SALM4 suppresses excitatory synapse development by cis-inhibiting trans-synaptic SALM3–LAR adhesion

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Synaptic adhesion molecules regulate various aspects of synapse development, function and plasticity. These functions mainly involve trans-synaptic interactions and positive regulations, whereas cis-interactions and negative regulation are less understood. Here we report that SALM4, a member of the SALM/Lrfn family of synaptic adhesion molecules, suppresses excitatory synapse development through cis inhibition of SALM3, another SALM family protein with synaptogenic activity. Salm4-mutant (Salm4−/−) mice show increased excitatory synapse numbers in the hippocampus. SALM4 cis-interacts with SALM3, inhibits trans-synaptic SALM3 interaction with presynaptic LAR family receptor tyrosine phosphatases and suppresses SALM3-dependent presynaptic differentiation. Importantly, deletion of Salm3 in Salm4−/− mice (Salm3−/−; Salm4−/−) normalizes the increased excitatory synapse number. These results suggest that SALM4 negatively regulates excitatory synapses via cis inhibition of the trans-synaptic SALM3–LAR adhesion.
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ynaptic adhesion molecules regulate synapse development, function and plasticity. Previous studies have identified a large number of synaptic adhesion molecules, including neuroligins and neurexins1–11. The known functions of these molecules include aspects of trans-synaptic interactions between pre- and postsynaptic adhesion molecules and positive synapse regulations (that is, promotion of synapse development, maturation and maintenance), although opposite aspects (cis-interactions between nearby synaptic adhesion molecules on pre- or postsynaptic membranes and negative regulations) are less well understood.

Synaptic cell adhesion-like molecules (SALMs; also known as Lrfns) represent a family of leucine-rich repeat (LRR)-containing synaptic cell adhesion molecules, with five known members: Salm1/Lrfn2, Salm2/Lrfn1, Salm3/Lrfn4, Salm4/Lrfn3 and Salm5/Lrfn5 (refs 12–15). SALMs share a similar domain structure, containing six LRRs, an immunoglobulin (Ig) domain, a fibronectin III (FNIII) domain in the extracellular region, a transmembrane domain and a cytoplasmic region. Despite these apparent similarities in their overall structures, however, SALMs also possess several structurally distinct features. For instance, their cytoplasmic regions differ substantially in their lengths and amino-acid (aa) sequences. In addition, Salm1–3 (but not Salm4 and -5) contain a C-terminal PDZ-binding motif that binds to the PDZ domains of postsynaptic density (PSD)-95 (refs 12–15).

Functionally, SALMs regulate neurite outgrowth and branching12,16,17. In addition, they have been implicated in the regulation of synapse development and function. For instance, Salm1 interacts with the GluN1 subunit of NMDA receptors (NMDARs), and promotes dendritic clustering of NMDARs in cultured neurons12. Salm2 associates with both NMDARs and AMPA receptors (AMPARs), and promotes the development of excitatory (but not inhibitory) synapses13. Salm3 and Salm5 (but not the other SALMs) have synaptogenic activities, promoting both excitatory and inhibitory presynaptic differentiation in contacting axons18 via trans-synaptic interactions with presynaptic LAR family receptor tyrosine phosphatases19. However, with the exception of Salm3, the functions of the SALMs have not been examined using in vivo approaches, such as mouse genetics.

Although individual SALM isoforms are structurally and functionally distinct, they have been shown to associate with one another and are likely to form larger complexes20. Specifically, Salm1–3 (but not Salm4 and -5) were found to form a co-immunoprecipitated complex in the rat forebrain. In heterologous cells, all of the SALMs can interact with one another, further extending the complexity of inter-SALM interactions20. In addition to these cis-interactions, SALMs can participate in trans-interactions, with Salm4 and -5 forming homophilic and trans-cellular adhesion complexes20. These results suggest that the individual SALM isoforms are unlikely to be physically or functionally isolated, but instead may participate in intricate interplays with other SALM isoforms. However, the details of such interactions remain largely unexplored.

In the present study, we generated and characterized Salm4-mutant (Salm4−/−) mice. To our surprise, we found that Salm4−/− mice display increased excitatory and inhibitory synapse numbers in the hippocampus. Our data suggest that Salm4 cis-interacts with Salm3, inhibits the trans-synaptic interaction of Salm3 with presynaptic LAR and suppresses Salm3-dependent presynaptic differentiation at excitatory synapses.

Results

SALM4 expression pattern in the CNS. In order to understand the functions of SALM4, we first determined the expression patterns of Salm4 in the nervous system. Salm4 mRNAs were detected in the mouse brain and spinal cord at embryonic days 16 and 18, as determined by in situ hybridization (Fig. 1a). In postnatal brains, Salm4 mRNAs were detected in various brain regions, including the neocortex, striatum and hippocampus.

Salm4 proteins (~95 kDa) were mainly detected in the rat brain, as determined by immunoblot analysis using Salm4-specific antibodies and different tissue lysates (Fig. 1b,c and Supplementary Fig. 1a–c). Salm4 protein expression gradually increased during postnatal brain development (Fig. 1d and Supplementary Fig. 1d). Salm4 proteins were detected in synaptic brain fractions, including crude synaptosomal, synaptic membranes and PSD fractions (Fig. 1e,f and Supplementary Fig. 1e,f), consistent with the previously reported ultrastructural localization of Salm4 proteins around cell junctions, including neuronal synapses20.

Generation and basic characterization of Salm4−/− mice. To explore the in vivo functions of Salm4, we generated Salm4−/− mice (Salm4−/− knockout (KO) mice) lacking Salm4 exons 2 and 3, which encode the full-length Salm4 protein (Fig. 2a,b). Salm4 mRNAs were undetectable in the Salm4−/− brain, as determined by in situ hybridization (Fig. 2c). Salm4 proteins were also undetectable, as determined by two different Salm4 antibodies (Fig. 2d and Supplementary Fig. 1g). The Salm4−/− brain had normal gross morphology and neuronal density in the hippocampus (Fig. 2e,f).

Because Salm4 is known to regulate neurite outgrowth and branching in cultured hippocampal neurons16,17, we examined the morphology of Salm4−/− CA1 pyramidal neurons in the hippocampus using biocytin dye injection and Sholl analysis. We found that Salm4−/− neurons had normal dendritic complexities in both apical and basal dendrites (Fig. 2g).

