

**Differential requirement for NMDAR activity in SAP97 $\beta$ -mediated regulation of the number and strength of glutamatergic AMPAR-containing synapses**

Mingna Liu<sup>1,2</sup>, Laura D. Lewis<sup>2,3</sup>, Rebecca Shi<sup>2,4</sup>, Emery N. Brown<sup>2,3</sup> and Weifeng Xu<sup>1,2</sup>

<sup>1</sup>Picower Institute for Learning and Memory, <sup>2</sup>Department of Brain and Cognitive Sciences, <sup>3</sup>MIT-Harvard Division of Health Science and Technology, <sup>4</sup>Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139

**Running Head:** Function of SAP97

**Corresponding Author:** W. Xu

**Corresponding Address:** Picower Institute for Learning and Memory, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139

**E-mail:** weifeng@mit.edu

Phone: 1-617-715-5392

Fax: 1-617-324-5543

## **Abstract**

PSD-95-like, disc-large (DLG) family membrane-associated guanylate kinase proteins (PSD/DLG-MAGUKs) are essential for regulating synaptic AMPA receptor (AMPA) function and activity-dependent trafficking of AMPARs. Using a molecular replacement strategy to replace endogenous PSD-95 with SAP97 $\beta$ , we show that the prototypic  $\beta$ -isoform of the PSD-MAGUKs, SAP97 $\beta$ , has distinct NMDA receptor (NMDAR)-dependent roles in regulating basic properties of AMPAR-containing synapses. SAP97 $\beta$  enhances the number of AMPAR-containing synapses in an NMDAR-dependent manner, while its effect on the size of unitary synaptic response is not fully dependent on NMDAR activity. These effects contrast with those of PSD-95 $\alpha$ , which increases both the number of AMPAR-containing synapses and the size of unitary synaptic responses, with or without NMDAR activity. Our results suggest that SAP97 $\beta$  regulates synaptic AMPAR content by increasing surface expression of GluA1-containing AMPARs, whereas PSD-95 $\alpha$  enhances synaptic AMPAR content presumably via increasing the synaptic scaffold capacity for synaptic AMPARs. Our approach delineates discrete effects of different PSD-MAGUKs on principal properties of glutamatergic synaptic transmission. Our results suggest that the molecular diversity of PSD-MAGUKs can provide rich molecular substrates for differential regulation of glutamatergic synapses in the brain.

**Key words:** PSD-MAGUK, DLG-MAGUK, hippocampus, excitatory synapse, mEPSC

## Introduction

AMPA receptors mediate fast excitatory synaptic transmission in the mammalian brain. Both the number of AMPAR-containing synapses (active synapses) and the size of the unitary active synapse response determine the strength of glutamatergic transmission. NMDAR activation leads to trafficking of AMPARs in and out of the synapse, resulting in long-term changes in the strength of AMPAR-mediated synaptic transmission. This mechanism underlies the Hebbian form of plasticity that is important for experience-dependent plasticity and learning and memory (Kerchner and Nicoll 2008; Malinow and Malenka 2002).

Glutamate receptors are embedded in the postsynaptic density (PSD), a highly organized protein network that links these membrane receptors to scaffold proteins, cytoskeletal components, and signaling molecules (Kennedy 1997; Malenka and Bear 2004; Scannevin and Huganir 2000). Among the scaffold proteins, the PSD-95-like, homolog of disc-large membrane-associated guanylate kinase proteins (PSD/DLG-MAGUK) family, comprising PSD-93, PSD-95, SAP97 and SAP102, plays an important role in regulating the trafficking and localization of synaptic AMPARs during development and plasticity (Craven and Bredt 1998; Elias and Nicoll 2007; Kim and Sheng 2004; Ryan and Grant 2009; Xu 2011). Understanding the functional diversity of PSD-MAGUKs in regulating basic synaptic properties and their interaction with NMDAR activity is important in elucidating the molecular mechanisms that underlie the wiring and plasticity of the excitatory circuit.

The PSD-MAGUKs differ primarily in the N-terminal region by alternative transcriptional start sites of the four genes (Chetkovich et al. 2002; Parker et al. 2004; Schlüter et al. 2006). Extensive work has been done on PSD-95, especially on the predominant isoform PSD-95 $\alpha$ , which can be modified in the N-terminal region of the protein by palmitoylation

(El-Husseini et al. 2002). It has been shown that levels of PSD-95 $\alpha$  are positively correlated with synaptic AMPAR-mediated synaptic strength, measured as increased AMPAR eEPSCs. This effect of increasing PSD-95 levels on AMPAR-mediated synaptic transmission is primarily due to a significant increase of the number and a modest increase of unitary strength of active synapses measured by miniature AMPAR EPSC analyses. The changes in NMDAR eEPSCs (to measure NMDAR-mediated transmission, including AMPAR-lacking, silent synapses) and paired pulse ratio (a crude measurement of presynaptic release probability), if any, are to a much lesser degree (Béïque and Andrade 2003; Béïque et al. 2006; Carlisle et al. 2008; Ehrlich et al. 2007; Ehrlich and Malinow 2004; El-Husseini et al. 2000; Elias et al. 2006; Nakagawa et al. 2004; Schlüter et al. 2006; Schnell et al. 2002).

