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SALM Synaptic Cell Adhesion-like Molecules Regulate the Differentiation of Excitatory Synapses

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Summary

Synaptic cell adhesion molecules (CAMs) are known to play key roles in various aspects of synaptic structures and functions, including early differentiation, maintenance, and plasticity. We herein report the identification of a family of cell adhesion-like molecules termed SALM that interacts with the abundant postsynaptic density (PSD) protein PSD-95. SALM2, a SALM isoform, distributes to excitatory, but not inhibitory, synaptic sites. Overexpression of SALM2 increases the number of excitatory synapses and dendritic spines. Mislocalized expression of SALM2 disrupts excitatory synapses and dendritic spines. Bead-induced direct aggregation of SALM2 results in coclustering of PSD-95 and other postsynaptic proteins, including GKAP and AMPA receptors. Knockdown of SALM2 by RNA interference reduces the number of excitatory synapses and dendritic spines and the frequency, but not amplitude, of miniature excitatory postsynaptic currents. These results suggest that SALM2 is an important regulator of the differentiation of excitatory synapses.

Introduction

The early stages of synaptic differentiation are thought to involve a series of events including the initial contact between the pre- and postsynaptic sides, *trans*-synaptic adhesion, and the recruitment of synaptic proteins to early synapses. Synaptic cell adhesion molecules (CAMs) play key roles in these processes and also function in the maintenance and activity-dependent changes of established synapses (Craig et al., 2006; Dean and Dresbach, 2006; Funke et al., 2004; Levinson and El-Husseini, 2005; Li and Sheng, 2003; Scheiffele, 2003; Waites et al., 2005; Washbourne et al., 2004; Yamagata et al., 2003; Yuste and Bonhoeffer, 2004).

Previous studies have identified several synaptic CAMs, including neuroligin, SynCAM, Sidekick, cadherin, protocadherin, and NCAM (Biederer et al., 2002; Ichtchenko et al., 1995; Kohmura et al., 1998; Washbourne et al., 2004; Wu and Maniatis, 1999; Yamagata et al., 2002, 2003). Each of these synaptic CAMs differ in terms of homo/heterophilic adhesion, calcium sensitivity, and synaptic/extrasynaptic localization and are thought to act in different processes, such as recognition of target domains within a neuron, synaptic differentiation, synaptic stability, and plastic changes in synapses.

Synaptic differentiation by CAMs is likely to involve *trans*-synaptic adhesion between pre- and postsynaptic CAMs and subsequent recruitment of synaptic proteins. An ideal synaptic CAM for synaptic differentiation may have two characteristics. First, it should mediate a heterophilic adhesion, because different CAMs in pre- and postsynaptic sides may allow dendrites and axons to efficiently find partners without self-adhesion. Second, the prototypical CAM should interact with an appropriate cytosolic scaffolding protein capable of recruiting a variety of synaptic proteins to the early synapse.

Neuroligin is an extensively studied family of CAMs that fits this profile (Ichtchenko et al., 1995). Members of the neuroligin family interact with β-neurexin, their presynaptic ligand, and with PSD-95, an abundant scaffolding protein in the postsynaptic density (PSD) (Ichtchenko et al., 1996; Irie et al., 1997). The β-neurexin-neuroligin complex has been shown to be sufficient to induce both pre- and postsynaptic differentiation (Chih et al., 2005; Dean et al., 2003; Graf et al., 2004; Nam and Chen, 2005; Scheiffele et al., 2000). Intriguingly, different isoforms of the neuroligin family display different subcellular localizations in neurons; for instance, neuroligin-1 distributes to excitatory synapses, while neuroligin-2 is detected at inhibitory synapses (Chih et al., 2005; Graf et al., 2004; Levinson et al., 2005; Song et al., 1999; Varoqueaux et al., 2004). Collective data from overexpression, dominant-negative inhibition, siRNA knockdown, neuron-fibroblast coculture, and bead aggregation experiments indicate that each neuroligin isoform plays a different role in the differentiation of excitatory and inhibitory synapses (Chih et al., 2005; Graf et al., 2004; Levinson et al., 2005; Nam and Chen, 2005; Prange et al., 2004; Sara et al., 2005). These results strongly suggest that the neuroligin family members are key regulators of synaptic differentiation. However, considering the huge diversity of neuronal synapses, the cell biological steps that link initial neuron-to-neuron contacts to the establishment of early synapses and their subsequent differentiation may be far more complex than our current understanding.

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PSD-95 is a key regulator of the structure and function of excitatory synapses (Funke et al., 2004; Kim and Sheng, 2004). In support of this, PSD-95 overexpression has been shown to promote the maturation of excitatory synapses (El-Husseini et al., 2000a). Conversely, down-regulation of PSD-95 by RNA interference and SNK (a polo-like inducible kinase) reduces excitatory synapses and synaptic responses (Nakagawa et al., 2004; Pak and Sheng, 2003; Prange et al., 2004). In addition, PSD-95 redirects neuroligin-2, which is mainly present at inhibitory synapses (Chih et al., 2005; Graf et al., 2004; Levinson et al., 2005; Prange et al., 2004), to excitatory synapses (Graf et al., 2004; Prange et al., 2004), inducing a shift in the balance between excitatory and inhibitory synapses. This suggests that the level of endogenous PSD-95 in neurons, which can be dynamically regulated by synaptic activity (Bao et al., 2004; Pak and Sheng, 2003), is a key determinant of the overall excitability of the neuron.

Of note, electron microscopic analysis has indicated that PSD-95 is located close to the postsynaptic membrane (Valtschanoff and Weinberg, 2001), suggesting that PSD-95 is located in an ideal position to link synaptic adhesion events to postsynaptic protein clustering and differentiation. Using the PDZ domains of PSD-95 as bait in yeast two-hybrid screens, we identified a family of CAM-like molecules, which was recently reported as SALM (Wang et al., 2006). Data from experiments including overexpression, bead aggregation, and siRNA knockdown of the SALM2 isoform strongly suggest that SALM2 selectively promotes the differentiation of excitatory synapses.

Results

Identification of SALM, a PSD-95-Interacting Family of Cell Adhesion-like Molecules

Using the PDZ domains of PSD-95 as bait in yeast two-hybrid screens of human cDNAs, we identified two different but highly related cell adhesion-like molecules (KIAA1246 and KIAA1484). Database searches using these two proteins identified two additional related proteins. Because these proteins were designated SALMs (for synaptic adhesion-like molecules) in the database and a recent paper (Wang et al., 2006), we adopted this nomenclature. However, our fourth protein was different from SALM4 and is thus designated SALM5. Invertebrate homologs of SALM were not found, suggesting that the SALM family has functions unique to vertebrates. The SALM family proteins are type I membrane proteins that share a similar domain structure. Extracellularly, they contain typical cell adhesion domains including leucine-rich repeats, an immunoglobulin domain, and a fibronectin type III domain. The transmembrane domain is then followed by a C-terminal PDZ domain binding motif (absent in SALM5) (Figure 1A and Figure S1A). Interestingly, the cytosolic regions of the SALM family proteins share little amino acid (aa) sequence identity (Figure S1B). The overall domain structure of SALM is similar, although not identical, to that of AMIGO, a family of CAMs implicated in axon tract development (Kuja-Panula et al., 2003), and NGL-1, a neuronal CAM that regulates the outgrowth of thalamocortical axons (Lin et al., 2003). AMIGO family members and NGL-1 have leucine-rich repeats and an immunoglobulin domain, but lack the fibronectin type III domain of SALM family members. The leucine-rich repeats of SALM resemble those found in the Slit family of axon-guidance proteins (Rothberg et al., 1988) and the Nogo-66 receptor (Fournier et al., 2001). Yeast two-hybrid, GST pull-down, in vitro coimmunoprecipitation, and coclustering assays consistently showed that SALM1, -2, and -3 (but not SALM5) interacted with PSD-95 and other PSD-95 family proteins (PSD-93/chapsyn-110, SAP97, and SAP102) (Figures 1B–1H). The ectodomain of AMIGO has been shown to mediate homophilic interactions (Kuja-Panula et al., 2003). However, the ectodomain of SALM2 fused to alkaline phosphatase did not interact with SALM2 displayed on the surface of heterologous cells (data not shown), suggesting that SALM2 does not mediate homophilic adhesion.

mRNA and Protein Expression Patterns of SALM

Northern blot analysis revealed that SALM2 mRNAs were mainly detected in rat brain and testis (Figure 2A). In situ hybridization showed that the mRNAs of the SALM isoforms were widely but distinctly expressed in various rat brain regions, including cerebral cortex, hippocampus, dentate gyrus, and olfactory bulb (Figure 2B). To study protein expression patterns of SALM2, we generated a SALM2-specific antibody that does not recognize other SALM isoforms in both immunoblot and immunohistochemistry conditions (Figure S2). An immunoblot analysis revealed that SALM2 proteins are mainly expressed in the brain (Figure 2C). Two major SALM2 protein bands were detected in rat brain (~105 and 80 kDa; Figure 2C). The top band, which is similar in size to SALM2 expressed in HEK293T cells (data not shown), appears to be N-glycosylated, as evidenced by the fact that the size was reduced following PNGase F digestion (Figure 2D). The expression levels of SALM2 proteins were found to increase steadily during postnatal rat brain development, parallel to the expression levels of PSD-95 (Figure 2E). SALM2 was mainly detected in synaptic plasma membrane (LP1) and light membrane (P3) brain fractions (Figure 2F) and was highly enriched in PSD fractions (Figure 2G).