Assessment of Salm4 protein expression by X-gal staining of Salm4−/− brain slices (5-week-old mice) revealed that Salm4 could be observed in various regions, including the neocortex, striatum, hippocampus and cerebellum (Fig. 2h). Notably, Salm4 X-gal signals in the hippocampus were stronger in the CA1 and CA3 regions than in the dentate gyrus. This contrasts with the similar levels of Salm4 mRNAs in the CA1/3 and dentate gyrus regions (Figs 1a and 2c), indicative of different expression levels of Salm4 mRNAs and proteins in different brain regions, similar to those observed in the cerebellum. Salm4 deletion had no effect on the levels of other SALMs or synaptic proteins, either in the whole brain or the hippocampus (Fig. 2i and Supplementary Fig. 1h).

Salm4−/− mice display increased synapse numbers. Electron microscopic (EM) studies previously detected Salm4 signals in axons, dendrites and synapses20, suggesting that Salm4 may regulate synapse development and function. We thus measured spontaneous excitatory synaptic transmission in hippocampal CA1 pyramidal neurons. We found that the frequency (but not the amplitude) of miniature excitatory postsynaptic currents (mEPSCs) was significantly increased in Salm4−/− neurons (Fig. 3a). A similar increase was also observed in the frequency (but not amplitude) of miniature inhibitory postsynaptic currents (mIPSCs; Fig. 3b). We could not detect any changes in the input-output relationship of evoked excitatory synaptic transmission and the paired pulse facilitation at Salm4−/−/− Schaffer collateral-CA1 pyramidal synapses (Supplementary Fig. 2), which is not in line with the increased frequency of mEPSCs (Fig. 3a). It is possible that mEPSCs in CA1 pyramidal neurons represent synaptic inputs other than those from the Schaffer collateral pathway (that is, the temporoammonic pathway), or that evoked

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EPSCs are less sensitive in detecting the changes in excitatory synapse number.

EM analysis of the Salm4−/− hippocampus (CA1 stratum radiatum) revealed significant increases in the density and length of the PSD apposed to presynaptic vesicles, whereas there was no change in the thickness or perforation (a measure of maturation) of the PSD (Fig. 3c). In addition, the density of symmetrically apposed to presynaptic vesicles, whereas there was no change in the number of excitatory synapses (Fig. 4b). The number of excitatory synapses in Salm4−/− hippocampal CA1 pyramidal neurons was increased compared to uninfected WT neurons expressing EGFP, with synaptic vesicle-perforation (a measure of maturation) (Supplementary Fig. 3d). These results suggest that Salm4 deletion increases the number of excitatory and inhibitory synapses in the hippocampus.

SALM4 re-expression rescues excitatory synapse numbers. To determine whether the increased synapse number observed in Salm4−/− neurons was caused by SALM4 deficiency, we re-expressed SALM4 in Salm4−/− CA1 pyramidal neurons by herpes simplex virus (HSV) infection on postnatal days (P) 15–18 (Supplementary Fig. 3a). We found that SALM4 re-expression, confirmed by immunoblot analysis (Fig. 3e and Supplementary Figs 1i and 3b), rescued the increased mEPSC frequency in Salm4−/− neurons to a normal range without affecting the amplitude (Fig. 3f). In contrast, SALM4 overexpression in wild-type (WT) neurons had no effect on the frequency or amplitude of mEPSCs, suggesting that endogenous levels of SALM4 proteins are sufficient to have the full negative effect on mEPSC frequency.

In contrast, SALM4 expression in Salm4−/− CA1 pyramidal neurons had no effect on the frequency or amplitude of mIPSCs (Supplementary Fig. 3c). Moreover, Salm4−/− neurons expressing enhanced green fluorescence protein (EGFP) showed mIPSC frequencies similar to those of WT neurons expressing EGFP, which is in contrast to the increased mIPSC frequency observed in uninfected Salm4−/− neurons. This might be attributable to the viral infection, which may have adverse effects on inhibitory (but not excitatory) synaptic transmission under our experimental conditions.

SALM4 cis-interacts with SALM2/3/5. To better understand the molecular mechanisms underlying the SALM4-dependent negative regulation of neuronal synapses, we performed an unbiased proteomic screen of SALM4-associated proteins in WT and Salm4−/− (control) mouse forebrains. We first enriched mature membrane proteins, including SALM4, using a wheat germ agglutinin (WGA) column (Fig. 4a). When these preparations were immunoprecipitated with SALM4 antibodies and subjected to proteomic analyses, we identified SALM2 as a major protein associated with SALM4 in WT brains but absent in Salm4−/− immunoprecipitates (of the total 20 peptides, 12 were SALM4 and 8 were SALM2; Fig. 4b,c).

This identification of an in vivo association between SALM4 and SALM2 contrasts with the previous reports that SALM1–3, but not SALM4 or -5, form complexes with one another in the rat brain. This discrepancy might reflect that our antibodies are somehow more efficient in pulling down SALM4 proteins in complex with SALM2. Indeed, a previous study reported that SALM1–3 exhibited antibody-dependent differential co-immunoprecipitation, wherein SALM1 immunoprecipitates contained almost undetectable amounts of SALM2 and SALM3, but SALM2 and SALM3 immunoprecipitates contained significant amounts of SALM1 (ref. 20).
To further characterize the interaction between SALM4 and SALM2, we performed co-immunoprecipitation experiments in heterologous cells. We found that SALM4 formed a complex with SALM2 (Fig. 4d and Supplementary Fig. 4a). In addition, the extracellular (ecto) domain of SALM4 (but not the cytoplasmic domain; SALM4-Ecto) could associate with SALM2, suggesting that the ecto domains of SALM4 and SALM2 are involved.

SALM2 forms a complex with SALM3 in vitro and in vivo and with SALM5 in vitro. In addition, SALM3 and SALM5 (but not the other SALMs) have synaptogenic activities. We therefore hypothesized that SALM4 might suppress neuronal synapses through a cis-interaction with SALM3 and/or SALM5. To examine this hypothesis, we first tested whether SALM4 could associate with SALM3 and SALM5, in addition to SALM2.

We found that SALM4 forms a complex with SALM3 in heterologous cells, but neither the ecto nor cytoplasmic domain of SALM4 was sufficient to mediate the interaction (Fig. 4e and Supplementary Fig. 4b), dissimilar to the domain requirement of the other SALMs.
the SALM4–SALM2 interaction. However, both full-length and ectodomain of SALM4 could associate with SALM5 (Fig. 4f and Supplementary Fig. 4b), similar to the SALM4–SALM2 interaction.

