Differential Roles of Postsynaptic Density-93 Isoforms in Regulating Synaptic Transmission

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>As Published</td>
<td><a href="http://dx.doi.org/10.1523/jneurosci.0019-12.2013">http://dx.doi.org/10.1523/jneurosci.0019-12.2013</a></td>
</tr>
<tr>
<td>Publisher</td>
<td>Society for Neuroscience</td>
</tr>
<tr>
<td>Version</td>
<td>Final published version</td>
</tr>
<tr>
<td>Accessed</td>
<td>Sat Dec 29 23:01:43 EST 2018</td>
</tr>
<tr>
<td>Citable Link</td>
<td><a href="http://hdl.handle.net/1721.1/89137">http://hdl.handle.net/1721.1/89137</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>Article is made available in accordance with the publisher’s policy and may be subject to US copyright law. Please refer to the publisher’s site for terms of use.</td>
</tr>
</tbody>
</table>

Detailed Terms
Cellular/Molecular

Differential Roles of Postsynaptic Density-93 Isoforms in Regulating Synaptic Transmission

Juliane M. Krüger, 1,2 Plinio D. Favaro, 1,2 Mingna Liu, 3 Agata Kitlińska, 4 Xiaojie Huang, 1,2 Monika Raabe, 4 Derya S. Akad, 1,5 Yanling Liu, 3 Henning Urlaub, 4,6 Yan Dong, 7 Weifeng Xu, 8 and Oliver M. Schlüter 1

1European Neuroscience Institute, 37077 Göttingen, Germany, 2Göttingen Graduate School for Neurosciences and Molecular Biosciences, 37077 Göttingen, Germany, 3Picower Institute for Learning and Memory, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02319, 4Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany, 5Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany, 6Bioanalytics, Department of Clinical Chemistry, 37075 Göttingen, Germany, and 7Department of Neuroscience, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

In the postsynaptic density of glutamatergic synapses, the discs large (DLG)-membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins coordinates a multiplicity of signaling pathways to maintain and regulate synaptic transmission. Postsynaptic density-93 (PSD-93) is the most variable paralog in this family; it exists in six different N-terminal isoforms. Probably because of the structural and functional variability of these isoforms, the synaptic role of PSD-93 remains controversial. To accurately characterize the synaptic role of PSD-93, we quantified the expression of all six isoforms in the mouse hippocampus and examined them individually in hippocampal synapses. Using molecular manipulations, including overexpression, gene knockdown, PSD-93 knock-out mice combined with biochemical assays, and slice electrophysiology both in rat and mice, we demonstrate that PSD-93 is required at different developmental synaptic states to maintain the strength of excitatory synaptic transmission. This strategy is differentially regulated by the six isoforms of PSD-93, including regulations of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor-active and inactive synapses, and activity-dependent modulations. Collectively, these results demonstrate that alternative combinations of N-terminal PSD-93 isoforms and DLG-MAGUK paralogs can fine-tune signaling scaffolds to adjust synaptic needs to regulate synaptic transmission.

Introduction

N-Methyl-D-aspartate (NMDA) receptor (R)-dependent long-term synaptic plasticity involves the recruitment and removal of AMPARs from the postsynaptic density (PSD) (Malinow and Malenka, 2002; Kerchner and Nicoll, 2008). Such dynamic trafficking as well as the basal turnover of AMPARs are regulated by multiple signaling pathways, which are coordinated by signaling scaffolds, particularly the DLG-MAGUK family of proteins (Craun and Bredt, 1998; Sheng and Kim, 2002; Xu et al., 2008; Ryan and Grant, 2009). The DLG-MAGUKs consist of PSD-93/Chapsyn-110, PSD-95/synapse associated protein (SAP)90, SAP97, and SAP102 to share the same domain structure of protein interaction modules, including alternative N-terminal domains, three consecutive PSD-95/discs large/ zona occludens-1 (PDZ) domains, and a src-homology 3 (SH3) domain linked to guanylate kinase (GK) like domain (Cho et al., 1992; Kistner et al., 1993; Müller et al., 1995; Brennan et al., 1996; Kim et al., 1996; Müller et al., 1996). The DLG-MAGUK isoforms and paralogs differ primarily by alternative N-terminal domains, which are coded for by alternative transcriptional start sites of the four DLG-MAGUK genes (Chetkovich et al., 2002; Parker et al., 2004; Schlüter et al., 2006). These different domains entail these isoforms/paralogs differential regulatory roles in excitatory synaptic transmission and potentially contribute to their evolutionary specialization in cognitive function (Nithianantharajah et al., 2013). For example, in hippocampal neurons, overexpression of N-terminally palmitoylated α-isozymes of PSD-95 (PSD-95α) or SAP97α increases AMPAR-mediated synaptic transmission (Schnell et al., 2002; Bégué and Andrade, 2003; Ehrlich and Malinow, 2004), whereas RNAi-mediated knockdown or genetic deletion of PSD-95 produces the opposite effect (Nakagawa et al., 2004; Bégué et al., 2006; Elias et al., 2006; Schlüter et al., 2006; Carlisle et al., 2008). On the other hand, the β-isozymes of PSD-95 and SAP97, which express an N-terminal L27 domain, govern activity-dependent enhancement of AMPAR function via...
signaling pathways similar to those that mediate long-term potentiation (LTP) of hippocampal excitatory synapses (Schütler et al., 2006).

In contrast to PSD-95 and SAP97, much less is known about PSD-93, which possesses more variations in its N-terminal domains. The PSD-93 gene codes six N-terminal domains, including two palmitoylated isoforms, an L27 domain containing isoform, and additionally three unique N-terminal isoforms (Parker et al., 2004). Similar to PSD-95α and SAP97α, overexpression of PSD-93α enhances AMPAR-mediated synaptic transmission in organotypic hippocampal slices (Elias et al., 2006). These and other results lead to a thought that PSD-93 and PSD-95 are redundant to some extent with similar cellular functionality. However, studies on the loss of function of all PSD-93 isoforms suggest a different conclusion. In hippocampal slices, genetic deletion of PSD-93 does not affect AMPARs, but RNAi-mediated knockdown induces a 50% decrease in AMPAR-mediated EPSCs (AMPA-R EPSCs) (Elias et al., 2006; Carlisle et al., 2008). Furthermore, a decrease in NMDAR EPSCs was observed at cortical synapses in PSD-93 knock-out (KO) mice (Tao et al., 2003), but a similar change is not observed in organotypic hippocampal slices upon RNAi-mediated knockdown of PSD-93 (Elias et al., 2006). Moreover, the phenotypes of PSD-95 and PSD-93 KO mice are opposite. In PSD-95-deficient mice, the induction of LTP is greatly facilitated, whereas long-term depression (LTD) is abolished (Migaud et al., 1998; Béïque et al., 2006; Carlisle et al., 2008). In contrast, in PSD-93 KO mice, the threshold for LTP induction is elevated and LTD is normal (Carlisle et al., 2008). These results suggest a different functional identity of PSD-93 from PSD-95, and the seemingly discrepant results may stem from contributions of different N-terminal PSD-93 isoforms in different studies. To address these discrepancies, we examined all these isoforms with a focus on their functionally relevant N terminus. Our results show that different PSD-93 isoforms played different regulatory roles in excitative synaptic transmission; they constitute a specific functional repertoire to modulate AMPAR synaptic function, which differs from the function of PSD-95 and SAP97.

