SLITRK2 variants associated with neurodevelopmental disorders impair excitatory synaptic function and cognition in mice

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SLITRK2 is a single-pass transmembrane protein expressed at postsynaptic neurons that regulates neurite outgrowth and excitatory synapse maintenance. In the present study, we report on rare variants (one nonsense and six missense variants) in SLITRK2 on the X chromosome identified by exome sequencing in individuals with neurodevelopmental disorders. Functional studies showed that some variants displayed impaired membrane transport and impaired excitatory synapse-promoting effects. Strikingly, these variations abolished the ability of SLITRK2 wild-type to reduce the levels of the receptor tyrosine kinase TrkB in neurons. Moreover, Slitrk2 conditional knockout mice exhibited impaired long-term memory and abnormal gait, recapitulating a subset of clinical features of patients with SLITRK2 variants. Furthermore, impaired excitatory synapse maintenance induced by hippocampal CA1-specific cKO of Slitrk2 caused abnormalities in spatial reference memory. Collectively, these data suggest that SLITRK2 is involved in X-linked neurodevelopmental disorders that are caused by perturbation of diverse facets of SLITRK2 function.
I
ntellectual disability (ID) affects about 3% of the general population and is the most common reason for referral to genetic centers. It is estimated that at least half of cases have a monogenic cause, with more than 1000 genes corresponding to an equivalent number of rare diseases currently identified. The most frequently identified genes are responsible for at most 0.5% of cases. All modes of transmission—autosomal dominant, recessive, and X-linked (dominant or recessive)—are observed. Because of the disproportionate number of males among individuals with ID and the possibility of performing genetic studies of X-linked patterns of inheritance in large families, special attention has been paid to identifying the genetic basis of X-linked ID (XLID). Prior to the advent of next-generation sequencing (NGS), major efforts by the EuroMRX consortium, the Sanger Center, and other international groups encompassing thousands of families, led to the identification of a large number of XLID genes. The generalization of NGS approaches, including exome sequencing and whole-genome sequencing has greatly accelerated the discovery of autosomal and XLID genes and increased the diagnostic yield of ID, resulting in a shortened diagnostic odyssey for families and expanded access to genetic counseling, including prenatal and pre-implantation genetic diagnosis. Thus, there are now about ~130 identified XLID genes, with an additional ~50 potential candidate XLID genes.

SLITRK2, a gene encoding a transmembrane protein that is highly expressed in the brain.

Slitrk- and Trk-like family proteins, composed of six members (Slitrk1-6), are type I transmembrane proteins that are strongly expressed in the central nervous system. Intriguingly, Slitrks positively regulate excitatory and inhibitory synapse development in cultured hippocampal neurons in a paralog-dependent manner. Extensive analyses have shown that Slitrks interact with distinct members of the LAR-RPTP (leukocyte common antigen-related receptor protein tyrosine phosphatase) family to promote presynaptic assembly. In addition, Slitrk5 interacts with receptor tyrosine kinase TrkB in cis to regulate their interaction with LAR-RPTPs. Structural analyses have further demonstrated that the N-terminal leucine-rich repeat 1 (LRR1) of Slitrks interacts with splicing variants of LAR-RPTPs that contain LRR cis to regulate their interaction.

Clinical manifestations in individuals with rare variants in SLITRK2.

We collected clinical information for the eight individuals with likely pathogenic variants. P1-5, P7-8, and P10 (Table 1 and Supplementary information). The mean age at examination was 16.9 years (range: 8–30 years). All but one patient had mild to severe intellectual disability and speech delay (mostly severe). Neurological regression was observed in one patient after the age of 6. Patients mainly presented with neuropsychiatric and behavioral issues, including major anxiety, ASD (4/8), and aggressiveness (2/8). Two patients had attention-deficit hyperactivity disorder (ADHD) with executive impairment, and two were diagnosed with hyperactivity or agitation.

Results
Identification of individuals with rare variants in SLITRK2.