In order to further narrow down the domains of SALM4 involved in the interactions with SALM2, SALM3 and SALM5, we generated additional deletion variants of SALM4 that lacks the LRR domain (SALM4-LRR), the FNIII domain (SALM4-FNIII) and a large portion of the cytoplasmic region (SALM4-ΔC44aa), which displayed normal total expression levels but markedly reduced surface expression levels in SALM4-ΔLRR and SALM4-ΔFNIII but not in SALM4-ΔC44aa (Fig. 5a,b and Supplementary Fig. 4c). Assuming that non-surface SALM4 proteins may still interact other SALMs in cytoplasmic compartments, we performed co-immunoprecipitation experiments and found that SALM4-ΔLRR interacted with SALM3 but not with SALM2 or SALM5 (Fig. 5c–e and Supplementary Fig. 4d). In addition, SALM4-ΔFNIII and SALM4-ΔC44aa interacted with all three SALMs (SALM2, SALM3 and SALM5; Fig. 5c–e). These results collectively suggest that the LRR domain of SALM4 is

**Figure 3 | Increased excitatory and inhibitory synapse numbers in the Salm4−/− hippocampal CA1 region.** (a,b) The frequency, but not amplitude, of mEPSCs (a) and mIPSCs (b) is increased in Salm4−/− hippocampal CA1 pyramidal neurons (P15–20). n = 15 cells (three mice) for WT and 14 (three mice) for KO for mEPSCs; n = 17 (three mice) for WT and 18 (three mice) for KO for mIPSCs, *P<0.05, **P<0.01, ns, not significant, Student’s t-test. (c,d) PSD density and length, but not thickness or perforation, are increased in the Salm4−/− hippocampal CA1 region (P14–21), as determined by EM analysis. Normal and perforated PSDs are indicated by arrows and arrowheads, respectively. Scale bar, 500 nm. n = 3 mice for WT and KO, **P<0.01, ns, not significant, Student’s t-test. (d) The density, but not the length or thickness, of symmetric, electron-dense synaptic structures is increased in the Salm4−/− hippocampal CA1 region (P14–21). GABAergic terminals, indicated by asterisks, are visualized by EM immunogold staining for GABA.

Postsynaptic dendrites (d) are indicated. Scale bar, 500 nm. n = 3 mice for WT and KO, **P<0.01, ns, not significant, Student’s t-test. (e,f) SALM4 re-expression rescues the increased mEPSC frequency in Salm4−/− CA1 neurons. Salm4−/− or WT neurons were infected with HSV carrying SALM4 + EGFP, or EGFP alone (P15–18), followed by mEPSC measurements (f). SALM4/EGFP protein expression was confirmed by immunoblotting (e). Note that SALM4 protein levels in the two WT neurons (lanes 1 and 2) are not significantly different (Supplementary Fig. 2b) because a small fraction of total neurons are infected, although this does not affect mEPSC measurements using EGFP-positive neurons. n = 14 cells (five mice) for WT-EGFP, 11 (five mice) for WT-SALM4, 12 (six mice) for KO-EGFP and 9 (three mice) for KO-SALM4, *P<0.05, ns, not significant, analysis of variance (ANOVA).
important for the interaction with SALM2 and SALM5, while the transmembrane domain of SALM4 is important for the interaction with SALM3.

SALM4 inhibits synapse-promoting effects of SALM2/3/5. Because SALM4 associates with SALM2, SALM3 and SALM5, we tested whether SALM4 regulates the functional properties of SALM2/3/5. SALM2 has been shown to facilitate excitatory synapse differentiation, as shown by that cultured hippocampal neurons overexpressing SALM2 show increased excitatory synapse number\(^1\). When we coexpressed SALM2 and SALM4 in cultured hippocampal neurons, SALM4 reversed the SALM2-dependent increase in excitatory synapse number, as indicated by the density of Shank1/2/3 (Fig. 6a), an excitatory postsynaptic marker protein. In contrast, the size and intensity of Shank clusters were not changed by either the overexpression of SALM2 or SALM2 + SALM4. This suggests that SALM4 suppresses excitatory synapse-promoting effect of SALM2.

