Cellular/Molecular

Essential Contribution of the Ligand-Binding βB/βC Loop of PDZ1 and PDZ2 in the Regulation of Postsynaptic Clustering, Scaffolding, and Localization of Postsynaptic Density-95

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Postsynaptic density-95 (PSD-95), a PSD-95/Discs large/zona occludens-1 (PDZ) domain-containing scaffold protein, clusters many signaling molecules near NMDA-type glutamate receptors in the postsynaptic densities. Although the synaptic localization of PSD-95 requires palmitoylation of two cysteines at the N terminus and the presence of at least one PDZ domain, how the clustering of PSD-95 is initiated and regulated remains essentially unknown. To address this issue, we examined PSD-95 clustering in primary cultured hippocampal neurons expressing full-length PSD-95 mutant proteins lacking the ligand-binding ability of PDZ1, PDZ2, and/or PDZ3. The formation of either excitatory or inhibitory synapses was unaffected. Combinations of individual mutations, however, significantly reduced the PSD-95 clustering index, in an approximately additive manner. The sensitivity to 2-bromo-palmitate and latrunculin A, reagents known to affect PSD-95 turnover, was also augmented. Furthermore, the synaptic recruitment of a PSD-95 ligand, synaptic GTPase-activating protein (synGAP), was significantly impaired, whereas the clustering of other scaffolding proteins, such as Homer 1c, Shank/Synamon, and PSD-93/Chapsin-110 was spared. Intriguingly, overexpression of the PSD-95 PDZ1/2/3 mutants caused the PSD-95 clusters to localize away from the dendritic shaft, resulting in the formation of elongated spines, in an inverse correlation with the overall PDZ-ligand affinity. Expression of a mutant synGAP lacking the PDZ-binding motif replicated both the clustering and spine morphology phenotypes. In conclusion, the ligand-binding affinity of the PDZ domains of PSD-95, contributed in part via its interaction with the C-terminal end of synGAP, plays a critical role in titrating the synaptic clustering of PSD-95 and controlling its tight association with the PSD scaffold, thereby affecting synapse maturation.

Key words: **PSD-95; clustering; spine; synGAP; NMDA receptor; PDZ domains; binding affinity**

Introduction

The postsynaptic density (PSD) is an electron-dense structure in which neurotransmitter receptors and associated signaling molecules are tightly clustered. This postsynaptic receptor complex plays an essential role in converting bouts of neurotransmitter release at chemical synapses into postsynaptic electrical and chemical signaling. Knock-in mice expressing a mutant NMDA-type glutamate receptor (NR) subunit 2B, lacking the intracellular C-terminal domain, displayed deficient synaptic properties reminiscent of NR1 knock-out mice (Sprengel and Single, 1999). Therefore, a physical link between the NMDA receptor and the downstream signaling and/or scaffolding molecules appears crucial for establishing normal synaptic function.

A major scaffold protein interacting with the NMDA receptor is PSD-95. This membrane-associated guanylate kinase (MAGUK) superfamily member possesses three N-terminal PSD-95/Discs large/zona occludens-1 (PDZ) domains, a central Src homology 3 (SH3) domain, and a C-terminal guanylate kinase (GK)-like domain (Cho et al., 1992; Kim et al., 1995; Kornau et al., 1995). Different ligand proteins interact with these three class I PDZ domains with various specificities and affinities (Sheng and Sala, 2001). For instance, synGAP (synaptic GTPase-activating protein) (Chen et al., 1998; Kim et al., 1998) binds to each PDZ equally. In contrast, the C-terminal PDZ-binding motif (–ESDV) of the NR2 subunit binds to PDZ1 and PDZ2 (Kornau et al., 1995), whereas ligands such as neuroligin (Irie et al., 1997) and cysteine-rich interactor of PDZ3 (CRIPT) (Niethammer et al., 1998) preferentially interact with PDZ3.

Extensive deletion mutagenesis studies established that the clustering of PSD-95 involves multimerization mediated by the

Received June 17, 2005; revised Nov. 18, 2005; accepted Nov. 22, 2005.

This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Y.F., H.B.) and by grants from the Human Frontier Science Program, Solution-Oriented Research for Science and Technology–Japan Science and Technology, and the Ministry of Health, Labor, and Welfare of Japan (H.B.). We thank Dr. Yutaka Hata (Tokyo Medical and Dental University, Tokyo, Japan) for providing the Shank antibody, Dr. Hideru Togashi (Center for Developmental Biology, RIKEN, Kobe, Japan) for assistance with low-density rat primary culture and electroporation techniques, and Dr. Toshifumi Tomoda (City of Hope National Medical Center, Beckman Research Institute, Duarte, CA) and Dr. Richard L. Huganir (Johns Hopkins University, Baltimore, MD) for the synGAP cDNAs. We also thank Dr. Makoto Goda for assistance with the light microscopy systems, Ayako Tanaka for cloning the CRIPT cDNA, Taichi Okamoto for technical assistance, and Dr. Hiroyuki Okuno for discussions on statistical data analysis.

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DOI:10.1523/JNEUROSCI.2489-05.2006

 0270-6474/06/260763-12$15.00/0

N-terminal region containing palmitoylated Cys-3 and Cys-5, and that the presence of one PDZ domain is sufficient for its synaptic targeting (Craven et al., 1999; Hsueh and Sheng, 1999; Christopherson et al., 2003). The importance of the tandem order of PDZ domains and of the short linker between them has also been suggested (Long et al., 2003). Furthermore, the PDZ-ligand interactions probably trigger and promote clustering via intermolecular interactions through the SH3–GK domains (Brenman et al., 1998; McGee et al., 2001; Tavares et al., 2001). However, the individual contribution of each PDZ domain and the impact of the series of ligand-binding sites on the clustering and scaffolding functions of PSD-95 have not been quantitatively examined.

To address these questions, we used PSD-95 mutants with an intact overall protein length and domain structure, in which specific mutations were introduced at the ligand-binding βB/βC loop of the PDZ1 and/or PDZ2 domains (Imamura et al., 2002). These PDZ1/2 ligand-binding site mutants were further combined with the N326S mutation in PDZ3, which greatly reduces the PDZ3–ligand affinity (Niethammer et al., 1998). By overexpressing these PSD-95 mutants with either individual or combined PDZ mutations in cultured hippocampal neurons, we probed the contributions of the individual ligand-binding sites in the clustering, membrane anchoring, and scaffolding functions of PSD-95. Our results suggested that multivalent ligand binding via the βB/βC loops of PDZ1 and PDZ2, in part by interacting with synGAP, not only plays a critical role in titrating the synaptic clustering and anchoring of PSD-95 but also, surprisingly, contributes to attracting the PSD-95-based scaffold to the proximity of dendritic shafts during synapse maturation.

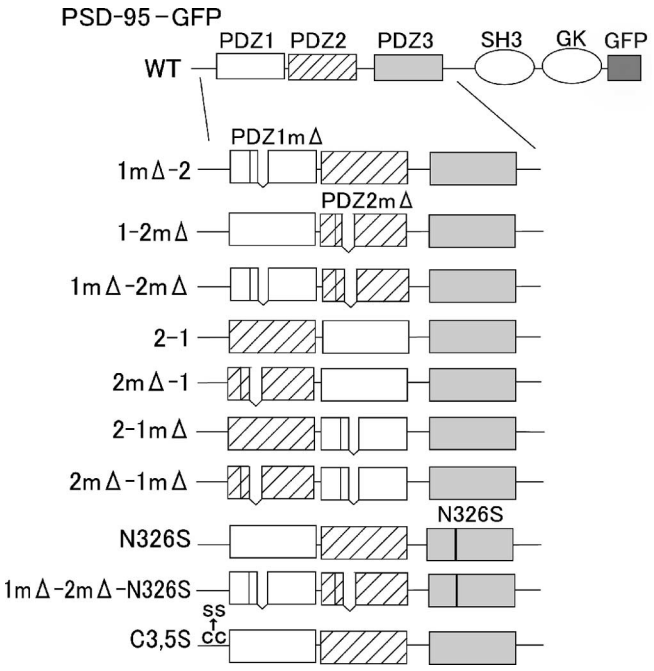

Figure 1. Schematic diagram of the domain structures of the series of PSD-95–GFP mutants and the wild type. The top panel shows the domain structure of PSD-95 tagged with GFP at the C terminus. The mutant illustrations show the N-terminal segment of PSD-95, including only the PDZ domains. PDZ1mΔ and PDZ2mΔ represent the PDZ1 and PDZ2 domains, respectively, with deletions of six amino acids (intervals) and point mutations that abolished their ligand-binding activity. The PDZ3 domain with the N326S point mutation (black vertical bar) has weak ligand-binding activity.

Materials and Methods

DNA constructs. The mutated PSD-95 cDNAs were constructed as described previously (Imamura et al., 2002). These were fused to enhanced green fluorescent protein (EGFP) via a linker sequence, Gly-Gly-Gly-Ser, using PCR in the EGFP-N1 vector (BD Biosciences-Clontech, Palo Alto, CA). The C3,5S and N326S mutations were introduced using a Quikchange mutagenesis kit (Stratagene, La Jolla, CA). The pRK5-synGAP(TRV) cDNA tagged with N-terminal Myc was obtained from Toshifumi Tomoda (City of Hope National Medical Center, Beckman Research Institute, Duarte, CA) with permission from Richard Huganir (Johns Hopkins University, Baltimore, MD), and mutagenesis was performed by PCR. All constructs were confirmed by DNA sequencing.

Primary culture, transfection, and immunocytochemistry. For immunocytochemical analyses, we prepared primary cultures of hippocampal neurons from either ICR mice or Wister rats, purchased from Japan SLC (Shizuoka, Japan). The high-density primary culture from postnatal days 0 to 1 mouse hippocampus was performed as described previously (Furuyashiki et al., 2002). Briefly, 4×10^4 cells were plated onto Matrigel (BD Biosciences, Bedford, MA)-coated spots of ~5 mm in diameter on glass coverslips, and the cultured neurons were fed with fetal calf serum-containing medium in the presence of Ara-C (Sigma, St. Louis, MO). Cultured neurons were transfected at 9 d *in vitro* (DIV) using the calcium phosphate method (Takemoto-Kimura et al., 2003), and then the cells were fixed at 12 DIV. In cotransfection experiments examining the effect of overexpressing wild-type (WT) or mutant synGAP, neurons were transfected at 8 DIV by lipofection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and cell phenotypes were examined at 10 DIV.

