postsynaptic density fraction and thus may be a component of type I postsynaptic densities.**

Many synaptic junctions in the central nervous system contain a prominent specialized structure called the "postsynaptic density" (PSD) (1–5). When viewed by electron microscopy, it is a fibrous, electron-opaque thickening lying opposite the presynaptic terminal, on the cytoplasmic side of the postsynaptic membrane. The morphology of postsynaptic densities is variable. Some are thick [20–60 nm (5)] and appear to cover the entire postsynaptic surface area, whereas others are thin, often discontinuous, patches (1, 6). The former have been termed type I PSDs, while the latter are called type II (1). Some investigators have suggested that different types of densities are associated with synapses of particular types. For example, type I PSDs most often occur in synapses that are thought to be excitatory (1, 7, 8), whereas type II PSDs are seen in synapses thought to be inhibitory (9, 10). This hypothesis suggests that morphologically distinct PSDs may also contain distinct proteins that serve specialized functions associated with their particular transmitter type.

In order to understand the structure and function of PSDs, various research groups have developed subcellular fractionation methods to isolate highly enriched preparations of PSD-like material (11–13). These procedures involve osmotic lysis of a subcellular fraction enriched in synaptosomes, followed by purification of junctional membrane complexes and extraction of membrane components by treatment with detergent. The detergent-insoluble residue, purified by density gradient fractionation, consists of electron-opaque, fibrous, disk-shaped structures, 20–60 nm thick and 200–500 nm in diameter (11–15). These structures are similar in appearance and staining characteristics to type I PSDs in intact fixed tissue, and they are not produced by detergent treatment of other subcellular organelles such as mitochondria or myelin (11). Thus, this subcellular fraction is considered to be highly enriched in type I PSDs.

The PSD fraction contains 20–30 proteins (13, 16). Among them are the cytoskeletal proteins tubulin (17, 18), actin (18, 19), and fodrin (20). It has been proposed that they are primarily structural, serving to anchor membrane or cytosolic proteins in the region of the postsynaptic membrane (21). The fraction is enriched in cyclic nucleotide phosphodiesterase activity (22, 23), calmodulin (24), and both cAMP- (25–27) and calmodulin-dependent protein kinase activities (27–29). Immunocytochemical evidence suggests that a calmodulin-dependent protein phosphatase, calcineurin, may also be located in PSDs *in situ* (30, 31). The presence of these regulatory enzymes suggests a role for the PSD in regulation of postsynaptic properties, such as receptor clustering, receptor sensitivity, or gating of ion fluxes. The major component of PSD fractions, making up 10–30% of the total protein, is a 50-kilodalton (kDa) protein of unknown function termed the "major PSD protein" (mPSDp) (18). This protein binds calmodulin (32, 33) and is also a substrate for a calmodulin-dependent protein kinase (28, 32).

We have recently purified and characterized a brain calmodulin-dependent protein kinase (34) that phosphorylates synapsin I (35, 36) as well as other brain proteins (34, 37). We will refer to it as synapsin I kinase to distinguish it from other calmodulin-dependent protein kinases. It is found in both soluble and particulate fractions of brain homogenates (37) and is a relatively abundant enzyme, making up as much as 0.3% of the total brain protein. When purified from the soluble fraction, it is a 650-kDa holoenzyme composed of three subunits with molecular masses of 50, 58, and 60 kDa (termed α, β', and β, respectively) (34). The α and β subunits are distinct peptides; however, β' may have been generated from β by proteolysis. The holoenzyme contains approximately nine α subunits and three β/β' subunits. All of the subunits bind calmodulin and are autophosphorylated under conditions in which the kinase is active. They are labeled with ^{32}P in endogenously phosphorylated brain homogenates (34, 37–39), where they account for a major portion of the endogenous "substrates" for calmodulin-dependent protein kinase in the 50- to 60-kDa region of NaDodSO$_4$/polyacrylamide gels (37).

