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Structure and Composition of the Postsynaptic Density during Development

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Abstract

In this study, we used electron tomography as well as immuno-gold labeling to analyze the morphology and distribution of proteins within PSDs isolated from rats before birth (embryonic day 19) and at post-natal days 2, 21 and 60. Our data provides direct evidence of distinct morphological and compositional differences in PSDs throughout development. Not all PSD components are present at the early stages of development, with a near lack of the scaffolding molecule PSD-95 at E19 and P2. The presence of NR1 and NR2b suggests that PSD-95 is not directly required for clustering of N-methyl-D-aspartic acid (NMDA) receptors in PSDs early in development. α -Actinin is abundant by E19 suggesting it is a core structural component of the PSD. Both α and β isoforms of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) are present early on, but then rise in labeling density approximately four-fold by P21. Of all the molecules studied, only calmodulin (CaM) was found in higher abundance early in PSD development and then fell in amount over time. Spatial analysis of the immuno-gold label shows a non-random distribution for all the proteins studied, lending support to the idea that the PSD is systematically assembled in an organized fashion. Morphological data from electron tomography shows that the PSD undergoes major structural changes through out development.

Keywords

synapse; electron tomography; synaptogenesis; CaMKII; dendritic spine; immuno-gold labeling

Introduction

Most excitatory synapses in the brain are formed on small protrusions from the cell dendrites known as dendritic spines, and much work has been done to study the structure and abundance of spines during development. At the ultrastructural level, electron microscopy has been instrumental in characterizing the formation, size and shape of dendritic spines during different stages of development as well as the arrangement of spine structures such as the postsynaptic density (PSD) and the actin cytoskeleton (Cohen and Siekevitz, 1978; Fiala et al., 1998; Harris et al., 1992; Rostaing et al., 2006). Light microscopy, on the other hand, has played a critical role in observing dynamics of dendritic spine morphology and spine turnover at different stages of development (for an excellent review see Bhatt et al, 2009). Based on a wealth of evidence it is generally thought that dendritic spines form in abundance during early postnatal development, but are then pruned to a lower density as the brain matures (Huttenlocher, 1979; Markus and Petit, 1987; Rakic et al., 1986), supporting a model where neural circuitry is first established with a large constellation of synaptic connections, and then honed through the removal of unnecessary

contacts. Although the structure of dendritic spines has been well studied during development, the detailed structural changes that occur in one of its most prominent architectural features, the PSD, have received considerably less attention.

The PSD is a cytoskeletal specialization organizing postsynaptic signaling machinery, and is a hallmark structure of an established synapse. First described by early electron microscopists studying cytoarchitecture of the central nervous system, it was named for its electron opaque appearance in negative stained micrographs of thinly sectioned neuronal tissue (Gray, 1959; Palay, 1956). Since then PSDs have been biochemically isolated and extensive work has been done to characterize their general morphology (Cohen et al., 1977; Cohen and Siekevitz, 1978; Cotman et al., 1974) as well as their biochemical composition (Dosemeci et al., 2006; Jordan et al., 2004; Li et al., 2004; Peng et al., 2004; Yoshimura et al., 2004). The heterogeneous nature of PSDs has posed a significant hurdle in describing the underlying structural arrangement of its protein components, and to make matters worse the protein composition of the PSD is dynamic and regulated in an activity-dependent manner (Ehlers, 2003). Fluctuations in protein composition presumably allow an individual PSD to reside in multiple structural states depending on the activation history of the synapse. Despite knowing much about the proteins in the PSD and how its composition is regulated, there is still work to be done to understand its molecular architecture and how this architecture changes over time. Available data already suggest an organized structure with proteins exhibiting both lateral and laminar modes of ordered distribution (Chen et al., 2008; Petersen et al., 2003; Valtschanoff and Weinberg, 2001).

There is very little known about how the structure of the PSD changes during development. Petralia et al (2005) showed that there are distinct compositional changes in PSDs in the hippocampus between post-natal day two (P2) and 35 (P35). For instance, they saw a switch between the scaffolding proteins SAP102 and PSD95 from P2 to P35, and a similar switch was seen in the N-methyl-D-aspartic acid (NMDA) receptor subunits NR2b and NR2a. While the methodology they used allowed them to study compositional changes *in vivo*, thin sections of the synapse provide only a cross-section of the PSD in any one image, making it difficult to study the molecular structure of the entire PSD. Petersen et al (2003) and DeGiorgis et al (2006) both used immuno-gold labeling to study the distribution of proteins within PSDs isolated from adult forebrain, but how distribution might change during development remains an open question. In this report, we use electron tomography to study morphological changes in PSDs isolated from embryonic day E19 and post-natal days P2, P21 and P60. We then use immuno-gold labeling of PSDs isolated from each time point to assess both changes in protein composition from one time point to the next as well as the spatial distribution of each molecule through development. The molecules studied range in function from molecular scaffolds (PSD-95 and α -actinin) to neurotransmitter receptors (NR1, NR2b and GluR1) and calcium signaling proteins (α CaMKII, β CaMKII and calmodulin (CaM)).

Methods

Isolation of Postsynaptic Densities

Subcellular fractions enriched in postsynaptic densities were obtained from rat brains by using a slightly modified version of the protocol developed by Cohen *et al.* (Cohen et al., 1977). Forebrains from female Sprague-Dawley rats at ~P60 (160-180 g) were harvested within 30 sec of decapitation and placed in ice cold isotonic sucrose solution (0.5 mM Hepes, pH 7.4, 0.32 M sucrose, 1 mM MgCl₂, 0.5 mM CaCl₂, and 1 μ g/ml leupeptin) followed by disruption in a motor driven glass/Teflon homogenizer. After crudely clarifying the homogenate by centrifugation at 1,400 $\times$ g for 10 min the supernatant was centrifuged again at 13,800 $\times$ g for 10 min and the pellet was resuspended in isotonic solution (0.5 mM

Hepes, pH 7.4, 0.32 M sucrose, and 1 µg/ml leupeptin). A synaptosomal preparation was obtained by centrifugation of the resuspended pellet through a discontinuous sucrose density gradient (1.0 M-->1.4 M) at 110,000 × g for 2 hr. The synaptosomal fraction was recovered from the interface between the 1.0 M and 1.4 M sucrose. Synaptosomes were then solubilized using 0.5% Triton X-100, followed by another centrifugation step on a second sucrose gradient (1.0 M-->1.5 M-->2.1 M) and synaptic junctions were recovered from the interface between the 1.5 M and 2.1 M sucrose. A second detergent extraction with 0.5% Triton X-100 plus 75 mM KCl strips away residual pre- and postsynaptic membranes, and centrifugation across a final sucrose gradient (1.0 M-->1.5 M-->2.1 M) resulted in a final fraction highly enriched in PSDs at the interface between the 1.5 M and 2.1 M sucrose. The PSDs were collected from the interface, diluted in 5 mM Hepes, pH 7.4, pelleted and then resuspended in 5 mM HEPES, pH 7.4. Samples were either used immediately or made into aliquots containing 20% glycerol and frozen for storage at -80°C. The same protocol was used to isolate PSDs from rats at E19, P2 and P21. In the case of E19 and P2, volumes were scaled down in size to compensate for the smaller amount of starting tissue. For P60 PSD preparations, 9 rat forebrains were routinely used. At P21, one litter was used which generally consists of 10-13 pups. Three litters (~36 pups) were used for a P2 preparation and embryos (~60) from five pregnant females were used as starting material for E19 preparations.