Exome sequencing, used as a diagnostic approach in individual P1, identified a nonsense p.655SD variant in the SLITRK2 gene (Table 1 and Fig. S1a). Sanger sequencing confirmed that this variant was present in his affected brother (P2), but absent from mother’s blood, suggesting a de novo occurrence in the maternal germ line (Fig. 1 and Fig. S1a). Because the SLITRK2 gene contains only one coding exon, we suspected that the mutant transcript does not undergo nonsense mRNA-mediated decay. Through an international data-sharing arrangement initiated through GenetMatcher, we identified six additional individuals with NDDs carrying rare non-synonymous SLITRK2 variants predicted to be damaging. Among those were six missense variants and one frameshift variant. These include SLITRK2 variants predicted to be damaging, based on analyses using SIFT and Polyphen2, with CADD scores between 4.1 and 28.2 (Table S1). The amino acid residues affected by the missense variants are all highly conserved across species and are located in the extracellular part of the SLITRK2 protein (Fig. 1 and Fig. S2).

Seven additional missense SLITRK2 variants were identified in seven unrelated male patients with NDDs. Among them: (i) a rare missense variant (p.E555SD) was identified in a male (P14) who also carried a pathogenic variant in CUL4B, encoding a ubiquitin E3 ligase subunit. This CUL4B variant appeared sufficient to explain the majority of his clinical manifestations and the SLITRK2 p.E555SD variant was therefore not considered as potentially disease-causing; (ii) four other missense variants occurred de novo in the unaffected mother. The remaining variants were maternally inherited. All of these missense variants were predicted to be damaging, based on analyses using SIFT and Polyphen2, with CADD scores between 4.1 and 28.2 (Table S1). The amino acid residues affected by the missense variants are all highly conserved across species and are located in the extracellular part of the SLITRK2 protein (Fig. 1 and Fig. S2).

Here, we report that eight individuals with seven unrelated families with moderate to severe ID and/or NDDs, including neuropsychiatric and behavioral problems, harbor rare potential disease-causing variants of the X-linked gene, SLITRK2. We systematically investigated the effects of SLITRK2 variants on biochemical properties, surface transport, ligand-binding activity, and synaptogenic activities in cultured neurons. In addition, we analyzed the synaptic and behavioral effects of a Slitrk2 deficiency using Slitrk2 conditional knockout (cKO) mice.
One patient had obsessive-compulsive behavior, vocal tics, “meltdowns” and tantrums. We also noted an unsteady gait (4/6), spasticity and/or dystonia (3/8), and seizures (3/8). Several patients (4/8) had kyphoscoliosis. Three patients had short stature (≤ 2 SDS). The patients for whom we were able to get photographs were not clearly dysmorphic except for one patient (Supplementary information). Occipitofrontal circumference measurements were highly variable, including two patients with macrocephaly and one patient with microcephaly. Brain magnetic resonance imaging (MRI) of most patients was normal or revealed nonspecific anomalies (4/8), including white matter reduction, ventricular dilation, and thin corpus callosum; three patients had white matter anomalies (Table 1).

Biochemical, structural, and ligand-binding phenotypes of disease-associated SLITRK2 variants. To investigate the structural effect of patient variants on human SLITRK2, we predicted the structure of SLITRK2 using AlphaFold222,23. Three variants (L74S, V201I, and E210K) are located in the LRR1 domain (aa LxxLxxNxL motif of LRR1-1 is expected to disrupt hydrophobic interactions with neighboring nonpolar residues (V54, L69, L77, L91, and L93). Similarly, mutation of P374 to arginine is expected to disrupt the LRRNT hydrophobic core of LRR2, comprising with V357 and L380. A point mutation, resulting in the replacement of a small, nonpolar valine by a long methionine (V511M), may collapse LRRCT folding of the LRR2 domain. The crystal structure of the human SLITRK1 LRR1 domain in complex with PTP8 Igl-3 has shown that E206 on the concave surface of SLITRK1 makes ionic interactions with the side chain of K135 and main chain of V13614. Mutation of SLITRK2 E210 corresponding to SLITRK1 E206 to positively charged lysine would be expected cause charge repulsion, thus interrupting the interaction of SLITRK2 LRR1 with LAR-RPTPs. Because cysteine residues are usually undesirable in exposed regions of a protein unless they acquire functions such as metal binding24, the R426C variant on the concave surface may adversely affect protein stability. However, other variants exposed on the surface (E461*, R484Q, and E555D in LRR2) and V2011, which form hydrophobic networks with P374 and V511M) are predicted to affect the structural stability of the protein (Table S2), possibly causing misfolding and aberrant trafficking. In particular, a point mutation in the nonpolar leucine, L74, to a polar serine residue in the consensus
V201I variant, we included two other variants—c.967A>G p.S323N and c.1765G>A p.V589I—present in 6 and 139 males from gnomAD respectively and therefore presumed to be benign (Table S1). Notably, P374 and V511 residues in human SLITRK2 are conserved in other human SLITRK proteins at equivalent positions, but none of the other residues exhibit complete sequence identity across the six SLITRK members (Fig. S2a). The two variants from Decipher, S9I and G15E, are located in the signal peptide of SLITRK2, which is expected to be cleaved after membrane targeting. Thus, for these two variants, we generated pCMV5-SLITRK2 vector harboring its own signal peptide, whereas the other variants were generated using the pDisplay-SLITRK2 vector (Fig. 3a). Most of these residues are quite evolutionarily conserved among various vertebrate species, implying their possible functional significance (Fig. S2b).