In Vivo Association of SALM2 and PSD-95 at Excitatory Synapses

In rat brain sections, SALM2 proteins were widely expressed in brain regions, including the cortex, hippocampus, and cerebellum (Figures S3A–S3C). SALM2 signals were detected in cortical pyramidal neurons, hippocampal CA3 and CA1 neurons, and cerebellar Purkinje neurons (Figures S3D–S3G). Subcellularly, SALM2 signals were associated with cell bodies, neurites, and punctate structures (Figures S3D–S3G). In high-resolution images, SALM2 clusters colocalized with or were closely apposed to synapsin I (a presynaptic marker; Figure S3H), suggesting that SALM2 proteins are present at synaptic sites.

In cultured hippocampal neurons, SALM2 colocalized with PSD-95 and vGlut1, which are excitatory post- and presynaptic markers, respectively (Figures 3A and 3B). In contrast, SALM2 did not colocalize with gephyrin and VGAT, which are inhibitory post- and presynaptic markers, respectively (Figures 3C and 3D). Notably, some SALM2 signals were detected in MAP2 (a marker

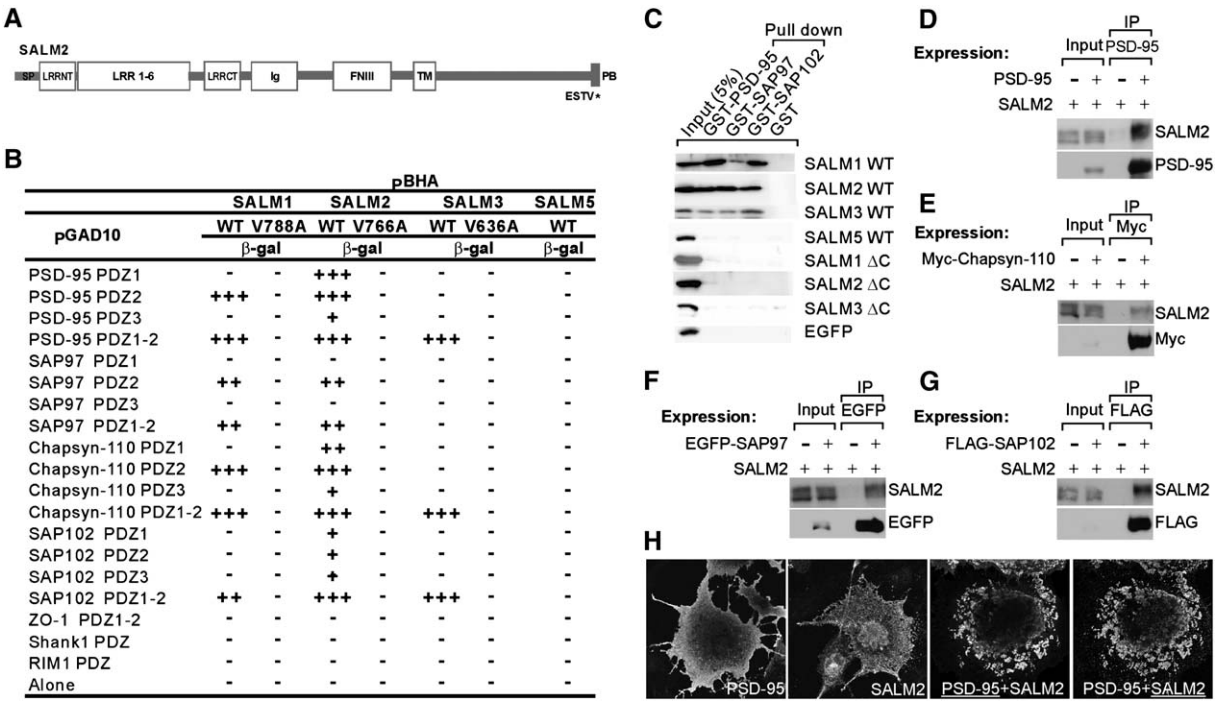

Figure 1. SALM Is a Family of Cell Adhesion-like Molecules that Interacts with PSD-95

(A) Domain structure of SALM2. SP, signal peptide; LRR, leucine rich repeat; NT, N-terminal domain; CT, C-terminal domain; Ig, immunoglobulin domain; FNIII, fibronectin type-III domain; TM, transmembrane domain; PB, PDZ domain binding motif; ESTV*, the last four aa residues of SALM2.

(B) Interaction of SALM isoforms with the PDZ domains of PSD-95 family proteins in the yeast two-hybrid assay. pBHA, bait vector; pGAD, prey vector; WT, wild-type; V788A, V766A, and V636A, mutant SALM isoforms in which the last residue was changed into alanine. Only β-gal results are shown for simplicity (histidine growth gave similar results).

(C) Interaction of SALM isoforms with PSD-95 family proteins in the pull down assay. EGFP-tagged cytosolic regions of SALM isoforms expressed in heterologous cells were pulled down by full-length PSD-95 family proteins fused to GST. ΔC, a mutant SALM that lacks the last 3 residues.

(D–G) Coimmunoprecipitation of SALM2 with PSD-95 family proteins in heterologous cells. HEK293 cells transfected (Expression) with SALM2 (untagged) and the indicated PSD-95 family protein constructs were immunoprecipitated (IP) and immunoblotted with the indicated antibodies. Input, 5%.

(H) Coclustering between SALM2 and PSD-95. COS7 cells expressing SALM2 (untagged), PSD-95, or both, were labeled with the antibodies indicated. In the case of double labeling, an active channel is indicated by underline.

for dendrites)-negative axons (data not shown), suggesting that SALM2 may perform some presynaptic functions perhaps by interacting with members of the PSD-95 family that are present in both dendrites and axons such as SAP97 and SAP102 (El-Husseini et al., 2000b; Muller et al., 1995).

Immunoprecipitation experiments showed that SALM2 forms an in vivo complex with PSD-95 family proteins, including PSD-95, chapsyn-110/PSD-93, and SAP97, but not with negative control PDZ proteins such as S-SCAM and CASK (Figures 3E–3H). In addition, SALM2 coprecipitated with GluR1 and GluR2 subunits of AMPA receptors and the NR1 subunit of NMDA receptors (Figure 3E). Together, these results indicate that SALM2 associates with PSD-95 at excitatory synapses.

SALM2 Expressed in Fibroblasts Does Not Induce Presynaptic Differentiation in Contacting Neurites

Because SALM2 contains typical adhesion domains, interacts with PSD-95, and is present at excitatory synapses, we hypothesized that SALM2 may promote excitatory synaptic differentiation. Previous reports have shown that heterologous cells expressing neuroligin or SynCAM can induce presynaptic differentiation in contacting axons of cocultured neurons (Biederer et al., 2002; Scheiffele et al., 2000). Conversely, heterologous cells expressing β-neurexin were shown to induce postsynaptic differentiation in contacting dendrites (Graf et al., 2004; Nam and Chen, 2005). When we tried similar experiments, however, SALM2-expressing COS cells did not induce detectable presynaptic differentiation in contacting axons of cocultured hippocampal neurons (data not shown), suggesting that SALM2 may not induce de novo presynaptic differentiation. Because SALM2 does not seem to mediate homophilic adhesion, and we have not yet identified any presynaptic SALM2 ligand, we could not test whether a presynaptic ligand could induce postsynaptic differentiation in this assay.