Materials and Methods

Dissociated neuronal cultures. Dissociated hippocampal cultures were prepared from postnatal day 0 (P0) or P1 mice or Wistar rats of either sex. The hippocampi were dissected and digested for 30 min at 37°C with papain, as previously detailed (Huetter and Baughman, 1986; Schütler et al., 2006). A total of 10^3 cells per well in a 24-well plate were plated into DMEM/Ham's F12 (Biochrom) supplemented with 2% B27 (Invitrogen) and 1% Glutamax on a poly-D-lysine-coated 24-well plate. Glial growth was inhibited with FUDR at 4 days in vitro (DIV). The cells were refed every 3–7 d with astrocyte-conditioned culture medium.

Hippocampal slice cultures. Organotypic hippocampal slices were prepared from P7–P9 Wistar rats of either sex, modified from previously published procedures (Stoppini et al., 1991; Schütler et al., 2006). Briefly, animals were anesthetized with isoflurane, and the hippocampi were dissected in ice-cold sucrose cutting buffer (in mM as follows: 204 sucrose, 26 NaHCO3, 10 glucose, 2.5 KCl, 1 NaH2PO4, 4 MgSO4, 1 CaCl2, 4 ascorbic acid). Using a guillotine with a wire frame, 300–μm-thick slices were cut and transferred into artificial CSF (ACSF) (containing in mM as follows: 119 NaCl, 26 NaHCO3, 20 glucose, 4 MgSO4, 2.5 KCl, 1 NaH2PO4, 4 CaCl2) for recovery. The slices were washed in BME (Biochrom) and plated onto MilliCell (Millipore) culture plate inserts in culture dishes containing BME-based culture medium supplemented with 20% heat-inactivated horse serum (Biochrom). The slices were incubated at 37°C in a 5% CO2 environment. On DIV 1, the slices were transferred to 34°C, and the culture medium was refreshed. On DIV 4, the culture medium was exchanged by BME-based culture medium supplemented with 5% heat inactivated horse serum, and the slices were refed every other day. Where indicated 20 μM bicuculline or 20 μM bicuculline and 50 μM APV was added to the feeding medium after virus infection.

Hippocampal slices. Coronal brain slices (400 μm) containing the dorsal hippocampus were prepared from mice of either sex, deeply anesthetized with isoflurane. The brain was rapidly removed in ice-cold NMDG cutting buffer (composition in mM as follows: 45 NMDG, 0.33 KCl, 0.5 MgCl2, 0.4 KH2PO4, 10 (–) glucose, 2 choline bicarbonate, 0.17 CaCl2, pH 7.4, with HCl). Slices were cut on a vibrating microtome recovered in ACSF with 1.3 mM MgSO4 and 2.5 mM CaCl2 at 35°C for 30 min, and then stored at room temperature until use (1–5 h).

Virus vectors and neuronal transduction. All lentiviral constructs were made from the lentiviral transfer vector FUGW (Lois et al., 2002) and its variant FHUG+W, which additionally contains an RNAi expression cassette driven by an H1 promoter (Schütler et al., 2006). For RNAi-mediated knockdown of rat PSD-93, a hairpin (sh93a) (Ogawa et al., 2008) targeting the sequence GCAAGCTTTGATTTGAAGA was inserted downstream to the H1 promoter (Elias et al., 2006; Schütler et al., 2006). A mouse-specific shRNA sh93m (target sequence: GACCTGCTAGCGAGACT) and sh93 (target sequence: CTCTATTGTTGGACTGTAT) driven by the mouse U6 promoter were subcloned into FHUG+W to replace the H1 promoter (Elias et al., 2006; Schütler et al., 2006). Dr. Robert J. Wenthold (National Institutes of Health) provided the rat PSD-93α2 coding sequence (Roche et al., 1999), and Dr. Guoping Feng (Massachusetts Institute of Technology) provided the mouse sequence of PSD-93α1, PSD-93β, PSD-93δ, and PSD-93ζ (Park et al., 2004). The EST clone AW060584 containing the N terminus of PSD-93y was purchased from Open Biosystems. For studying the effect of overexpression of PSD-93, the eGFP in FUGW was replaced by fusion proteins of C-terminally eGFP-tagged PSD-93 isoforms. To rule out an impact of the C-terminal eGFP on PSD-93 function, bicistronic constructs were generated using the CMV-IREs-GFP cassette of the plasmid pMES (Swartz et al., 2001).

Transduction of neurons in hippocampal slice cultures was achieved by injecting concentrated viral particles into the CA1 pyramidal cell layer on DIV 1 or DIV 2 using a Nanoinject II device (Drummond Scientific). Electrophysiology. Recordings were performed 4–9 d after viral infection from slice cultures, as previously described (Schütler et al., 2006) or acute hippocampal slices. A single slice was transferred into the recording chamber, which was constantly perfused with oxygenated ACSF (2–3 ml/min) with 4 mM MgSO4 and 4 mM CaCl2 for slice cultures, and 1.3 mM MgSO4 and 2.5 mM CaCl2 for acute slices, respectively. In slice cultures, 1–5 μM 2-chloroadenosine (Biolog) was added to the ACSF to reduce polysynaptic activity; 50 μM picrotoxin (Ascent Scientific) was added to block GABAergic transmission (Parker et al., 1999). The pipettes (open pipette resistance 3–6 MΩm) were filled with intracellular solution containing in mM as follows: 117.5 MeSO4H, 10 HEPES, 17.75 CaCl2, 10 TEA-Cl, 0.25 EGTA, 10 glucose, 2 MgCl2, 4 NaATP, 0.3 NaGTP, pH 7.3, and 290 mMOSm for slice cultures; or in mM as follows: 120 cesium gluconate, 200 HEPES, 4 QX-314/Cl, 0.4 EGTA, 5 TEACl, 4 MgATP, 0.3 NaGTP, pH 7.3, and 290 mMOSm for acute slices. For pairwise recording, an infected cell and its neighboring uninfected (control) cell were simultaneously recorded using whole-cell voltage-clamp configuration. EPSCs were simultaneously evoked in paired cells by a single bipolar stimulation electrode filled with ACSF (slice culture) or a concentric bipolar stimulating electrode (acute slice). AMPAR EPSCs were recorded at −60 mV. For NMDAR EPSCs, EPSCs were recorded at 40 mV, and the amplitude of NMDAR EPSCs was assessed by the amplitude of EPSCs at 60 ms after the peak currents, a time point at which AMPAR EPSCs are mostly absent. Signals were filtered at 4 kHz and digitalized at 10 kHz using an ITC-18 acquisition board (HEKA). The responses were monitored online and analyzed using custom-written acquisition and analysis software implemented in IGOR Pro (WaveMetrics). For each cell pair (slice culture) or cell (acute slice), a minimum of 40 sweeps was collected and averaged. The stimulation strength was adjusted such that the peak amplitude of AMPAR EPSCs in control cells was ~100 pA. A 5 mV hyperpolarizing step was used to monitor the input and series resistance over the record-
ing. For mEPSCs, the ratio of Ca$^{2+}$/Mg$^{2+}$ in ACSF was adjusted to 4/1, with 0.5 μM TTX and 50 μM sucrose supplemented. mEPSCs were recorded for 5–15 min, 3 min after break in at 13–17 DIV.

