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FAM81A Protein, a Novel Component of the Postsynaptic Density in Adult Brain

Ayse Dosemeci[1,*], **Hannah K. Loo**[1], **Dana Toy**[1], **Christine A. Winters**[1], **Thomas S. Reese**[1], and **Jung-Hwa Tao-Cheng**[2]

[1]Laboratory of Neurobiology, NINDS, NIH, Bethesda MD, United States

[2]EM Facility, NINDS, NIH, Bethesda MD, United States

Abstract

Analysis of affinity-purified PSD-95 complexes had previously identified a 'hypothetical protein', product of the gene *FAM81A* [1]. The present study examined the tissue and subcellular distribution of FAM81A protein and its expression levels during development. Comparison of different organs indicates selective expression of FAM81A protein in brain. FAM81A is expressed late in development, with a post-natal gradual increase in brain levels that parallels the expression of PSD-95. Comparison of subcellular fractions from adult brain shows that the distribution of FAM81A protein is similar to that of PSD-95, with a drastic enrichment in the postsynaptic density fraction. Immuno-electron microscopy of adult brain tissue reveals specific immunogold labeling for FAM81A protein at postsynaptic densities in the forebrain. The label for FAM81A protein is concentrated at the cytoplasmic edge of the electron-dense core of the postsynaptic density, with a mean distance of ~33 nm from the postsynaptic membrane. These observations firmly establish FAM81A protein as a component of the postsynaptic density in the adult brain, suggesting a role in synaptic function.

Keywords

postsynaptic density; PSD; synapse; FAM81A

1. Introduction

The postsynaptic density (PSD) is a large protein complex lining the intracellular side of the postsynaptic membrane in excitatory synapses, with an estimated mass of about a million kDa [2]. The protein complex anchors and organizes glutamate receptors within the synaptic area and is involved in postsynaptic signal transduction. Components of the PSD are involved in the induction of activity-induced changes in synaptic strength (review: [3]) and some PSD components may serve as messengers from the synapse to the nucleus [4], [5].

[*]Corresponding author: dosemeca@mail.nih.gov.

Historically, putative components of the PSD have been identified through analysis of PSD-enriched fractions, and for many of these proteins, localization at the PSD has now been verified by immuno-electron microscopy (review: [6]). These studies revealed that organizers of the PSD are specialized scaffold proteins that can bind to each other as well as to receptors and signaling molecules. The main scaffold protein at the PSD core is PSD-95. Proteomic analysis of affinity-purified PSD-95 complexes identified additional putative PSD components, including a novel protein, product of the FAM81A gene [1]. A recent quantitative mass spectrometric study indicated a stoichiometric ratio of one FAM81A protein to fourteen PSD-95 in PSD fractions [7].

FAM81A protein [Q8TBF8 (FA81A_HUMAN), Q3UXZ6 (FA81A_MOUSE), D4A7T8 (D4A7T8_RAT)] has a molecular weight of ~42 kDa and contains three coiled coil domains. To our knowledge, no published articles exist that focus on the FAM81A gene or protein. As a preliminary stage in defining the role of this protein in the mammalian organism, we undertook to examine its distribution among different organs, its expression during development and subcellular distribution in neurons. Our results indicate selective expression of FAM81A protein in adult forebrain and firmly establish its localization at the PSDs in excitatory neurons.

2. Materials and methods

Antibodies:

FAM81A antibody 1: Rabbit polyclonal from Novus (product # NBP2-33295), antigen is recombinant protein corresponding to amino acids 77-216 in human sequence (1:100 dilution for western). FAM81A antibody 2: Rabbit polyclonal from Atlas Antibodies (product # HPA065797), antigen is recombinant protein corresponding amino acids 253-359 in human sequence (1:100- 1:500 dilution for western; 1:100 dilution for immunoEM). GFAP: Mouse monoclonal from Sigma (clone G-A-5, product number G3893, 1:200 dilution for western). Actin: Mouse monoclonal from Thermo Fisher (MA1-744, 1:1000 dilution for western) PSD-95: Custom made rabbit polyclonal from New England Peptide (1:1000 dilution for western). Histone H3: Rabbit monoclonal from abcam (EPR16987, 1:400 dilution for western).