For immunocytochemistry, neurons were fixed in PBS(−) containing 4% paraformaldehyde/4% sucrose (or 2% paraformaldehyde/4% sucrose for PSD-93 staining). Fixed neurons were quenched in 0.1 M glycine/PBS(−), permeabilized with 0.2% Triton X-100/PBS for 10 min on ice, thoroughly washed with PBS(−), incubated in blocking solution [3% BSA/PBS(−)] for 30 min at room temperature, and then incubated overnight with primary antibodies (listed below) diluted in the blocking buffer at 4°C. The next day, the neurons were washed three times with PBS(−) and then were incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen) at a 1:500 dilution in blocking buffer. The antibody sources and dilutions are as follows: rabbit polyclonal anti-vesicular glutamate transporter-1 (VGLUT1) (1:1000; Synaptic Systems, Goettingen, Germany), monoclonal anti-vesicular GABA transporter (VGAT) (1:1000; Synaptic Systems), monoclonal anti-synaptophysin (1:1000; Sigma), monoclonal anti-PSD-95 [K28/43, 1:200 (Upstate Biotechnology, Lake Placid, NY); 7E3–1B8, 1:250 (Affinity BioReagents, Golden, CO)], monoclonal anti-synGAP (1:1000; Affinity BioReagents), rabbit polyclonal anti-Homer-1c (Irie et al., 2002), rabbit polyclonal anti-PSD-93 (1:250; Zymed, San Francisco, CA), rabbit polyclonal anti-Synamon/Shank (a kind gift from Yutaka Hata, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan), polyclonal anti-Myc (1:200; Cell Signaling Technology, Beverly, MA), and Alexa Fluor-conjugated anti-rabbit and anti-mouse antibodies (1:500; Invitrogen).

Pharmacological treatments. Pharmacological treatments were performed by low-density rat hippocampal cultures, and the resulting effects were examined by immunofluorescent (IF) staining, as described previously (Togashi et al., 2002). The PSD-95 mutant phenotypes reported in this study were essentially identical, regardless of the type of primary culture (data not shown). At 12 or 13 DIV, one-half of the culture medium was removed from the dish, and 2-bromo-palmitate (2-Br-Pal) or latrunculin A (LatA) (from a 1000× stock in DMSO) were mixed with this half, to concentrations of 200 and 10 μM, respectively. This medium was then returned to the culture dish and mixed well with the remaining medium by gentle rocking. After either a 2 or 8 h treatment, the cells were washed with cold PBS(−) and fixed immediately. Latrunculin A was obtained from Calbiochem (La Jolla, CA), and 2-Br-Pal (2-bromohexadecanoic acid) was from Sigma.

Quantitative microscopy and image analysis. All immunofluorescence images were acquired using a cooled CCD camera (Orca II; Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan), controlled by the MetaVue or MetaMorph software (Molecular Devices, Sunnyvale, CA) and mounted on either a BX50 upright or an IX-81 inverted microscope (Olympus Optical, Tokyo, Japan) with 100× [numerical aperture (NA), 1.35] and

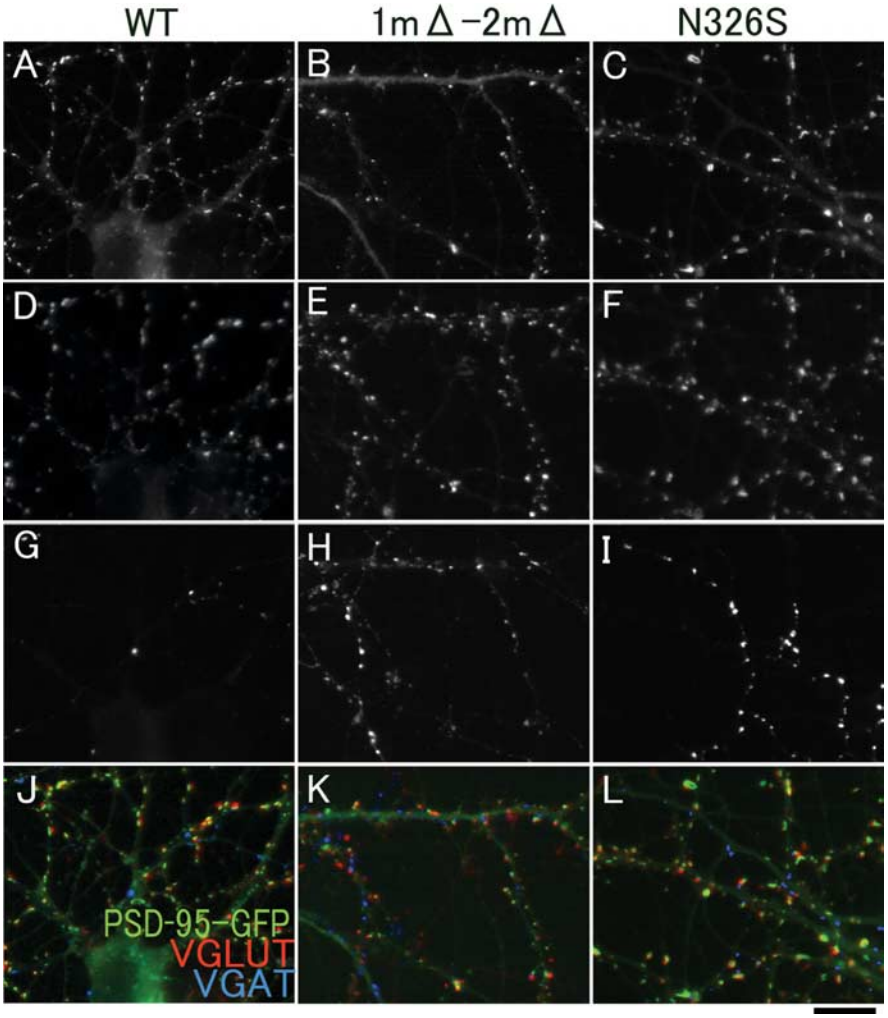

Figure 2. Wild-type and mutant PSD-95 form postsynaptic clusters apposed to the glutamatergic synaptic boutons. The PSD-95(WT)–GFP, 1mΔ-2mΔ, and N326S (***A–C*** and green in ***J–L***, respectively) form clusters juxtaposed to puncta immunopositive for an excitatory presynaptic marker, anti-VGLUT1 (***D–F*** and red in ***J–L***). No cluster colocalized with puncta positive for an inhibitory presynaptic marker, anti-VGAT (***G–I*** and blue in ***J–L***). The bottom images are merged images in which PSD-95–GFP staining is green, VGLUT1 staining is red, and VGAT staining is blue. Scale bar, 10 μm.

40× (NA, 0.85) objectives and filter cubes appropriate for costaining of GFP, Alexa 594, and Alexa 350, or GFP, Alexa 555, and Alexa 647. Immunostaining and image acquisition were performed using rigorously identical procedures within a set of experiments, which enabled us to obtain reproducible sets of immunofluorescence values, as detected by the cooled CCD camera.

For quantification purposes, only the neurons that expressed PSD-95–GFP within the average intensity values of 100–300 (in a 12-bit dynamics range, 0–4096 at a fixed gain setting) in the dendritic shafts were considered and randomly chosen, because most of the cells with normal morphology fell within this value range. In the preliminary series of experiments, the minimum and maximum intensity values, 100 and 300, were set as reliable thresholds to obtain a sufficient amount of mutant proteins to reveal a dominant phenotype and to avoid the toxic effects attributable to the overexpression of exogenous proteins, respectively. For each cell, one to four images were acquired to cover the main dendritic area. The dendritic segments (the apparent dendritic shaft diameter was >0.7 μm) were chosen randomly within 200 μm from the soma. For the synaptic clustering index (SCI), cluster density (the number of clusters per 20 μm dendritic segment), and cluster-shaft distance measurements, the PSD-95 clusters were defined as 0.3–1.0 μm diameter spots of increased GFP fluorescence, at least twice as bright as the dendritic shaft (to discriminate true clusters from stochastic local gradients; however, because of spatial resolution limitations, we were unable to reliably resolve clusters less than ~0.2 μm in diameter), which were closely juxtaposed to synaptophysin-immunopositive puncta. In the obtained ensemble dataset, both the cluster size and average cluster fluorescence intensity followed a Gaussian distribution, indicating that no systematic bias was introduced by these criteria.

The SCI was calculated off-line with the MetaVue or MetaMorph software, essentially as described previously (Arnold and Clapham, 1999), using images acquired with the 100× (NA, 1.35) objective. Briefly, after subtracting the background, the maximal intensity of each synaptic cluster was divided by the average intensity of the proximal parent dendritic shaft. The average dendritic shaft intensity was measured by drawing an orthogonal line traversing the dendritic area immediately nearest/adjacent to the cluster but excluding any outlying bright spots. Because we used wide-field microscopy, the inclusion of immunofluorescence values obtained from out-of-focus fields would inevitably result in underestimation and inexact SCI values. To avoid this, we determined the in-focus area of interest within each image and only analyzed the immunofluorescence values obtained from dendrites and clusters located within that area. In control experiments, the SCI analysis performed over the same fields of view, using a 40× objective lens, yielded quantitatively similar results, confirming that under our experimental conditions, the contribution of the out-of-focus photons was negligible, even with the 100× (NA, 1.35) objective (data not shown).