The statistical analysis of electrophysiological data was done with non-parametric tests using the software R Project (R Foundation of Statistical Computing). Comparison between pairs of control and infected cell responses was analyzed using the Wilcoxon Signed-Rank test. Averages of ratios of infected and uninfected cell pairs were calculated after logarithmic transformation and presented as back-transformed mean ± SEM. For nonpaired measures, the Wilcoxon Mann–Whitney two-sample rank test was used. Multiple groups were compared with the Kruskal–Wallis test with multiple comparisons. AMPAR/NMDAR ratios in acute slices were tested for normal distribution, and statistical significance was assessed using one-way or two-way ANOVA with Bonferroni post tests. All data are presented as mean ± SEM, which is shown as error bars in the graphs. For mEPSCs, statistical significance between distributions was calculated by the Kolmogorov–Smirnov test.

**Results**

**PSD-93 genomic structure and mRNA expression of the N-terminal isofoms in the mouse brain**

Multiple isoforms can be generated from the DLG-MAGUK genes by alternative promoter usage coding for the N-terminal domains (Brenman et al., 1996; Parker et al., 2004; Schlüter et al., 2006) and additionally by alternative splicing of exons in the linker sequence between the SH3 and GK domain (Lue et al., 1994; McLaughlin et al., 2002; Nikandrova et al., 2010). We analyzed the mouse gene for PSD-93 (dlgh2) and compared the exon–intron structure with the PSD-95 (dlgh4) and SAP97 (dlgh1) genes, two other functionally related DLG-MAGUKs, which share a similar gene structure (Fig. 1A) (Schlüter et al., 2006). The N-terminal domains of PSD-95 and SAP97 with a palmitoylation motif have been termed α, whereas the L27 domain containing isoforms have been termed β (Chetkovich et al., 2002; Schlüter et al., 2006). For easy comparison, we termed the palmitoylated PSD-93 isoforms α1 and α2, instead of α and β, and the L27 containing PSD-93 isoform β instead of α as originally suggested (Parker et al., 2004). For the N-terminal domains, five exons code for the L27 containing β-isofrom and two distinct exons for the palmitoylated α1- and α2-isofoms. The α or the β exons are spliced on the common exon 2, coding for a highly conserved KYRYQDED motif. 3’ of exon 2, the unique N-terminal isoforms γ, δ, and ε, are coded by single exons and spliced onto the alternative exons 3 and 4. The γ exon contains two putative start codons, which are contained in transcripts of the gene, as shown by the EST clone AW060584.

Using semiquantitative RT-PCR, we detected the expression of all six N-terminal isoforms in mouse hippocampus (Fig. 1B). The genes of β and ε were expressed homogeneously in hippocampus, cerebellum, cortex, olfactory bulb, and spinal cord. The expression patterns of the two α-isofoms overlapped, being highest in hippocampus, cortex, and olfactory bulb, followed by spinal cord and lowest in cerebellum. The expression levels of γ and δ-isofoms were highest in the cerebellum and spinal cord, respectively, and were similar in the other brain regions. The linker between the SH3 and GK domain is alternatively spliced in SAP97, and this splicing determines the association with the interaction partners and subcellular localization (Rumbaugh et al., 2003). The 13 insert is required for the interaction with 4.1N and AKAP79/150 (Lue et al., 1994; Nikandrova et al., 2010). The PSD-93 linker is coded by exons 16, 17a, 17b, and 18 (Fig. 1A). The coded amino acid sequence of exon 17a shared ~80% identity with the amino acid sequence coded by 13 of SAP97. The coded amino acid sequence of exon 17b is 90% identical to the translation of SAP97 15 (Fig. 1C). Using RT-PCR, we tested the expression pattern of these potentially alternatively spliced exons. Combining forward primers in exons 15 and 16 with reverse primers in exons 17a, 17, and 17b, we amplified one band for the combination of primers 15 and 16 with 17a or 17b and two bands for the combination of 15 and 16 with 17b (Fig. 1D, E). The individual bands indicated that the exon 17a and 17 are mutually exclusively expressed. In the lanes with two bands, the size difference indicated that exon 17b was expressed either in combination...
with exon 17a or 17. The bands with primer 16 were equally shifted upward on the gel compared with the bands with primer 15. The size difference matches the 76 nucleotide (nt) difference in the position of primer 15 and 16 on the transcript, indicating that exon 16 was included in all PSD-93 C-terminal transcripts.

To analyze the splicing onto exon 18, we combined forward primers in exon 16, and the alternative exon 17a and 17 with a reverse primer in exon 18 (Fig. 1C). The primer combination 16/18 yielded three PCR products, in which the smallest matched the calculated size of the exon combination 16-17-18. This observation indicated that each transcript contained either exon 17 or exon 17a. Using the primer combination 17a/18 and 17/18, we amplified two bands each, which match the calculated size of the exon combinations 17a-18 of 94 nt, 17a-17b-18 of 136 nt, 17-18 of 80 nt, and 17-17b-18 of 122 nt. Thus, the exons 17a and 17 are alternatively spliced and exon 17b is similar to exon I5 of SAP97 a cassette exon, which can be either spliced onto the exons 17 or 17a or excluded from the transcript. For the subsequent functional analysis, we used the N-terminal isoforms in combination with the C-terminal exon combination 16-17-18, which was described in one of the original clones of PSD-93 (Kim et al., 1996).

Protein levels of PSD-93 N-terminal isoforms in the mouse hippocampus

The RT-PCR experiment suggested that all N-terminal isoforms of PSD-93 are expressed in the hippocampus. To determine the protein level of these isoforms, we took two independent approaches: the quantitative Western blotting and MS. In the Western analysis, we took advantage of the size difference among the isoforms and antibodies that detect different subsets of the isoforms. The monoclonal mouse PSD-93 antibody recognized all recombinantly expressed eGFP-tagged isoforms from HEK293 cells with similar strength as judged by quantifying the expression level with double labeling with a GFP antibody (data not shown). Among the recombinantly expressed PSD-93 isoforms, the antibody detecting the \( \beta \)-H9251 and \( \beta \)-H9252 isoforms of the DLG-MAGUKs detected only PSD-93 \( \beta \)-H9251, \( \beta \)-H9251, and \( \beta \)-H9254, whereas a polyclonal rabbit antibody detected all PSD-93 isoforms as control (Fig. 2A). In total mouse hippocampus homogenates, four distinct bands were detected at molecular weights between 100 and 135 kDa (Fig. 2B).