Rat tissue:

The animal protocol was approved by the National Institute of Neurological Disorders and Stroke/ National Institute of Deafness and Communication Disorders/National Center for Complementary and Integrative Health Animal Care and Use Committee and conforms to NIH guidelines. Approval #Asp 1159-15. For comparison of levels of specific proteins in different organs, adult liver, kidney, spleen, heart, brain, gastrocnemius muscle and lung were harvested from pregnant Sprague Dawley rats within 30 min of euthanasia by CO2 inhalation. Tissue were homogenized and stored at −80°C until use. For the investigation of the expression of proteins during development, forebrains from Sprague Dawley rats at developmental stages P4, P7 and P10 and adult were collected and stored at −80°C until use. For subcellular fractionation, frozen adult rat brains custom collected (as described below) by Rockland Immunochemicals Inc. (Gilbertsville, PA) were used.

Subcellular fractionation from cerebral cortex

Brains from 7-12 weeks-old rats of both gender were custom collected by Rockland as follows: animals were anesthetized with isoflurane, brains were collected within two minutes after cervical dislocation and were immediately frozen in liquid nitrogen. Immediately before subcellular fractionation, brains were rapidly thawed in isotonic sucrose solution and dissected to remove white matter. Cerebral cortices were homogenized in isotonic sucrose. The homogenate was centrifuged at 850 g for 10 min to obtain pellet (P1) and supernatant (S1) fractions. P1 pellets were resuspended in isotonic sucrose and centrifuged at 600 g for 8 min (repeated two more times). The crude nuclear pellet was resuspended in 0.32M sucrose and layered on 2.3M sucrose and centrifuged at 40,000 rpm in a Beckman SW41 rotor for 1h. The nuclear fraction was collected as a pellet at the bottom. The supernatant (S1) fraction was centrifuged at 10,500 g for 12 min to obtain pellet (P2) and supernatant (S2) fractions. P2 pellets were resuspended in isotonic sucrose and fractionated on a sucrose gradient to isolate mitochondria (pellet through 1.2 M sucrose) and synaptosomes (1/1.2M sucrose interface). Synaptosomes were treated with 0.5 % TritonX-100 and the detergent insoluble pellet was fractionated on a sucrose gradient. Crude PSD fraction from the 1.5/2.1M sucrose interface was extracted with 0.5 % TritonX-100, 75 mM KCl and collected on a 2.1M sucrose cushion. Proteins from subcellular fractions were resolved by SDS-PAGE using 4-15% Mini-PROTEAN TGX Precast polyacrylamide gels (BioRad). Gels were transferred to PVDF membranes using the Trans-Blot Turbo Transfer System (BioRad), blocked, incubated with primary and secondary antibodies, and visualized via chemiluminescence.

Perfusion fixation of mouse brains and immunogold labeling for electron microscopy