Proteins of the same molecular weights are phosphorylated by a calmodulin-dependent protein kinase in purified PSD preparations (28, 29). The 52-kDa phosphoprotein is the major PSD protein referred to earlier. The identities of the 58- and 60-kDa phosphoproteins have not been established. These pro-

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Abbreviations: PSD, postsynaptic density; mPSDp, major postsynaptic density protein; kDa, kilodalton(s); INT, *p*-iodonitrotetrazolium violet; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; SM, synaptic membrane.

teins are similar to the phosphorylated proteins observed in crude brain particulate fractions (38). Consequently, Grab *et al.* (28) have suggested that the phosphorylated proteins in this molecular mass range in brain particulate fractions are probably PSD in origin.

The similarities between the 50-, 58-, and 60-kDa proteins phosphorylated in PSD fractions and the subunits of synapsin I kinase suggested that the two sets of proteins might be identical. To test this possibility, we have compared the proteins in PSD preparations with the subunits of synapsin I kinase by immunochemical and biochemical methods. We report here that the mPSDp is indistinguishable by three criteria from the α subunit of calmodulin-dependent synapsin I kinase. In addition, the β subunit of the kinase is present in the PSD fraction, although it is not the only protein present in the 60-kDa molecular mass range.

METHODS

Materials. $Na^{125}I$ (carrier free) was purchased from New England Nuclear. Dithiothreitol, *N,N*-bis(2-hydroxyethyl)glycine (Bicine), *p*-iodonitrotetrazolium violet (INT), sodium phosphate (mono- and dibasic), succinic acid, hemoglobin (bovine type II), mouse IgG, and napthol blue black (amido black) were purchased from Sigma. Trypsin treated with 1-tosylamido-2-phenylethyl chloromethyl ketone was purchased from Worthington, ultrapure sucrose from Schwarz-Mann, sodium *N*-lauroylsarcosinate from ICN, and staphylococcal protein A from Pharmacia. Cellulose-coated thin-layer chromatography plates were purchased from Eastman, and nitrocellulose membranes (BA85, 0.45 μm pore diameter) from Schleicher & Schuell. NS1/SP2 myeloma cells were a gift of Jeremy Brockes. BALB/c ByJ mice were purchased from The Jackson Laboratory, Simonsen albino rats (140- to 160-g males) from Simonsen Laboratories (Gilroy, CA), and New Zealand female rabbits from Lab Pets (Rosemead, CA).

Rabbit anti-mouse IgG antiserum was obtained from rabbits immunized by repeated subcutaneous injections with mouse IgG in Freund's adjuvant and was affinity purified by chromatography on Sepahrose 4B coupled with mouse IgG (40). ^{125}I-Labeled protein A (2–3 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) was prepared by the chloramine-T method (41). Calmodulin-dependent synapsin I kinase was prepared as described by Bennett *et al.* (34).

Preparation of Synaptic Membrane (SM) and PSD Fractions. SM and PSD fractions were prepared from 15 rats by the method of Cotman *et al.* (11). The PSD pellet adheres to glass and plastic; therefore, to maximize recovery of protein, it was resuspended in a small volume of 1% $NaDodSO_4$/2 mM Bicine. The reduction of INT by mitochondria that is used to increase their density produces an insoluble precipitate called formazan that adheres to the SM and PSD fractions. This precipitate interfered with determination of protein by the Lowry method (42). However, we estimate from Coomassie blue-stained gels that we obtained 40–60 mg of SM protein and 200–300 μg of PSD protein.

Selection of Hybridoma 6G9. Hybridomas that secrete monoclonal antibodies specific for synapsin I kinase were selected from a fusion of NS1/SP2 myeloma cells with spleen cells of BALB/c ByJ mice that had been immunized with kinase purified through the calmodulin-Sepharose step (34, 37). Details of the preparation and selection of these hybridomas will be published separately. Hybridoma 6G9 was initially selected by a solid-phase radioimmunoassay similar to that described by Moore *et al.* (43) in which culture supernatants were tested for the presence of antibodies that bound to the crude antigen. 6G9 culture supernatant was then tested for binding to individual peptides by the immunoblot procedure described below. The 6G9 hybridoma was subcloned, then ascites fluid was prepared by injection of $1–3 \times 10^6$ cells intraperitoneally into pristane-primed BALB/c ByJ mice. The antibody was partially purified by precipitation with 50% saturated ammonium sulfate, redissolved to a protein concentration of 20 mg/ml, and dialyzed against 20 mM Tris·HCl, pH 7.0.