In contrast, the role of the L-27 domain-containing  $\beta$ -isoforms of PSD-MAGUKs is less clear. Loss-of-function studies of SAP97 $\beta$  using genetic ablation showed no effect on basal glutamatergic transmission or long-term potentiation (Howard et al. 2010), presumably due to functional redundancy. However, overexpression studies in dissociated neuron culture suggest an active role of SAP97 $\beta$  in AMPAR trafficking (Leonard et al. 1998; Rumbaugh et al. 2003; Waites et al. 2009). When endogenous PSD-95 is knocked-down,  $\beta$ -isoforms of PSD-95 and SAP97 regulate synaptic AMPAR functions in an NMDAR- and CaMK-dependent manner (Schlüter et al. 2006). These studies have identified a role for SAP97 $\beta$  in AMPAR trafficking, but have not identified exactly how it affects synaptic properties. While analyzing evoked responses can provide quantitative measurements of effects of molecular manipulation on synaptic strength, it measures a sum of synaptic ensembles. How the  $\beta$ -isoform affects principal synaptic properties, such as the number of synapses and the unitary amplitude of synapses, is not easily accessible from evoked response measurements. This information is essential to

understand how the scaffold proteins organize and influence the connectivity of the postsynaptic neurons in the neural circuit. Additionally, the impact of synaptic activity on  $\beta$ -isoform-mediated modulation of these processes also remains to be elucidated, and is key to understanding the role of the  $\beta$ -isoform in synaptic plasticity.

To analyze the role of the  $\beta$ -isoform of SAP97 in regulating basic properties of synaptic glutamatergic transmission, we used a cell-restricted molecular replacement strategy to express specific PSD-MAGUKs against the background of RNAi-mediated acute knockdown of endogenous PSD-95. This strategy allowed us to avoid the dominant effect of the endogenous isoform. We compared the different contributions of SAP97 $\beta$  and PSD-95 $\alpha$  to basic characteristics of synaptic glutamatergic transmission, and examined how NMDAR-mediated activity influences the functional impact of each isoform on glutamatergic synaptic transmission.

## **Materials and Methods**

***Hippocampal Slice Cultures*** Hippocampi of 7-day old Sprague-Dawley rats were isolated, and 300  $\mu$ m slices were prepared using a Tissue Slicer (Siskiyou) in ice-cold sucrose-substituted artificial cerebrospinal fluid (ACSF). Slices were transferred onto MilliCell Culture Plate Inserts (MilliPore) and cultured in Neurobasal-A medium supplemented with 1  $\mu$ g/ml insulin, 0.5 mM ascorbic acid, and 20% horse serum. Media was changed every second day. When slices were treated pharmacologically, 25  $\mu$ M D-APV (Tocris) was included in the media 1 day after virus injection and was present until the day of recording.

***Virus preparation and infection*** All lentiviral constructs were modified from the original lentiviral transfer vector FUGW (Lois et al. 2002) and its variant FHUG+W, which contains an RNAi expression cassette driven by an H1 promoter (Schlüter et al. 2006).

In the acute knockdown experiment, the shRNA targeting PSD-95 mRNA (sh95) is expressed under the H1 promoter, and 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-95, or N-terminally eGFP-tagged SAP97. Silent mutations were introduced in the sh95 target region of the PSD-95 construct to prevent shRNA knockdown of the replacement constructs that were expressed under regulation of the ubiquitin promoter. For the production of the lentiviral vectors, the transfer vectors, the HIV-1 packaging vectors, pRSV/REV and pMDLg/pRRE, and the envelope glycoprotein vector VSV-G (Dull et al. 1998) were cotransfected into HEK293 fibroblasts using FUGENE6 transfection reagent (Roche, Basel, Switzerland). Supernatants of culture media were collected 60 hours after transfection, and centrifuged at 50,000 x g to concentrate the viral particles. To infect hippocampal slice cultures, concentrated viral solutions were injected into the CA1 pyramidal cell layer using a nanojector (Drummond). To infect cortical cultures, 3  $\mu$ l of concentrated viral supernatant were dispensed into 3 ml of culture media for each 35 mm dish.

***Electrophysiology*** All experiments were performed 4–8 days after infection and done at 29–30°C. For mini EPSC recordings, neurons were recorded under voltage-clamp configuration in ACSF containing (in mM): 119 NaCl, 26 NaHCO<sub>3</sub>, 10 glucose, 2.5 KCl, 1 NaH<sub>2</sub>PO<sub>4</sub>, 4 MgSO<sub>4</sub>, and 4 CaCl<sub>2</sub>, saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub> and supplemented with 1  $\mu$ M tetrodotoxin, 50  $\mu$ M picrotoxin, 50  $\mu$ M D-APV and 50 mM sucrose. The patch pipette (4.5–7 M $\Omega$ ) solution contained (in mM): 130 CsMeSO<sub>3</sub>, 20 CsCl, 10 HEPES, 6 MgCl<sub>2</sub>, 2 NaATP, 0.3 NaGTP, 5 Na phosphocreatine, 5 QX-314 and 5 EGTA, pH 7.3. Some experiments were recorded in the presence of 10 or 50  $\mu$ M i-naphthyl acetyl spermine (NASPM) as indicated. Data were collected using a MultiClamp 700B amplifier

(Axon Instruments) digitized at 10 kHz with the A/D converter ITC-18 computer interface (Heka Instruments). Data were acquired and analyzed on-line using custom routines written by Richard Gerkin, Ph.D, with Igor Pro software (Wavemetrics). Input and series resistances were monitored throughout the recordings. mEPSCs were analyzed off-line with Mini Analysis Program (Synaptosoft), using a threshold of 6 pA.