The animal protocol was approved by the National Institute of Neurological Disorders and Stroke/ National Institute of Deafness and Communication Disorders/National Center for Complementary and Integrative Health Animal Care and Use Committee and conforms to NIH *guidelines*. Approval #: ASP1142). Adult mice were deeply anesthetized with isoflurane, and intracardially perfusion fixed with 4% paraformaldehyde in PBS via a Masterflex L/S Economy Pump System (Cole-Parmer, Vernon Hills, IL). In order to avoid over-fixation, total fixation time was about 30 min from the beginning of flow of the fixative into the heart until the time the brain was vibratomed into 100 μm thick coronal sections. The brain sections were washed and stored in PBS at 4°C, then immuno-labeled, free-floating in 24-well cell culture plates. All steps were carried out at room temperature unless otherwise indicated. Samples were made permeable and blocked with 0.1% saponin and 5% normal goat serum in PBS for 30 min, incubated with primary and then secondary antibodies (Nanogold, at 1:200, Nanoprobes, Yaphand, NY) for 1 hr, and fixed with 2% glutaraldehyde in PBS overnight. Controls for immunolabeling included omitting primary antibodies or using other primary antibodies in the same immunolabeling runs. Samples were then washed thoroughly in deionized water, silver enhanced (HQ kit, Nanoprobes), treated with 0.2% osmium tetroxide in 0.1M phosphate buffer at pH 7.4 for 30 min on ice, then block stained with 0.25% uranyl acetate in acetate buffer at pH 5.0 for 1 hr at 4°C, dehydrated in graded ethanol, and embedded in epoxy resin. Thin sections were counter stained with uranyl acetate and lead citrate, and examined with a JEOL 1200 EXII transmission electron

microscope. Images were photographed with a bottom-mounted digital CCD camera (AMT XR-100, Danvers, MA, USA).

3. Results

Levels of FAM81A protein in different rat organs were compared by western immunoblotting. Out of the seven different tissues tested, only brain was found to be immunopositive for FAM81A, a profile parallel to that of glial fibrillary acidic protein (GFAP), but in contrast to another protein, actin, which was expressed in all tissues tested, albeit at differing levels (Figure 1).

Next, we examined the expression of FAM81A protein in brain during development. Forebrains were harvested from animals within an age range from postnatal day 4 (P4) to adult. Two antibodies (Ab1 and Ab2) raised against unique peptides corresponding to non-overlapping sequences on the FAM81A protein were used. Both Ab1 and Ab2 recognized a main band with a molecular weight in the forty kDa range (figure 2 arrow) in adult brain. Another ~50 kDa band recognized by both antibodies was prominent in samples from P4-P10. However, mass spectrometric analysis of the 50 kDa band (from P10 animals) did not yield any peptides corresponding to FAM81A (whereas peptides corresponding to over a thousand other proteins were detected). Comparison of the ~42 kDA bands on lanes corresponding to P4, P7, P10 and Ad (adult) indicates that FAM81A expression increases with age, peaking in adult brain. Developmental profile of FAM81A protein closely resembles that of PSD-95, a major PSD scaffold, considered to be a marker of mature PSDs. On the other hand, levels of actin in brain showed a slight decrease between postnatal day 4 and adult.

While presence of FAM81A protein in PSD-95 complexes [1] points to possible localization at the PSD in neurons, the protein may also be present in other cellular compartments. Based on immunohistochemistry, Human Protein Atlas reports presence of FAM81A protein in neuronal nuclei. Also supporting a nuclear localization were results from an NCBI ‘conserved domain search’ we conducted for FAM81A protein. Searches with sequences for human, mouse and rat FAM81A protein identified a domain that resembles the ‘structural maintenance of chromosome N terminal’ (SMC_N) superfamily (E-values of 1.29e-04, 6.53e-05, 1.61e-05 respectively) between amino acids 79-334 in human and amino acids 76-317 in the mouse and rat. SMC proteins are generally involved in DNA metabolism and dynamics [8].

Subcellular localization of FAM81A protein was explored by conventional subcellular fractionation through a series of centrifugation steps as described in Methods. Fractions enriched in nuclei, mitochondria, synaptosomes and PSDs were obtained. Fractionation of nuclei and PSDs were monitored by western immunoblotting using antibodies for organelle-specific markers histone H3 and PSD-95 respectively. The distribution of FAM81A protein was monitored using two antibodies (Ab1 and Ab2) as before. FAM81A immunopositive band at ~42 kDa was found to be highly enriched in the PSD fraction, in parallel with PSD-95, whereas the nuclear fraction showed very little labeling at that position with either Ab1 or Ab2 (Figure 3). Other bands in the nuclear fraction that were immunopositive with

both Ab1 and Ab2 were further analyzed by mass spectrometry, but no peptides corresponding to FAM81A were identified (data not shown) indicating that these bands are not modified forms of FAM81A protein.

