The X-Linked Intellectual Disability Protein TSPAN7 Regulates Excitatory Synapse Development and AMPAR Trafficking

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SUMMARY

Mutations in TSPAN7—a member of the tetraspanin protein superfamily—are implicated in some forms of X-linked intellectual disability. Here we show that TSPAN7 overexpression promotes the formation of filopodia and dendritic spines in cultured hippocampal neurons from embryonic rats, whereas TSPAN7 silencing reduces head size and stability of spines and AMPA receptor currents. Via its C terminus, TSPAN7 interacts with the PDZ domain of protein interacting with C kinase 1 (PICK1), to regulate PICK1 and GluR2/3 association and AMPA receptor trafficking. These findings indicate that, in hippocampal neurons, TSPAN7 regulates AMPA receptor trafficking by limiting PICK1 accessibility to AMPA receptors and suggest an additional mechanism for the functional maturation of glutamatergic synapses, whose impairment is implicated in intellectual disability.

INTRODUCTION

The TM4SF2 gene on Xp11.4 encodes tetraspanin 7 (TSPAN7), member of the tetraspanin superfamilly of evolutionarily-conserved membrane proteins that associate dynamically with numerous partner proteins in tetraspanin-enriched microdomains (TEMs) of the plasma membrane (Boucheix and Rubinstein, 2001). Tetraspanins regulate cell morphology, motility, and signaling in brain, immune system, tumors, and elsewhere (Boucheix et al., 2001). Mutations in tetraspanins leading to loss of function phenotype are relatively rare probably because many tetraspanins overlap functionally (Hemler, 2005). Nonetheless, specific tetraspanins play critical roles in oocytes during fertilization, fungi during leaf invasion, Drosophila embryos during neuromuscular synapse formation, T and B lymphocyte activation, retinal degeneration, and brain function (Hemler, 2005). Some TM4SF2 mutations, including TM4SF2 inactivation by X;2 balanced translocation, a premature stop codon TGA (gly218-to-ter) (Zemni et al., 2000), and a 2-bp deletion (564 delGT) resulting in a premature stop codon at position 192 (Abidi et al., 2002) are directly associated with nonsyndromic intellectual disability. The gly218-to-ter nonsense mutation and the 2-bp deletion predict a truncated protein lacking the fourth transmembrane domain and cytoplasmic C-terminal tail.

Tetraspanins consist of four transmembrane domains, a short extracellular loop (EC1), a very short intracellular loop (IL), a longer extracellular loop (EC2), and short N- and C-terminal cytoplasmic tails. The EC2 has a constant and a variable region, the latter contains several protein interaction sites (Berditchevski, 2001). All known tetraspanins contain the Cys-Cys-Gly sequence in the EC2, and >50% of tetraspanins include a Pro-x-x-Cys-Sys sequence, that forms disulfide bonds important for correct EC2 folding (Berditchevski, 2001). The N and C termini of individual tetraspanins are highly conserved across vertebrates, but differ markedly from one tetraspanin to the next; the C-terminal tail is especially divergent (Hemler, 2008). This suggests that, despite their short lengths, the N and C termini have specific functions, including linkage to cytoskeletal and signaling proteins.

Tetraspanins regulate the signaling, trafficking and biosynthetic processing of associated proteins (Hemler, 2008), and may link the extracellular domain of α chain integrins with intracellular signaling molecules, including PI4K and PKC, both of which regulate cytoskeletal architecture (Chavis and Westbrook, 2001; Hemler, 1998; Yauch and Hemler, 2000). TM4SF2 transcripts are present in colon, muscle, heart, kidney, and spleen of mice, but are expressed most strongly in brain (Hosokawa et al., 1999), primarily in neurons of frontal cortex, olfactory bulb, cerebellar cortex, caudoputamen, dentate gyrus, and hippocampal CA3 (Zemni et al., 2000). Kainic acid treatment up-regulates TM4SF2 mRNA, suggesting that TSPAN7 is involved in synaptic plasticity (Boda et al., 2002). However, the function of TSPAN7 in the brain is unknown, and it is unclear how mutations affect neuronal development and function, and cause intellectual disability.

To clarify TSPAN7’s role in the brain, we examined its influence on the morphology and synaptic organization of developing...
hippocampal neurons. We focused on dendritic spines—main sites of excitatory synapses in the brain—because changes in spine morphology and density are associated with synaptic plasticity and learning (Kasai et al., 2010), and defects in spine morphology are associated with neurological disorders including intellectual disability (Humeau et al., 2009). We show that TSPAN7 promotes filopodia and dendritic spine formation in cultured hippocampal neurons, and is required for spine stability and normal synaptic transmission. We also identify PICK1 (protein interacting with C kinase 1) as a TSPAN7 partner. PICK1 is involved in the internalization and recycling of AMPA receptors (AMPA R) (Perez et al., 2001). Remarkably, TSPAN7 regulates the association of PICK1 with AMPARs, and controls AMPAR trafficking. These findings identify TSPAN7 as a key player in the morphological and functional maturation of glutamatergic synapses, and suggest how TSPAN7 mutations can give rise to intellectual disability.

RESULTS

TSPAN7 Overexpression Promotes Synaptic Maturation and Filopodia and Spine Formation in Hippocampal Neurons

We first examined effects of TSPAN7 overexpression in rat hippocampal neurons transfected at days in vitro (DIV) 1 and fixed at DIV5–7, before synaptogenesis. Because two TSPAN7 mutations linked to intellectual disability predict a protein lacking the fourth transmembrane domain and C terminus (Abidi et al., 2002; Zemni et al., 2000), we also analyzed the expression of TSPAN7ΔC, truncated 6 amino acids upstream of the fourth transmembrane domain. In TSPAN7-overexpressing neurons at DIV5, the density (number/10 μm) of filopodia-like protrusions on axons (identified by Tau-1 staining, not shown) was ~1.5 times greater than in EGFP controls (1.22 ± 0.05 versus 0.9 ± 0.03; ***p < 0.001) and TSPAN7ΔC-overexpressing cells (1.22 ± 0.05 versus 0.82 ± 0.02; ***p < 0.001; Figure 1A). At DIV7, when dendrites are clearly evident, the density of filopodia-like structures on dendrites was ~1.4 times greater in TSPAN7-overexpressing neurons than EGFP controls (2.94 ± 0.14 versus 2.19 ± 0.14; **p = 0.002) and TSPAN7ΔC neurons (2.94 ± 0.14 versus 1.91 ± 0.10, ***p < 0.001) (Figure 1B).

Expression of full length TSPAN7, but not the ΔC mutant, also promoted the formation of actin-enriched filopodia in COS7 cells (see Figure S1 available online). No differences between TSPAN7-overexpressing, controls and TSPAN7ΔC-overexpressing neurons, in terms of filopodia width were found at DIV5 or DIV7 (DIV5: 4.39 ± 0.31 versus 4.72 ± 0.33 versus 4.45 ± 0.27, ANOVA p > 0.05; DIV7: 2.21 ± 0.10 versus 2.02 ± 0.12 versus 2.32 ± 0.10, ANOVA p > 0.05) (Figures 1A and 1B). Given the importance of filopodia in synapse formation, these findings suggest that TSPAN7 is involved in synaptogenesis.