***Statistical Analysis of Mini Events. Model fitting:*** We observed that neither inter-event intervals (IEIs) nor event amplitudes followed a normal distribution, and thus proceeded with a model-based analysis method to accurately represent the data structure (adapted from (Phillips et al. 2011)). An exponential model was fit to inter-event interval data, and a left-truncated gamma model was fit to event amplitudes to account for the rightwards skew and thresholding of amplitudes. The exponential model was fit with a maximum likelihood calculation of the rate parameter estimate, providing an estimate of the event frequency. Goodness-of-fit was assessed with the time-rescaling theorem (Brown et al. 2002). The truncated gamma model could not be fit analytically due to the truncation point, so maximum likelihood parameters were estimated numerically using the interior-point algorithm (MATLAB, Mathworks). Goodness-of-fit for the truncated gamma model was assessed using a two-sample Kolmogorov-Smirnov test to compare the empirical and theoretical distributions.

***Hypothesis testing and confidence intervals:*** After modeling the IEI and amplitude distributions, the estimated parameters and mean amplitudes were compared using the Wilcoxon two-sample rank sum test. If two groups had paired samples, the one-sample signed rank test was used to calculate whether the difference between the pairs was significantly different from zero. To compute 95% confidence intervals for the differences between groups, the asymptotic normal approximation to the rank sum statistic was used with a critical value of  $p = 0.05$ .

*Cumulative distribution functions and confidence intervals:* Cumulative distribution functions (CDFs) were computed by a nonparametric bootstrap: the events in a single cell were resampled with replacement, and new model parameter estimates were computed from the resampled data. This resampling was repeated 500 times to obtain a distribution of 500 parameter estimates for a single cell. Each set of parameter estimates was used to compute a CDF. The 2.5<sup>th</sup>, 50<sup>th</sup>, and 97.5<sup>th</sup> percentiles of this CDF were then displayed to show the median CDF with 95% confidence intervals.

When calculating a CDF for a typical cell in a group, the resampling procedure was computed as above for each cell in the group. For each resampling, a group parameter was then calculated as the mean parameter estimate across all cells. These group parameter estimates were used to generate a CDF that represented a typical cell within the group, yielding 500 CDFs from the bootstrap procedure. As in the previous case, the 2.5<sup>th</sup>, 50<sup>th</sup>, and 97.5<sup>th</sup> percentiles of the CDF were used to calculate the median and a 95% confidence interval for the CDF.

Group comparisons were visualized with box plots of the parameter estimates for each cell within a group. Boxplots mark the 25<sup>th</sup>, 50<sup>th</sup>, and 75<sup>th</sup> percentiles of the parameter estimates within a group, with whiskers extending to the most extreme data points that were not considered outliers. Cells that were more than 3 times the 25-75 percentile distance from the 25<sup>th</sup> or 75<sup>th</sup> percentile were labeled as outliers in the display with a cross, but all cells were included in the statistical analysis and the outlier label was for display purposes only.

***Dissociated Cortical Neuronal Culture*** Dissociated cortical cultures were prepared from P1 Sprague-Dawley rats. The cortical hemispheres were dissected and digested for 20 minutes at 37°C with papain according to published protocols (Schlüter et al. 2006). Cells were plated on poly-D-lysine-coated 35 mm cell culture dishes or 12-well plates



with Neurobasal media (Invitrogen) supplemented with B27 (Invitrogen). Glial growth was inhibited with FUDR at DIV4. Neurons were infected with respective lentiviruses at DIV7 and collected after DIV17.

***Biotinylation Assay*** The biotinylation assay was performed following the method described (Ehlers 2000). Cortical neuron cultures were preincubated with TTX (1  $\mu$ M) and leupeptin (200  $\mu$ g/ml) for 30 min at 37°C to avoid lysosomal degradation of internalized AMPARs. Cultures were cooled with ice-cold DPBS (+ 1 mM MgCl<sub>2</sub> and 2.5 mM CaCl<sub>2</sub>) and incubated at 4°C for 10 minutes to stop membrane trafficking, then treated with a cleavable biotin solution (sulfo-NHS-biotin,) 1.5 mg/ml, in DPBS (+ 1 mM MgCl<sub>2</sub> and 2.5 mM CaCl<sub>2</sub>) for 30 minutes at 4°C to label surface receptors. Unbound biotin was quenched with cold DPBS containing 50 mM glycine at 4°C for two 5-minute washes. To determine total biotinylated receptors, cultured neurons were immediately scraped into 0.5%SDS-PIRA lysis buffer containing (in mM): 50 Tris, 150 NaCl, 2 EDTA, 50 NaF, and 1% TritonX-100, pH7.4 and a protease inhibitor mixture. To determine endocytosis rate, another set of neurons were incubated with conditioned media for 30 minutes at 37°C to allow constitutive trafficking of AMPARs. As a control, a final subset of neurons was subjected to glutathione-containing cleavage buffer immediately. The cultures subjected to the 30-minute incubation with conditioned media were then cooled to 4°C and incubated with glutathione-containing cleavage buffer containing (in mM): 50 glutathione, 75 NaCl, 10 EDTA, 75 NaOH, and 1%BSA in H<sub>2</sub>O, pH8.5, for three 15-minute washes at 4°C. The cleavage reaction was then quenched with two 5-minute washes of 5mg/ml iodoacetamide in cold DPBS (pH 8.5). Neurons were then lysed for 3 minutes in 0.5%SDS-RIPA buffer. The lysates were centrifuged at 16,000 x g for 15 minutes at 4°C. The volume of the lysate containing 300  $\mu$ g of protein from the supernatant was brought to 1 ml of volume with 0.1%SDS-RIPA buffer. Biotinylated