Enrichment of FAM81A protein at the PSD fraction strongly suggests that it is localized at the PSD. However, because artifactual redistribution of proteins during cell lysis and fractionation cannot be ruled out, verification by an independent technique is necessary. Thus, immuno-electron microscopy was used to visualize the distribution of FAM81A protein in the intact cell. Figure 4 shows electron-micrographs of excitatory synapses from adult mice hippocampus (Figure 4 A-C) and cerebral cortex (Figure 4 D,E) labelled using FAM81A antibody. More than half of PSDs from cerebral cortex and hippocampus showed specific labeling for FAM81A protein.

The label was concentrated at the cytoplasmic edge of the PSD core, with a mean distance of 34.1±1.1 nm from the postsynaptic membrane in hippocampus and 32.6±1.1 nm from the postsynaptic membrane in cerebral cortex (Figure 5). The localization of FAM81A label within the PSD was similar to that of GKAP, suggesting that the two proteins occupy the same laminar layer of the PSD, between PSD-95 at the PSD core and Homer at the PSD pallium (Supplementary Figure 3). Although some degree of hinderance to the penetration of the antibody may be expected when using the pre-embedding technique for immunolabeling, distinct localization of the label for PSD-95 at the dense core of the PSD (Supplementary Figure 3) indicates that this area of the PSD is still largely accessible.

In contrast to cerebral cortex and hippocampus, very little if any label on PSDs was observed in the cerebellum. Apart from PSDs, label for FAM81A was dispersed randomly in neuronal cytoplasm and did not appear to be associated with particular cytoplasmic structures. Labeling in nuclei was variable. About one third of the neuronal nuclei showed labeling densities greater or equal to that of the cytoplasm, while the rest of nuclei had labeling densities lower than that of the cytoplasm.

Discussion

FAM81A protein was detected only in brain tissue by western immunoblotting (Figure 1), in agreement with data from MaxQuant data base (http://maxqb.biochem.mpg.de/mxdb/), using mass spectrometry. On the other hand, presence of FAM81A mRNA, albeit at low levels compared to brain, is reported in several organs (Human Protein Atlas, https://www.proteinatlas.org) suggesting that the protein could be expressed at low levels in other tissues. Altogether our data and information from data bases suggest selectively high, but not exclusive, expression of FAM81A in brain.

Comparison of brain tissue from different developmental stages in rat shows increasing levels of FAM81A protein from postnatal day 4 to adult (Figure 2). Late expression coinciding with PSD-95 suggests a function for FAM81A protein in mature synapses in adult brain. It should be noted that another MAGUK, SAP102, with a structure similar to PSD-95 is expressed early in development [9], [10] and is thought to fulfill a similar

function to PSD-95 in immature brain. It is not known whether a distinct protein of the same family as FAM81A is expressed in immature brain.

FAM81A protein is found to be highly enriched in PSD fractions (Figure 3) and PSDs in cerebral cortex and hippocampus show distinct labelling for the protein (Figure 4). FAM81A label is mostly concentrated between the electron dense PSD core and the PSD pallium that contains the Shank-Homer scaffold (review: [6]). Specific localization of FAM81A protein at the PSD strongly implies a synaptic function for the protein. On the other hand, very low levels of expression in the immature brain suggest that FAM81A protein is not essential for synapse formation or for the basic organization of PSD. Rather, the present data implies a role of the protein in adult synaptic function.

