Activity-dependent regulation of synaptic strength by PSD-95 in CA1 neurons

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Zhang P, Lisman JE. Activity-dependent regulation of synaptic strength by PSD-95 in CA1 neurons. J Neurophysiol 107: 1058–1066, 2012. First published November 23, 2011; doi:10.1152/jn.00526.2011.—CaMKII and PSD-95 are the two most abundant postsynaptic proteins in the postsynaptic density (PSD). Overexpression of either can dramatically increase synaptic strength and saturate long-term potentiation (LTP). To do so, CaMKII must be activated, but the same is not true for PSD-95; expressing wild-type PSD-95 is sufficient. This raises the question of whether PSD-95’s effects are simply an equilibrium process [increasing the number of AMPA receptor (AMPA) slots] or whether activity is somehow involved. To examine this question, we blocked activity in cultured hippocampal slices with TTX and found that the effects of PSD-95 overexpression were greatly reduced. We next studied the type of receptors involved. The effects of PSD-95 were prevented by antagonists of group I metabotropic glutamate receptors (mGluRs) but not by antagonists of nonsynaptic glutamate receptors. The inhibition of PSD-95-induced strengthening was not simply a result of inhibition of PSD-95 synthesis. To understand the mechanisms involved, we tested the role of CaMKII. Overexpression of a CaMKII inhibitor, CN19, greatly reduced the effect of PSD-95. We conclude that PSD-95 cannot itself increase synaptic strength simply by increasing the number of AMPAR slots; rather, PSD-95’s effects on synaptic strength require an activity-dependent process involving mGlur and CaMKII.

AMPA receptor current; metabotropic glutamate receptor; Ca2+/calmodulin-dependent protein kinase II

POSTSYNAPTIC DENSITY PROTEIN 95 (PSD-95) is a major component of the postsynaptic density (PSD) (Chen et al. 2005; Cheng et al. 2006; Sugiyama et al. 2005). The protein interacts with a wide variety of membrane and cytoplasmic proteins to form a large signaling complex (for reviews, see Kim and Sheng 2004; Xu 2011; Zheng et al. 2011a). A major functional role of PSD-95 is to act as a “slot” protein for AMPA receptors (AMPARs): It binds to the stargazin-like transmembrane AMPAR regulatory protein (TARP) and thereby anchors AMPARs at the synapse (particularly the GluR1-containing forms) (Ehrlich and Malinow 2004; Elias et al. 2006). Recent work has directly visualized the complex of PSD-95 with AMPAR (Chen et al. 2008).

Overexpression of PSD-95 strongly affects synaptic function; the amplitude of the excitatory postsynaptic current (EPSC) is increased up to fourfold (Beique and Andrade 2003; Ehrlich and Malinow 2004; Elias et al. 2008; Schnell et al. 2002). This increase involves the same strengthening processes that occur during long-term potentiation (LTP), because synapses strengthened by PSD-95 overexpression cannot be further potentiated by LTP induction protocols (Ehrlich and Malinow 2004; Stein et al. 2003). Conversely, reducing PSD-95 expression decreases the EPSC (Ehrlich et al. 2007; Elias et al. 2006). These results indicate that changing the expression of PSD-95 alters core processes that control synaptic strength, making it important to understand the mechanisms involved.

As with PSD-95, overexpression of CaMKII can increase synaptic strength in a manner that occludes LTP (Lledo et al. 1995; Pettit et al. 1994; Pi et al. 2010a, 2010b). However, unlike CaMKII, which must be activated to produce strengthening (Lledo et al. 1995; Pi et al. 2010b), overexpression of unmodified PSD-95 is sufficient to produce its dramatic effects. One possible explanation is that PSD-95 produces strengthening by a direct process in which PSD-95 is a limiting factor in the structural assembly of the PSD (Xu 2011). Thus the effects of PSD-95 might be unrelated to the ongoing activity in slice culture (Barria and Malinow 2005; Hayashi et al. 2000). Alternatively, the strengthening produced by PSD-95 may depend on this activity. Although the effect of the β-form of PSD-95 on synaptic strength has been suggested to depend on NMDA receptor (NMDAR) activity, the effects of the predominant form (α-PSD-95, >90%) do not depend on NMDAR activity (Schluter et al. 2006). The effects of other forms of neural activity have not been examined. In this study, we demonstrate that the synaptic strengthening produced by α-PSD-95 is dependent on activity that can be blocked by tetrodotoxin (TTX). In further experiments, we identify some of the mechanisms involved; these include critical roles for metabotropic glutamate receptor (mGluR) and CaMKII.