Antibody Characterization

Please see Table 1 for a list of all antibodies used. In our immuno-gold labeling experiments a negative control in the absence of primary antibody was run in every case and the small amount of labeling was quantified and subtracted as background. Western blots for each of the antibodies are shown in supplemental Fig. 1.

The PSD-95 antibody recognized only a single band at the expected molecular weight (95 kD) on Western blot of rat brain homogenate, the synaptosomal fraction and the PSD fraction. Immunocytochemical staining of cultured neurons revealed fluorescent puncta on dendrites and dendritic spines (Bressler et al., 2004), and immuno-gold labeling of isolated PSDs showed labeling distributions and density similar to that shown in the present study (Petersen et al., 2003 and DeGiorgis et al., 2006).

The αCaMKII antibody recognized only a single band at the expected molecular weight (51 kD) on Western blots of rat brain homogenate, the synaptosomal fraction and the PSD fraction. The manufacturing of the antibody is described and Western blot evidence of its specificity for the α and not the β isoform of CaMKII is presented in Waxham et al. (1989).

The βCaMKII antibody recognized only a single band at the expected molecular weight (60 kD) on Western blots of rat brain homogenate, the synaptosomal fraction and the PSD fraction. Scholz et al. (1988) were the ones to develop and characterize the specificity of this antibody.

The NR1 antibody recognized only a single band at the expected molecular weight (120 kD) on Western blots of rat brain homogenate, the synaptosomal fraction and the PSD fraction. Seigel et al., (1994) developed the antibody, showed isoform specificity via Western blot and used it to localize NR1 distribution through different brain regions.

The NR2b antibody recognized only a single band at the expected molecular weight (170 kD) on Western blots of rat brain homogenate, the synaptosomal fraction and the PSD fraction. Laurie et al., (1977) raised antibodies against all four isoforms of the NR2 subunit, and then showed specificity via Western blot and usefulness in immunocytochemistry by looking at differences in the distribution of all four isoforms in rat brain.

The GluR1 antibody recognized only a single band at the expected molecular weight (106 kD) on Western blot of rat brain homogenate, the synaptosomal fraction and the PSD fraction. Martin et al., (1993) developed antibodies to all GluR subunits and showed specificity via Western blot, as well as localization to unique cell populations and subcellular regions by immunocytochemical staining in rat brain. Craig et al, (1993) showed postsynaptic localization of GluR1 using this antibody in cultured neurons.

The α -actinin antibody recognized only a single band at the expected molecular weight (100 kD) on Western blots of rat brain homogenate, the synaptosomal fraction and the PSD fraction. Abd-El-Basset et al., (1991) first established the immunohistochemical use of this antibody by studying the distribution of actin binding proteins in developing astroglia.

The calmodulin antibody recognized only a single band at the expected molecular weight (17 kD) on Western blots of rat brain homogenate, the synaptosomal fraction and the PSD fraction. Sacks et al., (1991) developed and characterized this antibody. They showed its specificity for calmodulin across various species, and calculated affinity for the interaction as well.

Immunolabeling of Postsynaptic Densities and Electron Microscopy

Four microliters of isolated PSD sample (~1 $\mu\text{g}/\mu\text{l}$) was placed on a formvar/carbon coated copper EM grid (freshly glow-discharged) and allowed to sit for one minute. Excess sample was blotted away with filter paper and the grid was floated sample-side-down on top of 40 μl of blocking buffer (5% BSA in PBS, pH 7.4) for 10 minutes. The grid was then moved to 30 μl of blocking buffer containing a dilution of the primary antibody for 30 minutes at RT. In these experiments, primary antibodies included monoclonal antibodies targeted to PSD-95 (ABR, MA1-046, 1:20), α -actinin (Sigma, 1:20), α CaMKII (produced in-house, 1:20), β CaMKII (CB- β 1, 1:100), NR1 (Millipore, 1:10), NR2b (Chemicon, MAB5778, 1:20), GluR1 (UpState, 1:10) and CaM (Upstate, 05-173, 1:5). After incubation with the primary antibody, grids were washed three times (5 minutes each) on drops of blocking buffer before a 30 minutes incubation on 30 μl of blocking buffer containing a 1:5 dilution of 12 nm gold-conjugated secondary antibodies (Jackson ImmunoResearch). After incubation with the gold-conjugated secondary, grids were washed three times as before but with water to remove residual salt, and negatively stained with Nanovan (Nanoprobes). Control PSDs were treated in the exact same way however no primary antibodies were applied. Prior to all immuno-labeling for quantification a titration with each primary antibody was performed to insure a concentration that provided a near asymptotic average labeling density. A similar titration was also performed using the 12 nm gold-conjugated secondary antibody. It is important to point out that the concentrations of antibodies used provided asymptotic average labeling densities. Many individual PSDs showed a higher labeling density than average, indicating that the system was not saturated in its ability to label additional target protein.

After immuno-labeling and negative staining, PSDs were imaged in the electron microscope (either with a JOEL CX100 or a FEI Polara F30) and digital micrographs were collected using a CCD. Micrographs of approximately 20 randomly selected PSDs were collected for each antibody at each developmental time point for subsequent quantitative analyses. PSDs were only imaged if they were between 0.2 and 1.2 μm across at their widest point. PSDs also needed to demonstrate a reasonably defined boundary, to avoid ambiguities in determining a perimeter needed to calculate their surface area. PSDs were most often roughly circular in shape, but this was not a requirement for inclusion in the analysis. Many PSDs were elongated or had protrusions from the side, making their boundaries irregular.

Electron Tomography and Image processing

For tomography, four microliters of PSD fraction were placed on a formvar/carbon coated copper EM grid (200 mesh) and allowed to sit for one min. At which point, excess sample was blotted away and the grid was washed twice with 5 μ l of Milli-Q water, before negative staining with Nanovan. The staining solution contained 1 μ g/ml bacitracin for even wetting on the grid surface and a 1:20 dilution of 10 nm colloidal gold balls (Sigma) for use as fiducial markers during alignment. PSDs that met morphological criteria were used to generate tomographic tilt-series on a 300kV FEI Polara F30 electron microscope at 63,000x magnification under low dose conditions. The total electron dose of each tomographic series was approximately 500 $e^-/\text{\AA}^2$. Images were collected at two degree tilt increments from -60 to +60° with 2X binning on a 4K $\times$ 4K Tietz CCD, which generated a final pixel size of 1.15 nm/pixel. Comparison of single images collected at the beginning and end of a tilt series confirmed that there was minimal radiation damage using these low dose conditions. Using the 10 nm gold fiducial markers included in the sample the images in the tilt series were aligned and tomographic reconstructions were generated using the IMOD software package (<http://bio3d.colorado.edu/imod/>). Tomograms were denoised by a single round of anisotropic nonlinear diffusion filtering using TOMOAND (software and references available at <http://www.ual.es/~jjfdez/SW/tomoand.html>).

