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Ontogeny of Postsynaptic Density Proteins at Glutamatergic Synapses

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Abstract

In glutamatergic synapses, glutamate receptors (GluRs) associate with many other proteins involved in scaffolding and signal transduction. The ontogeny of these postsynaptic density (PSD) proteins involves changes in their composition during development, paralleling changes in GluR type and function. In the CA1 region of the hippocampus, at postnatal day 2 (P2), many synapses already have a distinct PSD. We used immunoblot analysis, subcellular fractionation, and quantitative immunogold electron microscopy to examine the distribution of PSD proteins during development of the hippocampus. Synapses at P2 contained substantial levels of NR1 and NR2B and most GluR-associated proteins, including SAP102, SynGAP, the chain of proteins from GluRs/SAP102 through GKAP/Shank/Homer and metabotropic glutamate receptors, and the adhesion factors, cadherin, catenin, neuroligin, and Nr-CAM. Development was marked by substantial decreases in NR2B and SAP102 and increases in NR2A, PSD-95, AMPA receptors and CaMKII. Other components showed more moderate changes.

Introduction

Postsynaptic density (PSD) proteins are necessary for excitatory glutamatergic postsynaptic responses, and include scaffolding and functional elements that modulate/mediate signal transduction and trafficking of synaptic proteins (reviewed by Sheng and Kim, 2002; Wenthold et al., 2003; Bredt and Nicoll, 2003; Ehrengreuber et al., 2004). In addition to glutamate receptors (GluRs) such as α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors, numerous other proteins associate with GluRs in the postsynaptic membrane and PSD. GluRs associate via their C-termini with anchoring proteins including membrane-associated guanylate kinases (MAGUKs). The best-studied MAGUKs belong to the PSD-95 group, containing PSD-95, PSD-93, SAP102 and SAP97. Both NMDA and AMPA receptors can bind directly to MAGUKs; AMPA receptors also can bind to them indirectly via stargazin and other transmembrane AMPA receptor regulatory proteins (TARPs). Other associated proteins that affect GluR distribution and function at the synapse include tyrosine receptor kinase B (TrkB), calmodulin-dependent protein kinase II (CaMKII), proteins binding to MAGUKs such as synaptic GTPase-activating protein (SynGAP), spine-associated RapGAP (SPAR), guanylate kinase-associated protein (GKAP), proteins forming links between MAGUKs/GKAP through Shank and Homer dimers to metabotropic glutamate receptors (mGluRs) and receptors regulating internal calcium stores, plus a number of adhesion proteins including the cadherin/catenin complex, neuroligin (which can bind to MAGUKs), and cell-adhesion molecules (CAMs) such as NCAM, L1 and Nr-CAM.

Most studies of these various proteins have concentrated on adult synapses in vivo and developing synapses in vitro, while surprisingly little is known about ontogeny of these proteins in synapses in vivo (see reviews by Garner et al., 2002, Goda and Davis, 2003, Li and Sheng, 2003; Ziv and Garner, 2004). A basic questions that needs to be addressed is: Do changes in protein composition of the PSD, during postnatal development, involve mainly changes in

amounts of most proteins, or are there radical changes in protein types (e.g., replacement of one receptor subtype for another), as needed during different stages of development? In this study, we examined the developmental changes in GluRs and several associated proteins in synapses. Previous studies showed that 1) NMDA receptors are high from P2-adult, while AMPA receptors increase with age (Petralia et al., 1999), and 2) the NMDA receptor-associated MAGUK, SAP102, decreases with age while other MAGUKs, PSD-95 and PSD-93, increase with age (Sans et al., 2000). Here we show that those early postnatal synapses that display ultrastructural signs of maturity already have acquired substantial levels of many GluR-associated proteins. Further postnatal development involves major reciprocal switches in levels of similar proteins (SAP102 versus PSD-95; NR2B versus NR2A) and moderate increases or decreases in other proteins. This study provides immunogold data for the hippocampus, showing the developmental switch in NMDA receptor type, and showing that developmental changes occur for other proteins that are associated functionally with these receptors and that contribute to the maturation of learning and memory.

Materials and methods

Antibodies and their characterization

Data from 27 antibodies are presented in the immunochemical and immunocytochemical experiments in this study. Of these, the 24 antibodies used for new immunogold experiments carried out in this study have been well-characterized in previous studies, using mainly light microscope level examination and, in some cases, immunogold localization at synapses in adults.

Mouse NR2A and NR2B antibodies (affinity-purified; C-terminal, i.e., recognizes intracellular domain) were characterized in general and in hippocampus in Watanabe et al. (1998) and for immunogold in Yamada et al. (2001); these NR2A (amino acids 1126–1408) and NR2B (amino acids 1353–1432) antibodies were made from equivalent mouse subunits, GluR ϵ 1 and GluR ϵ 2. The latter antibodies were used to quantify NR2A and NR2B immunogold labeling in synapses, but were not used for immunoblots because our supply of these antibodies was low. TrkB antibody (affinity-purified) recognizes the extracellular domain of TrkB (prepared against amino acids 76–96) and was characterized in hippocampus and cerebral cortex (Wu et al., 1996; Aoki et al., 2000). Shank 3 (amino acids 1379–1675) and Homer 1b/c (amino acids 340–357) antibodies were characterized for immunogold of hippocampus of adult rats by the authors (RSP/RJW) in Tu et al. (1999; also for Homer in cerebellum in Xiao et al., 1998). We also used a pan Homer antibody that was made against the full-length GST-Homer 1a fusion protein (Xiao et al., 1998). SynGAP was characterized in hippocampus in Kim et al. (1998; affinity-purified; last 20 amino acids). In addition to the antibody from Dr. Huganir, used for immunogold localization, another SynGAP antibody (affinity-purified; amino acids 947–1167) was purchased from Affinity BioReagents, Inc. (Golden, CO, USA) and used in the present study for both immunogold and immunoblot analyses. A C-terminal GKAP antibody (affinity-purified; amino acids 744–964) was characterized in hippocampus (and in adult cerebral cortex for immunogold) by Kim et al. (1997) and Naisbitt et al. (1997). SPAR antibody (affinity-purified; amino acids 1744–1760) was characterized in adult brain and hippocampal neurons in vitro (Pak et al., 2001, 2003). Nr-CAM (837; recognizes extracellular domain) antibody distribution in developing brain (light microscopy; including hippocampus) was characterized in Lustig et al. (2001). Another Nr-CAM antibody that was used in our study was characterized by Suter et al. (1995; affinity-purified; recognizes extracellular domain; this did not work as well for immunogold and was used in our study for biochemistry only). L1 antibody was characterized by Appel et al. (1995; rat monoclonal; amino-acids 818–832; recognizes extracellular domain); L1 distribution in hippocampus was described using a different antibody (Matsumoto-Miyai et al., 2003). Purchased antibodies included SynGAP