We next examined the effects of TSPAN7 overexpression in more mature neurons after the initial wave of synaptogenesis is complete. We transfected neurons at DIV11 with HA-TSPAN7 or HA-TSPAN7ΔC, and analyzed dendritic spines at DIV21. HA-TSPAN7 but not HA-TSPAN7ΔC increased spine density. Spine density was 1.8 times greater in HA-TSPAN7 neurons than in EGFP controls (9.32 ± 0.71 versus 5.06 ± 0.19; **p = 0.009) and 1.6 times greater than in HA-TSPAN7ΔC (5.75 ± 0.88; *p = 0.024). Spine length was unaffected (1.90 ± 0.08 versus 1.85 ± 0.05 versus 1.80 ± 0.06 μm; ANOVA p > 0.05) but HA-TSPAN7ΔC reduced spine head width versus control (0.99 ± 0.02 versus 1.12 ± 0.03 μm, *p = 0.012) and HA-TSPAN7 neurons (0.99 ± 0.02 versus 1.13 ± 0.03 μm, ***p = 0.007) (Figure 1C). Furthermore, TSPAN7ΔC overexpressing neurons had greater staining intensity for GluA2 (1.5 ± 0.15-fold relative to control **p < 0.05) more GluA2-positive clusters (1.27 ± 0.07-fold relative to control, *p < 0.05), greater staining intensity for PSD-95 (1.27 ± 0.04-fold relative to control **p < 0.01) and more PSD-95 positive clusters (1.28 ± 0.06-fold relative to control, *p < 0.05). By contrast, TSPAN7ΔC overexpressing neurons had significantly lower staining intensity (0.75 ± 0.04 and 0.83 ± 0.03) and reduced cluster density (0.64 ± 0.12 and 0.70 ± 0.01) for GluA2 and PSD-95, respectively (*p < 0.05, **p < 0.01, ***p < 0.001; p values relative to controls).

Overexpression of TSPAN7 and TSPAN7ΔC had no effects on staining intensity or cluster density for β1 integrin, Bassoon, or NMDA receptor (NMDAR) subunit 1 (GluN1) (Figure 2A). Because of these effects on spines and excitatory synapses, we next examined colocalization of endogenous TSPAN7 with various synaptic markers (Figure 2B). TSPAN7 colocalized mainly with GluA2 (51.61% ± 1.11%) and surface β1 integrin (50.46% ± 1.03%), and to a lesser extent with Bassoon (40.30% ± 1.28%), PSD-95 (35.25% ± 1.59%) and GluN1 (28.27% ± 1.50%) in neurons at DIV18. These results suggest that, like integrins and GluA2 (Petralia and Wenthold, 1992; Shi and Ethell, 2006), TSPAN7 is present at both synaptic and extra-synaptic sites.

TSPAN7 Knockdown Impairs Excitatory Synapse Development and Enhances Spine Motility

To further explore the role of TSPAN7, we used siRNAs to silence it in mature hippocampal neurons. We designed siRNA14 and siRNA47 specific for human and rat TSPAN7, and showed by western blot that they were effective in COS7 cells cotransfected with HA-TSPAN7 (Figure S2A). The two siRNAs were also effective in inhibiting endogenous TSPAN7 expression in hippocampal neurons (Figures 3A, S2C, and S2D). Specifically, in siRNA14- and siRNA47-transfected neurons, endogenous TSPAN7 levels were significantly lower than in control by immunofluorescence (Figure S2D; EGFP: 1.00 ± 0.09, siRNA14: 0.17 ± 0.04, siRNA47: 0.35 ± 0.06, ***p < 0.001, values normalized to EGFP). In neurons transfected with siRNA14 at DIV11 and analyzed at DIV18, spine head width was significantly lower than in scrambled siRNA14-transfected neurons (Figures 3A and 3B; 0.86 ± 0.02 μm versus 1.08 ± 0.01; ***p < 0.001), whereas spine density and length were unchanged. Notably, the effect of TSPAN7’s silencing on spine width was rescued by coexpressing a wild-type TSPAN7 variant resistant to siRNA14 (rescue WT), but not by coexpressing TSPAN7ΔC variant resistant to siRNA14 (rescue ΔC) (Figures 3A and 3B; rescue WT: 1.04 ± 0.02, p = 0.58; rescue ΔC: 0.82 ± 0.03, ***p < 0.001). The efficacy of the expression of the rescue constructs, rescue WT and rescue ΔC, was first verified by western blot in COS7 cells and immunofluorescence in hippocampal neurons (Figures S2B–S2D).

TSPAN7 Regulates Synapse and AMPAR Trafficking

We transfected neurons at DIV11 with HA-TSPAN7 or HA-TSPAN7ΔC, and analyzed synaptic spines at DIV21. HA-TSPAN7 neurons had 1.3 times greater than in HA-TSPAN7ΔC (2.94 ± 0.14 versus 2.19 ± 0.14; **p = 0.002) and TSPAN7ΔC neurons (2.94 ± 0.14 versus 1.91 ± 0.10, ***p < 0.001) (Figure 1B). By contrast, TSPAN7ΔC-overexpressing neurons had significantly lower staining intensity (0.75 ± 0.04 and 0.83 ± 0.03) and reduced cluster density (0.64 ± 0.12 and 0.70 ± 0.01) for GluA2 and PSD-95, respectively (*p < 0.05, **p < 0.01, ***p < 0.001; p values relative to controls).

Overexpression of TSPAN7 and TSPAN7ΔC had no effects on staining intensity or cluster density for β1 integrin, Bassoon, or NMDA receptor (NMDAR) subunit 1 (GluN1) (Figure 2A). Because of these effects on spines and excitatory synapses, we next examined colocalization of endogenous TSPAN7 with various synaptic markers (Figure 2B). TSPAN7 colocalized mainly with GluA2 (51.61% ± 1.11%) and surface β1 integrin (50.46% ± 1.03%), and to a lesser extent with Bassoon (40.30% ± 1.28%), PSD-95 (35.25% ± 1.59%) and GluN1 (28.27% ± 1.50%) in neurons at DIV18. These results suggest that, like integrins and GluA2 (Petralia and Wenthold, 1992; Shi and Ethell, 2006), TSPAN7 is present at both synaptic and extra-synaptic sites.
Time-lapse imaging of spine turnover showed that the numbers of spines that disappeared and appeared ex novo were significantly greater in siRNA14 than scrambled siRNA14 neurons (Figure 3C; disappeared spines: 16.20% ± 1.02% versus 6.12% ± 3.21%; ***p < 0.001; new spines: 15.32% ± 1.66% versus 7.21% ± 2.82%; ***p < 0.001 ANOVA followed by Tukey). Spine turnover was fully rescued by rescue WT (Figure 3C; disappeared spines: 5.81% ± 3.49% versus 6.12% ± 3.21%, p > 0.05; new spines: 6.33% ± 3.53% versus 7.21% ± 2.82%, p > 0.05; ANOVA relative to scrambled). By contrast, in TSPAN7 overexpressing neurons, the number of spines that appeared ex novo was significantly greater than in scrambled control (Figure 3C; disappeared spines: 8.9 ± 2.18 versus 6.12% ± 3.21%, p > 0.05; new spines: 17.85% ± 2.81% versus 7.21% ± 2.82%, p < 0.001 ANOVA followed by Tukey).
Dendritic spine motility and remodeling over 40 min were also significantly greater in TSPAN7-knockdown than scrambled controls (Figure S3; cumulative fluorescence intensity change at 10 min: 27.24% ± 3.18% versus 13.42% ± 2.11%, **p = 0.004; at 20 min: 39.10% ± 4.52% versus 16.78% ± 1.59%, ***p < 0.001; at 30 min: 41.63% ± 5.71% versus 18.41% ± 1.39%, **p = 0.003; at 40 min: 46.89% ± 6.62% versus 21.08% ± 2.86%, ***p < 0.001). Taken together, these findings indicate that TSPAN7 is important for the stability and maturation of dendritic spines. Notably, TSPAN7ΔC failed to rescue the effect of TSPAN7 knockdown.

