Slitrks control excitatory and inhibitory synapse formation with LAR receptor protein tyrosine phosphatases

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The balance between excitatory and inhibitory synaptic inputs, which is governed by multiple synapse organizers, controls neural circuit functions and behaviors. Slit- and Trk-like proteins (Slitrks) are a family of synapse organizers, whose emerging synaptic roles are incompletely understood. Here, we report that Slitrks are enriched in postsynaptic densities in rat brains. Overexpression of Slitrks promoted synapse formation, whereas RNAi-mediated knockdown of Slitrks decreased synapse density. Intriguingly, Slitrks were required for both excitatory and inhibitory synapse formation in an isoform-dependent manner. Moreover, Slitrks required distinct members of the leukocyte antigen-related receptor protein tyrosine phosphatase (LAR-RPTP) family to trigger synapse formation. Protein tyrosine phosphatase σ (PTPσ), in particular, was specifically required for excitatory synaptic differentiation by Slitrks, whereas PTPδ was necessary for inhibitory synapse differentiation. Taken together, these data suggest that combinatorial interactions of Slitrks with LAR-RPTP family members maintain synapse formation to coordinate excitatory–inhibitory balance.

leucine-rich repeat | neuropsychiatric disorder | synaptic cell-adhesion

Synaptic cell-adhesion molecules (CAMs) direct various stages of synaptogenesis, the process of synapse creation involving the assembly, maturation, validation, and specification of specialized sites of asymmetrical junctions between neurons (1). The list of known synaptic CAMs has expanded rapidly, although the precise synaptic functions of most CAMs remain incompletely understood. Among the various CAMs, neuronal transmembrane proteins containing extracellular leucine-rich repeat (LRR) domains, in particular, have received considerable research attention (2–4).

The Slit- and Trk-like (Slitrk) proteins constitute one such LRR domain-containing family. Originally identified in a screen for genes that were differentially expressed in mice with neural tube defects (5), Slitrks are type I transmembrane proteins with extracellular domains containing two clusters of LRRs. Members of the Slitrk family, which consists of six proteins (Slitrk1–6) (5), are highly and widely expressed in the CNS (6). Intriguingly, Slitrk isoforms have been associated with multiple neuropsychiatric disorders. For example, Slitrk1 variants are linked to the spectrum of obsessive-compulsive disorders (OCDs), Tourette syndrome, and trichotillomania (7, 8), and Slitrk2 is associated with schizophrenia and bipolar disorder (9, 10). Moreover, Slitrk1 mutant mice show anxiety-like behaviors and Slitrk5-deficient mice display OCD-like behaviors (11, 12). Recently, Slitrk3 was shown to control inhibitory synapse development selectively (13). Despite this progress, little about the synaptic functions of other Slitrk isoforms, apart from their ability to regulate neurite outgrowth (14), has been studied in detail.

The leukocyte antigen-related receptor protein tyrosine phosphatase (LAR-RPTP) family is composed of three members: LAR, protein tyrosine phosphatase δ (PTPδ), and PTPσ, all of which share a similar domain organization comprising three Ig domains and four to eight fibronectin type III repeats. LAR-RPTP family members are evolutionarily conserved and are functionally required for axon guidance and synapse formation (15). Recent studies have shown that netrin-G ligand-3 (NGL-3), neurotrophin receptor tyrosine kinase C (TrkC), and IL-1 receptor accessory protein-like 1 (IL1RAPL1) bind to all three LAR-RPTP family members or distinct members of the family; however, the functional significance of these multifaceted interactions remains elusive (16–18).

Here, we systematically investigated the effects of hippocampal Slitrk isoforms on synapse structure and function in cultured hippocampal neurons using both gain-of-function and loss-of-function strategies. Slitrk expression was detected in the postsynaptic density in brains. Strikingly, we found that a subset of Slitrk isoforms (Slitrk1, Slitrk2, Slitrk4, and Slitrk5) specifically acted at excitatory synapses in various functional assays. In contrast, Slitrk3 acted exclusively at inhibitory synapses. Importantly, we found that distinct members of the LAR-RPTP family mediated different outcomes: PTPσ was required for triggering excitatory presynaptic differentiation, whereas PTPδ was necessary for inhibitory presynaptic differentiation. Taken together, our data suggest that Slitrk isoforms collaborate with distinct members of the LAR-RPTP family to specify the development of specific synapse types in cultured hippocampal neurons.