(see above), N-cadherin (amino acids 802–819; recognizes intracellular domain) and β -catenin (amino acids 571–781); BD/Transduction Laboratories; Lexington, KY, USA and Mississauga, ON, Canada), α -CaMKII (alpha subunit; Stressgen Biotechnologies; Victoria, British Columbia, Canada), and neuroligin (amino acids 1–695; recognizes extracellular domain; Synaptic Systems, Göttingen, Germany); the latter 4 antibodies are mouse monoclonals. Immunogold labeling of N-cadherin and β -catenin at synapses (α N-catenin and β -catenin in adult mouse cerebral cortex and cerebellum, α N-catenin in the P7 and P10 mouse cerebellum, and N-cadherin and α N-catenin in the P2 chick midbrain) was described by Uchida et al. (1996) using a preembedding immunogold method. The purchased CaMKII antibody has been characterized fully in the brain, and specifically in hippocampus, by Erondy and Kennedy (1985). Neuroligin labeling was described in developing brain (immunoblot), in hippocampus (light microscopy) and for immunogold in adult neocortex by Song et al. (1999; their antibody was made similarly to our purchased antibody). Three antibodies to mGluR5 were obtained from Chemicon International (Temecula, CA, USA); another from Chemicon was made to the last 13 amino acids and recognizes both mGluR5 and mGluR1 α . Antibodies to AMPA receptors, NR1, NR2A/B, PSD-95, PSD-93, SAP97 and SAP102 were characterized in general and for immunogold labeling in hippocampus by our lab (Petralia and Wenthold, 1992; Petralia et al., 1994a,b, 1999; Sans et al., 2000, 2001). Some new labeling studies were carried out for NR1 (amino acids 909–938; affinity-purified; recognizes the intracellular domain), SAP97 (amino acids 1–105; previous published studies used both this antiserum and an identical affinity-purified antibody), and SAP102 (amino acids 1–119; antiserum; previous published studies used an identical affinity-purified antibody).

Immunoblot analysis and subcellular fractionation

For preparation of tissue extracts for immunoblots, hippocampi from E18 (embryonic day 18), P2, P5, P10, P15, P35 and P50 rats were homogenized in phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors (2 mM EDTA, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 50 μ g/ml Leupeptin, 10 μ g/ml Pepstatin, 10 μ g/ml Aprotinin), diluted in 2x SDS sample buffer and boiled. Protein concentrations were measured using a BCA assay (Pierce, Rockford, IL, USA) or a Bio-Rad protein assay (BioRad, Hercules, CA, USA). Proteins were separated with SDS-PAGE (4–20% gradient gels) and transferred to Immobilon-P membranes and treated as described (Sans et al., 2000, 2001). After chemiluminescence detection, films were scanned using a Molecular Dynamics densitometer.

Subcellular fractionation of P2 rat brains was performed as described in Sans et al. (2001). The synaptosome fraction, isolated by discontinuous sucrose gradient centrifugation, was solubilized in cold 0.5% Triton X-100 and centrifuged to obtain the PSD I pellet. This pellet was resuspended, solubilized and centrifuged to obtain the PSD II pellet. Pellets were resuspended in 50 mM Tris-HCl (pH 8) before measuring protein concentrations.

Immunogold methods

All animal procedures were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85–23) under NIDCD protocol #1022-01. The postembedding method has been described (Petralia et al., 1998, 2001, 2002, Wang et al., 1998, Zhao et al., 1998, Petralia & Wenthold, 1999, Sans et al., 2000, 2001; the most recent modifications have been published in Darstein et al. (2003) and Kim et al. (2003)), and the method is modified from a previous study (Matsubara et al., 1996). Studies described in this paper were done on hippocampus, mainly on stratum radiatum (apical dendrite subregion) of the CA1 region of the rat (two or three animals per age; see below), at postnatal days P2, P10 and P35. Rats were anesthetized with a 1:1 mixture of ketamine and xylazine (approximately 0.1 ml/100 g body weight injected into the biceps femoris muscle of the right hind leg) and perfused first with 0.12 M phosphate buffer (PB) for

15–30 seconds to wash out blood, then perfused with 4% paraformaldehyde plus 0.5% glutaraldehyde in 0.12 M PB; then brains were post-fixed in the same fixative for 2 hours at 4°C. Parasagittal sections of hippocampus were cut at 300 μ m in 0.1 M PB plus 4% glucose on a Microslicer (Leica VT 1000S; Vienna, Austria). These sections were cryoprotected in 30% glycerol and frozen in liquid propane in a Leica EM CPC (cryopreparation chamber; Vienna, Austria). Frozen sections were immersed in 1.5% uranyl acetate in methanol at –90°C in a Leica AFS (automatic freeze-substitution instrument), infiltrated with Lowicryl HM-20 resin at –45°C, and polymerized with ultraviolet light. Thin sections were incubated in 0.1% sodium borohydride plus 50 mM glycine in Tris-buffered saline plus 0.1% Triton X-100 (TBST), followed by 10% normal goat serum in TBST, primary antibody in 1% normal goat serum in TBST (2 hours for earlier studies [GKAP, SynGAP, SAP97] and overnight at 4°C for other antibodies), immunogold (10 nm; IgG gold for earlier studies [GKAP, SynGAP, SAP97; Amersham Biosciences, Piscataway, NJ]; F(ab')₂ gold for other antibodies [Ted Pella, Redding, CA]) in 1% normal goat serum in TBST plus 0.5% polyethylene glycol (20,000 MW); and sections finally were stained in 1% uranyl acetate and 0.3% lead citrate (with 1% sodium hydroxide). Final figures were processed with Adobe Photoshop to optimize brightness and contrast, especially to enhance gold particle intensity relative to tissue staining, as in our previously published studies. All scale bars are 100 nm.

Quantitative analysis

For quantification, large areas of neuropil of CA1 stratum radiatum were photographed from randomly selected regions. At least 2 animals were examined for the 3 ages. Random micrographs were taken of synapses, and data are shown as gold/synapse but the included area of the synapse varied. Thus, gold was measured in 2 area-categories: synapse (cleft and PSD) and within 100 nm below (perpendicular to) the postsynaptic membrane (PSM). The latter category was adopted to account for large accumulations of gold labeling seen just below (=subadjacent to) the density using some antibodies, especially CaMKII, Shank and Homer (for our previous descriptions of this for Shank and Homer in adult synapses, see Xiao et al., 1998, Tu et al., 1999). Both area-categories were counted, and are presented as “synapse,” and “synapse+100 nm” (equals the sum of both area-categories). A complete listing of area-categories counted is presented as tables of counts and statistical probabilities and developmental trends (supplementary Tables 1 and 2). Also, some data were included, for comparison, from published studies on GluR1, GluR2/3, NR1, and PSD-95; the only data available for these included the synapse category (published in Petralia et al., 1999, Sans et al., 2000) for all 4 antibodies (in addition to these, new studies were done for SAP102 and NR1, as described in the Results). Immunogold labeling for adhesion proteins, including cadherin, catenin, neuroligin, and Nr-CAM, as well as TrkB (see Results and Discussion), was examined in the postsynaptic, presynaptic and perisynaptic areas, although quantification was included only for the postsynaptic compartment.

Statistical significance was determined using a two-tailed t-test assuming unequal variances. Differences in immunogold means were considered significant if $p < 0.05$ and highly significant if $p \leq 0.001$. No correction was made for multiple comparisons because such corrections can be problematic (Rothman, 1990; Perneger, 1998, 1999). Our sampling and statistical analysis methods have been described in more detail in Petralia et al. (2004). Synapse counts from animals were combined; in most cases there was no significant difference between 2 animals. However, if those from 2 animals showed a significant difference, then a third animal was included in the count. Immunogold labeling was not counted per unit length of the synapse, because we found in our previous study that the values of gold per synapse versus gold per micrometer length of the synapse were similar for these ages (Petralia et al., 1999; lack of significant change of synapse length during development is noted also for hippocampal neurons in vitro by Renger et al. [2001] and reviewed in general by Ziv and Garner [2004]). Thus, the

gold per synapse values given here can be compared readily with those already published using the same method (Petralia et al., 1999; Sans et al., 2000); this is the most efficient method of comparing results among proteins found in the same region and during the same developmental sequence.