SALM3 and SALM5 have been shown to promote presynaptic differentiation in contacting axons\(^18\). We thus tested whether SALM4 could inhibit SALM3- or SALM5-dependent presynaptic differentiation, using neuron–fibroblast-mixed culture assays\(^21\). We found that coexpression of SALM4 with SALM3 in...
immunoblotted with the indicated antibodies. Transfected with HA-SALM4 (WT and deletion variants) (presynaptic differentiation, we next tested whether SALM4 couldBecause SALM4 suppresses SALM3- and SALM5-dependent
SALM4 inhibits the interaction between SALM3/5 and LAR.
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presynaptic differentiation, we next tested whether SALM4 could
inhibit the interaction between SALM3 and its known presynaptic
ligand, LAR\(^\text{19}\), or that between SALM5 and LAR\(^\text{24}\). To this end,
we tested whether the binding of LAR (a soluble form) to SALM3
displayed on heterologous cell surfaces could be inhibited by
SALM4 coexpressed in the same cell. Indeed, the interaction of
SALM3 with increasing concentrations of soluble LAR
(extracellular Ig1-3 domains; LAR-Ig1-3-Fc) was significantly
reduced by SALM4 coexpression (Fig. 7a). Similarly, LAR binding
to SALM5 was significantly inhibited by SALM4 coexpression (Fig. 7b).
In control experiments, we used a mutant SALM4 that lacks
SALM3 binding (SALM4-Ecto; see Fig. 4e) to see whether it fails to
inhibit LAR binding to SALM3. We found that SALM4-Ecto
partially reversed full-length SALM4-dependent suppression of
LAR binding to SALM3 (Fig. 7c). SALM4-Ecto did not affect the
surface expression of SALM3 (Fig. 7d and Supplementary Fig. 5b).
Figure 6 | SALM4 inhibits SALM2-dependent excitatory synapse facilitation and SALM3/5-dependent presynaptic differentiation. (a) SALM4 inhibits SALM2-dependent promotion of excitatory synapse number. (Top panels) Representative images of cultured hippocampal neurons transfected with control (EGFP alone), SALM2 and EGFP (SALM2), SALM4 and EGFP (SALM4) or co-transfected with SALM2 and SALM4 (SALM2 + SALM4) at DIV10. Neurons were analysed at DIV14 by triple immunofluorescence for EGFP (green), Shank (an excitatory postsynaptic marker protein; red) and SALM4 (blue). Scale bar, 10 μm, applies to all images. (Bottom panels) Bar graphs summarizing the effects of SALM4 overexpression on SALM2-induced postsynaptic development, quantified using Shank immunoreactivity (pan-Shank). n = 15 for control (EGFP alone), SALM2, SALM4 and SALM2 + SALM4, **P < 0.01, ***P < 0.001, ns, not significant, ANOVA with Tukey’s test. (b) SALM4 suppresses SALM3/5-dependent presynaptic differentiation. (Top panels) Representative images of cocultures. Hippocampal neurons were cocultured for 2 days (DIV 10–12) with HEK293T cells expressing EGFP alone (control), SALM3/5, SALM3/5 + SALM4, LRRTM2 or LRRTM2 + SALM4, and stained for EGFP/HA (blue), SALM4 (green) and synapsin I (red). Scale bar, 10 μm. (Bottom panels) Quantification of heterologous synapse-forming activities of SALM3/5 and LRRTM2, by measuring the ratio of synapsin I to surface HA immunofluorescence (absolute red/synapsin and blue/HA fluorescence values are also indicated). Note that SALM4 coexpression does not affect the surface expression of SALM3, SALM5 or LRRTM2, as indicated by fluorescence intensity of HA signals. n = 12 for control, 11 for SALM3, 10 for SALM3 + SALM4, 10 for SALM5, 10 for SALM5 + SALM4, 12 for LRRTM2 and 11 for LRRTM2 + SALM4, **P < 0.01, ***P < 0.001, ns, not significant, ANOVA with Tukey’s test. (c) SALM4 does not affect surface levels of SALM3 or SALM5, as determined by surface biotinylation assays. HEK293T cells transfected with SALM3/5 alone, or SALM3/5 + SALM4, were biotin-labelled, followed by avidin precipitation and immunoblotting. Input and surface, 1% and 8%, respectively. n = 3 for SALM3 and SALM3 + SALM4, ns, not significant, Student’s t-test.
Figure 7 | SALM4 inhibits the interaction between SALM3/5 and LAR. (a) Soluble LAR binding to SALM3 on heterologous cells is reduced by coexpression of SALM4. HEK293T cells transfected with HA-CD8 (control), SALM3 alone or SALM3 + SALM4 were incubated with LAR-Ig1-3-Fc (Ig1-3 domains of LAR fused to human Fc) at increasing concentrations, followed by staining for Fc (bound LAR-Ecto-Fc), HA (CD8 or SALM3; red) and SALM4 (blue). For quantification, signals for bound LAR-Ig1-3-Fc were normalized by surface HA-SALM3 signals. Surface expression of HA-SALM3 is not affected by SALM4 coexpression (bottom right). n = 18 cells for CD8, 23 for S3, 20 for S3 + S4 and 19 for S4 (0.25 mM); 8, 13, 18 and 18 (0.5 mM); 17, 27, 26 and 22 (1 mM); 13, 27, 19 and 19 (1.5 mM); 23, 27, 32 and 15 (2 mM). *** P < 0.001 (relative to SALM3 + SALM4 or SALM4 alone), ns, not significant, ANOVA. Scale bar, 20 μm. (b) LAR binding to SALM5 is reduced by coexpression of SALM4. Experiments were performed as in a, except using SALM5 rather than SALM3 and a single concentration of LAR-Ig1-3-Fc (2 mM) for simplicity. n = 53 for CD8, 46 for S5 and 41 for S5 + S4. *** P < 0.001, ns, not significant, ANOVA. Scale bar, 20 μm. (c, d) SALM4-Ecto (SALM4e) that lacks SALM3 binding, replacing full-length SALM4, shows partially reverses SALM4-dependent suppression of LAR binding to SALM3. SALM4 or SALM4e does not affect surface expression of SALM3, as shown by biotinylation of surface proteins (d). n = 34 for S3, 28 for S3 + S4 and 30 for S3 + S4e, *** P < 0.001, ns, not significant, ANOVA and ** P < 0.01, Student’s t-test. Scale bar, 20 μm. (e) SALM4-Ecto, replacing full-length SALM4, substantially reverses SALM4-dependent rescue of the increased mEPSC frequency in Salm4−/− CA1 neurons. WT neurons were infected with HSV carrying EGFP, and Salm4−/− neurons were infected with HSV carrying EGFP, EGFP + HA-SALM4 and EGFP + HA-SALM4-Ecto (P15-18), followed by mEPSC measurements. n = 9 cells (three mice) for WT-EGFP, 9 (three mice) for KO-EGFP, 8 (three mice) for KO-SALM4 and 9 (three mice) for KO-SALM4e, * P < 0.05, ** P < 0.01, ns, not significant, ANOVA and ** P < 0.01, Student’s t-test.
Figure 8 | Double KO of SALM3 and SALM4 normalizes excitatory synapse numbers in both Salm4−/− and Salm3−/− neurons. (a) Comparison of SALM3 and SALM4 protein expression patterns in sagittal brain sections by X-gal staining of Salm3+/− and Salm4+/− single KO slices (5 weeks). Note that the hippocampal CA3 and CA1 signals are stronger than those in the dentate gyrus. Scale bar, 1 mm. (b) Salm3−/−; Salm4+/− CA1 pyramidal neurons display mEPSC frequencies comparable to those of WT neurons (P18–21), indicative of normalization in both in Salm4−/− and Salm3−/− neurons, although such effects are weaker in Salm3−/− neurons. Note that the double KO has no effect on mEPSC amplitudes. n = 12 cells (four mice) for WT, 11 (three mice) for Salm3−/−, 12 (three mice) for Salm4−/−, 13 (four mice) for Salm3−/−; Salm4−/−, *P < 0.05, **P < 0.01, ***P < 0.001, ns, not significant, ANOVA with Bonferroni multiple comparison test; #P < 0.05, Student’s t-test. (c) The double KO does not rescue the increased mIPSC frequency in Salm4−/− CA1 pyramidal neurons. n = 15 (four mice) for WT, 13 (three mice) for Salm3−/−, 12 (three mice) for Salm4−/− and 12 (four mice) for Salm3−/−; Salm4−/−, *P < 0.05, ns, not significant, ANOVA with Bonferroni multiple comparison test. (d) The increased PSD density in the Salm4−/− hippocampus is normalized by the double KO, as shown by the EM analysis of the hippocampal CA1 stratum radiatum region (P20). Normal and perforated PSDs are indicated by arrows and arrowheads, respectively. Scale bar, 500 nm. n = 3 mice for WT, Salm4−/−, and Salm3−/−; Salm4−/−; ***P < 0.001, ns, not significant (P = 0.1551 for PSD thickness and 0.3996 for perforated spines), ANOVA. (e) The increased density of symmetric, electron-dense synaptic structures in the Salm4−/− hippocampus is not normalized by the double KO, as shown by the EM analysis of the hippocampal CA1 stratum radiatum region (P20). GABAergic terminals (asterisks) are indicated by EM immunogold staining for GABA. Postsynaptic dendrites (d) are also indicated. Scale bar, 500 nm. n = 3 mice for WT, Salm4−/−, and Salm3−/−; Salm4−/−, ***P < 0.001, ns, not significant, ANOVA.
Double *Salm3*-Salm4 KO normalizes excitatory synapse numbers. The cis-regulation of Salm3/5 by Salm4 requires their expression in the same cells. In line with this, X-gal staining revealed that the expression patterns of Salm3 and Salm4 proteins significantly overlapped in mouse brain regions, including the neocortex, hippocampus and striatum (Fig. 8a and Supplementary Fig. 6a), and also in hippocampal CA3 and CA1 regions. The potential overlap between Salm4 and Salm5 could not be tested because Salm5−/− mice that allow X-gal staining were unavailable; however, we note that the known mRNA distribution pattern of Salm5 substantially overlap with those of Salm3 and Salm4 (ref 13,14). Next, we hypothesized that if Salm4-dependent suppression of excitatory synapse number involves the inhibition of Salm3/5, removal of Salm3/5 might rescue the increased excitatory synapse number observed in Salm4−/− neurons. To this end, we crossed Salm3−/− mice, which we recently reported to exhibit reduced excitatory synapse numbers in the hippocampus19, with Salm4−/− mice to obtain double KO mice (Supplementary Figs 6b and 5e). Salm3−/−/Salm4−/− mice and Salm3−/− mice (but not Salm4−/− mice) displayed slightly lower body weights than controls (Supplementary Fig. 6c).