receptors were precipitated with a 70  $\mu$ l NeutrAvidin-agarose (Thermo Scientific) slurry overnight at 4°C, then washed with 0.1%SDS-RIPA buffer three times. NeutraAvidin-agarose-bound receptors were eluted into the sample buffer (6 M Urea, 0.8 M  $\beta$ -mercaptoethanol, 6%SDS, 20% glycerol, 25 mM Tris-HCl, pH 6.8, 0.1% bromophenol blue). Biotinylated receptors were detected by western blot analyses. The levels of surface receptors and internalization ratio were compared by normalizing the signal to that of GFP-expressing lentivirus infected sister cultures with equal protein loading. Antibodies were used at 1:500 dilution with anti-GluA1 (Abcam), anti-SAP97 (Neuromab), and anti-TfR (Invitrogen); at 1:1000 dilution with anti-PSD-95 (Thermo Scientific), and at 1:5000 dilution with anti-tubulin (Sigma). IRDye 800CW and 680LT Secondary antibodies (Licor) were used at 1:5000 dilution for detection on an Odyssey IR laser Scanner (Licor). One-way ANOVA followed by a Tukey-Test was used for data analyses.

## **Results**

### **SAP97 $\beta$ rescues the decrease in mini frequency caused by PSD-95 knockdown**

To study the effect of SAP97 $\beta$  on basic synaptic properties, we overexpressed SAP97 $\beta$  while concurrently knocking down the expression of endogenous PSD-95 using a lentivirus-mediated molecular replacement strategy in CA1 neurons in organotypic hippocampal slice cultures (Schlüter et al. 2006). We recorded miniature AMPAR excitatory postsynaptic currents (AMPA mEPSCs) simultaneously from pairs of cells consisting of an infected cell and an uninfected neighboring cell. We used a newly developed routine, adapted from (Phillips et al. 2011), to analyze mini data by comparing the distributions of mini amplitude and frequency of mini events. Consistent with

previous studies, acute knockdown of PSD-95 (by sh95) decreased mini frequency and did not affect mini amplitude (Figure 1A, values and subsequent mEPSC analyses values were shown in Table 1). The mini frequency of neurons with PSD-95-to-SAP97 $\beta$  replacement was maintained at a level similar to that of uninfected neighboring cells, and the mini amplitude was increased (Figure 1B). SAP97 $\beta$  replacement therefore rescued the effects of acute knockdown of PSD-95, restoring mini frequency to baseline levels. This rescue of mini frequency could be due to an effect on either release probability or the number of active synapses. To test whether the presynaptic release probability was affected by the manipulations of scaffold proteins in the postsynaptic neurons, we measured the paired pulse ratio (PPR) of postsynaptic responses from simultaneously recorded pairs of an uninfected neuron and an infected neighboring neuron. The paired pulse stimulation was delivered to the Schaffer Collateral afferents with a 50 ms inter-stimulus interval. Neither acute knockdown of PSD-95 nor the PSD-95-to-SAP97 $\beta$  replacement affected the PPR (sh95, n = 17 pairs, uninfected,  $1.29 \pm 0.08$ , infected,  $1.31 \pm 0.12$ ,  $p > 0.5$ , Figure 1C; PSD-95-to-SAP97 $\beta$  replacement, n = 15 pairs, uninfected,  $1.31 \pm 0.07$ , infected,  $1.27 \pm 0.10$ ,  $p > 0.5$ , Figure 1D). Consistent with previous studies (Schlüter et al. 2006), these results suggest that the postsynaptic manipulations do not influence presynaptic release properties with these expression and recording conditions. Therefore, the effects on mini frequency by acute knockdown of PSD-95 and by PSD-95-to-SAP97 $\beta$  replacement were most likely due to changes in the number of active synapses.

**The SAP97 $\beta$ -mediated rescue in mini frequency is dependent on NMDAR activity**

Previous work has shown that the SAP97 $\beta$ -mediated rescue of the reduction of eEPSCs induced by PSD-95 knockdown depends on NMDAR activity (Schlüter et al. 2006). We therefore asked whether both the rescue of mini frequency and the increase in mini amplitude depend on NMDAR activity. We analyzed mini events in slices treated with D-APV to block NMDAR activity (treated from the day after virus injection until the time of recording). Chronic D-APV treatment in organotypic slice cultures increased both mini frequency and mini amplitude in CA1 pyramidal cells (Figure 2A). Neurons with knockdown of PSD-95 had significantly lower mini frequency and mini amplitude compared to their uninfected neighbors (Figure 2B). The mini frequency in neurons with PSD-95-to-SAP97 $\beta$  replacement was lower than that of simultaneously recorded uninfected neighboring neurons, whereas the mini amplitude was not significantly different (Figure 2C).

To test whether the difference in mini frequency was due to changes in release probability, we measured PPR from simultaneously recorded infected and uninfected neuron pairs. With the chronic D-APV treatment, the PPR was not affected by the knockdown of PSD-95 or the PSD-95-to-SAP97 $\beta$  replacement (sh95,  $n = 15$  pairs, uninfected,  $1.42 \pm 0.10$ , infected,  $1.58 \pm 0.10$ ,  $p > 0.05$ , Figure 2D; SAP97 $\beta$  replacement,  $n = 21$  pairs, uninfected,  $1.61 \pm 0.11$ , infected,  $1.54 \pm 0.10$ ,  $p > 0.5$ , Figure 2E), suggesting that these molecular manipulations do not influence presynaptic release properties. The changes in mini frequency are most likely due to changes in the number of active synapses. Overall, our results suggest that the rescue of active synapse numbers by PSD-95-to-SAP97 $\beta$  replacement is dependent on NMDAR activity. This NMDAR-dependent rescue of active synapse numbers can account for the NMDAR-dependent rescue of AMPAR eEPSCs by PSD-95-to-SAP97 $\beta$  replacement in previous studies (Schlüter et al. 2006).

