Molecular Determinants for Subcellular Localization of PSD-95 with an Interacting K$^+$ Channel

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Summary

Ion channels and PSD-95 are colocalized in specific neuronal subcellular locations by an unknown mechanism. To investigate mechanisms of localization, we used biolistic techniques to express GFP-tagged PSD-95 (PSD-95:GFP) and the K$^+$-selective channel Kv1.4 in slices of rat cortex. In pyramidal cells, PSD-95:GFP required a single PDZ domain and a region including the SH3 domain for localization to postsynaptic sites. When transfected alone, PSD-95:GFP was present in dendrites but absent from axons. When cotransfected with Kv1.4, PSD-95:GFP appeared in both axons and dendrites, while Kv1.4 was restricted to axons. When domains that mediate the interaction of Kv1.4 and PSD-95 were disrupted, Kv1.4 localized nonspecifically. Our results provide evidence that Kv1.4 itself may determine its subcellular location, while an associated MAGUK protein is a necessary but not sufficient cofactor.

Introduction

Immunolocalization studies have revealed that K$^+$ channels are expressed within neurons in a striking variety of spatial distributions. Different K$^+$ channels are localized in specific compartments within axons, dendrites, the cell body, or combinations of these areas (Sheng et al., 1992; Wang et al., 1994; Veh et al., 1995). In some neurons, K$^+$ channels are colocalized with PSD-95 (Kim et al., 1995), a protein purified from postsynaptic densities that is a homolog of the Drosophila protein Disc-large (Dlg), (Cho et al., 1992). PSD-95 contains three motifs known as PDZ domains that are approximately 90 amino acids in length and were first identified in PSD-95, Dlg, and ZO-1. In biochemical assays, PDZ domains have been found to bind the C terminus of many channels including outward (Kim et al., 1995) and inward rectifier K$^+$ channels (Cohen et al., 1996), NMDA receptors (Kornau et al., 1995), and kainate receptors (Garcia et al., 1998). The last four amino acids on the C terminus of these channels determine in large part which PDZ domains they bind (Kim et al., 1995; Kornau et al., 1995; Doyle et al., 1996; Songyang et al., 1997), although binding is also influenced by amino acids further from the C terminus (Niethammer et al., 1998).

PSD-95 is a member of the MAGUK (membrane-associated guanylate kinase) family of genes, which encode proteins containing a guanylate kinase domain, an SH3 domain, and at least one PDZ domain (Sheng, 1996).

There are several lines of evidence that point to a role of MAGUK proteins in determining the subcellular distribution of ion channels. In heterologous cells, PSD-95 mediates clustering of ion channels into raft-like structures (Kim et al., 1995). In Drosophila, there is evidence that interaction with MAGUK proteins is necessary not only for clustering but also for subcellular localization of membrane proteins. The MAGUK protein Discs-large, for instance, is necessary for correct localization of Fasciclin II and Shaker to the neuromuscular junction (Tejedor et al., 1997; Thomas et al., 1997; Zito et al., 1997). In addition, disrupting the PDZ-containing protein INAD prevents the TRP channel from localizing to the rhabdome of the photoreceptor (Shieh and Zhu, 1996; Chevesich et al., 1997; Tsunoda et al., 1997). In Caenorhabditis elegans, mutating the PDZ domain-containing protein lin-10 prevents the glutamate receptor gll-1 from localizing to synapses in interneurons (Rongo et al., 1998).

In the mammalian CNS, evidence that PDZ domain-containing proteins are necessary for ion channel localization is more indirect. Transfecting the C terminus of the GluR2 receptor prevents the endogenous receptor from localizing to postsynaptic densities (Dong et al., 1997). In addition, a targeted deletion of the C terminus of NR2 in a transgenic mouse prevented that receptor from localizing to postsynaptic sites (Mori et al., 1998). The C terminus of NR2 interacts with PSD-95 and that of GluR2 interacts with the PDZ domain-containing proteins ABP (Srivastava et al., 1998) and GRIP (Dong et al., 1997). Thus, the interaction of these channels with PDZ-containing proteins might be necessary for localization. On the other hand, a separate study in transgenic mice found that NR2 receptors with deleted C termini did localize (Sprengel et al., 1998).