Overexpression of SALM2 Leads to Increases in the Number of Excitatory Synapses and Dendritic Spines

Another way of demonstrating the function of a synaptic CAM is to overexpress the CAM in cultured neurons and monitor changes in excitatory and inhibitory synapses (Chih et al., 2005; Levinson et al., 2005; Prange et al., 2004; Sara et al., 2005). To this end, we examined the effect of SALM2 overexpression in cultured hippocampal neurons (days in vitro or DIV12–18), by using an expression construct that increases SALM2 expression by 130% in neurons (n = 13; data not shown). SALM2

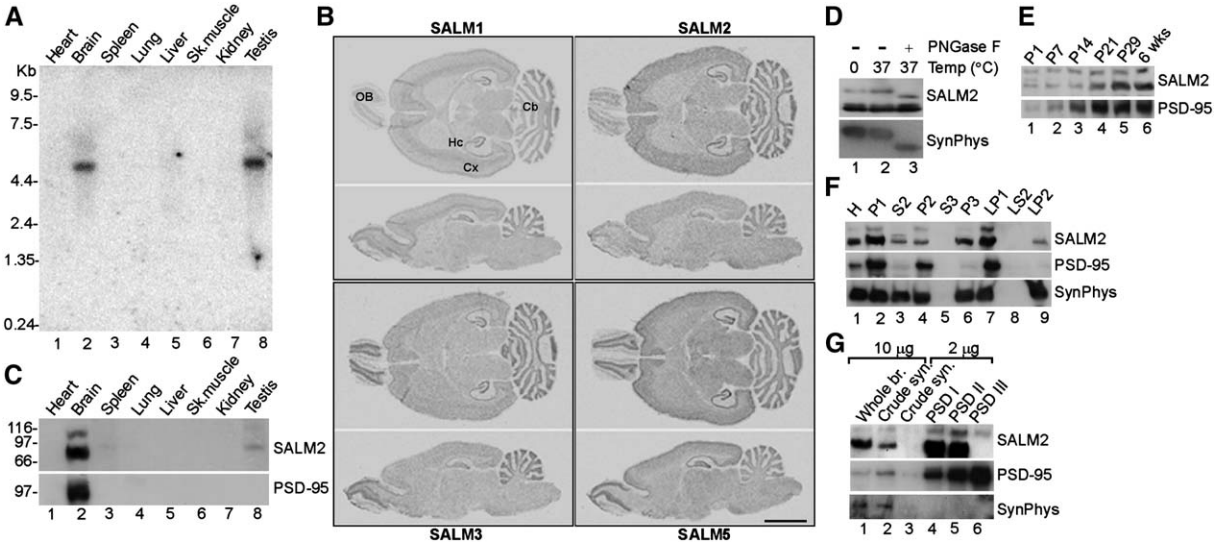

Figure 2. mRNA and Protein Expression Patterns of SALM

(A) Tissue distribution of SALM2 mRNAs in Northern blot analysis. Sk. muscle, skeletal muscle.

(B) Distribution of SALM family mRNAs revealed by in situ hybridization analysis. OB, olfactory bulb; Cx, cortex; Hc, hippocampus; Cb, cerebellum. Scale bar, 6 mm.

(C) Tissue distribution pattern of SALM2 proteins in immunoblot analysis.

(D) N-glycosylation of SALM2. The crude synaptosomal fraction of rat brain was subjected to PNGase F digestion, followed by immunoblotting with the indicated antibodies. SynPhy, synaptophysin (positive control).

(E) Expression levels of SALM2 during postnatal rat brain development. P, postnatal; wks, weeks. Identical amounts of proteins were loaded for each lane.

(F) Distribution of SALM2 in biochemical rat brain fractions. H, homogenates; P2, crude synaptosomes; S2, supernatant after P2 precipitation; S3, cytosol; P3, light membranes; LP1, synaptosomal membranes; LS2, synaptosomal cytosol; LP2, synaptic vesicle-enriched fraction. Identical amounts of proteins were loaded for each lane.

(G) Enrichment of SALM2 in PSD fractions; extracted with Triton X-100 once (PSD I) or twice (PSD II) or with Triton X-100 and Sarkosyl (PSD III). Br., brain; syn, synaptosome. The amounts of loaded proteins are indicated.

overexpression significantly increased the number of excitatory synapses, which were defined by vGlut1 (an excitatory presynaptic marker)-positive PSD-95 clusters (Figures 4A and 4B), and dendritic spines, defined by dendritic protrusions (0.5–3.0 μm in length) positive for both PSD-95 and vGlut1 (Figures 4A and 4C). In contrast, expression of a SALM2 mutant lacking the C-terminal PDZ binding motif (SALM2 ΔC) in neurons did not increase the number of excitatory synapses and even reduced the number of spines in a dominant-negative manner (Figures 4A–4C). The limited dominant effect of SALM2 ΔC on excitatory synapses (Figures 4A–4C) suggests that SALM2 may be more important for the differentiation of dendritic spines than excitatory synapses, although both parameters were reduced by high-level overexpression of SALM2 ΔC (see below). While affecting excitatory synapses, overexpression of SALM2 or SALM2 ΔC had no effect on the number of inhibitory synapses, defined by VGAT (an inhibitory presynaptic marker)-positive gephyrin (an inhibitory postsynaptic marker) (Figures 4D and 4E). These results suggest that SALM2 promotes the differentiation of excitatory, but not inhibitory, synapses through a mechanism that requires PSD-95 interaction.

In contrast to its positive effects on the number of excitatory synapses and dendritic spines, SALM2 overexpression did not significantly change the frequency or amplitude of miniature excitatory postsynaptic currents (mEPSCs) or miniature inhibitory postsynaptic currents (mEPSCs) (Figures 4F–4I). In addition, SALM2 overexpression did not increase the number of GluR1 (surface) or NR1 clusters (Figure S4). These results suggest that SALM2 overexpression preferentially promotes the morphological differentiation of excitatory synapses.

The lack of neuroligin-like synaptogenic activity in SALM2 suggests that it might function as a synapse differentiating factor, which should have a greater influence on neurons at late stages than at early stages. To explore this possibility, we compared the effects of SALM2 overexpression on early-stage versus late-stage neurons. Of note, SALM2 overexpression at early stages (DIV6–12) did not significantly increase the number of excitatory synapses (Figures S5A–S5C), in contrast to the significant effects at late stages (DIV12–18; Figures 4A–4C). These results suggest that SALM2 has a greater influence on excitatory synaptic differentiation at late stages.

Mislocalized Expression of SALM2 Disrupts Excitatory Synapses and Dendritic Spines

In a previous study, mislocalized expression of neuroligin-2 was shown to disrupt postsynaptic protein clustering and synaptic transmission (Graf et al., 2004). We hypothesized that, if SALM2 is an important regulator of synaptic differentiation, high-level and mislocalized expression of SALM2 should disperse postsynaptic proteins into extrasynaptic sites and thus inhibit the differentiation of excitatory synapses. To this end, we first generated a SALM2 expression construct that drives approximately 5- and 4-fold higher SALM2 expression in heterologous cells and neurons, respectively

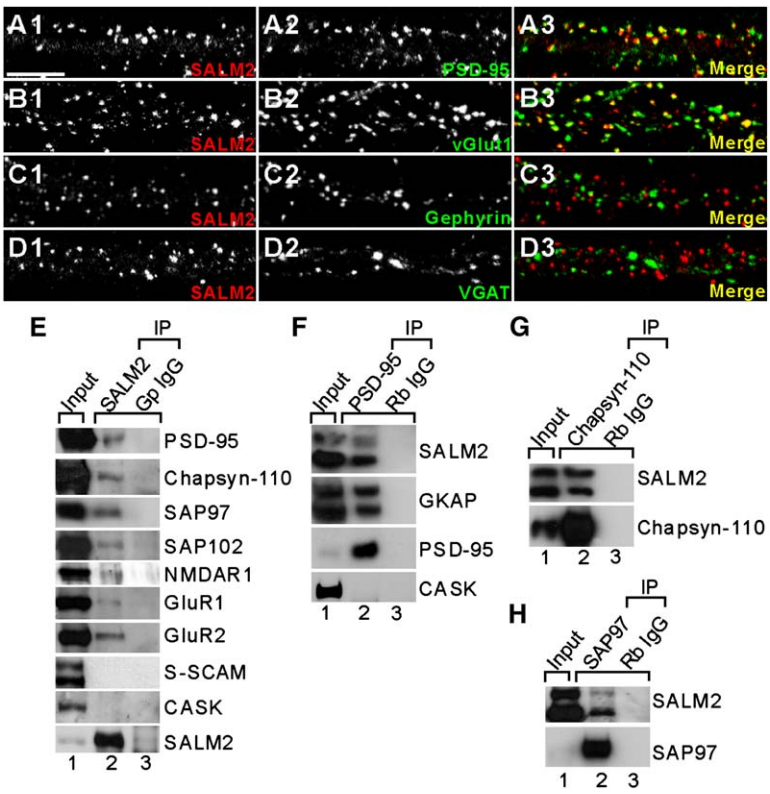

Figure 3. SALM2 Colocalizes and Associates with PSD-95 at Excitatory Synapses

(A–D) Localization of SALM2 at excitatory synapses. Cultured hippocampal neurons (DIV21) were labeled by double immunofluorescence staining for SALM2 and the indicated subcellular markers. Scale bar, 10 μm. (E–H) In vivo coimmunoprecipitation between SALM2 and PSD-95 family proteins. Detergent lysates of the crude synaptosomal fraction of adult rat brain were immunoprecipitated with antibodies against SALM2 (E), PSD-95 family proteins (F–H). Rb or Gp, rabbit and guinea pig control antibodies; NMDAR1, NR1 subunit of NMDA glutamate receptors; GluR1 and GluR2, subunits of AMPA glutamate receptors. Input, 5%.