Western Blotting

First, 12 μ g of total protein from forebrain homogenate, the synaptosomal and PSD fractions were separated by SDS-PAGE using a 10% polyacrylamide gel. Separated bands of protein were then transferred to a nitrocellulose membrane and incubated overnight in blocking buffer (5% dry milk in wash buffer) at 4°C. After blocking, membranes were incubated in a dilution of primary antibody (typically 1:1000) in blocking buffer for 1 hr at room temperature on an orbital shaker. Membranes were washed 3 $\times$ for 5 min in wash buffer (10 mM Tris, pH 8.0, 150 mM NaCl and 0.05% NP40) before application of the secondary antibody (Alexa 488-conjugated goat anti-mouse or goat anti-rabbit antibodies) at a dilution of 1:5000 in blocking buffer for 1 hr at room temperature on an orbital shaker. Incubation in the secondary antibody was followed by three more 5 min washes prior to imaging on a Typhoon laser scanner (GE Healthcare Life Sciences).

For Western blots of CaM it was necessary to add 10 mM EDTA to the SDS sample buffer and 1 mM EDTA to the 15% acrylamide gel and running buffer. Without the addition of EDTA the band detected by the CaM antibody does not migrate at the expected molecular weight as it is likely still in complex with other protein components or bound to calcium.

Point Pattern Analysis of Gold Particle Distribution

We employed Ripley's K-function analysis to assess the distribution of gold particles (Ripley, 1977). The K-function, $K(r)$, expresses the expected number of neighboring gold balls within a given distance r . $K(r)$ represents an analysis of inter-gold particle distribution patterns in a given region at all distance scales.

For this point pattern analysis, the X-Y coordinates corresponding to the center of each gold ball are needed. To do this a binarized image of the gold balls associated with each PSD was generated and the coordinates for the centroid of each gold particle was extracted using ImageJ (NIH). Using these coordinates for each set of points on a PSD, a convex hull encompassing all of the internal points was generated.

We used Ripley's K-function to compare experimentally determined patterns to the null hypothesis of complete spatial randomness (CSR). In order to do so, we need to know the K-function for a random particle distribution for each PSD. Except for a regular shaped

boundary (e.g., a square or circle), such a function is usually not known, and taking into account the edge effect of a complicated boundary, like the edge of the PSD, is not an easy task. One way to overcome this problem is to simulate the inter-particle distance distribution and evaluate the K-function numerically via Monte Carlo simulation (Yamada and Rogerson, 2003) and we employed this approach. One hundred sets of randomly distributed gold particles were simulated and then the mean, upper, and lower envelopes of the K-function for each set of simulations for a given convex hull were calculated. We then compared these simulation results (i.e., the inter-particle distribution) with the experimental data.

Simulated gold particles were generated as follows. As mentioned earlier, we calculated a convex hull for each particle set. We then used the boundary of this convex hull to generate a PSD of 60 nm thickness. Simulated gold particles were then randomly distributed within this 3D volume at the same labeling density (of internal points) as those in the given PSD particle set. We impose the volume exclusion principle, i.e., the distance between two simulated gold particles must be larger than the diameter of the actual gold particle (12 nm). In addition, the minimum distance of 6 nm was imposed on the inter-particle distance projected on to the 2D plane, as this is the minimal distance (the radius of the gold ball) at which two gold balls are easily distinguished in the raw 2D image data.

Finally, we compared the distribution of gold balls for each PSD to the upper or lower envelopes of the K-function defined by the 100 simulations of complete spatial randomness (Fig 8). The null hypothesis was rejected when the experimental data do not lie entirely between the upper and lower envelope of the simulated data (the envelope test, see Chapter 7 in Illian et al., 2008). All of the analysis and the simulations were done using MATLAB (The MathWorks, Natick, MA).

When deciding how to analyze the spatial distribution of the gold balls we also evaluated the Pair Correlation function as well as nearest neighbor analysis. Illian et al. (2008, Chapter 3) suggests that RKF was the most appropriate method to use when the data is confined within limited boundaries such as in the PSD. Our final decision to use Ripley's K function was based on the fact that the small size of some of our data sets caused both of the other techniques to produce plots with substantial fluctuations that were difficult to interpret.

Results

Isolation of Postsynaptic Densities

PSDs were isolated from rat forebrain using the same basic protocol developed by Cohen et al in 1977 (Cohen et al., 1977). As described in the Materials and Methods, forebrain tissue was homogenized and then fractionated by centrifugation through a sucrose gradient to obtain synaptosomes, a fraction enriched in pinched off presynaptic terminals with the postsynaptic membrane and PSD still attached. PSDs were then extracted from the synaptosomal fraction by exploiting their insolubility in Triton X-100. Fig. 1A shows a Coomassie stained gel with lanes containing protein from homogenate, synaptosomes and PSDs. There was not a large difference evident when comparing the protein pattern between homogenate and synaptosomes; only a few bands were visible in the homogenate that do not appear in the synaptosomes (arrowheads next to bands in homogenate lane). However, comparing the synaptosomes to PSDs, a relatively large subset of bands disappears (arrow heads next to bands in the synaptosome lane). There were bands present in the PSD fraction that were obviously distinct from the previous fractions (arrows). There were also bands that looked similar to ones in the previous fractions but upon closer inspection were not the same molecular weight (arrows with asterisks). It is not clear whether they are different proteins

or if they are just different isoforms or post-translationally modified versions of the same parent protein.

Figs. 1B and C show Western blots of the same biochemical fractions with antibodies against the PSD scaffolding molecule PSD-95 and the presynaptic vesicle protein SV2, respectively. As expected, PSD-95 showed a subtle enrichment between the homogenate and synaptosomal fractions, but a robust enrichment between the synaptosomal and PSD fractions. In contrast, SV2 enriched slightly between the homogenate and synaptosomal fraction, but then nearly disappeared in the PSDs. This was anticipated due to the disruption of membranes and release of pre-synaptic components, which are removed in the purification step between synaptosomes and PSDs. In total, these protein patterns in the different fractions are consistent with those reported previously and establish that by global protein patterns our PSD preparations are consistent with other studies (Cohen et al., 1977; Cotman et al., 1974; Ehlers, 2003).

Comparison of PSDs isolated from E19, P2, P21 and P60

There have been numerous studies examining the morphology of PSDs isolated from forebrain of young adult rat and other species (Cohen et al., 1977; Cotman et al., 1974; Petersen et al., 2003). We evaluated the protein composition and morphology of PSDs isolated from rats at four developmental time points (E19, P2, P21 and P60). The protein yield of PSD fractions from early in development (P2 and E19) was low, which made Commassie staining an inefficient method for visualizing protein separated by SDS-PAGE. Because of this, silver staining was used to compare protein profiles of PSDs from the four developmental time points (Fig. 2). At first inspection, the samples can be split into two groups based on the protein profiles. One group is made up of the two early time points (E19 and P2) and the other is made up of the two later time points (P21 and P60). There were obvious major bands shared by PSDs within each of the two groups. Boxes in Fig. 2 represent major bands that are shared within the early and late groups of PSDs, but are absent or only minor bands in the other group. Although there are differences in the profiles of the late and early groups they also share many alike bands that appear in similar abundance. These bands are indicated by arrowheads along the right side of the gel shown in Fig. 2.