While data from subcellular fractionation and immunoEM presented here firmly establish FAM81A protein as a component of the PSD, our assays could not demonstrate a consistent selective localization in neuronal nuclei. Subcellular fractionation showed less FAM81A in the nuclear fraction compared to parent fractions, whereas immunoEM data showed variable labeling for the protein at the nucleus. The very low levels of FAM81A observed in the nuclear fraction may be due to diffusion of the relatively small protein out of the nuclei during subcellular fractionation. In contrast to our observations by immunoEM that indicated higher levels of label in nuclei compared to the cytoplasm in only a minority of neurons, immunohistochemistry data presented in Human Protein Atlas shows predominant labeling for FAM81A in nuclei. Although the reasons for this discrepancy are not entirely clear, technical factors, such as fixation conditions and the activity level of neurons prior to fixation may have played a role.

Although our biochemical and immunoEM data fail to show an enrichment of FAM81A in nuclei, these observations do not negate presence of the protein in the nucleus. Actually, at least a transient localization of FAM81A in the nucleus is suggested by its putative interactions with nuclear elements: (i) our NCBI conserved domain search on the FAM81A sequence reveals a ‘structural maintenance of chromosome N terminal’ (SMC_N)-like domain; (ii) FAM81A is reported to interact with minichromosome maintenance complex component 2 (MCM2; https://thebiogrid.org/126935/summary/homo-sapiens/fam81a.html: [11]).

In summary, data presented here indicates that FAM81A protein is selectively expressed in adult brain and localizes at the PSD in excitatory neurons in the cerebral cortex and hippocampus. While biochemical and immunoEM data establishes FAM81A as a PSD component, sequence analysis and data mining hints at interactions with nuclear components, opening the possibility for a role of the protein in synapse to nucleus signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- FAM81A protein is selectively expressed in brain
- FAM81A protein levels are highest in adult brain
- FAM81A protein is located at the postsynaptic density

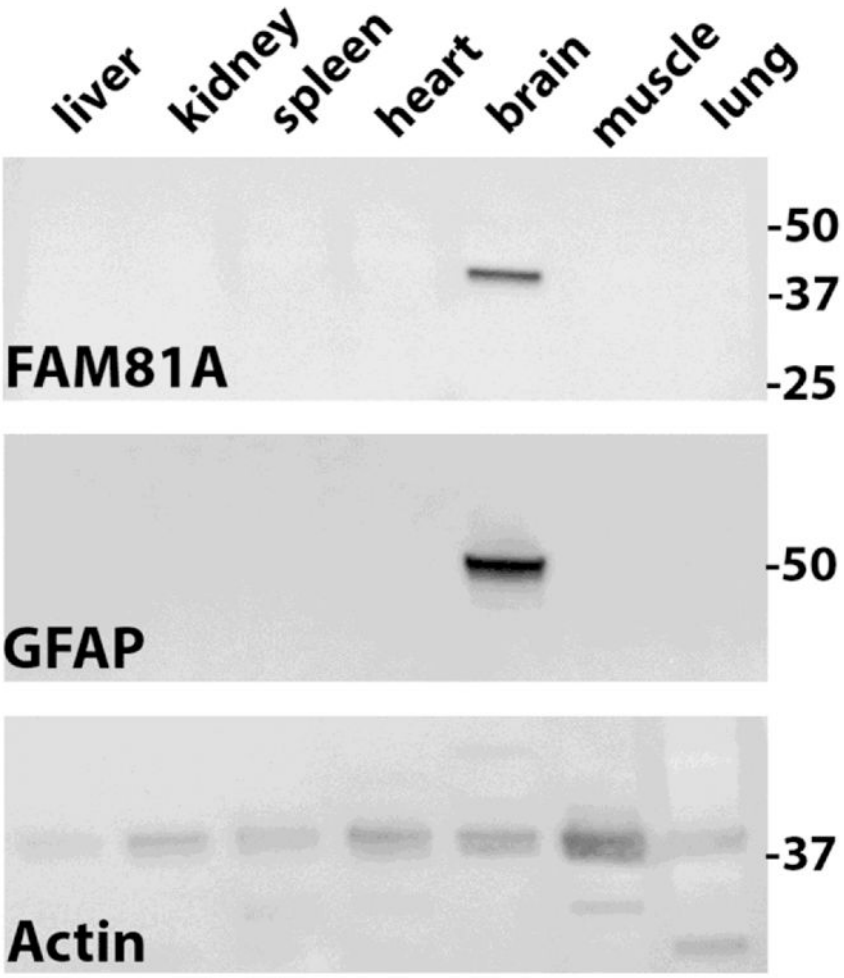

Figure 1: FAM81A protein is selectively expressed in brain.