(data not shown). High-level and mislocalized expression of SALM2 by this construct in cultured hippocampal neurons (DIV14–16) caused a marked reduction in the number of excitatory synapses and dendritic spines, compared to the overexrepssion of CD8 (an unrelated T cell surface antigen), often leading to the dispersion of very small clusters of PSD-95 in the dendritic trunk (Figures 5A–5C). Notably, mislocalized expression of SALM2 resulted in an increase in the number of inhibitory synapses (Figures 5D and 5E). It has been shown that PSD-95 promotes redistribution of neuroligin-2 from inhibitory synapses to excitatory synapses, changing the ratio of excitatory to inhibitory synapses (Graf et al., 2004; Prange et al., 2004). A possible explanation for the increase in inhibitory synapses is that SALM2 overexpression-induced disruption of excitatory synapses might have caused the release of inhibitory synapse-enhancing molecules sequestered at excitatory synapses such as neuroligin-2.

In addition to wild-type SALM2, we also examined the effects of high-level overexpression of SALM2 ΔC. Similar to the effects of wild-type SALM2, SALM2 ΔC caused a marked decrease in the number of excitatory synapses and dendritic spines as well as an increase in the number of inhibitory synapses (Figures 5A–5E). This suggests that regions of SALM2 other than the PSD-95 binding C terminus are sufficient to exert the dominant-negative effects, although the importance of the C terminus cannot be excluded. Together, these results, along with the C terminus-dependent promotion of excitatory synapses by moderate overexpression of SALM2 (Figures 4A–4C), suggest that both the C terminus and the non-C-terminal regions of SALM2 contribute to SALM2-dependent synaptic differentiation.

Direct Aggregation of SALM2 Induces Clustering of Excitatory Postsynaptic Proteins

As our results suggested that SALM2 promotes excitatory synaptic differentiation, we used a bead aggregation assay to examine whether SALM2 is capable of driving postsynaptic differentiation at excitatory synapses. Cultured neurons expressing N-terminally ECFP-tagged SALM2 were incubated with beads coated with EGFP antibodies. Robust clustering of SALM2 was observed at sites of bead aggregation on dendrites (61.1% ± 7.2% of aggregated beads were SALM2-positive, n = 20 cells; Figure 6). This SALM2 clustering induced coclustering of PSD-95 (34.5% ± 5.5% of bead-induced SALM2 clusters were PSD-95-positive; n = 15 cells; Figure 6A). The SALM2 and PSD-95 clusters were largely negative for synapsin I (5.6% ± 2.9% of bead-induced SALM2 clusters were synapsin I-positive, n = 10 cells; Figure 6A), indicating that the SALM2 and PSD-95 clusters were not induced by interneuronal synapses. In contrast, SALM2 ΔC, which does not bind PSD-95, minimally induced coclustering of PSD-95 (3.3% ± 1.3% of bead-induced SALM2 ΔC clusters were PSD-95-positive, n = 10; Figure 6B), suggesting that PSD-95 coclustering occurs through the interaction of the SALM2 C terminus with PSD-95. In addition to PSD-95, SALM2 aggregation induced coclustering of GKAP (36.3% ± 6.7%, n = 10; Figure 6C), a PSD-95-associated PSD protein (Kim et al., 1997). SALM2 also induced coclustering of AMPA receptors (GluR1) and, to a lower level, NMDA receptors (NR1), but their coclustering efficiencies were smaller than those of PSD-95 and GKAP, as determined by their fluorescence intensities normalized to the average dendrite intensity (GluR1, 1.77 ± 0.14, n = 20 cells; NR1, 1.36 ± 0.26, n = 18; PSD-95, 3.02 ± 0.49, n = 16;

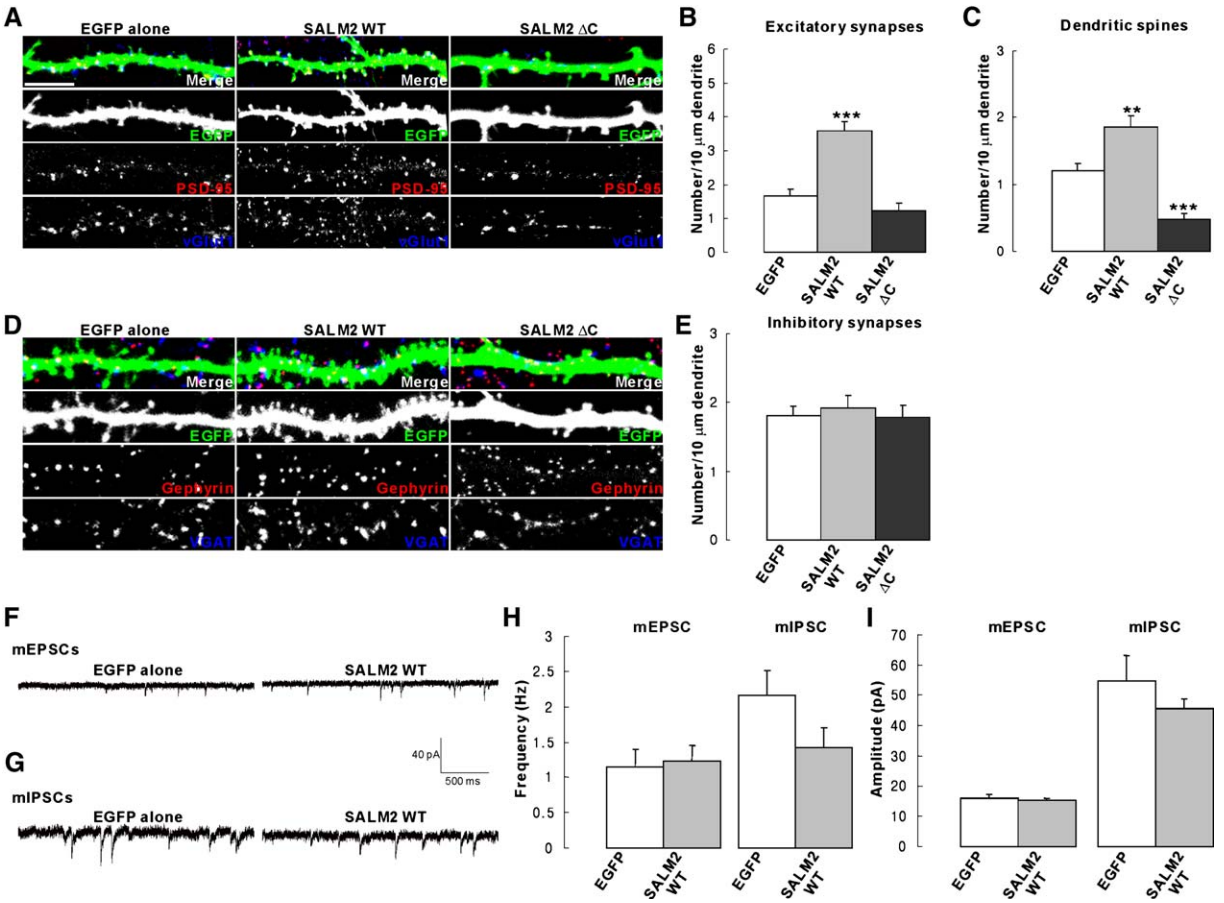

Figure 4. Overexpression of SALM2 Leads to Increases in the Number of Excitatory Synapses and Dendritic Spines

(A) Cultured hippocampal neurons were transfected with pIRES-EGFP- SALM2, pIRES-EGFP- SALM2 ΔC, or pIRES-EGFP alone (DIV 12-18), and immunostained triply for EGFP, PSD-95, and vGlut1. Scale bar, 10 μm.

(B and C) Quantitation of the effect of SALM2 expression on the number of excitatory synapses (vGlut1-positive PSD-95 clusters; B) and dendritic spines (vGlut1- and PSD-95-positive dendritic protrusions; [C]). Histograms show mean ± SEM (n = 46 cells for pIRES-EGFP alone and pIRES-EGFP-SALM2 and 20 cells for pIRES-EGFP-SALM2 ΔC, **$p < 0.01$; ***$p < 0.0001$, ANOVA, Tukey's test).

(D) Cultured neurons were transfected with pIRES-EGFP-SALM2, pIRES-EGFP alone, or pIRES-EGFP-SALM2 ΔC, and immunostained triply for EGFP, gephyrin, and VGAT.

(E) Quantitation of the effect of SALM2 expression on the number of inhibitory synapses (VGAT-positive gephyrin clusters). Mean ± SEM (n = 66 cells for pIRES-EGFP alone, 66 for pIRES-EGFP-SALM2, and 30 for pIRES-EGFP-SALM2 ΔC).