Results

Slitrks Are Expressed in Postsynaptic Density Fractions in Rat Brains. To examine Slitrk protein expression in brains, we first generated a Slitrk1-specific antibody that does not cross-react with other Slitrks (Fig. S1 *A* and *B*). Using this antibody as well as commercially available Slitrk antibodies (for Slitrk2, Slitrk3, and Slitrk4), we examined the expression patterns of Slitrk proteins in rat tissues. Slitrk protein expression was mainly detected in the brain and not in other tissues (Fig. 1*A*). The expression of Slitrks steadily increased during embryonic and postnatal brain development (Fig. 1*B*), a pattern similar to that of postsynaptic density protein 95 kDa (PSD-95). Slitrk proteins were widely distributed in rat brains (Fig. 1*C*) and were detected in various subcellular fractions, including

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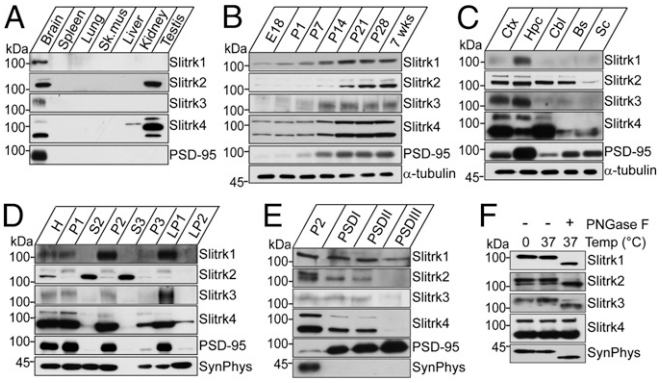

Fig. 1. Expression patterns of Slitrk proteins in rat brains. (*A*) Tissue expression of Slitrk proteins, revealed by immunoblot analysis with anti-Slitrk antibodies. mus., muscle; Sk., skeletal. (*B*) Expression levels of Slitrk proteins during development. E, embryonic day; P, postnatal day. α-tubulin was used for normalization. (*C*) Regional distribution of Slitrk proteins in various rat brain areas, revealed by immunoblotting of brain homogenates. Bs, brainstem; Cbl, cerebellum; Ctx, cerebral cortex; Hpc, hippocampus; Sc, spinal cord. α-Tubulin was used for normalization. (*D*) Distribution of Slitrk proteins in subcellular fractions of rat brains. H, homogenates; LP1, synaptosomal membrane fraction; LP2, synaptic vesicle-enriched fraction; P1, nuclear pellet; P2, crude synaptosomes; P3, light membrane fraction; S2, supernatant after P2 precipitation; S3, cytosol. A total of 15 μg of each fraction was loaded in immunoblot experiments. PSD-95 and synaptophysin (SynPhys) were used as positive controls. (*E*) Enrichment of Slitrk proteins in PSD fractions, extracted with Triton X-100 once (PSDI) or twice (PSDII), or with Triton X-100 plus sarkosyl (PSDIII). A total of 5 μg of crude synaptosomes (P2) and PSD fraction samples was loaded in immunoblot experiments. Note that Slitrk2 was not enriched in PSD fraction samples. PSD-95 and SynPhys were used as positive controls. (*F*) *N*-glycosylation of Slitrk proteins in rat brains. The crude synaptosome (P2) fraction of adult rat brain was subjected to PNGase F digestion, followed by immunoblot analyses with the indicated antibodies. SynPhys was used as a positive control. Temp, temperature (°C). Note that the anti-Slitrk4 antibody detected only immature species of Slitrk4 proteins, because PNGase F digestion did not shift the band position. Molecular mass markers are labeled in kilodaltons in *A*–*F*.

synaptosomes and synaptic membrane fractions (Fig. 1*D* and Fig. S1*C*). Notably, Slitrk2 exhibited unique subcellular localizations, with its immature form mainly distributed to the cytosolic fractions. Slitrk1 was enriched in PSD fractions up to PSDIII, which is highly detergent-resistant (Fig. 1*E*). Other Slitrks showed a similar enrichment in PSD fractions, albeit to a lesser extent; however, Slitrk2 was not enriched in PSD fractions. The used antibodies detected mature (i.e., *N*-glycosylated) Slitrks in both rat brains and cultured neurons, with the exception of anti-Slitrk4 (19) (Fig. 1*F* and Fig. S1 *D* and *E*).

Overexpression of Slitrks Increases Synapse Density. Because Slitrks are biochemically detected in synaptic fractions, we next asked whether Slitrks regulate synapse formation and/or maturation. As a gain-of-function approach, we cotransfected neurons at 10 d in vitro (DIV10) with expression vectors encoding full-length Slitrks [Slitrk1–5 excluding Slitrk6; an explanation is provided by Beaubien and Cloutier (6), as well as below] and EGFP to visualize the cellular morphology of transfected neurons, and we immunostained the transfected neurons for the synaptic markers Synapsin I, vesicular glutamate transporter 1 (VGLUT1; an excitatory presynaptic marker), glutamic acid decarboxylase 67 kDa (GAD-67; an inhibitory presynaptic marker), PSD-95 (an excitatory postsynaptic marker), and/or gephyrin (an inhibitory postsynaptic marker) at DIV14 (Fig. 2 and Fig. S2). For each of the five Slitrks, overexpression in cultured hippocampal neurons drastically increased synapse density, monitored as the number of Synapsin I puncta, producing effects similar to those of previously discovered synaptic adhesion molecules, such as neuroligins (NLs), leucine-rich repeat transmembrane neuronal proteins (LRRTMs), and NGLs (20–22) (Fig. 2 *A* and *D*). Whether the increases in synapse density were general or specific to a particular synapse type was determined by evaluating the number of presynaptic (VGLUT1 and GAD-67) or postsynaptic (PSD-95 and gephyrin) puncta (Fig. 2 *B*–*D* and Fig. S2 *A*–*C*). Interestingly, overexpression of Slitrk1, Slitrk2, Slitrk4, or Slitrk5 specifically promoted excitatory synapse formation, whereas overexpression of Slitrk3 did not (Fig. 2 *B* and