One hypothesis is that MAGUK proteins provide signals that localize different ion channels to specific subcellular locations. An argument against this hypothesis is that Kv1.3 and Kv1.4, which have very different subcellular localizations, will both bind PSD-95 in heterologous cells (Kim and Sheng, 1996). This is not particularly strong evidence, however, since it is not known which MAGUK protein either channel binds in vivo. One way of testing this hypothesis would be to eliminate MAGUK proteins that interact with a particular channel and then determine whether the channel could still localize. This is difficult in mammalian systems, because channels can interact directly or indirectly (through other channel subunits) with a number of different MAGUK proteins, some of which may not have been identified.

Here, we further examine the role of the MAGUK protein, PSD-95, in localization of an ion channel. We show that exogenous PSD-95 tagged with GFP (PSD-95:GFP) localizes to excitatory postsynaptic sites when transfected into pyramidal cells of a cortical slice. Deletion analysis shows that postsynaptic localization of PSD-95:GFP requires the presence of at least one PDZ domain. This result suggests that interactions with ion channels or other membrane proteins may be necessary for localization of PSD-95. Can interaction with an ion
channel direct the localization of PSD-95? To address this question, we cotransfected PSD-95 with an axonally localized K⁺ channel, Kv1.4, with which it has been shown to interact in vitro. We found that PSD-95 was now localized in the axon as well as the dendrite, while Kv1.4 was present mainly in the axon. These data support a model in which PSD-95 can be localized to specific subcellular compartments by interactions via PDZ domains with ion channels and other membrane proteins.

Results

GFP-Tagged PSD-95 Is Localized to Dendritic Spines of Cortical Pyramidal Cells

PSD-95 tagged with GFP (PSD-95:GFP) was cotransfected with the reporter gene alkaline phosphatase into cortical slices from P12-P14 rat brain using the biolistic particle delivery system (Arnold et al., 1994; Arnold and Heintz, 1997). Figure 1A shows that alkaline phosphatase filled the entire cell, delineating its anatomical features. The axons of cortical pyramidal cells can be readily distinguished from dendrites by their length and orientation. The axons project away from the surface of the cortex in a direction 180 degrees from the apical dendrite and extend far beyond any of the dendrites. Figure 1B shows a punctate pattern produced by PSD-95:GFP overlaid on the alkaline phosphatase image. We obtained similar patterns of expression of PSD-95:GFP when it was transfected alone, insuring that alkaline phosphatase did not affect its expression (data not shown). One particularly conspicuous feature of the localization of PSD-95:GFP is that it was virtually absent from the axon (Figure 1D, white arrows), confirming the pattern of expression found in vivo (Hunt et al., 1996). In contrast, alkaline phosphatase was clearly present in the same axon (Figure 1C, white arrows). GFP fluorescence was barely detectable in the axons of other cells transfected with full-length PSD-95:GFP (n = 5) as well as those transfected with deletion mutants (n = 30). PSD-95:GFP was localized to the distal tips of dendritic spines (Figures 1E-1G) consistent with PSD-95:GFP localizing to postsynaptic sites (Boyer et al., 1998).

To further examine the localization of PSD-95:GFP,
we counterstained transfected slices with antibodies to α-actinin 2 (Figure 2), a marker for excitatory postsynaptic sites (Wyszynski et al., 1997, 1998). Each punctum of PSD-95:GFP colocalized with a punctum of α-actinin 2 staining. Since α-actinin 2 is localized to excitatory postsynaptic sites, it is likely that PSD-95:GFP is as well. Note that there are more puncta of α-actinin 2 visible than of PSD-95:GFP because PSD-95:GFP is only in transfected cells, whereas α-actinin 2 is present in untransfected cells as well. Scale bar, 5 μm.

The data in Figure 2 provide evidence that PSD-95:GFP localizes to excitatory postsynaptic sites, similar to endogenous PSD-95 (Hunt et al., 1996; Rao et al., 1998).

Inspection of the localization pattern of PSD-95:GFP suggests that the intensity of puncta does not diminish at distal points along the dendrite. To test this point quantitatively, we measured average intensities of PSD-95:GFP fluorescence of 150-200 individual puncta and plotted them as a function of distance from a point in the center of the cell body. Although there was considerable variability, the intensity of PSD-95:GFP showed no systematic variation with distance from the soma (Figure 3A). We performed similar measurements for alkaline phosphatase by determining the average intensity of fluorescence produced by Texas red dye in spines in circular regions of approximately the same size as the PSD-95:GFP puncta. As can be seen in Figure 3B, the average intensity of PSD-95:GFP fluorescence did not change with distance from the soma, unlike fluorescence associated with alkaline phosphatase that showed a systematic decrease. Similar results were found in other cells (n = 5). The fact that the intensity of PSD-95:GFP puncta did not vary even over large distances raises intriguing questions about the mechanisms by which its transport and targeting are achieved.