Based on the differential protein profiles it was expected that PSDs from different times during development would exhibit distinct morphologies. In order to compare the morphology of isolated PSDs from each time point tomographic reconstructions were produced from negative stained samples. An example of a typical PSD isolated at E19 is shown in Fig. 3A, which is a display of the entire tomographic reconstruction viewed parallel to the Z-axis. In Fig. 3B, a 10 nm cross-section of the same PSD is shown. These two views are contrasted to illustrate the internal structural information gained through tomographic reconstruction, as well as to correlate the internal structure with its appearance in a full projection image. The same display is used subsequently for each of the developmental time points. PSDs isolated at E19 displayed a relatively lacey appearance compared to those at P2 (compare Fig. 3 A and B with Fig. 4 A and B). There appears to be more “empty” space (asterisks) distributed through the E19 structure, which was made primarily of rope-like strands of protein (horizontal arrows in Fig. 3B) intertwined to form a lattice structure. The lattice structure is evident in both the full projection image (panel A) and the 10 nm cross-section (panel B), but the individual strands are most easily distinguished in the cross-section (also see supplemental movie 1). Fig. 3C is a blow-up of the region bounded by the dashed box in the upper portion of 3B. It shows a closer view of the protein strand indicated by the arrow. At this higher zoom it is possible to see that the strands are made up of smaller protein components. Fig. 3D is a blow-up of the region bounded by the dashed box in the lower portion of 3B. It illustrates a region of the E19 PSD

that is made of a mesh of small protein filaments. One of the filaments is indicated by the arrow.

Fig. 4A shows a typical PSD isolated at P2. The overall appearance was different from the E19 PSD in that the strands of protein were not such a prominent feature (especially in the full projection image). However, they are still visible in the 10 nm cross-section (horizontal arrow in Fig. 4B). Fig. 4C is a blow-up of the region bound by the dashed box on the left of panel B. The arrow points to one of the rope-like strands of protein similar to the ones seen prominently in the E19 PSD. The lattice structure was less apparent at P2 as the PSD appeared more densely packed with protein, and there was less “empty” space visible in both the full projection and the 10 nm cross-section (asterisks). Despite the denser protein packing the P2 PSD was still mostly mesh-like compared to PSDs from P21 and P60. The lattice was visible, but studded with more regions of high protein density (vertical arrows in Fig. 4B and supplemental movie 2). Fig. 4D is a blow-up of the dense region of protein in Fig. 4B. Filaments like those seen in the E19 PSD are visible in the patch of protein, indicated by the arrow.

Fig. 5 and 6 show typical PSDs isolated at P21 and P60, respectively. PSDs isolated at these later developmental time points generally exhibited a denser protein packing although there are often perforations or pockets of low density (asterisks in Fig. 5B and 6B). The PSDs in Fig. 5 and 6 both display a variety of morphological features that were common to PSDs isolated from both P21 and P60, including protein rings (vertical arrows pointing downward in Fig. 5 and 6) and large solid regions of densely packed protein (horizontal arrows). The protein rings can be seen in the full projection, but their hollow center is more evident in the tomographic cross-section. This is due to the fact that, while they look like rings in cross-section, they are often fully enclosed by protein in all three dimensions and the edges appear densest from this angle. Fig. 5C-G shows a series of cross-sections through the protein ring bound by the dashed box in Fig. 5B. Moving from left to right through panels C to G the cross-sections move down through the Z-axis of the tomogram, showing that the protein ring is enclosed on the top and bottom and hollow in the middle. Hence, while it looks like a ring in projection it is actually roughly spherical (see also supplemental movies 3 and 4). Large areas of densely packed protein were more common to both P21 and P60 suggesting that large areas of the lattice seen routinely at E19 and P2 have been filled in by protein components recruited later in development. Vertical arrows pointing upward in Fig. 6B point to strands of protein that resemble those seen more routinely in PSDs isolated at E19 and P2, suggesting that the lattice structure seen at these early developmental time points remains at P21 and P60, but is obscured by additional protein packing. The letter “M” in Fig. 5 and 6 denotes pieces of membrane that were apparently not solubilized during the isolation process. These were common at P21 and P60, but were not seen at E19 and P2. It is not clear whether this is due to changes in the PSD that alter its interaction with the membrane or if it is a change in membrane composition later in development. While it is possible that the holes/perforations described in tomograms above could represent areas where stain failed to penetrate the PSD, we do not believe this is the case. If the stain had failed to penetrate, these areas would appear as light (or lighter) in intensity than the surrounding protein.

The dimensions of the PSDs in our preparations ranged from 0.2 to 1.2 μm when measured across their widest points, which is typical of that reported previously (Cohen et al., 1977; Cotman et al., 1974; Petersen et al., 2003). Due to their highly variable shape, measuring their area ended up being a more useful assessment on which to compare their relative sizes over the four developmental time points. Fig. 7 shows histograms of PSD surface area for each of the four developmental time points. Mean surface areas were $0.195 \pm 0.009 \mu\text{m}^2$ at E19, $0.313 \pm 0.017 \mu\text{m}^2$ at P2, $0.259 \pm 0.017 \mu\text{m}^2$ at P21 and $0.251 \pm 0.012 \mu\text{m}^2$ (surface

area $\pm$ SEM) at P60. All four time points show a peak around $0.15 \mu\text{m}^2$. The distribution of PSD sizes for all time points can be loosely described as a long-tailed marginal distribution.

Immuno-gold Labeling of PSDs Isolated at E19, P2, P21 and P60

We investigated the abundance of a subset of proteins in PSDs isolated from E19, P2, P21 and P60, utilizing immuno-gold staining in combination with electron microscopy. In all cases, negative controls were run in the absence of primary antibody and any gold present on PSDs was calculated as background labeling and subtracted from the values for each experimental condition. Across all experiments the background labeling density was consistently low and ranged from $0.7 - 7.0$ gold particles/ $0.1 \mu\text{m}^2$. Figs. 8A-D show representative PSDs immuno-gold labeled for PSD-95 at all four time points. We discuss the labeling of each class of proteins below, but can make the general conclusion that there was a trend toward increased labeling densities for most of the molecules probed with the exception of CaM. Also, some showed a peak in labeling density at intermediate time points (Fig. 8E). A summary of all the quantified data is presented in Table 2, and below is a detailed description of the results broken down by protein category.

Scaffolding Molecules—Of immediate interest is that not all components were present in PSDs isolated during early stages of development. In our preparations, PSD-95 labeling is very low in PSDs isolated early in development (0.2 gold/ $0.1 \mu\text{m}^2$ at E19 and 1.1 gold/ $0.1 \mu\text{m}^2$ at P2). In both cases roughly half of the PSDs imaged showed labeling above background (for actual values see supplemental table 1) suggesting that many of the PSDs isolated at E19 and P2 do not contain significant amounts of PSD-95. If only the PSDs that showed labeling were used to calculate a mean labeling density the values increase slightly to 0.4 gold/ $0.1 \mu\text{m}^2$ at E19 and 2.5 gold/ $0.1 \mu\text{m}^2$ at P2, which was still strikingly low.