We then examined the expression levels and intracellular transport properties of the various SLITRK2 variants following expression in human embryonic kidney 293 T (HEK293T) cells (Fig. 3). Immunoblot analyses of lysates of HEK293T cells transfected with vectors encoding HA-tagged full-length SLITRK2 wild-type (WT) or the respective variants showed that total protein expression levels of all SLITRK2 point mutants (except E461*) were comparable to those of SLITRK2 WT (Fig. 3b). The E461* variant, which resulted in a premature termination codon, yielded a truncated protein of ~60 kDa in SDS-PAGE analyses (Fig. 3b). Notably, unlike SLITRK2 WT, which is expressed as both N-glycosylated and non-glycosylated protein species, three SLITRK2 variants (L74S, P374R, and R426C) in HEK293T cells were detected as immature, non-glycosylated forms (Fig. S3). We next examined the surface and intracellular protein levels of SLITRK2 WT and the indicated SLITRK2 variants in HEK293T cells (Fig. 3c, d). In keeping with the glycosylation profiles (Fig. S3), only three SLITRK2 variants (L74S, P374R, and R426C) displayed significantly reduced surface levels, reflecting their complete entrapment in an intracellular compartment (Fig. 3c, d), in the absence of altered cellular membrane integrity (as monitored by transferrin receptor expression; Fig. S4). The E461* variant also exhibited reduced surface levels (Fig. 3c, d), in agreement with the absence of a transmembrane region in this variant; thus, this truncated SLITRK2 is likely secreted (Fig. S5). Results obtained using surface biotinylation assays were consistent with these findings, showing markedly lower surface expression levels of L74S, P374R, and E461* variants (Fig. S6). Note that, although S9I and G15E variants are located in the SLITRK2 signal peptide, they did not affect the membrane transport of SLITRK2 (Fig. 3c, d).

To determine whether the various SLITRK2 variants affected the interactions with PTPδ, an extracellular ligand of SLITRK2, we assayed cell-surface binding of recombinant Ig-fusion proteins of PTPδ (IgC-PTPδ) or IgC alone (negative control) with HEK293T cells expressing HA-tagged SLITRK2 variants (Fig. S7). IgC-PTPδ proteins avidly bound to HEK293T cells expressing SLITRK2 variants, except those expressing a subset of SLITRK2 variants with defective surface transport properties (Fig. S7). IgC did not bind to any tested SLITRK2 variants (Fig. S7).