For the cluster-shaft distance measurement, the images were displayed at the same magnification, and the synaptic clusters were chosen using the same criteria as for the SCI measurement. A line was manually drawn off-line from the center of each synaptic cluster to the nearest edge of their parent dendritic shafts, and the length was measured using the MetaVue or MetaMorph software. A distance value of zero was applied to those clusters formed directly on the dendritic shaft (shaft clusters). We acknowledge the caveat that such data obtained from projection images strongly underestimate the protrusion into the *z*-axis but this does not alter the main results of our analyses, because this *z*-axis underestimation factor is equally distributed across the *x*–*y* plane, although the correlation factor obtained in Figure 7*A* may be slightly underrepresented.

The SCI and cluster-shaft distance values were thus calculated for 20–100 clusters from at least 100 μm of dendritic segments per cell, and the average values for each cell were further subjected to a multicomparison analysis. Six to 11 cells were analyzed in total for each individual PSD-95 mutant construct. For the statistical analysis, the SCI measurement was evaluated by one-way ANOVA, with the *post hoc* Dunnett's test, using the JMP software (SAS Institute, Toronto, Ontario, Canada), and the cumulative distribution of the cluster-shaft distances was analyzed by the Kolmogorov–Smirnov test, using the PAST software (Hammer et al., 2001). Graphs were made with the Prism software (GraphPad Software, San Diego, CA) and Excel (Microsoft, Seattle, WA).

Results

Construction of full-length PSD-95 mutants containing PDZ domains with reduced binding affinity

The mutant PDZ domains, PDZ1mΔ and PDZ2mΔ, in which the ligand-binding βB/βC loop of either the PDZ1 or PDZ2 domain

of PSD-95 was altered, were shown previously to be unable to bind to NR2B and Kv1.4, both *in vitro* and in a heterologous system (Imamura et al., 2002). We further confirmed that they are also unable to bind CRIPT, a well known neuronal PDZ3 ligand, despite the apparent homology between the mutated PDZ1/2 sequences and PDZ3 (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). To further disrupt the ligand binding at the PDZ3 domain, we introduced an additional point mutation, N326S (Niethammer et al., 1998). This significantly reduced the binding of mutated PDZ3 to CRIPT and allowed only weak binding to Kv1.4 (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Based on these results, we next systematically replaced the PDZ1, PDZ2, and PDZ3 of PSD-95 with PDZ1mΔ, PDZ2mΔ, and PDZ3 (N326S) and fused EGFP at their respective C termini. Figure 1 shows the series of PSD-95–GFP mutants used in this study. The PDZ1–PDZ2 inverted mutants were also constructed to test whether the relative positions of the PDZ domains were as important in cultured neurons as in a heterologous expression system (Imamura et al., 2002).

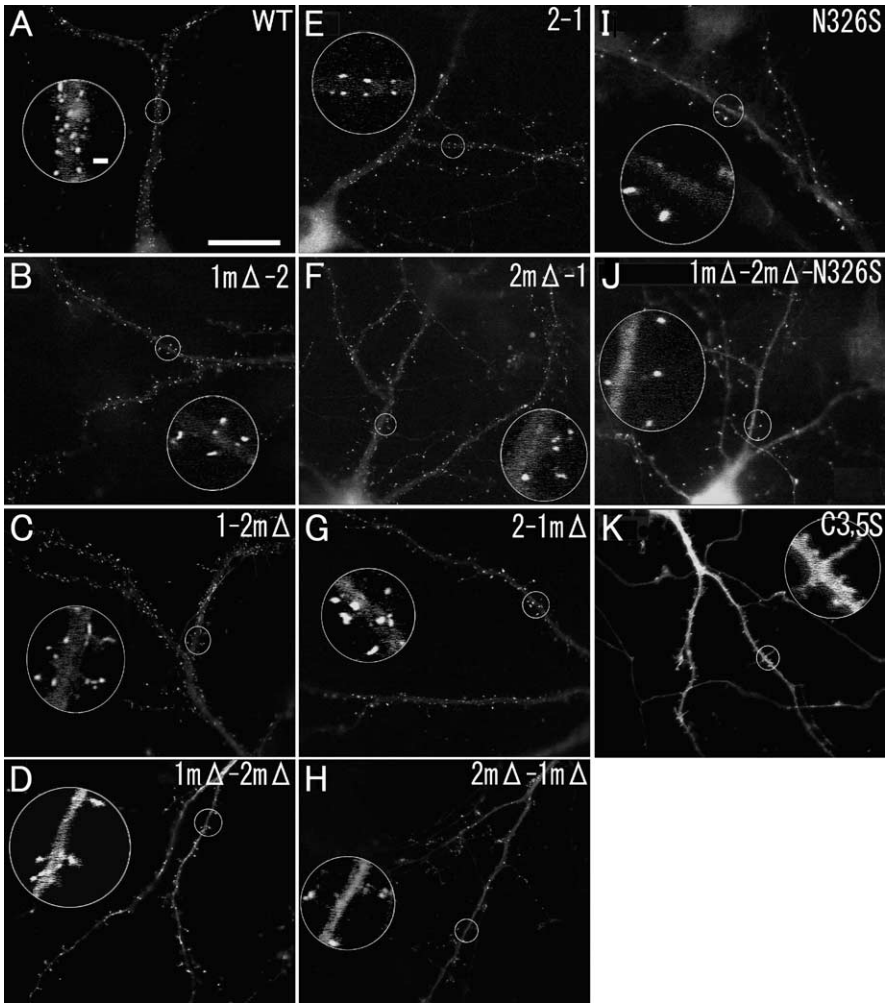

Figure 3. Synaptic clustering of PSD-95 is diminished by PDZ-ligand-binding mutations. GFP fluorescent images were taken from transfected hippocampal neurons expressing the wild-type (***A***) and the series of mutant PSD-95–GFPs (***B–K***). ***A***, ***E***, The wild-type and the 2–1 (inversion of PDZ1 and PDZ2, with intact ligand-binding affinity) mutant formed bright shaft clusters. Note that the majority of the clusters are shaft clusters. ***B***, ***C***, ***F***, ***G***, When either the PDZ1 or PDZ2 domain is mutated, the clustering activity is slightly reduced. A significant number of clusters are formed at some distance from the dendritic shaft, indicating their presence on spine-like protrusions. ***D***, ***H***, Mutations in both PDZ1 and PDZ2 reveal clusters located farther away, on the tips of longer protrusions. In contrast to the WT, the fluorescence intensity of these clusters is not substantially higher compared with the mean dendritic shaft intensity. ***I***, The N326S mutation in the PDZ3 domain also reduces the clustering efficiency. ***J***, 1mΔ–2mΔ–N326S, the mutant that lacks functional PDZ domains, forms discernible clusters juxtaposed to the presynaptic markers, but the clustering efficiency is extremely low. The clusters are even farther from their parent dendritic shafts. All of the ligand-binding-deficient mutants are distinct from the C3,5S mutant (***K***) in that they form punctate clusters. Scale bars: 20 μm; inset, 1 μm. The insets are the magnified images of the areas outlined by white circles in the images. All of the insets have the same magnification.

Individual PDZ domains independently and additively contribute to the synaptic clustering of PSD-95

To examine the role of ligand binding to each individual PDZ domain in postsynaptic clustering during synaptic maturation, we transfected the cDNAs for each PSD-95–GFP mutant in cultured mouse hippocampal neurons at 9 DIV. Neurons were fixed at 12 DIV and immunostained with a presynaptic marker. We first confirmed that exogenous PSD-95(WT)–GFP and PSD-95(1mΔ–2mΔ)–GFP formed clusters in puncta of ~0.3–0.4 μm in diameter. These PSD clusters were juxtaposed to puncta immunoreactive to VGLUT1, a marker for excitatory glutamatergic termini, but were far from puncta immunoreactive to VGAT, a marker for inhibitory GABAergic termini (Fig. 2). We initially calibrated the expression levels of the PSD-95–GFP constructs by comparing the anti-PSD-95-immunoreactive signals of untransfected neurons with those of transfected neurons containing both exogenous and endogenous PSD-95. We established that, at a fixed gain setting, using our cooled CCD acquisition system, neurons with mean GFP intensities in dendritic shafts between 100 and 300 had a dendritic morphology indistinguishable from that of untransfected neurons. The anti-PSD-95 immunoreactivity completely overlapped with the GFP puncta, and the GFP fluorescence intensities of 100–300 corresponded to ~3.4- to 5.6-fold higher amounts of PSD-95 immunoreactivity compared with the basal, endogenous levels in untransfected cells. In the mutant PSD-95–GFP containing PDZ1mΔ, the distribution of endogenous PSD-95, stained using a PSD-95 antibody (7E3–1B8) that did not recognize the PDZ1mΔ, was indistinguishable from the GFP fluorescence from the mutant PSD-95 molecules (data not shown). Furthermore, we confirmed that the overexpression of the full-length wild-type or mutant PSD-95–GFP constructs in neurons apparently had minimal effects on the integrity of the full-length protein (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). The wild-type and mutant PSD-95–GFP were able to form complexes when expressed in COS cells, because they coimmunoprecipitated together (data not shown). Thus, the exogenously expressed PSD-95–GFP freely intermingled with the endogenous PSD-95. Therefore, any phenotypic change resulting from overexpressing the mutant PSD-95, as opposed to overexpressing the wild-type PSD-95, was likely to result from a dominant effect triggered by the reduced binding affinity of the mutated PDZ domain(s).