METHODS

Organotypic hippocampal slice cultures. Experiments utilized Sprague-Dawley rats (postnatal days 6–7). Animals were maintained in the Animal Care Facility at Brandeis University, and all procedures were approved and performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. Rats were decapitated, and the hippocampus was removed under sterile conditions. Hippocampal slices of 400-μm thickness were cut with a tissue chopper. The slices were quickly transferred onto the membrane of six-well plate inserts (0.4-μm pore size; Falcon) and incubated at 35°C in 5% CO2. The culture medium was minimum essential medium (MEM; Invitrogen 11700-077) supplemented with 20% horse serum (Sigma, St. Louis, MO), 1 μg/ml insulin, 2 mM L-glutamine (or glutaMAX; Invitrogen), 25 mM HEPES, 0.2 mM CaCl2, 2 mM MgSO4, 26 mM NaHCO3, 30 mM d-glucose, and 0.5 mM L-ascorbate. The culture medium was changed three times per week.

Single-cell electroporation. Single-cell electroporation (SCE) was performed at 10–16 days in vitro (DIV), as previously described (Haas et al. 2001; Rathenberg et al. 2003). In brief, slices were placed in a sterile SCE chamber filled with rat Ringer solution containing (in mM) 160 NaCl, 5.4 KCl, 12 MgCl2, 2 CaCl2, and 5 HEPES. Glass micropipettes (4–5 MΩ) were filled with 150 ng/μl mCherry (mRFP) plasmid DNA (Asrican et al. 2007) and 200 ng/μl Dendra2-PSD-95 (α-form, abbreviated as D-PSD-95) (Blanpied et al. 2008), 200 ng/μl green fluorescent protein (GFP)-CaMKIIα (Hudmon et al. 2005), or...
200 ng/μl GFP-CN19 plasmid in Ringer solution. CN19 plasmid was constructed by inserting CN19 sequence (Coultrap and Bayer, unpublished observations) into an enhanced GFP (EGFP)-C1 vector (Clontech) between BamHI and XhoI restriction sites. The tip of the micropipette was placed next to the soma of CA1 neurons, and one hundred 1-ms pulses of 10-V amplitude were delivered at 200 Hz. After transfection, hippocampal slices were transferred back to the incubator and incubated for 1–2 days to allow the expression of proteins. In some experiments, drugs were added immediately after transfection and were present for the following 1–2 days. The drugs used include 1 μM TTX (Sigma), 100 μM DL-2-amino-5-phosphopentonic acid (DL-APV; Ascent Scientific, Ellisville, MO), 10 μM 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[2,3]quinoxaline-2,3-dione (NBQX; Sigma), 50 μM (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)propanoic acid (LY341495; Ascent Scientific), 500 μM (RS)-α-methyl-4-carboxyphenylglycine [(RS)-MCPG; Tocris Bioscience, Ellisville, MO), 25 μM 2-methyl-6-(phenylethyl)pyridine hydrochloride (MPEP; Tocris Bioscience), and 100 μM (S)-(+)α-amino-4-carboxy-2-methylbenzeneacetic acid (LY367385; Tocris Bioscience). In some experiments, APV was refreshed after 24 h to ensure its efficacy. Prior to imaging or electrophysiological recording, these drugs were removed by extensive perfusion.