Because synaptic activity induces various changes in neurons, ranging from transient posttranslational modifications to modulation of gene expression (Flavell and Greenberg, 2008), we next examined whether TSPAN7 is required for
activity-dependent spine remodeling following chemically induced long-term potentiation (LTP) in hippocampal neurons. As expected (Fortin et al., 2010), induction of chemical LTP in hippocampal neurons transfected with scrambled siRNA14 resulted in spine head enlargement (1.43 ± 0.03 μm LTP versus 1.06 ± 0.01 μm non-LTP; ***p < 0.001), and increased spine
density: 6.63 ± 0.19 LTP versus 5.55 ± 0.15 non-LTP; *p < 0.05) (Figure 3D). By contrast, TSPAN7 knockdown not only reduced spine head size under basal conditions but also prevented both spine enlargement (0.83 ± 0.01 μm LTP versus 0.81 ± 0.01 μm non-LTP; p > 0.05) and increased spine density due to LTP (number of spines per 10 μm: 5.92 ± 0.17 LTP versus 5.87 ± 0.16 non-LTP; p > 0.05). These results show that TSPAN7 is required for the activity-dependent morphological changes that occur during chemically-induced LTP.

**TSPAN7 Knockdown Reduces the Accumulation of Synaptic Markers**

We next examined the effect of TSPAN7 knockdown on the expression of synaptic proteins. Compared to neurons expressing scrambled siRNA14, those expressing siRNA14 had significantly lower staining intensity for GluA1 (0.75 ± 0.05 versus 1.00 ± 0.08; *p = 0.019, values normalized to scrambled siRNA), GluA2/3 (0.49 ± 0.04 versus 1.00 ± 0.07; **p < 0.001), and PSD-95 (0.78 ± 0.04 versus 1.00 ± 0.05; **p < 0.01), but not for GluN1, surface β1 integrin, or Bassoon (Figures 4A and 4B and data not shown). Compared to neurons expressing scrambled siRNA14, those expressing siRNA14 also had significantly fewer clusters per unit dendritic length for GluA1 (number of puncta relative to scrambled siRNA14: 0.50 ± 0.10; **p = 0.003), GluA2/3 (0.58 ± 0.09; **p = 0.004), and PSD-95 (0.52 ± 0.06; **p < 0.001), but not for GluN1, surface β1 integrin, or Bassoon (Figures 4A and 4B, and data not shown). The effects of siRNA14 on the density and intensity of individual GluA1, GluA2/3, and PSD-95 clusters were reversed by expressing siRNA14 together with TSPAN7 resistant to siRNA14 (rescue WT). Specifically, staining intensities for GluA1, GluA2/3, and PSD-95 were 0.99 ± 0.07, 0.97 ± 0.03, and 0.89 ± 0.06 times that of scrambled siRNA14 (p > 0.05 ANOVA) and cluster densities for GluA1, GluA2/3 and PSD-95 were 0.94 ± 0.06, 0.95 ± 0.05, and 0.97 ± 0.04 times scrambled siRNA14, respectively (p > 0.05, ANOVA; Figures 4A and 4B).

In stark contrast, expression of siRNA14 together with the TSPAN7 ΔC variant resistant to siRNA14 (rescue ΔC) did not reverse the reduction in the expression of GluA1, GluA2/3, and PSD-95 caused by TSPAN7 silencing. Specifically, staining intensities for GluA1, GluA2/3, and PSD-95 were 0.79 ± 0.06 (*p = 0.027), 0.71 ± 0.06 (*p = 0.008), and 0.80 ± 0.03 (**p = 0.002) times that of scrambled siRNA14, and cluster densities for GluA1, GluA2/3, and PSD-95 were 0.70 ± 0.05 (**p = 0.004), 0.65 ± 0.07 (*p = 0.013), and 0.75 ± 0.05 (**p < 0.001, ANOVA) times that of scrambled siRNA14, respectively (Figures 4A and 4B). Expression of siRNA14 together with either rescue WT or rescue ΔC had no effect on GluN1, β1 integrin, or Bassoon expression (Figures 4A and 4B, and data not shown).

To further probe TSPAN7’s role in synapse development, we examined whether TSPAN7 knockdown prevented thrombospondin-1 (TSP-1)-induced synaptic maturation (Christopherson et al., 2005). Immature hippocampal neurons (DIV8) transfected with scrambled siRNA or siRNA14 were treated for 12 days with TSP-1, and effects on synapse density were assessed in terms of colocalization of synapsin and PSD-95 (Garcia et al., 2010). Scrambled siRNA14 neurons treated with TSP-1 had significantly higher levels of synapsin/PSD-95 colocalization than control untreated neurons (Figure S4, colocalized clusters per 50 μm dendrite: 23.94 ± 2.64 for scrambled siRNA14 treated versus 17.45 ± 1.32 for scrambled siRNA14 untreated; **p < 0.01). By contrast, in TSPAN7-silenced neurons, the colocalization of synapsin and PSD-95 was modestly but significantly reduced under basal conditions (colocalized clusters per 50 μm dendrite: 12.84 ± 0.66 for siRNA14 untreated versus 17.45 ± 1.32 for scrambled siRNA14 untreated, *p < 0.05), and unaffected by TSP-1 treatment (15.44 ± 2.33 for siRNA14 treated versus 12.84 ± 0.66 for siRNA14 untreated, p > 0.05; Figure S4). These findings are further evidence that TSPAN7 is required for synapse maturation, and are consistent with the observed reduction in spine head size when TSPAN7 is knocked down; they also indicate that the C terminus of TSPAN7 is involved in synapse maturation.

**TSPAN7 Knockdown Compromises Excitatory Synaptic Transmission**

Having demonstrated morphological and molecular changes caused by TSPAN7 silencing, we next investigated whether TSPAN7 affected excitatory synaptic transmission by recording spontaneous miniature excitatory postsynaptic currents (mEPSCs) in primary hippocampal pyramidal neurons. We took advantage of the low transfection efficiency in primary neuron cultures and restricted the electrophysiological recordings to transfected neurons surrounded only by nontransfected cells. Thus, the patched neuron received synaptic inputs from control cells expressing normal levels of TSPAN7. We were therefore able to examine the consequences of silencing TSPAN7 selectively in the postsynaptic compartment. The amplitude (Figures 5A and 5B) and frequency (Figures 5C and 5D) of mEPSCs were markedly reduced by TSPAN7 knockdown, both with siRNA14 and siRNA47. These effects were reversed by expressing siRNA14 together with rescue WT (rescue, Figures 5A–5D). Next, we tested whether TSPAN7 knockdown affected AMPAR subunit composition by using phlanthotoxin-433 (PhTx), a specific blocker of AMPARs lacking GluA2. PhTx had no effect on mEPSC amplitude in control neurons (Figure S5A), as reported previously (Thiagarajan et al., 2005). Likewise, PhTx did not reduce mEPSC amplitude in neurons transfected with siRNA14 or siRNA47 (Figure S5B), suggesting that TSPAN7 knockdown does not preferentially deplete synapses of AMPARs containing GluA2, consistent with our finding that both GluA1 and GluA2/3 staining is reduced (Figure 4). These findings therefore show that TSPAN7 knockdown results in markedly impaired postsynaptic excitatory transmission. Because reduced mEPSC frequency in TSPAN7 knockdown pyramidal cells (Figures 5C and 5D) could be due to either reduced release probability or reduced number of functional synapses, we discriminated between these possibilities by studying evoked synaptic AMPAR and NMDAR currents between pairs of primary hippocampal pyramidal neurons (Figure S6). As in the mEPSC recordings, only the postsynaptic neuron was transfected with siRNA14, whereas the presynaptic cell expressed normal levels of TSPAN7. Under these conditions, evoked AMPAR currents were strongly reduced (Figure S6A, bottom), consistent with...
the effects on mEPSCs (Figure 5). By contrast, evoked NMDAR currents were not significantly affected by TSPAN7 knockdown (Figure S6A, top), consistent with our findings on GluN1 immunolocalization (Figure 4). As a consequence, the NMDA/AMPA ratio was markedly and significantly increased in TSPAN7 knockdown neurons (Figure S6B, right). By contrast, the paired-pulse ratio—a measure related to presynaptic release probability—was not significantly affected (Figures S6A and S6B, left). These findings therefore suggest that postsynaptic loss of TSPAN7 compromises excitatory synaptic transmission by selectively impairing AMPAR over NMDAR currents, with negligible effects on presynaptic release probability. As a consequence, TSPAN7-deprived neurons have more silent synapses.
Association between TSPAN7, PICK1, and AMPARs in Neurons