At Least One PDZ Domain Is Necessary for Localization of PSD-95

A systematic deletion of each of the five modular domains of PSD-95:GFP (Figure 4) was carried out in order to determine which domains were necessary for localization. We found that deletion of individual PDZ domains had no effect on the ability of PSD-95:GFP to localize to dendritic spines (Figures 4B-4D; Figure 6). We also tested constructs that had all three possible combinations of two PDZ domains deleted. We found that any single PDZ domain was sufficient to localize PSD-95:GFP to dendritic spines (Figures 4E-4G; Figure 6). However, deletion of all three PDZ domains prevented PSD-95:GFP from localizing to dendritic spines (Figures 4H-4K; Figure 6). These results indicate that while no single PDZ domain was preferred, at least one was required for localization. Thus, PSD-95 might require interaction with ion channels and other membrane proteins through its PDZ domains in order to localize.

The SH3 Domain Is Also Necessary for Localization

We tested whether the PDZ domains by themselves were sufficient to localize PSD-95:GFP to dendritic spines by deleting the SH3 domain, guanylate kinase (GK) domain, the linker domain between PDZ3 and SH3 (L1), and the linker domain between SH3 and GK (L2) to create the construct ΔSH3.GKPSD-95:GFP. Constructs

![Figure 2. PSD-95:GFP Colocalizes with an Excitatory Postsynaptic Marker](image)

Cortical slices transfected with PSD-95:GFP were frozen, cut into sections with a thickness of 15 μm, and stained for α-actinin 2 and GFP. Each punctum of PSD-95:GFP overlaps with a punctum of α-actinin 2. Since α-actinin 2 is localized to excitatory postsynaptic sites, it is likely that PSD-95:GFP is as well. Note that there are more puncta of α-actinin 2 visible than of PSD-95:GFP because PSD-95:GFP is only in transfected cells, whereas α-actinin 2 is present in untransfected cells as well. Scale bar, 5 μm.

![Figure 3. Intensity of Fluorescence Related to PSD-95:GFP and to Alkaline Phosphatase as a Function of Distance from the Cell Body](image)

(A) The intensity of PSD-95:GFP puncta did not vary systematically with distance from the soma. The line shown was calculated by linear regression. (B) Intensity of alkaline phosphatase staining diminished with increasing distance from cell body.
Figure 4. At Least One PDZ Domain Is Necessary for Localization of PSD-95:GFP

(A) PSD-95 contains five modular domains. GFP was added to the C terminus. (B) PSD-95:GFP was able to localize to dendritic spines with PDZ1 deleted. Scale bar, 10 µm. (C–G) Deletion of up to two PDZ domains did not affect localization. The chart on the right indicates which PDZ domains have been deleted (−) or spared (+) for each construct tested. Scale bar, 5 µm. (H–K) ΔPDZ1.2.3PSD-95:GFP (all three PDZ domains deleted) did not localize. (H) Alkaline phosphatase and ΔPDZ1.2.3PSD-95:GFP. Scale bar, 10 µm. (I) ΔPDZ1.2.3PSD-95:GFP. (J) Alkaline phosphatase. Scale bar, 5 µm. (K) ΔPDZ1.2.3PSD-95:GFP.

containing these deletions were no longer localized to any specific region (Figures 5A–5C). We found that ΔSH3.GKPSD-95:GFP clustered Kv1.4 following coexpression of the two proteins in COS-7 cells (data not shown), indicating that ΔSH3.GKPSD-95:GFP is probably a functional protein. When we deleted just the GK domain (ΔGKPSD-95:GFP), localization of PSD-95:GFP was unaffected (Figures 5D–5F). We were unable to test whether deleting the SH3 region (SH3, L1, and L2 domains together) prevented PSD-95:GFP from localizing, because these constructs did not express. We conclude that the PDZ domains alone are not sufficient to localize PSD-95.

