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Alternative N-Terminal Domains of PSD-95 and SAP97 Govern Activity-Dependent Regulation of Synaptic AMPA Receptor Function

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Summary

PSD-95 and SAP97 are scaffolding proteins that have been implicated in regulating AMPA receptor incorporation and function at synapses. Gain- and loss-of-function approaches, however, have generated conflicting results. To minimize adaptations during development and potential dominant-negative effects of overexpression, we have combined silencing of endogenous PSD-95 in mature neurons with heterologous expression of specific SAP97 or PSD-95 isoforms. We find that both PSD-95 and SAP97 contain alternative N termini expressing either double cysteines that normally are palmitoylated (α-isoforms) or an L27 domain (β-isoforms). Whereas α-isoforms of PSD-95 and SAP97 influence AMPA receptor-mediated synaptic strength independent of activity, the effects of β-isoforms are regulated by activity in a CaMKII-dependent manner. Importantly, the synaptic effects of the β-isoforms are masked by the endogenous α-isoform of PSD-95. These results demonstrate that the different N termini of the predominant endogenous forms of PSD-95 (α-isoform) and SAP97 (β-isoform) govern their role in regulating synaptic function.

Introduction

Changes in strength at glutamatergic synapses are considered to be fundamentally important for information processing and storage in the brain. One important mechanism for the regulation of synaptic strength is the synaptic incorporation and retrieval of AMPA receptors (AMPARs), the principal receptors for fast excitatory neurotransmission in the mammalian central nervous system (Bredt and Nicoll, 2003; Malinow and Malenka, 2002; Song and Huganir, 2002). Recent evidence suggests a key role for postsynaptic scaffolding proteins from the disc-large (DLG) subfamily of membrane-associated guanylate kinases (MAGUKs) in regulating synaptic AMPAR trafficking (Beique and Andrade, 2003; Ehrlich and Malinow, 2004; El-Husseini et al., 2000; Funke et al., 2005; Kim and Sheng, 2004; Montgomery et al., 2004; Nakagawa et al., 2004; Rumbaugh et al., 2003; Scannevin and Huganir, 2000; Schnell et al., 2002; Stein et al., 2003). Family members include PSD-95/SAP90, PSD-93/Chapsyn110, SAP97, and SAP102, with the most extensively studied family members being PSD-95 and SAP97. While overexpression of PSD-95 in dissociated cultured neurons or hippocampal slice cultures consistently enhances AMPAR-mediated excitatory postsynaptic currents (AMPAR EPSCs), but not NMDA receptor-mediated EPSCs (NMDAR EPSCs) (Beique and Andrade, 2003; Ehrlich and Malinow, 2004; Nakagawa et al., 2004; Schnell et al., 2002), the synaptic consequences of overexpressing SAP97 have been inconsistent. An enhancement of the frequency of AMPAR miniature EPSCs in dissociated neuronal cultures and the amplitude of AMPAR EPSCs in hippocampal slice cultures has been reported by some investigators (Nakagawa et al., 2004; Rumbaugh et al., 2003) while others observed no effect in this latter preparation (Ehrlich and Malinow, 2004; Schnell et al., 2002).

Genetic deletions or loss-of-function mutants of DLG-MAGUK family members, on the other hand, consistently have been found to have minimal effects on basal synaptic transmission. Specifically, genetic knock out of PSD-95 at synapses was reported to have no effect on basal synaptic transmission in the CA1 region of the hippocampus, although long-term depression was absent (Migaud et al., 1998). Similarly, knock out of PSD-93, the only family member normally present in cerebellar Purkinje cells, had no detectable effect on synaptic structure or function in the cerebellum (McGee et al., 2001). Although loss-of-function mutants of SAP97 and Dlg, its *Drosophila* ortholog, are lethal due to major defects in cell-cell contact formation during embryogenesis (Caruana and Bernstein, 2001; Lahey et al., 1994; Woods and Bryant, 1991), neurons cultured from the SAP97 mutant embryos develop normally and form synapses with normal levels of AMPARs and no detectable abnormalities (Klocker et al., 2002). These findings raise the possibility that DLG-MAGUK family members share redundant functions in neurons and loss of one can be partially compensated by other family members. Alternatively, other adaptive processes during synaptogenesis and synapse maturation might compensate for the loss of the normal function of DLG-MAGUKs in basal synaptic transmission.

The detailed structures of the various DLG-MAGUKs provide clues as to whether family members are redundant or have functionally distinct roles. All share a common modular structure of five protein-protein interaction domains; specifically, three N-terminal PDZ domains are followed by an SH3 and an enzymatically inactive GK domain (Funke et al., 2005; Kim and Sheng, 2004; Montgomery et al., 2004; Scannevin and Huganir, 2000). However, SAP97 and PSD-95, the most extensively studied family members, have two important differences. First, they interact with AMPAR subunits via distinct mechanisms: SAP97 appears to directly bind to the AMPAR subunit GluR1 (Cai et al., 2002; Leonard et al., 1998) while PSD-95 requires stargazin to interact with AMPARs (Fukata et al., 2005). Second, their N-terminal domains can differ (Cho et al., 1992; Muller et al., 1995). The predominant isoform of PSD-95 has an N-terminal domain, which is palmitoylated and also capable of multimerizing PSD-95 with itself and other MAGUKs with a palmitoylated N terminus, such as PSD-93 (Hsueh

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et al., 1997; Kim et al., 1996). The palmitoylated N terminus of PSD-95 appears to be particularly important for its synaptic targeting (Craven et al., 1999). In contrast, SAP97 contains a longer N-terminal L27 domain, capable of heteromultimerization and homomultimerization with itself and other L27 domain-containing proteins, such as CASK (Lee et al., 2002). Although synaptic targeting of SAP97 is thought to be dependent on the presence of the alternatively spliced exon I3, which is localized in the SH3-GK linker (Rumbaugh et al., 2003), the multimerization of SAP97 via its L27 domain has recently been suggested to also be important for mediating its synaptic effects (Nakagawa et al., 2004). Interestingly, a small proportion of PSD-95 in the brain contains an N-terminal L27 domain (Chetkovich et al., 2002), but the functions of this isoform have not been examined.

To further examine and clarify the roles of PSD-95 and SAP97 in the regulation of basal excitatory synaptic transmission and provide explanations for some of these apparently conflicting results, we have taken a different approach to study the molecular basis of synaptic function. Specifically, we have used a cell-restricted, molecular replacement strategy, in which expression of endogenous PSD-95 is acutely reduced via RNAi and replaced by specific isoforms of the DLG-MAGUK family. Our results suggest that the specific roles of PSD-95 and SAP97 in synaptic function are governed by differences in their N-terminal domains, which impart activity dependence or independence on their function.

Results

SAP97, PSD-95, and PSD-93 Have Similar Alternative N-Terminal Domains

Both PSD-95 and PSD-93 are expressed in brain with one of two alternative N-terminal domains (Chetkovich et al., 2002; Parker et al., 2004) containing either an N-terminal cysteine doublet that is palmitoylated (Topinka and Bredt, 1998) or an N-terminal L27 domain, similar to SAP97. To determine whether such alternative N termini are a feature common to all DLG-MAGUK family members, we first searched the National Center for Biotechnology Information (NCBI) expressed sequence tag (EST) database. This resulted in the identification of one mouse EST clone (BY131151) that originates from the SAP97 gene and codes for N-terminal double cysteines identical to the α-isoform of PSD-95. We next analyzed the rat gene structures of all four DLG-MAGUKs, PSD-95, PSD-93, SAP97, and SAP102, in more detail to reveal common elements of the gene structures (Figure 1A). For their N-terminal regions, SAP97 and PSD-95 share a common gene structure that contains three β-exons that code for an L27 domain and one α-exon that codes for the cysteine doublet (Figure 1A). Either the β-exons or the α-exon are spliced into the common exon 2. PSD-93 shares a common gene structure with PSD-95 and SAP97 but also has an additional α-exon spliced into exon 2 and three unique alternative N termini coding exons, which are spliced into exon 3 (Figure 1A) (Parker et al., 2004). On the other hand, compared to other DLG-MAGUK family members, the N terminus coding region of SAP102 is unique in that it lacks any of the α- or β-exons that are spliced into exon 2 (Figure 1A).

To determine whether SAP97 is indeed transcribed in rat brain with two alternative 5′ sequences, we performed RT-PCR analysis. This confirmed the presence of different species of SAP97 mRNA, one coding for a potentially palmitoylated cysteine pair (SAP97α) and the other coding for an L27 domain (SAP97β) (Figure 1B). Thus both PSD-95 and SAP97, the two most extensively studied DLG-MAGUKs, are present in brain in two isoforms; the α-isoforms contain an N-terminal cysteine doublet that is normally palmitoylated, and the β-isoforms contain an L27 domain. To date, virtually all studies on these proteins have focused only on either the prototypic PSD-95α or the prototypic SAP97β. We were therefore interested in how these domains might underlie differences in the synaptic function of these molecules.