**Electrophysiology.** Slices at 10–18 DIV were continuously superfused (1.8 ml/min) at room temperature with an artificial cerebrospinal fluid (ACSF) solution containing (in mM) 124 NaCl, 2.5 KCl, 4 CaCl₂, 4 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 0.05 picrotoxin, balanced with 95% O₂ and 5% CO₂ (pH 7.4). Glass pipettes of 2–6 MΩ were pulled from borosilicate micropipettes (53432–921; VWR Scientific, West Chester, PA) with a micropipette puller (P-87 Flaming/Brown Micropipette Puller; Sutter Instrument, Novato, CA). The patch pipettes were filled with a solution containing (in mM) 120 Cs-methanesulfonate, 20 CsCl, 10 HEPES, 0.3 Na₃GTP, 0.2 EGTA, and 10 phosphocreatine, pH 7.3. For cell-pair recordings, one transfected CA1 neuron and one neighboring nontransfected neuron within 20-μm distance were selected for whole cell recordings. Cells were held at −65 mV in voltage-clamp mode, and EPSCs were evoked by stimulating Schaffer-collateral pathway via a stimulation electrode placed at 100–200 μm from the cell body layer. The stimuli were delivered every 6 s with a 0.2-ms duration. The intensity of the stimuli (usually between 50 and 300 μA) was adjusted to induce 50- to 100-pA AMPAR EPSC at ~65 mV. The location and intensity of stimuli were always kept the same between a pair of neighboring cells. Series and input resistances were monitored throughout each experiment, and data were rejected if the series resistance varied by >20%. EPSC magnitudes were calculated by averaging a 5-ms time window centered at the peak of the EPSC, and 20–50 EPSCs were averaged.

**Fig. 1.** Overexpression of PSD-95 potentiates AMPA receptor (AMPA) excitatory postsynaptic currents (EPSCs) in hippocampal CA1 neurons. A: CA1 neurons from hippocampal slice culture were transfected to overexpress D-PSD-95 and mCherry (a morphological marker). Cell-pair recordings were performed from a transfected neuron (mCherry fluorescence is shown, left) and a nearby untransfected neuron (right). B1: control, untransfected nearby neuron pairs had similar levels of AMPAR EPSCs (48.1 ± 7.8 pA vs. 54.9 ± 12.1 pA; n = 15, P = 0.40). B2: as another control, overexpression (Oxpr) of green fluorescent protein (GFP)-CaMKIIα and mCherry for 2 days did not affect AMPAR EPSCs (69.7 ± 18.7 vs. 57.3 ± 20.4 pA for untransfected and transfected cells, respectively; n = 6, P = 0.53), consistent with previous findings. WT, wild type. B3–4: in contrast, AMPAR EPSCs were greatly increased by overexpression of D-PSD-95 and mCherry for 1 (31.1 ± 17.7 vs. 77.9 ± 14.8 pA for untransfected and transfected cells respectively; n = 5, P = 0.002) or 2 (41.2 ± 9.3 vs. 156.9 ± 22.3 pA for untransfected and transfected cells, respectively; n = 11, P < 0.001) days. Red dots and crosses in each graph represent means and SE of the AMPAR currents. Insets on right of each graph show sample EPSCs from a pair of nearby cells. EPSCs of transfected cells are red and EPSCs of untransfected cells are black in B2–4. Calibration: 100 pA, 100 ms. C: summary of the effect of PSD-95 overexpression on amplitudes of AMPAR EPSCs in cell-pair recordings. Paired Student’s t-tests were performed to evaluate statistical significance. **P < 0.01; ***P < 0.001. NS, P > 0.05.
For LTP recordings, the slices were immersed in the same ACSF solution, except with a lower picrotoxin concentration (1 μM), which does not induce burstlike responses but allows reliable LTP and long-term depression (LTD) induction (Pi et al. 2010b). LTP was induced by delivering 200 stimulation pulses to the Schaffer collaterals at 2 Hz with a holding potential of 0 mV. EPSCs before and after LTP induction were recorded as described above.