### **SAP97 $\beta$ rescue of mini amplitudes is not fully dependent on NMDAR activity**

We next examined the effect of SAP97 $\beta$  on mini amplitudes. We observed a decrease in mini amplitudes in neurons with knockdown of PSD-95 and with chronic treatment of D-APV. This decrease was rescued in neurons with PSD-95-to-SAP97 $\beta$  replacement, which showed mini amplitudes at similar levels to those in simultaneously recorded uninfected neurons (Figure 2C). However, these neurons did not exhibit increased mini amplitudes relative to control neurons (Figure 1C), in contrast to the results observed without D-APV. These results suggest that the effect of PSD-95-to-SAP97 $\beta$  replacement on mini amplitudes is at least partially independent of NMDAR activity, as it could rescue mini amplitudes even during NMDAR blockade. However, because mini amplitudes did not increase compared to control neurons when NMDAR activity was blocked, these results suggest that NMDAR activity is required for PSD-95-to-SAP97 $\beta$  replacement to enhance mini amplitudes. These findings suggest a complex dependency on NMDAR activity, where SAP97 $\beta$  can rescue mini amplitude to baseline levels independently of NMDARs, but not enhance it further.

### **SAP97 $\beta$ replacement increases mini amplitude via recruiting GluA1-homomeric receptors**

Previous studies have suggested that SAP97 specifically interacts with the C-terminus of the GluA1 subunit and facilitates trafficking of GluA1-containing AMPARs to the neuronal surface (Leonard et al. 1998; Rumbaugh et al. 2003). We therefore tested whether this increase in mini amplitudes was sensitive to NASPM, an AMPAR receptor blocker that specifically blocks calcium permeable, GluA2-lacking (presumably GluA1-homomeric) AMPARs. Slices were treated with 10 or 50  $\mu$ M NASPM for 30 minutes before recording,

and NASPM was present throughout the recordings. We found that NASPM treatment blocked the increase in mini AMPAR amplitudes seen previously in neurons with PSD-95-to-SAP97 $\beta$  replacement, but did not influence the mini frequency (Figure 2F). These results suggest that in neurons with PSD-95-to-SAP97 $\beta$  replacement, all active synapses contained a proportion of GluA2 containing heteromeric AMPARs at the comparable level to that in uninfected control neurons. However, they also contained a small proportion of GluA1-homomeric receptors, leading to the increase in mini amplitude.

Our results so far show that SAP97 $\beta$  replacement of endogenous PSD-95 specifically maintains the number of active synapses dependent on NMDAR activation, while its effect on enhancing the unitary synaptic response is not fully dependent on NMDAR activity. The enhanced unitary synaptic response most likely is due to an increase in the number of NASPM-sensitive AMPARs at each active synapse, whereas the number of NASPM-insensitive AMPARs remains at control levels.

### **PSD-95-to-PSD-95 $\alpha$ replacement increases both the number and strength of active synapses independently of NMDAR activity**

How does SAP97 $\beta$  differ from PSD-95 $\alpha$  in regulating basic characteristics of AMPAR-mediated synaptic transmission? To test this, we overexpressed PSD-95 $\alpha$  while simultaneously knocking down the expression of endogenous PSD-95. Consistent with previous overexpression studies (Béïque and Andrade 2003; Ehrlich and Malinow 2004; El-Husseini et al. 2000; Stein et al. 2003), PSD-95-to-PSD-95 $\alpha$  replacement increased mini frequency dramatically (Figure 3A). We also observed a modest but significant increase in mini amplitude (Figure 3A). Chronic D-APV treatment did not block the increase in mini frequency and mini amplitudes in cells with PSD-95-to-PSD-95 $\alpha$  replacement

(Figure 3B). Our results suggest that the effects of PSD-95 $\alpha$  on both the number of active synapses and the unitary strength of active synapse are independent of NMDAR activation, consistent with previous studies. In fact, PSD-95 may have contributed to the enhancement of mini amplitude caused by chronic NMDAR blockade, as acute knockdown of endogenous PSD-95 blocked this increase (Figure 2B).

We next tested the NASPM sensitivity of mini amplitudes, and found that with PSD-95-to-PSD-95 $\alpha$  replacement, NASPM treatment blocked the increase in the amplitude of AMPAR mEPSCs but did not affect the increase the mini frequency, similar to PSD-95-to-SAP97 $\beta$  replacement (Figure 3C). These results suggest that PSD-95-to-PSD-95 $\alpha$  replacement recruits GluA1 homomeric receptors to active synapses, as was seen with PSD-95-to-SAP97 $\beta$  replacement.

Our data thus far delineates the distinct effects of replacing endogenous PSD-95 with either SAP97 $\beta$  or PSD-95 $\alpha$  on regulating basic synaptic properties. PSD-95-to-SAP97 $\beta$  replacement can only create and maintain active synapses in the presence of NMDAR activity; whereas PSD-95-to-PSD-95 $\alpha$  replacement can do so regardless of NMDAR activity. Both SAP97 $\beta$  and PSD-95 $\alpha$  replacement increased mini amplitude by recruiting NASPM-sensitive AMPARs to active synapses. This increase was not fully dependent on NMDAR activity, as mini amplitudes were either maintained or increased by SAP97 $\beta$  or PSD-95 $\alpha$  replacement of endogenous PSD-95 when NMDAR activity was chronically blocked.