We found that the increased mEPSC frequency observed in Salm4−/−/CA1 pyramidal neurons was normalized to near-WT levels in Salm3−/−/Salm4−/−; Salm4−/−/CA1 pyramidal neurons (Fig. 8b). This result also indicates that the reduced mEPSC frequency in Salm3−/− neurons was normalized in the double KO mice, although the extent of normalization was weaker than that for Salm4−/− neurons. The double KO had no effect on the amplitude of mEPSCs.

When mIPSCs were compared, we found that the double KO did not rescue the increased mIPSC frequency seen in Salm4−/− mice (Fig. 8c), nor did it affect the mIPSC frequency in Salm3−/−/CA1 pyramidal neurons. Moreover, the observed mIPSC amplitudes were comparable across the four genotypes. Together, these results indicate that the double KO of Salm3 and Salm4 rescues the increased frequency of mEPSCs (but not mIPSCs) in Salm4−/− mice.

We next performed EM analysis to see whether the double KO normalizes the increased excitatory and inhibitory synapse densities in Salm4−/− mice. We found that the Salm3−/−/Salm4−/− hippocampus (CA1 stratum radiatum) shows the density of the PSD significantly smaller than that of Salm4−/− hippocampus but comparable to that of WT hippocampus (Fig. 8d). In contrast, the increased inhibitory synapse density in Salm4−/− hippocampus was not normalized by the double KO (Fig. 8e). These results further suggest that Salm3 mediates the Salm4-dependent inhibition of excitatory synapse number.

Discussion

In the present study, we report that Salm4−/− mice display increased excitatory and inhibitory synapse numbers in the hippocampus, and that Salm4 cis-interacts with Salm3, inhibits the Salm3–LAR interaction and suppresses Salm3-dependent presynaptic differentiation.

Our data indicate that a Salm4 mutant that lacks Salm3 binding (Salm4-Ecto), unlike full-length Salm4, loses the ability to inhibit LAR binding to Salm3 in HEK293T cells, and to rescue the increased mEPSC frequency in CA1 pyramidal cells (Fig. 7d–f). However, the extent of these reversals are partial, especially in the LAR-binding experiments in vitro. One possibility is that Salm4–Ecto, although it no longer associates with Salm3 in HEK293T cells under co-immunoprecipitation conditions (Fig. 4e), may still weakly associate with, or get sufficiently close to, Salm3 on the surface membrane through its intact extracellular domain and inhibit LAR binding to Salm3; the fact that Salm4–LRR binds to Salm3 does not exclude the possibility that the LRR domain of Salm4 may still weakly bind to Salm3. This nonspecific inhibition, if present, might be stronger when Salm4–Ecto is highly expressed in HEK293T cells, whereas it is weaker when Salm4–Ecto is weakly expressed in neurons, or Salm4–Ecto cannot easily have an access to endogenous Salm3 at excitatory synaptic sites.

Our hypothesis (Salm3 inhibition by Salm4) is genetically supported by our observation that double KO of Salm3 and Salm4 normalizes mEPSC frequency and excitatory synapse number phenotypes. This suggests that Salm3, but not Salm5, may act as a major downstream effector of Salm4 for the inhibition of excitatory synapse number in the hippocampus. In line with this possibility, Salm3 and Salm4 show substantial overlaps in their protein distribution patterns in the brain (Fig. 8a). Moreover, a previous study showed that the temporal expression patterns of Salm3 and Salm4 mRNAs along the embryonic developmental stages are more similar than those of the other SALMs14. These results, however, do not exclude the possibility that Salm5 may closely interact with Salm4 in some other brain regions.

Our data, however, provide relatively few clues as to how Salm4 negatively regulates inhibitory synapses. The increased inhibitory synapse number in Salm4−/− mice might merely represent a compensatory change induced by the increase in excitatory synapse number. Alternatively, it could be a more direct effect involving Salm4. In line with this, a previous study showed that Salm3 and Salm5, which associate with Salm4, can induce both excitatory and inhibitory presynaptic differentiation in cocultured neurons18. In addition, the LAR family proteins, which bind to Salm3 and Salm5, can induce both excitatory and inhibitory postsynaptic differentiations by interacting with NGL-3, TrkC, IL1RAPL1, IL1RacP and Slitrk2,6.

It is likely that ‘postsynaptic’ Salm4 cis-interacts with Salm3 to suppress excitatory synapses. Salm4 is detected at both pre- and postsynaptic sites, and is thought to mediate homophilic and trans-synaptic adhesions20. This homophilic adhesion, however, is unlikely to contribute to the Salm4-dependent negative regulation of Salm3 because we herein showed that HSV-mediated re-expression of Salm4 in Salm4−/−/CA1 pyramidal neurons (postsynaptic side) was sufficient to rescue the increased mEPSC frequency in a cell-autonomous manner.