Organs from adult rat were harvested and homogenized. Brain consisted of forebrain and cerebellum, and muscle was gastrocnemius. Equal amounts of protein (10 μg) were applied to each lane (Coomassie stained protein profiles in Supplementary figure 1). Western immunoblots were obtained using antibodies specific for FAM81A (Ab 1), GFAP and Actin.

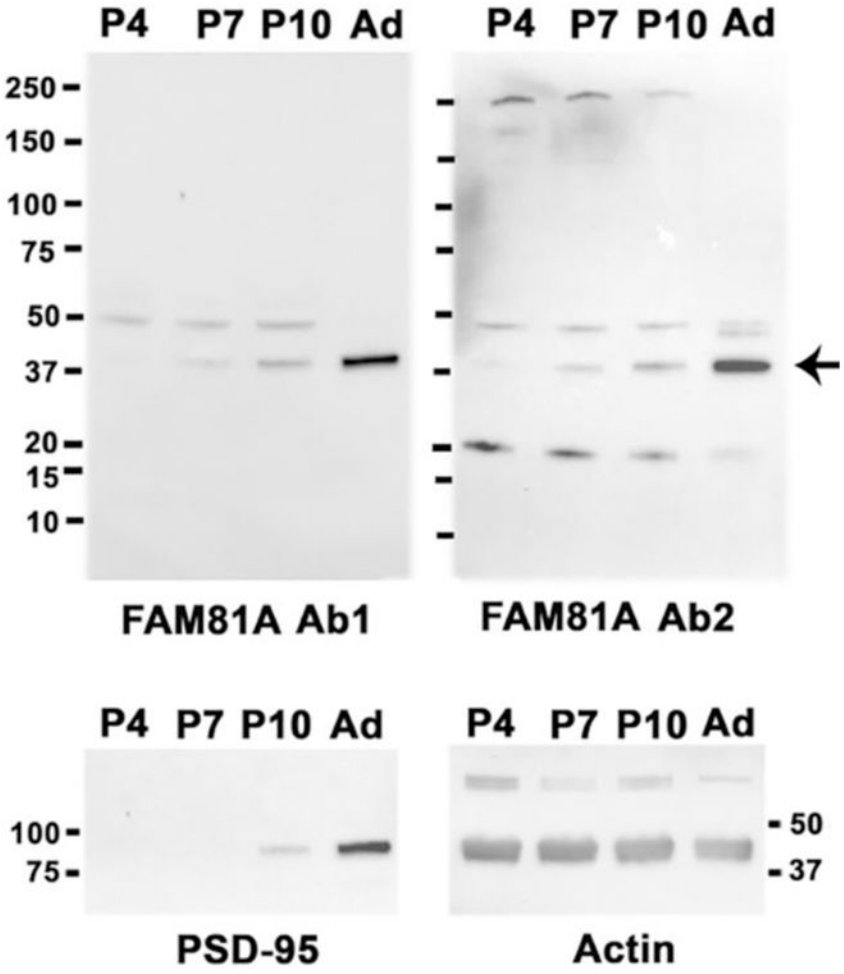

Figure 2: FAM81A protein in brain is expressed late in development, with a post-natal gradual increase in levels that parallels PSD-95.

Forebrains were collected from rats at postnatal days 4, 7 and 10 (P4, P7, P10) and adult (Ad) and homogenized. Equal amounts of protein (10μg) were applied to each lane (Coomassie stained protein profiles in Supplementary figure 2). Western immunoblots were obtained using two antibodies (Ab1 and Ab2) raised against peptides corresponding to non-overlapping sequences of FAM81A, as well as antibodies specific for PSD-95 and Actin.