Endogenous PSD-95 could complicate our studies since PSD-95 can homomultimerize through interactions mediated by residues near the N terminus (Hsueh et al., 1997). However, ΔPDZ1.2.3PSD-95:GFP and ΔSH3.GKPSD-95:GFP did not localize in pyramidal cells despite having intact N-terminal regions. These experiments suggest that PSD-95:GFP is not significantly localized through interaction with endogenous PSD-95. They also suggest that palmitoylated residues on the N-terminal region, which are necessary for PSD-95 to associate with cell membranes and to interact with ion channels in vitro (Topinka and Bredt, 1998), are not sufficient to mediate localization of PSD-95 to synapses.

In order to investigate the degree of localization of the various deletion constructs, the intensity of PSD-95:GFP fluorescence was measured both at the puncta at the ends of dendritic spines and at points on the dendrite immediately adjacent to the spine. The ratio of these two numbers was calculated and the results tabulated for five neurons for each construct. PSD-95:GFP fluorescence was approximately 10 times more intense at the tips of spines than in the adjacent dendrite (Figure 6). To compare the expression pattern of alkaline phosphatase to that of PSD-95:GFP, we calculated the ratio of alkaline phosphatase fluorescence on the same
PSD-95 and Kv1.4 Localization

developed an experiment in which we tested not just whether a MAGUK protein localized, but where it would localize if it interacted with a particular ion channel. Given a MAGUK protein that normally localizes to the dendrite and a channel that normally localizes to the axon, what would be the localization of a MAGUK/channel complex? Would the channel protein retain its targeting, bringing the MAGUK protein with it to the axon, or would the MAGUK protein retain its targeting and bring the channel to the dendrite? One approach to this experiment would be to fuse a motif that was recognized by the PDZ domains of the dendritically localized MAGUK protein onto the C terminus of an axonally targeted channel. Fortunately, the naturally occurring channel, Kv1.4, and the MAGUK protein, PSD-95, represent such a combination without requiring modification.

Kv1.4 Localizes PSD-95

Deletion analysis suggested that PSD-95:GFP requires interaction with membrane proteins through its PDZ domains in order to localize to dendritic spines. We found that the SH3 domain including the adjacent linker regions was also necessary for localization. One explanation of our results is that the SH3 region might be permissive for localization of PSD-95, while the actual subcellular location of PSD-95 is determined by interactions with membrane proteins through its PDZ domains. Conversely, the SH3 region might determine the subcellular localization of PSD-95, while interactions with membrane proteins through PDZ domains are permissive. To distinguish between these two possibilities, we designed an experiment in which we tested not just whether a MAGUK protein localized, but where it would localize if it interacted with a particular ion channel. Given a MAGUK protein that normally localizes to the dendrite and a channel that normally localizes to the axon, what would be the localization of a MAGUK/channel complex? Would the channel protein retain its targeting, bringing the MAGUK protein with it to the axon, or would the MAGUK protein retain its targeting and bring the channel to the dendrite? One approach to this experiment would be to fuse a motif that was recognized by the PDZ domains of the dendritically localized MAGUK protein onto the C terminus of an axonally targeted channel. Fortunately, the naturally occurring channel, Kv1.4, and the MAGUK protein, PSD-95, represent such a combination without requiring modification.

Kv1.4 associates with PSD-95 through its first and second PDZ domains in vitro (Kim et al., 1995). In addition, immunohistochemistry of brain sections (Sheng et al., 1992) and immuno-electron microscopy (Cooper et al., 1998) demonstrated that Kv1.4 is specifically localized to axons (Kv1.4 is normally present only at low levels in the cortex (Sheng et al., 1992; Rhodes et al., 1995). Furthermore, PSD-95 specifically localized to dendrites in vivo (Hunt et al., 1996; Rao et al., 1998),

Figure 5. The SH3 Domain (Including Linkers) Is Necessary for Localization of PSD-95:GFP

(A–C) △SH3.GKPSD-95:GFP (SH3, GK domains and linkers between PDZ3 and SH3 [L1] and between SH3 and GK [L2] deleted) did not localize to dendritic spines. (A) Alkaline phosphatase and △SH3.GKPSD-95:GFP. Scale bar, 10 μm. (B) Alkaline phosphatase. Scale bar, 5 μm. (C) △SH3.GKPSD-95:GFP. (D–F) △GKPSD-95:GFP (GK domain deleted) localized to dendritic spines. (D) Alkaline phosphatase and △GKPSD-95:GFP. Scale bar, 10 μm. (E) Alkaline phosphatase. Scale bar, 5 μm. (F) △GKPSD-95:GFP.

neurons at identical locations. The ratio of alkaline phosphatase fluorescence intensity in spines versus dendrites was approximately 1. The PSD-95:GFP deletion constructs yielded similar ratios except for the △1.2.3 and △SH3.GK. Neither △1.2.3 nor △SH3.GK had ratios that were significantly different from that of alkaline phosphatase (p < 0.05, p < 0.05, respectively; Figure 6).