To gain insights into how TSPAN7 influences postsynaptic organization and synaptic transmission, we next identified proteins that bind TSPAN7. We used a yeast two-hybrid system, with the cytoplasmic C terminus of TSPAN7 (aa 234–249; Figure 6A) as bait, to screen a human fetal brain cDNA library. Four prey cDNA clones were isolated (clones 40, 48, 15, and 28, Figure 6A), all of which encoded PICK1. Interaction between TSPAN7 and PICK1 was further studied in a yeast two-hybrid system with, as bait, the full length TSPAN7 C terminus (wt) or the TSPAN7 C terminus lacking the last four amino acids (D4, aa 234–245 without YEMV)—a putative PDZ binding motif (Figure 6B). The following constructs were tested as potential prey (Figure 6B): full length PICK1 (aa 1–415), N terminus + PDZ domain (aa 1–108), PDZ domain alone (aa 14–108), N terminus + PDZ + linker region (aa 1–147), BAR domain alone (aa 142–367), and N terminus + PDZ + linker region + BAR domain (aa 1–367). We also used two constructs (full length PICK1 and PDZ domain alone) containing the KD to AA mutation in the PDZ domain (PDZ*), which is expected to abolish PDZ-dependent interactions of PICK1 (Staudinger et al., 1997).

Only two PICK1 constructs (full length PICK1, and N terminus + PDZ + linker region + BAR domain construct) interacted with the wt TSPAN7 C terminus (Rescue). None of the TSPAN7 constructs missing the last four amino acids of the C terminus (D4) interacted with the PICK1 constructs (Figure 6B). These data show that TSPAN7 and PICK1 interact, and that the last four C terminus amino acids of TSPAN7 and the PDZ domain of PICK1 are necessary for interaction in the two-hybrid system.

We further investigated TSPAN7/PICK1 interaction by coimmunoprecipitation. In transfected COS7 cells, HA-TSPAN7 coimmunoprecipitated with myc-PICK1 (Figure 6C, top). To home in on the regions of interaction, we performed GST pull-down in COS7 cells. GST was fused to the TSPAN7 C terminus (ct wt, aa 234–249), or the TSPAN7 C terminus with the last three amino acids (ct wt, aa 234–245).
(ct Δ3) or four (ct Δ4) amino acids removed, and tested for binding to full length PICK1, the PICK1 PDZ domain, and two PICK1 mutants: PICK1 PDZ domain mutated (KD → AA), and PICK1 without the first 121 amino acids (Δ121), and thus lacking the PDZ domain. As expected, the wt C terminus of TSPAN7 pulled down full length PICK1, and also the PICK1 PDZ domain alone, whereas TSPAN7 C terminus Δ3 and Δ4 pulled down nothing. These data indicate that the PICK1 PDZ domain is not only necessary but also sufficient to bind the last four amino acids of TSPAN7, in contrast to the findings in the two-hybrid experiments where the BAR domain was also required. We hypothesize that, in the two hybrid system, the PDZ flanking region (containing BAR) stabilizes the interaction or allows correct PDZ domain folding, whereas this is not necessary for binding to the full length PICK1.

Figure 6. TSPAN7 Directly Interacts with PICK1, Associates with β1 Integrin and GluA2/3, and Regulates PICK1-GluA2/3 Interaction

(A) Representation of TSPAN7 showing transmembrane domains (red) and TSPAN7 C-terminal tail (aa 234–249). The latter was used as bait in two-hybrid screen of a human fetal brain cDNA library. Four prey cDNA clones were isolated (clones 40, 48, 15, and 28)—all encoded PICK1.

(B) Yeast two-hybrid test with, as bait, the full length C terminus of TSPAN7 (wt) or a truncated version of it (Δ4, aa 234–245) without the last four aas (putative PDZ binding motif). As potential prey, full length PICK1 (aa 1–415), N terminus + PDZ (aa 1–108), PDZ domain (aa 14–108), N terminus + PDZ + linker region (aa 1–147), BAR domain (aa 142–367), N terminus + PDZ + linker + BAR (aa 1–367), and two constructs mutated at sites expected to abolish PDZ-dependent binding: PDZ (KD → AA) (aa 14–108) and full length PICK1 (KD → AA) were probed. Full length PICK1, and N terminus + PDZ + linker + BAR (aa 1–367) constructs interacted with the TSPAN7 C-terminal tail (wt) (3+).

(C) Coimmunoprecipitation and pull-down experiments with TSPAN7 and PICK1. In COS7 cells HA-TSPAN7 coimmunoprecipitated with myc-PICK1 (top). In GST pull-downs in COS7 cells (bottom), the C terminus of TSPAN7 (ct wt, 234–249 aa) pulled down PICK1 (myc-PICK1) and PICK1's PDZ domain (myc-PDZ) but not the mutated PDZ domain (KD → AA; myc-PDZ*) or the PICK1 fragment lacking the first 121 aas (flag-D121).

(D) PICK1 and TSPAN7 interaction in neurons. Immunofluorescence labeling of hippocampal neurons (top) shows that endogenous TSPAN7 and PICK1 co-localize to some extent in dendrites and spines. Coimmunoprecipitation experiments on brain extracts using monoclonal PICK1 antibody (bottom) show that TSPAN7 and PICK1 are associated.

(E) The C-terminal tail of TSPAN7 (ct wt; 234–249 aa) pulls down PICK1, GluA2/3, and β1 integrin (left) from primary hippocampal neuron extracts. Coimmunopurification of AMPAR complexes using anti-GluA2/3 antibodies (middle and right panels) shows that PICK1 and GluA2/3 associate more in siRNA14-infected neurons than scrambled siRNA14-infected neurons (1.22 ± 0.05 versus 1.01 ± 0.01, **p = 0.004; PICK1/GluA2/3 ratio in siRNA14-expressing neurons relative to that in scrambled siRNA14-expressing neurons). β1 integrin associates with GluA2/3 only in the presence of TSPAN7 (middle).
GST pull-down. The Δ121 and KD → AA mutated PDZ constructs were not pulled down, showing that the PDZ domain of PICK1 mediates the interaction with the TSPAN7 C-terminal tail (Figure 6C).