(F and G) Sample traces of mEPSCs (F) and mIPSCs (G) in cultured hippocampal neurons expressing pIRES-EGFP-SALM2 and pIRES-EGFP alone (DIV12–18).

(H and I) Quantitation of the effects of SALM2 expression on the frequency (H) and amplitude (I) of mEPSCs and mIPSCs. Mean ± SEM (n = 11 cells for mEPSCs and 10 cells for mIPSCs). The change in mIPSC frequency was nonsignificant ($p = 0.128$; Student's t test).

GKAP, 2.22 ± 0.18, n = 10; Figures 6D and 6E). In contrast to excitatory postsynaptic proteins, the beads minimally induced gephyrin coclustering (4.9% ± 2.4% of SALM2 clusters were gephyrin positive, n = 10 cells; Figure 6F). These results suggest that aggregation of SALM2 is sufficient to drive the clustering of excitatory, but not inhibitory, postsynaptic proteins.

Knockdown of SALM2 by siRNA Leads to the Loss of Excitatory Synapses and Dendritic Spines

We then tested the effects of siRNA knockdown of SALM2 on excitatory and inhibitory synapses. A SALM2 siRNA construct reduced SALM2 expression in heterologous cells by 35% (Figure S6A), and decreased the expression of exo- and endogenous SALM2 in cultured neurons by 47% and 72%, respectively (Figures S6B and S6C). SALM2 knockdown by this siRNA construct caused a significant decrease in the number of excitatory synapses and dendritic spines, often leading to the dispersion of very small clusters of PSD-95 in the dendritic trunk (Figures 7A–7C; DIV16/17–21), but did not affect the number of inhibitory synapses (Figures 7D and 7E). SALM2 knockdown also reduced the total amount of PSD-95 immunofluorescence signals normalized to the dendritic area by ~3 folds (control, 1022.03 ± 121.18 in arbitrary unit, n = 19 cells; SALM2 knockdown, 332.63 ± 113.48, n = 18, $p < 0.001$), suggesting that SALM2 might be involved in the synaptic localization and stabilization of PSD-95, although the signals for synaptically localized PSD-95 clusters are often exaggerated compared with dispersed signals. In addition, SALM2 knockdown significantly reduced the number of both GluR1 (surface) and NR1 clusters, showing a greater effect on GluR1 (Figure S7).

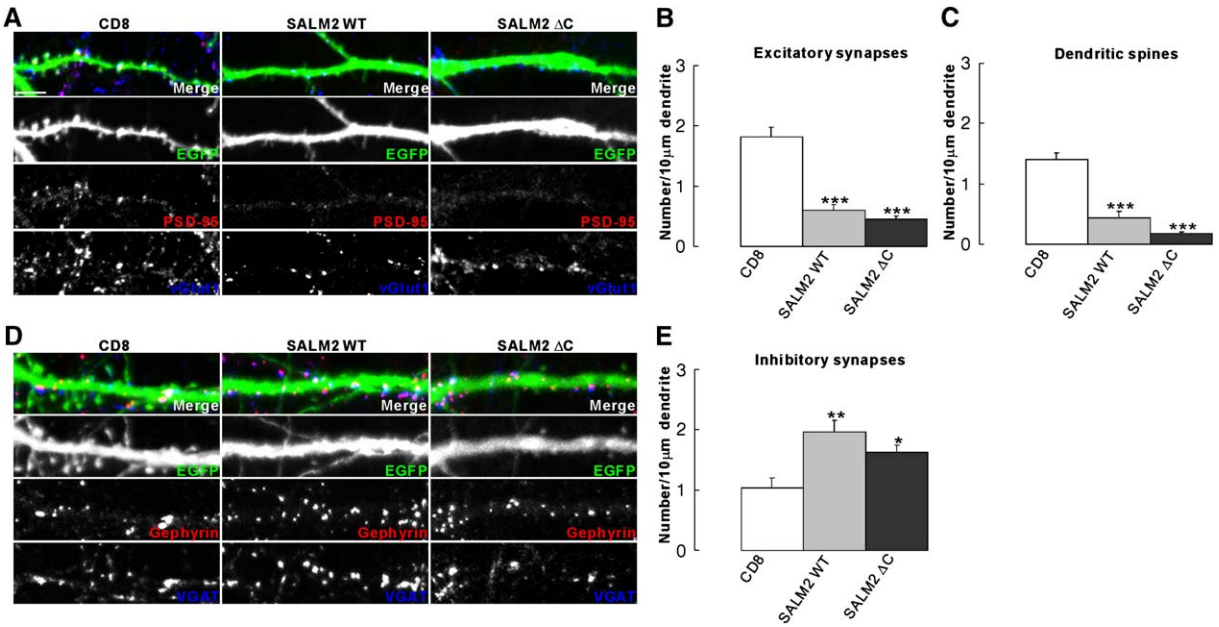

Figure 5. Mislocalized Expression of SALM2 Disrupts Excitatory Synapses and Dendritic Spines

(A) Cultured hippocampal neurons transfected with GW1 SALM2 (a high-level expression construct; DIV14–16), HA-CD8, or GW1 SALM2 ΔC, were triply stained for SALM2, PSD-95, and vGlut1. Scale bar, 5 μm.

(B and C) Quantitation of the effects of mislocalized expression of SALM2 and SALM2 ΔC on excitatory synapses (B) and dendritic spines (C). Mean ± SEM (n = 25 cells for CD8, 30 for SALM2, and 28 for SALM2 ΔC, ***p < 0.0001, ANOVA Tukey's test).

(D) Cultured hippocampal neurons transfected with GW1 SALM2, HA-CD8, or GW1 SALM2 ΔC (DIV14–16) were triply stained for SALM2, gephyrin, and VGAT.

(E) Quantitation of the effects of mislocalized expression of SALM2 and SALM2 ΔC on inhibitory synapses. Mean ± SEM (n = 16 cells for CD8, 20 for SALM2, and 17 for SALM2 ΔC, *p < 0.05, **p < 0.01, ANOVA Tukey's test).

Consistent with our data from morphological measurements, SALM2 knockdown in cultured hippocampal neurons resulted in a decrease in the frequency, but not amplitude, of mEPSCs (Figures 7F, 7H, and 7I; DIV 16/17–21). In contrast, SALM2 knockdown had no effect on the frequency or amplitude of mIPSCs (Figures 7G–7I). Taken together, these results suggest that SALM2 is important for the maintenance of excitatory, but not inhibitory, synapses.

These results appear to be caused by specific degradation of SALM2 mRNAs, as evidenced by the observation that the SALM2 siRNA-induced decreases in the number of excitatory synapses and dendritic spines could be reversed by cotransfection of an siRNA-resistant SALM2 rescue construct (Figures S6D and S8). In addition, a SALM2 siRNA with a point mutation, which does not reduce SALM2 expression in heterologous cells (data not shown), did not induce any significant changes in the number of excitatory synapses or dendritic spines or the frequency or amplitude of mEPSCs (Figure S9), further supporting the specific action of the SALM2 siRNA.

We also compared the effects of SALM2 knockdown on early-stage neurons with those on late-stage neurons. SALM2 knockdown in neurons at early stages (DIV7–12) significantly reduced the number of excitatory synapses (Figure S10), but to a lesser extent than late stages (DIV16/17–21; Figures 7A and 7B). These results suggest that SALM2 is more important for the maintenance of excitatory synapses in neurons at late stages.

Discussion

Regulation of the Differentiation of Excitatory Synapses by SALM2

We herein identified a family of cell adhesion-like molecules directly associating with PSD-95. Several lines of evidence indicate that SALM2 is an important regulator of the differentiation of excitatory, but not inhibitory, synapses: (1) SALM2 is present at excitatory synapses; (2) SALM2 is enriched in the PSD and biochemically associates with PSD-95, a key scaffolding molecule at excitatory synapses; (3) moderate overexpression of SALM2 increases the number of excitatory synapses and dendritic spines; (4) mislocalized expression of SALM2 reduces the number of excitatory synapses; (5) bead aggregation of SALM2 is sufficient to induce the clustering of excitatory postsynaptic proteins including PSD-95, GKAP, and AMPA receptors; and (6) siRNA knockdown of SALM2 reduces the number of excitatory synapses and dendritic spines and the frequency of mEPSCs. These results strongly suggest that SALM2 is involved in the regulation of excitatory, but not inhibitory, synaptic differentiation.