We next determined the identity of these isoforms using a “parallel comparison” strategy. Specifically, in dissociated mouse neuronal cultures, we expressed the untagged isoforms. The endogenous PSD-93 banding pattern of cultures matched the one from hippocampal extracts. To identify specifically the size of the recombinant proteins, the endogenous PSD-93 expression was silenced by the simultaneous expression of an shRNA against mouse PSD-93, resulting in ~90% knockdown (sh93ms band intensity normalized to control neurons: 9.8 ± 1.1%, n = 3; Fig. 2B). Using the PSD-93 antibody, we compared the size of individual isoforms with the bands of the hippocampal extracts. The 135 kDa band matched the size of the PSD-93β isoform, whereas the strongest band at 110 kDa matched the size of PSD-93a1, \( \alpha \), and \( \delta \). Expression of PSD-93γ resulted in two bands, which matched the two lower bands between 100 and 110 kDa in the hippocampal extracts. The size of PSD-93ε also matched the low-
Figure 2. Western blot and mass spectroscopy analysis of the expression of different PSD-93 isoforms in mouse hippocampus. A, Test of the mouse PSD-93 antibody (left) and an α/β MAGUK (right) to recognize heterologously expressed GFP-tagged PSD-93 isoforms (PSD-93::GFP) in dissociated neuronal cultures. The PSD-93 antibody also recognized endogenous PSD-93 (left blot) and the α/β MAGUK antibody endogenous SAP97 (110 kDa), PSD-93α (110 kDa), and PSD-95α (95 kDa). B, Identification (mouse PSD-93 antibody) of the PSD-93 isoforms by size by comparing untagged heterologously expressed PSD-93 isoforms in mouse neuronal cultures with homogenates of mouse hippocampus. Endogenous expression of PSD-93 in culture was knocked down (sh93ms). C, Identification of PSD-93 in mouse hippocampus homogenates of PSD-93 KO and WT control samples with the α/β MAGUK antibody. D–G, MS analysis of the expression of different PSD-93 isoforms in mouse hippocampus reveals isoform-specific peptides after cleavage with endoproteinase trypsin. D, MSMS spectrum of isoform α-specific peptide ALSLLEEYCK. E, MSMS spectrum of isoform β-specific peptide AENYQLLWDTIASLK. F, MSMS spectrum of isoform δ-specific peptide LVTLWDSVR. G, MSMS spectrum of isoform ε-specific peptide FASIWYAK. The Mascot scores for the database search are 43, 62, 62, and 5, respectively. The sequences of the peptides are listed within the spectra. Y-type and b-type fragment ions that unambiguously identify the peptides in the database search are listed in the sequence and at the corresponding fragment ions. C*, Carbamyl cysteine.
est band detected in hippocampal extracts. Using the α/β MAGUK antibody, we found that the bands at 110 kDa and 135 kDa were present in hippocampal extracts of wild-type (WT) mice but absent in PSD-93 KO mice, consistent with the above analysis that the 135 kDa band was PSD-93β and the 110 kDa band contained PSD-93α1 and/or α2 (Fig. 2C). Using densitometric quantification of the bands, we observed that PSD-93β contributed 10% to the total PSD-93, the band containing PSD-93α1, α2, and δ contributed 50%, and the two lower bands contributed 20% each.

Results from the above Western analysis were informative but not clear-cut; some bands appeared to contain more components (Fig. 2B). In an attempt to resolve the uncertainties, we next used MS analysis. We immunoprecipitated PSD-93 from the hippocampal extracts and subjected the excised bands after tryptic digestion to LC-coupled tandem MS analysis. The in silico tryptic digest generated one unique peptide for PSD-93α1, two unique peptides for α2, 12 unique peptides for β, three for γ, nine for δ, and two for ε. Altogether, 9 of the 29 predicted peptides were identified with the MS analysis. Five peptides were specific for the subgroups of PSD-93 we detected altogether 5 peptides, which are specific for the two forms (Fig. 2).

Using dissociated rat neuronal cultures, we first verified the effectiveness of these constructs (Fig. 3A). Seven days after infection, the levels of all PSD-93 isoforms were reduced. The sh93a and sh93b reduced endogenous PSD-93 by >70% in different independent cultures (sh93a: 21.4 ± 8.6%, n = 3; sh93b: 10.6 ± 1.7%, n = 3). The sh93c was most efficient and reduced endogenous PSD-93 by ~95% (sh93c: 2.0 ± 1.0%, n = 2). The levels of other synaptic proteins, including PSD-95, GluA2, and GluN2B, did not change significantly. The mouse PSD-93-specific shRNA (sh93ms) did not affect PSD-93 isoforms in rat neuronal cultures but was effective in mouse neuronal cultures (Figs. 2B, 3A).

We then tested the effect of the shRNAs in organotypic rat slice cultures. Using parallel dual whole-cell patch-clamp recordings, we measured the AMPAR EPSCs at a holding potential of -60 mV and the NMDAR EPSCs at a holding potential of 40 mV (60 ms after the AMPAR EPSC peak) simultaneously from a transduced fluorescent neuron and an untransduced neighboring control neuron. This approach allows the quantification of the effect of molecular manipulations on basal synaptic transmission (Hayashi et al., 2000; Schlüter et al., 2006). Expression of these shRNAs had no significant effect on basal synaptic transmission; both the AMPAR and NMDAR EPSCs were not affected (sh93a AMPAR, n = 25; control, -39.8 ± 3.4 pA vs infected, -36.2 ± 3.5 pA, p = 0.51; NMDAR, control, 35.5 ± 3.9 pA vs infected, 30.9 ± 3.4 pA, p = 0.39; n = 22; sh93b AMPAR, n = 9; control, -32.3 ± 3.8 pA vs infected, -30.7 ± 5.0 pA, p = 0.73; NMDAR, n = 8; control, 33.4 ± 5.2 pA vs infected, 23.5 ± 5.2 pA, p = 0.25; sh93c AMPAR, n = 20; control, -52.1 ± 5.2 pA vs infected, -46.2 ± 4.0 pA, p = 0.37; NMDAR, n = 16; control, 31.0 ± 3.8 pA vs infected, 31.8 ± 4.2 pA, p = 0.71; Fig. 3B). In the interleaved controls using an shRNA against PSD-95, we observed a 50% reduction of AMPAR EPSCs (see Fig. 5A), an effect similar to that previously reported (Schlüter et al., 2006). These results were apparently at odds with a previous study, showing a 50% reduction of AMPAR EPSCs in rat organotypic slice cultures (Elias et al., 2006). In an attempt to resolve this discrepancy, we measured AMPAR/NMDAR EPSC ratios in acute slices from PSD-93 KO and WT mice (McGehee et al., 2001). To test whether developmental differences occur in the strength of basal synaptic transmission, we analyzed slices from three different age groups: P13-P17 during hippocampal development, P25-P30 shortly after hippocampal development, and P60-P74 mature hippocampus. The AMPAR/NMDAR EPSC ratio gradually increased during the three time points in WT mice (F(2,41) = 18.80, p < 0.01; P13-P17, 1.31 ± 0.14 vs P25-P30, 2.87 ± 0.28, p < 0.05; P25-P30 vs P60-P74, 4.36 ± 0.52, p < 0.05; Fig. 3C,D), consistent with the maturation of AMPAR-silent synapses into AMPAR-containing ones (Durand et al., 1996). In PSD-93 KO mice, the AMPAR/NMDAR EPSC ratio increased from P13-P17 to P25-P30 but then remained on that level (F(2,38) = 20.87, p < 0.01; P13-P17, 1.33 ± 0.13 vs P25-P30, 2.88 ± 0.24, p < 0.01; P25-P30 vs P60-P74, 2.74 ± 0.23, p = 0.88; Fig. 3C,D). Consequently, the AMPAR/NMDAR EPSC ratio was larger in WT mice on P60-P74 compared with that of PSD-93 KO mice, although we did not measure any difference at the time points earlier (F(2,80) = 5.22, p < 0.01, two-way ANOVA; WT P60-P74 vs KO P60-P74, p < 0.01). Thus, the lack of change in AMPAR/NMDAR ratios in young mice was consistent with previous reports showing no difference in basal synaptic transmission in PSD-93 KO mice (Migaud et al., 1998; Elias et al., 2006). The difference in older animals (P60-P74) suggested, that during late development in the hippocampus, the lack of PSD-93 caused impairments in glutamatergic synapses. Apparently, this condition might be phenocopied under some culturing conditions in organotypic slice
cultures because it was previously reported that knockdown of PSD-93 caused a selective reduction in AMPAR function (Elias et al., 2006). Notably, the lack of effect of the sh93 constructs in our slice cultures mimicked the phenotype in young up to P30 PSD-93 KO mice.

Together, these results suggested that loss of PSD-93 did not affect young synapses in the Schaffer-collateral CA1 pyramidal neuron pathway significantly but was required for proper glutamatergic synaptic transmission in adult synapses.