Immunoblots. Proteins from $NaDodSO_4$/polyacrylamide gels were transferred onto nitrocellulose paper as described by Towbin *et al.* (44). After transfer, the nitrocellulose sheets were incubated with (*i*) buffer D (50 mM Tris·HCl, pH 7.4/0.9% NaCl/0.1% NaN_3) containing 5% hemoglobin (2 hr); (*ii*) 6G9 partially purified ascites fluid diluted into buffer D containing 1% hemoglobin (8–12 hr); (*iii*) wash buffer (buffer D containing 0.5% hemoglobin) (1 hr with three changes); (*iv*) rabbit anti-mouse IgG (2–7 μg/ml) in wash buffer (2 hr); (*v*) wash buffer (1 hr with three changes); (*vi*) ^{125}I-labeled protein A ($2–3 \times 10^5$ cpm/ml) in wash buffer (2 hr); (*vii*) wash buffer (1 hr with three changes). The nitrocellulose sheet was dried, and bands containing ^{125}I were detected by autoradiography.

Other Procedures. Peptide mapping was performed by the method of Elder *et al.* (45) as modified by Kelly and Cotman (18). Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was performed by the method of Laemmli (46). Stacking gels were 3.5% acrylamide/0.09% bisacrylamide. Running gels were 10% acrylamide/0.27% bisacrylamide. Molecular mass standards were phosphorylase *b*, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa. Crude rat brain homogenate was prepared as described by Bennett *et al.* (34) and subjected to centrifugation at $10,000 \times g$ for 10 min to remove unbroken cells and nuclei.

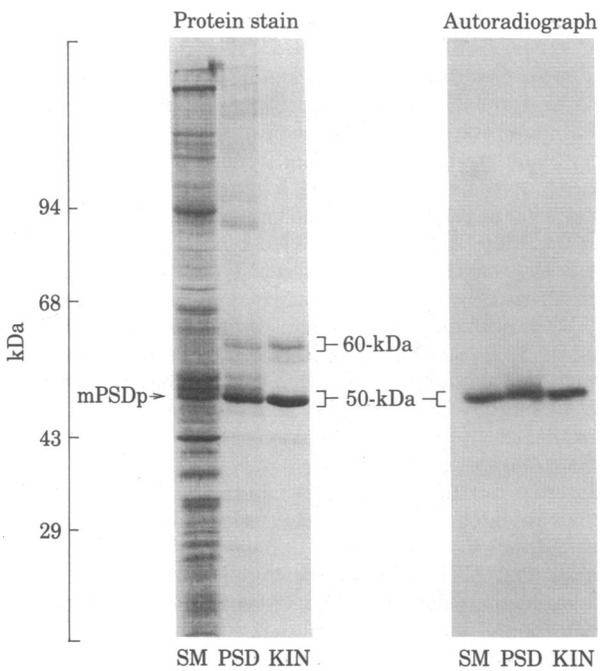

FIG. 1. Immunochemical relationship between the α subunit of synapsin I kinase and the mPSDp. (*Left*) The SM fraction (5 μl, ≈70 μg), the PSD fraction (30 μl, ≈25 μg), and synapsin I kinase (KIN, 6 μg) purified to 95% homogeneity (34) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and stained with Coomassie blue. The position of mPSDp is noted. (*Right*) The SM fraction (5 μl), the PSD fraction (7 μl, ≈6 μg), and purified synapsin I kinase (1 μg) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, transferred to nitrocellulose, and tested for reaction with antibody 6G9 (diluted 1:500).