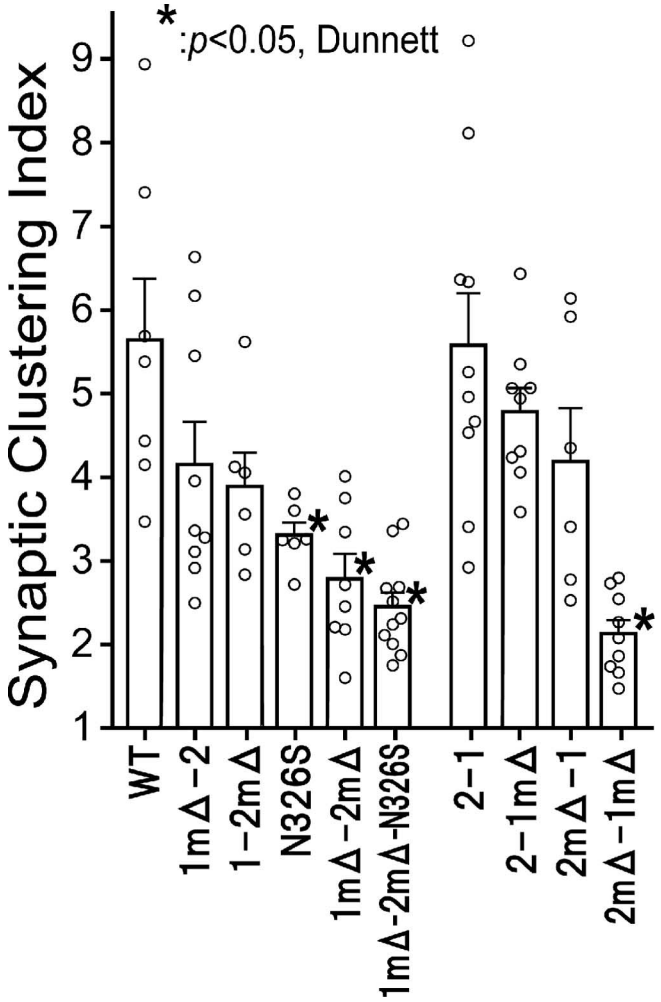

Figure 4. Individual PDZ domains independently and additively contribute to postsynaptic clustering of PSD-95. The SCI was measured as the peak GFP intensity of the synaptic PSD-95–GFP clusters divided by the average intensity of the parent dendritic shaft. Wild-type PSD-95 forms clusters that are 5.64 ± 0.73 times as bright as the parent dendritic shaft, on average. In contrast, the 1mΔ–2mΔ mutant forms clusters with an SCI of approximately half of the wild type (SCI, 2.79 ± 0.298; $p < 0.05$ vs WT). Open bars and error bars are means ± SEM of the cell averages ($n = 6$–11 cells), and open circles represent the average of each cell (20–100 clusters per cell). We confirmed that the SCI values of each cluster in a cell conformed to a normal distribution. Each dataset was statistically analyzed by one-way ANOVA, with the *post hoc* Dunnett's test. *$p < 0.05$ versus WT.

To quantitatively measure the clustering efficiency at the postsynaptic site, we used the SCI (Arnold and Clapham, 1999). Basically, the SCI was calculated as the peak GFP pixel intensity at the clusters juxtaposed to the presynaptic markers divided by the average GFP pixel intensity of the adjacent dendritic shaft (see Materials and Methods). The SCI value varied from cluster to cluster and cell to cell, but the values exhibited a normal distribution (data not shown). The average SCI of the wild type was 5.64 ± 0.73 (mean ± SEM) (Figs. 3*A*, 4). The C3,5S mutant, which lacks the two palmitoylation sites at the N terminus, was localized diffusely (Fig. 3*K*), as reported previously (Craven et al., 1999). In contrast to the C3,5S mutant, all of the ligand-binding-deficient mutants formed distinguishable clusters (Fig. 3*B–J*) juxtaposed to the presynaptic marker synaptophysin. However, the synaptic clusters of these mutants were less dense, with a substantial portion of the PSD-95–GFP molecules remaining unclustered in dendritic shafts (Fig. 3*D*,*H*,*J*). Noticeable degradation of the PSD-95–GFP proteins in transfected neurons overexpressing either the wild-type or mutant PSD-95–GFP proteins was not detected (supplemental Fig. 2, available at www.jneurosci.org as supplemental material), indicating that the loss of synaptic clustering probably resulted from dispersion rather than protein degradation. The postsynaptic clustering activity was significantly attenuated in the 1mΔ–2mΔ mutant compared with the wild type (Fig. 4) (SCI, 2.79 ± 0.30; $p < 0.05$, ANOVA with the *post hoc* Dunnett's test). The N326S mutation in PDZ3 also significantly reduced the clustering efficiency (SCI, 3.18 ± 1.87; $p < 0.05$, ANOVA with the *post hoc* Dunnett's test), to an extent approximately comparable with that of the 1mΔ–2mΔ mutant. These results suggested that synaptic clustering is not only governed by ligand binding to the PDZ3 domain, whose contribution was shown previously in a peptide inhibition experiment (Passafaro et al., 1999), but also by ligand binding to both PDZ1 and PDZ2. Consistent with this idea, the 1mΔ–2mΔ–N326S mutant exhibited the lowest clustering efficiency of all of the non-inverted mutants (SCI, 2.44). The inversion of the mutated PDZ domains had no discernable effect per se on synaptic clustering in hippocampal neurons ($SCI_{2m\Delta\text{-}1}$, 4.19 ± 0.64, $SCI_{2\text{–}1m\Delta}$, 4.78 ± 0.28 vs $SCI_{1m\Delta\text{-}2}$, 4.15 ± 0.51, $SCI_{1\text{–}2m\Delta}$, 3.89 ± 0.40), unlike the results seen previously in a heterologous expression system with only Kv1.4 as a ligand (Imamura et al., 2002). This probably reflects the complexity of the stoichiometry and the competition among multiple PDZ ligands for PSD-95 binding within the small volume of the PSD near physiological synapses.

Collectively, these observations support the notion that the ligand binding to PDZ1, PDZ2, and PDZ3 independently contributes to the postsynaptic clustering of PSD-95 at the PSD, and that this regulation operates in an approximately additive manner.

Postsynaptic clustering of a PSD-95 ligand, synGAP, is severely altered in PSD-95 mutant expressing neurons, but the clustering of Shank/Synamon, Homer, and PSD-93 remains intact

We next tested whether a loss in PDZ binding affinity in PSD-95 affected the recruitment of a nontransmembrane PDZ ligand or PSD scaffolds distinct from PSD-95 by performing immunocytochemical analyses in synaptic clusters of wild-type and mutant expressing neurons. We found no significant difference in the immunoreactivity (IF) intensity at synaptic clusters for either Shank/Synamon, Homer 1c/Vesl-L/PSD-Zip45, or PSD-93/Chapsin-110, three postsynaptic molecular scaffold proteins distinct from PSD-95, between the neurons expressing wild-type PSD-95 and the 1mΔ–2mΔ mutant ($p > 0.5$ in all IF experiments) (Fig. 5*C–E*). In contrast, the staining intensity for synGAP, a major PSD-95 ligand, was significantly reduced in the neurons expressing the 1mΔ–2mΔ and 1mΔ–2mΔ–N326S PSD-95 mutants compared with the wild-type expressing neurons (Fig. 5*A*,*B1*,*B2*) [mean ± SEM; WT, 2.88 ± 0.20; 1mΔ–2mΔ, 1.9 ± 0.17; $p < 0.05$ (*t* test) vs WT; and WT, 3.4 ± 0.62; 1mΔ–2mΔ–N326S, 1.7 ± 0.12; $p < 0.05$ (*t* test) vs WT]. These results suggest that the impact of the reduced PDZ-ligand affinity of PSD-95 is limited to the primary PSD-95 ligands and may not extend to other PSD proteins indirectly associated with PSD-95 via the scaffolding complexes present in the PSD.

Loss of the ligand-binding affinity in PDZ domains destabilizes the PSD-95 association with the PSD

Previous work examining PSD-95 turnover at and near synapses demonstrated that only a limited pool of PSD-95 undergoes active turnover (Okabe et al., 1999), and an EM study further suggested that PSD-95 is deeply anchored to the core fraction (<12 nm from the postsynaptic membrane) of the PSD (Petersen et al., 2003). To investigate whether the PDZ-ligand binding plays a role in the tight integration of PSD-95 in the PSD, we treated neurons with 100 μM 2-bromo-palmitate, a reagent that blocks palmitoylation (El-Husseini Ael et al., 2002) and thereby inhibits the delivery of PSD-95 to plasma membranes. Within 2 h after treatment, the synaptic clustering of PSD-95 in the wild-type expressing neurons, measured using SCI, only decreased by 14.2% compared with the vehicle-treated control, whereas the SCI in the mutant (1mΔ–2mΔ) PSD-95 expressing neurons significantly decreased to almost half of that of the control (55.7%; $p < 0.01$, Student's t test) (Fig. 6*A*). After an 8 h incubation, the SCI values of the wild-type expressing neurons were reduced to a level similar to that of the 1mΔ–2mΔ mutant. Thus, the removal of PSD-95 from the synaptic clusters was facilitated in the 1mΔ–2mΔ mutant as a result of the reduced delivery of new palmitoylated PSD-95 to the surface membranes. This result suggests that intact PDZ1/2 ligand binding may contribute to synaptic clustering by either promoting the aggregation of palmitoylated PSD-95 molecules or facilitating the maintenance of the palmitoylated form of PSD-95.