By P21, labeling for PSD-95 increased to 38.5 gold/ $0.1 \mu\text{m}^2$, the highest mean density for PSD-95 across all four time points, which decreased to 27.5 gold/ $0.1 \mu\text{m}^2$ by at P60 (Fig. 8D and E). In this case, each change in density from one time point to the next was shown to be significant ($P < 0.05$). Of all PSDs analyzed across both P21 and P60 only one did not show labeling above background, suggesting that by P21 nearly all PSDs have incorporated PSD-95 as a stable scaffolding component. It is worth noting that the labeling density we observed in adult PSDs is consistent with that reported earlier using a similar labeling protocol (Petersen et al., 2003).

Interestingly, labeling for the actin binding protein α -actinin was relatively high at the two early time points (E19 and P2, Fig. 8E). Labeling density at E19 was 12.1 gold/ $0.1 \mu\text{m}^2$ and by P2 it had more than doubled to 32.1 gold/ $0.1 \mu\text{m}^2$. This abundant labeling early in development suggests that α -actinin likely plays a role in building the core lattice of the PSD and likely forms an important point of contact for the actin cytoskeleton. As one would expect from a core PSD molecule, all but one PSD across all four time points labeled positively for α -actinin (see supplemental table 1). Labeling density for α -actinin at P21 and P60 was 17.8 and 45.8 gold/ $0.1 \mu\text{m}^2$, respectively. These results indicate that α -actinin is an abundant protein component of the PSD across all time points, solidifying its role as an integral structural component of the PSD.

Glutamate Receptors—Three different glutamate receptor subunits were probed in this study; two subunits of the NMDA-type receptor (NR1 and NR2b) and one from the AMPA-type receptor (GluR1). The NMDA receptor subunit NR1 (the subunit required to form a functional ion channel) was present at a labeling density of 5.3 gold/ $0.1 \mu\text{m}^2$ at E19 and 4.4 gold/ $0.1 \mu\text{m}^2$ at P2. Although there is a slight decrease it is not significant, suggesting that there is little change in NR1 composition from the late embryonic stage (E19) through the

early postnatal stage (P2). In the P21 PSDs NR1 labeling increased five-fold from that of P2 to 22.3 gold/ $0.1\mu\text{m}^2$ and then dropped back down to 8.7 gold/ $0.1\mu\text{m}^2$ at P60. It is not clear why there is a spike in the abundance of NR1 during adolescence (P21), but the same pattern was seen in the labeling density for another NMDA receptor subunit NR2b, suggesting that the two receptor components could be up-regulated in concert at this stage of development. In fact, NR2b labeling densities are similar to NR1 across all four time points with a labeling density of 7.9, 8.5, 24.3 and 12 gold/ $0.1\mu\text{m}^2$ at E19, P2, P21 and P60, respectively.

The AMPA receptor exhibited low labeling densities at all developmental time points studied. Early labeling for its subunit GluR1 was as low as 1.0 gold/ $0.1\mu\text{m}^2$ at E19 and only 1.5 gold/ $0.1\mu\text{m}^2$ at P2. This number approximately doubled by P21 to 2.7 gold/ $0.1\mu\text{m}^2$ and 3.1 gold/ $0.1\mu\text{m}^2$ at P60. Despite the low density, the majority of PSDs at all time points exhibited labeling above background for GluR1 (see supplemental table 1).

Signaling Proteins—CaMKII is one of the most well studied molecular components of the PSD. The data in Fig.8B show that both α - and β CaMKII were present in PSDs isolated early (E19 and P2) and later (P21 and P60) in development. Labeling for both isoforms of the kinase approximately tripled from E19 (3.1 gold/ $0.1\mu\text{m}^2$ for α CaMKII and 2.1 gold/ $0.1\mu\text{m}^2$ for β CaMKII) to P2 (7.8 gold/ $0.1\mu\text{m}^2$ for α CaMKII and 9.0 gold/ $0.1\mu\text{m}^2$ for β CaMKII), which was followed by a three- and four-fold increase, respectively, by P21 (28 gold/ $0.1\mu\text{m}^2$ for α CaMKII and 35 gold/ $0.1\mu\text{m}^2$ for β CaMKII). The density of both isoforms of CaMKII did not show a significant change from P21 to P60, suggesting that by P21 labeling for CaMKII had become asymptotic. While the majority of PSDs showed labeling for both isoforms of CaMKII across all time points (96% for α CaMKII and 94% for β CaMKII), a small number of them contained a very low amount (seven PSDs from both E19 and P2 showed < 1 gold/ $0.1\mu\text{m}^2$ and four from P21 and P60 showed < 10 gold/ $0.1\mu\text{m}^2$). This variability in labeling for CaMKII is in agreement with that seen by Petersen et al (2003), in their immuno-gold labeling of PSDs. These high levels of both CaMKII isoforms suggest the holoenzyme not only plays an enzymatic role, but also functions as a structural component within the PSD.

CaM is a small protein that senses changes in intracellular calcium and transduces this signal to downstream molecules such as CaMKII. Interestingly, CaM was the only molecule in this study that followed an inverse trend and showed its highest PSD labeling at E19 (28.1 gold/ $0.1\mu\text{m}^2$) with a significant downward progression out to P21 (8.0 gold/ $0.1\mu\text{m}^2$). The decrease in labeling density from P21 to P60 was not statistically significant. This decline in labeling through development suggests that the increase seen in the other molecules is not a general rule for all proteins in the PSD and also suggests a greater demand for signaling through CaM during early stages of PSD development.

Spatial Analysis of Immuno-Gold Labeling

We next tested whether the distribution of gold labeling across the PSDs was random or clustered by subjecting the data for each PSD to spatial analysis using Ripley's K function (RKF). A typical example of the analysis for an individual PSD is shown in Fig. 9. The first step to doing the analysis was to extract the XY coordinates corresponding to the center of each gold ball and calculate the convex hull surrounding each data set for use as a boundary (Fig. 9B). The convex hull is a polygon whose vertices are defined by the outer most points of the data set, and because of this it is not a perfect representation of the PSD surface area. However, it has been used previously to examine the distribution of immuno-gold labeling in PSDs (Petersen et al., 2003). In general it is slightly smaller than the actual area as the gold markers do not usually follow the exact edge of the PSD, but it can, on occasion, incorporate area outside of the PSD's edge. Despite these potential differences, it does

represent the area labeled by immuno-gold markers and is free of subjectivity. Next, the points falling on the interior of the convex hull were compared to a simulated random distribution of gold particles at the same density placed within the same boundary (the red points in Fig. 9C). Because the labeling on PSDs actually occurs in three dimensions, we wanted to capture this in our simulation, so we added a third dimension (60 nm thickness) to the convex hull and randomly placed gold within the volume (details of the simulation process can be found in the materials and methods section). The XY coordinates of the randomly distributed gold particles were then extracted as with our experimental data and the simulation process was repeated for 100 iterations to provide a basis for analysis of non-random distribution.