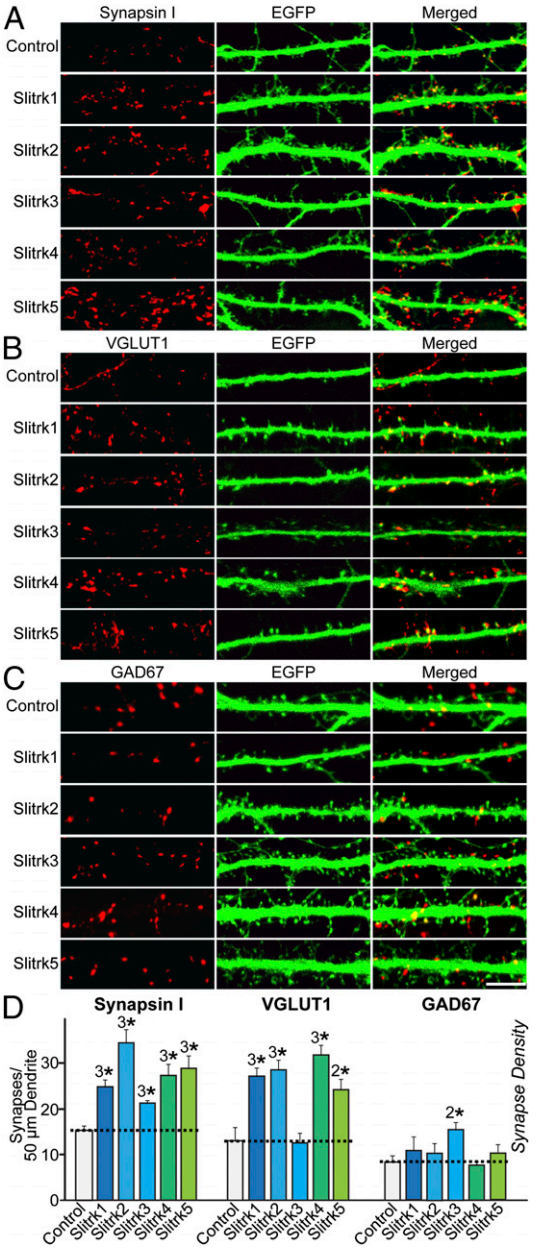

Fig. 2. Overexpression of Slitrks increases synapse density in cultured hippocampal neurons. Representative images of hippocampal neurons transfected with the indicated expression vectors at DIV10 and analyzed by double immunofluorescence with antibodies to GFP and Synapsin I (*A*), VGLUT1 (*B*), or GAD-67 (*C*) at DIV14. (Scale bar: *A*–*C*, 5 μm.) (*D*) Quantitative bar graphs of synapse density in images in *A*–*C*. Data shown are means ± SEMs (two to three dendrites per transfected neuron were analyzed and group-averaged). Statistical significance was assessed by comparing the various conditions with controls using the Student *t* test ($^2*P < 0.01$; $^3*P < 0.001$).

D and Fig. S2 *A*–*C*). Instead, overexpression of Slitrk3 led to a modest increase in inhibitory synapse density (Fig. 2 *C* and *D*), indicating that gain-of-function manipulations of Slitrk isoforms exert differential effects on synapse numbers in cultured hippocampal neurons. Consistent with this observation, investigation of subcellular localization of recombinant Slitrk1 and Slitrk2 in cultured hippocampal neurons, visualized by monitoring expression of Slitrk1 monomeric Venus (mVenus) fluorescent protein-fused Slitrk1 (Slitrk1-mVenus) and Slitrk2 (Slitrk2-mVenus) showed that Slitrk1-mVenus and Slitrk2-mVenus puncta were present in excitatory, but not inhibitory, synapses of transfected neuronal dendrites (Fig. S3 *A*–*C*). Recombinant Slitrk3 was shown to localize exclusively to inhibitory synapses (13). Slitrk overexpression did not change other morphological parameters, such as synapse size or synapse strength, assessed by measuring puncta size and intensity, respectively (Fig. S3*D*). Taken together, these results suggest that Slitrks are differentially localized to distinct synapse types and regulate the formation of the respective synapses in an isoform-dependent manner.