We then tested the PSD-95 mutants for a potential alteration in the susceptibility of postsynaptic clustering to 5 μM LatA, a reagent that favors filamentous actin (F-actin) depolymerization within spines (Allison et al., 1998). In the wild-type PSD-95 expressing neurons, LatA treatment for 17 h was required to substantially reduce the SCI. Even after 17 h of treatment, distinct clusters remained on dendrites ($SCI_{0\ h}$, 6.31 ± 0.30; $SCI_{17\ h}$, 4.12 ± 0.20; $p < 0.01$) (Fig. 6*B*). In contrast, in the 1mΔ–2mΔ mutant expressing neurons, the clusters readily dissociated within 1.5 h ($SCI_{0\ h}$, 3.63 ± 0.086; $SCI_{1.5\ h}$, 2.61 ± 0.23; $p < 0.01$). However, when scaled to the untreated cells, the SCI values of the wild-type and 1mΔ–2mΔ expressing neurons both decreased, to 65.3 and 61.8% of the original levels, respectively, after 17 h of treatment. Therefore, similar to the

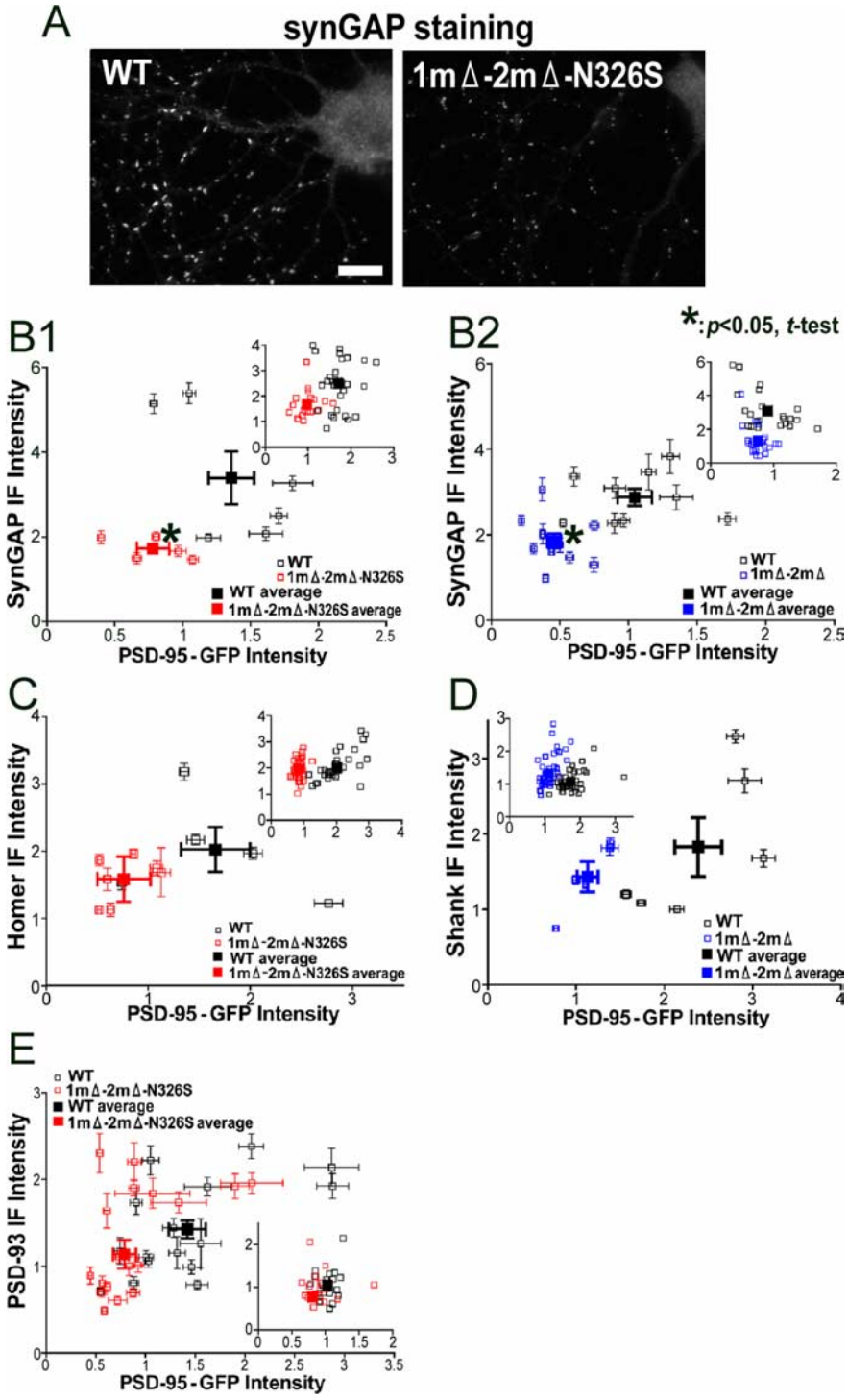

Figure 5. SynGAP immunoreactivity is significantly reduced in the postsynaptic clusters of 1mΔ–2mΔ PSD-95-expressing neurons compared with the wild-type PSD-95-expressing neurons. ***A***, SynGAP IF images of the cells expressing PSD-95(WT)–GFP and 1mΔ–2mΔ–N326S are shown in grayscale. The staining signal is significantly reduced in the postsynaptic clusters of the 1mΔ–2mΔ PSD-95-expressing neurons compared with the wild-type PSD-95-expressing neurons. Scale bar, 10 μm. ***B–E***, Graphs represent the plots of the IF intensities of synGAP (***B***), Homer (***C***), Shank (***D***), and PSD-93 (***E***) versus the PSD-95–GFP intensities of the clusters in the neurons expressing wild-type or mutant PSD-95–GFP (black open squares, wild type; blue open squares, 1mΔ–2mΔ mutant; red open squares, 1mΔ–2mΔ–N326S mutant; filled squares, their averages). Insets are the plots of each cluster observed in one representative neuron. In the main graphs, each small square with bars indicates the averaged value ± SEM of each neuron [GFP intensity to the *x*-axis (arbitrary units) and IF intensity to the *y*-axis (arbitrary units)]. All of the cells analyzed expressed approximately similar amounts of PSD-95–GFP in the dendritic shaft, regardless of the mutant type. Therefore, the PSD-95–GFP intensities reflect the SCI values. ***B***, The IF intensities of a major PSD-95 ligand, synGAP, are significantly lower in the 1mΔ–2mΔ–N326S clusters (***B1***, red) and the 1mΔ–2mΔ clusters (***B2***, blue) compared with the PSD-95(WT)–GFP clusters (black). ***C–E***, In contrast, using the same assay, the IF intensities for other scaffold proteins distinct from PSD-95, such as Homer-1c (***C***), Shank/Synamon (***D***), and PSD-93/Chapsin-110 (***E***), are not significantly altered in the postsynaptic clusters ($p > 0.5$). *$p < 0.05$ versus WT by Student's t test.

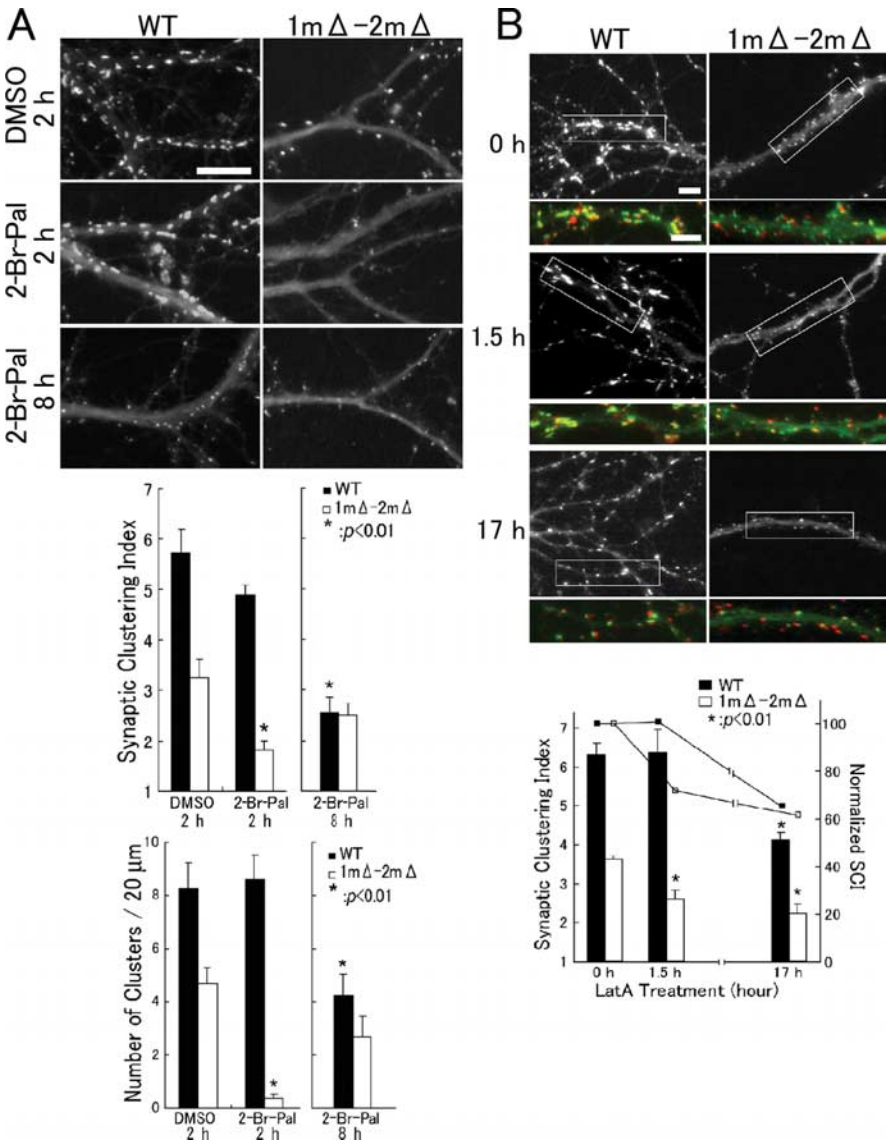

Figure 6. Ligand-binding-deficient mutants are more loosely anchored to the postsynaptic membrane than the wild type. ***A***, Synaptic clusters in neurons expressing ligand-binding-deficient PSD-95 mutants are rapidly dispersed in response to treatment with a palmitoylation blocker, 2-Br-Pal. 2-Br-Pal dramatically reduces the clustering efficiencies in neurons expressing the mutant PSD-95 (1mΔ–2mΔ–N326S) but only moderately in neurons expressing the wild-type PSD-95. Grayscale images show the PSD-95–GFP fluorescence of the wild-type and 1mΔ–2mΔ-expressing neurons treated with DMSO or 2-Br-Pal for 2 or 8 h. The top graph summarizes the results of the SCI analysis of the DMSO-treated or 100 μM 2-Br-Pal-treated wild-type and 1mΔ–2mΔ-expressing neurons. The bottom graph shows the cluster density (number of clusters per 20 μm dendritic segment) of the same datasets. *$p < 0.01$ versus the SCI of DMSO 2 h by the Student's *t* test. ***B***, Increased susceptibility to an actin-depolymerizing drug (LatA) in neurons expressing a ligand-binding-deficient mutant. Actin depolymerization by 5 μM latrunculin A caused much faster cluster dissociation in the 1mΔ–2mΔ mutant PSD-95-expressing neurons. Grayscale images of the wild-type and 1mΔ–2mΔ-expressing neurons treated with LatA for 0, 1.5, and 17 h. The small images below are the colored images (PSD-95–GFP, green) merged with synaptophysin staining (red) of the areas outlined by the white rectangles in the grayscale images. The graph shows the SCI values of the LatA-treated and nontreated wild-type and 1mΔ–2mΔ-expressing neurons. Raw values are shown as bar graphs (left *y*-axis), and scaled values (normalized to SCI at 0 h) are shown as line graphs (right *y*-axis). *$p < 0.01$ versus the SCI of nontreated (0 h) WT and 1mΔ–2mΔ, respectively, by the Student's *t* test. Scale bars, 10 μm.

previous experiments using 2-bromo-palmitate treatments, the mutant (1mΔ–2mΔ) PSD-95, during actin depolymerization, seemed to be more likely than the wild type to move away from synaptic clusters, although the presynaptic marker staining remained intact. Thus, the PDZ1/2 binding activity probably plays a critical role in facilitating postsynaptic clustering, in conjunction with a postsynaptic actin polymerization-dependent process.