Analysis by RKF is accomplished by calculating the average number of gold markers that fall within increasing radii extending from the center of each gold marker in the set (see supplemental Fig. 2). If clustering exists within the data set more gold particles will fall within a smaller radius when compared to the simulated random distributions. We considered the experimental data to be clustered if the plot of its RKF function fell outside of the minimum or maximum envelopes defined by the 100 iterations of simulated random distributions (Fig. 9D). Because we are dealing with point pattern distributions within a finite space (convex hull) the RKF plot is always a sigmoidal curve. This is because eventually the radius of measurement becomes large enough to capture all of the gold balls and the number of gold balls within increasing radii becomes asymptotic.

Among the eight different molecules probed in this study, at all four developmental time points, RKF analysis showed significant clustering in the majority of PSDs analyzed. Table 3 summarizes the number of PSDs analyzed in each group and those that showed a significant deviation from random. If a PSD did not have a minimum of 10 gold balls that fell within the convex hull we chose to remove it from the analysis, because the small number of data points led to RKF plots with large fluctuations. Because of this criterion, some PSDs at E19 and P2 could not be analyzed since labeling densities were generally lower early in development. CaM is an exception to this as labeling was high at E19 and was minimal by P60.

Discussion

To our knowledge, this is the first time that PSDs isolated from different developmental time points have been directly visualized in the electron microscope and their protein composition examined at the ultrastructural level. While isolating PSDs does remove them from their cellular environment it also provides the unique ability to image the entire structure from an *en face* view. This also makes them amenable to analysis by electron tomography, which permits 3D visualization of macromolecular complexes. In doing so, distinct morphological characteristics were evident between PSDs isolated before birth (E19) and during early postnatal development (P2). By P21 the overall structure of the PSD seems to have stabilized and no distinguishable morphological features were evident between PSDs isolated at P21 and P60. The tomographic reconstructions suggest that early in development (E19) the majority of PSDs are characterized by a lattice-like matrix of protein. The notion of a postsynaptic lattice or mesh is not new, as previous studies have reported an underlying mesh within the PSD (Matus and Taff-Jones, 1978; Gulley and Reese, 1981; Landis, 1987, (Petersen et al., 2003). In fact, Matus and Taff-Jones (1978) reported the appearance of mesh-like PSDs when isolated using the detergent deoxycholate as opposed to TritonX-100. They showed that PSDs isolated with deoxycholate were stripped of more protein revealing an underlying lattice-like structure they suggested to be the frame upon which the rest of the PSD was built (Matus and Taff-Jones, 1978). Shortly after birth (P2) the protein matrix is still mostly visible but less prominent, presumably

because the PSD is beginning to be filled in by the recruitment of additional proteins. Three weeks post-natal (P21), the lattice structure evident in early development is difficult to discern, and the appearance of structures such as dense rings of protein as well as large areas of densely packed protein are typical. The same structural characteristics are seen in PSDs isolated at P60 suggesting that by P21 the majority of large structural changes have already occurred.

Along with the structural changes, immuno-gold labeling of PSDs isolated at each time point revealed distinct compositional changes associated with each stage of development. First of all, not all PSD components are present early on, such as the scaffolding protein PSD-95. It was not unexpected that levels of PSD-95 are low as previous work has shown there is little PSD-95 in PSD fractions collected from rats two days postnatal (Petralia et al., 2005). However, quantification of PSD-95 in intact PSDs provides direct evidence for its near absence in immature complexes, and suggests that it is not critically involved in the initial stages of PSD formation. This means that postsynaptic densities in early stages of development may be missed by using PSD-95 as a postsynaptic marker and suggests that PSD-95 is not required for the initial clustering of NMDA receptors within postsynaptic densities. This supports previous findings that also suggest PSD-95 is not necessary for clustering of NMDA receptors (Migaud et al., 1998; Passafaro et al., 1999). While PSD-95 labeling was virtually absent at E19 and P2, it is likely that other members of the same protein family, such as chapsyn 110, SAP-102 or SAP-97 play an analogous scaffolding role at this early developmental time point (Petralia et al., 2005) and this possibility will be evaluated in future analyses.

Also of interest was the high level of α -actinin early in development, making it a likely candidate as one of the core PSD components involved in establishing the protein lattice described above. This observation fits nicely with current data showing that α -actinin interacts with both CaMKII and NMDA receptors (Leonard et al., 1999; Walikonis et al., 2001; Robison et al., 2005a), which would allow it to act as a point of recruitment within the PSD. The network of interactions between these three molecules is actually quite extensive with CaMKII having distinct binding sites for both α -actinin and the C-terminal tail of the NMDA receptor. The NMDA receptor subunits NR1 and NR2b both contain binding sites for α -actinin and CaMKII, and allows for the formation of a ternary protein complex (Walikonis et al., 2001; Robison et al., 2005). Multiple interactions within a complex like this would allow for increased stability once recruited to the PSD. The most well-recognized role for α -actinin is its ability to bind to and cross-link actin filaments and α -actinin may also be the attachment point for actin filaments often seen projecting from the PSD into the cytoplasm of dendritic spines (Cohen and Siekevitz, 1978; Rostaing et al., 2006; Korobova and Svitkina, 2010). It is also possible that the cytoskeletal protein actin is involved in the formation of the underlying PSD mesh, and further immuno-labeling experiments for actin and other cytoskeletal components will be pursued in future studies.

The labeling density for NMDA receptor subunits NR1 and NR2b changed in concert through all four time points with a peak at P21. While immuno-gold labeling cannot be used to quantitate the amount of these subunits, it is still an interesting trend recognizing that they are typically part of the same receptor complex. It has been shown that the NR2b subunit is important for synaptic plasticity early in development (Barria and Malinow, 2005; Sheng et al., 1994) and that the CaMKII binding domain in its C-terminal tail allows it to recruit CaMKII to the PSD (Gardoni et al., 1998; Shen and Meyer, 1999). This interaction is critical for long term potentiation and it is hypothesized that the switch from NR2b to NR2a (which has lower binding affinity for CaMKII) is a mechanism for regulating synaptic plasticity (Barria and Malinow, 2005). Even though our data show an increase in NR2b over time it is possible that the ratio of NR2b to NR2a has still gone down and further experiments to

assess this developmental switch in NMDA receptor subunit composition will be required to speak to this issue directly.

Recent work has shown that GluR1 containing AMPA receptor complexes account for approximately 80% of receptors at CA1 synapses in the hippocampus (Lu et al., 2009), which makes the low labeling of GluR1 at all time points a bit surprising. While the labeling density might not represent all copies of GluR1 in the PSD, the small amount of gold label seen at all time points suggests that a sub-population of the AMPA receptors at postsynaptic sites is not stably anchored within the framework of the PSD. This would be consistent with theories about the relatively rapid cycling of AMPA receptors at synapses (Ehlers, 2000; Lee et al., 2004; Malinow and Malenka, 2002).