Role of N-terminal PSD-93 isoforms in regulating AMPA receptor function

The α-isomers of PSD-93, PSD-95, and SAP97 enhanced AMPAR function when overexpressed in hippocampal organotypic slices (Schnell et al., 2002; Béïque and Andrade, 2003; Ehrlich and Malinow, 2004; Nakagawa et al., 2004; Elias et al., 2006; Schlüter et al., 2006). However, the role of other isoforms, which made up to 50% of the total PSD-93 in the hippocampus (Fig. 2B,C), is not well understood. To generate a thorough understanding about PSD-93, we examined all PSD-93 isoforms side by side in their regulation of excitatory synaptic transmission.

We first tested the effects of gain of function of the individual PSD-93 isoforms. Using rat organotypic hippocampal slices, we overexpressed the individual isoforms as eGFP-tagged fusion proteins from lentiviral expression vectors (Schlüter et al., 2006). Neither of the two α isoforms enhanced AMPAR function when overexpressed (PSD-93α1, n = 14; control, −48.6 ± 4.0 pA; infected, −43.7 ± 6.2 pA, p = 0.33; PSD-93α2, n = 13; control, 65.9 ± 9.5 pA; infected, −58.7 ± 5.3 pA, p = 0.64; Fig. 4A,B,H).

This result was seemingly in contradiction to a previous study, in which overexpression of PSD-93α1 as well as PSD-95α enhances AMPAR function (Elias et al., 2006). To rule out that our eGFP tag compromised the function of the tagged protein, we expressed PSD-93α2 in a bicistronic vector separated from eGFP. Overexpression of PSD-93α2 by this strategy did not enhance AMPAR EPSCs either; indeed, it slightly but significantly reduced them (n = 11, control, −38.9 ± 5.1 pA; infected, −25.8 ± 2.4 pA, p < 0.05; Fig. 4C,H).

We then moved on to other PSD-93 isoforms. Consistent with results from overexpression of PSD-95β and SAP97β, overexpression of PSD-93β did not affect AMPAR EPSCs (n = 14; control, −44.6 ± 5.7 pA; infected, −46.4 ± 4.9 pA; p = 0.67; Fig. 4D,H) (Schnell et al., 2002; Schlüter et al., 2006). Similarly, overexpression of the long γ-isofrom did not affect AMPAR EPSCs (n = 8, control −33.4 ± 2.2 pA; infected, −42.0 ± 7.1 pA, p = 0.25; Fig. 4E,H). On the other hand, overexpression of either PSD-93δ or e increased the peak amplitude of AMPAR EPSCs (PSD-93δ, n = 18; control, −37.4 ± 4.1 pA; infected, −53.5 ± 4.9 pA, p < 0.001; PSD-93e, n = 11; control, −41.6 ± 6.5 pA; infected, −75.0 ± 9.6 pA, p < 0.001; Fig. 4F–H). Because these two isoforms do not contain the N-terminal conserved region, which is important for the N-terminal multimerization of the α-DLG-MAGUKs, their mechanism of enhancing AMPAR function is likely to be different from the α-isofrom-mediated effect (Xu et al., 2008).

Similar to other DLG-MAGUKs, NMDAR function was not affected by overexpression of either of the PSD-93 isoforms (Fig. 4) (Schnell et al., 2002; Elias et al., 2006; Schlüter et al., 2006).
However, overexpression of PSD-93Δ caused a significant increase in the amplitude of NMDAR EPSCs (amplitude measured at 60 ms after the peak, see Materials and Methods: n = 18; control, 24.8 ± 3.8 pA; infected, 33.2 ± 5.1 pA, p < 0.05; Fig. 4).

To verify this effect on NMDARs, we isolated NMDAR EPSCs by blocking AMPAR EPSCs with 10 μM NBQX. With this approach, no significant change was observed in neurons overexpressing PSD-93Δ (n = 7; control, 32.3 ± 3.4 pA; infected, 35.5 ± 1.9 pA, p = 0.38). Because prolonged overexpression of PSD-95 can change presynaptic release of glutamate (Futai et al., 2007), which may affect the amplitude of EPSCs as well as the decay kinetics of EPSCs, we tested whether overexpression of PSD-93Δ affected the paired-pulse ratios (PPRs; interpulse interval, 100 ms), which is often altered by changes in presynaptic release. However, overexpression of PSD-93Δ did not significantly affect the PPR (PPR control, 1.78 ± 0.12; infected, 1.76 ± 0.13; p = 1.0; n = 5). In conclusion, our results suggested that overexpression of the δ and γ isoforms, but not other isoforms of PSD-93, enhanced AMPAR function.

Interaction between of PSD-93 and PSD-95

DLG-MAGUKs can interact with each other when regulating synaptic transmission. For example, the enhancement of AMPAR responses by SAP97β is modulated by PSD-95 (Schütler et al., 2006). Because PSD-95 and PSD-93 can be coimmunoprecipitated from brain extracts and colocalized in isolated PSDs, we speculated that endogenous PSD-95 might mask the effects of PSD-93 on AMPARs (Sans et al., 2000; DeGiorgis et al., 2006). To test this hypothesis, we overexpressed the PSD-93 isoforms while simultaneously knocking down the expression of endogenous PSD-95 with a lentiviral molecular replacement vector (Schütler et al., 2006). Consistent with previous findings, knockdown of PSD-95 alone (by sh95) selectively reduced the amplitude of AMPAR EPSCs by 50% and had no significant effect on NMDAR EPSCs (n = 16; AMPAR EPSCs control, 46.7 ± 6.2 pA; infected, −23.8 ± 2.7 pA, p < 0.01; NMDAR EPSCs control, 26.2 ± 4.4 pA; infected, 24.0 ± 2.9 pA, p = 0.54; Fig. 5A, H) (Nakagawa et al., 2004; Elias et al., 2006; Schlüter et al., 2006). A previous study attributed these effects to an increase in the fraction of AMPAR-silent synapses (Elias et al., 2006). Consistent with this notion, we detected a decrease in the frequency, but not the amplitude, of miniature EPSCs (mEPSCs) in the sh95-expressing neurons (mEPSC amplitude: control, −19.2 ± 0.4 pA, n = 24; infected, −19.6 ± 0.6 pA, n = 19, Kolmogorov–Smirnov, p = 1.0; mEPSC interevent interval (Δt): control, 1.0 ± 0.1 s; infected: 1.5 ± 0.2 s, Kolmogorov–Smirnov, p < 0.05; Fig. 6A).
Similar to SAP97β, PSD-95 replacement with the PSD-93 isoforms α1, α2, β, or γ rescued AMPAR function to the control cell levels (α1, n = 8; AMPAR EPSCs control, −61.7 ± 8.3 pA; infected, −50.0 ± 7.4 pA, p = 0.38; α2, n = 10; AMPAR EPSCs control, 51.1 ± 7.6 pA; infected, 60.1 ± 6.8 pA, p = 0.28; β, n = 11; AMPAR EPSCs control, −46.7 ± 5.4 pA; infected, −61.8 ± 14.4 pA, p = 0.52; γ, n = 13; AMPAR EPSCs control, −44.7 ± 5.8 pA; infected, −39.4 ± 5.9 pA, p = 0.43; Fig. 5B–E,H). These results suggested that these PSD-93 isoforms can compensate for the loss of PSD-95 function but did not enhance AMPAR function above control level in either the presence or absence of PSD-95. To test whether the rescue was mediated by increasing the fraction of synapses with AMPARs or the number of AMPARs in active synapses, we measured mEPSCs for the PSD-93α2 replacement. PSD-95-to-PSD-93α2 replacement did not rescue the shβ5-caused decrease in frequency of mEPSCs and had no influence on the amplitude either (mEPSC amplitude: control, −19.1 ± 0.4 pA, n = 22; infected, −19.1 ± 0.6 pA, n = 16; Kolmogorov–Smirnov, p = 0.82; mEPSC Δt: control, 0.91 ± 0.1 s; infected, 1.6 ± 0.3 s, Kolmogorov–Smirnov, p < 0.01; Fig. 6C). These results suggested that expression of PSD-93α2 did not increase the number of synapses with AMPARs. On the other hand, the PSD-95-to-PSD-93α replacement increased the frequency of mEPSCs above the control cell levels with a small increase of the amplitude as well (mEPSC amplitude: control, −18.8 ± 0.5 pA, n = 20; infected, −21.3 ± 1.2 pA, n = 15, Kolmogorov–Smirnov, p = 0.13; mEPSC Δt: control, 1.6 ± 0.2 s; infected, 0.7 ± 0.1 s, Kolmogorov–Smirnov, p < 0.01; Fig. 6B), indicating primarily an increase of synapses with AMPARs (Stein et al., 2003). When we compared the average amplitudes of the PSD-95-to-PSD-93α replacement and control neurons, we measured a statistically significant increase (Wilcoxon Mann–Whitney, p < 0.05), consistent with the trend observed with the amplitude distribution comparison. Notably, the lentivirus-mediated PSD-95-to-PSD-93α replacement results in higher levels of PSD-95 than that in noninjected cells, resulting in the increased responses in evoked transmission (Schlüter et al., 2006).