Knockdown of Slitrks Decreases Synapse Density. To address whether Slitrks are required for the formation of synapse structure, we first generated a series of lentiviral vectors expressing shRNA targeting individual Slitrks (Table S1). We then infected cultured rat cortical neurons with each of these knockdown (KD) lentiviruses and assessed endogenous target mRNA and protein levels by quantitative real-time RT-PCR and quantitative immunoblotting, respectively (Fig. 3 *A*–*C* and Fig. S4 *A* and *B*). We excluded Slitrk6 because it exhibits little or no expression in the hippocampus (6). The shRNA sequences used suppressed endogenous mRNA by ∼80% for Slitrk1, ∼75% for Slitrk2, ∼75% for Slitrk3, ∼70% for Slitrk4, and ∼75% for Slitrk5 (Fig. S4*A*), and they reduced the levels of endogenous proteins by ∼75% for Slitrk1, ∼70% for Slitrk2, ∼70% for Slitrk3, and ∼65% for Slitrk4 (Fig. 3 *B* and *C* and Fig. S4*B*). We also found that each Slitrk shRNA sequence specifically affected the level of only the targeted isoform and not off-target isoforms (Fig. S5). We next investigated whether single KD of Slitrk1, Slitrk2, Slitrk3, Slitrk4, or Slitrk5 altered synapse number and/or size in cultured hippocampal neurons. To accomplish this, we transfected cultured neurons at DIV8 with lentiviral expression vectors that expressed only EGFP (control) or coexpressed EGFP with shRNAs against Slitrk1 (K4), Slitrk2 (K11), Slitrk3 (K15), Slitrk4 (K17), or Slitrk5 (K21), and we immunostained neurons at DIV14 for Synapsin I, VGLUT1, and GAD-67 (Fig. 3 *D*–*I*). We found that single KD of each Slitrk significantly reduced synapse numbers (Fig. 3 *D*–*E*), indicating that all five Slitrks contribute to the formation of synapse structure. Moreover, single KD of Slitrk1, Slitrk2, Slitrk4, or Slitrk5 caused a modest but significant decrease in excitatory synapse density but not inhibitory synapse density (Fig. 3 *F* and *G*). In contrast, single KD of Slitrk3 led to a specific reduction in inhibitory synapse density (13) (Fig. 3 *H* and *I*). Slitrk KD did not affect synapse size under any experimental conditions (Fig. S4*C*). More importantly, coexpression of a shRNA-resistant form of Slitrk1, Slitrk2, Slitrk3, Slitrk4, or Slitrk5 (i.e., rescue vectors) completely eliminated the deficits in synapse density observed with the corresponding Slitrk KDs (Fig. 3 *D* and *E*, Fig. S6*A*, and Table S2). Expression of a scrambled shRNA (sc-shRNA) vector against each of the Slitrk isoforms tested (Slitrk1–5) had absolutely no effect on synapse density, confirming that the observed phenotypes are due to specific isoform KD and not off-target effects (23) (Figs. S5 and S6 *B* and *C* and Table S3). Collectively, these results corroborate the notion that individual Slitrks play critical roles in maintaining synapse structure at distinct synapse types.

Slitrks Interact with PTPδ or PTPσ but Not with LAR. To identify presynaptic ligands of postsynaptic Slitrks, we performed affinity chromatography of solubilized rat brains using recombinant Slitrk1