For the immature versus mature synaptic contact study at P2, immature contacts were defined as described previously (Petralia et al., 2003) and are described in more detail in the Results. For the latter study, one author did all identifications of synapses and counts. In addition, to check the validity of the counting method, a blind study of one group of the counts was carried out by another author; the resulting counts matched closely with those seen for the full immature/mature study with that antibody. Control sections, which lacked the primary antibody, had only rare gold. No gold was seen in the “total area” of the synapse (a large area extending 100 nm for postsynaptic, presynaptic and total perisynaptic (on both presynaptic and postsynaptic sides)) for rabbit antibody controls at P2 (n=15), P10 (n=30) and P35 (n=100) and mouse antibody controls at P2 (n=15) and P10 (n=30) while the latter at P35 had 0.02 gold/“total area” of the synapse (n=100). In addition, some of the test antibodies produced only rare gold in sections and can be considered as controls relative to labeling produced by other test antibodies. These include both mouse (P2 and P10 of CaMKII) and rabbit (P2 of NR2A and all 3 ages for mGluR1/5) antibodies. Serum controls were not done for most antibodies used in this study since they are affinity-purified; however, serum controls were carried out for the antisera that were quantified in this study, including Nr-CAM, SAP102, Shank and Homer. Values of serum controls were subtracted from counts obtained with Nr-CAM, SAP102 and Shank antisera (the difference for Homer was less than five percent). As a further control for the validity of antisera data, the labeling trend for SAP102 antiserum at synapses, as shown in this study, was compared to previously published values using an affinity-purified batch of the same antibody, published in Sans et al. (2000; available at that time); antiserum and affinity-purified antibody produced very similar results.

Results

Immunoblots

Immunoblots of the developing hippocampus showed at least 3 trends (Fig. 1): 1) protein already abundant before birth, for the adhesion proteins, N-cadherin, β -catenin, and Nr-CAM, and for Shank and Homer; 2) protein present before birth and abundant by P2, for neuroligin, NR1, NR2B (i.e., as extrapolated from findings for NR2A/B {high} and NR2A {very low}), TrkB, SAP102, and mGluR5; 3) protein increases from little or nothing at P2 to high levels in adult, for NR2A, PSD-95, SAP97, and CaMKII. Examination of the PSD fraction at P2 showed that, in most cases, the amounts of the protein seen in the whole hippocampus immunoblots at P2 correlate well with the amounts found in the PSD. Exceptions to this are SynGAP and GKAP, which showed very abundant protein in the PSD at P2 in spite of relatively low overall levels of protein seen in hippocampus at P2. This suggests that most SynGAP and GKAP protein in the neuron is strongly associated with PSDs at P2. Another exception is Nr-CAM, which showed abundant protein in the overall level of protein at P2 in spite of a low level seen in the PSDs at P2; this might reflect the abundance of Nr-CAM in presynaptic terminals. Further details of immunoblot protein distribution will be discussed with specific immunogold results for each protein (Figs. 2–9).

Immunogold studies – general

Immunogold labeling of synapses in the CA1 stratum radiatum of hippocampus (Figs. 2–10; Tables 1,2, supplementary Tables 1,2) was examined in 2 area-categories of synapse profiles, including synapse and synapse+100 nm, as described in detail in the Methods. Analyses of more than 13,000 synapses were included in new data presented here. In general, findings for

different area-categories were similar and trends seen during development were similar to trends indicated in immunoblot studies (Fig. 1). Ultrastructural details appeared normal in sections from all ages, and showed similar characteristics as those described in our previous developmental studies of this region of the hippocampus (Petralia et al., 1999,2003;Sans et al., 2000).

Comparison of major trends for synaptic labeling

When all of the antibodies used in this study were compared using the same area-category, synapse only (Fig. 2; as defined in Methods), some major developmental trends were evident (also Tables 1,2, supplementary Tables 1,2). This category was preferred for the initial comparison because it can be compared readily to previously published data. There was a major increase through development in NR2A, PSD-95, and CaMKII (similar to the previously published increase in synaptic AMPA receptors; Petralia et al., 1999), and there was a major decrease in NR2B and SAP102. Other proteins showed more modest changes with age, including an increase in cadherin, catenin, neuroligin, SynGAP, Shank and Homer. In most cases, developmental trends seen in synaptic labeling (Fig. 2) were similar to those seen in immunoblots (i.e., comparing synaptic protein versus whole hippocampus protein; Fig. 1). This also was evident when the overall change of immunogold labeling from P2 to P35 was compared (Table 2). Dramatic increases (11-12x) were evident for NR2A and CaMKII and substantial decreases (to about half) were seen for NR2B and SAP102. Interestingly, this decrease in synaptic SAP102 is not reflected in changes in overall protein levels for SAP102, probably because of abundant cytoplasmic levels of SAP102, as discussed in Sans et al. (2000). Other proteins showed modest increases for synapses between these ages. Also for this change, there generally was little difference between the 2 categories, synapse versus synapse +100 nm. A more detailed analysis of both immunoblot and immunogold labeling is presented in the following sections. We also compared immunogold labeling in the synapse (cleft and PSD) with that seen subjacent to the PSD, as indicated in the synapse+100 nm category, specifically for those proteins in the molecular chain, NR2-SAP102-GKAP-Shank-Homer, as illustrated in figure 2. Immunogold labeling for some proteins such as NR2A, NR2B, and SAP102 was concentrated at the synapse, while Shank and Homer showed substantial labeling in the area subjacent to the PSD; GKAP, which occupies the middle position of the chain, showed middle values (Table 1). Thus, the immunogold data support a model for an arrangement of the proteins in a chain that may be similar for all ages. In addition, some of the subjacent labeling for these and other proteins examined in this study probably represent these proteins in transit to or from the synapse. Overall, the ratio of labeling in synapse versus synapse +100 nm did not change much over development (Table 1; supplementary Table 1).

Detailed analyses arranged by protein group

Intercellular adhesion proteins

Intercellular adhesion proteins were considered first because these may be the first components of developing synapses (Garner et al., 2002; Li and Sheng, 2003; Sytnyk et al., 2004). Both N-cadherin (135 kD) and β -catenin (110 kD) produced a dense band at all ages and in the P2 PSD II fraction (Fig. 1). At synapses, N-cadherin and β -catenin were examined in areas surrounding the synapse (pre, post, peri) as described in the Methods; immunogold labeling was prominent at all ages on both the presynaptic and postsynaptic sides of the synapse (Figs. 2,3). Both cadherin and catenin increased in postsynaptic labeling from P10 to P35 (only postsynaptic labeling was quantified). Immunoblots (Fig. 1) of neuroligin and Nr-CAM (837 antibody; the other Nr-CAM antibody produced similar immunoblot results – data not shown) showed prominent labeling throughout development (as shown previously for neuroligin; Song et al., 1999). Neuroligin was high in the P2 PSD fraction; in contrast Nr-CAM was evident but relatively low. For Nr-CAM, the densest band was at 140 kD (with a lighter band at about 200

kD); this 140 kD band represents the alpha-chain (Kayyem et al., 1992), which matches the region selected to make the 837 antibody. Immunogold labeling for neuroligin (Fig. 4) was seen mainly on the postsynaptic side of the synapse, consistent with studies showing that it is primarily a postsynaptic protein (Song et al., 1999). Levels of neuroligin within the synapse remained fairly constant through the ages. Presynaptic and postsynaptic labeling for Nr-CAM (837; Figs. 2,4) was prevalent at all ages and postsynaptic labeling at least, showed an increase from P2 to P10. It was seen typically as clusters of gold particles adjacent to or crossing the synaptic cleft. In addition, in a few examples, gold labeling was associated with uncoated pits (presumably exocytotic) associated with the presynaptic or postsynaptic membrane (Fig. 4); this supports suggestions that this group of adhesion factors are cycled via exocytosis at the point of cell-cell contacts to reinforce and stabilize the contacts (Alberts and Galli, 2003). Labeling for L1 at synapses was generally similar to that seen for Nr-CAM but was not as prevalent (data not shown).