We next investigated PICK1/TSPAN7 interaction in neurons. Immunofluorescence labeling of hippocampal neurons showed that endogenous TSPAN7 and PICK1 colocalized to some extent in dendrites and spines (Figure 6D). Coimmunoprecipitation experiments on brain extracts using a monoclonal PICK1 antibody showed that TSPAN7 and PICK1 were associated (Figure 6D). Finally, the C terminus of TSPAN7 (ct wt) pulled down PICK1, GluA2/3, and β1 integrin—the latter known to interact with several tetraspanins (Berditchevski, 2001)—but not GluN1, Neuriligin-1, GRIP, or PKCα from extracts of primary hippocampal neurons (Figure 6E, left). These findings show that TSPAN7 and PICK1 interact in neurons. Because the C-terminal tail of TSPAN7 also pulls down GluA2/3 and β1 integrin, it is likely that TSPAN7, PICK1, AMPAR, and β1 integrins associate to form macromolecular complexes in neurons.

Because PICK1 is a ligand of AMPAR GluA2/3 subunits and is involved in internalizing and recycling AMPARs (Hanley, 2008b; Perez et al., 2001), we next investigated PICK1/AMPAR interaction in neurons in presence and absence of TSPAN7. From primary neuron extracts expressing siRNA14 or scrambled siRNA14, we immunopurified AMPAR complexes using GluA2/3 C-terminal antibodies, assessing the results by western blot. In TSPAN7-knockdown neurons, PICK1 and GluA2/3 associated together more strongly than in neurons expressing scrambled siRNA14 (Figure 6E, right; 1.22 ± 0.05 versus 1.01 ± 0.01, **p = 0.004, PICK1/GluA2/3 ratio in siRNA14-expressing neurons normalized to the ratio in scrambled siRNA14 neurons). Furthermore β1 integrin associated with AMPARs only in the presence of TSPAN7 (Figure 6E, middle). These findings indicate that, in rat hippocampal neurons, TSPAN7 regulates the extent of interaction between GluA2/3 subunits, PICK1 and β1 integrin, possibly by acting as a macromolecular organizer.

TSPAN7 Regulates AMPAR Trafficking via PICK1

Because TSPAN7 is important for the morphological and functional maturation of excitatory synapses (Figures 1, 2, 3, 4, and 5), and because it interacts dynamically with other synaptic proteins (Figure 6), we next investigated whether TSPAN7 interactions are required for regulating excitatory synaptic function. In view of the well-established role of PICK1 in AMPAR turnover (Hanley, 2008a) and the direct interaction between PICK1 and TSPAN7 (Figure 6), we first addressed whether TSPAN7 and PICK1 cooperate in regulating GluA2 trafficking. Neurons expressing siRNA14 or scrambled siRNA14 were first incubated for 10 min with antibody against an extracellular epitope of GluA2. The time course of GluA2 internalization was estimated from the ratio of intracellular to total fluorescence (internalization index) (Passafaro et al., 2001) in neurons fixed 0, 5, and 10 min after antibody incubation. The GluA2 internalization index was significantly higher in TSPAN7 knockdown than scrambled siRNA14 neurons at all times (Figures 7A and 7B; 0 min: 1.26 ± 0.05 versus 1.00 ± 0.08, *p = 0.04; 5 min: 1.74 ± 0.04 versus 1.46 ± 0.04, ***p < 0.001; 10 min: 1.27 ± 0.07 versus 1.08 ± 0.05, *p = 0.04; values normalized to the levels in scrambled siRNA14 neurons at time 0).

To ascertain whether these effects were due to increased GluA2 internalization, we repeated the experiments in the presence of the dynamin inhibitor dynasore (80 μM for 30 min before internalization assay). As expected, dynasore abolished all differences in the internalization index between TSPAN7–knockdown and scrambled siRNA14 neurons at the three times (Figures 7A and 7B; 0 min: 1.00 ± 0.17 versus 1.06 ± 0.17, p = 0.81; 5 min: 1.04 ± 0.28 versus 1.07 ± 0.18, p = 0.93; 10 min: 1.04 ± 0.09 versus 0.97 ± 0.09, p = 0.64). These findings indicate that, in TSPAN7 absence, AMPAR internalization is increased. Given the uniform effects of TSPAN7 knockdown on GluA2 internalization over the 10 min period, in successive experiments (Figure 8), a single incubation period of 5 min was used. In the first set of experiments (Figure 8A), we further characterized TSPAN7’s effect on GluA2 trafficking. We checked the specificity of TSPAN7 knockdown on GluA2 internalization by expressing siRNA14 alone or together with rescue WT. Rescue WT fully restored GluA2 internalization to control levels (EGFP: 1.00 ± 0.03, siRNA14: 1.21 ± 0.09, *p = 0.01, rescue WT: 1.01 ± 0.04, p = 0.86; values normalized to EGFP). However, when siRNA14 was expressed with rescue ΔC, GluA2 internalization was not restored to control levels (rescue ΔC 1.19 ± 0.07 **p = 0.008) (Figure 8A). We next investigated TSPAN7 overexpression, finding it had opposite effects to TSPAN7 knockdown: reduced GluA2 internalization compared to control (EGFP: 1.00 ± 0.03, TSPAN7: 0.70 ± 0.05, ***p < 0.001, values normalized to EGFP). By contrast, TSPAN7ΔC overexpression had no effect on GluA2 internalization (TSPAN7ΔC: 0.91 ± 0.06 relative to EGFP, p = 0.17), clearly showing that the TSPAN7 C terminus is involved in regulating AMPAR trafficking (Figure 8A).

In the next set of experiments (Figures 8B–8D), we investigated the combined influence of TSPAN7 and PICK1 on GluA2 trafficking, by directly manipulating expression of the two proteins. We knocked down PICK1 using a previously characterized siRNA (siPICK1) (Citri et al., 2010). As expected, PICK1 silencing decreased GluA2 internalization relative to EGFP. When siPICK1 was coexpressed with siRNA14, GluA2 internalization was reduced as effectively as with siPICK1 alone, fully preventing the increase expected with TSPAN7 knockdown (Figures 8B and 8D, EGFP: 1.00 ± 0.04, siPICK1: 0.85 ± 0.01, ***p = 0.02, siPICK1+siRNA14: 0.77 ± 0.07, **p = 0.006, values normalized to EGFP). Next, we overexpressed PICK1 (myc tagged) either alone or with TSPAN7 (pIRES-EGFP-TSPAN7). Neurons overexpressing only PICK1 had greater GluA2 internalization than EGFP controls, consistent with findings showing that PICK1 overexpression decreases GluA2 surface levels (Terasima et al., 2004). When PICK1 and TSPAN7 were overexpressed together, PICK1 prevented the decrease in GluA2 internalization expected with TSPAN7 overexpression (Figures 8C and 8D, EGFP: 1.00 ± 0.04, PICK1: 1.26 ± 0.04, ***p < 0.001, PICK1+TSPAN7: 1.29 ± 0.05, ***p < 0.001 Tukey after ANOVA). These findings lead us to suggest a model whereby expression levels of TSPAN7 regulate PICK1-mediated AMPAR trafficking, possibly because TSPAN7 competes with AMPARs for PICK1 binding (Figure 6E) at the PDZ domain (Figures 6A–6D) (Dev et al., 1999; Xia et al., 1999). Because PICK1 also regulates
Figure 7. TSPAN7 Knockdown Results in Increased GluA2 Internalization

(A) Representative images of GluA2 internalization experiments 0, 5, and 10 min after a 10 min incubation with anti-GluA2 antibody, in TSPAN7 knockdown and scrambled siRNA14 neurons, untreated (left) or treated with dynasore (right).