### **SAP97 $\beta$ rescues surface AMPARs whereas PSD-95 $\alpha$ impacts synaptic AMPARs**

SAP97 has been shown to directly interact with the AMPAR subunit GluA1 (Leonard et al. 1998; Rumbaugh et al. 2003; Sans et al. 2001), and regulate the dynamics of synaptic

AMPA receptors (Waites et al. 2009). We therefore used western blots to test the influence of acute knockdown of PSD-95, PSD-95-to-SAP97 $\beta$  replacement, and PSD-95 $\alpha$  replacement on total GluA1 levels and surface GluA1 levels. Such quantification of changes in total protein and surface levels was not feasible with conventional transfection approaches because of the low transfection efficiency in neuronal cultures. In this case, because lentiviral infection could reach over 95% transducing efficacy visualized by the percentage of GFP positive neurons, quantitative western blot analysis was possible. Acute knockdown of PSD-95 or its replacement with PSD-95 $\alpha$  or SAP97 $\beta$  did not significantly influence total GluA1 levels compared to GFP infected cultures, even though there was a trend of decrease with acute knockdown of PSD-95, and a trend of increase with PSD-95-to-SAP97 $\beta$  and PSD-95-to-PSD-95 $\alpha$  replacement (Figure 4A and B, SAP97 $\beta$  replacement v.s. GFP,  $p > 0.05$ ,  $n = 10$ ,  $1.22 \pm 0.28$ ; PSD-95 $\alpha$  replacement v.s. GFP,  $p > 0.05$ ,  $n = 10$ ,  $1.31 \pm 0.31$ ; sh95 v.s. GFP,  $p > 0.05$ ,  $n = 9$ ,  $0.83 \pm 0.31$ , one-way ANOVA, followed by Tukey Test). Although acute knockdown of PSD-95 did not significantly influence the total GluA1 level, the manipulation significantly decreased surface GluA1 levels as measured using a biotinylation assay in dissociated cortical neuron cultures (Figure 4C and D, sh95 v.s. GFP,  $p < 0.05$ ,  $n = 7$ ,  $0.55 \pm 0.10$ , one-way ANOVA, followed by Tukey Test), correlated with the decrease of synaptic AMPAR-mediated response measured in slice culture preparations. PSD-95-to-SAP97 $\beta$  replacement rescued the decrease of surface GluA1 levels caused by acute knockdown of endogenous PSD-95 (Figure 4C and D, PSD-95-to-SAP97 $\beta$  replacement v.s. sh95,  $p < 0.05$ ,  $n = 8$ ,  $1.23 \pm 0.15$ , one-way ANOVA, followed by Tukey Test). In contrast, the influence of PSD-95-to-PSD-95 $\alpha$  replacement on surface GluA1 levels was not as pronounced (PSD-95-to-PSD-95 $\alpha$  replacement v.s. sh95,  $p > 0.05$ , v.s. GFP,  $p > 0.05$ ,  $n = 8$ ,  $0.85 \pm 0.10$ , one-way ANOVA, followed by Tukey Test). Acute knockdown of PSD-95, PSD-95-to-SAP97 $\beta$  or PSD-95 $\alpha$  replacement



did not influence surface TfR levels when compared to GFP-infected neurons (Figure 4C and E, sh95,  $n = 7$ ,  $1.23 \pm 0.15$ ; SAP97 $\beta$  replacement,  $n = 8$ ,  $1.29 \pm 0.09$ ; PSD-95 $\alpha$  replacement,  $n = 8$ ,  $1.33 \pm 0.07$ , one-way ANOVA). Constitutive endocytosis of GluA1-containing receptors was also not affected by acute knockdown of PSD-95 or replacement with SAP97 $\beta$  or PSD-95 $\alpha$  when compared to GFP-infected neurons (Figure 4F and G, sh95,  $n = 6$ ,  $0.91 \pm 0.25$ ,  $p > 0.05$ ; SAP97 $\beta$  replacement,  $n = 6$ ,  $1.32 \pm 0.43$ ,  $p > 0.05$ ; PSD-95 $\alpha$  replacement,  $n = 6$ ,  $1.10 \pm 0.40$   $p > 0.05$ , one-way ANOVA). These results suggest that PSD-95-to-SAP97 $\beta$  replacement may rescue the decrease of synaptic AMPARs caused by loss of PSD-95 by rescuing surface GluA1-containing AMPARs, whereas PSD-95-to-PSD-95 $\alpha$  replacement may have significant impact on synaptic AMPARs rather than on total surface AMPAR levels.

## Discussion

Taken together, we show that PSD-95 $\alpha$  or SAP97 $\beta$  have overlapping but distinct effects in regulating AMPAR synaptic properties. First, SAP97 $\beta$ -mediated rescue of active synapse numbers is dependent on NMDAR activity, whereas PSD-95 $\alpha$ -mediated rescue is not. Notably, the effect of PSD-95-to-PSD-95 $\alpha$  replacement on AMPAR synaptic transmission was not as pronounced during NMDAR blockade as it was in the control condition. This difference may be due to the significant increase in the mEPSC amplitude and frequency in uninfected cells in the D-APV-treated condition (Figure 3A). We did not detect significant differences between cells with PSD-95-to-PSD-95 $\alpha$  replacement in the D-APV-treated condition and in the control condition ( $p = 0.24$  for the mEPSC amplitude comparison,  $p = 0.69$  for the mEPSC frequency, Student's t-test). Furthermore, there was a significant increase in the mEPSC amplitude and frequency