PSD-95 is the Principal MAGUK in the Hippocampus

Although PSD-95, SAP97, and PSD-93 are known to be expressed in the hippocampus (Sans et al., 2000), before examining potential functional differences between the α- and β-isoforms of PSD-95 and SAP97, it was important to have some knowledge about their relative abundance. We first expressed GFP-tagged PSD-95α, PSD-93α, and SAP97β in HEK293 cells, and using an anti-GFP antibody, their expression levels were adjusted to provide reference points for the quantification of different DLG-MAGUKs in crude synaptosomal fractions of juvenile hippocampus (Figure 1C, middle panel). An antibody that recognizes both α- and β-isoforms of DLG-MAGUKs detected prominent bands at 95 kDa and 120 kDa, the former representing PSD-95 and the latter, according to its size, SAP97β (Figure 1C, left panel). To determine whether DLG-MAGUK family members other than PSD-95 might contribute to the 95 kDa band, we examined extracts from PSD-95 knockout mice and found no DLG-MAGUK close to this molecular weight (Figure 1C, right panel). Thus, PSD-95 and SAP97β appear to be the only DLG-MAGUKs with either a palmitoylated N terminus or an L27 domain detectable in the hippocampus by Western blotting. Although this analysis did not allow independent measurement of the α- and β-isoforms of PSD-95, previous work has found that the majority of brain PSD-95 is the α-isoform, with only ~10% of brain PSD-95 being the β-isoform with an N-terminal L27 domain (Chetkovich et al., 2002). Assuming that the single band at 95 kDa represents PSD-95α, this indicates that PSD-95α was far more abundant than SAP97β, the levels of which were ~16% of PSD-95α levels in the hippocampus.

RNAi-Mediated Acute Reduction in PSD-95 Decreases Synaptic Strength

There are striking inconsistencies between the synaptic effects of overexpressing PSD-95, which greatly enhances basal synaptic transmission (Beique and Andrade, 2003; Ehrlich and Malinow, 2004; Nakagawa et al., 2004; Schnell et al., 2002), versus genetically deleting it, which has minimal effects on synaptic strength (Migaud et al., 1998). To examine the synaptic function of the different isoforms of PSD-95 and SAP97, we therefore reasoned it would be advantageous to express them in cells in which endogenous PSD-95 was acutely reduced. This approach minimizes compensatory adaptations due to the lack of PSD-95 during synaptogenesis

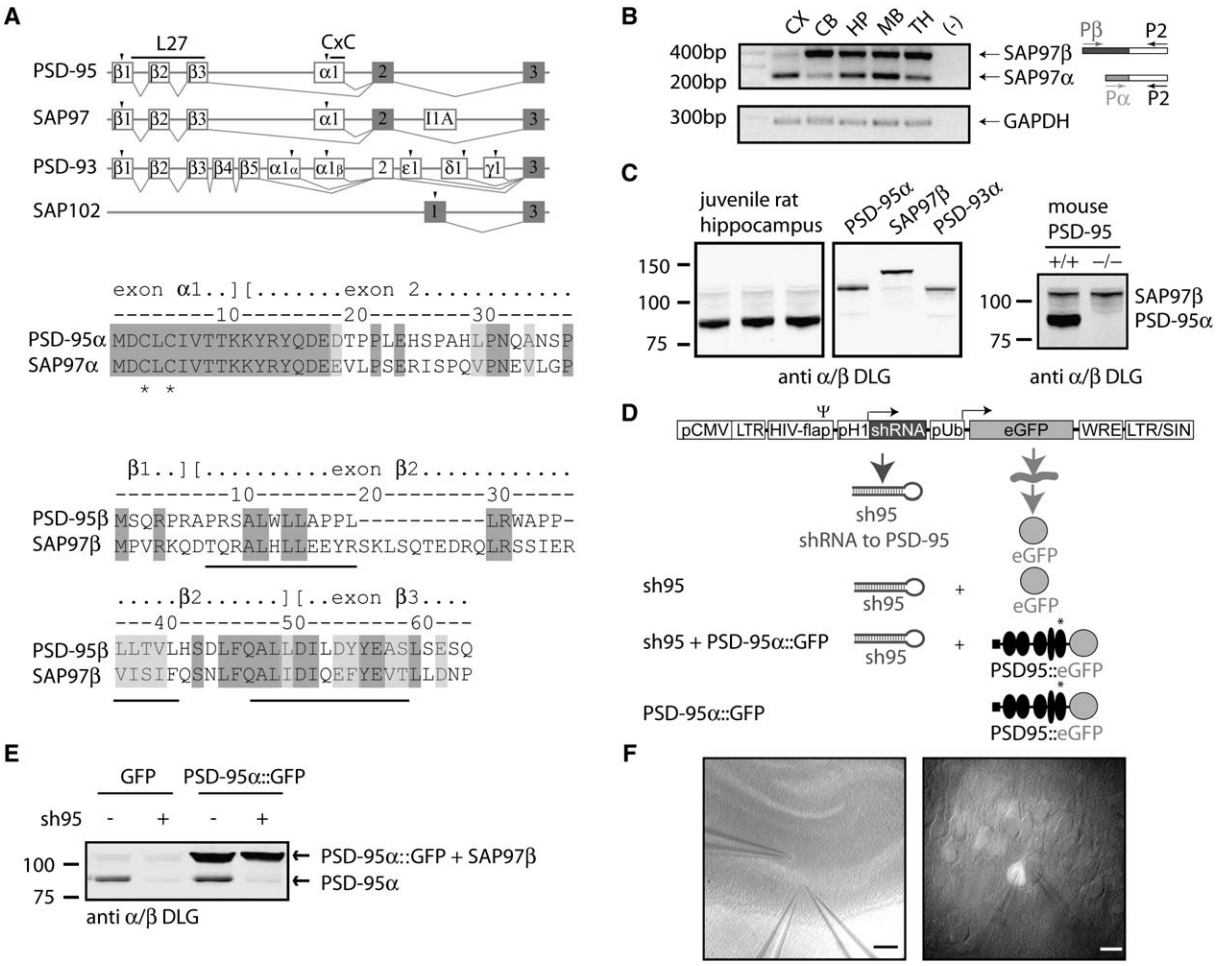

Figure 1. Analysis of Endogenous DLG-MAGUKs and Lentivirus Preparation

(A) (Top panel) Comparison of genomic structures of the N termini of DLG-MAGUKs. Gray boxes represent constitutive exons; open boxes, alternatively spliced exons. (Bottom panels) sequence alignment of predicted N-terminal amino acid sequences of α- and β- isoforms of PSD-95 and SAP97 and indicated intron-exon boundaries. Gray areas highlights identical amino acid residues; light gray highlights similar residues.

(B) SAP97α was transcribed in different brain regions as detected by RT-PCR (CX, cerebral cortex; CB, cerebellum; HP, hippocampus; MB, midbrain; TH, thalamus; [-], negative control). One common downstream primer P2 and two upstream primers Pα and Pβ, specific to SAP97α and SAP97β, respectively, were used in the PCR reaction. GAPDH specific primers were used as a control for total RNA level.

(C) Quantitative western blots probed with anti-α/β-DLG antibody show that PSD-95α is the predominant brain DLG-MAGUK. (Left panel) Samples from three rat hippocampus P2 fractions. (Middle panel) Homogenates from HEK293 cells expressing GFP-tagged forms of indicated proteins. (Right panel) Hippocampus homogenates from wild-type and PSD-95 knockout mice.

(D) Diagram of lentivirus vectors for shRNA, molecular replacement, and overexpression (LTR, long terminal repeat; ψ, packaging signal; Flap, flap element from HIV-1; pH1, H1 promoter; pUb, ubiquitin promoter; WRE, woodchuck hepatitis virus posttranscriptional regulatory element). Below are shown the products of the various cDNAs (the [*] above PSD95::eGFP indicates silent mutations).

(E) Western blot of cortical neuron cultures infected with the indicated lentiviruses shows effective knockdown of endogenous PSD-95 via sh95 and successful replacement with PSD-95::GFP (blotted with anti-α/β-DLG antibody).