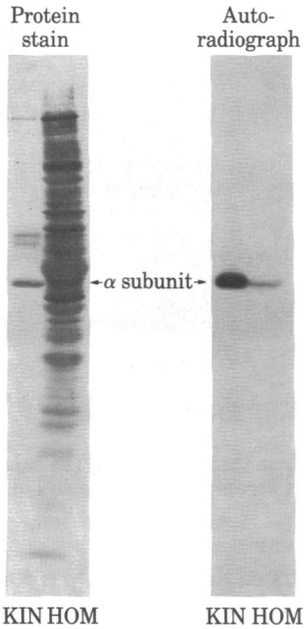

FIG. 2. Binding specificity of antibody 6G9 tested by the immunoblot method. Synapsin I kinase (KIN, 6 μg), partially purified through the calmodulin-Sepharose step (34, 37), and crude rat brain homogenate (HOM, 150 μg) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis in duplicate lanes and transferred to nitrocellulose paper. One pair of lanes (*Left*) was stained with amido black (44). The other pair (*Right*) was tested for reaction with antibody 6G9 (diluted 1:1000) by the immunoblot method. The calmodulin-Sepharose purified kinase is approximately 50% pure. The major band in the 50-kDa region is the α subunit (37). This is the preparation that was originally used as antigen for the generation of antibody 6G9.

RESULTS

The SM and PSD fractions prepared as described in *Methods* had protein compositions (Fig. 1 *Left*) similar to those published by Kelly and Cotman (16). The protein previously defined as mPSDp migrated with the α subunit of purified synapsin I kinase (marked 50 kDa).

For immunochemical comparison of the α subunit and the mPSDp, we used a monoclonal antibody (6G9) that binds with high affinity to the α subunit of the kinase on immunoblots (Figs. 1 and 2) and recognizes a single protein band of the same molecular weight in crude brain homogenates (Fig. 2). Proteins from the SM and PSD fractions were tested for binding to 6G9 as described for Fig. 1 *Right*. The results indicated that the epitope recognized by 6G9 is contained in the mPSDp as well as the α subunit of the kinase. Since 6G9 appears to react with only one protein band even in crude brain homogenates, its binding to both the α subunit and the mPSDp is strong evidence that the two are chemically related.

Additional and independent evidence that the two proteins are closely related was obtained by comparison of their iodinated tryptic peptide maps. Gel pieces containing the two proteins were cut from gels similar to those pictured in Fig. 1. The proteins were iodinated within the gel and digested with trypsin as described (18). Maps of the resulting iodinated peptides were indistinguishable (Fig. 3). In two separate experiments, both major and minor peptides were identical. The maps resemble those published by Kelly and Cotman of the mPSDp cut from $NaDodSO_4$ gels of the PSD fraction (18). This experiment provides further evidence that the mPSDp and the α subunit of the kinase are closely related and suggests that they may be identical.

The PSD fraction contained protein bands in the 60-kDa region that migrated near the position of the β subunit of the purified synapsin I kinase (Fig. 1, marked 60-kDa). We do not yet have a monoclonal antibody that reacts with the β subunits of the kinase on immunoblots, so we were unable to make an immunochemical comparison of the β subunits and the 60-kDa proteins of the PSD fraction. However, we have compared the two by peptide mapping. The regions marked by the 60-kDa bracket in Fig. 1 were cut from gels of the PSD fraction and the purified kinase. Iodinated tryptic peptide maps of the proteins are shown in Fig. 4. The eight major peptides in maps of the β subunit (marked with arrows) appeared to be prominent in maps of the 60-kDa proteins from the PSD fraction. Thus, a protein related to the β subunit is apparently present in the PSD fraction, but it is not the only protein present in this molecular mass range.