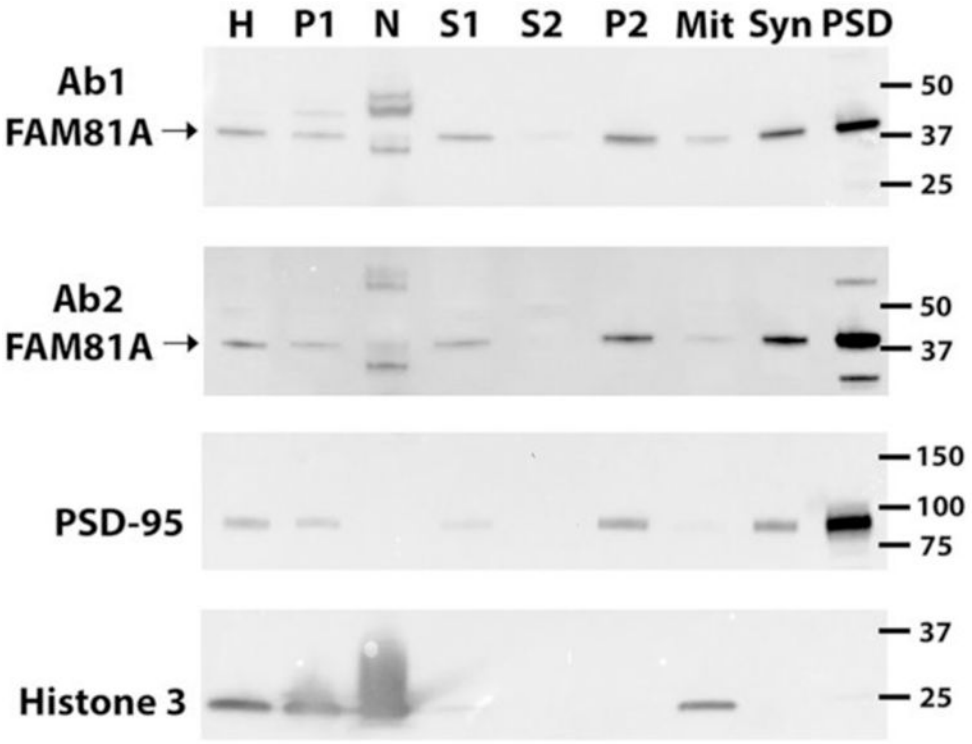

Figure 3: FAM 81A protein is enriched in PSD fractions.

Subcellular fractions were obtained from adult rat cerebral cortex. Equal amounts of protein were applied to each and western immunoblotting was carried out using antibodies for FAM81A (Ab1 and Ab2), PSD-95 (a marker for the PSD) and Histone H3 (a marker for the nucleus). H: homogenate; P1, S1: pellet and supernatant from first (low speed) centrifugation; N: nuclear fraction; P2, S2: pellet and supernatant from second (higher speed) centrifugation; Mit: mitochondrial fraction; Syn: synaptosome fraction; PSD: postsynaptic density fraction.