PSD-95-to-PSD-93β replacement also rescued AMPAR EPSCs to the control cell level (n = 14; control, −50.5 ± 3.9 pA; infected, −60.1 ± 6.6 pA, p = 0.15; Fig. 5F,H). Similar to that of PSD-95α, PSD-93β-rescued neurons showed an increase in mEPSC frequency, but not amplitude (amplitude: control, −19.6 ± 0.5 pA, n = 15; infected, −21.7 ± 1.1 pA, n = 14, Kolmogorov–Smirnov, p = 0.13; Δt: control, 2.0 ± 0.2 s; infected, 1.6 ± 0.3 s, Kolmogorov–Smirnov, p < 0.05; Fig. 6D). These results together suggested that, similar to PSD-95α, PSD-93β primarily increased the number of synapses with AMPARs, but not as efficiently, because the evoked responses of the transduced neurons did not increase above control cell level.

Finally, PSD-95-to-PSD-93ε replacement increased the amplitude of AMPAR EPSCs by approximately twofold, similar to the overexpression in naive neurons (with normal level of endogenous PSD-95) (n = 13, control, −43.9 ± 4.8 pA, infected, −78.5 ± 8.2 pA, p < 0.01; Fig. 5G,H). Furthermore, PSD-93ε increased both the frequency and amplitude of mEPSCs (amplitude: control, −18.5 ± 0.4 pA, n = 15; infected, −20.9 ± 0.5 pA, n = 13, Kolmogorov–Smirnov, p < 0.05; Δt: control, 1.9 ± 0.3 s; infected, 1.1 ± 0.2 s, Kolmogorov–Smirnov, p < 0.05; Fig. 6E). This effect of PSD-93ε on mEPSCs differed from PSD-95-to-PSD-93β replacement. These results together suggested that PSD-93ε enhanced AMPARs similar to PSD-95ε despite its different N-terminal structure, lacking the palmitoylation signal and multimerization domain.

**Excitatory synaptic activity modulates the effect of PSD-93β and δ on AMPARs**

The β-isoforms of PSD-95 and SAP97 function to enhance AMPAR EPSCs in an activity-dependent manner (Schlüter et al., 2006). To increase the excitatory drive on neurons, inhibitory GABAergic transmission was blocked by adding the GABA-A receptor antagonist bicuculline (BIC, 20 μM) to the culture me-

---

**Figure 5.** Difference in efficiency of PSD-93 isoforms to rescue AMPAR EPSCs after PSD-95 knockdown. A–G, Amplitude of AMPAR EPSCs (A, top) and NMDAR EPSCs (A, bottom) of neurons expressing (A) shRNA against PSD-95, and (B–G) eGFP-tagged PSD-95 replacement constructs of (B) PSD-93α1, (C) PSD-93α2, (D) PSD-93β, (E) PSD-93γ, (F) PSD-93δ, and (G) PSD-93ε were plotted against those of simultaneously recorded uninjected neighboring neurons in hippocampal slice cultures. H, Summary bar graph of AMPAR EPSC ratio of infected versus uninjected control cell Calibration: 100 pA and 100 ms.
predominated in our interface slice culture system, we measured mEPSC frequency and amplitude in chronic BIC-treated cultures. Similar to the roller tube slice cultures, we measured a twofold increase in mEPSC frequency without a change in the mEPSC amplitude (amplitude: control, $-23.3 \pm 1.0$ pA, $n = 17$; + BIC, $-24.9 \pm 1.3$ pA, $n = 19$, Wilcoxon Mann–Whitney, $p = 0.40$; $\Delta t$: control, $0.7 \pm 0.1$ s; + BIC, $0.4 \pm 0.04$ s; Wilcoxon Mann–Whitney, $p < 0.01$; Fig. 7). However, the distribution of the mEPSC amplitude was shifted with BIC treatment, such that smaller mEPSCs were overproportionally increased (amplitude: Kolmogorov–Smirnov, $p < 0.01$; $\Delta t$: Kolmogorov–Smirnov, $p < 0.01$; Fig. 7). Thus, in contrast to dissociated neuronal cultures, chronic BIC treatment in organotypic slice cultures primarily caused an increase in excitatory synaptic transmission either by increasing release probability and/or increasing the number of synapses with AMPARs. Importantly, BIC treatment rescues the sh95-mediated decrease in AMPAR EPSCs selectively without affecting NMDAR EPSCs, a result, which precludes a presynaptic mechanism (Schlüter et al., 2006). Hence, we conclude that BIC primarily increased synaptic strength by increasing the number of synapses with AMPARs.