TrkB

TrkB may act both as an intercellular adhesion factor and a receptor. It may be one of the earliest components of developing glutamatergic synapses, and is found on both the presynaptic and postsynaptic sides (Wu et al., 1996; Margotti et al., 2002; R.A. Gomes, C.M. Hampton, and A.K. McAllister, Soc. Neurosci. Abs. 2003; Yamada and Nabeshima, 2003; Elmariah et al., 2004). In immunoblots of TrkB (Fig. 1), labeling for both full-length (145 kD) and truncated (95 kD) TrkB was seen throughout development, as noted previously for adult hippocampus and cerebral cortex (Wu et al., 1996). The full-length TrkB protein decreased slightly, after an initial increase from E18 to P2, and the truncated protein increased during development. The increase in the full-length TrkB from embryo to early postnatal ages, and the increase in the truncated protein through postnatal development are consistent with published findings (Escandón et al., 1994; Fryer et al., 1996). Only a low level was evident in the P2 PSD II fraction; the only band in this fraction was the full-length form as described for adult brain PSDs (Wu et al., 1996). For immunogold analyses, labeling was examined in the areas surrounding the synapse (pre, post, peri) as described in the Methods, since TrkB may be both presynaptic and postsynaptic (Wu et al., 1996; Drake et al., 1999; R.A. Gomes, C.M. Hampton, and A.K. McAllister, Soc. Neurosci. Abs. 2003). Labeling for the TrkB receptor was found on both the presynaptic and postsynaptic sides of the synapse (Fig. 5). Only postsynaptic labeling was quantified, and the major changes in postsynaptic labeling were in the synapse+100 nm category, with a decrease from P2 to P10 and an increase from P10 to P35 (Figs. 2,5, supplementary Table 2; see Methods).

Glutamate receptors and MAGUKs

According to current models, once adhesion proteins establish a stable contact between axon and dendrite, GluRs and their associated anchoring proteins can colonize the postsynaptic membrane. In immunoblots (Fig. 1), antibodies to NMDA receptors showed patterns similar to those described in Sans et al. (2000). Antibodies to NR1 (120 kD) and NR2A/B (180 kD) showed a strong band at P2 through P50. Antibody to NR2A did not produce a distinct band until P10 and increased with age. An intense band was seen in the P2 PSD II fraction with antibodies to NR1 and NR2A/B, while that to NR2A produced only a faint band. These data, along with our previous immunoblot data showing that NR2B is highest at P2 and decreases with age (Sans et al., 2000), demonstrate that NR2B is high in the PSD at P2. Immunogold labeling of synapses (Figs. 2,6) matched closely with immunoblot results; NR2B showed high levels at P2 and P10 and a decrease in adult, while NR2A showed only low levels at synapses at P2, a small increase at P10, and a large increase in adult. As expected, gold labeling for these NR2 subunits was concentrated within the synapse and was relatively uncommon subjacent to the density, as compared to some other proteins like Shank and Homer (Fig. 2; Table 1).

Labeling patterns for the MAGUKs, including SAP102 and PSD-95/SAP97, paralleled those of NR2B and NR2A, respectively. Thus, immunoblots (Fig. 1) for SAP102 labeling were high from P2 to adult, while immunolabeling for PSD-95 and SAP97 did not produce a distinct band until P10 and increased with age; all produced bands at about 100 kD. Likewise, the band in the P2 PSD II fraction was intense for SAP102 and light or absent for PSD-95 or SAP97, respectively. In synapses, SAP97 showed an increase in labeling with age (one animal for P2 and P10 and two for P35; data not shown; see Sans et al., 2001 for micrographs of immunogold labeling in P35 synapses), similar to that shown previously for PSD-95 and PSD-93. This is in contrast to that shown previously for SAP102, which showed a decrease in labeling with age (Sans et al., 2000). In addition, for this study, we performed new immunogold labeling for SAP102 at P2, P10 and P35 (data not shown). As in our published study, a significant decrease was seen during development (for both categories, including gold per synapse and synapse +100 nm – the latter was not done in the published study; Fig. 2; Table 2 and supplementary Table 2).

Proteins binding to glutamate receptors and MAGUKs

Using antibody to CaMKII, a distinct band (55 kD) was seen first at P5 and this increased with age (Fig. 1). The P2 PSD II fraction showed only a faint band. Similarly, in synapses, immunogold labeling for CaMKII (Figs. 2,7) was absent at P2, very low at P10, and showed a large increase from P10 to P35 (i.e., consistent with early biochemical studies of CaMKII showing that it was low in synapse protein of the early postnatal forebrain; Kelly and Vernon, 1985). The high amount of labeling subjacent to the PSD (both at P10 and P35) is consistent with the findings of studies on hippocampal synapses in vitro (Dosemeci et al., 2001).

In immunoblot (Fig. 1), SPAR antibody produced a light band at P2 and this increased in density with age. This band was about 170 kD, corresponding to the cytoplasmic component of SPAR labeling (Pak et al., 2001). The 190 kD band, reported previously to be present in the PSD, was much lighter in our material at all ages. However, no band was seen in the P2 PSD II fraction. Immunogold labeling of synapses with SPAR antibody (Figs. 2,7) did produce some moderate synaptic labeling at all ages studied, as well as labeling in the postsynaptic and presynaptic cytoplasm associated with presumptive actin filaments (<10 nm; similar to actin filaments identified with actin antibodies – Petralia et al., 2001 and unpublished data); the latter is consistent with the higher labeling for cytoplasmic SPAR, seen at 170 kD in blots, and a low ratio of gold/synapse divided by gold/synapse+100 nm (see supplementary Table 1) indicating high labeling subjacent to the PSD (gold/synapse divided by gold/synapse+100 nm). In this context, the immunogold labeling seen within the PSD could be associated with actin filaments that pass through the PSD, or perhaps it is due to some weak binding to other components of the PSD.

In immunoblot (Fig. 1), antibody to SynGAP produced a light band (135 kD) at P2 and this increased with age. A dense band was seen in the P2 PSD II fraction. SynGAP labeling from 2 antibodies produced similar results at synapses at all ages; only the antibody from Dr. Huganir was used for quantification. SynGAP labeling at synapses showed a wide variation at P2 (Figs. 2,8; quantification of 3 animals in 5 experiments). However, the average was moderate at this and subsequent ages. There was an increase from P10 to adult.

Immunoblot with a C-terminal antibody to GKAP (Fig. 1) showed light bands at P2 and strong bands at later ages and in the P2 PSD II fraction. The higher molecular weight band (130 kD) was the most prevalent in the P2 hippocampus and P2 PSD II fraction; both the 95 and 130 kD bands are high in adult brain PSDs (Kim et al., 1997) and the developmental pattern seen for these bands was nearly identical to that shown previously for the visual cortex (Yoshi et al., 2003). Immunogold labeling at synapses (Figs. 2,8) with antibody to GKAP appeared moderately high at all ages and showed a slight decrease with age, but it was not significant.