(F) (Left panel) Image of the recording setup from hippocampal slice culture at low magnification. The double-barreled stimulation electrode was placed ~150 μm away from the CA1 cell body layer in stratum radiatum (scale bar, 100 μm). (Right panel) Overlay of DIC image and GFP fluorescence at high magnification showing the GFP-infected cell and the uninfected neighboring cell, from which simultaneous recordings were made (scale bar, 15 μm).

and synapse maturation and, in addition, opens the possibility of analyzing the function of heterologously expressed constructs without the necessity of a dominant effect as required by a standard overexpression approach. To accomplish this task, we constructed a dual promoter lentiviral vector in which the human H1 promoter drove expression of a shRNA to PSD-95, while an ubiquitin promoter simultaneously drove expression of GFP or a GFP-fusion protein (Figure 1D). The shRNA to PSD-95 (sh95) was highly effective in silencing heterologous PSD-95 expression in HEK293 cells (data not shown) as well as endogenous PSD-95 when introduced into dissociated cortical cultures using lentivirus-mediated gene transfer (Figure 1E). Indeed, Western blot analysis showed that endogenous PSD-95 expression could be silenced efficiently when examined over a week after infection (Figure 1E). Furthermore, immunocytochemical examination of endogenous PSD-95 in cells expressing sh95 revealed no detectable puncta (data not shown).

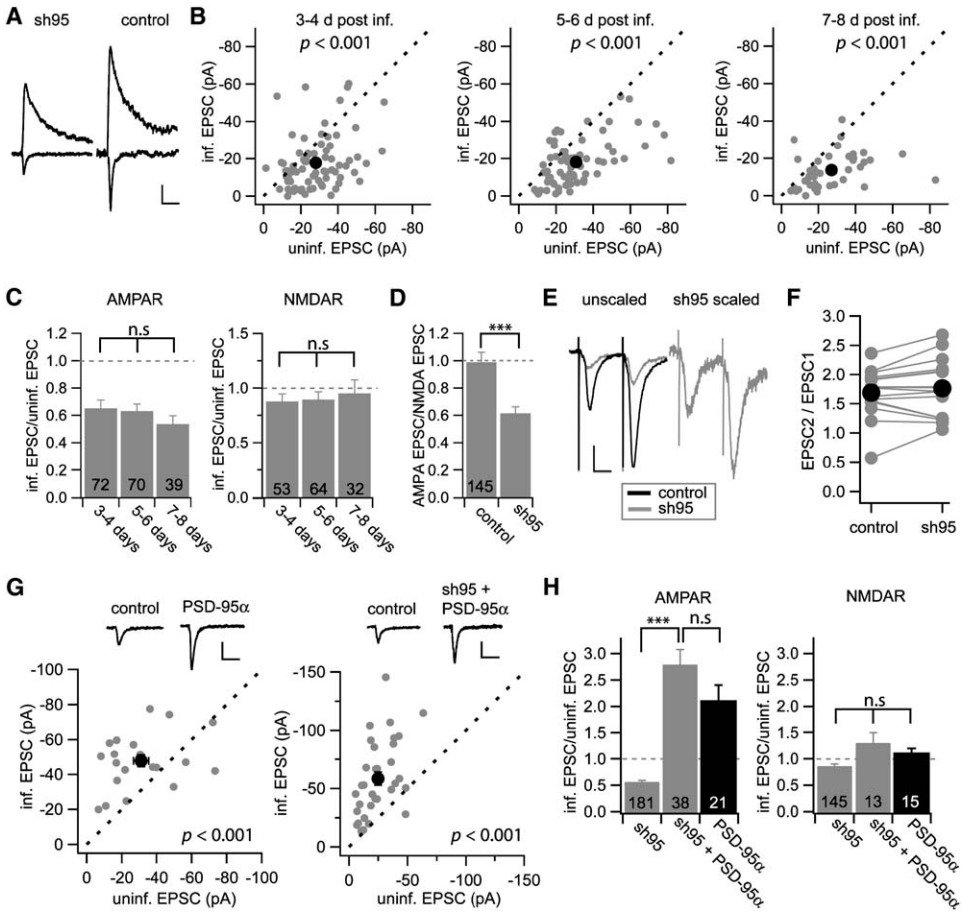

Figure 2. Acute Knockdown of PSD-95 Depresses AMPAR EPSCs and Its Replacement by PSD-95α Enhances AMPAR EPSCs

(A) Sample traces simultaneously recorded from sh95-expressing and control cells (scale bars, 50 ms, 10 pA).

(B) Amplitude of AMPAR EPSCs of neurons expressing sh95 for various durations are plotted against those of simultaneously recorded uninfected neighboring neurons. (Gray symbols represent single pairs of recordings; black symbols show mean ± SEM; p values were calculated with a paired Student's t test comparing absolute values of paired recordings).

(C) Summary (mean ± SEM) of effects of sh95 on AMPAR- and NMDAR EPSCs calculated as the averaged ratios obtained from pairs of infected and uninfected neighboring neurons (numbers of pairs analyzed are indicated in the bar; n.s. indicates $p > 0.05$ using an ANOVA Tukey HSD t test).

(D) Summary of the normalized ratios of AMPAR EPSCs to NMDAR EPSCs in sh95-expressing and uninfected neighboring cells (***$p < 0.001$, paired t test).

(E) Sample traces showing EPSCs in response to paired-pulse stimulation (scale bars, 20 pA, 50 ms).

(F) Paired-pulse ratios of AMPAR EPSCs for pairs of recordings (black symbol shows mean ± SEM, which was less than diameter of symbol).

(G) Amplitudes of AMPAR EPSCs of neurons expressing PSD-95α alone (left panel) or sh95 and PSD-95α (right panel) versus control neurons. Sample traces are shown above. Scale bars, 20 pA, 50ms.

(H) Summary of effects on AMPAR EPSCs (left panel) and NMDAR EPSCs (right panel) of expressing sh95 alone, PSD-95α alone, and both together (n.s. $p > 0.05$, ***$p < 0.001$).

To allow for expression of recombinant PSD-95 in the same cells expressing sh95, silent mutations were introduced into the sh95-targeted region of the GFP-tagged PSD-95 cDNA (Figure 1D). In cortical cultures infected with this lentivirus, endogenous PSD-95 is efficiently silenced, whereas the recombinant GFP-tagged protein is expressed at levels comparable to cultures infected with a lentivirus lacking the shRNA expression cassette (Figure 1E). These results demonstrate that endogenous PSD-95 in neurons can be efficiently knocked down and replaced by a recombinant protein using this bicistronic lentiviral expression system.

To investigate the acute effect of knocking down endogenous PSD-95 on AMPAR EPSCs, we injected concentrated sh95-expressing lentivirus into the CA1 cell body layer of hippocampal slice cultures the day after plating. GFP expression in individual cells could be detected as early as two days after infection. This allowed us to make simultaneous whole-cell voltage-clamp recordings from an infected and neighboring uninfected cell while stimulating the same Schaffer collateral axons (Figure 1F). Consistent with a previous study (Nakagawa et al., 2004), AMPAR EPSCs were reduced on average by 35%–45% in cells expressing sh95 (n = 181 pairs) (Figures 2A–2C). The magnitude of the reduction in AMPAR EPSCs was examined at three different time points from 3 to 8 days after infection and was not significantly different (Figures 2B and 2C), demonstrating that the synaptic effect of silencing PSD-95 is constant over this time period. In contrast to the clear effects on

AMPAR EPSCs, NMDAR EPSCs were not significantly affected by acute reduction of PSD-95 (n = 149 pairs) (Figure 2C). Consistent with these results, the ratio of AMPAR EPSCs to NMDAR EPSCs was significantly reduced in cells expressing sh95 (n = 145 pairs) (Figure 2D). The lack of effect of sh95 on NMDAR EPSCs is important not only because it supports a specific role for PSD-95 in AMPAR-mediated synaptic transmission, but also because it rules out the possibility that the lentivirus-mediated expression of this shRNA had some nonspecific deleterious effect on cell or synapse health. As an additional control, an ineffective shRNA to PSD-95 was expressed and had no detectable effect on either AMPAR or NMDAR EPSCs (n = 15 pairs, $p > 0.05$).

Overexpression of PSD-95 during synaptogenesis modulates presynaptic maturation (El-Husseini et al., 2000), and the paired-pulse ratio (PPR) of AMPAR-mediated synaptic responses, a measure of presynaptic function, was altered in PSD-95 knockout mice (Migaud et al., 1998). It was therefore of interest to examine the PPR in cells expressing sh95. In contrast to these previous results, however, acute knockdown of PSD-95 had no detectable effect on the PPR (n = 16 pairs) (Figures 2E and 2F). Thus there are at least two differences in the synaptic consequences of acutely reducing PSD-95 with shRNA versus genetically deleting it: the acute knockdown of PSD-95 depresses AMPAR EPSCs but has no detectable effect on presynaptic function, while knocking out PSD-95 leaves basal synaptic transmission intact but leads to changes in presynaptic function (Migaud et al., 1998).