To test whether excitatory synaptic activity also affects PSD-93-mediated synaptic regulation, we increased the excitatory drive in slice cultures after viral transduction. PSD-95 replacement with PSD-93α1, α2, or γ rescued the PSD-95 knockdown-induced decrease in AMPAR EPSCs to the control cell levels ($\alpha_1$: $n = 9$, control, $-52.3 \pm 7.6$ pA; infected, $-52.6 \pm 9.9$ pA, $p = 1.00$; $\alpha_2$: $n = 10$, control, $-65.9 \pm 13.8$ pA; infected: $-58.9 \pm 7.6$ pA, $p = 0.90$; γ: $n = 10$, control, $-36.2 \pm 7.3$ pA; infected, $-40.5 \pm 5.4$ pA, $p = 0.49$; Fig. 8A,B,E), similar to the culturing condition without BIC (Fig. 5B,C,E). In contrast, incubation with BIC enhanced the effect of PSD-95-to-PSD-93β replacement on AMPARs by fourfold ($n = 9$, control, $-27.2 \pm 2.5$ pA; infected, $-113.9 \pm 16.2$ pA, $p < 0.01$; Fig. 8C). We then tested whether the BIC-mediated enhancement was NMDAR-dependent. AMPAR EPSCs in PSD-95-to-PSD-93β replacement cells were not significantly different compared with control cells when, after BIC treatment, the NMDAR-selective antagonist APV was applied 2 d before analysis ($n = 7$; control, $-57.8 \pm 6.8$ pA; infected, $75.2 \pm 12.2$ pA, $p = 0.22$; Fig. 8D). Thus, similar to PSD-95β and SAP97β, this BIC-mediated enhancement was predominant in our interface slice culture system, we measured mEPSC frequency and amplitude in chronic BIC-treated cultures. Similar to the roller tube slice cultures, we measured a twofold increase in mEPSC frequency without a change in the mEPSC amplitude (amplitude: control, $-23.3 \pm 1.0$ pA, $n = 17$; + BIC, $-24.9 \pm 1.3$ pA, $n = 19$, Wilcoxon Mann–Whitney, $p = 0.40$; $\Delta t$: control, $0.7 \pm 0.1$ s; + BIC, $0.4 \pm 0.04$ s; Wilcoxon Mann–Whitney, $p < 0.01$; Fig. 7). However, the distribution of the mEPSC amplitude was shifted with BIC treatment, such that smaller mEPSCs were overproportionally increased (amplitude: Kolmogorov–Smirnov, $p < 0.01$; $\Delta t$: Kolmogorov–Smirnov, $p < 0.01$; Fig. 7). Thus, in contrast to dissociated neuronal cultures, chronic BIC treatment in organotypic slice cultures primarily caused an increase in excitatory synaptic transmission either by increasing release probability and/or increasing the number of synapses with AMPARs. Importantly, BIC treatment rescues the sh95-mediated decrease in AMPAR EPSCs selectively without affecting NMDAR EPSCs, a result, which precludes a presynaptic mechanism (Schlüter et al., 2006). Hence, we conclude that BIC primarily increased synaptic strength by increasing the number of synapses with AMPARs.

To test whether excitatory synaptic activity also affects PSD-93-mediated synaptic regulation, we increased the excitatory drive in slice cultures after viral transduction. PSD-95 replacement with PSD-93α1, α2, or γ rescued the PSD-95 knockdown-induced decrease in AMPAR EPSCs to the control cell levels ($\alpha_1$: $n = 9$, control, $-52.3 \pm 7.6$ pA; infected, $-52.6 \pm 9.9$ pA, $p = 1.00$; $\alpha_2$: $n = 10$, control, $-65.9 \pm 13.8$ pA; infected: $-58.9 \pm 7.6$ pA, $p = 0.90$; γ: $n = 10$, control, $-36.2 \pm 7.3$ pA; infected, $-40.5 \pm 5.4$ pA, $p = 0.49$; Fig. 8A,B,E), similar to the culturing condition without BIC (Fig. 5B,C,E). In contrast, incubation with BIC enhanced the effect of PSD-95-to-PSD-93β replacement on AMPARs by fourfold ($n = 9$, control, $-27.2 \pm 2.5$ pA; infected, $-113.9 \pm 16.2$ pA, $p < 0.01$; Fig. 8C). We then tested whether the BIC-mediated enhancement was NMDAR-dependent. AMPAR EPSCs in PSD-95-to-PSD-93β replacement cells were not significantly different compared with control cells when, after BIC treatment, the NMDAR-selective antagonist APV was applied 2 d before analysis ($n = 7$; control, $-57.8 \pm 6.8$ pA; infected, $75.2 \pm 12.2$ pA, $p = 0.22$; Fig. 8D). Thus, similar to PSD-95β and SAP97β, this BIC-mediated enhancement was...
NMDAR-dependent and could be fully reversed (H$_{2.24}$ = 13.16, p < 0.001; BIC/APV, 1.3 ± SEM (0.3; 0.2) vs control, p < 0.05; BIC vs BIC/APV, p > 0.05; Figure 8H). PSD-95-to-PSD-93β replacement also induced enhanced upregulation of AMPAR EPSCs upon incubation of BIC (n = 9; control, −44.3 ± 3.7 pA; infected, −93.2 ± 13.1 pA, p < 0.01; Fig. 8F,J). Incubation of BIC had no significant effect on PSD-93ε-mediated upregulation of AMPAR EPSCs with and without BIC, PSD-95-to-PSD-93ε replacement enhanced AMPAR EPSCs by approximately twofold (n = 13; control, −35.4 ± 3.0 pA; infected, −90.0 ± 9.0 pA, p < 0.01; Fig. 8G,J). These results show that, when excitatory synaptic activity was high, PSD-93β was exceedingly more effective in enhancing AMPAR function than other DLG-MAGUK β-isofoms. Additionally, PSD-93δ shared properties of DLG-MAGUK α- and β-isofoms by enhancing AMPAR EPSCs directly through its synaptic levels and additionally through an activity-dependent mechanism.

**Discussion**

DLG-MAGUK scaffolding proteins critically regulate excitatory synaptic transmission. Whereas the synaptic roles of PSD-95, SAP-97, and SAP102 have been characterized, the role of PSD-93, the most complex paralog of DLG-MAGUKs, remains not well understood. The available results related to PSD-93 are often theoretically inconsistent and sometimes even contradicting. To provide a comprehensive understanding of PSD-93 in excitatory synaptic transmission, we examined all six PSD-93 isoforms in the mouse hippocampus and identified their differential functions. We first demonstrated that the PSD-93 gene shared a similar exon–intron structure as the PSD-95 and SAP97 genes (Fig. 1A); separate promoters and exons downstream of the common exon 2 of the α- and β-isofoms coded for the additional N-terminal isoforms γ, δ, and ε. All six PSD-93 isoforms were expressed in the hippocampus but with different expression levels (Figs. 1, 2). We next demonstrated that these N-terminal isoforms of PSD-93 enhanced AMPAR function by different mechanisms: (1) PSD-93β shared functional properties with the two other DLG-MAGUK β-isofoms of PSD-95 and SAP97 (Figs. 4, 5, 8); (2) despite their high sequence homology, the two α-isofoms of PSD-93 did not exhibit similar enhancing effect on AMPARs as mediated by PSD-95α and SAP97α (Figs. 4, 5); (3) PSD-93ε was comparably potent as PSD-95α in enhancing AM-
PAR function, despite its structural difference (Figs. 4, 5, 6); (4) PSD-93δ enhanced AMPAR function when overexpressed, and this enhancement was potentiated by increased excitatory synaptic activity (Figs. 4, 5, 8). These results reveal that different isoforms of PSD-93 expressed in the hippocampus allow for specific and selective modes to regulate synaptic transmission and plasticity and further contributing to the evolutionary broadening of the signaling scaffolds to regulate more complex behaviors (Nithianantharajah et al., 2013).

Abundance of PSD-93 isoforms in the hippocampus

Using a combination of RT-PCR, Western blotting, and MS, we determined the abundance of all six PSD-93 N-terminal isoforms in the mouse hippocampus. The N-terminal domain of PSD-93 derived from different transcription start sites. Our results show that the transcripts of all six N-terminal isoforms were expressed in the hippocampus. With the combination of isoform group-specific antibodies, the molecular size, and the fingerprint in MS, we identified unambiguously the expression of PSD-93α, δ, and γ in the hippocampus. In addition, both transcripts of the two α-isoforms are expressed. On the protein level, however, results are not unambiguous. The corresponding band amounted to 50% of the total PSD-93 protein; thus, the α-isoforms are the most abundant of the PSD-93 isoforms (Fig. 2). The presence of PSD-93δ could be derived from the pattern of Western blot bands, although intrinsic uncertainty may be involved in this pattern-based analysis (Fig. 2). The abundance of the other isoforms was estimated to range between 5% and 20% based on quantitative Western blots. The relative abundance of these isoforms appeared to differ between regions of the CNS (e.g., the relative abundance of the δ-isoform is low in hippocampus but high in spinal cord) (Leyland and Dart, 2004). These differences in relative abundance may contribute to the functional diversity of synapses in different brain regions and potentially during different developmental stages.