Our results indicate that moderate overexpression of SALM2 increases the number of excitatory synapses and dendritic spines but does not increase the frequency or amplitude of mEPSCs. This suggests that additional SALM2 expression above the physiological range is sufficient to induce morphological differentiation of excitatory synapses. In addition, SALM2 siRNA knockdown decreases both the number of excitatory synapses and dendritic spines and the frequency of mEPSCs, suggesting

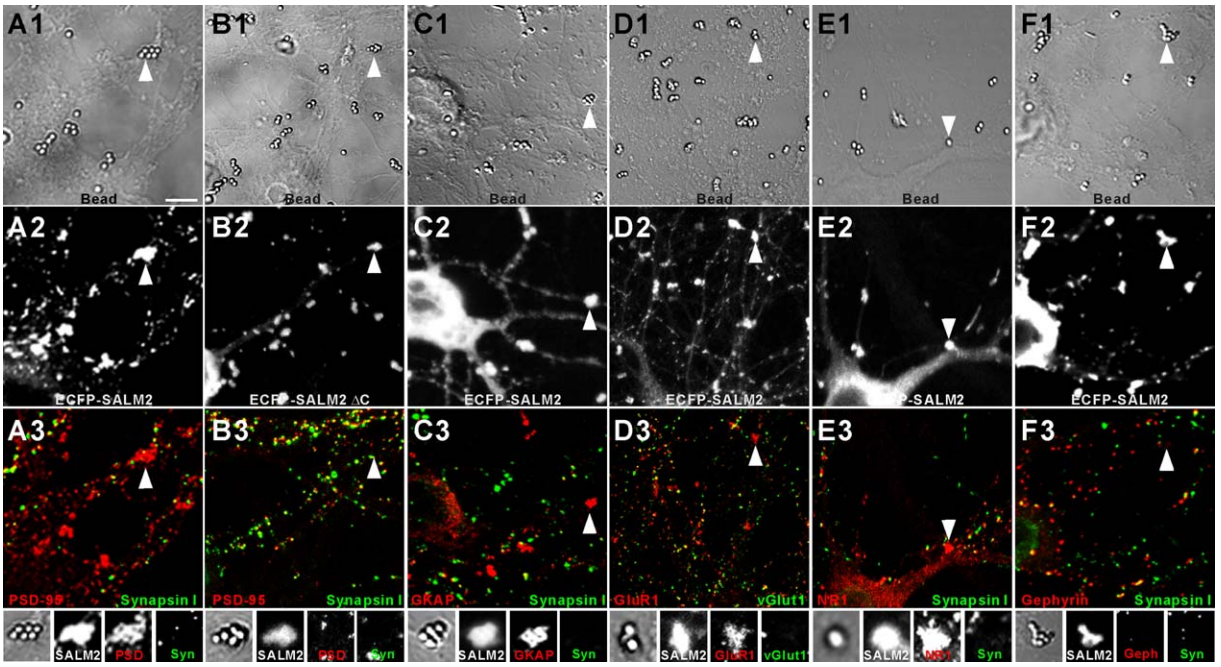

Figure 6. Direct Aggregation of SALM2 Induces Clustering of Excitatory Postsynaptic Proteins

Cultured hippocampal neurons expressing N-terminally ECFP-tagged SALM2 (A and C–F), or SALM2 ΔC ([B]; DIV12–14), were incubated with neutravidin beads coated with biotin-conjugated EGFP antibodies and visualized at DIV15 by triple immunofluorescence staining for ECFP, synapsin I (Syn), and the indicated proteins including PSD-95 ([A and B]; PSD), GKAP (C), GluR1 (D), NR1 (E) and gephyrin ([F]; Geph). Synapsin I staining was replaced with vGlut1 staining in GluR1 coclustering experiments (D) due to antibody incompatibility in triple staining. Beads were visualized by DIC imaging. The area indicated by an arrow in the upper panels is enlarged in the bottom panels. Scale bar, 5 μm.

that SALM2 is required for both morphological and functional differentiation of excitatory synapses. Together, these data suggest that SALM2 is both necessary and sufficient for the differentiation of excitatory synapses.

Mechanisms by which SALM2 Regulates Excitatory Synaptic Differentiation

How might SALM2 regulate the differentiation of excitatory synapses? Given that the extracellular portion of SALM2 contains cell adhesion domains, and the C-terminal tail of SALM2 interacts with PSD-95, SALM2 might plausibly promote synaptic differentiation by simultaneously interacting with both a presynaptic ligand and PSD-95 in a fashion similar to that of neuroligin (Chih et al., 2005; Dean et al., 2003; Graf et al., 2004; Ichtchenko et al., 1996; Irie et al., 1997; Nam and Chen, 2005; Scheiffele et al., 2000). A result contradicting this hypothesis is that SALM2 expressed in heterologous cells does not induce presynaptic differentiation in contacting axons of cocultured neurons, suggesting that SALM2 may not induce presynaptic differentiation through a *trans*-synaptic adhesion. However, this negative result could also be caused by the choice of an incorrect type of cocultured neuron, an inappropriate SALM2 splice variant, or insufficient posttranslational modification of SALM2 in heterologous cells. Furthermore, it remains possible that as yet unknown presynaptic ligands could induce the clustering of SALM2 and SALM2-associated postsynaptic proteins. Indeed, this possibility is not inconsistent with the results from our overexpression, bead aggregation, and siRNA knockdown experiments. If this sort of *trans*-synaptic interaction exists, our observation that the ectodomain of SALM2 does not bind to SALM2 indicates that SALM2 may mediate heterophilic adhesion.

It is also possible that, rather than functioning in parallel with neuroligin, SALM2 promotes excitatory postsynaptic differentiation after *trans*-synaptic adhesion is established by "early" CAMs such as neuroligin. In support of this possibility, overexpression or siRNA knockdown of SALM2 had stronger effects on neurons at late stages. If this is the case, the presence of PSD-95 in the neuroligin-PSD-95 complex at early synapses may promote SALM2 recruitment through the PDZ interaction. In agreement with this, the C terminus of SALM2 was required for synaptic differentiation in the overexpression experiments. Once docked at synaptic sites, SALM2 and PSD-95 may mutually promote their synaptic recruitment and stabilization, as suggested by the SALM2 knockdown-induced dispersion of PSD-95 into small dendritic clusters and reduction in the total amount of PSD-95. In addition, PSD-95 may couple SALM2 to various PSD-95-associated postsynaptic proteins to facilitate the differentiation of early synapses. This possibility is supported by the fact that bead aggregation of SALM2 induces coclustering of PSD-95-associated GKAP.

In addition to the PSD-95 binding C terminus, other regions of SALM2 seem to mediate SALM2-dependent postsynaptic differentiation. In support of this, the dominant-negative effects of high-level SALM2 overexpression were still observed with the mutant lacking the C terminus, which may induce dispersion of some proteins that bind to the non-C-terminal regions of SALM2 and