We next examined the synaptic effects of overexpression of PSD-95α using lentiviral-mediated gene transfer with and without simultaneous expression of sh95. Under these two conditions, expression of PSD-95α enhanced AMPAR EPSCs to approximately the same degree (n = 21 and 38 pairs, respectively) (Figures 2G and 2H). The magnitude of this enhancement (2- to 2.5-fold) was less than previously reported using gene gun-mediated gene transfer (3.5- to 8-fold; Nakagawa et al., 2004; Schnell et al., 2002), presumably because the levels of recombinant PSD-95α are lower with lentivirus, which causes random integration of few copies of the cDNA into genomic DNA (Lois et al., 2002). The "rescue" of the sh95-mediated synaptic depression by PSD-95α suggests that this isoform alone is responsible for mediating the increase in synaptic strength, as the sh95 silences both α- and β-isoforms of PSD-95. We also examined NMDAR EPSCs in cells expressing recombinant PSD-95α, and consistent with previous results (Beique and Andrade, 2003; Ehrlich and Malinow, 2004; Nakagawa et al., 2004; Schnell et al., 2002), they were not significantly affected (Figure 2H).

SAP97β Rescues the Decrease of AMPAR EPSCs Due to PSD-95 Knockdown

The prototypic SAP97 is the β-isoform (SAP97β), which contains a L27 domain in its N-terminal region (Lee et al., 2002; Muller et al., 1995). In previous work using hippocampal slice cultures, overexpression of SAP97β produced either no effect on AMPAR EPSCs (Ehrlich and Malinow, 2004; Schnell et al., 2002) or an increase, which was much less than that observed with overexpression of PSD-95α (Nakagawa et al., 2004). Consistent with these findings, overexpression of SAP97β containing the I3 insert, which is required for synaptic targeting in dissociated neuronal cultures (Rumbaugh et al., 2003), had no significant effect on either AMPAR EPSCs (n = 19 pairs) or NMDAR EPSCs (n = 15 pairs) (Figures 3A and 3C). We also examined two additional SAP97β isoforms containing different inserts in the SH3-GK linker region (I2 or I2I5I4 containing) (McLaughlin et al., 2002; Wu et al., 2000), and these also had no detectable effect on AMPAR EPSCs (data not shown).

Surprisingly, however, when SAP97β was expressed with sh95 such that endogenous PSD-95 levels were severely reduced, it had a very clear effect; AMPAR EPSCs were no longer depressed by the sh95 (n = 35 pairs) (Figures 3B and 3C). NMDAR EPSCs, on the other hand, were unaffected (n = 30 pairs) (Figures 3B and 3C). Thus there was a dramatic difference in the effects of recombinant SAP97β on AMPAR EPSCs depending on whether normal or reduced levels of PSD-95 were present. These results suggest that endogenous PSD-95 has a dominant effect over SAP97β such that the functional role of SAP97β may best be revealed on a background of greatly reduced PSD-95. Furthermore, they demonstrate that the lack of effect of SAP97β when overexpressed in this and previous work (Ehrlich and Malinow, 2004; Schnell et al., 2002) is not due to some interference in its function by the GFP tag (see Nakagawa et al., 2004). Instead, whether or not synaptic effects of SAP97β are observed may depend on the levels of PSD-95 present at individual synapses and the magnitude of SAP97β overexpression.

The N-Terminal Domains of SAP97 and PSD-95 Govern Their Effects on AMPAR EPSCs

Our results thus far demonstrate that SAP97β and PSD-95α have different effects on AMPAR-mediated synaptic transmission. To determine whether these differences are due to their distinct N-terminal domains, we examined the effects of overexpressing the α-isoform of SAP97, which contains a cysteine doublet similar to that in PSD-95α rather than an L27 domain, and also the effects of replacing endogenous PSD-95 with SAP97α. Similar to PSD-95α, SAP97α dramatically increased AMPAR EPSCs 2- to 3-fold when either overexpressed or replacing endogenous PSD-95 (n = 26 and 18 pairs, respectively) (Figure 3D). We next examined the effects of replacing endogenous PSD-95 with PSD-95β, which contains an L27 domain. Like SAP97β, this rescued the AMPAR EPSCs only back to control cell levels (n = 23 pairs), in contrast to the 2- to 3-fold increase caused by PSD-95α (Figure 3E). Similar to all previous experiments, NMDAR EPSCs were not significantly affected by either SAP97α or PSD-95β replacement constructs (data not shown).

These results demonstrate that the N-terminal domains of SAP97 and PSD-95 are responsible for their different effects on AMPAR EPSCs. Whereas the double cysteine-containing α-isoforms have a much greater effect on AMPAR-mediated synaptic transmission, the L27 domain-containing β-isoforms are capable of rescuing to control levels the reduced AMPAR EPSCs caused by the silencing of endogenous PSD-95. These results also suggest that additional sequence differences between PSD-95 and SAP97 not involving the N-terminal

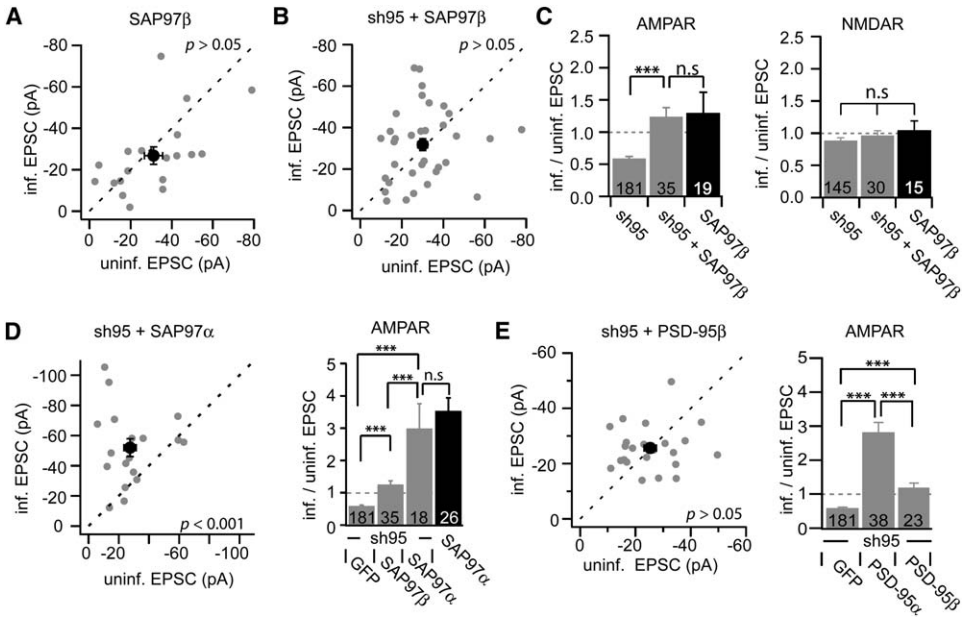

Figure 3. Isoform-Specific Regulation of AMPAR EPSCs by SAP97 and PSD-95

(A) Amplitudes of AMPAR EPSCs expressing SAP97β versus control neurons.

(B) Amplitudes of AMPAR EPSCs expressing sh95 + SAP97β versus control neurons.

(C) Summary of effects on AMPAR EPSCs (left panel) and NMDAR EPSCs (right panel) of expressing sh95 alone, SAP97β alone, and both together (n.s., $p > 0.05$; ***$p < 0.001$).

(D) Amplitudes of AMPAR EPSCs expressing sh95 + SAP97α versus control neurons (left panel) and summary of effects of replacing PSD-95 with SAP97β versus SAP97α as well as overexpression of SAP97α (right panel) (***$p < 0.001$).

(E) Amplitudes of AMPAR EPSCs expressing sh95 + PSD-95β versus control neurons (left panel) and summary of effects of replacing PSD-95 with PSD-95β versus PSD-95α (right panel) (***$p < 0.001$).

Gray symbols represent single pairs of recordings; black symbols show mean ± SEM; p values were calculated with a paired Student's t test comparing absolute values of paired recordings.

domains are unlikely to account for their functional differences in this assay system.

Regulation of AMPAR EPSCs by L27 Domain β-Isoforms Is Activity Dependent

Data from both overexpression of PSD-95α and acute knockdown with sh95 suggest that PSD-95α plays an important role in regulating AMPAR EPSCs (Figure 2). However, the ~40% reduction of AMPAR EPSCs by acutely silencing endogenous PSD-95 was relatively small, given that in cortical cultures, PSD-95 protein level was decreased by >90% (Figure 1E). In addition, in PSD-95 knockout mice, AMPAR-mediated synaptic transmission is unaltered, despite a lack of upregulation of other DLG-MAGUK protein levels (Migaud et al., 1998; Yao et al., 2004) (Figure 1C). These data suggest that there may be other factors that can regulate AMPAR-mediated synaptic responses in the absence of PSD-95, one obvious candidate being neural activity, which dramatically influences the trafficking of AMPARs, often via activation of NMDARs (Bredt and Nicoll, 2003; Malinow and Malenka, 2002; Song and Huganir, 2002). To examine the effects of activity on the reduction in AMPAR EPSCs caused by sh95, we applied the NMDAR antagonist D-APV (25 μM) to reduce activity or the $GABA_A$ receptor antagonist bicuculline (20 μM) to increase excitatory activity to our culture media beginning the day after virus infection. Treating slice cultures with D-APV significantly enhanced the decrease in AMPAR EPSCs caused by decreasing PSD-95 levels with sh95 (n = 34 pairs) compared with interleaved control slice cultures in which sh95 was expressed (n = 64 pairs) (Figure 4A). In contrast, the addition of bicuculline to the culture media rescued AMPAR EPSCs in sh95 expressing cells to control cell levels (n = 59 pairs) (Figure 4A), demonstrating that synaptic activity by itself is capable of rescuing synaptic strength when PSD-95 levels are greatly reduced. These results also suggest that the ~40% reduction in AMPAR EPSCs caused by the sh95 under control condition is not larger because of some activity-dependent compensation.