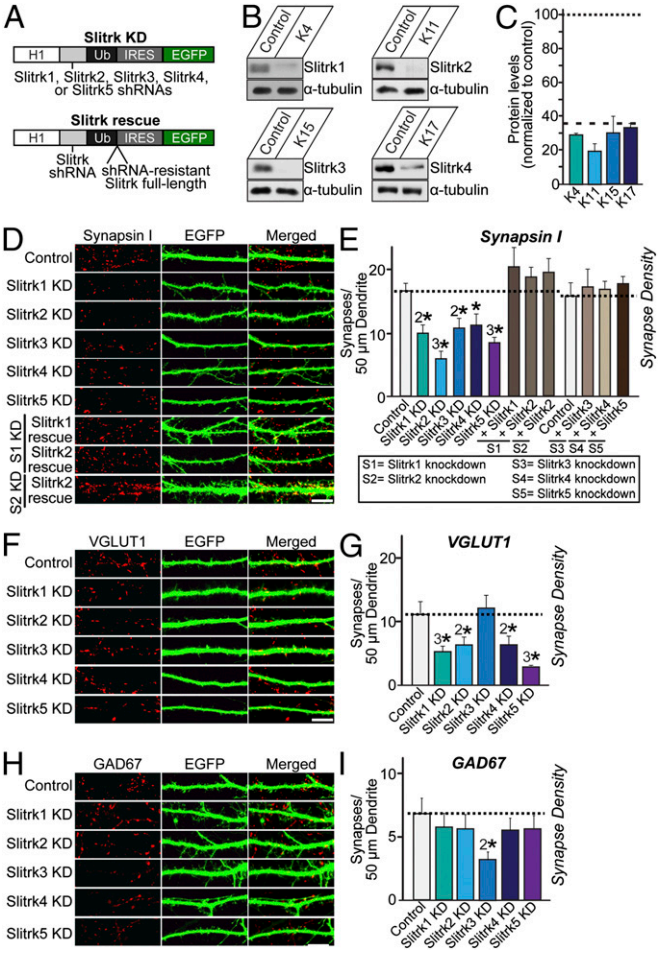

Fig. 3. KD of Slitrks reduces synapse numbers in cultured hippocampal neurons. (*A*) Design of lentiviral shRNA vectors for KD of Slitrk1, Slitrk2, Slitrk3, Slitrk4, or Slitrk5. H1, human H1 promoter; IRES, internal ribosome entry sequence; Ub, ubiquitin promoter. Slitrk rescue vectors were constructed by inserting shRNA-resistant, full-length Slitrk1 or Slitrk2 in-frame into the corresponding Slitrk KD vector. (*B*) Representative immunoblots of lysates of cortical neurons infected with potent lentiviral shRNAs (K4, Slitrk1; K11, Slitrk2; K15, Slitrk3; K17, Slitrk4) at DIV2, harvested at DIV12, and probed using anti-Slitrk antibodies (Fig. S5*B*). Slitrk5 was not investigated due to the lack of suitable antibodies. α-tubulin was used for normalization. (*C*) Levels of the target proteins, Slitrk1 (K4), Slitrk2 (K11), Slitrk3 (K15), and Slitrk4 (K17) in *B* measured by semiquantitative immunoblotting. The dotted line indicates the 65% KD cutoff level for tests of biological effects. (*D*) Representative images of cultured hippocampal neurons infected at DIV8 with a lentiviral vector expressing EGFP only (Control) or coexpressing EGFP and Slitrk1-KD (with/without Slitrk1/2 rescue vectors), Slitrk2-KD (with/without Slitrk2 rescue vector), Slitrk3-KD, Slitrk4-KD, or Slitrk5-KD, and analyzed by double immunofluorescence with antibodies to GFP and Synapsin I at DIV14. (Scale bar: 5 µm.) (*E*) Summary graphs of the effects of single KD of Slitrk1, Slitrk2, Slitrk3, Slitrk4, or Slitrk5 on synapse density (quantified using Synapsin I immunoreactivity) and phenotypic restoration by Slitrk1 (+Slitrk1), Slitrk2 (+Slitrk2), Slitrk3 (+Slitrk3), Slitrk4 (+Slitrk4), or Slitrk5 (+Slitrk5) rescue vectors. S1, S2, S3, S4, and S5 denote the Slitrk1, Slitrk2, Slitrk3, Slitrk4, and Slitrk5 single-isoform KD condition. The rest of rescue images is presented in Fig. S6*A*. (*F*) Same as in *D*, except that anti-VGLUT1 antibodies were used for immunocytochemistry analyses. (Scale bar: 5 µm.) (*G*) Summary graphs of the effects of single KD of Slitrk1, Slitrk2, Slitrk3, Slitrk4, or Slitrk5 on excitatory synapse density, quantified using VGLUT1 immunoreactivity. (*H*) Same as *D*, except anti–GAD-67 antibodies were used for immunocytochemical analyses. (Scale bar: 5 µm.) (*I*) Summary graphs of the effects of single KD of Slitrk1, Slitrk2, Slitrk3, Slitrk4, or Slitrk5 on inhibitory synapse density, quantified using GAD-67 immunoreactivity. Data shown in *E*, *G*, and *I* are means ± SEMs. Statistical significance was assessed by comparing the various conditions with controls using the Student *t* test (**P* < 0.05; ^{2}**P* < 0.01; ^{3}**P* < 0.001).

fusion proteins immobilized on protein A-Sepharose (Ig-Slitrk1). Mass spectroscopy analyses revealed seven peptides encoding the type II receptor PTPδ (details are provided in Table S4). Recently, Takahashi et al. (13) also reported that PTPδ binds to Slitrks in vitro, consistent with this observation. However, whether other members of the type IIb RPTP family (i.e., LAR and PTPσ; collectively termed LAR-RPTPs hereafter) can also bind Slitrks has not been tested.

Using cell-adhesion assays to address this possibility, we found that Slitrk1-, Slitrk2-, or Slitrk3-expressing L cells specifically aggregated with PTPδ-expressing L cells (Fig. 4 *A* and *B*), consistent with data obtained in cell surface labeling assays using IgC-PTPδ recombinant proteins (13). Similarly, Slitrk1-, Slitrk2-, or Slitrk3-expressing cells aggregated with PTPσ-expressing cells (Fig. 4 *A* and *B*). Strikingly, Slitrk-expressing cells did not aggregate with LAR-expressing cells; in contrast, NGL-3–expressing cells showed strong adhesive activities