Together, these data demonstrated that the clusters containing PDZ-binding-deficient PSD-95 mutants were more sensitive to treatment with a palmitoylation inhibitor or an actin depolymerization reagent. These results are consistent with the idea that efficient PDZ-ligand binding strongly promotes the clustering of PSD-95 and stabilizes the membrane anchoring, subsequent to a step after the activity-dependent palmitate cycling and the actin cytoskeleton-dependent attachment of the PSD-95 to the PSD complex.

These experiments alone may not formally rule out the possibility that the apparently increased susceptibility of the 1mΔ–2mΔ mutant-containing clusters to disperse during various manipulations (treatments with either a palmitoylation inhibitor or an actin depolymerization reagent) might be at least partially elicited by subtle reductions in the absolute amounts of expressed proteins in various local dendritic compartments rather than by a decrease in PDZ-ligand binding. We do not favor this interpretation, however, because the low expression level of wild-type PSD-95–GFP (less than the cutoff threshold of 100; see Materials and Methods) was not accompanied by a reduction in the SCI (data not shown).

To obtain independent support for the PDZ-ligand-binding-mediated stabilization of the localization of PSD-95 to the PSD, we performed additional quantitative measurements of PSD alterations, including spine morphology.

The PDZ-ligand-binding deficiency is correlated with a severe defect in the dendritic localization of PSD-95 clusters and may affect spine morphology

Consistent with the suggestion that the stability of PSD-95 may be compromised at postsynaptic clusters expressing PDZ-ligand-binding mutants, the overexpression of PSD-95–GFP with dysfunctional PDZ domains generated a significantly large number of synaptic clusters that were located away from the parent dendritic shaft (Figs. *3D*,*H*,*J*, insets, *7A*, 8) ($p < 0.001$, Kolmogorov–Smirnov test). In contrast, the wild-type PSD-95 clusters were formed directly on the dendritic shaft (Figs. *3A*, *7A*, 8), consistent with previous reports. To quantify this phenotype, we reanalyzed the same dataset of cells shown in Figures 3 and 4, measured the projected distance between each synaptic cluster and the closest dendritic shaft (cluster-shaft distance; when clusters were formed within the boundary of the dendritic shaft in a maximal projection image, the distance was considered as zero), and compared this index among the various types of mutants (Fig. *7A*).

Most of the wild-type PSD-95 clusters were formed directly on

the dendrite (80.7% of the clusters were shaft clusters), and the average ± SEM apparent distance was 0.18 ± 0.029 μm, within the limit of the optical spatial resolution. Untransfected neurons analyzed with the anti-PSD-95 antibody staining produced a qualitatively similar value (0.10 ± 0.030 μm vs WT; $p > 0.05$, Kolmogorov–Smirnov test; data not shown), again within the limit of spatial resolution. In contrast, the average cluster-shaft distance of 1mΔ–2mΔ was significantly increased, to 0.47 ± 0.093 μm. In these 1mΔ–2mΔ expressing neurons, the cluster-shaft distance values showed a non-Gaussian distribution, which was quite distinct from that of the wild-type expressing neurons ($p < 0.001$, Kolmogorov–Smirnov test), with >40% of the clusters being significantly distant (this distance ranged from 0.2 to 3 μm) from the nearest shaft (Figs. 7*A*, 8). The clusters that formed far from dendrites did not form on immature filopodia/spines, because they were all juxtaposed with presynaptic markers, such as synaptophysin and VGLUT1 (Fig. 2). Thus, the formation and attraction of presynaptic boutons to the sites of PSD-95 clusters were not impaired at all, even in the PSD-95 mutant expressing neurons.

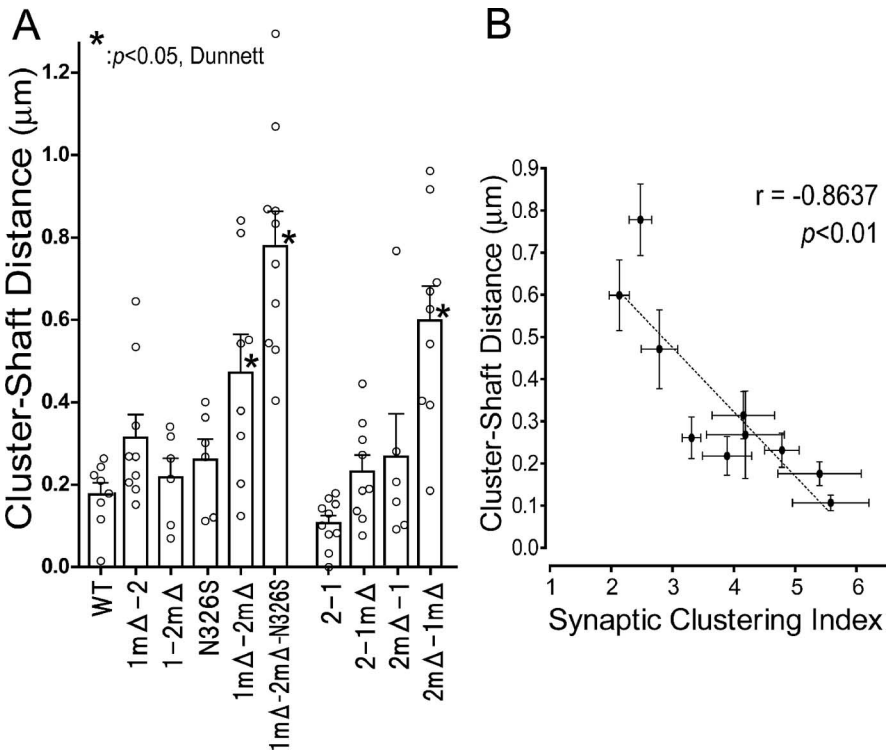

Figure 7. The cluster-shaft distance is increased in the mutant-expressing neurons compared with the wild-type-expressing neurons and negatively correlates with the SCI value. ***A***, Ligand-binding-deficient PSD-95 mutants formed clusters on tips of protrusions (spines) away from the dendritic shafts. The distance from the center of the synaptic clusters to the edge of the parent dendritic shaft was measured on the same dataset of cells used to calculate the SCI (Figs. 3, 4). To the shaft clusters (clusters that appeared to be formed directly on the dendritic shaft), the value 0 was arbitrarily assigned. Bars and error bars represent the averages and the SEMs of the calculated means of each cell (open dots). The error bars of WT and 2–1 are exceptionally small, because these cells predominantly have shaft clusters to which we applied the same values equally, and the large fraction of the shaft clusters will result in average distances below 0.2 μm. *$p < 0.05$ versus WT. ***B***, The SCI is negatively correlated with the cluster-shaft distance. The average SCI and the average cluster-shaft distance, the distance from the PSD-95 clusters to the dendritic shaft, of the wild type and each mutant type ($n = 6–10$ cells per mutant type), are plotted on the *x*-axis and *y*-axis, respectively. Error bars indicate SEM. The broken line shows the fitted regression line ($r = -0.8637$; $p < 0.01$).

We wondered whether the PDZ-ligand-binding affinity might regulate this distance between the PSD-95 cluster and the shaft. To examine this possibility, we measured the cluster-shaft distance in all of the available PSD-95 mutants. Single PDZ mutants (1mΔ–2 or 1–2mΔ) had fewer shaft clusters (69.3 and 73.2%, respectively) compared with the wild type, and, in these cells, many clusters were found on the heads of spine-like protrusions. Similarly, in the PDZ3 N326S mutant expressing neurons, some of the clusters were formed on the spines, whereas 70.7% of the clusters were on the shafts (Fig. 8). The severest phenotype was found in the 1mΔ–2mΔ–N326S mutant expressing neurons, which had only 43.8% of the shaft clusters. Some of these were farther from the dendritic shafts and seemed to form on the cross-sections of thin, long protrusions from the dendrites.

Together, these data indicated that the average cluster-shaft distance was qualitatively (Fig. 3) and quantitatively (Figs. 7*A*, 8) longer in the mutant type with a lower SCI value. In fact, a statistically significant negative correlation was found between the SCI and the cluster-shaft distance (the coefficient of correlation, $r = -0.8637$; $p < 0.01$) (Fig. 7*B*).