Because we only observed an effect of recombinant SAP97β on AMPAR EPSCs when PSD-95 was greatly reduced, we speculated that the influence of activity on the effectiveness of sh95 in reducing synaptic strength might be mediated through the β-isoforms of endogenous SAP97. To examine this hypothesis, we replaced endogenous PSD-95 with SAP97β and cultured the slices in the presence of D-APV. In contrast to control conditions in which SAP97β rescues the decrease in AMPAR EPSCs caused by sh95 (n = 35 pairs) (Figure 4B), in the presence of D-APV this rescue was prevented (n = 30 pairs) (Figure 4B). To test whether this influence of activity is an intrinsic property of SAP97β or simply of the N-terminal L27 domain, we repeated the experiment with PSD-95β. Again the rescue of synaptic AMPAR function that normally occurs when PSD-95β replaces endogenous PSD-95 (n = 23 pairs) was blocked by D-APV treatment (n = 18 pairs) (Figure 4C). It should be noted that the reduction in synaptic strength caused

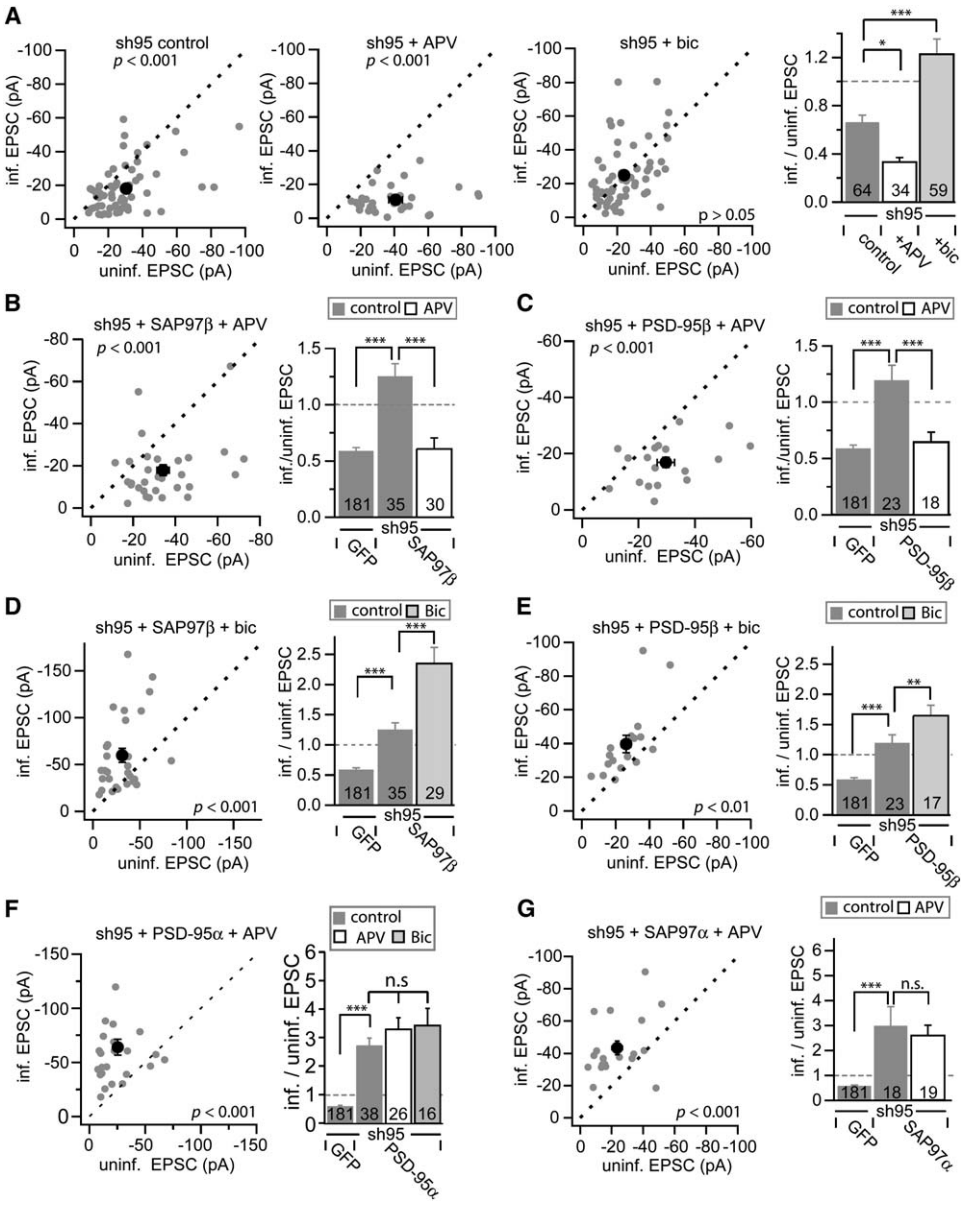

Figure 4. Activity Regulates Effects of Both Acute Knockdown of PSD-95 and Its Replacement by β- but Not α-Isoforms of SAP97 and PSD-95

(A) Amplitudes of AMAPR EPSCs in cells expressing sh95 under various conditions versus control neurons (left panels) and summary of effects of treatment with D-APV (25 μM) or bicuculline (20 μM).

(B) Amplitudes of AMAPR EPSCs in cells expressing sh95 + SAP97β in the presence of D-APV versus control neurons (left panel) and summary of effects of replacement of PSD-95 with SAP97β under control conditions versus in the presence of D-APV. Effects of sh95 alone are also shown for comparison.

(C) Amplitudes of AMPAR EPSCs in cells expressing sh95 + PSD-95β in the presence of D-APV versus control neurons (left panel) and summary of effects of replacement of PSD-95 with PSD-95β under control conditions versus in the presence of D-APV.

(D and E) Same as (B) and (C), respectively, except slices were treated with bicuculline rather than D-APV.

(F) Same as (C), except PSD-95 was replaced with PSD-95α rather than PSD-95β. Right panel shows that neither D-APV nor bicuculline treatments influenced the synaptic effects of PSD-95α.

(G) Same as (B), except PSD-95 was replaced with SAP97α and not SAP97β.

Gray symbols represent single pairs of recordings; black symbols show mean ± SEM; p values were calculated with a paired Student's t test comparing absolute values of paired recordings.

For all panels, *p < 0.05; **p < 0.01; ***p < 0.001; n.s. p > 0.05.

by sh95 alone in D-APV (Figure 4A) was greater than that observed when SAP97β and PSD-95β were expressed (Figures 4B and 4C; p < 0.02). This suggests SAP97β and PSD-95β still could exert some effects on synaptic function even in the presence of D-APV.

As increased excitatory activity could rescue AMPAR-mediated synaptic transmission in sh95-expressing cells (Figure 4A), we next tested whether the rescue mediated by L27 domain-containing β-isoforms of SAP97 and PSD-95 (Figure 3) could similarly be enhanced by

increasing activity with bicuculline. Indeed, in the presence of bicuculline, replacement of endogenous PSD-95 with either SAP97β (n = 29 pairs) or PSD-95β (n = 17 pairs) caused a significantly larger enhancement of AMPAR EPSCs than that observed in control conditions (Figures 4D and 4E). These results demonstrate that activity and the β-isoforms of SAP97 and PSD-95 act additively on AMPAR-mediated synaptic function and suggest that increased activity enhances the effect of these β-isoforms on AMPAR EPSCs.