Confocal imaging. Images of transfected CA1 neurons were acquired with an Olympus confocal microscope (FluoView FV300/BX61W1) equipped with a ×60 water immersion objective (1.1 NA, Olympus). Dendra2 and mCherry were sequentially excited with 488- and 543-nm lasers, and 500–550 and 580–680 nm emissions were collected, respectively. The laser intensities and detector gains were kept at the same level for all imaging experiments. Stacks of 10–30 images with 2-μm focal steps were collected at 1× digital zoom. The pixel size for imaging was 0.46 μm. All images in figures are projections of three-dimensional stacks using maximal intensity. For each Z stack, the fluorescent intensities of D-PSD-95 were calculated by averaging intensity in a region of interest in soma. The Z stack with the maximal D-PSD-95 intensity was selected, and the background intensity in a region near to the soma and at the same depth was also calculated. The PSD-95 intensity was corrected by subtracting the background intensity. The ratio of the corrected D-PSD-95 intensity over the background intensity was used to estimate the D-PSD-95 expression level changes, with and without TTX/MCPG. It should be noted that the method used only quantifies the expression levels of ectopically expressed protein. The method is a sensitive measurement of relative PSD-95 level, if we assume that the fraction of ectopically expressed protein is much higher than the endogenous protein.

Statistics. All results are presented as means ± SE. Unpaired or paired Student’s t-tests were performed, and a P value < 0.05 was considered to be statistically different between test groups.

RESULTS

We overexpressed D-PSD-95 (α-form) and a morphological marker (mCherry) in CA1 neurons for 1–2 days. A transfected neuron and an untransfected nearby neuron (distance <20 μm) were recorded in whole cell mode to measure the AMPAR EPSCs in both neurons evoked by the same presynaptic stimulation (Zhu et al. 2000) (at −65 mV; Fig. 1A). If neither cell was transfected, the responses in neighboring cells were similar (average amplitude ratio: 1.14 ± 0.25; Fig. 1, B1 and C). PSD-95 overexpression greatly potentiated AMPAR EPSCs: after 1 day of expression, AMPAR EPSCs from transfected cells were 2.58 ± 0.48 times those of those from untransfected cells (Fig. 1B3); after 2 days of expression, AMPAR EPSCs were increased nearly fourfold (3.81 ± 0.54, Fig. 1, B3–4 and C). In contrast, although activated CaMKII also potentiates AMPAR EPSCs (Lledo et al. 1995; Pi et al. 2010b), overexpression of CaMKIIα in unactivated form had no significant effect (0.82 ± 0.29 times controls; Fig. 1, B2 and C), as previously reported (Pi et al. 2010b). Therefore, the strengthening produced by PSD-95 is a specific result of PSD-95 overexpression.

To test whether neural activity is required for α-PSD-95 to produce strengthening, we incubated transfected slices in 1 μM TTX for 2 days and then measured AMPAR EPSCs (TTX was removed when measurements were made). Under these conditions, overexpression of PSD-95 failed to potentiate AMPAR EPSCs of untransfected cells are black. B: summary of the amplitude of EPSCs in pairs of transfected and untransfected neurons. Overexpression of PSD-95 for 2 days potentiates synaptic strength (left, also shown in Fig. 1B4; n = 11), whereas TTX abolished the effect of PSD-95 overexpression (right). C: PSD-95 overexpression occluded LTP in CA1 neurons (n = 15, control; n = 8, PSD-95). Insets on right show EPSCs from a representative cell before LTP induction (1) and after LTP induction (2). D: the presence of TTX (1 μM) restored the ability to induce LTP in PSD-95-overexpressing neurons (n = 8 for PSD-95-overexpressing cells in TTX, n = 6 for untransfected cells in TTX). Insets on right show EPSCs from a representative cell before LTP induction (1) and after LTP induction (2). E: LTP was occluded by PSD-95 overexpression without the presence of TTX during incubation, but LTP could be induced when slices were treated with TTX during incubation. Traces were extracted from C and D to show the role of TTX in LTP induction of PSD-95-expressing cells. Calibration for A, C, and D: 100 pA, 100 ms. Paired Student’s t-tests were performed to evaluate statistical significance. ***P < 0.001. NS, P > 0.05.
EPSCs (1.25 ± 0.17 times controls; Fig. 2, A and B), arguing for a role of activity in the strengthening process. Since overexpression of PSD-95 also occludes LTP (Ehrlich and Malinow 2004; Stein et al. 2003), we tested whether this occlusion was prevented by TTX. Figure 2D shows that, in TTX, LTP of PSD-95-overexpressing cells was similar to that of untransfected cells. Thus the LTP occlusion produced by PSD-95 overexpression is also activity dependent.