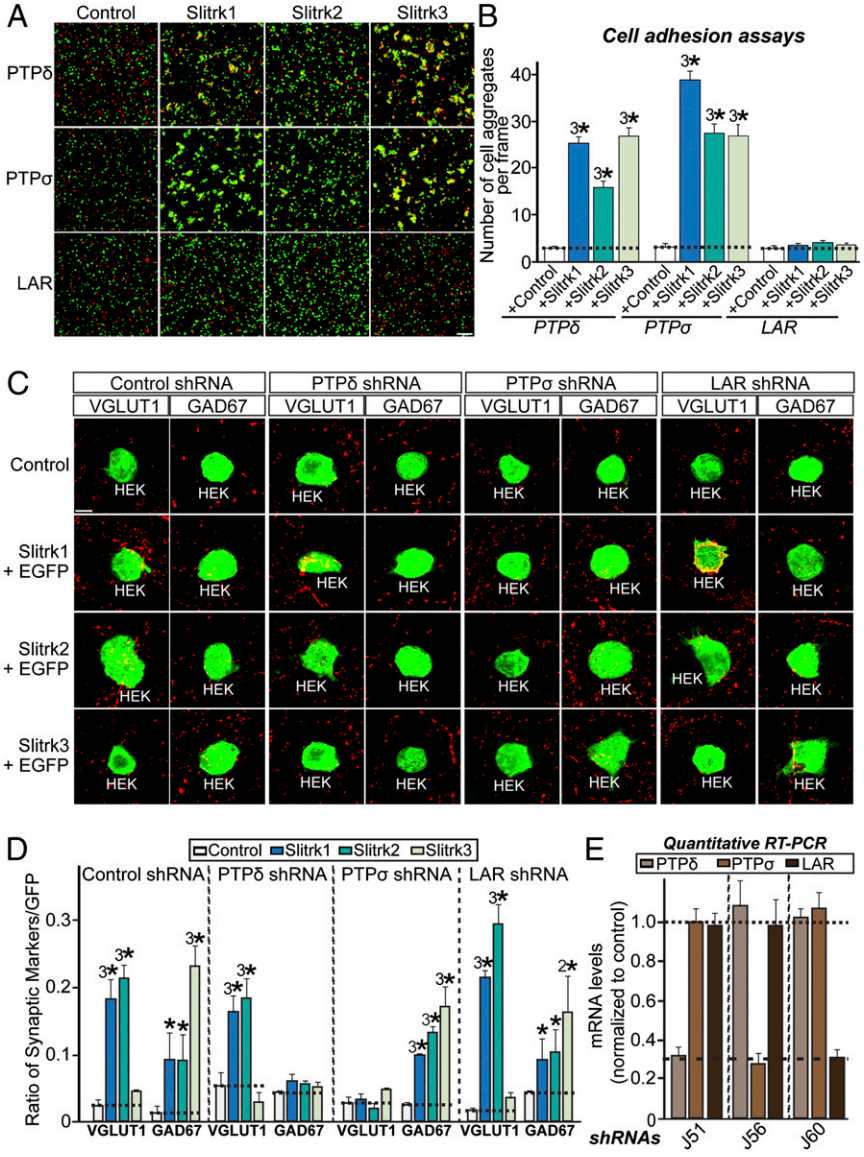

Fig. 4. Interaction of Slitrks with LAR-RPTPs and effects of individual LAR-RPTP KD on Slitrk activities in artificial synapse-formation assays. (*A*) Representative images of cell-adhesion assays using L cells doubly transfected with expression constructs for EGFP and Slitrk isoforms (Slitrk1, Slitrk2, or Slitrk3) or CD8 (negative control), mixed with a separate group of L cells doubly transfected with DsRed and LAR-RPTP isoforms (PTPδ, PTPσ, or LAR). (Scale bar: 100 μm.) (*B*) Quantification (average number of clusters per frame) of results shown in *A*. Data shown are means ± SEMs. Statistical significance was assessed by comparing the various conditions with controls using the Student *t* test (^{3}*$P < 0.001$). (*C*) Hippocampal neurons infected at DIV1–3 with control lentiviruses (Control shRNA) or shRNA-expressing PTPδ-KD (PTPδ shRNA), PTPσ-KD (PTPσ shRNA), or LAR-KD (LAR shRNA) were cocultured for 3 d (DIV10–13) with HEK293T cells expressing EGFP alone (Control) or coexpressing EGFP and Slitrk1 (Slitrk1 + EGFP), Slitrk2 (Slitrk2 + EGFP), or Slitrk3 (Slitrk3 + EGFP). Panels show representative images of cocultures stained with antibodies to EGFP (green) and excitatory (VGLUT1) or inhibitory (GAD-67) synaptic markers (red). Coincident green and red signals are shown in yellow. (Scale bar: 25 μm.) (*D*) Quantification of the artificial synapse-formation activity of Slitrks shown in *C*. Activity was quantified by measuring the ratio of synaptic marker staining to EGFP fluorescence (for absolute red and green fluorescence values). Statistical significance was assessed by comparing the various conditions with controls using the Student *t* test (*$P < 0.05$; ^{2}*$P < 0.01$; ^{3}*$P < 0.001$). (*E*) Levels of target mRNAs (PTPδ, PTPσ, and LAR) measured by quantitative RT-PCR in cultured cortical neurons infected at DIV1–3 with lentiviruses expressing the indicated shRNAs (J51, PTPδ; J56, PTPσ; J60, LAR). mRNA levels were determined at DIV12 (dotted line: 70% KD cutoff level). Note that each shRNA vector specifically suppressed the mRNA levels of its target isoform but not those of off-target isoforms.

toward LAR-expressing cells [consistent with the findings of Woo et al. (6)] (Fig. S7 *A* and *B*). To confirm that Slitrks do not interact with LAR, we incubated HEK293T cells coexpressing EGFP and Slitrk isoforms (Slitrk1, Slitrk2, and Slitrk3) or NGL-3 (positive control) with LAR-Ig fusion or IgC (negative control) and found that LAR did not bind to any Slitrks examined but did bind to NGL-3 (Fig. S7*C*). We could not perform similar analyses with PTPδ or PTPσ because the yield of recombinant PTPδ and PTPσ proteins was insufficient. These results are puzzling when viewed in light of the structural similarities among LAR-RPTP family members. However, it is not unreasonable to suppose that only PTPδ and PTPσ bind to Slitrks, considering the existence of multiple postsynaptic ligands that bind only to distinct LAR-RPTPs (21, 22).