To further test whether the N-terminal L27 domain in these β-isoforms of DLG-MAGUKs was critical for the activity dependence of their function, we examined whether reducing activity influenced the synaptic effects of the α-isoforms of PSD-95 and SAP97. In contrast to the clear effects that D-APV treatment had on the rescue of AMPAR EPSCs by the β-isoforms (Figures 4B and 4C), identical D-APV treatments had no effect on the large enhancement of AMPAR EPSCs caused by replacement of endogenous PSD-95 with PSD-95α (n = 26 pairs) or SAP97α (n = 19 pairs) (Figures 4F and 4G). Similarly, bicuculline treatment also did not affect the increase of AMPAR EPSCs caused by replacement of endogenous PSD-95 with PSD-95α (Figure 4F, n = 16 pairs). These results provide further evidence that the activity dependence of the synaptic function of DLG-MAGUKs is due to the N-terminal L27 domains that are only present in the β-isoforms.

Regulation of Synaptic Strength by β-Isoforms of SAP97 and PSD-95 Is CaMKII Dependent

We have shown that SAP97β can rescue AMPAR-mediated synaptic transmission to control levels when endogenous PSD-95 is greatly reduced (Figure 3B) and that this effect of SAP97β is significantly enhanced by activity (Figure 4D). However, overexpression of SAP97β in the presence of normal levels of PSD-95 had no effect (Figure 3A), suggesting that PSD-95α normally has a dominant effect in the regulation of AMPAR EPSCs. To test whether increasing excitatory activity could override the dominant effect of PSD-95α and allow SAP97β to exert some synaptic effects, we treated slices with bicuculline during the expression of SAP97β. However, under this condition SAP97β still had no detectable effect on AMPAR EPSCs (n = 16 pairs) (Figure 5A). Thus, the dominant effect of PSD-95α on synaptic function was independent of the level of activity.

How does activity regulate the synaptic effects of the β-isoforms of SAP97 and PSD-95? Because blockade of NMDARs with D-APV had clear effects, we focused on the possible role of one of the major targets of NMDAR-mediated Ca^{2+} influx, calcium/calmodulin-dependent protein kinase II (CaMKII) (Lisman et al., 2002). This is a particularly intriguing candidate because CaMKII phosphorylates SAP97 in vitro (Gardoni et al., 2003; Mauceri et al., 2004), a modification that may influence its synaptic targeting (Mauceri et al., 2004). To test for the involvement of CaMKII, we asked whether treatment of slices with the inhibitor KN-93 (20 μM) affected the rescue of AMPAR EPSCs by SAP97β when sh95 is expressed. This treatment had the same effect as D-APV (n = 30 pairs) in that it prevented the return of AMPAR EPSCs to control levels (n = 18 pairs, Figure 5B). Similarly, KN-93 prevented the rescue of AMPAR EPSCs

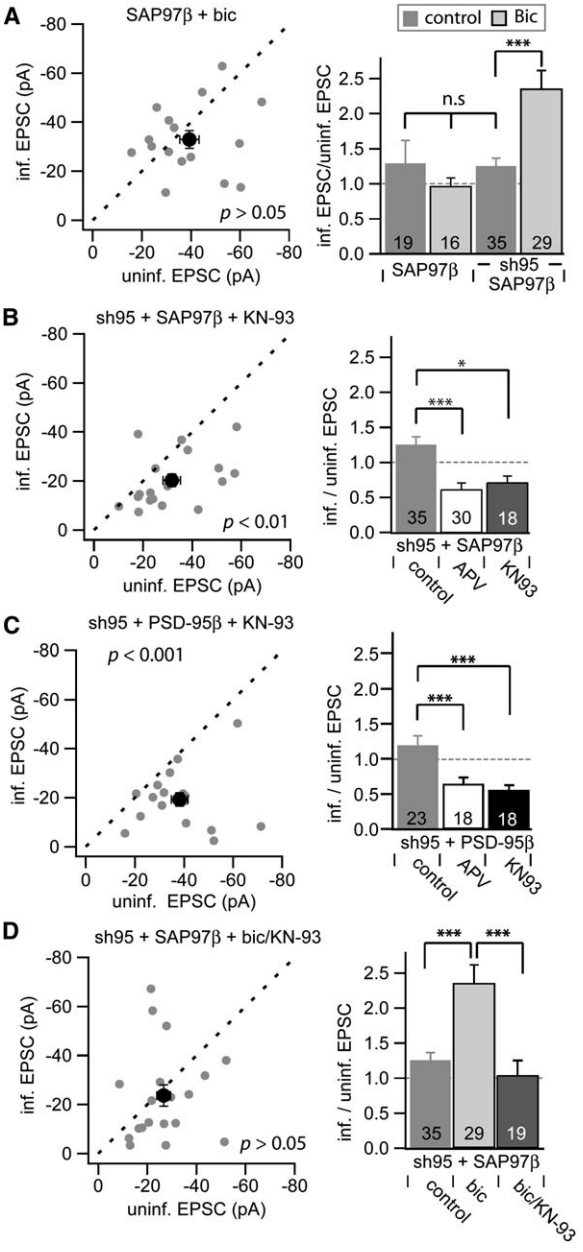

Figure 5. CaMKII Is Involved in the Regulation of AMPAR EPSCs by SAP97β and PSD-95β

(A) (Left panel) Amplitudes of AMPAR EPSCs in cells expressing SAP97β versus control neurons from slices treated with bicuculline. (Right panel) Summary of effects of bicuculline treatment on cells expressing SAP97β alone or sh95 + SAP97β (n.s. $p > 0.05$; *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$).

(B) (Left panel) Amplitudes of AMPAR EPSCs in cells expressing sh95 + SAP97β versus control neurons from slices treated with KN-93. (Right panel) Summary of effects of treatment of slices with D-APV or KN-93 on cells expressing sh95 + SAP97β.

(C) Same as (B), except that PSD-95β was examined.

(D) (Left panel) Amplitudes of AMPAR EPSCs in cells expressing sh95 + SAP97β versus control neurons from slices treated with bicuculline and KN-93. (Right panel) Summary of effects of treatment of slices with bicuculline alone or bicuculline and KN-93 on cells expressing sh95 + SAP97β.

Gray symbols represent single pairs of recordings; black symbols show mean ± SEM; p values were calculated with a paired Student's t test comparing absolute values of paired recordings.

by PSD-95β when endogenous PSD-95 was reduced by sh95 (n = 18 pairs, Figure 5C). To further examine the importance of CaMKII, we expressed SAP97β and sh95 in slices treated with both KN-93 and bicuculline to see if inhibition of CaMKII could prevent or decrease the large activity-dependent enhancement of the effects of SAP97β on AMPAR EPSCs. Again, inhibition of CaMKII (n = 19 pairs) greatly reduced the synaptic consequences of expressing SAP97β (Figure 5D). These results suggest that CaMKII is one of the downstream signaling molecules by which activity influences the regulation of synaptic strength by SAP97β.

Discussion

Restructuring the molecular architecture of excitatory synapses is thought to be critically important for many forms of activity- and experience-dependent plasticity. In this context, critical components of the postsynaptic density are members of the PDZ domain-containing, DLG-MAGUK family of scaffolding proteins (Beique and Andrade, 2003; Ehrlich and Malinow, 2004; El-Husseini et al., 2002; Funke et al., 2005; Kim and Sheng, 2004; Montgomery et al., 2004; Scannevin and Huganir, 2000; Schnell et al., 2002; Stein et al., 2003). Using a molecular replacement strategy, we demonstrate that the α- and β-isoforms of the two most well-characterized DLG-MAGUKs, PSD-95 and SAP97, regulate basal AMPAR-mediated synaptic strength via different mechanisms. The cysteine doublet-containing, palmitoylated α-isoforms of PSD-95 and SAP97 increased AMPAR EPSCs independent of the level of both endogenous PSD-95 and activity. In contrast, the function of the L27-containing β-isoforms was activity dependent and completely masked by PSD-95α, the principle endogenous isoform of PSD-95. Furthermore, the influence of activity on the functional effects of SAP97β required CaMKII activity. Thus, differences in the functional synaptic effects of PSD-95α and SAP97β, the major endogenous isoforms of these DLG-MAGUKs, can be attributed solely to their N-terminal domains. Our results also demonstrate that a molecular replacement strategy is an important complement to more standard methodologies as it can reveal functions of a protein that cannot be observed with overexpression or knockout/knockdown approaches.