We next investigated whether the activity that is necessary for PSD-95-induced strengthening involves ionotropic glutamate receptors, the block of which may affect PSD-95 expression and spine stability (De Roo et al. 2008). We found that blocking NMDARs (100 μM APV) and AMPARs (10 μM NBQX) did not block PSD-95-induced strengthening (3.33 ± 0.42 times greater than controls, close to the 3.81 ± 0.54-fold strengthening without APV and NBQX; Fig. 3, A1–2 and B). This is consistent with the report by Schluter et al. (2006) showing that the effects of the α-form (but not the less prevalent β-form) of PSD-95 are insensitive to NMDAR blockade.

We next examined the role of mGluRs. Group I and II mGluRs are the predominant forms in the hippocampus, with group I mostly in CA1 neurons and group II mostly in CA3 neurons (Petralia et al. 1996; Rafael et al. 1996; Shigemoto et al. 1997).

**Fig. 3. Inhibiting group I/II metabotropic glutamate receptors (mGluRs) prevents PSD-95-induced synaptic strengthening.** A: CA1 neurons were transected to overexpress D-PSD-95 and mCherry and were incubated in various glutamate receptor inhibitors for the following 2 days. Cell-pair recordings were performed from a transfected neuron and a nearby untransfected neuron. A1: NMDA receptor (NMDAR) inhibitor (100 μM APV) did not prevent PSD-95-induced synaptic strengthening (19.0 ± 5.9 pA vs. 117.9 ± 16.4 pA; n = 7, P < 0.001). A2: blocking both NMDAR (100 μM APV) and AMPAR (10 μM NBQX) also did not block synaptic strengthening by PSD-95 (45.2 ± 6.5 vs. 150.4 ± 19.9 pA; n = 11, P < 0.001). A3: blocking NMDAR (APV), AMPAR (NBQX), and mGluR (primarily group II; 50 μM LY341495) did not block synaptic strengthening by PSD-95 (95.3 ± 17.0 and 186.5 ± 17.6 pA for untransfected and transfected cells, respectively; n = 9, P = 0.006). A4: blocking mGluR (primarily group I; 500 μM MCPG) significantly reduced synaptic strengthening by PSD-95 (57.0 ± 12.3 and 88.3 ± 9.9 pA for untransfected and transfected cells, respectively; n = 22, P = 0.014). A5: blocking NMDAR (APV), AMPAR (NBQX), and mGluR (MCPG) almost completely abolished synaptic strengthening by PSD-95 (85.7 ± 15.2 and 112.0 ± 12.1 pA for untransfected and transfected cells, respectively; n = 15, P = 0.19). Insets on right of each graph show sample EPSCs from a pair of nearby cells. Calibration for A1 and C: 100 pA, 100 ms. Paired Student’s t-tests were performed to evaluate statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001. NS, P > 0.05.
al. 1997). We found that the nonselective group I and II mGluR inhibitor MCPG (500 μM) had a large inhibitory effect on PSD-95-induced strengthening (Fig. 3B): In MCPG, PSD-95 still significantly increased synaptic strength (P < 0.05), but the synaptic strength was only 1.40 ± 0.16 times the control, which is much smaller than 3.81 ± 0.54 times without MCPG (Fig. 1B4). The effect of PSD-95 overexpression was also blocked when combining MCPG, APV, and NBQX (1.31 ± 0.14, a value not significantly different from the untransfected controls). These results suggest that the activity of mGluR is critical to the effects of PSD-95, and that the role of AMPA and NMDA is either small or nonexistent (the data are not sufficient to distinguish these possibilities). By contrast, another mGluR inhibitor, 50 μM LY341495 (a group II mGluR inhibitor but also a relatively weak inhibitor for group I/III mGluR), in addition to blockade of NMDAR/AMPAR, only modestly reduced the effect of PSD-95: Synaptic strength of PSD-95-overexpressing cells was 3.33 ± 0.42 times greater in APV/NBQX but only 2.17 ± 0.19 times greater in APV/NBQX/LY341495 (Fig. 3B). Because MCPG has an inhibitory effect on PSD-95-induced strengthening similar to that of TTX, we asked whether MCPG could similarly block the occlusion of LTP by PSD-95 overexpression. Indeed, we were able to elicit near-normal LTP in PSD-95-overexpressing cells when MCPG was present during incubation (Fig. 3C).