The Shank-Homer-mGluR chain

Proteins associated with the PSD can organize into extensive chains beginning with a complex of glutamate receptor-MAGUK-GKAP that can link through GKAP to Shank-Homer and continue to metabotropic GluRs and other proteins (Naisbitt et al., 1999; Xiao et al., 2000; Wenthold et al., 2003; Ehrenguber et al., 2004). Also, Shank may link directly with the GluRs (mGluR1 and GluR δ 2), and indirectly to AMPA receptors via GRIP (Tu et al., 1999; Uemura et al., 2004). Previous immunoblots for Shank proteins show that they are present in the early postnatal brain (Lim et al., 1999). In our study, Shank 3 antibody produced multiple bands (Fig. 1) ranging from about 120 kD to 240 kD, as expected due to the recognition of multiple shank variants (Tu et al., 1999). The major band at 240 kD and most other bands were prominent at all ages and appeared to increase slightly with age, while a single band at 140 kD was highest at E18 and decreased with age. A 240 kD band was especially dense in the P2 PSD II fraction. Shank 3-immunogold labeling (Figs. 2,9) was prevalent at synapses at all ages and showed an increase from P2 to adult. This immunogold labeling formed mainly as distinct clusters both in the PSD and subjacent to it (i.e., as we described previously in the adult; Tu et al., 1999); this pattern was similar at all ages (Figs. 2,9, Table 1).

Homer 1a has been described during development; mRNA for the latter is present in the early postnatal hippocampus and peaks at P14 (Kato et al., 1997). However, since Homer 1a is an immediate early gene product and lacks the coiled-coil domain needed for Homer dimerization, its distribution may not coincide with that of other Homers such as the 1b,c form studied here. Immunolabeling for other Homers has been described with light microscopy in P14 hippocampus; both Homer 1b/c and Homer 2a/b are prevalent in the CA1 region, while Homer 3a/b is prevalent mainly in the CA3 region (Shiraishi et al., 2004). Homer 1b,c antibody produced a prominent band at 45 kD at all ages (Fig. 1); this band appeared to increase slightly with age (shown previously in immunoblots for later postnatal development in Shiraishi et al., 2004), and also was prominent in the P2 PSD II fraction. In synapses (Figs. 2,9), immunogold labeling for Homer 1b,c antibody was abundant at all ages; the only increase seen was from P2 to P10. Labeling for Homer 1b,c was prominent both in the PSD and subjacent to it, where it often formed clusters of gold labeling (as we described previously in the adult only; Xiao et al., 1998; Tu et al., 1999), roughly similar to the pattern seen with Shank antibody (Figs. 2,9, Table 1). After SAP102 antibody, this Homer antibody produced the highest immunogold labeling of the antibodies examined in this study for P2 (0.913 gold/synapse+100 nm), and produced the highest at P10 (Shank antibody produced the highest at P35; Fig. 9). The percentage of synapses labeled with immunogold for this antibody to Homer was 44% at P2 and reached its peak of 71% at P10. A second Homer antibody, pan Homer, produced similar density and distribution of immunogold labeling at all ages (Fig. 9).

The Shank/Homer dimer chain likely associates with the metabotropic GluRs, mGluR1 and mGluR5, which are found in hippocampus throughout postnatal development (Shigemoto et al., 1992; López-Bendito et al., 2002; Di Giorgi Gerevini et al., 2004). As in adults, these mGluRs often appear to be concentrated in perisynaptic regions (López-Bendito et al., 2002). In immunoblots (Fig. 1), multiple bands represent monomers and dimers as noted previously (Romano et al., 2001, 2002). The peak in labeling intensity seen at P10 is consistent with previous findings (e.g., Romano et al., 2002; Di Giorgi Gerevini et al., 2004). However, the antibodies produced only low gold labeling and were not quantified (data not shown).

Mature versus immature synaptic contacts at postnatal day 2

Immature synaptic contacts were defined as described previously (Petralia et al., 2003); these lacked a substantial PSD (less than about 10 nm thick). For example, see figure 3 for catenin at P2 (upper right micrograph; note that the presynaptic terminal contains few distinct vesicles). Another good example can be seen in figure 4 for Nr-CAM at P2 (upper left micrograph; several

presynaptic vesicles are evident); these immature synaptic contacts were much less common at P10 compared to P2 (although not quantified). Mature synapses (about half of all synapses) showed several distinct vesicles near the active zone and a well-developed PSD. Occasional examples of synaptic contacts with a well-developed PSD but with no more than two presynaptic vesicles were excluded from this comparison because of their low number. For this study of mature versus immature synaptic contacts at P2, we chose four representative antibodies: NR2B and SAP102 antibodies were chosen because they are the abundant NR2 and MAGUK types seen at P2; Homer antibody was chosen because, of all the antibodies studied here except for SAP102, it produced the most abundant immunogold labeling at P2; catenin antibody was chosen because of substantial evidence for a role of cadherin/catenin complexes in particular (of the proteins included in our study; reviews by Garner et al., 2002, Goda and Davis, 2003, Li and Sheng, 2003) in the earliest events of synaptogenesis, and because catenin labeling was more robust than cadherin labeling. Distribution of catenin (Fig. 10) was similar in immature and mature synaptic contacts at P2. This is consistent with the abundant immunogold labeling for α N-catenin seen at immature synaptic contacts in the mouse cerebellum (Uchida et al., 1996). In contrast, immunogold labeling for NR2B, SAP102 (new labeled sections for this study), and Homer 1b,c in immature synaptic contacts was very low relative to that seen in mature synapses. Two sets of experiments for SAP102 were done (total of 220 synapses for the first test {data not shown} and 172 synapses for the second test {Fig. 10}); the two tests produced nearly identical results. Counts for NR1 (1 animal; new labeled section for this study) showed a similar pattern (data not shown).

Note that most synapses at P2 showed basic features (asymmetric synapse with thickened PSD and round presynaptic vesicles) of glutamatergic synapses, which in adult make up the vast majority of synapses in CA1 stratum radiatum. However, the identity of nascent glutamatergic synaptic contacts is less certain and unlikely to be resolved definitively. For example, identification of glutamatergic synapses by using glutamate immunolabeling can be problematic (Broman et al., 2000). The earliest stages in synapse structural development may vary (Vaughn, 1989); in hippocampal neurons in vitro, the earliest synaptic contacts lack a distinct PSD and have vesicles of variable forms (Ahmari et al., 2000; Ziv and Garner, 2004). Furthermore, spine synapses, typical of adult glutamatergic synapses in the CA1 stratum radiatum, are rare at P2, with most synapses formed directly on dendrite shafts (Fiala et al., 1998; Petralia et al., 2003); physiological studies confirm that functional glutamatergic synapses form directly on dendrite shafts of CA1 pyramidal neurons at P2 (Durand et al., 1996). Thus, the major characteristics of adult glutamatergic synapses of the CA1 stratum radiatum, including distinct PSDs, clusters of round vesicles, and synaptic spines, would not be expected features of nascent excitatory synapses. Preferential labeling for catenin in immature synaptic contacts may indicate that some of these are GABAergic, inhibitory synapses, which have abundant catenin in vitro (Benson and Tanaka, 1998) and are present in CA1 stratum radiatum at birth (Tyzio et al., 1999). Nevertheless, the evidence overall suggests that the majority of immature synaptic contacts at P2 represent early stages in glutamatergic synapse development.