(B) Extent of GluA2 internalization was quantified as the ratio of intracellular to total fluorescence (internalization index) in untreated and dynasore-treated neurons. The internalization index is significantly higher in TSPAN7 knockdown neurons than scrambled siRNA14 neurons, at all times, in untreated conditions (left panels) (0 min: 1.26 ± 0.05 versus 1.00 ± 0.09, *p = 0.04; 5 min 1.74 ± 0.04 versus 1.46 ± 0.04, ***p < 0.001; 10 min: 1.27 ± 0.07 versus 1.08 ± 0.05, *p = 0.04; Tukey after ANOVA; values normalized to scrambled siRNA14 neuron levels at time 0). Dynasore treatment (80 μM, 30 min; right panels) abolishes all differences in the internalization index between TSPAN7 knockdown and scrambled siRNA14 neurons (0 min: 1.00 ± 0.17 versus 1.06 ± 0.17, p = 0.81; 5 min: 1.04 ± 0.28 versus 1.07 ± 0.18, p = 0.93; 10 min: 1.04 ± 0.09 versus 0.97 ± 0.09, p = 0.64; Tukey after ANOVA). AMPARs are internalized more efficiently in the absence of TSPAN7.
spine size (PICK1 knockdown increases and PICK1 overexpression decreases spine size), in both cases without altering density (Nakamura et al., 2011), we investigated whether TSPAN7’s effects on spine morphology were dependent on PICK1. To this end, we did double knockdown and double overexpression experiments of TSPAN7 and PICK1 to change TSPAN7 levels but maintain similar relative amounts of the two proteins (Figure S7). As expected, TSPAN7 knockdown reduced spine width, mimicking the effect of PICK1 overexpression. However, when TSPAN7 and PICK1 were knocked down simultaneously, spine size was larger than in siRNA14 neurons but smaller than in siPICK1 neurons, suggesting no interdependence between TSPAN7 and PICK1 in regulating spine size (EGFP: 0.94 ± 0.02 μm, siRNA14: 0.73 ± 0.02 μm, siPICK1: 1.07 ± 0.02 μm, siRNA14 + siPICK1: 0.85 ± 0.02 μm, p = 0.003 against EGFP and p < 0.001 against siPICK1 and siRNA14, Tukey after ANOVA). Furthermore, the PICK1 knockdown-induced increase in spine length (Nakamura et al., 2011) was unaffected by simultaneous TSPAN7 knockdown (EGFP: 1.69 ± 0.04 μm, siRNA14: 1.76 ± 0.03 μm, p = 0.22, siPICK1: 1.84 ± 0.03 μm, p = 0.016, siRNA14 + siPICK1: 1.84 ± 0.04 μm, p = 0.009). Similarly, in double overexpression

Figure 8. Effects of TSPAN7 Knockdown and Overexpression on GluA2 Trafficking Are Dependent on PICK1
(A) Representative images of GluA2 internalization experiments on transfected neurons 5 min after completion of 10 min incubation with anti-GluA2. Neurons were transfected with EGFP (control), siRNA14, siRNA14 plus wild-type TSPAN7 resistant to siRNA14 (rescue WT), siRNA14 plus TSPAN7ΔC resistant to siRNA14 (rescue ΔC), TSPAN7, or TSPAN7ΔC. The extent of GluA2 internalization is shown in the histograms on the right. TSPAN7 knockdown (siRNA14) increases the internalization index, rescue WT rescues the effect of TSPAN7 knockdown by reducing the internalization index close to control levels, whereas rescue ΔC does not. Overexpression of TSPAN7, but not of TSPAN7ΔC, reduces the GluA2 internalization index compared to control.

(B–D) Representative images of GluA2 internalization experiments performed as in (A). Neurons were transfected with EGFP (control), siPICK1, siPICK1 plus siRNA14 (B), PICK1 or PICK1 plus TSPAN7 (C). Extent of GluA2 internalization (internalization index) is shown in histograms on the right (D). PICK1 knockdown (siPICK1) decreases the GluA2 internalization index and, when cotransfected with siRNA14, prevents the siRNA14-dependent increase in GluA2 internalization. PICK1 overexpression increases the GluA2 internalization index and, when cotransfected with TSPAN7, prevents TSPAN7-induced reduction in GluA2 internalization. Scale bar represents 10 μm.
experiments (Figure S7), TSPAN7 and PICK1 did not interfere with each other. PICK1 overexpression reduced spine width and length also in the presence of exogenous TSPAN7 (width: TSPAN7: 0.90 ± 0.02 μm p = 0.32, PICK1: 0.73 ± 0.02 μm **p < 0.001, PICK1+TSPAN7: 0.74 ± 0.01 μm ***p < 0.001; length: TSPAN7: 1.75 ± 0.05 μm p = 0.43, PICK1: 1.54 ± 0.05 μm *p = 0.04, PICK1+TSPAN7: 1.52 ± 0.02 μm *p = 0.001), whereas TSPAN7 overexpression increased spine density also in the presence of exogenous PICK1 (spine number/10 μm: EGFP: 3.43 ± 0.21, siRNA14: 3.55 ± 0.21 p = 0.09, siPICK1: 3.61 ± 0.23 p = 0.56, siRNA14+siPICK1: 3.62 ± 0.25 p = 0.55, TSPAN7: 4.11 ± 0.22 *p = 0.04, TSPAN7+PICK1: 4.41 ± 0.47 *p = 0.04). These findings suggest that TSPAN7 and PICK1 regulate spine morphology by independent molecular pathways.

**DISCUSSION**

We have shown that TSPAN7 is a key molecule in synapse maturation and function: it regulates spine density and size, and the expression of the postsynaptic proteins PSD-95 and AMPAR; it directly interacts with PICK1 to control the extent of PICK1’s association with GluA2/3 and hence AMPAR trafficking. These findings delineate an additional molecular mechanism for the regulation of AMPAR currents and synaptic strength, and suggest a functional explanation for the involvement of TM4SF2 in XLID.

**Role of TSPAN7 in Dendritic Spine Stabilization and Filopodia Production**

We found in COS7 cells that overexpression of TSPAN7, but not TSPAN7ΔC (mutant lacking C terminus, as in XLID) induced the formation of filopodia-like structures. TSPAN7 overexpression in immature hippocampal neurons (before synapse formation) also increased the density of dendritic and axonal filopodia, suggesting that TSPAN7 is a strong inducer of filopodia, irrespective of cell type or subcellular compartment in which it is expressed. Because TSPAN7 expression remains high in adult brain (Zemni et al., 2000), we investigated whether TSPAN7 regulates dendritic spines in more mature neurons. We found that TSPAN7 overexpression increased the number of dendritic spines. Other molecules, such as CamKII, syndecan-2, and parallemmin-1 also upregulate filopodia and spine number when overexpressed in neurons (Arstikaitis et al., 2011; Ethell and Yamaguchi, 1999; Jourdain et al., 2003). By contrast TSPAN7 knockdown reduced spine head width without affecting spine density. This was surprising because despite some reports of signaling pathways regulating spine size without affecting spine density (Woolfrey et al., 2009), in general, spine density reduction occurs together with spine shrinkage. To probe why TSPAN7 overexpression and knockdown do not have reciprocal effects on spines, we analyzed spine dynamics by time-lapse imaging. Knockdown markedly increased spine motility and turnover, but—as before—had no effect on density. Reduced spine stability on TSPAN7 loss appears pertinent to intellectual disability because spine stabilization is required for synaptogenesis during development and also for strengthening synaptic connections in mature neurons—for example in response to LTP-inducing stimuli (Bourne and Harris, 2008).