DISCUSSION

We have shown that the protein termed the major PSD protein (18) has several similarities to the α subunit of a recently purified brain calmodulin-dependent protein kinase, synapsin I

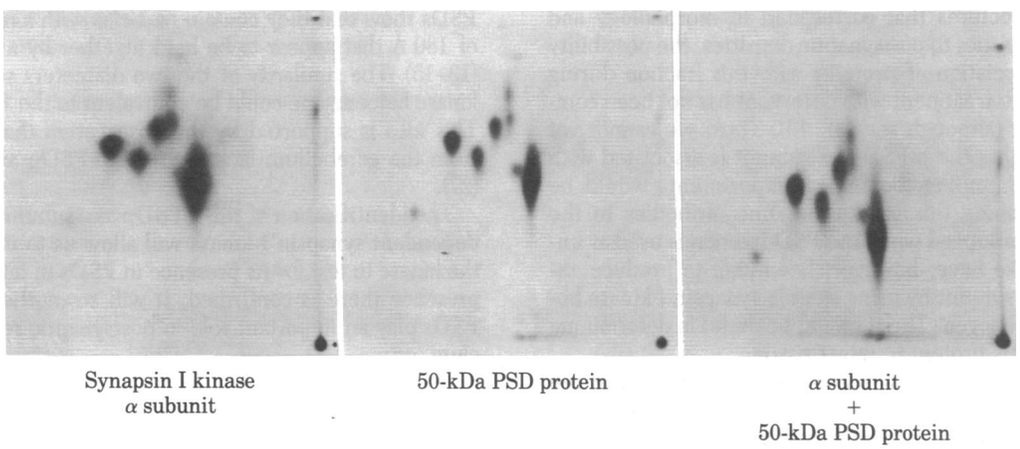

FIG. 3. Iodinated tryptic peptides of the α subunit of synapsin I kinase and the mPSDp. The PSD fraction (30 μl, ≈25 μg) and purified synapsin I kinase (6 μg) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. The bands marked 50 kDa in Fig. 1 were cut from the gel. Iodinated tryptic peptide maps of each were prepared as described (18). Two microliters of peptide solution was applied to the first two plates; 1.5 μl of each was applied to the third. The origin was at the lower right. The anode was to the right and the cathode to the left; chromatography was from bottom to top.

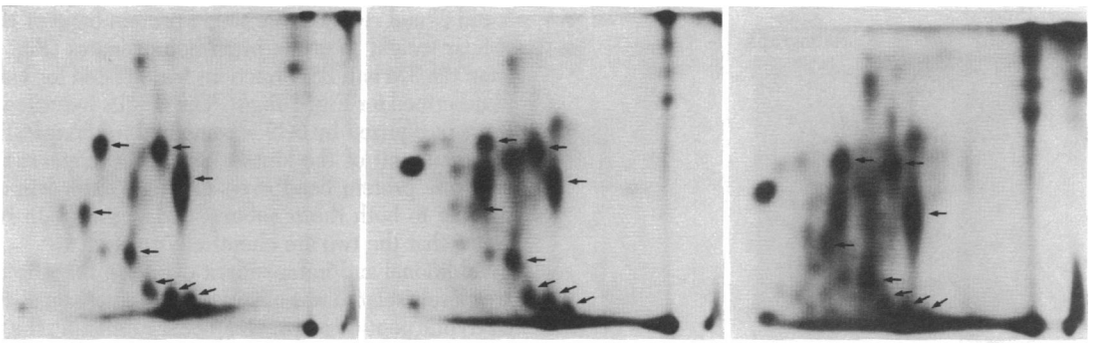

FIG. 4. Iodinated tryptic peptides of the β subunit of synapsin I kinase and 60-kDa proteins from the PSD fraction. The bands marked 60-kDa in Fig. 1 were cut from the same gel used in the experiment of Fig. 3. Iodinated tryptic peptide maps were prepared from each; 2 μl of peptide solution was applied to the plates. Electrophoresis and chromatography were as in Fig. 3.

kinase (34). They have the same mobility on $NaDodSO_4$/polyacrylamide gels. They both bind calmodulin (28, 34) and are phosphorylated in the presence of calcium, calmodulin, Mg^{2+}, and ATP (30, 34). They are both recognized by monoclonal antibody 6G9. Finally, iodinated tryptic peptide maps of the two are identical. These similarities do not rule out small differences between them in amino acid sequence or covalent modification. Nevertheless, they indicate that the two proteins are closely related and may be identical. One of the other two subunits of synapsin I kinase, β, also appears to be present in the PSD fraction. A third subunit, β′, occurs in lower amounts than the other two in the kinase holoenzyme and may be a proteolytic product of β (34, 37). We have preliminary evidence, based on peptide maps, that β′ is also present in the PSD fraction (data not shown).