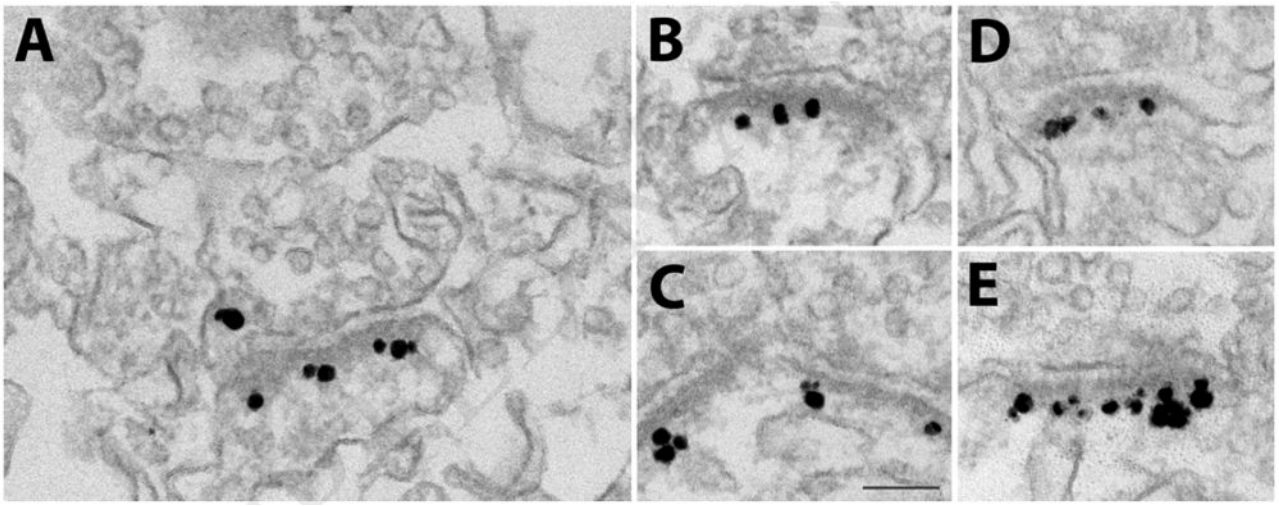

Figure 4: FAM81A protein label is concentrated at the PSD.

Electron micrographs of excitatory synapses in adult mouse brain immunogold labeled for FAM81A, using antibody 2. Dark grains of heterogeneous size are silver enhanced gold particles representing FAM81A label.

A,B,C: Hippocampus; **D,E**: Cerebral cortex. Scale bar = 0.1 μm.

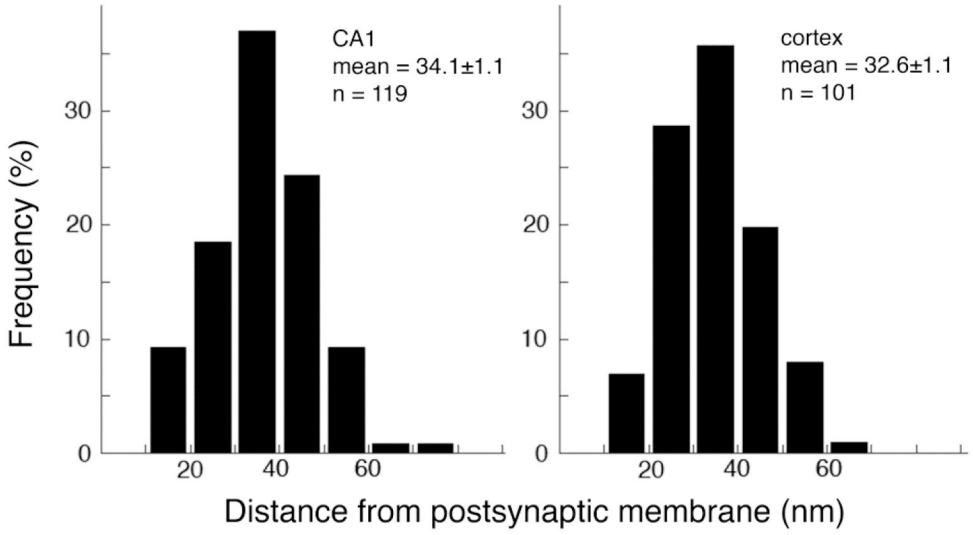

Figure 5: Laminar distribution of FAM81A label within the PSD.

Distances of gold particles from the postsynaptic membrane were measured in electron micrographs from adult mouse brain. Histograms show the percentages of label in consecutive laminar layers (10 nm bins).