The loss of PDZ binding in synGAP replicates the clustering and morphological phenotypes of PDZ-ligand-binding-deficient PSD-95

One candidate PDZ ligand that may be involved in the phenotypes associated with the overexpression of mutant PSD-95 is synGAP (Fig. 5). To directly test whether synGAP binding to PSD-95 is required for the efficient PSD-95 clustering and mature spine formation, we next examined the effects of overexpressing a synGAP C-terminal deletion mutant with diminished binding ability to the PDZ domains [synGAP(ΔSXV)]. We cotransfected the wild-type synGAP(TRV) with the intact C-terminal PDZ-binding motif or the mutant synGAP(ΔSXV), which lacks five C-terminal residues, QQTRV (Vazquez et al., 2004), together with the wild-type PSD-95–GFP. The expression of the synGAP(ΔSXV) caused the PSD-95(WT)–GFP to become distributed in an aberrant pattern with a reduced SCI, very similar to that of the ligand-binding-deficient PSD-95, i.e., 1mΔ–2mΔ (Fig. 9*A*,*B*). The localization of synGAP(ΔSXV) no longer overlapped with that of PSD-95, although many nonsynaptic synGAP clusters were detectable (Fig. 9*A*), presumably because of interactions with proteins other than PSD-95 (Tomoda et al., 2004). In contrast, the expression of the wild-type synGAP(TRV) did not change the pattern of PSD-95 localization, and the synGAP(TRV) colocalized well with the PSD-95 clusters (Fig. 9*A*,*B*). Similarly, the defect in synaptic clustering and the increase in cluster-shaft distance found in neurons expressing the 1mΔ–2mΔ mutant (Figs. 4, 7) were primarily replicated by the coexpression of PSD-95(WT)–GFP with the synGAP(ΔSXV) mutant (Fig. 9*C*,*D*). Consistently, the cluster density (the number of clusters per 20 μm dendritic segment) was greatly reduced in parallel (Fig. 9*C*), and, conversely, the cluster-shaft distance was elongated (Fig. 9*D*). This effect was specific for the PDZ-binding motif mutant synGAP(ΔSXV) and was not found with a mutant

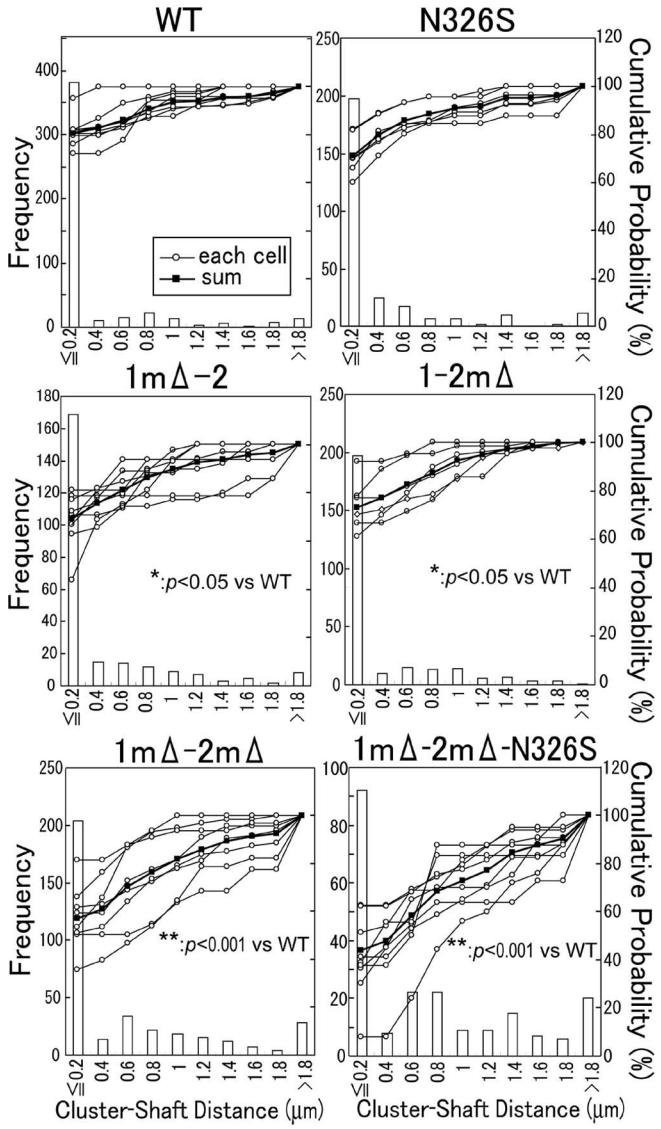

Figure 8. Histograms of the cluster-shaft distance distributions in the PSD-95 wild-type- and mutant-expressing neurons. The cumulative probability (right *y*-axis) of the cluster distances for each individual cell is traced (lines with open circles), and the aggregate data are traced (thicker lines with filled squares). The frequency histogram of the aggregate data were superimposed (left *y*-axis). Note that, in the mutant-expressing neurons, the cluster-shaft distance distribution cannot be fitted with a single Gaussian distribution, because they have a substantial portion of nonshaft clusters. This discrepancy is statistically significant. $*p < 0.05$ and $**p < 0.001$ versus WT by Kolmogorov–Smirnov test.

synGAP lacking GAP activity synGAP(GAP*) (data not shown). These data are consistent with the notion that the interaction of synGAP with the PDZ domains of PSD-95 may be essential for the efficient cluster formation at a higher density along the dendrites and the proper localization of the PSD-95 clusters closer to the dendritic shafts.

Additionally, we noticed that the combined expression of the 1mΔ–2mΔ mutant of PSD-95 and the synGAP(ΔSXV) mutant enhanced the phenotypes (reduced SCI, diminished synaptic clusters, and larger cluster-shaft distances) compared with neurons coexpressing the PSD-95(WT)–GFP in the presence of the synGAP(ΔSXV) mutant (Fig. 9*B–D*). Thus, primary PDZ ligands of PSD-95 distinct from synGAP may also contribute to the synaptic clustering and morphological phenotypes associated with decreased PDZ binding.

These findings together raise the possibility that the PDZ-ligand binding of PSD-95, in part via synGAP, may play a direct role in the synaptic clustering of PSD-95, while also directing the localization of PSD-95 containing clusters toward the vicinity of the dendritic shafts, perhaps during the final step of spine maturation.

Discussion

The localization and function of many essential synaptic proteins are regulated by molecular interactions with PDZ-containing scaffolding proteins. However, the molecular mechanisms that regulate the clustering of such PSD scaffolds at synapses are not fully understood. In this report, PDZ-ligand-binding-deficient, full-length PSD-95 mutants were used to probe the contribution of each of the three PDZ-ligand-binding affinities in various aspects of PSD-95-mediated PSD organization.

Independent and additive contributions of each PDZ domain to PSD-95 clustering and recruiting PDZ ligands to the PSD scaffold

Previous studies showed that at least one PDZ domain was needed to target the PSD-95 to the synapse, in addition to the requirement of the N-terminal region including the palmitoylated pair of cysteines, which is essential for multimerization and membrane targeting (Craven et al., 1999; Hsueh and Sheng, 1999; Christopherson et al., 2003). Furthermore, the inhibition of the PDZ3 binding to the microtubule-binding protein CRIPT impaired the synaptic clustering of the PSD-95 (Passafaro et al., 1999). We also found that overexpression of a ligand-binding-deficient PDZ3 mutant of PSD-95 with a single point mutation, in cultured hippocampal neurons, significantly reduced the clustering efficiency of PSD-95 at synapses. This indicated that the interaction between the PDZ3 and its ligand(s) plays an important role in clustering PSD-95 at the synapses. Importantly, by extending the mutagenesis to the ligand-binding sites of PDZ1 and PDZ2, we demonstrated that the ligand-binding activities of PDZ1 and PDZ2 were equally critical for the efficient clustering of PSD-95. Furthermore, the impact of losing the PDZ-binding affinities one by one accumulated in an approximately additive manner. Thus, our data indicated that each individual binding event at each PDZ domain of PSD-95 independently and approximately additively contributes to the additional clustering of PSD-95 at the synapses.

We further verified this conclusion by a mutant study using one of the major ligand proteins, synGAP. The absence of the PDZ-binding motif of synGAP produced a pattern of results quite similar to those obtained with the ligand-binding-deficient PSD-95 mutants and strongly affected both the PSD-95 clustering and the spine morphology. In keeping with the results obtained by a systematic introduction of the mutations to the PDZ domains, the synGAP mutant data suggested that the presence of PSD-95 ligand-binding motifs is not sufficient, but rather an intact synGAP-PSD-95 interaction, mediated via PDZ-binding, is necessary for the clustering and the functions of PSD-95.

Our results also revealed that the ligand-binding-deficient mutants exhibited significantly weaker associations with the PSD. The 2-bromo-palmitate treatment easily dispersed the mutant PSD-95–GFP molecules from their clusters. These results support the notion that PSD-95 is anchored to the PSD by both the palmitate modification and the ligand-PDZ domain interactions. Furthermore, the clusters expressing PSD-95 mutants became extremely labile after F-actin depolymerization by the LatA treatment, consistent with the possibility that the mutant PSD-95

was unable to reach the core fraction of the PSD and thus became mislocalized toward the peripheral, cytoskeleton-dependent fraction of the PSD (Allison et al., 2000; Zhang and Benson, 2001). Together, the data suggested that the tight and stable association of PSD-95 with the PSD requires the interactions of the PDZ domains with their ligands, as well as the appropriate PSD-95 lipidification and actin polymerization.

Our results also suggested that each ligand-binding event at the PDZ domains may additionally fulfill two other separate functions in parallel: efficient targeting of PSD-95 to the spines, and stable recruitment and incorporation of PSD-95 ligands into the PSD. In this regard, it may be useful to consider two distinct classes of PSD-95 ligands: one class including PSD-95-specific ligands, such as synGAP, and another class that includes scaffold-independent ligands, such as NMDA receptor subunits, which, for example, can tightly interact with synapse-associated protein 102 (SAP102) as well. Although the latter may still become localized to the synapses in the absence of PSD-95 (Migaud et al., 1998; Rao et al., 1998), our data suggested that PDZ-ligand binding strongly contributes to the tight association of these two classes of proteins as stable multiprotein networks in the vicinity of synapses.