Differential Requirement of LAR-RPTPs in Promoting Excitatory vs. Inhibitory Synapse Development. We then addressed whether LAR-RPTP family members are important for the synaptogenic activities of Slitrks in artificial synapse-formation assays (24). First, we transfected HEK293T cells with pDisplay-Slitrk containing only extracellular regions of Slitrks or untagged Slitrk expression constructs, and we cocultured these cells with hippocampal neurons (Fig. 4 *C* and *D* and Fig. S8). We confirmed that all Slitrks strongly recruited the presynaptic markers Synapsin I, VGLUT1, and/or GAD-67, but not the postsynaptic marker PSD-95, to HEK293T cells (Fig. 4 *C* and *D* and Fig. S8).

To examine whether LAR-RPTPs are required for the synaptogenic activities conferred by Slitrks, we developed shRNA lentiviruses that specifically knocked down individual LAR-RPTP family members (Fig. 4*E*). We then infected cultured neurons with lentiviruses expressing either an empty shRNA vector (control shRNA) or an shRNA KD construct targeting LAR (LAR shRNA), PTPδ (PTPδ shRNA), or PTPσ (PTPσ shRNA), and we undertook an extensive series of artificial synapse-formation assays using infected neurons and HEK293T cells expressing Slitrk1, Slitrk2, or Slitrk3 vectors (Fig. 4 *C* and *D*). Intriguingly, PTPδ KD resulted in failure of a variety of Slitrks to induce GAD-67 recruitment but did not affect VGLUT1 clustering (Fig. 4 *C* and *D*). In contrast, PTPσ KD resulted in a failure of Slitrk1 and Slitrk2 to induce VGLUT1 clustering on contacting axons of cocultured hippocampal neurons but did not affect GAD-67 recruitment (Fig. 4 *C* and *D*). Moreover, infection of cultured neurons with lentiviruses expressing the scrambled version of either PTPδ KD (PTPδ sc-shRNA) or PTPσ KD (PTPσ sc-shRNA) had no noticeable effects on the synaptogenic activities of Slitrks, indicating no off-target biological actions of the shRNA vectors used in this study (Fig. 4*E* and Fig. S9). In parallel experiments, LAR KD did not alter the synaptogenic activities of Slitrks, suggesting that LAR is not a major presynaptic receptor for the synaptogenic actions of Slitrks. These results are consistent with the binding data showing that LAR does not bind any Slitrks examined (Fig. 4 *A* and *B* and Fig. S7). Taken together, these data indicate that Slitrks physiologically use distinct LAR-RPTP isoforms to trigger excitatory and inhibitory synapse formation selectively.