MCPG blocks both group I (mGluR1 and mGluR5) and group II (mGluR2 and mGluR3) mGluRs. To find the specific subtypes of mGluR involved, we used two more specific mGluR inhibitors: MPEP (25 μM, inhibits mGluR5) and LY367385 (100 μM, inhibits mGluR1) (Fig. 4). We found that MPEP but not LY367385 prevented PSD-95 from potentiating synaptic strengthening (synaptic strength of PSD-95-overexpressing cells was 0.80 ± 0.16 times in MPEP but 1.91 ± 0.21 times greater in LY367385; Fig. 4). Therefore, mGluR5 is the mGluR subtype that is required for PSD-95 to potentiate synaptic transmission. This also explains why the nonspecific mGluR inhibitor LY341495 at 50 μM failed to block PSD-95 effects: In slices, 100 μM LY341495 is necessary to block mGluR5 (Fitzjohn et al. 1998).

A few studies suggest some correlation between PSD-95 expression level and either neuronal activity changes (De Roo et al. 2008; Ehlers 2003; Sun and Turrigiano 2011) or mGluR/FMRP (fragile X mental retardation protein) interactions (Muddashetty et al. 2007; Todd et al. 2003; Zalfa et al. 2007). Thus a possible mechanism for the effects of TTX or MCPG could be by limiting PSD-95 expression. To test this possibility, we cotransfected cells with mCherry and D-PSD-95 (Fig. 5). The ratio of corrected (by subtracting background) PSD-95 intensity over background intensity was used to estimate the relative protein expression level. We did find a nearly 40% decrease of the PSD-95 intensity-to-background ratio by comparing controls (17 cells from 5 slices) and TTX-treated cells (19 cells from 5 slices, P = 0.04; Fig. 5, A, B, and D); by contrast, there was a moderate increase of PSD-95 expression by MCPG (~30%), although not reaching statistical significance (31 cells from 4 slices, P = 0.25; Fig. 5, A, C, and D). These changes in the expression are moderate and cannot explain why the effect of PSD-95 is almost totally absent in TTX- or MCPG-treated slices.

CaMKII is activity sensitive and can interact with PSD-95 (Steiner et al. 2008). To test whether CaMKII activity is necessary for PSD-95-induced strengthening, we overexpressed CN19, a CaMKIIe-derived CaMKII inhibitor (Chang et al. 1998; Vest et al. 2007). We transfected two neighboring CA1 neurons, one with CN19 and the other with CN19 plus PSD-95 (this strategy was chosen because CN19 by itself can decrease synaptic strength) (Sanhueza et al. 2011). We found that PSD-95 + CN19 did not significantly increase the synaptic strength, compared with that of CN19-expressing cells (1.35 ± 0.32 times; Fig. 6). Therefore, inhibition of CaMKII activity largely prevents PSD-95 from increasing the synaptic strength, although small effects of PSD-95 may be present because of incomplete blockade of CaMKII.

**DISCUSSION**

In the simplest model, the strengthening produced by PSD-95 would occur because the protein is rate limiting in the assembly of the structure that anchors AMPARs at the synapses. Here, we demonstrate that this is not the case: If synaptic activity is blocked by TTX or MCPG during the period of PSD-95 overexpression (α-form), abundant PSD-95 is still expressed (Fig. 5), consistent with a recent report (Sun and Turrigiano 2011); however, synaptic strength is not increased.
Furthermore, the occlusion of LTP produced by PSD-95 overexpression (presumably because of a saturation effect) is also prevented by TTX or MCPG. Our study is the first direct demonstration that the potentiation produced by PSD-95 overexpression depends on activity. Our findings complement the finding by Ehrlich et al. (2007) showing that decreasing PSD-95 weakens synaptic strength and that this effect is blocked by TTX.