between cells with PSD-95-to-PSD-95 $\alpha$  replacement and cells with knockdown of PSD-95 in the D-APV-treated condition ( $p < 0.001$  for the mEPSC amplitude comparison, and  $p < 0.01$  for the mEPSC frequency comparison, Student's t-test). These analyses suggest that the effect of PSD-95 $\alpha$  on AMPAR synaptic transmission is likely independent of NMDAR activity. The lack of further increase in the D-APV-treated condition may be due to the limited level of lentivirus-mediated PSD-95 $\alpha$  expression. If postsynaptic calcium influx is required for the effect of PSD-95 $\alpha$  on synaptic transmission, other calcium sources may be at play such as voltage-gated calcium channels and intracellular calcium store. Acute NASPM treatment did not influence the effects of the replacement strategy on mini frequency, but decreased mini amplitudes to the level of control cells, suggesting that only a fraction of synaptic AMPARs at any given active synapse are NASPM-sensitive, presumably GluA1 homomeric receptors. Because our recordings were performed several days after the virus injection, and the overexpression of PSD-95 $\alpha$  or SAP97 $\beta$  happened concurrently if not preceding the knockdown of PSD-95, our analyses only reflected the end results of the molecular replacement on synaptic properties. Future experiments with precise temporal control of knocking-down PSD-95 and replacement with other MAGUK proteins will provide more insight on how MAGUK proteins regulate the creation and maintenance of active synapses.

Our western blot analyses showed that acute knockdown of PSD-95 significantly reduced surface levels of GluA1. This decrease was rescued by PSD-95-to-SAP97 $\beta$  replacement, suggesting that SAP97 $\beta$  replacement can upregulate the surface level of GluA1-containing AMPARs. PSD-95-to-PSD-95 $\alpha$  replacement can presumably increase synaptic anchoring points, thereby increasing synaptic AMPAR content without influencing the level of surface GluA1-containing AMPARs as significantly as PSD-95-to-SAP97 $\beta$  replacement.

Recent studies have identified several proteins physically associated with pore-forming GluA subunits in the AMPAR complex, including the transmembrane AMPAR regulatory proteins (TARPs) (Chen et al. 2000; Tomita et al. 2003), the cornichons (CNIHs) (Schwenk et al. 2009), CKAMP44 (von Engelhardt et al. 2010) and GSG1-I (Schwenk et al. 2012), all of which change the processing of AMPARs (Coombs and Cull-Candy 2009; Kato et al. 2010; Schwenk et al. ; Schwenk et al. 2009; Straub and Tomita 2011; von Engelhardt et al. 2010). Moreover, using quantitative proteomic analyses, Schwenk et al. have shown that native AMPAR complexes display a high degree of molecular diversity regarding the abundance and stability of associated proteins. It has been suggested that interactions between different AMPAR subunits and CNIHs vs TARPs may control subunit-specific trafficking of AMPARs and the strength and kinetics of AMPAR-mediated synaptic transmission (Herring et al. 2013). It is possible that PSD-95 $\alpha$  and SAP97 $\beta$  recruit different AMPAR complexes with distinct constituents of AMPAR subunits and associated proteins that exhibit different trafficking properties. Further studies will determine whether there is a differential association of PSD-95 $\alpha$  and SAP97 $\beta$  with different AMPAR complexes.

In conclusion, our results demonstrate that PSD-95 $\alpha$  and SAP97 $\beta$  have distinct effects on glutamatergic synaptic properties, including activity-dependent regulation of active synapse number and unitary synaptic AMPAR response. SAP97 $\beta$  enhances the number of AMPAR-containing synapses in an NMDAR-dependent manner, while its effect on the size of unitary synaptic response is not fully dependent on NMDAR activity. These effects contrast with those of PSD-95 $\alpha$ , which increases both the number of AMPAR-containing synapses and the size of unitary synaptic responses, with or without NMDAR activity. The molecular diversity of PSD-MAGUK family proteins provides rich molecular substrates for differential regulation of experience-dependent plasticity in glutamatergic synapses in the brain.

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## Figure Legends

**Figure 1.** Replacement of endogenous PSD-95 with SAP97 $\beta$  rescued AMPAR mEPSC frequency and enhanced mini amplitudes due to changes in postsynaptic properties. Sample traces of AMPAR mEPSCs (left panel), cumulative distribution function (CDF) plots of AMPAR mEPSC amplitude (middle panel) and inter-event interval (right panel) of neurons expressing sh95+eGFP (**A**) or sh95+eGFP::SAP97 $\beta$  (**B**) in red were shown in comparison to those of uninfected control neurons in black in hippocampal slice cultures. Acute knockdown of PSD-95 with sh95 decreased mEPSC frequency but did not influence mEPSC amplitude (**A**). Replacement of endogenous PSD-95 with SAP97 $\beta$  rescued AMPAR mEPSC frequency and enhanced mEPSC amplitude (**B**). Acute knockdown of endogenous PSD-95 (**C**) and replacement of endogenous PSD-95 with SAP97 $\beta$  (**D**) did not affect paired pulse ratio. Sample traces (left panel) of AMPAR eEPSCs of an infected neuron (in red) were shown in comparison to those of the simultaneously recorded, uninfected control neuron (in black). Paired pulse ratios from simultaneously recorded pairs of neurons were shown in black, the mean values ( $\pm$  S.E.M) were shown in red in the right panel. In this and subsequent figures, unless otherwise indicated, sample traces were taken from a pair of simultaneously recorded neurons unless otherwise indicated. For mini frequency and mini amplitude analyses, a typical cell CDF was plotted, showing the median (thick trace) and 95% confidence interval (2.5<sup>th</sup> and 97.5<sup>th</sup> percentile boundary with thin traces with shaded area) for each group, computed with non-parametric bootstrap. Amplitudes (inset of the middle panel) and frequencies (inset of the left panel) of paired cells were plotted with black symbols connected with lines. Box plots (insets) are shown with median, 25<sup>th</sup>, and 75<sup>th</sup> percentiles, with whiskers extending to the most extreme data points that are not considered outliers. Numbers of pairs and *p* values calculated with signed rank test are

shown in the insets. For paired pulse ratio analyses, paired student's t-test was used to calculate  $p$  values.