Discussion

This study shows that major changes occur in GluR-associated proteins during development of synapses in CA1 stratum radiatum of the hippocampus. Intercellular adhesion proteins showed the greatest abundance in immunoblot in late embryonic and early postnatal hippocampus relative to later ages, of proteins studied here. In addition, a comparison of immature and mature-appearing synapses at P2, for 4 of the proteins in this study, showed that only the adhesion protein, catenin, was prevalent in the most immature-appearing synaptic contacts. In contrast, only synapses that appeared to be basically mature showed abundant labeling for the other 3 proteins - Homer 1b,c, SAP102, and NR2B. These data support

suggestions that intercellular adhesion proteins are among the first components of the earliest synaptic contacts in hippocampus (reviews by Garner et al., 2002, Goda and Davis, 2003, Li and Sheng, 2003, Ziv and Garner, 2004).

The second major finding is that many of the proteins in the synapses in this study followed patterns that are correlated with the previously described decrease of SAP102 and increase of PSD-95 with development (Sans et al., 2000). Thus, the decrease in NR2B followed a pattern that was similar to that seen for SAP102. Other proteins that may have some decrease with development in synapses included TrkB and GKAP; interestingly, both of these exist in 2 different forms, perhaps suggesting that there is some change in prevalence of forms with age (discussed below), similar to the more obvious switchover, from NR2B and SAP102, to NR2A and PSD-95. The most striking change was the distinctive increase in a number of proteins associated with the latter developmental switch. This increase in NR2A and PSD-95 was correlated with increases in the AMPA receptors (Petralia et al., 1999), and with at least 2 other MAGUKs, PSD-93 (Sans et al., 2000) and SAP97, as well as with CaMKII. In addition, a number of other proteins, such as Shank, Homer, and catenin, which already are abundant at P2, showed some increases that may be correlated with this pattern. For most antibodies, differences between the counts for the synapse versus the synapse+100 nm area did not vary much between ages. This suggests that there is no major shift in distribution for most of the proteins considered, consistent with qualitative observations. Thus, for example, large accumulations of gold-labeling subjacent to the PSD, seen for Shank and Homer, were common at all ages studied.

Finally, another finding of this study is that, in most cases, there were close correlations between the changes in protein levels seen in immunoblots during development, the levels of proteins seen in PSD fractions at P2, and the changes seen in synaptic labeling during development. It suggests that regulation of levels of these proteins expressed at synapses in hippocampus is connected directly to regulation of synthesis of these proteins in neurons of the hippocampus. Exceptions to this may be SAP102, SynGAP, and GKAP. These showed distinct increases from P2 to P10 in immunoblots although the synaptic labeling showed a distinct decrease at this time for SAP102 and no significant change for SynGAP or GKAP. This probably reflects a prevalent cytoplasmic pool of these proteins, as noted previously (Sans et al., 2000; cytoplasmic labeling for SAP102 is illustrated in Sans et al., 2003 [and unpublished data]). Another exception involves SPAR, which showed synaptic labeling but no band in the PSD fraction at P2 (discussed in the Results and below).

The abundance of various adhesion factors, including cadherins/catenins, neuroligin, Nr-CAM, L1, and TrkB (TrkB may act as an adhesion factor, as explained below), in early postnatal synapses is expected, since at least some of these are likely participants in the earliest stages of synaptogenesis (Garner et al., 2002, Li and Sheng, 2003, Sytnyk et al., 2004). For example, N-cadherin appears to be a component of a protein complex transported to the presynaptic side of early synaptic contacts (Zhai et al., 2001). Neuroligin binds directly to PSD-95 in the PSD and to neuroligin in the presynaptic terminal (Fig. 2D), yet targeting of neuroligin to synapses is independent of this binding, supporting suggestions that neuroligin is recruited early in synaptogenesis (Dresbach et al., 2004). Labeling for Nr-CAM, a member of the L1 family of cell adhesion molecules (Crossin and Krushel, 2000; Hortsch, 2000), often was seen in clusters of gold particles in the synaptic cleft, suggesting that the antibodies label molecules linked together across the synaptic cleft. Previous studies have indicated that both Nr-CAM and L1 are presynaptic (Lustig et al., 2001; Matsumoto-Miyai et al., 2003), and these proteins commonly form homophilic bonds between cell processes (Hortsch, 2000; Alberts and Galli, 2003). Furthermore, postsynaptic expression of another L1-family member, neurofascin, may be necessary for the establishment of basket cell axon terminal synapses on cerebellar Purkinje cells (Ango et al., 2004); also another family member is found in dendrites of developing

cortical neurons and modulates neuronal positioning and dendrite orientation (Demyanenko et al., 2004). Indeed, members of a closely related family of cell adhesion molecules, the NCAMs, associate with both the presynaptic and postsynaptic sides of early synaptic contacts and may play a major role in synaptogenesis and synapse maturation (Sytnyk et al., 2002; Polo-Parada et al., 2004). In fact, NCAM180 associates with NR2A-containing NMDA receptors in the central region of the postsynaptic membrane in hippocampus; this distribution changes following induction of long-term potentiation in vivo in adult rodents (Fux et al., 2003) where NCAMs help mediate synaptic plasticity (Bukalo et al., 2004). Similar to NCAMs, members of the L1 family are important both in synaptogenesis (Faivre-Sarrailh et al., 1999; Suh et al., 2004) and for synaptic plasticity in adult brain (Law et al., 2003).

We also showed that TrkB is prevalent at all ages. This is consistent with previous light microscope studies; TrkB and its ligand, BDNF, are found in hippocampus and cerebellum prior to birth and through adult (Dieni and Rees, 2002). TrkB is found in axonal and dendritic filopodia and growth cones and in mature synapses (Wu et al., 1996; Drake et al., 1999; R.A. Gomes, C.M. Hampton, and A.K. McAllister, Soc. Neurosci. Abs. 2003) and may control expression and function of NMDA receptors (Margotti et al., 2002; Yamada and Nabeshima, 2003; Elmariah et al., 2004). Conversely, TrkB signaling may be dependent on AMPA receptor function (Hashimoto et al., 1999). Also, TrkB/BDNF (brain-derived neurotrophic factor) combinations may act as homophilic adhesion factors at synapses via BDNF dimerization; this adhesion factor function has been suggested in studies of TrkB function in adult hippocampus (Beck et al., 1993) and vestibular epithelium (Montcouquiol et al., 1998). TrkB labeling showed a decrease from P2 to P10 and increase from P10 to P35 that were significant for the synapse +100 nm category but not for the synapse only category, suggesting that the change is mainly subjacent to the PSD. This may be related to the increase of the truncated form of TrkB, as indicated in the immunoblots. The truncated form is found in the homogenate and synaptic membranes but not PSD fractions in hippocampal and cortical neurons (Wu et al., 1996; Aoki et al., 2000), although both forms are concentrated at the postsynaptic region of the neuromuscular junction (Gonzalez et al., 1999).

The developmental switch in NMDA receptor composition from mainly NR1/NR2B to mainly NR1/NR2A was predicted from a number of earlier studies (reviewed by Wenthold et al., 2003). Also, in our previous study (Sans et al., 2000), we showed in immunoblots that NR2B protein decreases and NR2A protein increases with development in hippocampus. Here we show definitive ultrastructural evidence of this switch in hippocampal synapses. This appears to be the common pattern in the central nervous system (Erisir and Harris, 2003; Liu et al., 2004) although there are exceptions, such as for some motoneurons that express abundant NR2A early in development (Oshima et al., 2002). In Sans et al. (2000), we also showed the switch in the MAGUK composition of hippocampus using both immunoblots and immunogold localization at synapses (i.e., SAP102 decreases and PSD-95 and PSD-93 increase). Thus, there may be a correlation between SAP102 and NR2B distribution in synapses, versus PSD-95 and NR2A distribution in synapses, and this correlation may reflect patterns of receptor trafficking and stabilization (Sans et al., 2003; Wenthold et al., 2003).