To ensure that the subset of the SLITRK2 variants with impaired surface transport properties exhibited similar trafficking defects in neurons, we transfected cultured hippocampal neurons with WT or variant SLITRK2 and analyzed them by immunocytochemistry. Whereas most HA-tagged SLITRK2 variants were transported into
dendrites as efficiently as SLITRK2 WT (Fig. 4), three SLITRK2 variants (L74S, P374R, and R426C) were either completely retained in the cell body of transfected neurons, or displayed markedly impaired trafficking into dendrites (Fig. 4), as judged relative to immunofluorescence signals in the somatic compartment of transfected neurons. Puzzlingly, HA immunoreactive intensity in the somatic compartment was not decreased in neurons transfected with L74S, P374R, or R426C (see Fig. 4b), possibly reflecting imprecise evaluation owing to saturation of HA immunoreactivity in transfected neurons. We thus repeated the same experiments using a subset of SLITRK2 variants and quantified HA immunoreactive intensity in transfected neurons imaged using settings that prevented saturation of immunofluorescence signals (Fig. S8a, b). We found that HA immunoreactivity was significantly increased in somatic compartments of neurons expressing L74S or P374R, but not in those of neurons expressing R426C (Fig. S8a, b). Similar findings were also obtained using Slitrk2-deficient cultured neurons (Fig. S9). These findings are also consistent with the slight increase in R426C entrapment in an intracellular compartment (i.e., cis-golgi) of cultured neurons, as assessed by its colocalization with anti-GM130 (Fig. S10). No alterations in dendritic branching were observed in neurons expressing SLITRK2 WT or the indicated SLITRK2 variants (Fig. S8c). The E461* variant also exhibited impaired dendritic trafficking and reduced levels of HA immunofluorescence signals in the somatic compartment (Fig. 4), in agreement with the absence of a transmembrane region; in E461*, again suggesting that the truncated SLITRK2 protein is likely secreted. These observations suggest that the surface transport deficiency observed in heterologous cells is similarly recapitulated in cultured hippocampal neurons. Taken together, these data suggest that one subset of SLITRK2 variants observed in patients with neurodevelopmental disorders causes improper biochemical processing and abnormal trafficking.

Synaptic phenotypes of disease-associated SLITRK2 missense variants. SLITRK2 was previously shown to trigger presynaptic differentiation when expressed in heterologous cells and cocultured in contact with axons. To test whether the surface transport-defective SLITRK2 variants exhibit differences in presynaptic differentiation-inducing ability, we performed heterologous synapse-formation assays using HEK293T cells expressing SLITRK2 WT or the indicated SLITRK2 variants (Fig. S11). SLITRK2 WT robustly recruited clustering of both the glutamatergic presynaptic marker VGLUT1 (vesicular glutamate transporter 1) and GABAergic presynaptic marker GAD67 (glutamic acid decarboxylase 67 kDa)
around SLITRK2-expressing HEK293T cells, whereas L74S, P374R, R426C, and E461* were functionally inactive in recruiting both VGLUT1 and GAD67, as expected given their impaired surface expression levels and PTP-binding activities (Fig. S11; see Figs. 3c, d, S6 and S7).

**Generation of Slitrk2-cKO mice.** To evaluate the cellular effects of SLITRK2 variants, we generated conditional Slitrk2-KO (Slitrk2-cKO) mice. Transgenic mice with a deletion of Slitrk2 were generated by crossing homozygous Slitrk2-flxed mice, in which exon 2 and exon 3 are flanked by loxP sites, with a Cre
recombinant driver line under control of the Nestin promoter (Nestin-Cre) (Fig. 5a, b). Quantitative RT-PCR analyses showed that mRNA levels of Slitrk2, but not those of the other Slitrks, were completely eliminated in Nestin-Slitrk2 mice (Fig. 5c). Nestin-Slitrk2 male mice weighed marginally (but significantly) less at postnatal day 30 (P30), P40, and P50 compared with control mice, a generalized metabolic phenotype of the Nestin-Cre driver line25 (Fig. 5d), although their brain weights and size were comparable (Fig. 5e, f). Gross morphology, cortical thickness and neuron numbers, including interneuron numbers, were comparable between Nestin-Slitrk2 and neuron numbers, including interneuron numbers, were comparable between Nestin-Slitrk2 mice and control littersmates, as assessed by Nissl staining (Fig. 5g) and immunostaining for the neuron-specific marker NeuN (Fig. 5h, i) and GABAergic interneuron marker GAD67 (Fig. 5j, k). In addition, semi-quantitative immunoblot analyses confirmed complete elimination of detectable Slitrk2 protein in Nestin-Slitrk2 mice (Fig. 5l, m), as assessed with the Slitrk2-specific polyclonal antibody, JK177 (Fig. S12), and further showed that expression levels of various synaptic proteins in the cortex of Nestin-Slitrk2 mice were comparable to those of littermate control mice (Fig. 5l, m). Moreover, ventricle volumes in Nestin-Slitrk2 mice were comparable to those of control mice (Fig. S13).