Functional differences of the PSD-93 isoforms

To test for functional differences among the PSD-93 N-terminal isoforms, we overexpressed them in organotypic hippocampal slice cultures and measured their gain of function on AMPARs. Both PSD-93δ and γ enhanced AMPAR function. This result adds new isoforms in the DLG-MAGUK subfamily that may upregulate AMPARs during synaptic plasticity. Importantly, the mechanisms underlying these isoform-mediated AMPAR enhancements are different (Figs. 4, 5, 6, 7, 8), and these differential mechanisms allow for specialized modes of synaptic potentiation under different conditions or different brain regions. Specifically, PSD-93δ-mediated enhancement of AMPARs exhibited similar magnitude when overexpressed in either the presence or absence of endogenous PSD-95 and thus was independent of endogenous PSD-95 (Figs. 4, 5). This enhancement is mediated both by increased numbers of synapses with AMPARs and increase in AMPAR potency, measured as changes in mEPSC frequency and amplitude, respectively (Fig. 6). On the other hand, expression of PSD-95α or SAP97α also enhances synaptic responses (Schlüter et al., 2006), but at least for PSD-95α the enhancement is primarily achieved by delivering new AMPARs to silent synapses (Fig. 6) (Stein et al., 2003). It is also worth noting that the increase in mEPSC frequency in PSD-93-expressed cells is not likely mediated by presynaptic changes because all our manipulations were specific for the postsynaptic site. Rather, as shown previously, upregulating PSD-95 increases the frequency of mEPSC, a process that is mediated by increasing the number of synapses with AMPARs, potentially via unsilencing of silent synapses (Stein et al., 2003; Elias et al., 2006). When the molecular manipulations last longer, changes in presynaptic release probability are induced by PSD-95 overexpression (Futai et al., 2007). Thus, the increase in mEPSC frequency may partly be mediated by presynaptic mechanisms. Notably, PSD-95α-mediated enhancement requires N-terminal multimerization, whereas PSD-93δ lacks the N-terminal multimerization motif (Parker et al., 2004; Xu et al., 2008). Together, these results suggest a unique role of PSD-93δ, as a monomer, in enhancing AMPAR function.

PSD-93δ and γ did not enhance AMPAR function when overexpressed in neurons with normal expression of PSD-95, but they rescued AMPAR function to the control cell levels when endogenous PSD-95 was knocked down. This function is similar to PSD-95β and SAP97β (Schlüter et al., 2006). In contrast to the γ-isoform of PSD-93, expression of the β-isoforms enhanced AMPAR function-dependent on excitatory synaptic activity. This function of PSD-93β, although often masked by endogenous PSD-95, may play critical roles under certain conditions. For example, in the developing sensory cortex, immature synapses lack PSD-95 (Yoshii et al., 2003). After eye opening and the start of sensory input, PSD-95 is recruited to these synapses. Thus, there is a time window during which the β-isoforms actively regulate AMPARs. Given that PSD-95-lacking neurons often possess a high level of silent synapses (Béïque et al., 2006), by delivering/stabilizing AMPARs, these β-isoforms may also function to increase the number of synapses with AMPARs, thus fully activating certain neural circuits (Fig. 8).

PSD-93δ-mediated AMPAR enhancement required endogenous PSD-95; when PSD-95 was absent, expression of PSD-93δ rescued AMPAR-mediated synaptic transmission only to the control cell levels. The AMPAR enhancement mediated by PSD-93δ was achieved primarily by increasing the number of synapses with AMPARs (Fig. 6). For PSD-93δ, the enhancement was dependent on excitatory synaptic activity in the slice culture, similar to the β-isoforms (Fig. 8). Thus, the primary physiological function of PSD-93δ and δ is to regulate synaptic transmission in an activity-dependent manner. This notion is partially supported by the observation that in animals in which all PSD-93 isoforms are knocked out, LTP induction is reduced in hippocampal slices (Carlisle et al., 2008). Given that expression of PSD-93α1 and α2 did not change AMPAR function (Fig. 4), the reduced LTP may be attributable to other isoforms, including β and δ.

Overexpression of PSD-93α1 and α2 did not enhance AMPAR function. This finding was unexpected, as the α-isoforms of the DLG-MAGUKs share a high degree of sequence identity and expression of other DLG-MAGUKs with similar N terminus does enhance AMPAR function (Schnell et al., 2002; Béïque and Andrade, 2003; Ehrlich and Malinow, 2004; Nakagawa et al., 2004; Schlüter et al., 2006). Upon the PSD-95-to-PSD-93a2 replacement, the number of synapses with AMPARs (unsilent synapses) was significantly reduced as a consequence of the PSD-95 knockdown (Fig. 6). It remains to be addressed how PSD-93a2 in this replacement experiment could still rescue AMPAR EPSCs to control cell levels. Nonetheless, the observed minimal effect of the PSD-93a2 on AMPARs is consistent with our other findings. For example, when all PSD-93 isoforms were knocked down, we did not detect any changes in AMPAR function in young synapses (slice culture and acute slice up to P30), whereas knockdown of PSD-95 resulted in a 50% reduction of AMPAR EPSCs (Figs. 3, 5) (Nakagawa et al., 2004; Elias et al., 2006; Schlüter et al., 2006). Given the lower abundance of all PSD-93 isoforms compared with PSD-95 and particularly the AMPAR-regulating isoforms...
(e.g., PSD-93δ and ε), a minimal effect on AMPARs was expected upon PSD-93 knockdown (Peng et al., 2004; Cheng et al., 2006). Accordingly, there is no change in AMPAR-mediated synaptic transmission in young PSD-93 KO mice (Elias et al., 2006; Carlisle et al., 2008). In contrast, upon PSD-95 KO or knockdown, AMPAR-mediated synaptic transmission is reduced (Fig. 5) (Nakagawa et al., 2004; Bégué et al., 2006; Elias et al., 2006; Schlüter et al., 2006; Carlisle et al., 2008). Although these results consistently suggest a lack of basal regulation of AMPARs in the young hippocampus by PSD-93 as discussed above, discrepant results are observed. It has been shown in rat hippocampal slice cultures that overexpression of PSD-93α1 enhances AMPAR function, and knockdown of PSD-93 reduces AMPAR function by ~50% (Elias et al., 2006), suggesting that PSD-93α1 may become more “active” under certain experimental conditions. Our results in P60–P74 PSD-93 KO mice show that the AMPA/NMDAR ratio was decreased compared with that of control mice (Fig. 3). Thus, at different developmental synaptic states, PSD-93 may become essential to maintain the synaptic strength.

In conclusion, our results demonstrate differential effects of different PSD-93 isoforms on AMPAR function. These results provide a comprehensive understanding of PSD-93 in regulating synaptic transmission, differentiate the functional difference among DLG-MAGUKs, such as PSD-93 versus PSD95, and help establish a conceptual basis for exploring the roles of other DLG-MAGUKs in regulating excitatory synaptic transmission and plasticity.

References