We found that the type of activity required for PSD-95 to strengthen synapses was dependent on TTX but had no clear dependence on AMPARs or NMDARs, consistent with a previous report (Schluter et al. 2006). This suggests that TTX blocks some activities insensitive to AMPAR/NMDAR blockade. Indeed, it has been shown that the intrinsic conductances in guinea pig hippocampal neurons generate spiking even when glutamatergic excitation is blocked (Hablitz and Johnston 1981); a study in rat hippocampal slice culture also shows that there is remaining network activity when synaptic transmission is blocked (Sasaki et al. 2007).

We also find that the effect of PSD-95 overexpression is almost completely blocked by mGluR inhibitors MCPG and MPEP. Both group I and II mGluRs are expressed in CA1 (Petralia et al. 1996; Rafael et al. 1996; Shigemoto et al. 1997). Group I mGluR is required for various types of LTP, including LTP of AMPAR-mediated transmission in hippocampal interneurons (Lapointe et al. 2004), and principal cells of CA1 and granule cells of the dentate gyrus (Francesconi et al. 2004) and LTP of NMDAR-mediated transmission in principal cells of CA1, CA3, and the dentate gyrus (Kwon and Castillo 2008). mGluR is also implicated in regulating PSD-95 expression. A recent study suggests that inhibiting mGluR destabilizes the binding of PSD-95 mRNA and FMRP (fragile X mental retardation protein), resulting in degradation of PSD-95 mRNA and a decrease of PSD-95 expression (Muddashetty et al. 2007; Todd et al. 2003; Zalfa et al. 2007). However, our imaging did not detect a decrease of PSD-95 expression by MCPG.

CaMKII is a major component of PSD and the key regulator of LTP/LTD processes (Lisman et al. 2002). Previous studies show that the CaMKII inhibitor KN-93 does not affect PSD-95-induced synaptic strengthening (Ehrlich and Malinow 2004; Schluter et al. 2006). However, the interpretation of the data is complicated by limitations of the KN family of CaMKII inhibitors. In spines, KN-62 (10 μM) only decreases CaMKII activity by ~40% (Lee et al. 2009). This could be due to the extremely high concentration (~100 μM) of calmodulin (Faas et al. 2011) and CaMKII (Feng et al. 2011) in spines. Since these inhibitors work through competitive binding and their concentration is lower than that of calmodulin and CaMKII, KN compounds will only be partially effective in spines. In the present study, we used CN19, a peptide derivative from the endogenously present CaMKII inhibitor, CaMKII, and a CN19-P95 overexpressing cell (red). Calibration: 50 pA, 50 ms. Paired Student’s t-tests were performed to evaluate statistical significance.

Fig. 6. Inhibition of CaMKII prevents PSD-95’s effects on synaptic strength. A and B: AMPAR EPSCs in CN19+PSD-overexpressing cells are not significantly different from those in CN19-overexpressing cells (CN19: 47.4 ± 6.7, CN19+PSD-95: 64.2 ± 15.3, n = 14, P = 0.24). Insert in A shows representative EPSC recordings from a CN19-overexpressing cell (black) and a CN19+PSD-95-overexpressing cell (red). Calibration: 50 pA, 50 ms. Paired Student’s t-tests were performed to evaluate statistical significance.
activation are critical for the process by which PSD-95 induces strengthening.