Consistent with these data, we also found that TSPAN7 knockdown in mature neurons prevented spine enlargement in response to chemical LTP, suggesting that the thin, highly motile spines that were present, were unable to mature into mushroom “memory” spines in response to synaptic activation. Spine dynamics when TSPAN7 was overexpressed were characterized by an appearance rate of new spines that exceeded the disappearance rate, so density increased, but spine head width did not change. This suggests that the primary function of TSPAN7 is to promote new spine (or filopodia) formation, and that it has only a permissive role in spine maturation.

**Role of TSPAN7 in Functional Maturation of Synapses**

Because spine width and stability increase with postsynaptic density (PSD) size and glutamate receptor number (Bourne and Harris, 2008), we also investigated the effect of TSPAN7 on the expression of synaptic markers. TSPAN7 overexpression increased, and knockdown decreased, PSD-95 and GluR2 expression whereas GluN1 and j1 integrin were unchanged. Moreover, PSD-95/synapsin colocalization was significantly reduced after TSPAN7 knockdown, indicating that the number of synapses (i.e., containing pre- and postsynaptic markers) was reduced, despite an apparent lack of change in spine density. TSPAN7 silencing also reduced spontaneous and evoked AMPAR currents, but did not affect NMDAR currents or presynaptic release probability, consistent with the selective reduction in AMPAR subunits observed by immunofluorescence, and strengthening the idea that TSPAN7 loss increases the number of weak (containing few AMPARs) and silent synapses (lacking AMPARs). Evidence indicates that spine growth can precede synapse formation, and that new spines initially lack PSDs and presynaptic partners (Arellano et al., 2007; Knott et al., 2006; Nägerl et al., 2007). The maturation and stabilization of new synapses depend on the activity-driven formation of PSDs (De Roo et al., 2008; Fischer et al., 2000), with PSD-95 being particularly important for stabilizing nascent spines by anchoring receptors and scaffolding proteins (Ehrlich et al., 2007; Marrs et al., 2001). We found that in the absence of TSPAN7, the morphological and functional maturation of spines and synapses was impaired: dendritic spines remained small and unstable, with low PSD-95 content and reduced ability to support synaptic transmission. Furthermore, as shown by synapsin/PSD-95 colocalization, synapses did not mature in response to the synaptogenic factor TSP-1, an astrocyte-derived protein important in synaptogenesis (Christopherson et al., 2005) and spine formation (Garcia et al., 2010). In our experiments, the TSPAN7 mutant lacking the fourth transmembrane domain and C-terminal tail (TSPAN7ΔC) behaved as a dominiant negative construct in terms of spine morphology and expression of synaptic proteins. This is likely because TSPAN7ΔC was not efficiently transported to spines (Figures 1C and 3A). Because tetraspanins organize proteins in multimolecular complexes (Boucheix and Rubinstein, 2001), TSPAN7ΔC may lose the ability to interact with key molecules (e.g., PICK1), while keeping the remaining interacting proteins, including endogenous TSPAN7 and other tetraspanins (Stipp et al., 2003), away from the spines.
**TSPAN7 Regulates AMPAR Trafficking via PICK1**

Using the C-terminal tail as bait in a yeast two-hybrid screen, we showed that TSPAN7 directly binds PICK1 and that the interaction involves PICK1’s PDZ domain and the last four amino acids (YEMV) of TSPAN7-putative PDZ binding motif. GST pull-down from brain homogenates and communoprecipitation from neuronal lysates confirmed this interaction and provided evidence that it occurred physiologically. TSPAN7 also associated with GluA2/3 and, as expected (Berditchevski, 2001) with β1 integrin. Surprisingly, we found that PICK1 associated more tightly with GluA2/3 following TSPAN7 knockdown. These findings suggest that TSPAN7 may help organize macromolecular signaling complexes at the synapse, consistent with data that tetraspanins form TEMs regulating the assembly and clustering of membrane proteins on the cell surface (Hemler, 2005).

Because PICK1 is a known regulator of AMPAR trafficking (Hanley, 2008b) as well as spine size (Nakamura et al., 2011), we wondered whether TSPAN7 regulates the structural and functional maturation of spines via its interaction with PICK1. Our experiments showed that TSPAN7 knockdown increased, and TSPAN7 overexpression reduced, GluA2 internalization, whereas TSPAN7ΔC—which does not bind PICK1—had no effect on GluA2 distribution. Because PICK1 is known to promote GluA2/3 internalization (Hanley, 2008b), these findings are consistent with a model whereby TSPAN7 regulates AMPAR trafficking via PICK1, possibly because it binds and sequesters PICK1 by competing for the same PDZ domain recognized by GluA2/3 (Dev et al., 1999; Xia et al., 1999).

According to this model, TSPAN7 knockdown increases the amount of available PICK1 to bind GluA2/3, with consequent increase in AMPAR retention intracellularly. Importantly—as the model predicts—simultaneous knockdown of PICK1 and TSPAN7 lowered the GluA2 internalization index (Figures 8B and 8D). Exogenous TSPAN7 probably reduces free PICK1 levels because PICK1 overexpression reverses TSPAN7-dependent reduction in GluA2 internalization (Figures 8C and 8D). These data therefore identify TSPAN7 as a modulator of AMPAR trafficking via its interaction with PICK1.

PICK1 is also important for restricting spine size by inhibiting Arp2/3-mediated actin polymerization (Rocca et al., 2008). However, unlike the case with AMPAR trafficking, our other findings indicate that TSPAN7 and PICK1 are not involved cooperatively in regulating spine morphology (Figure S7), suggesting that the two proteins regulate structural synaptic plasticity via independent signaling pathways. We found, for example, that knockdown of TSPAN7 and PICK1 in the same cell did not affect spine width in the same way as knockdown of either alone, whereas overexpression of both only had the same effect on spine width as PICK1 overexpression alone (Figure S7).

TSPAN7’s involvement with PICK1-dependent regulation of AMPAR trafficking but not with PICK1-dependent spine regulation is consistent with what is known of the mechanisms of PICK1 regulation: it restricts spine size by inhibiting Arp2/3-mediated actin polymerization (Nakamura et al., 2011), binding to Arp2/3 via its C terminus (Rocca et al., 2008), whereas the N terminus PDZ domain is responsible for binding to GluR2/3 (Dev et al., 1999) and TSPAN7. These findings are also in line with the expectation that structural and functional synaptic plasticity can be decoupled (Cingolani et al., 2008).

**EXPERIMENTAL PROCEDURES**

Most experiments were on cultured hippocampal neurons prepared from rat embryos at gestational age 18 days or from rat pups at postnatal day 0. Some experiments were on African green monkey kidney (COS7) cells. Animals were obtained from Charles River, Italy, and were killed in accordance with European Communities Council Directive 86/609/EEC.