Another implication of our work, in view of the approximately additive effect of the three PDZ domains in the clustering of PSD-95, is that the tightness of the PSD association with the PSD protein complex may be tuned and controlled as a function of the number of PDZ-ligand-binding events. The intermolecular MAGUK interaction is reportedly activity regulated, at least in the case of SAP97 (Nakagawa et al., 2004). Whether this activity dependence in MAGUK clustering and dispersion is actually mediated by the PDZ-ligand binding still remains to be demonstrated. If this is the case, then the clustering efficiency of the MAGUK-like scaffolds, such as PSD-95, could be dynamically modulated depending on the local synaptic activity, thereby accounting for the requirement for multivalent scaffolding proteins in the regulation of synaptic plasticity (Migaud et al., 1998; Ehrlich and Malinow, 2004; Nakagawa et al., 2004).

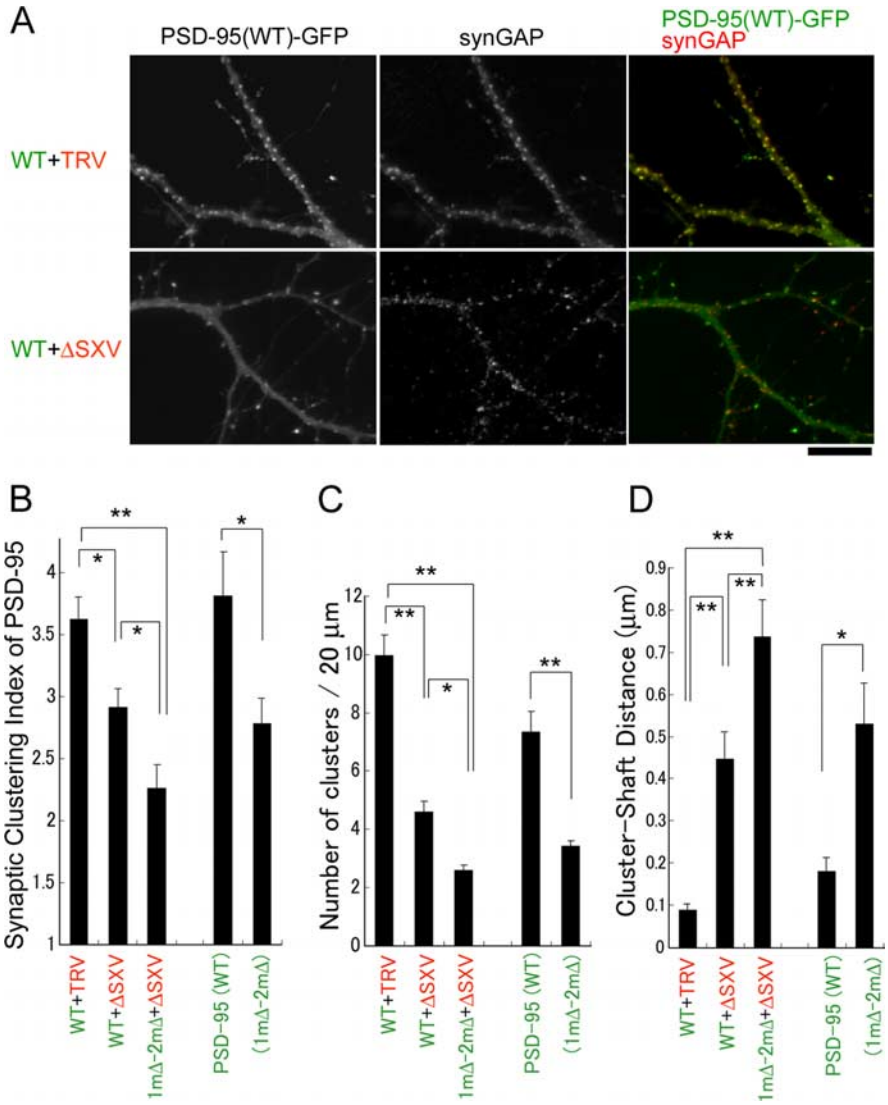

Figure 9. Overexpression of a mutant synGAP lacking the C-terminal PDZ-binding motif results in a severe defect in the PSD-95 cluster formation and a significant increase in the PSD-95 cluster-shaft distance. ***A***, Immunolocalization of PSD-95 clusters [PSD-95(WT)–GFP] and synGAP in neurons coexpressing the wild-type PSD-95 and either the wild-type synGAP (TRV, top panels) or the PDZ-binding motif mutant synGAP (ΔSXV, bottom panels). Neurons were cotransfected at 8 DIV and fixed at 10 DIV. The coexpression of wild-type synGAP(TRV) with wild-type PSD-95 did not result in a detectable change in the clustering efficiency and cluster density compared with the overexpression of PSD-95(WT)–GFP alone. Remarkably, during coexpression with synGAP(ΔSXV), PSD-95(WT)–GFP displayed an aberrant dendritic distribution that was similar to the phenotypes seen with the ligand-binding-deficient PSD-95 mutant (i.e., 1mΔ–2mΔ). ***B–D***, Quantification of the defects seen in synaptic cluster formation and in spine morphology. Based on the images of PSD-95–GFP, the SCI values (***B***), the cluster density (the number of synaptic clusters per 20 μm dendritic segment) (***C***), and the cluster-shaft distance (***D***) were measured in the neurons expressing the indicated constructs and are shown as bar graphs. We confirmed that the PSD-95 clusters analyzed were all juxtaposed to the synaptophysin-staining puncta. Scale bar, 10 μm. *$p < 0.05$, **$p < 0.01$, by one-way ANOVA with *post hoc* Tukey's test (n = 12–16 neurons); or *$p < 0.05$, **$p < 0.01$, by the Student's t test in the experimental pairs of PSD-95(WT)–GFP only and PSD-95(1mΔ–2mΔ)–GFP only.

Possible recruitment of a morphogenic signaling complex via PSD-95 during synapse development

In our hands, the ligand-binding-deficient PSD-95 not only reduced the clustering efficiency and altered the composition of the PSD but also localized its own clusters far from the dendritic shafts in hippocampal neurons (Figs. 3, 7). The aberrant PSD cluster location and the higher frequency of elongated spine morphology were consistently observed in both a transient plasmid expression protocol by gene delivery at 8–9 DIV and after a long-term expression protocol by electroporation at 0 DIV, with subsequent 12–13 d cultures. Because no gross morphological change was reported in the PSD-95 knock-out mouse (Migaud et al., 1998), it may appear, at first glance, that the defect in the PSD cluster localization seen in our culture could be interpreted as an experimental artifact. However, we believe that this is not the case for two reasons. First, the PSD location and the spine morphol-

ogy defects are not observed in all spines but only in a minority of the spines within a mutant PSD-95 expressing neuron (at most, 20.8% of spines longer than 1 μm, even in the severest case). Therefore, a large-scale quantification study in the knock-out mice brain, using serial electron microscopy over entire dendritic trees, would be needed to corroborate our findings, but such an effort was not undertaken in the initial screen (Migaud et al., 1998). Second, we observed correlations not only between the synaptic clustering index and the cluster-shaft distance (a negative correlation) but also between the PSD-95 cluster formation and the synGAP recruitment (a positive correlation). Indeed, synGAP, a GTPase-activating protein for Ras, was less densely localized at postsynaptic sites in the 1mΔ–2mΔ–N326S expressing neurons, whereas Shank/Synamon, Homer 1c/Ves1-L/PSD-Zip45, and PSD-93/Chapsin-110 were normally localized, as in the wild-type PSD-95 expressing neurons. Most critically, a synGAP mutant lacking the C-terminal PDZ-binding motif showed effects similar to those seen with PSD-95 mutants. These results are consistent with the idea that synGAP, in part downstream of PSD-95, may function in the clustering and movement of the PSD proteins in the spines. Furthermore, synGAP reportedly also negatively regulates spine formation and limits spine head expansion and filopodial extension (Vazquez et al., 2004). Together, alterations in the distribution and the PSD clustering of synGAP may at least partially account for the aberrant phenotype observed in cultured neurons. Thus, PSD-95 may play a role in recruiting a morphogenic signaling complex within the PSD complex.

Such a view is consistent with the following experimental results. (1) The morphological changes associated with the wild-type PSD-95 overexpression were shown to increase both the number and size of the spines, together with the accelerated synaptic maturation (El-Husseini et al., 2000). (2) Several partners interacting with PSD-95, such as Citron (Furuyashiki et al., 1999), CRIPT (Niethammer et al., 1998), and SPAR (spine-associated RapGAP) (Pak et al., 2001), were shown to interact with cytoskeletal elements. The overexpression of related scaffold proteins, such as Homer and Shank, which closely interact with actin cytoskeletal components (Naisbitt et al., 1999; Shiraishi et al., 1999), reportedly induces spine head enlargement (Sala et al., 2001). (3) A growing list of morphogenic signaling molecules has been identified within the PSD complex, such as FMR1 (the fragile X mental retardation gene) (Comery et al., 1997; Nimchinsky et al., 2001), LIM kinase (Meng et al., 2002), N-cadherin (Togashi et al., 2002), ephrinA3-EphA4 (Murai et al., 2003), cortactin (Hering and Sheng, 2003), Rho-GEF (guanine nucleotide exchange factor) Kalirin-7 (Penzes et al., 2001), and EphB2 (Murai et al., 2003). Because PSD-95-ligand binding is critical for maintaining proper PSD organization, impairments of the scaffolding functions of PSD-95 might indirectly disrupt the PSD localization and the spine maturation by triggering the misorchestration of a morphogenic signaling complex.

In summary, this work demonstrates that the ligand-binding activities of each of the three PDZ domains are essential for PSD-95 to organize the PSD properly and are likely to be involved in achieving normal spine development, in part via interactions with synGAP. We found that the multivalent binding nature of PSD-95 plays a key role in the dynamic regulation of PSD protein clustering. Our study thus provides new insights into the structural basis of the role of PSD-95 in synaptic development and plasticity.

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