Our study extends the reported complexity of interactions among the Salm family members. The proteomic data derived from WT and KO (control) brain samples suggest that there is a strong biochemical association between Salm4 and Salm2 in vivo. In addition, Salm4 associates with Salm3 and Salm5 in addition to Salm2 in heterologous cells. Given that Salm3−/− Salm4−/− Salm5−/− form a strong co-immunoprecipitable complex in the brain20, Salm4, together with Salm3s 1–3 and Salm5, may form combinations of protein complexes larger than previously revealed in different brain regions. This complex might be further stabilized by interactions with PSD-95 through Salm4s 1–3, and with LAR family proteins through Salm3s and 5.
Inhibition of neuronal synapses via cis-interactions of Salm4 is reminiscent of the previously reported cis-interaction between neurelin-1 and neurexin-1β, which suppresses neurelin-1-dependent presynaptic differentiation in contacting axons35. In addition, postsynaptic MDGA1 was shown to cis-interact with neurexin-2 and thereby suppress neurexin-2-dependent inhibitory presynaptic differentiation36,27. These results suggest that postsynaptic adhesion molecules may cis-interact with one another to regulate their trans-interactions. In addition, we recently showed that a trans-interaction between postsynaptic NGL-1 and presynaptic netrin-G1 (a glycosylphosphatidylinositol (GPI)-anchored synaptic adhesion molecule) induces the cis-interaction of netrin-G1 with LAR28. These results suggest that there may be functional interplays between cis- and trans-adhesions at the synapse.

Salm4 have been implicated in autism spectrum disorders, intellectual disability and schizophrenia39,33. In addition, LAR family proteins are implicated in autism spectrum disorders, attention deficit/hyperactivity disorder, restless leg syndrome and schizophrenia2,6. Therefore, the mechanisms revealed in the present study might help us better understand these disorders.

In conclusion, our results suggest that postsynaptic Salm4 negatively regulates excitatory synapse numbers through a cis-interaction with Salm3 and the inhibition of both the Salm3–Lar interaction and Salm3-dependent presynaptic differentiation. Our study provides genetic support for the emerging notion that neighbouring adhesion molecules cis-interact with one another to regulate neuronal synapses.

**Methods**

**Generation of Salm4+/− mice.** A mouse ES cell clone (15252A-A12; Lrnf3_A1A2), derived from the C57BL/6N strain, was obtained from Velocigene (VG15252). The cassette (ZEN-Ub1) for an allele deletion contained LacZ and 6N) were microinjected into the blastocyst of the C57BL/6J-Tyr c-2J. The PCR products for WT and KO alleles are 617 and 417 bp long, respectively.

**cDNA constructs.** Full-length untagged mouse Salm4 (aa 1–626) expression construct was generated by amplifying the insert from a rat brain cDNA library (BD Biosciences Clontech) by PCR and subcloning it into Gateway vector. Haemagglutinin (HA)-tagged full-length mouse Salm3 (aa 28–636) was subcloned into pDisplay vector. Full-length untagged mouse Salm4, Myc-tagged Salm4s, EGFP-tagged Salm4s and Salm4/S-50-Ecto constructs have been described previously38. Cytoplasmic regions of mouse Salm4 (aa 561–626) were subcloned into pEGFP-C1. The pDisplay-LRRTM2 construct has been described39. Ha-tagged full-length mouse Salm4 (aa 28–627), Salm4-ALRR (aa 287–627), Salm4-ACFNI (aa 28–400, 530–627), Salm4-AC44aa (aa 28–583) with their own transmembrane domains, cytoplasmic domains and stop codons were subcloned into pDisplay, and Salm4-Ecto (aa 28–530) was subcloned into a modified pDisplay vector lacking the Myc epitope but with an intact HA epitope and transmembrane domain. pRES2-SALM2-WT-EGFP has been described previously43.

**In situ hybridization.** Mouse brain sections (12 μm thick) at embryonic day E16 and E18 and postnatal days (P7, P14, P21 and P56) were prepared using a cryostat (Leica CM 1950). Mouse brain sections from WT and Salm4+/− mice (P56) were also generated to visualize the lack of Salm4 mRNAs in Salm4+/− mice. Hybridization probe specific for mouse Salm4 mRNA was prepared using the following primers: nt 1,429–1,481 of Salm4 (GenBank DQ078787). Antisense riboprobe was generated using 35S-uridine triphosphate (UTP) and the Riboprobe system (Promega).

**Antibodies.** Salm4 antibodies (1,820, 1:500 for western blot (WB); 2026, 1:1,000 for WB or purified form 1:200 for immunocytochemistry (ICC); both guinea pigs) were generated by using GST-Salm4 aa 561–626 and 596–626 (last 30 aa; NM_175478) as immunogens, respectively. Salm1 (2,022, 1:500 for WB; guinea pig), Salm2 (2,058, 1:500 for WB; guinea pig) and Salm3 (2,024, 1:500 for WB; guinea pig) antibodies were generated using the last 30 aa of mouse proteins. Salm5 (1,943, 1:500 for WB; guinea pig) antibodies generated using the H2-ctoplasmic domain of mouse Salm5 (aa 551–716). EGFP antibodies (1,168, 1:1,000 for ICC; rabbit) were generated using the full-length EGFP protein as immunogen. Rabbit polyclonal Shank antibodies, which are specific for all three Shanks (Shank1/2/3, 1:500 for ICC), were used.

**Fluorescent images.** Fluorescent images were acquired using confocal laser scanning microscope (Leica TCS SP5 II). We performed immunoblot analysis using the following primary antibodies: p38 (Cell Signaling, 9212S, 1:1,000 for WB), GluN2B (NeuroMab, N59/36, 1:1,000 for WB), synaptin 1 (Syny, 106, 1:1,001, 1:1,000 for WB, 1:500 for ICC), SLC4 (SySy, 940, 1:1,000 for WB), Myc (Sigma, M4439, 1:500 for WB), GluN2A (Invitrogen, A-6473, 1:500 for WB), GluN2B (NeuroMab, N59/36, 1:1,000 for WB), synaptan 1 (Syny, 106, 1:1,001, 1:1,000 for WB, 1:500 for ICC), SLC4 (SySy, 294, 1:500 for WB), Myc (Sigma, M4439, 1:1,000 for WB, 1:200 for ICC) and EGFP (Rockland, 1:500 for ICC).

**Subcellular and PSD fractions.** Subcellular fractions of the rat brain were prepared as described previously40. PSD fractions were purified as described previously41.