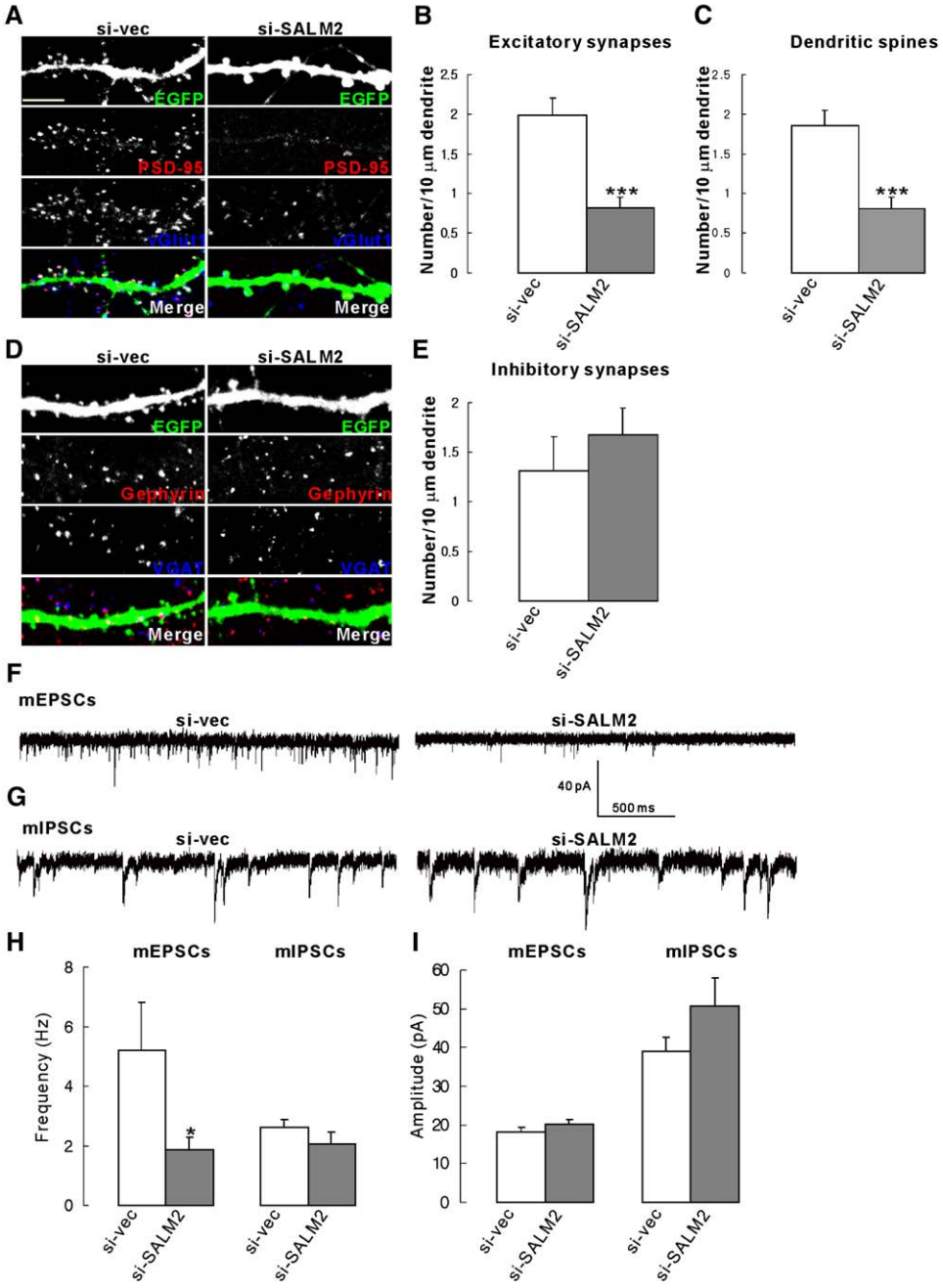

Figure 7. Knockdown of SALM2 by siRNA Leads to the Loss of Morphological and Functional Excitatory Synapses

(A) Cultured hippocampal neurons expressing pSUPER SALM2 (si-SALM2), or pSUPER alone (si-vec; DIV16/17–21), were triply stained for EGFP, PSD-95, and vGlut1. Scale bar, 5 μm.

(B and C) Quantitation of the effects of SALM2 knockdown on the density of excitatory synapses (B) and dendritic spines (C). Mean ± SEM (n = 15 for si-vec and 21 for si-SALM2, ***p < 0.0001, Student's t test).

(D) Cultured neurons expressing si-SALM2, or si-vec (DIV16/17–21), were triply stained for EGFP, gephyrin, and VGAT. Scale bar, 10 μm.

(E) Quantitation of the effect of SALM2 knockdown on the density of inhibitory synapses. Mean ± SEM (n = 19 for si-vec and 18 for si-SALM2, p = 0.41, Student's t test).

(F and G) Sample traces of mEPSCs (F) and mIPSCs (G) in cultured hippocampal neurons expressing si-SALM2 or si-vec (DIV16/17–21).

(H and I) Quantitation of the effects of SALM2 knockdown on the frequency (H) and amplitude (I) of mEPSCs and mIPSCs. Mean ± SEM (n = 10 cells for mEPSCs and 7 cells for mIPSCs, *p < 0.05, Student's t test).

contribute to the maintenance of excitatory synapses. Such mechanisms have been seen in other synaptic CAMs, i.e., N-cadherin and NCAM, which associate with the actin cytoskeleton and various signaling molecules through their cytosolic domain (Juliano, 2002; Scheiffele, 2003; Yap and Kovacs, 2003). In this context, it should be noted that the cytosolic regions of the SALM family proteins share essentially no sequence identity except for their extreme PSD-95 binding C termini, suggesting that they may have distinct functions.

Interestingly, our bead aggregation and siRNA knockdown results indicate that SALM2 preferentially associates with AMPA receptors and, to a lesser extent, with NMDA receptors, suggesting that this mechanism may contribute to SALM2-dependent postsynaptic differentiation. Acquisition of AMPA receptors at NMDA receptor-only silent synapses are implicated in synaptic plasticity and postnatal development of excitatory synapses (Bredt and Nicoll, 2003). Considering that SALM2 overexpression and knockdown have stronger influences on neurons at late stages, SALM2 may promote the maturation of excitatory synapses primarily during the late stages of neuronal development through mechanisms including synaptic enrichment of AMPA receptors. In support of the notion that adhesion molecules can regulate synaptic maturation, Dasm1, an Ig superfamily protein, has been shown to regulate excitatory synaptic maturation by selectively acting on AMPA receptors, but not NMDA receptors (Shi et al., 2004). It remains to be determined whether synaptic localization of AMPA receptors is one of the major mechanisms for SALM2-dependent synaptic differentiation and, if so, how SALM2 promotes synaptic AMPA receptor localization.

Other Implications of the Association of SALM2 with PSD-95

Several other inferences may be drawn from our observation of a direct association between SALM2 and PSD-95. First, although the ultrastructural localization of SALM2 could not be determined in the present study due to the lack of a suitable antibody, the fact that SALM2 is enriched in PSD fractions and associates with PSD-95 suggests that SALM2 is likely to be present at synaptic sites rather than in the puncta adherentia junctions, which flank synaptic junctions and are similar to epithelial adherence junctions (Yamagata et al., 2003).

Second, it should be noted that while both SALM and neuroligin bind to PSD-95, the SALM family proteins bind more strongly to the first and second PDZ domains of PSD-95, whereas neuroligin preferentially binds to the third PDZ domain (Irie et al., 1997; Song et al., 1999). Thus, it is possible that SALM and neuroligin undergo parallel and noncompetitive binding to PSD-95 to perform their specific functions.

And third, recent studies have shown that overexpression of PSD-95 promotes the localization of neuroligin-1 at excitatory synapses as well as the redistribution of neuroligin-2 from inhibitory to excitatory synapses (Graf et al., 2004; Prange et al., 2004). Conversely, siRNA knockdown of PSD-95 decreased the number of excitatory synapses and concomitantly increased the number of inhibitory synapses (Prange et al., 2004), suggesting that the amount of PSD-95 in a neuron is a key factor in controlling the balance between excitatory and inhibitory synapses (Prange et al., 2004). Because our data indicate that SALM2 binds to PSD-95 and regulates the differentiation of excitatory synapses, it is conceivable that SALM2, along with neuroligin, might be involved in the PSD-95-dependent regulation of overall neuronal excitability.

Differential Characteristics of the SALM Family Proteins

While we were revising the current paper, which identifies the SALM family and mainly focuses on SALM2, a similar study identifying the same SALM family was published (Wang et al., 2006), in which SALM1 was mainly characterized. Comparison of the two studies reveals that although SALM1 and SALM2 share various features, including domain structure and interaction with PSD-95, they also have interesting differences. Specifically, SALM1 mainly associates with NMDA receptors, but not with AMPA receptors, whereas SALM2 associates with both NMDA and AMPA receptors, with a stronger association with AMPA receptors. In addition, SALM1 promotes neurite outgrowth in young neurons but not in late-stage neurons. By contrast, SALM2 exerts a greater influence on excitatory synapses of late-stage neurons. Postnatal expression of SALM1 in rat brain plateaus at approximately postnatal day 1 whereas that of SALM2 increases steadily during postnatal development. Structurally, the cytosolic regions of SALM1 and SALM2 share essentially no identity except for their extreme C termini. Therefore, SALM1 and SALM2 may differentially act on different stages of synaptic development (SALM1 on early and SALM2 on late stages) through their differential interaction with glutamate receptors and cytosolic binding partners. These results are reminiscent of the differential characteristics of the isoforms in the PSD-95 or neuroligin family (Craig et al., 2006; Dean and Dresbach, 2006; Kim and Sheng, 2004; Levinson and El-Husseini, 2005).

In sum, our data suggest that SALM2 is an important regulator of the differentiation of excitatory synapses. A direction to pursue would be to understand detailed mechanisms underlying the SALM2-dependent synaptic differentiation. Other future studies may seek to identify and characterize novel SALM ligands and explore whether SALM is involved in the regulation of structural and functional plasticity of synapses.