**cDNA Constructs**

TSPAN7 constructs were obtained by PCR from the full-length human TSPAN7 coding sequence (cDNA clone IRATp970H0323D, imaGenes GmbH, Germany) and cloned into the appropriate vectors. See Supplemental Experimental Procedures for details on TSPAN7 cDNA constructs and siRNAs. Flag-ΔT121 PICK1 was a gift from Prof. E. B. Ziff (New York University School of Medicine). Full-length myc-PICK1 and myc-PICK1 with PDZ domain mutated (KD → AA) were a gift from Prof. R. Huganir (Howard Hughes Medical Institute, Baltimore). PDZ and mutated PDZ fragments were made by PCR amplification with appropriate oligonucleotides and subcloned into the GW1 vector together with myc-tag (British Biotechnology, UK). SiPICK1 (FUGWsh18b) and GFP-PICK1 (FUGWsh18b-GFP-PICK1) were a gift from Prof. R.C. Malenka (Nancy Pritzker Laboratory, Stanford University School of Medicine, Palo Alto, CA).

**Yeast Two-Hybrid Screening**

For two-hybrid experiments, fragments corresponding to the TSPAN7 C terminus were cloned in frame with the GAL4 binding domain, and used as bait to screen a human fetal brain cDNA library (ProQuest Pre-made cDNA Libraries) and test interaction with PICK1 domains. See Supplemental Experimental Procedures for details.

**Cell Cultures and Transfection**

Dissociated hippocampal neurons were plated at 75,000/well for immunocytochemistry and 300,000/well for biochemistry. Neurons were transfected by the calcium phosphate method as described (Lois et al., 2002). See Supplemental Experimental Procedures for details.

**Lentiviral Infection**

Hippocampal neurons were infected at DIV8 with siRNA14 or scrambled siRNA14 as described by Lois et al. (2002) and used at DIV13.

**GST Pull-Down, Immunoprecipitation, and Immunopurification**

GST fusion proteins were prepared in *Escherichia coli* strain BL21, isolated and immobilized on Sepharose beads which were then incubated with cell lysates or rat brain homogenates. Pulled down proteins were analyzed by SDS-PAGE and western blot with appropriate antibodies. Band intensity was measured with ImageQuant software (Bio-Rad). For immunoprecipitation cell lysates or rat brain homogenates were incubated with specific antibodies conjugated with protein A-agarose. The beads were centrifuged and supernatants incubated with protein-A beads conjugated with anti-myc, anti-PICK1 or IgG (control). The beads were washed with lysis buffer and PBS plus protease inhibitors, re-suspended in sample buffer and boiled for SDS-PAGE. For immunopurification, soluble neuron extracts were loaded onto a cyagen bromide-activated Sepharose 4B column bound with anti-GluR2/3. After incubation, the column was washed and GluR2/3-binding complexes were eluted and re-suspended in buffer for SDS-PAGE. Band intensity was measured with...
ImageQuant software (Bio-Rad). See Supplemental Experimental Procedures for details.

**Immunostaining and Antibodies**

COS7 cells and hippocampal neurons were fixed in 4% paraformaldehyde/4% sucrose. Fluorescent images were acquired with a BioRad MRC1024 confocal microscope or an LSM 510 Meta confocal microscope (Carl Zeiss; gift from F. Monzino). Morphological analysis and fluorescent staining intensity were quantified with Metamorph image analysis software (Universal Imaging) as described (Passafaro et al., 2003). For antibodies used and further details see Supplemental Experimental Procedures.

**Image Analysis and Quantification**

Live time-lapse imaging was done in an environmentally controlled chamber with 5% carbon dioxide at 37°C, using an Axiovert 200M (Zeiss) confocal system equipped with spinning-disc (Perkin Elmer). The 100x objective and the 561 nm laser line were used for acquisition. Cultured hippocampal neurons (DIV20) were imaged every 10 min for 40 min. Z-space slices (0.5 μm) were captured and flattened by maximum projection. Image analysis was performed with the Velocity High-Performance Imaging System.

**TIME-LAPSE IMAGING**

Live time-lapse imaging was performed in an environmentally controlled chamber with 5% carbon dioxide at 37°C, using an Axiovert 200M (Zeiss) confocal system equipped with spinning-disc (Perkin Elmer). The 100x objective and the 561 nm laser line were used for acquisition. Cultured hippocampal neurons (DIV20) were imaged every 10 min for a total period of 40 min. Z-space slices (0.5 μm) were captured and flattened by maximum projection. Image analysis was performed with the Velocity High-Performance Imaging System.

**AMPAR Internalization Assay**

Live hippocampal neurons (DIV15–18) were incubated (10 min, 37°C) with antibody against the GluA2 extracellular region (Chemicon, concentration 10 μg/ml). After washing in PBS with 1 mM MgCl2 and 0.1 mM CaCl2, neurons were returned to growth medium at 37°C for 0, 5, or 10 min, fixed for 7 min at room temperature in 4% paraformaldehyde/4% sucrose without permeabilization, and stained with a Cy5-conjugated secondary antibody for 1 hr at room temperature to visualize surface receptors. The neurons were then stained with a Cy5-conjugated secondary antibody for 1 hr at room temperature under permeabilizing conditions with GDB buffer (50 mM phosphate buffer pH 7.4 containing 0.2% gelatin, 0.5% Triton X-100, and 0.8 M NaCl), to visualize internalized receptors. In some experiments, dynasore (80 μM, Tocris Bioscience) was added for 30 min to block receptor internalization; primary antibody was then applied.

**Electrophysiology**

Whole-cell patch clamp recordings were performed at room temperature on 10–13 DIV primary hippocampal pyramidal neurons perfused continuously with artificial cerebrospinal fluid (aCSF).

mEPSCs were recorded at holding potential –70 mV over 5–15 min. The 10%–90% rise time (Rt) and weighted decay time constant (Dt) of mEPSCs were calculated as described (Cingolani et al., 2008), and were unaffected by TSPAN7 knockdown: Rt/Dt: 0.58 ± 0.02/2.72 ± 0.14 (control) 0.57 ± 0.02/2.90 ± 0.17 (siRNA14); 0.58 ± 0.03/2.86 ± 0.13 (siRNA47); 0.54 ± 0.03/2.73 ± 0.24 (rescue WT), Whole-cell paired-recordings from monosynaptically connected primary hippocampal pyramidal neurons were performed at 11–15 DIV. See Supplemental Experimental Procedures for details.

**Chemical LTP**

Hippocampal neurons were infected at DIV11 with siRNA14 or scrambled siRNA14. Chemical LTP was induced at DIV18 by treating the neurons for 3 min with an extracellular solution (140 mM NaCl, 1.3 mM CaCl2, 5 mM KCl, 25 mM HEPES, 33 mM glucose) containing 200 μM glycine, 1 μM strychnine, and 20 μM bicuculline. After treatment, the neurons were incubated for 90 min at 37°C and then fixed for spines analysis (Lu et al., 2001).

**Treatment with Thrombospondin-1**

Hippocampal pyramidal neurons were transfected at DIV8 with either scrambled siRNA or siRNA14, and treated with thrombospondin-1 (TSP-1; 250 ng/ml, added every 3 days), or vehicle. After 12 days, the effects of TSP-1 on synapse formation was assessed by quantifying the colocalization of the presynaptic marker synapsin and the postsynaptic marker PSD95 (Christopherson et al., 2005; Garcia et al., 2010).

**Statistical Analyses**

Data are expressed as means ± standard error of the mean (SEM). Statistical significance was assessed using the paired and unpaired Student’s t test as appropriate (for two group comparisons) or ANOVA followed by the Tukey post test (for more than two group comparisons). Analysis was performed with GraphPad Prism Version 4.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2012.01.021.

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