**Mouse genotyping.** Mouse genotyping was performed using the following three PCR primers: 5′-TCTCTGTAGGTTGTTACAG-3′ (5′ forward primer for both WT and KO alleles), 5′-CAGTCTTTTACCATCATCAGGC-3′ (5′ reverse primer for WT) and 5′-GTCGTCCTACGTTCGCTACG-3′ (5′ reverse primer for KO). The PCR products for WT and KO alleles are 167 and 417 bp long, respectively.

**Immunohistochemistry and immunoblotting.** Three-week-old mouse brain slices (50 μm) were permeabilized with 0.5% Triton X-100 in 1× PBS for 1 h followed by primary and secondary antibody incubations. Fluorescent images were acquired using confocal laser scanning microscope (M1000, Zeiss) and analysed using the M1000 software (series 7.4). Light microscopic images were taken using a camera (DAGE-MTI, MRC-590) and fluorescent images were acquired using confocal laser scanning microscope (LSM780, Zeiss).

**X-gal staining.** Mice were transcardially perfused with heparinized 1× PBS and 4% formaldehyde in 1× PBS and postfixed at 4°C for overnight. For X-gal staining, brain slices (250 μm) were sectioned using vibratome (Leica VT 1000S) and incubated in X-gal staining solution containing 1 mg ml−1 X-gal at 37°C for overnight.

**Sholl analysis.** Biocytin hydrochloride (0.5%; Sigma) was added to the pipette solution after measurements of basal transmission for general health check for 2–3 min. Then, the slices were fixed with 4% formaldehyde at 4°C overnight and permeabilized with 0.5% Triton X-100 in 1× PBS. Alexa Fluor 555-conjugated streptavidin (2 mg ml−1, Invitrogen) in 1× PBS was used for staining (1:300 for 1–2 h). Fluorescent images were acquired using confocal laser scanning microscope (LSM780, Zeiss).

**Electrophysiology.** To measure mEPSCs and mIPSCs, CA1 pyramidal cells (P15–21, 250 μm with cold, oxygenated, 95% O2 and 5% CO2, sucrose-substituted ACSF (SCSF) (in mM): 212 sucrose, 10 glucose, 25 NaHCO3, 5 KCL, 1.25 NaH2PO4, 1.2-ascorbate, 2 pyruvate with 3.5 MgCl2 and 0.5 CaCl2) were held at −70 mV (6–8 min), using a Multiclamp 700B amplifier (Clampex 9.2, Molecular Devices). Pipette solutions contained (in mM) 117 CsMeSO4, 8 NaCl, 10 Mg-ATP, 0.3 Na-GTP and 10 EGTA (295 mOsm) for mEPSC measurements; or 115 CsCl, 10 TEACl, 8 NaCl, 10 HEPES, 5 Qx-314Cl,
4 Mg-ATP, 0.3 Na-GTP and 10 EGTA (294 mOsm) for mIPSC measurements. TTX (0.5 mM; Tocris) and picrotoxin (100 mM; Sigma-Aldrich) were added to oxygenated artificial cerebrospinal fluid (ACSF) (in mM: 125 NaCl, 10 glucose, 25 NaHCO3, 2.5 KCl, 1.25 NaH2PO4 with 1.3 MgCl2 and 2.5 CaCl2 for mEPSC recordings. TTX (0.5 mM), NBQX (10 mM; Tocris) and AP5 (25 mM; Tocris) were added for mIPSC measurements. Synaptic currents were analysed using a customized macro in Igor Pro 4.01 (WaveMetrics) and Clampfit (pCLAMP 10.4).

For field recordings, transverse hippocampal slices (400 μm) were prepared from 3- to 4-week-old male littersmates. The brain was rapidly isolated and placed in ice-cold, oxygenated dissecting buffer containing (in mM) 212.7 sucrose, 5 KCl, 1.23 NaH2PO4, 0.5 CaCl2, 10 MgCl2, 26 NaHCO3, 10 and glucose. Hippocampal slices were prepared by cutting with a Leica VT1000S vibratome (Leica) and transferred for recovery to a holding chamber containing oxygenated ACSF consisting of (in mM) 124 NaCl, 5 KCl, 1.23 NaH2PO4, 2.5 CaCl2, 1.5 MgCl2, 26 NaHCO3 and 10 glucose at 28–30°C for at least 1 h before recording. After recovery, slices were transferred to a recording chamber where they were perfused continuously with oxygenated ACSF (27–28°C) at a flow rate of 2 ml/min. Hippocampal CA1 field excitatory postsynaptic potentials were evoked by afferent collateral stimulation (0.2 ms current pulses) with concentric bipolar electrode. Synaptic responses were recorded with ACSF-filled microelectrodes (1–2 MΩ) and were quantified as the initial slope of field excitatory postsynaptic density.

Data from slices with stable recordings (<5% change over the baseline period) were included in the analysis. All data are presented as mean ± s.e.m. normalized to the preconditioning baseline (at least 20 min of stable responses). The experiments were blind to mouse genotypes. Recordings were performed using an AM-1800 Microelectrode amplifier (A-M systems) and the IGOR software (WaveMetrics, Lake Oswego, OR, USA) was used for digitizing and analysing the responses.

Electron microscopy. WT, Salm4+/− and Salm3+/−/Salm4−/− mice were deeply anaesthetized with sodium pentobarbital (80 mg kg−1, intraperitoneally (i.p.)) and were intracardially perfused with 10 ml of heparinized normal saline, followed by 50 ml of a freshly prepared fixative of 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 m phosphate buffer (PB, pH 7.4). The hippocampus was removed from the brain, postfixed in the same fixative for 2 h and stored in PB (0.1 M) at 4°C. Sections were cut transversely to a 70 μm. The sections were osmicated with 0.5% osmium tetroxide (in 0.1 M PB) for 1 h, dehydrated in graded alcohols, flat embedded in Durcupan ACM (Fluka) and cured for 48 h at 60°C. Small pieces containing the stratum radiatum of hippocampal CA1 region were cut out of the waters and glued on the plastic block by cyanoacrylate. Ultrathin sections were cut and mounted on Formvar coated single-slot grids. For excitatory synapses, sections were stained with uranyl acetate and lead citrate, and examined with an electron microscope (Hitachi H-7500; Hitachi) at 80 kV accelerating voltage. For inhibitory synapses, sections were stained with uranyl acetate and lead citrate, and examined with an electron microscope (Hitachi H-7500; Hitachi) at 80 kV accelerating voltage. For inhibitory synapses, sections were stained with uranyl acetate and lead citrate, and examined with an electron microscope (Hitachi H-7500; Hitachi) at 80 kV accelerating voltage. For inhibitory synapses, sections were stained with uranyl acetate and lead citrate, and examined with an electron microscope (Hitachi H-7500; Hitachi) at 80 kV accelerating voltage.