Previous work on the synaptic consequences of expressing SAP97 in hippocampal slice cultures has yielded conflicting results (Ehrlich and Malinow, 2004; Nakagawa et al., 2004; Schnell et al., 2002). It was previously suggested that the lack of effect of SAP97 may be due to the disruption of its 3D structure by fusion to GFP (Nakagawa et al., 2004). Two of our results indicate that this is unlikely to be the case: (1) the same recombinant SAP97β fused to GFP that had no effect on basal synaptic strength when expressed alone had a clear effect when endogenous PSD-95 was reduced by shRNA, (2) PSD-95β with GFP fused at its C terminus had the same effects as SAP97β with GFP fused to its N terminus. Instead, we suggest that two other factors—the magnitude of its overexpression and the "state" of the synapses in which it is expressed—contribute to the functional synaptic consequences of expressing SAP97β. With regard to the former, it is likely that using a eukaryotic expression vector with a CMV promoter (pGW1; (Nakagawa et al., 2004) will result in greater expression than lentivirus-mediated gene transfer. Consistent with this proposal, the magnitude of increase in synaptic strength caused by overexpression of PSD-95 was much higher in the previous work using pGW1 to express PSD-95 and SAP97 (7- to 8-fold; Nakagawa et al., 2004) than in the present work (2- to 3-fold) or previous work that used eukaryotic expression vectors (3.5-fold; Schnell et al., 2002) or Sindbis virus to express PSD-95 (2- to 3-fold; Ehrlich and Malinow, 2004). With regard to the "state" of the synapse, our results demonstrate that the level of endogenous PSD-95 is one factor that greatly influences the functional synaptic effects of SAP97β.

The previous study that reported effects of SAP97 expression on synaptic strength in slice cultures (Nakagawa et al., 2004) suggested that its L27 domain was important because of its ability to multimerize with itself or other proteins. However, PSD-95 also multimerizes (Christopherson et al., 2003; Hsueh et al., 1997) and the importance of PSD-95 multimerization for its synaptic functions has not been tested. Therefore, it remains unclear whether SAP97 multimerization underlies any special features of its functions that distinguish it from PSD-95. Here we demonstrate that the N-terminal L27 domain has the important role of conferring activity dependence and CaMKII dependence to the functional synaptic effects of SAP97 as well as PSD-95. In contrast, the synaptic effects of the major endogenous isoform of PSD-95, PSD-95α, as well as SAP97α, were unaffected by activity manipulations. Thus differences in the PDZ, SH3, and GK domains of PSD-95 and SAP97 as well as differences in their intervening sequences are not involved in conferring activity dependence. These results also suggest that PSD-95 may not be a primary target of the signaling pathways that mediate various forms of activity-dependent synaptic delivery of AMPARs (Ehrlich and Malinow, 2004; Stein et al., 2003). Consistent with this conclusion, LTP is robustly expressed in PSD-95 knockout animals (Migaud et al., 1998).

Instead, our results implicate SAP97β as a possible key target of the NMDAR/CaMKII signaling pathway that is required for many forms of experience- and activity-dependent synaptic plasticity (Lisman et al., 2002). Indeed, SAP97 can be phosphorylated by CaMKII at S39, and this has been reported to enhance its targeting to dendritic spines in dissociated cultured neurons (Mauceri et al., 2004). However, several observations make it unlikely that such a mechanism accounts for the activity dependence and CaMKII dependence of the effects of SAP97β on synaptic strength. First, an S39D mutation in SAP97β, which enhanced spine localization of SAP97 (Mauceri et al., 2004), did not result in an increase in AMPAR EPSCs when overexpressed in hippocampal slice cultures (data not shown). Second, a search of the GenBank database revealed that only the rat isoform of SAP97β contains a serine residue at position 39; mouse and human isoforms do not, making it less likely that phosphorylation of S39 of this isoform of SAP97β (containing the I3 insert) is functionally important. Third, the synaptic effects of PSD-95β, which does not contain serine 39, were blocked by inhibition of NMDARs and CaMKII in a manner identical to SAP97β. Fourth, whereas the α-isoforms of SAP97 and PSD-95

displayed a highly punctate pattern in dendrites and were localized preferentially in spine-like structures in our slices, the β-isoforms displayed a diffuse somatodendritic staining pattern (J. Tsui and R.M., unpublished data), results that are consistent with experiments examining endogenous SAP97β and PSD-95α (Cho et al., 1992; Kim et al., 1996; Sans et al., 2001). This was an intrinsic property of the β-isoforms themselves as the placement of the GFP tag (N-terminal in SAP97β and C-terminal in PSD-95β) made no difference in their subcellular localization. Furthermore, the activity manipulations that affected the magnitude of the effects of SAP97β and PSD-95β on AMPAR EPSCs had no discernable effect on their synaptic localization (J. Tsui and R.M., unpublished data). However, we cannot rule out that undetectable activity-dependent changes in the synaptic localization of SAP97β and PSD-95β and/or their CaMKII-dependent phosphorylation on other residues contributed to their functional effect on synaptic strength. Alternatively, CaMKII-dependent phosphorylation of other proteins, such as transmembrane AMPA receptor regulatory proteins (TARPs) or AMPAR subunits, might mediate the activity-dependent enhancement of AMPAR EPSCs via SAP97β or PSD-95β.

Our results also address the key issue of the functional interplay between PSD-95 and SAP97 given that their predominant endogenous isoforms are PSD-95α and SAP97β. Although the synaptic effects of PSD-95α were not influenced by activity, it had both a dominant and restrictive role in controlling synaptic strength, presumably by influencing the incorporation of AMPARs into the postsynaptic density (PSD) (Chen et al., 2000; Ehrlich and Malinow, 2004; Schnell et al., 2002). It is dominant in that in the present work and all studies performed to date, the level of PSD-95α correlates with AMPAR-mediated synaptic strength independent of other variables such as activity or the preparation in which it is studied (Beique and Andrade, 2003; Ehrlich and Malinow, 2004; El-Husseini et al., 2002; Funke et al., 2005; Kim and Sheng, 2004; Montgomery et al., 2004; Scannevin and Huganir, 2000; Schnell et al., 2002; Stein et al., 2003). It is restrictive in that SAP97β could only affect synaptic function in the absence of endogenous PSD-95. How does PSD-95 subserve these functions? One possibility is that PSD-95 serves as a "slot" protein (Malinow et al., 2000) which, via interacting with AMPAR/TARP complexes, controls the number of synaptic AMPARs in the PSD (Schnell et al., 2002). Two lines of evidence argue against PSD-95 being the sole slot protein. First, in the absence of PSD-95, AMPAR EPSCs can be normal. Second, during chronic activity manipulations in dissociated neuronal cultures, synaptic PSD-95 levels change in a direction diametrically opposite to that of miniature excitatory postsynaptic current (mEPSC) amplitude (Ehlers, 2003; O'Brien et al., 1998; Turrigiano et al., 1998). Therefore, other MAGUK family members must also be able to fulfill this putative function. SAP97β is unlikely to be one such MAGUK; in marked contrast to PSD-95, its synaptic levels, as assayed by light microscopy in our slice cultures, were not correlated with synaptic strength. Therefore, a limitation of this "slot" model is that it does not explain why decreasing the level of PSD-95α is required for SAP97β to exert a functional synaptic effect. To account for these observations we propose that PSD-95α may also "gate" the synaptic incorporation of AMPARs and thereby limit the access of extrasynaptic AMPARs to the PSD. Increasing its levels would provide more "gates" (and perhaps more "slots;" the two roles are not mutually exclusive), while in its absence, there would be free access of extrasynaptic AMPARs to the PSD, the final level of synaptic AMPARs still being dictated by the number of "slot" proteins.

In this model, SAP97β functions in an activity- and CaMKII-dependent fashion to either facilitate the delivery of AMPARs to extrasynaptic membrane from intracellular pools or trigger the release of AMPARs that are retained at an extrasynaptic pool so they can be transported into synapses. In either scenario, translocation of the SAP97β into the PSD, and perhaps into spines, is not required. Consistent with the first proposal is the observation that endogenous SAP97β associates with AMPARs in an early step of the biosynthetic pathway and is not bound to AMPARs localized on the plasma membrane (Sans et al., 2001). Furthermore, SAP97β overexpression in dissociated neuronal cultures leads to an increase in surface synaptic AMPARs, suggesting a role either in mobilizing the receptors from an intracellular pool or diffusion of receptors from extrasynaptic to synaptic sites (Rumbaugh et al., 2003).

We have assumed that the acute knockdown of PSD-95 using RNAi and its simultaneous replacement with recombinant forms of PSD-95 and SAP97 are less likely to cause compensatory synaptic adaptations than traditional genetic knockout approaches. In this context there are three major differences between RNAi and knockout approaches. First, genetic deletion results in loss of the protein throughout development and therefore may affect synaptic properties by influencing synaptogenesis, whereas viral approaches allow the manipulation of mature neurons. Second, the duration over which the protein is absent and adaptations can occur is longer when standard knockout approaches are used. Using RNAi, the effects of PSD-95 knockdown were clear after 3 days and stable for an additional 5 days. Third, knockout approaches involve deleting the protein in all cells in a given brain area whereas the lentivirus-mediated shRNA expression affects small numbers of scattered cells, limiting the molecular manipulation to the postsynaptic cells under investigation. However, this latter approach is not without its own limitations. It still takes several days to take effect and theoretically may cause changes in network activity, which in turn differentially influences synaptic function on the molecularly modified cells. Thus, the results from experiments using this molecular replacement strategy must be considered in the context of the results obtained from other approaches, each of which has its own distinct set of advantages and limitations.