Slitrk2-cKO induces an impairment in excitatory synaptic development that is not reversed by reintroduction of a subset of SLITRK2 patient variants. Slitrk2 was previously shown to specifically promote excitatory, but not inhibitory, synapse maintenance in cultured neurons and hippocampal CA1 neurons10,17,19. To address this, we first examined whether Slitrk2 deletion induced a specific decrease in excitatory synapse numbers and synaptic transmission in cultured hippocampal neurons. Immunostaining neurons at DIV14 with antibodies to VGLUT1 and SHANK, markers for excitatory postsynaptic density, revealed a reduction in the density of excitatory synaptic puncta immunoreactive to both VGLUT1 and SHANK (Fig. S14a, b). As expected, neither the density nor size of inhibitory synaptic puncta was changed in neurons immunostained with antibodies to VGAT and GABA<sub>A</sub>γ2, an inhibitory postsynaptic marker (Fig. 5a, b, d).

To assess whether the potential disease-causing SLITRK2 variants affect the excitatory synapse-promoting function of SLITRK2 WT, we infected cultured hippocampal Slitrk2-cKO neurons with lentiviruses expressing either inactive Cre (ΔCre; Control) or active Cre recombinase at DIV5 and co-transfected them with human SLITRK2 expression variants and EGFP at DIV8–9. We subsequently immunostained these infected/cotransfected neurons with antibodies to synaptophysin, SHANK and EGFP (to visualize the transfected neurons) at DIV13–14. The density, but not the size, of excitatory synaptic puncta immunoreactive to both synaptophysin and SHANK was significantly reduced in Slitrk2-deficient neurons (Fig. 6a, b; Fig. S14e). Co-expression of SLITRK2 WT completely reversed the excitatory synaptic deficits observed in Slitrk2-deficient neurons (Fig. 6a, b). In contrast, co-expression of the SLITRK2 variants, L74S, P374R, R426C, or E461<sup>*</sup>, consistently failed to rescue the deficits in excitatory synapses (Fig. 6a, b), in agreement with the results obtained in heterologous synapse-formation assays (Fig. S11). Strikingly, expression of T312A also failed to rescue the deficits in excitatory synapses (Fig. 6a, b), despite the fact that this SLITRK2 variant exhibited expression levels, surface transport, and presynaptic differentiation-inducing abilities comparable to those of Slitrk2 WT (see Figs. 3, 4, S4, S6, and S11).

To corroborate these immunocytochemical results, we measured miniature excitatory postsynaptic currents (mEPSCs) in cultured hippocampal neurons using whole-cell electrophysiological recordings (Fig. 6c–e). We found that the frequency, but not amplitude, decay time or rise slope, of mEPSCs was markedly decreased in Slitrk2-deficient cultured neurons (Fig. 6c–g); see also Fig. S15). In contrast, Slitrk2 deletion did not affect the frequency or amplitude of miniature inhibitory postsynaptic currents (mIPSCs) in cultured hippocampal neurons (Fig. S15). Co-expression of SLITRK2 WT or the indicated SLITRK2 variants completely reversed decrease in mEPSC frequency (Fig. 6c, e), whereas co-expression of SLITRK2 variants defective in rescuing anatomical deficits failed to rescue mEPSC frequency (Fig. 6c, e). Consistent with results obtained in cultured hippocampal neurons, immunohistochemical analyses using Nestin-Slitrk2 mice revealed that the density of VGLUT1 and PSD-95 puncta was significantly decreased in a subset of hippocampal CA1 layers (stratum oriens [SO] and stratum radiatum [SR]) (Figs. S16). These results collectively suggest that the synaptic phenotypes of the various SLITRK2 variants likely reflect perturbation of diverse facets of SLITRK2 functions.