Cultured neuron transfection and imaging. Cultured hippocampal neurons were prepared from E18 rat brains, as described previously47,48, on coverslips coated with poly-D-lysine and grown in Neurobasal medium supplemented with B-27 (Invitrogen), 0.5% fetal bovine serum, 0.5 mM Glutamax (Invitrogen) and sodium pyruvate (Invitrogen). For the overexpression of SALMs in cultured neurons, hippocampal neurons were transfected with pBES-SALM2-EGFP, pGW1-Salm4-Mycl-M4EGFP or EGFP-Control using a CalPhos Kit (Clontech) at DIV10 and immunostained at DIV14. For the ICC, cultured neurons were fixed with 4% paraformaldehyde/4% sucrose, permeabilized with 0.2% Triton X-100 in PBS, immunostained with primary antibodies (against EGFP, Shank1 and SALM4 to identify the triply transfected hippocampal neurons), and Cy3-, Cy5- and fluorescein isothiocyanate-conjugated secondary antibodies (Jackson Immunoresearch). The images were acquired using a confocal microscope (LSM710, Carl Zeiss) with a × 63 objective lens. All image settings were kept constant. The Z-stacked images were converted to maximal projection and analysed to obtain the size, intensity and density of the puncta immunoreactivities derived from marker proteins. The quantification was performed in a blind manner using MetaMorph (Molecular Devices).

Mixed culture synapse-formation assays. Mixed culture assays were performed with HEK293T cells (American Type Culture Collection) as described22. Cultured hippocampal neurons were incubated until HEK293T cells transfected with EGFP (Control), or the indicated SALM3, SALM5 or LRTM2 constructs, were added at 10 days of culture (DIV 10) for further coculture experiments. HEK293T cells were transfected with FuGene (Roche, USA) as indicated. After 48 h, transfected HEK293T cells were trypsinized, seeded on the hippocampal neuron cultures at DIV 10, further cocultured for 48 h and double-immunostained with synapsin I, SALM4 and HA/GFP antibodies at DIV 12 as described previously49. All images were acquired using a confocal microscope. For quantifications, the contours of the transfected HEK293T cells were chosen as the region of interest. The fluorescence intensity of synapsin puncta normalized to each HEK293T cell area was quantified for red, green and blue channels with the MetaMorph Software (Molecular Device).

Recombinant protein expression and purification. The Ig1-3 domains (aa 30–318) of human LAR-RPTP was cloned into the BamHI and XbaI sites of the pET-21a vector (Invitrogen) to express a recombinant protein with a 6xHis tag. The High Five insect cells (Invitrogen) were infected with protein A-Sepharose column (GE Healthcare Life Science) for the purification of 6xHis tagged proteins. The affinity resin was washed with the buffer containing 50 mM Tris-HCl (pH 8.0) and 200 mM NaCl. Fc-tagged proteins immobilized in the protein A-Sepharose column were eluted with glycine buffer (100 mM glycine, pH 2.1).

Recombinant protein-binding assays. For recombinant LAR-binding assays, HEK293T cells expressing combinations of SALMs were incubated with extracellular solution containing (in mM): 118 NaCl, 2.6 KCl, 10 HEPS, 2 CaCl2, 2 MgCl2, 10 Mg-ATP, 10 EGTA and 100 μg/ml BSA (pH 7.2) for 1 h at 4°C, and then pelleted. The cell pellet was resuspended in LAR-1g1-3-Fc and incubated with 0.25, 0.5, 1.5 and 3 mg/ml of purified soluble LAR-Ig1-3-Fc proteins at 0.25, 0.5, 1, 1.5 and 2 mM concentrations were added for mIPSC measurements. Synaptic currents were analysed using a cell-attached configuration where a single 2 mM was used for SALM5 binding. After washing of LAR-1g1-3-Fc and fixation of the...
cells, LAR binding was visualized by incubating the cells with anti-human-Fc antibodies (Sigma; 2 μg/ml, goat). Then, the cells were incubated with HA antibodies (Sigma; 1:500, mouse or rabbit), or Myc antibodies (Sigma; 1:500, mouse), without permeabilization for surface HA staining (HA-SALM3/CDB), followed by permeabilization and staining with SALM4 C-terminal antibodies (#2026; purified, 1:500, Gp).

**Mass spectrometric screen of SALM4-associated proteins.** Preparation of protein samples, affinity chromatography and proteomic analysis were performed as described previously38. Briefly, crude synaptosomes were prepared from mouse forebrains (15 WT and KO mice; 3 weeks), and solubilized in 1% PBS containing 0.5% Triton X-100, 0.1% SDS, 0.5 mM EDTA, 0.5 mM EGTA and protease inhibitor cocktail (Roche). From the solubilized proteins, mature membrane proteins were enriched using WGA column chromatography (WGA bead, Vector Laboratories) and eluted using N-acetyl-d-glucosamine (Sigma). The samples were incubated with SALM4 antibodies (2026, purified, Gp) prebound to protein A-Sepharose beads (GE Healthcare), followed by protein elution with 2.5 × XT MOPS buffer (BIO-RAD). Boiled samples were resolved using 4 – 12% Criterion XT Precast Gels (BIO-RAD) followed by silver staining. From the stained gels, protein bands were excised and subjected to mass spectrometric analyses. Mass spectrometry was performed using LTQ-Orbitrap XL ETD (Thermo Scientific) at the Korea Basic Science Institute.

**Statistics.** The results of statistical analysis are indicated in Supplementary Table 1.

**Data availability.** The authors confirm that all relevant data are available from the authors.

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Author contributions
D.L. conducted in situ hybridization experiments; E.L. and J.N. conducted biochemical experiments; E.L. and Y.L. conducted single and double knockout mouse generation; E.L. conducted immunohistochemistry experiments; E.L., J.D.R., T.-Y.C. and W.M. conducted slice electrophysiology experiments; Y.S.C. conducted electron microscopy experiments and analysis; R.N. and E.L. conducted in vivo co-immunoprecipitation and mass spectrometry experiments; J.S.K. and H.K. conducted neuron culture and coculture assay experiments; Su-Yeon Choi, E.L., D.K. and S.-G.L. conducted Fc assay experiments; E.L. conducted all the other experiments; H.M.K., H.K., Se-Young Choi, J.W.U., M.G.K., Y.C.B., J.K. and E.K. supervised the project and wrote the manuscript.

Additional information
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