The absence of CaMKII from early postnatal hippocampal synapses was predicted from studies of CaMKII in synapse protein of the early postnatal forebrain, where CaMKII is largely cytosolic (Kelly and Vernon, 1985). This may have been represented in our study by the occasional gold particles seen in the cytoplasm but not near the synapse itself. In addition, CaMKII β , which is not recognized by our antibody, is very abundant in the brain after birth (mRNA; Bayer et al., 1999), and has been implicated in filopodia extension and synapse formation (Fink et al., 2003). Since CaMKII forms a major portion of the adult PSD, it would not be surprising that similarly structured PSDs at P2 have abundant levels of a developmentally important form of CaMKII, such as CaMKII β .

Most of the SPAR protein that we saw in the hippocampus was the 170 kD form, which is cytosolic (Pak et al., 2001). Since SPAR associates with actin and we found gold labeling associated with presumptive actin filaments, it is possible that the labeling that we saw associated with the PSD represents SPAR (perhaps associated with actin) that is only loosely attached to the PSD. The development of SynGAP and GKAP has not been studied previously, although we mentioned SynGAP briefly in Sans et al. (2000), i.e., SynGAP is seen commonly in nonsynaptic surface specializations, probably representing bare PSDs (Petralia et al., 2003), at P2. GKAP appears early in the development of hippocampal synapses in culture (Rao et al., 1998). Our results suggest that the higher molecular weight form of GKAP (Kim et al., 1997) is the major synaptic form in early postnatal development, as noted previously (Yoshi et al., 2003; van Zundert et al., 2004); subsequently both forms of GKAP become prevalent at synapses in adult.

It is not clear why there is substantial labeling for several proteins subjacent to the PSD, up to 100 nm from the postsynaptic membrane as examined in this study. Such a distribution has been described previously for part of Homer and Shank labeling (Xiao et al., 1998; Tu et al., 1999). In a detailed study of the laminar organization in adult synapses of cerebral cortex, Valtschanoff and Weinberg (2001) illustrated modest, accessory peaks of perpendicular (axodendritic) distribution for GKAP and Shank between 50–100 nm; in comparison, these were not seen for PSD-95. Also, for CaMKII, high labeling subjacent to the PSD was shown in cultured hippocampal synapses; again, as in the previous example, this contrasted with labeling for PSD-95, which was restricted to a “compact region on the cleft side of the PSD” (Dosemeci et al., 2001). Labeling for proteins in the area subjacent to the PSD may reflect protein molecules in this region that are unattached, attached to some other subjacent protein, e.g., Shank+Homer or SPAR+actin (as noted above), or form chains of proteins extending from the PSD to the subjacent area (see Fig. 2C and an illustration in Wenthold et al., 2003). For example, Homer, perhaps linked to PSD chains of proteins, may make contact with perisynaptic metabotropic glutamate receptors and TRPC cation channels and with receptors on internal reticular membranes, and may form links between them; this has been best studied in Purkinje cell spines of the adult cerebellum (Xiao et al., 1998; Petralia et al., 2001; Kim et al., 2003). By P2, synapses already contain substantial amounts of all of the components of a chain of proteins including GluRs, MAGUKs, GKAP, Shank, Homer dimers, and mGluRs, leading from the active zone of the synapse to the perisynaptic membrane (Fig. 2). Such physical links between varied receptors in postsynaptic, perisynaptic and intracellular membranes may mediate precise control of glutamatergic synapse function and development (Xiao et al., 2000; Ehrengreuber et al., 2004). In these chains, changes with development may involve NMDA receptors and MAGUKs, so that most of the early chains probably include NR2B-containing receptors and SAP102 (an association of the latter in these chains is based on yeast two-hybrid screens of GKAP - Kim et al., 1997; see also review by van Zundert et al., 2004). SAP102, in turn would link, in these early chains, to the higher molecular weight form of GKAP (as noted above; van Zundert et al., 2004).

Our study shows that glutamatergic synapses develop relatively rapidly in the early postnatal hippocampus. Even the earliest ultrastructural signs of a maturing PSD indicate the presence of some amount of most of the proteins involved in function of the “mature” glutamatergic synapse. This rapid acquisition of these proteins is consistent with findings in vitro; new synaptic sites accumulate substantial levels of Shank in less than 30 minutes and NR1 within about 80 minutes (Bresler et al., 2004). We conclude that synapses in the early postnatal animal can possess most of the components necessary to carry out many of the complex functions attributed to glutamatergic synapses. Nevertheless, we show here that these synapses undergo many profound changes in relative protein composition and abundance during development; specifically, synaptic maturation involves mainly the substitutions of some key components, especially PSD-95 for SAP102 and NR2A for NR2B.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Immunoblots of proteins during the development of the hippocampus from embryonic day 18 (E18) and postnatal days 2 to 50 (P2-P50) and in isolated postsynaptic density (PSD) fractions from rat brains. PSD II represents the final PSD isolate. The same amount of protein was applied to all lanes. Hom, homogenate; P2frac, P2 fraction. Proteins are listed in the same order as used for the description of the immunogold Results, and include: intercellular adhesion proteins, TrkB, glutamate receptors and MAGUKs, proteins associated directly with these, and the Shank-Homer-mGluR5 chain.

Figure 2.

Histogram and diagram showing major changes in proteins in synapse development. The Y-axis of the histogram shows gold per synapse (expressed as percent of the highest value per antibody) for antibodies used in this study, as defined in the Methods. Detailed data for each antibody study are included in supplementary Tables 1 and 2. In addition, percentages based on previously published values for GluR1, GluR2/3, NR1 and PSD-95 (published in Petralia et al., 1999 and Sans et al., 2000) are included for comparison. Note the large increase in NR2A (compare to the published data for PSD-95 and AMPA receptors) and CaMKII and decrease in NR2B and SAP102 with age (all highly significant at $p \leq 0.001$), while there is no significant change in some such as GKAP and SPAR. Other proteins change more modestly with age and include an increase in cadherin, catenin, neuroigin, SynGAP, Shank and Homer. Diagram illustrates a hypothetical synapse at P2. Note that mature-appearing synapses at P2 contain most major component proteins of the PSD. The light color of AMPARs and CaMKII indicate their low density at this age. TARPs, which may bind AMPARs to MAGUKs, are not discussed in this paper. Not evident in this diagram are profound changes in MAGUKs and NMDA receptor types that occur during further postnatal development (evident in the histogram above); also not illustrated are the earlier stages in synaptogenesis when the only major proteins at the contact may be intercellular adhesion proteins (see text for details; this diagram is based partially on a diagram in the review of Wenthold et al., 2003, and has been modified extensively for the present study).

Figure 3.

Immunogold labeling for cadherin and catenin in synapses during postnatal development of hippocampus CA1 stratum radiatum. Both were moderately high at all ages and showed a significant increase in labeling from P10 to P35. For cadherin, 28%, 21%, and 36% of synapses (postsynaptic (synapse+100 nm)) were labeled for P2, P10, and P35, respectively. For catenin, 26%, 33%, 41% of synapses (postsynaptic (synapse+100 nm)) were labeled for P2, P10, P35, respectively. The Y-axis indicates gold per synapse or per synapse+100 nm (postsynaptic), as defined in the Methods. Scale bars for micrographs are 100 nm, arrows in micrographs indicate gold labeling associated with the PSD (or presynaptic active zone), and histograms show values plus standard errors. Vertical bars on one synapse on the right illustrate the vertical distances included in the categories of “synapse” and “synapse+100 nm”, as defined in the text.