The high labeling density of α CaMKII in PSDs at P60 was to be expected as previous studies have quantified its abundance and studied its distribution by immuno-gold labeling (Chen et al., 2005; Petersen et al., 2003). However, the large amount of labeling for β CaMKII was a surprise. Normally α CaMKII is thought of as the PSD enriched isoform, but looking at the Western blots in supplemental Fig. 1 it would appear that, if anything, the β isoform is more enriched relative to the amounts seen in homogenate and the synaptosomal fractions during isolation. It is important to note that CaMKII is a holoenzyme typically composed of mixtures of both α and β subunits and so targeting of one or the other subunit would be expected to also carry its partner into the PSD. That being said, one major difference between the two isoforms is β CaMKII's ability to directly bind actin (Fink et al., 2003; Ohta et al., 1986; Sanabria et al., 2009; Shen et al., 1998). Because of this, perhaps β CaMKII is involved more in regulating the interface between the PSD and the actin cytoskeleton in the spine, while α CaMKII regulates PSD structure through interactions with its many binding partners (e.g., α -actinin and NMDA receptors). Consistent with this hypothesis is that fact that β CaMKII is known to regulate spine growth (Fink et al., 2003; Lin and Redmond, 2008; Okamoto et al., 2007), a process that is highly dependent on alterations in the actin cytoskeleton. It is also interesting to note that by P21 and through P60 the labeling density for CaMKII rises considerably, suggestive of it playing a structural role in the PSD in addition to an enzymatic one. The unique structure of CaMKII (Kolodziej et al., 2000) suggests that it could be capable of binding multiple partners at the same time through its radial distribution of subunits.

Except for CaM, labeling for all of the other proteins studied increased in its abundance through development. This indicates that an increase in amount is not a rule for all proteins in the PSD. The most direct explanation for the increased abundance of CaM early in development is that synapses at this stage of development may depend more heavily on the Ca^{2+} /CaM-signaling pathways than synapses that have attained a mature state. One possibility is that as CaM-dependent proteins are recruited into the PSD and become integrated with their various binding partners, their activity is no longer entirely dependent on CaM, so CaM is no longer required in such abundance (Robinson et al., 2005b). Alternatively, because CaM can regulate binding between CaMKII and its binding partners (both positively and negatively), it is possible that higher levels of CaM early in development could function to promote certain interactions while suppressing others during the early stages of PSD assembly (Ohta et al., 1986; Merrill et al., 2007).

Results from the RKF analysis of immunogold labeling indicate that all of the proteins studied show a non-random distribution within the PSD. This suggests that the assembly of the PSD also follows a non-random process, although from the present data, it is not possible to identify specific protein components within the PSD. We are presently pursuing tomographic reconstructions of cryo-preserved PSDs at these different developmental stages which may permit visualization and identification of specific proteins or protein complexes

within the PSD. From the present spatial analysis we can conceive of two ways that proteins would be non-randomly distributed. Proteins could be regularly distributed within sub-regions of the PSD as described for CaMKII (Petersen et al., 2003), or molecules could be clustered together through shared protein-protein interactions. For example, several NMDA receptors could be clustered near one another through interactions with the same CaMKII holoenzyme. While such interactions likely contribute to clustering, from the present data we cannot make such conclusions, as only one protein species was labeled at a time. To better address such modular organization, future studies will require dual labeling various combinations of proteins with different sized gold balls.

In summary, the results of the present study illustrate that the three-dimensional structure of PSDs isolated at different times during development exhibit distinct morphological characteristics. While this is not surprising, this is the first time that PSDs isolated at various stages of development have been visualized and compared at the ultrastructural level using electron tomography. While no two PSDs are exactly alike there are general morphologies that are shared, and it appears that by P21 the majority of PSDs in rat forebrain are structurally mature by morphological criteria. As expected with structural changes, there are also accompanying changes in protein composition. While it is often assumed that the PSD plays an organizational role at the synapse, this assumption is largely intuitive and there is only a limited amount of data to support it (Petersen et al., 2003; Valtschanoff and Weinberg, 2001). The eight different proteins analyzed in this study all show non-random distributions providing evidence for systematic and organized assembly of the PSD. However, this is a small sampling of the over 100 protein components now thought to compose the PSD (Dosemeci et al., 2007; Dosemeci et al., 2006; Jordan et al., 2004; Li et al., 2004; Peng et al., 2004; Yoshimura et al., 2004) and there is still much work to be done to fully understand this organization and how exactly it supports synaptic function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Isolation of adult PSDs

(A) Coomassie stained SDS-PAGE gel loaded with forebrain homogenate (Hom.), synaptosomes (Syn.) and PSDs. The arrowheads in the homogenate and synaptosome lanes indicate bands that are not present in the subsequent fraction. Arrows next to bands in the PSD lane indicate proteins that are not present in previous fractions. Arrows with asterisks indicate bands that look similar to one in the previous fractions but upon closer inspection are not identical in molecular weight. Numbers (in kDa) to the left of the gel indicate the location of molecular weight standards run on the same gel. (B) Western blot illustrating the enrichment of the PSD scaffolding protein PSD-95 through the isolation process. (C) Western blot illustrating the loss of synaptic vesicle protein SV2 after solubilization of synaptosomes. All lanes in all panels were loaded equally with 12 μ g of total protein.

Figure 2. Comparison of Protein Profiles of PSDs Isolated at E19, P2, P21 and P60

Silver stained gel of SDS-PAGE separated proteins from PSDs isolated at E19, P2, P21 and P60. Based on the profiles the four lanes are categorized as early (E19 and P2) and late (P21 and P60) and major bands shared by PSDs within each group are indicated by boxes. Protein bands that are present across all four time points are indicated by arrowheads along the right side of the gel. Molecular weights (kDa) are indicated to the left of the gel. One microgram of total protein was loaded in each lane.

Figure 3. Tomographic Reconstructions of PSD isolated at E19

(A) Full projection image of the entire tomographic reconstruction at E19. (B) 10 nm cross-section through the center of the tomographic reconstruction shown in (A). Horizontal arrows indicate rope-like strands of protein that comprise the majority of the PSD structure. Asterisks indicate large areas of empty space within the structure. (C) Blow-up of the region bounded by the dashed box in the upper portion of (B). The arrow indicates one of the strands of protein seen making up the PSD lattice. In this panel it is possible to see the smaller protein components that make up the larger strands of protein. (D) Blow-up of the region bounded by the dashed box in the lower portion of (B). The arrow points to a smaller filament that can be seen in the meshwork of protein. Gold balls in the images are fiducial markers used to align images during the tomographic reconstruction. Scale bars represent 100 nm in (A) and (B) and 20 nm in (C) and (D).

Figure 4. Tomographic Reconstructions of PSDs isolated at P2

(A) Full projection image of the entire tomographic reconstruction at P2. (B) 10 nm cross-section through the center of the tomographic reconstruction shown in (A). The protein is more densely packed than at E19, but similar strands of protein can still be seen (horizontal arrow). The PSD lattice is studded with regions of densely packed protein as indicated by the vertical arrow. Large empty spaces are less abundant than at E19, but can still be seen (marked by the asterisk). (C) Blow-up of the region bounded by the dashed box on the left in (B). The arrow indicates one of the strands of protein seen making up the PSD lattice, and again, smaller protein components that make up the larger strands can be seen. (D) Blow-up of the region bounded by the dashed box on the right in (B). A dense region of protein packing can be seen, and the arrow points to a smaller filament like the ones seen in Fig. 3D. Gold balls in the images are fiducial markers used to align images during the tomographic reconstruction. Scale bars represent 100 nm in (A) and (B) and 20 nm in (C) and (D).