In summary, we have presented evidence of additional important differences in the functional roles of the predominant isoforms of PSD-95 and SAP97. Most importantly, we have found that the N-terminal L27 domain in SAP97β confers activity dependence and CaMKII dependence on both SAP97 and PSD-95. By use of a molecular replacement approach, we have also provided explanations for conflicting results in the literature and the functional interplay between PSD-95

and SAP97. While PSD-95 appears to be a dominant and presumably more stable component of the PSD (in that its function is not affected by chronic activity), the functional effects of SAP97 are highly dependent on activity. Further work involving molecular replacement of SAP97 will be required to test its specific role in various forms of synaptic plasticity.

Experimental Procedures

Dissociated Neuronal Cultures

Dissociated neuronal cultures were prepared from newborn Sprague-Dawley pups as previously described with minor changes (Stellwagen et al., 2005). Briefly, trypsinized hemispheres of cortex were triturated and plated on poly-L-lysine coated 10 cm culture dishes in B-27 supplemented Neurobasal media, and then refed subsequently with N-2 supplemented MEM plus GlutaMax (Invitrogen). Glial growth was inhibited by FUDR at 3 DIV.

Antibodies and Western Blots

The following antibodies were used: mouse PSD-95 antibody (Affinity Bioreagents), mouse α/β-DLG (SAP97) antibody (Stressgen), mouse GFP antibody (JL-8, Clontech), rabbit synaptophysin antibody (Zymed), goat anti-mouse conjugated with Alexa 680 (Rockland), and goat anti-rabbit conjugated with Alexa 800 (Rockland). Neuronal cultures were collected in ice-cold RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 50 mM Na_2HPO_4, 150 mM NaCl, 2 mM EDTA and protease inhibitor cocktail [Roche], [pH 7.4]) and diluted with SDS-PAGE sample buffer (BioRad). HEK293 cells were transfected at 60%–80% confluency with expression constructs for PSD-95, SAP97, and PSD-93 (Parker et al., 2004). Cells were harvested and lysed 24 hr after transfection in ice-cold RIPA buffer and diluted with SDS-PAGE buffer. Hippocampi were collected and homogenized in ice-cold homogenization buffer. A crude synaptosomal fraction P2 was solubilized in SDS-PAGE sample buffer after protein concentrations were adjusted (Schlüter et al., 1999). Samples were separated on 4%–12% gradient Bis-Tris gels (Invitrogen), transferred onto nitrocellulose, and decorated with the indicated antibodies (Schlüter et al., 1999). Signals of fluorescent dye-labeled secondary antibodies were detected and quantified with an Odyssey Infrared Imaging System (Li-cor Biotechnology).

RT-PCR

Protocols were adapted from Lin et al. (Lin et al., 1997) with minor modifications. Total RNA was isolated from different brain regions of 21–23 day old rats using Trizol reagent (Invitrogen), and 5 μg of each RNA sample was used in each 20 μl of randomly primed reverse transcription (RT) reactions for first strand cDNA synthesis according to the manufacturer's protocol (SuperscriptII reverse transcriptase; Invitrogen). Two microliters of cDNA from each RT reaction was used for PCR amplification with annealing temperature of 58ºC. Two upstream primers, Pα (5′-TTGTTGTCGTTGTTGAAATA GGCAT) and Pβ (5′-ACTAAGCCAAACCGAAGACAGACAG) and one downstream primer P2 (5′-ACAAACCCGTGGACATTGTGAATGT) were used in PCR amplification reactions to recognize either the α- or β-isoform of SAP97 with the predicted PCR products of 238 bp and 393 bp, respectively. The pair of GAPDH primers in control reactions was PGup (5′- CAAGATGGTGAAGGTCGGTGTGAA) and PGdn (5′-CAGCACCAGCATCACCCCCATTT) that amplified a product of 275 bp. Negative controls that lacked template confirmed that reagents were not contaminated with cDNAs. The primers flanked at least one intron so contamination by genomic DNA could be ruled out.

Hippocampal Slice Cultures

Procedures for preparation of hippocampal slice cultures were essentially as described (Stoppini et al., 1991), with minor modifications. Briefly, hippocampi of 8 day old rats were isolated, and 220–300 μm slices were prepared using a Vibratome (Leica Microsystems) in ice-cold sucrose substituted artificial cerebrospinal fluid (ACSF; see Electrophysiology section). Slices were transferred onto MilliCell Culture Plate Inserts (MilliPore) and cultured in Neurobasal-A medium supplemented with 1 μg/ml insulin, 0.5 mM ascorbic acid, and 20% horse serum. Media was changed every second day. When slices were treated pharmacologically, 25 μM D-APV (Tocris), 20 μM bicuculline (Tocris), and/or 20 μM KN-93 (Calbiochem) were included in the media 1 day after virus injection.

Virus Preparation and Infection

All lentiviral transfer vector constructs were modified from the original FUGW vector backbone (Lois et al., 2002). For shRNA expression, an H1 promoter cassette from pSuper vector, including a hairpin (sh95) targeting the PSD-95 sequence GGA CAT CCA GGC ACA CAA G, was cloned between the HIV-flap and ubiquitin promoter (Figure 1D) (Brummelkamp et al., 2002). When the effect of shRNAs was tested, the ubiquitin promoter-driven eGFP expression was used to identify infected cells. For molecular replacement studies, eGFP was replaced by fusion proteins of either C-terminally, eGFP-tagged PSD-95α, PSD-95β, and SAP97α, or N-terminally, eGFP-tagged SAP97β. Silent mutations were introduced in the PSD-95 construct target region of sh95 (GGAtATtCAaGCgCAtAAG), so that the shRNA did not knock down the replacement constructs that were expressed under regulation of the ubiquitin promoter. For the production of lentiviral vectors, the transfer vector, the HIV-1 packaging vector Δ8.9, and the VSVG envelope glycoprotein vector were cotransfected into HEK293 fibroblasts using FUGENE6 transfection reagent (Roche, Basel, Switzerland). Supernatants of culture media were collected 48 hr after transfection and centrifuged at 50,000 × g to concentrate the viral vector. To infect hippocampal slice cultures, concentrated viral solutions were injected into the CA1 pyramidal cell layer using a Picospritzer II (General Valve). To infect cortical cultures, 500 μl of unconcentrated viral supernatant was used for each 100 mm dish.

Electrophysiology

A single slice was removed from the insert and put in the recording chamber where it was constantly perfused with ACSF containing (in mM) 119 NaCl, 26 $NaHCO_3$, 10 glucose, 2.5 KCl, 1 NaH_2PO_4, 4 $MgSO_4$, and 4 $CaCl_2$, constantly bubbled with 95% O_2 and 5% CO_2. Picrotoxin (50 μM) was included to isolate EPSCs and chloroadenosine (1–2 μM) was added to reduce polysynaptic activity. All experiments, except for those illustrated in Figures 2A–2C, were performed 4–8 days after infection. A single, double-barreled glass pipette filled with ACSF was used as a bipolar stimulation electrode and was placed within 300 μm of the recording pipettes in stratum radiatum. The pipette solution for whole-cell voltage-clamp recordings contained (in mM) 117.5 $CsMeSO_3$, 10 HEPES, 10 TEACl, 8 NaCl, 15.5 CsCl, 1 $MgCl_2$, 0.25 EGTA, 4 MgATP, 0.3 NaGTP, and 5 QX-314. Data were collected using an Axopatch 700A amplifier or two Axopatch 1D amplifiers (Axon Instruments), digitized at 5 kHz with the A/D converter PCI-MI0-16E-4 (National Instruments). Data were acquired and analyzed on-line using custom routines written with Igor Pro software (Wavemetrics). AMPAR EPSCs were recorded at −60 mV and measured using a 2 ms window around the peak. NMDAR EPSCs are recorded at +40 mV and measured 60–65 ms after the initiation of the EPSC, a time point at which AMPAR-mediated currents are absent or minimal. Small, hyperpolarizing voltage steps were given before each afferent stimulus allowing on-line monitoring of input and series resistances. Stimulation pulses were provided at 0.2 Hz. Simultaneous, dual whole-cell recordings were established from an infected and closely adjacent uninfected cell (as indicated by GFP expression and lack thereof). AMPAR EPSCs were collected after adjusting stimulation strength so that AMPAR EPSCs in control cells were 10–50 pA and 40 to 70 traces were averaged to obtain the basal AMPAR EPSCs from the two cells. Cells were then depolarized to +40 mV and another 25 to 50 dual component EPSCs were collected to obtain a measurement of NMDAR EPSCs. Comparisons between infected and uninfected cell responses were done using a paired t test. Statistical analyses among different constructs and conditions were done using one-way analysis of variation (ANOVA) and Tukey's HSD t test.

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