Figure 4.

Immunogold labeling for neuroligin and Nr-CAM in synapses during postnatal development of hippocampus CA1 stratum radiatum. Both neuroligin and Nr-CAM were moderately high at all ages; there were no significant changes between ages for neuroligin and there was an increase in Nr-CAM from P2 to P10. For neuroligin, 37%, 44%, 48% of synapses (postsynaptic (synapse+100 nm)) were labeled for P2, P10, P35, respectively. For Nr-CAM, 24%, 42%, 32% of synapses (postsynaptic (synapse+100 nm)) were labeled for P2, P10, P35, respectively. Histograms are for neuroligin (top) and Nr-CAM (bottom). The Y-axis indicates gold per synapse or per synapse+100 nm (postsynaptic), as defined in the Methods. Scale bars for micrographs are 100 nm, arrows in micrographs indicate gold labeling associated with the PSD (or presynaptic active zone), and histograms show values plus standard errors. Asterisks indicate gold associated with possible exocytotic pits.

Figure 5.

Immunogold labeling for TrkB in synapses during postnatal development of hippocampus CA1 stratum radiatum. There were significant changes in postsynaptic labeling from P2 to P10 (decrease for synapse+100 nm; see Methods) and P10 to P35 (increase for synapse+100 nm). There were 30%, 17%, and 27% of synapses (postsynaptic (synapse+100 nm)) labeled for P2, P10, and P35, respectively. The Y-axis indicates gold per synapse or per synapse+100 nm (postsynaptic), as defined in the Methods. Scale bars for micrographs are 100 nm, arrows in micrographs indicate gold labeling associated with the PSD, and histograms show values plus standard errors.

Figure 6. Immunogold labeling for NR2A and NR2B in synapses during postnatal development of hippocampus CA1 stratum radiatum. Micrographs illustrate the decrease in NR2B and increase in NR2A at synapses during development. For NR2B, there was a significant decrease from P2 to P35 and from P10 to P35; 30%, 33%, and 23% of synapses were labeled for P2, P10, and P35, respectively. For NR2A, there was a significant increase from P2 to P10 and P10 to P35; 3%, 11%, and 29% of synapses were labeled for P2, P10, and P35, respectively. The Y-axis indicates gold per synapse or per synapse+100 nm as defined in the Methods. Scale bars for micrographs are 100 nm, arrows in micrographs indicate gold labeling associated with the PSD, and histograms show values plus standard errors.

Figure 7.

Immunogold labeling for CaMKII and SPAR in synapses during postnatal development of hippocampus CA1 stratum radiatum. For CaMKII, note labeling within and subjacent to density. There was no synaptic labeling at P2, and a significant increase from P10 to P35. For synapse+100 nm (see Methods for details), 9% and 55% of synapses were labeled at P10 and P35, respectively. For SPAR, gold labeling was moderate at synapses at all ages; there were no significant differences between ages. For synapse+100 nm, 29%, 33%, and 31% of synapses were labeled at P2, P10, and P35, respectively. In addition, cytoplasmic labeling often was associated with <10 nm filaments (small arrows; presumably actin). The Y-axis indicates gold per synapse or per synapse+100 nm as defined in the Methods. Scale bars for micrographs are 100 nm, arrows in micrographs indicate gold labeling associated with the PSD, and histograms show values plus standard errors.

Figure 8.

Immunogold labeling for SynGAP and GKAP in synapses during postnatal development of hippocampus CA1 stratum radiatum. For SynGAP, gold labeling was moderately prevalent at all ages, and increased significantly from P10 to P35. Upper left micrograph (P2) is from the ABR SynGAP antibody, which was not used for quantification; other micrographs are from the quantitative studies using the Haganir SynGAP antibody. For synapse+100 nm, 19%, 18%, and 30% of synapses were labeled for P2, P10, and P35, respectively. For GKAP-C labeling, there were no significant changes with age. For synapse+100 nm, 20%, 21%, and 19% of synapses were labeled for P2, P10, and P35, respectively. The Y-axis indicates gold per synapse or per synapse+100 nm as defined in the Methods. Scale bars for micrographs are 100 nm, arrows in micrographs indicate gold labeling associated with the PSD, and histograms show values plus standard errors.

Figure 9.

Immunogold labeling for Shank and Homer (1b,c and pan antibodies) in synapses during postnatal development of hippocampus CA1 stratum radiatum. Shank and Homer showed similar patterns with each other at all ages, with labeling prevalent both in the PSD and subjacent to it. There was a significant increase in Shank from P2 to P35 and in Homer 1b,c from P2 to P10. For Shank, 33%, 41%, and 52% of synapses (synapse+100 nm) were labeled for P2, P10, and P35, respectively. For Homer 1b,c, 44%, 71%, and 61% of synapses (synapse+100 nm) were labeled for P2, P10, and P35, respectively. The Y-axis indicates gold per synapse or per synapse+100 nm as defined in the Methods. Scale bars for micrographs are 100

nm, arrows in micrographs indicate gold labeling associated with the PSD, and histograms show values plus standard errors.

Figure 10.

Comparison of immunogold labeling in immature versus mature synaptic contacts at P2 in hippocampus CA1 stratum radiatum. Immature synapses included here lacked a substantial PSD (for details, see Results). Note that there was no significant change in catenin labeling, while there was a highly significant increase ($p \leq 0.001$) in labeling for NR2B, SAP102, and Homer 1b,c. The Y-axis indicates gold per synapse or per synapse+100 nm as defined in the Methods (values plus standard errors).

Table 1

Ratio of immunogold labeling at synapse/synapse+100 nm at P2, P10 and P35 for the NMDA receptor-Homer chain of proteins (illustrated in Fig. 2).*

P2	P10	P35
NR2B (0.8), SAP102 (0.7), NR2A (0.6), GKAP (0.5), Shank/Homer (0.4)	NR2B (0.8), NR2A/SAP102/GKAP (0.7), Shank/Homer (0.5)	NR2B/NR2A/SAP102 (0.8), GKAP (0.7), Homer (0.5), Shank (0.4)

* High ratios indicate that there was little gold subjacent to the PSD (NR2A, NR2B, and SAP102), while low ratios indicate that there was substantial gold subjacent to the PSD (Shank and Homer). GKAP shows a mid-value ratio, consistent with its middle position in the chain.

Table 2

Changes in immunogold labeling at synapses between P35 and P2 indicating developmental trends of protein increase (↑) or decrease (↓).

Antibody	P2 vs. P35
	Synapse (P35÷P2)
Cadherin	1.4x
Catenin	1.7x↑*
Neurologin	1.2x
Nr-CAM	1.6x↑*
TrkB	0.8x
NR2A	12x ↑**
NR2B	0.5x ↓**
SAP102	0.4x ↓**
CaMKII	11.2x #↑
SPAR	1.1x
SynGAP	1.8x↑*
GKAP	1x
Shank	1.8x↑*
Homer	1.9x↑*

Based on averages shown in supplementary Table 1.

* Changes were considered significant if $p < 0.05$ (2-tailed t-test assuming unequal variables)

** $p \leq 0.001$ changes are indicated with bold type). Synapse number ranged from 499–1403. Notable trends are the highly significant increase in NR2A and CaMKII (#comparison is for P10 vs. P35 since there was no gold labeling at P2 for CaMKII) and the highly significant decrease in NR2B and SAP102.