The α subunit and the mPSDp are similar in two additional respects not described in this report. Immunochemical measurements indicate that, like the mPSDp (27, 47), the α subunit is much less concentrated in cerebellum than in the forebrain (48). Also, like the mPSDp (18), the α subunit streaks in the isoelectric focusing dimension of two-dimensional gels, and its recovery is low (data not shown). This probably occurs because the kinase holoenzyme is not completely dissociated by nonionic detergents.

Although several laboratories have shown that the PSD fraction contains structures that correspond in morphology and staining characteristics to postsynaptic densities, the possibility of artifactual association of proteins with this fraction during homogenization or treatment with detergent has not been completely ruled out (although see ref. 13). Thus, we cannot yet firmly conclude that the mPSDp/α subunit is associated with PSDs *in vivo*. Immunocytochemical experiments would be helpful in confirming this conclusion, but antibodies to the mPSDp are not produced when the PSD fraction is used as immunogen (49). We have, however, been able to produce antibodies to the α subunit by using soluble synapsin I kinase holoenzyme as immunogen. These should be useful in determining the location of this protein in intact tissue.

When it was originally described, it was postulated that the mPSDp might be a specific marker for the PSD (18, 50). However, Flanagan *et al.* (51) recently presented evidence that it may exist in soluble as well as particulate form. Our results support this idea, since we have previously shown that the α subunit is present in both soluble and particulate fractions of brain homogenates and appears to exist in a dynamic equilibrium between them (37). Kelly and Cotman (52) and Ratner and Mahler (21) have demonstrated that the mPSDp is crosslinked to PSD proteins by disulfide bonds. Thus, disulfide bonds are probably involved in the association of the kinase with the PSD fraction. They have proposed that control of formation of these bonds may be involved in regulation of the assembly and function of PSDs *in vivo*. It is possible that the extent of disulfide bonding may be influenced by autophosphorylation or dephosphorylation of the kinase subunits.

Evidence from other laboratories suggests that the synapsin I kinase is also associated with synaptic vesicles. For example, synapsin I, a principle substrate of the kinase, is primarily associated with vesicles (25, 53). Moreover, highly enriched vesicle fractions contain an endogenous protein kinase activity that phosphorylates synapsin I at the sites that are phosphorylated by purified synapsin I kinase (54, 55). In addition, partially purified synaptic vesicles from rat brain (56) and *Torpedo* electric organ (55) contain endogenous substrates for calmodulin-dependent protein kinase that have the same molecular weights as the autophosphorylated subunits of synapsin I kinase. The presence of the synapsin I kinase in several subcellular compartments would be consistent with the notion that it is involved in the regulation of many functions (34).

The Stokes radius of the synapsin I kinase holoenzyme is 95 Å, as determined by gel filtration (34), thus its diameter in solution is approximately 200 Å. Electron micrographs of isolated PSDs show that they contain particles with a median diameter of 180 Å that appear to be held together by a lattice of fibers (13–15). The similarity of the two diameters suggests that the kinase holoenzyme could be equivalent to the PSD "particles." This idea is supported by the observation that PSDs isolated from the cerebellum lack both the mPSDp and the particles (27).

The identification of the mPSDp as a subunit of calmodulin-dependent synapsin I kinase will allow us to use antibodies to the kinase to test for its presence in PSDs in intact tissue. If its presence there is confirmed, it will strengthen the idea that PSDs play an important role in postsynaptic regulation by calcium ion.

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