Interestingly, blockade of glutamatergic synapse functions with NMDAR and AMPAR inhibitors had little effect on the function of PSD-95 (Fig. 3), although NMDARs allow Ca\(^{2+}\) entry and CaMKII activation (Lee et al. 2009). By contrast, inhibition of mGluR effectively inhibits PSD-95’s effects (Figs. 3 and 4). It is reported that synaptic activation of mGluR, paired with backpropagating action potentials, induces large, wavelike Ca\(^{2+}\) releases in neurons (Nakamura et al. 1999). Such Ca\(^{2+}\) increase can reach a few micromolar, much larger than the Ca\(^{2+}\) elevation induced by an action potential (Nakamura et al. 1999). Furthermore, there is evidence for synaptically induced large Ca\(^{2+}\) elevations in spines. The elevations are mediated by Ca\(^{2+}\) release from synaptic endoplasmic reticulum and are dependent on mGluR rather than NMDAR activity (Holbro et al. 2009). Recent studies show that activation of group I mGluRs can increase CaMKII activity and CaMKII-dependent protein synthesis (Mockett et al. 2011).

Therefore, the Ca\(^{2+}\) elevation by mGluR may cause the CaMKII activation needed for PSD-95-induced strengthening.

Although we have identified some of the molecular processes involved in PSD-95-induced strengthening, there are likely other mechanisms. Nitric oxide (NO) has been implicated in the structural changes induced by PSD-95 overexpression (Nikonenko et al. 2008), and this may occur by CaMKII-dependent phosphorylation of nitric oxide synthase (NOS), the NO synthesizing enzyme (Hayashi et al. 1999; Komeima et al. 2000). Other reactions might involve changes in PSD-95 itself. For instance, there is activity-dependent palmitoylation of PSD-95 (El-Husseini et al. 2002). Also, Kim et al. (2007) demonstrated that TTX activates Rac1-JNK1 activity, leading to phosphorylation of PSD-95 at S295; this induces clustering of PSD-95. Stein et al. (2008) found that CaMKII phosphorylates S73 of PSD-95 (Gardoni et al. 2006), resulting in the activity-dependent trafficking of PSD-95. TTX and MCPG might affect some or all of these processes.

One alternative explanation for the effect of TTX is that the synaptic potentiation by PSD-95 is occluded by TTX-induced synaptic scaling (Turrigiano and Nelson 2004). However, this is unlikely: The effect of PSD-95 on synaptic strength is 300–400% and it is almost totally blocked by TTX. This is much too large an effect to be attributed to known effects of TTX on scaling. In primary cell culture, the effect of TTX on quantal size was only ~100% (Turrigiano et al. 1998). In slice culture preparations similar to that used in our study, TTX- and APV-induced synaptic scaling is much smaller [20–40% increase of miniature EPSC (mEPSC) amplitude] than that in primary cell culture (Aoto et al. 2008; Soden and Chen 2010). Subsequent efforts to observe this scaling in intact tissue have shown it to be small or nonexistent (Echegoyen et al. 2007). Therefore, any TTX-induced scaling is unlikely sufficient to occlude the large effect of PSD-95 overexpression.

PSD-95 is highly concentrated in the PSD area, unlike other PSD proteins, such as SAP102, which are also present in the cytoplasm of spines (Zheng et al. 2011b). Therefore, PSD-95 is an excellent marker that allows visualization of excitatory synapses. However, PSD-95 overexpression has been problematic as a synapse marker because it also alters the structure and function of synapses, leading to LTP saturation (Ehrlich and Malinow 2004; Elias et al. 2008; Schnell et al. 2002; Stein et al. 2003). To avoid synaptic strengthening and morphological changes, some studies have used low-level expression of PSD-95 in labeling synapses (Gray et al. 2006). Low-level expression of PSD-95 makes imaging of synapses difficult and can still affect synaptic strength. Our study provides a labeling method that avoids these problems; by expressing fluorescent PSD-95 while activity is inhibited, efficient labeling can be achieved without saturating synaptic strength.

In conclusion, our results show that PSD-95 affects synaptic strength in an activity-dependent manner, rather than simply increasing the number of AMPAR slots through an equilibrium process. The activity of two key signaling molecules, mGluR and CaMKII, is required for PSD-95 to produce its dramatic increase in synaptic strength.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: P.Z. and J.E.L. conception and design of research; P.Z. and J.E.L. drafted manuscript; P.Z. and J.E.L. edited and revised manuscript; P.Z. and J.E.L. approved final version of manuscript.

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