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and PSD-95:GFP specifically localized to dendrites when transfected into cortical slices (Figure 1D). We altered the extracellular domain between S3 and S4 of Kv1.4 to encode a FLAG epitope, which could be identified by a commercially available anti-FLAG antibody. When PSD-95:GFP and Kv1.4-FLAG were cotransfected into slices, Kv1.4 staining was almost entirely restricted to the axon (Figures 7A, 7C, 7E, and 7F), while PSD-95:GFP staining was found in both the axon and at synapses in the dendrites (Figures 7B, 7C, 7E, and 7F). The mean intensity of fluorescence associated with Kv1.4-FLAG was found to be an average of 16 times more in the axon than in the dendrites (n = 3 cells). Because PSD-95:GFP localized to the axon only when it associated with Kv1.4, it is likely that the signal for axonal localization was on Kv1.4.

PSD-95 Is Necessary for Localization of Kv1.4
If Kv1.4 contains information vital for PSD-95 localization to axons, is PSD-95 necessary for localization of Kv1.4? Transfection of Kv1.4 alone showed no specific localization (Figure 8E), indicating that coexpression with PSD-95 was necessary for Kv1.4 to localize specifically to the axon. To further test whether interaction with PSD-95 was necessary for localization of Kv1.4, we abolished the interaction domains on each protein individually and examined the effect on localization. Kim et al. (1995) showed that changing the Kv1.4 C-terminal valine to alanine could abolish the interaction with PSD-95. Similarly, we mutated the C terminus of the FLAG-tagged Kv1.4 from valine to alanine to obtain a mutant, Kv1.4 V655A, that would not interact with PSD-95. Unlike cotransfection of Kv1.4 and PSD-95:GFP (Figures 7A, 7C, 7E, and 7F), Kv1.4 V655A cotransfection with PSD-95:GFP resulted in PSD-95:GFP's absence from the axon and Kv1.4 V655A's diffuse spread throughout the entire cell (Figures 8A and 8B). In the dendrites, PSD-95:GFP was distributed in the characteristic punctate dendritic pattern. Also, since PSD-95 binds to Kv1.4 through PDZ1 and PDZ2 (Kim et al., 1995), we cotransfected Kv1.4 with a PSD-95 mutant lacking these domains (ΔPDZ1.2PSD-95:GFP). In this case, Kv1.4 was not localized exclusively to the axon, as when it was cotransfected with PSD-95:GFP, but instead was distributed throughout the entire cell (Figure 8C). As expected, Δ1.2PSD-95:GFP produced the characteristic punctate distribution in the dendrites (Figure 8D). These results indicate that PSD-95 is necessary for localizing Kv1.4 to the axon in this system.

Discussion
The localization of PSD-95 is a complex process that is likely to include the transport and targeting of the protein...
PSD-95 and Kv1.4 Localization

Our experiments suggest that PSD-95:GFP must interact with a membrane protein via its PDZ domains to localize to postsynaptic sites and that Kv1.4 must interact with a MAGUK protein via its C terminus to localize to axons. They also suggest that Kv1.4 can localize PSD-95:GFP to the axon. The simplest interpretation of our results is that Kv1.4 contains a signal that specifies an axonal localization but that localization also requires a permissive signal from a MAGUK protein with which it interacts.

Localization of PSD-95:GFP to dendritic spines required the presence of the SH3 region in addition to a single PDZ domain. In this respect, PSD-95 is similar to the Drosophila MAGUK protein Discs-large, which requires a single PDZ domain and the HOOK region to localize to septate junctions in epithelial cells (Hough et al., 1997). Localization in neurons and epithelial cells is, therefore, distinct from clustering in heterologous cells, where only a single PDZ domain is required (Hsueh et al., 1997). It is interesting to note that any of the three PDZ domains was sufficient to localize PSD-95 (provided the non-PDZ domain portion of the PSD-95 was intact). Since nonchannel membrane proteins such as CRIP1 and neurogliin bind to PDZ3 (Irie et al., 1997; Niethammer et al., 1998), it has been suggested that PDZ3 might play a special role in the localization of PSD-95. However, our results do not support this interpretation.