Figure 5. Tomographic Reconstructions of PSDs isolated at P21

(A) Full projection image of the entire tomographic reconstruction at P21. (B) 10 nm cross-section through the center of the tomographic reconstruction shown in (A). The vertical arrows in (A) point to protein ring structures that are commonly seen by P21 and the vertical arrows in (B) point to the same structures in the cross-section. The horizontal arrows indicate a large dense protein structure that is common by P21. The asterisk in (B) indicates an empty space that perforates the PSD and the “M” indicates a piece of membrane that is still attached. (C to G) A montage of slices moving down through the Z-axis of the region bounded by the dashed box in (B). Moving from left to right the panels show that the protein rings seen by P21 are actually fully enclosed. Gold balls in the images are fiducial markers used to align images during the tomographic reconstruction. Scale bars represent 100 nm.

Figure 6. Tomographic Reconstructions of PSDs isolated at P60

(A) Full projection image of the entire tomographic reconstruction at P60. (B) 10 nm cross-section through the center of the tomographic reconstruction shown in (A). The horizontal arrows point to a large dense protein structure similar to the one seen at P21. The downward-facing vertical arrows point to a protein ring structure. The upward-facing arrows in (D) point to protein strands that are similar to those seen at E19 and P2. Scale bars represent 100 nm.

Figure 7. Histograms of PSD surface area at E19, P2, P21 and P60

Histograms depicting the distribution of surface areas of PSDs isolated at E19 (A), P2 (B), P21 (C) and P60 (D). Comparison of the four histograms shows that all four developmental time points share a peak around $0.15 \mu\text{m}^2$ and similar size ranges.

Figure 8. Protein labeling density of target proteins at 48hr vs. adult

Electron micrographs of PSDs isolated at E19 (A), P2 (B), P21 (C) and P60 (D) immunogold labeled for PSD-95. Scale bars represent 100 nm. (E) Graph depicting the average labeling density for each antibody at each of the four developmental time points. For convenience, the graph has been organized by the functional category of each protein (scaffolds, receptors, signaling molecules). 14-23 PSDs were analyzed for each condition and the error bars represent the SEM. Asterisks indicate time points that show a significant change (increase or decrease) with respect to the previous time point.

Figure 9. Point pattern analysis of the spatial distribution of immunogold PSD labeling
 (A) Electron micrograph of a P60 PSD immuno-gold labeled for PSD-95. (B) Convex hull (black line surrounding points) of the extracted data points in (A). (C) Simulated random distribution of points (red dots) within the boundary. (D) The Ripley's K-function (RKF) of the experimental data (black solid line) is compared to the simulated complete spatial randomness (red solid line). The blue and green dashed lines represent the maximum and minimum envelopes, respectively, defined by the 100 iterations of simulated random distributions. The inset in (D) is a blow-up of the early portion of the plot, bounded by the black dashed line, where it is clear that the experimental plot (black) is outside of the maximum envelope of the simulated random data (blue dashed line).

Table 1

Antigen	Immunogen	Manufacturer, species antibody was raised in, mono- vs. polyclonal, catalog or lot number	Dilution used
PSD-95	Purified recombinant rat PSD-95.	Thermo Scientific (Rockford, IL), mouse monoclonal, MA1-046	Western: 1:1,000 Immuno-Gold: 1:20
α CaMKII	CaMKII purified from rat forebrain. The recognition site is somewhere within the first 342 amino acids based on immunoreactivity to deletion mutants of α CaMKII.	Waxham Lab, mouse monoclonal	Western: 1:1,000 Immuno-Gold: 1:20
β CaMKII	CaMKII purified from rat brain cytosol.	Invitrogen (Carlsbad, CA), mouse monoclonal, 13-9800	Western: 1:1,000 Immuno-Gold: 1:100
NR1	Recombinant fusion protein containing GST and NMDAR1 AA 660-811.	Millipore (Billerica, MA), mouse monoclonal, MAB363	Western: 1:1,000 Immuno-Gold: 1:10
NR2b, C-terminal tail	Recombinant protein from rat NR2b.	Millipore (Billerica, MA), mouse monoclonal, MAB5778	Western: 1:1,000 Immuno-Gold: 1:20
GluR1	KLH-conjugated synthetic peptide corresponding to AA 858-868 (TLPRNSGAGAS) of human GluR1 (AA 840-850 of mature GluR1).	Millipore (Billerica, MA), rabbit monoclonal, 05-855	Western: 1:1,000 Immuno-Gold: 1:10
α -Actinin	Cytoskeletal fraction of bovine mammary gland epithelium (BMGE) cultured cells.	Sigma-Aldrich (St. Louis, MO), mouse monoclonal, A 5044	Western: 1:1,000 Immuno-Gold: 1:20
Calmodulin	AA 128-148, bovine CaM conjugated with thyroglobulin.	Millipore (Billerica, MA), rabbit monoclonal, 05-855	Western: 1:1,000 Immuno-Gold: 1:5

Table 2

Summary of immuno-gold labeling results

	Gold/0.1um ² ± SEM			Gold/PSD ± SEM					
	E19	P2	P21	P60	E19	P2	P21	P60	
α -Actinin	12 ± 1	32 ± 3	18 ± 2	46 ± 2	17 ± 2	50 ± 10	21 ± 3	119 ± 13	
α CaMKII	3 ± 1	8 ± 1	28 ± 3	31 ± 3	10 ± 2	39 ± 8	49 ± 8	105 ± 16	
β CaMKII	2 ± 0.5	9 ± 2	35 ± 5	27 ± 3	7 ± 1	56 ± 13	35 ± 6	76 ± 10	
CaM	28 ± 4	15 ± 2	8 ± 2	6 ± 1	31 ± 6	32 ± 6	6 ± 1	14 ± 2	
GluR1	1 ± 0.2	2 ± 0.3	3 ± 1	3 ± 1	3 ± 0.5	9 ± 2	6 ± 1	14 ± 3	
NR1	5 ± 1	4 ± 1	22 ± 3	9 ± 1	12 ± 2	28 ± 3	21 ± 3	63 ± 10	
NR2b	7 ± 1	9 ± 1	24 ± 2	12 ± 2	19 ± 3	33 ± 7	22 ± 3	46 ± 7	
PSD95	0.2 ± 0.06	1 ± 0.5	38 ± 4	27 ± 3	2 ± 0.5	8 ± 3	56 ± 9	96 ± 16	

Table 3

Number of individual PSDs that showed clustering by RKF analysis for each antibody and developmental time point.

	E19	P2	P20	P60
α-Actinin	6 of 7	12 of 15	8 of 10	15 of 16
αCaMKII	1 of 4	10 of 12	14 of 16	14 of 17
βCaMKII	1 of 1	10 of 11	14 of 15	12 of 15
CaM	8 of 10	10 of 14	NA	5 of 6
GluR1	NA	2 of 3	NA	4 of 7
NR1	2 of 4	9 of 15	5 of 8	13 of 16
NR2b	3 of 6	11 of 16	2 of 11	14 of 16
PSD95	NA	6 of 6	12 of 13	15 of 16