Experimental Procedures

Antibodies

GST- SALM2 (aa 551–766 containing the entire cytoplasmic region) was used for immunization of a guinea pig (1348) and affinity-purified using SulfoLink columns (Pierce). The following antibodies were described previously: PSD-95 1402 (Choi et al., 2005), Chapsyn-110 (Kim et al., 1996), SAP97 (Kim et al., 1996), S-SCAM 1146 (Mok et al., 2002), EGFP 1173 (Ko et al., 2003a), GKAP 1443 (Ko et al., 2003b), CASK (Hsueh and Sheng, 1999), NMDAR1 (Sheng et al., 1994). The other antibodies were purchased commercially: Myc (Santa Cruz), FLAG (Sigma), PSD-95 (Affinity BioReagents), gephyrin (Synaptic Systems), vGlut1 (Synaptic Systems), NR1 (Pharmingen), GluR1 (Calbiochem), surface GluR1 (Oncogene), GluR2 (Chemicon), VGAT (Synaptic Systems), synaptophysin (Sigma), synapsin I (Chemicon), and α-tubulin (Sigma).

cDNA Constructs

The PDZ2 domain of PSD-95 (aa 89–299) in the pBHA bait vector was used to screen a human brain yeast two-hybrid cDNA contained in the pACT2 prey vector (Clontech). Full-length rat SALM2 (aa 1–766) was PCR-amplified from a rat brain cDNA library (Clontech) and subcloned into GW1 (British Biotechnology) for high-level and mislocalized expressions. SALM2 ΔC (aa 1–762) was also subcloned into GW1. For low-level expressions, rat SALM2 cDNAs (full-length and ΔC) were subcloned into pIRES2-EGFP (Clontech). ECFP-tagged SALM2 was generated by adding the ECFP cassette between aa 33 and 34 of GW1 SALM2. SALM1 (aa 586–788), SALM2 (aa 551–766), SALM3 (aa 566–636), and SALM5 (aa 559–719) were subcloned into pBHA. pGAD10 plasmids were previously described (Choi et al., 2005; Mok et al., 2002). For pull-down assay, the cytoplasmic regions of SALM1 (aa 586–788), SALM2 (aa 551–766), SALM3 (aa 546–636),

and SALM5 (aa 559–719) were subcloned into pEGFP-C1 (Clontech). For short-interfering RNA (siRNA) knockdown of SALM2, pSUPER SALM2 was generated by annealing oligonucleotides containing nt 245–263 (with the first nucleotide of the start codon as nucleotide 1) of the rat SALM2 cDNA (accession number, XM_344874; critical 19 nt sequence, GTCGAGACTTCGCCAATAT) and subcloning into pSUPER.gfp/neo (OligoEngine) vector. A pSUPER SALM2 variant with a point mutation was generated by mutating the 19-nt sequence into GTCGAGACTTTGCCAATAT. SALM2 expression construct resistant to siRNA was generated by introducing a point mutation at residue 255 (C to T) using the QuickChange kit (Stratagene). The following expression constructs have been described previously: GW1-PSD-95 (Kim et al., 1995), GW1-Myc-chapsyn-110 (Hsueh et al., 1997), EGFP-SAP97 (Choi et al., 2005), pFLAG-CMV2 SAP102 (Choi et al., 2005).

Deglycosylation Assays in Brains

Enzymatic deglycosylations were performed with rat P2 (crude synaptosomes) fractions extracted with 1% deoxycholate (DOC) using N-glycosidase/PNGase F (New England Biolabs) according to the manufacturer's instructions. Deglycosylations were analyzed by immunoblotting with SALM2 (1348), or synaptophysin antibodies.

In Situ Hybridization Analysis

In situ hybridization was performed as described previously (Kim et al., 2003). Brain sections (12 μm thick) from adult rats (6 weeks) were used. SALM isoform-specific hybridization probes encompassing the C-terminal region and the 3′ untranslated region were prepared from the following constructs: pGEM7zf containing nt 2126-2486 of rat SALM2 (coding region is nt 1–2301) and rat SALM5 (the entire sequence of the BF544867 EST clone, corresponding to the last 174 nt of the coding region and the first 301 nt of the 3′ untranslated region; full-length sequence of rat SALM5 cDNA is not available yet); pBluscript II containing nt 2214–2755 of rat SALM1 (coding region is nt 1–2415) and rat SALM3 (the entire sequence of the CB608382 EST clone, corresponding to the last 246 nt of the coding region and the first 286 nt of the 3′ untranslated region). Antisense riboprobes were prepared by RNA polymerase transcription using a Riboprobe System (Promega) in the presence of α-^{35}S-UTP.

Northern Blot Analysis

pGEM7zf SALM2 used in situ hybridization assays was used to generate SALM2-specific probe, which was labeled using α-^{32}P-dCTP and the Prime-a-Gene labeling system (Promega). This probe was used to hybridize the rat multiple-tissue Northern membrane (Clontech) in ExpressHyb solution following the manufacturer's instructions (Clontech).

Brain Fractionation and Coimmunoprecipitation

Subcellular and PSD fractions of adult rat brain were prepared as described (Carlin et al., 1980; Huttner et al., 1983). For developmental studies, whole brain homogenates were prepared from the rat brains at the ages of P1, P7, P14, P21, P29, and P42. In vivo coimmunprecipitation was performed as described (Wyszynski et al., 1999). In brief, the P2 fraction of adult rat brain was extracted in buffer containing 1% sodium deoxycholate and 50 mM Tris-HCl, pH 9.0, followed by incubation with immunoprecipitation antibodies. For coimmunoprecipitation in heterologous cells, cells were extracted in phosphate-buffered saline containing 1% Triton X-100.

Bead Aggregation Assays

Cultured neurons were transfected with ECFP-tagged SALM2 (wild-type or ΔC) at DIV12. Neutravidin labeled FluoSpheres (Molecular Probes; 1 μm diameter) were incubated with biotin-conjugated anti-EGFP antibodies (Rockland) for 2 hr at room temperature. The beads were resuspended with the conditioned medium and seeded onto ECFP-SALM2 expressed neurons at DIV14. Neurons were maintained for 24 hr before triple immunofluorescence staining and DIC imaging at DIV15.

Neuron Culture, Transfection, and Immunocytochemistry

Primary hippocampal cultures were prepared from embryonic day (E) 18–19 rat hippocampi described previously (Ko et al., 2003a). Cultured neurons were transfected using mammalian transfection kit (Invitrogen) and fixed with 4% paraformaldehyde/4% sucrose, permeabilized with 0.1% Triton X-100 in phosphate buffered saline, and incubated with the specific primary and Cy3-, Cy5-, or FITC- conjugated secondary antibodies. For in vivo immunocytochemical analysis, adult rat brain sections (50 μm) were permeabilized by incubation in 50% ethanol. The following antibodies were used for immunocytochemistry of transfected neurons and brain sections: SALM2 (3 μg/ml), EGFP (1 μg/ml), PSD-95 (Affinity BioReagent, 1:500), vGlut1 (1 μg/ml), gephyrin (1 μg/ml), and VGAT (1 μg/ml).

Image Acquisition and Quantification

Z-stacked fluorescent images were acquired using a confocal microscope (LSM510 or PASCAL; Zeiss). The same parameter settings were used for all scanning. Each experiment was repeated from three to five times, and neuronal images for analysis were randomly selected. All the morphometric measurements were performed using the MetaMorph image analysis software (Universal Imaging). To determine the density of synaptic protein clusters, one or two proximal dendrites with the largest caliber were chosen and analyzed. Clusters or puncta were defined as discrete regions of immunoreactivity with at least 2-fold higher intensity than the background. The numbers of clusters were counted and normalized to 10 μm length of dendrites after manual tracing and measurement in MetaMorph software. Excitatory and inhibitory synapses were defined by vGlut1-positive PSD-95 and vGAT-positive gephyrin clusters, respectively. PSD-95 and VGAT clusters that are localized outside the transfected neurons were not counted because they likely represent synaptic sites of nearby untransfected neurons. To determine the spine density, spines were defined as dendritic protrusions (0.5∼3.0 μm length; with or without a head) that are positive for both PSD-95 and vGlut1 (excitatory post- and presynaptic markers). The density of spines from a single image frame were grouped and averaged; means from multiple individual neurons were averaged to obtain a population mean and SEM. All the values were expressed as mean ± SEM. Statistical significance was determined by Student's t test or ANOVA Tukey test.

Electrophysiology

EGFP-positive cultured hippocampal neurons transfected with SALM2 moderate overexpression (pIRES-EGFP-SALM2) or SALM2 siRNA knockdown (pSUPER neo/GFP SALM2) constructs were whole-cell voltage clamped at −70 mV. The extracellular solution for whole-cell voltage clamp experiments contained (in mM): 150 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 HEPES (pH 7.4). The pipette solution for mEPSC measurements contained (in mM): 115 $CsMeSO_3$, 10 CsCl, 5 NaCl, 10 HEPES, 10 EGTA, 4 Mg-ATP, 0.3 Na-GTP (pH 7.35). TTX (1 μM; Tocris) and bicuculine (40 μM; Tocris) were added to the extracellular solution to block spontaneous action potentials and mIPSCs. The pipette solution for mIPSC measurements contained (in mM): 144 CsCl, 10 HEPES, 10 EGTA, 4 Mg-ATP, 0.3 Na-GTP (pH 7.35). TTX (1 μM), APV (50 μM), and CNQX (10 μM) were added to the extracellular solution to block action potentials and mEPSCs. All measurements were made using an Axopatch 200B amplifier (Axon instruments) at room temperature. Data were acquired at 10 kHz and low-pass filtered at 1 kHz. Pipette resistances were 4∼8 MOhm, and input resistances were >100 MOhm. Data were analyzed using Mini Analysis Program (Synaptosoft).

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/50/2/233/DC1/.

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