**Figure 2.** Replacement of endogenous PSD-95 with SAP97 $\beta$  rescued AMPAR mEPSC frequency dependent on NMDAR activity, and enhanced mini amplitudes via recruiting NASPM-sensitive AMPARs. Chronic NMDAR blockade (Chronic D-APV treatment) increases AMPAR mEPSC frequency and amplitude (**A**). Sample traces of AMPAR mEPSCs (left panel), cumulative distribution function (CDF) plots of AMPAR mEPSC amplitude (middle panel) and inter-event interval (right panel) of neurons in slice cultures treated with D-APV (in blue) were shown in comparison to those of uninfected neurons in control slice cultures (in black). Amplitudes (inset of the middle panel) and frequencies (inset of the left panel) were plotted with box plots.  $p$  values were calculated with unpaired signed rank test. Sample traces of AMPAR mEPSCs (left panel), cumulative distribution function (CDF) plots of AMPAR mEPSC amplitude (middle panel) and inter-event interval (right panel) of neurons expressing sh95+eGFP (**B**) or sh95+eGFP::SAP97 $\beta$  (**C** and **F**) in red were shown in comparison to those of uninfected control neurons in black in hippocampal slice cultures treated with D-APV (**B** and **C**), or recorded in NASPM (**F**). With chronic blockade of NMDARs (D-APV treatment), acute knockdown of endogenous PSD-95 decreased AMPAR mEPSC frequency and amplitude (**B**), and replacement with SAP97 $\beta$  failed to rescue mEPSC frequency and occludes the increase of mEPSC amplitude (**C**). Acute knockdown of endogenous PSD-95 (**D**) and replacement of endogenous PSD-95 with SAP97 $\beta$  (**E**) did not affect paired pulse ratio with chronic blockade of NMDARs. The symbol “+” indicates the outlier of the data points which was included in the data analyses. NASPM blocked the increase of AMPAR mEPSC amplitude caused by SAP97 $\beta$  replacement of endogenous PSD-95, but not the rescue of AMPAR mEPSC frequency (**F**).

**Figure 3.** Replacement of endogenous PSD-95 with PSD-95 $\alpha$  enhanced AMPAR mEPSC frequency independent of NMDAR activity, and enhanced mini amplitude via recruiting NASPM-sensitive AMPARs. Sample traces of AMPAR mEPSCs (left panel), cumulative distribution function (CDF) plots of AMPAR mEPSC amplitude (middle panel) and inter-event interval (right panel) of neurons expressing sh95+ PSD-95 $\alpha$ ::eGFP in red were shown in comparison to those of uninfected control neurons in black in hippocampal slice cultures (**A**). Amplitudes (inset of the middle panel) and frequencies (inset of the left panel) of paired cells were plotted with black symbols connected with lines. With chronic blockade of NMDARs (D-APV treatment), replacement with PSD-95 $\alpha$  still enhanced mEPSC frequency and amplitude (**B**). NASPM blocked the increase of AMPAR mEPSC amplitude caused by PSD-95 $\alpha$  replacement of endogenous PSD-95, but not the enhancement of AMPAR mEPSC frequency. The symbol “+” indicates the outlier of the data points which is included in the data analyses (**C**).

**Figure 4.** Replacement of endogenous PSD-95 with SAP97 $\beta$  compensated the decrease of surface GluA1 caused by acute knockdown of endogenous PSD-95. Surface proteins in cultured cortical neurons were biotinylated at DIV 18-21 and pulled down using NeutrAvidin-agarose beads. Total Cell Lysate (**A** and **B**) and Biotinylated receptors (**C-E**) were assayed using western blots. Total GluA1 levels were not significantly affected by infections with GFP, acute knockdown of PSD-95 (sh95), and replacement with SAP97 $\beta$  and PSD-95 $\alpha$  (SAP97 $\beta$ RP and PSD-95 $\alpha$ RP, **A** and **B**). Acute knockdown of PSD-95 decreased surface GluA1 levels and replacement with SAP97 $\beta$  compensated the decrease of surface GluA1 levels (**C**). Acute knockdown of PSD-95 and replacement with SAP97 $\beta$  and PSD-95 $\alpha$  did not influence surface transferrin receptor (TfR) levels (**C** and **E**). Constitutive endocytosis of GluA1-containing receptors was not significantly affected by acute knockdown of PSD-95 and replacement with

SAP97 $\beta$  and PSD-95 $\alpha$  (**F**, sample blots, **G**, accumulative data). Bar graphs show mean  $\pm$  SEM. \*\*,  $p < 0.01$ , with one-way ANOVA, followed by Tukey test.

### **Table Legend**

Mean amplitudes and frequencies are the maximum likelihood estimates from model-based analyses. The difference between 2 groups, 95% confidence intervals, and  $P$  values are shown. The Wilcoxon signed rank test was used for pairwise comparisons to calculate whether the difference between the pairs was significantly different from zero. The Wilcoxon rank sum test was used for unpaired 2-group comparisons. Statistical significance was assessed at  $p < 0.05$ . D-APV, D-aminophosphonovaleric acid; EPSC, excitatory postsynaptic current; NASPM, 1-naphthyl acetyl spermine. See text for detailed description of groups.