Discussion

Recent studies have established that a host of neuronal transmembrane proteins containing LRR domains play important roles in synapse development, although the precise functions of these proteins are only slowly being uncovered (3, 13, 17, 20, 25–27). Six Slitrk family members (Slitrk1–6) share similar domain organizations and have been shown to regulate neurite outgrowth in Pheochromocytoma Cell Line 12 (PC12) cells (5, 28). Intriguingly, various Slitrk-KO mice exhibit a range of neurological behaviors, implying differential functions of these proteins in the CNS (12, 29, 30). However, potential isoform-specific synaptic functions of Slitrk family members have not yet been systematically explored. In the present study, we utilized a series of functional approaches to explore the synaptic functions of Slitrk proteins using cultured hippocampal neurons as a model system. We also performed an extensive array of artificial synapse-formation assays in conjunction with loss-of-function manipulations of the LAR-RPTP family to probe the mechanisms underlying the synaptogenic activities of Slitrks. We made four principal observations. First, immunoblot analyses revealed that Slitrks are widely expressed in rat brains. Specifically, we found that Slitrk1, Slitrk3, and Slitrk4 are enriched in PSD fractions (Fig. 1). However, considering the inherent limitations of the protocol used to isolate PSD fractions in this study (31), caution should be applied in interpreting biochemical enrichment of Slitrk isoforms in PSD fractions as definitive evidence for localization of these isoforms at specific synapse types. Indeed, Slitrk3 was shown to be specifically localized to and to function at inhibitory synapses, but it appeared to be biochemically enriched in the PSD fractions in this study, as well as in the study by Takahashi et al. (13). Second, overexpression of individual Slitrk isoforms markedly increased the density of both excitatory and inhibitory synapses in cultured hippocampal neurons (Fig. 2). These findings are reminiscent of observations of other synaptic proteins, such as NLs, LRRTMs, and NGLs (16, 25, 32). Although LRRTMs and NGLs act specifically on excitatory synapses, Slitrks were found to function in both excitatory and inhibitory synapses, with Slitrk1, Slitrk2, Slitrk4, and Slitrk5 acting on excitatory synapses and Slitrk3 acting on inhibitory synapses. These results provide strong evidence that Slitrks play a central role in general synapse formation, similar to that of NLs (21) (Fig. 2). Notably, Slitrk3 functioned like NL2, indicating that a distinct molecular determinant in Slitrk3 mediates its exclusive targeting to inhibitory synapses. Third, KD of Slitrks led to impairments in synapse structure (Fig. 3). Individual KD of Slitrk1, Slitrk2, Slitrk4, or Slitrk5 decreased the number of excitatory synapses. In contrast, KD of Slitrk3 specifically reduced the number of inhibitory synapses (Fig. 3). Systematic analyses of KO mice deficient in the expression of Slitrk(s) should be performed to validate the RNAi phenotypes documented in this study. Fourth, Slitrks appeared to interact with both PTPδ and PTPσ but not with LAR, at least in vitro (Fig. 4 *A* and *B* and Fig. S7). Strikingly, Slitrks required distinct presynaptic receptors to trigger specific types of presynapses: KD of PTPσ specifically attenuated the synaptogenic activities of Slitrk1 and Slitrk2 on excitatory synapses, whereas KD of PTPδ completely abolished the synaptogenic activities of Slitrks on inhibitory synapses (13) (Fig. 4 *C* and *D*). In contrast to a previous report that PTPδ is responsible for mediating induction of excitatory presynapses by IL1RAPL1 (18), these results suggest that individual Slitrks functionally use a different set of extracellular ligands to accelerate presynaptic development. We speculate that individual members of the LAR-RPTP family link to unique sets of cytoplasmic proteins that mediate activation of intracellular signaling cascades, leading to formation of either excitatory or inhibitory synapses, depending on signals from the postsynaptic side. In support of speculation, it has been shown that LAR-RPTP family members are differentially localized to distinct synapse types (13, 17, 33). PTPδ is exclusively localized to axons of inhibitory synapses, whereas PTPσ is exclusively localized to axons of excitatory synapses in cultured hippocampal neurons (13, 17). The differential localization of LAR-RPTP members partly explains why KD of each LAR-RPTP isoform exerts distinct effects in artificial synapse-formation assays (Fig. 4 *C* and *D*). However, these results cannot fully account for why overexpression of Slitrk isoforms affects distinct synapse types. One possible explanation can be found in the presynaptic ligand interaction-independent activities of Slitrk isoforms, which are quite reminiscent of NL1 (22). Therefore, structural information detailing how Slitrks associate with LAR-RPTP family members should provide mechanistic insight into why different LAR-RPTPs have unique catalogs of postsynaptic receptors.

Overall, our results confirm the notion that Slitrks are bona fide synaptic CAMs. It is likely that Slitrks collaborate with other

known synaptic CAMs to maintain the structure and function of synapses, particularly together with other synaptic CAMs that also interact with LAR-RPTPs in a combinatorial manner (16–18). In addition, our data establish that different Slitrk isoforms have differential functions, a finding reminiscent of the previous observation that overexpression of each Slitrk isoform differentially affects neurite outgrowth in PC12 cells, whose activities are conferred by their unique cytoplasmic regions (5). Although the precise mechanism governing synaptic adhesion between Slitrks and LAR-RPTPs remains elusive, the fact that Slitrks and LAR-RPTPs serve double duty as inducers of either excitatory or inhibitory synapses places these protein families at center stage in the control of excitatory–inhibitory balance, which is critical for neuronal function (34). Indeed, genetic mutations of a subset of Slitrks have been associated with multiple neuropsychiatric diseases (7, 9, 10). In support of this idea, Slitrk1 is expressed in neural circuits of basal ganglia implicated in Tourette syndrome (35). Although many neuropsychiatric disorders, particularly autism spectrum disorders, are thought to occur as a result of an imbalance between excitatory and inhibitory synapses, whether the spectrum of OCDs reflects a similar excitatory–inhibitory imbalance has not yet been established (36). Our work underscores the notion that Slitrks and their *trans*-synaptic signaling pathways are linked to pathophysiological mechanisms underlying related neuropsychiatric diseases.

Our study raises a number of questions that need to be addressed. Can KO of other Slitrk isoforms recapitulate the excitatory–inhibitory imbalance observed in Slitrk3-KO mice? Why do very similar proteins within the same family (i.e., Slitrks, LAR-RPTPs) activate different functional *trans*-synaptic pathways? Do Slitrks function similarly or differently in other brain regions that are particularly relevant for the occurrence of the spectrum of OCDs (i.e., cortex, striatum, thalamus)? The definitive answers to these questions will shed light on the detailed molecular mechanisms underlying the function of Slitrks in synapse formation and could unveil the pathophysiological mechanisms by which Slitrk dysfunction contributes to the behavioral and cognitive deficits in related neuropsychiatric conditions.

Methods

Expression constructs and antibodies used in this study were described in detail in *SI Text*. Artificial synapse-formation assays, cell-adhesion assays, and cell surface labeling assays were performed with HEK293T cells as previously described (22, 30). Generation of lentiviral shRNA plasmids and production and characterization of recombinant lentiviruses were performed as previously described (37) and are detailed in *SI Text*.

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