Our results suggest that interaction with a MAGUK protein is necessary for Kv1.4 to localize to the axon. This is consistent with experiments in Drosophila that show that the Shaker channel and Fasciclin II must interact with Discs-large in order to localize to the neuromuscular junction (Tejedor et al., 1997; Thomas et al., 1997; Zito et al., 1997). Although the experiments in this study indicate that the signal for localization to the axon is most likely a part of Kv1.4, they do not address the nature of the signal motif. Recent work has suggested that localization motifs may be found on the cytoplasmic tail of $K_1$ channels. In epithelial cells, a sequence on the membrane proximal cytoplasmic tail of Kv2.1 localized the channel (Scannevin et al., 1996). In addition, work in Drosophila muscle indicated that the last 11 amino acids on the C terminus of the Shaker K$^+$ channel were sufficient to mediate localization of CD-8 to the neuromuscular junction (Zito et al., 1997).

Some neuronal messenger RNAs are transported to remote locations outside the cell body, suggesting that mRNAs may contain sequences that determine subcellular localization (Steward et al., 1998). Our experiments do not support this mechanism for PSD-95. Any single PDZ domain is capable of localizing PSD-95 to synapses, and these domains are considerably more homologous at the amino acid level than at the nucleotide level. It seems more likely that PSD-95 is localized as a result of protein-protein interactions through its PDZ domains.

It has been suggested that ion channels may localize within cells by first diffusing throughout the cell and then being selectively stabilized at specific subcellular locations. This possibility is supported by observations of chimeras consisting of CD-8 fused to the cytoplasmic tail of the Shaker $K^+$ channel that were expressed in Drosophila muscle. These chimeras were observed shortly in a specific site, clustering with other proteins into an aggregate, and stabilization of that aggregate.
after expression to localize diffusely throughout the muscle, but at later times after the production of mRNA had ceased, to cluster only at the neuromuscular junction (Zito et al., 1997). Results from our study suggest that PSD-95:GFP might localize through a different mechanism following transfection into cortical slices. In the experiments described here, PSD-95:GFP mRNA was continually produced and yet the protein was still able to localize in a specific manner. Furthermore, when slices were examined as early as 12 hr after transfection, PSD-95:GFP was found in a punctate pattern (D. B. A. and D. E. C., unpublished data). These results suggest that, unlike the CD8/Shaker chimeras, PSD-95 protein is targeted directly to synapses. Future studies involving observation of PSD-95-GFP expression in real time may further elucidate mechanisms by which PSD-95 is localized.

Our experimental results support the conclusion that neither PSD-95 nor Kv1.4 by itself contains sufficient information to mediate subcellular localization. Thus, it is likely that localization of these proteins is more complex than that of proteins targeted to the nucleus or endoplasmic reticulum, where a single domain is necessary and sufficient for localization. We propose a model in which PSD-95 is localized as a result of interactions with membrane proteins. In this model, PSD-95 plays a permissive role, but the ion channels or membrane proteins that bind to the PDZ domains play an instructive role in determining subcellular localization.

Experimental Procedures

DNA Constructs

PSD-95:GFP was made as follows: EGFP (Clontech) was amplified by PCR so that three glycines, an EcoRI, and NheI site were added of the cell body at dendritic spines. In both cases, the average circular area was measured along with the distance to the center of the cell body at dendritic spines. In the case of alkaline phosphatase, the average intensity of Texas red fluorescence inside the same circular area was measured along with the length from the center of the punctum to the center of the cell body. In the case of alkaline phosphatase, the average intensity of Texas red fluorescence inside the same circular area was measured along with the distance to the center of the cell body at dendritic spines. In both cases, the average background fluorescence was subtracted from each measurement.

In order to estimate the degree of localization of PSD-95:GFP constructs, average fluorescence intensity was measured in puncta on dendritic spines and at points on the dendritic shaft immediately adjacent to the puncta. At least 25 measurements were made and averaged together following subtraction of background for both spines and dendritic shafts. The ratio was then calculated and averaged over five cells. In order to calculate a similar ratio for alkaline phosphatase, Texas red fluorescence intensity was measured at the identical points used to measure PSD-95:GFP.

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