Reduced TrkB levels induced by SLITRK2 WT, but not by disease-associated SLITRK2 variants. Previous studies have shown that SLITRK5 acts as a co-receptor for TrkB that orchestrates brain-derived neurotrophic factor (BDNF)-TrkB-dependent trafficking and signaling13, and that an obsessive-compulsive disorder (OCD)-associated SLITRK5 missense variant impairs SLITRK5 binding to TrkB25. Thus, we next analyzed whether the identified SLITRK2 variants also altered TrkB binding, assessed by communoprecipitation assays in HEK293T cells. We found that disease-linked SLITRK2 variants with impaired excitatory synapse maintenance did not affect the ability of SLITRK2 to bind TrkB (Fig. S17a, b).

BDNF plays critical roles in neuronal survival and synaptic plasticity through activation of a full-length receptor (TrkB-FL) while truncated TrkB isoforms (TrkB-T) are presumed to competitively inhibit BDNF-mediated TrkB signaling in certain contexts27. Excitotoxic stimulation of cultured neurons with glutamate downregulated TrkB-FL, resulting in downregulation of BDNF-TrkB signaling28. In addition, calpain activation triggers the truncation of TrkB-FL, leading to downregulation of BDNF-
TrkB signaling. These findings suggest that disease-linked SLITRK2 variants could alter BDNF-TrkB signaling. To test this possibility, we infected DIV3 cortical cultured neurons with lentiviruses expressing SLITRK2 WT or the indicated variants, and collected the infected neuronal lysates at DIV14. Immunoblot analyses revealed that overexpression of SLITRK2 WT significantly decreased TrkB-FL levels, but markedly increased TrkB-T levels (Fig. 7a, b), suggesting that SLITRK2 might reduce forward trafficking of TrkB and/or trigger the truncation of TrkB-FL. Strikingly, T312A abolished the ability of SLITRK2 WT to decrease TrkB-FL levels (Fig. 7a, b). In addition, the effects of L74S, P374R, R426C, or E461* were similar to those of T312A.
whether a SLITRK2 deletion affects the levels of TrkB-FL and TrkB-(Fig. 7a, b). To corroborate these observations, we examined effects. These results underscore the contribution of a normal cellular stress responses, such as the endoplasmic reticulum (ER) ionic stress response and unfolded protein response30 stress. 

The accumulation of misfolded proteins is well known to trigger accumulation of misfolded proteins is well known to trigger cellular stress responses, such as the endoplasmic reticulum (ER) stress response and unfolded protein response30–32. Thus, we hypothesized that expression of a subset of SLITRK2 variants (i.e.,

BDNF-TrkB activation range to the regulation of SLITRK2-mediated excitatory synaptic functions.

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(Fig. 7a, b). To corroborate these observations, we examined whether a SLITRK2 deletion affects the levels of TrkB-FL and TrkB-T in hippocampal CA1 lysates (Fig. 7c, d). We found that a deletion of SLITRK2 induced a significant increase in TrkB-FL and decrease in TrkB-T (Fig. 7c, d), confirming the SLITRK2 gain-of-function effects. These results underscore the contribution of a normal cellular stress responses, such as the endoplasmic reticulum (ER) stress response and unfolded protein response30–32. Thus, we hypothesized that expression of a subset of SLITRK2 variants (i.e.,

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Fig. 4 Impaired dendritic targeting of a subset of SLITRK2 variants in cultured hippocampal neurons. a Representative images of hippocampal neurons transfected with the indicated WT or mutant forms of SLITRK2 at DIV10. The transfected neurons at DIV14 were double-immunostained for antibodies against the somatodendritic marker, MAP2 (cyan), and HA (magenta). Scale bar, 20 μm (applies to all images). b, c Somatic (b) or dendritic (c) targeting of WT or the indicated mutant forms of SLITRK2 in hippocampal neurons was quantified by measuring average intensity of HA immunofluorescence in primary dendrites. The average intensities of SLITRK2 WT and variants in the soma region of transfected neurons were also quantified. All data are shown as means ± SEMs (‘n’ denotes the number of neurons from three independent experiments; WT, n = 12; L745, n = 10; E210K, n = 10; P337A, n = 10; T312A, n = 10; P374R, n = 10; R426C, n = 10; E461*, n = 11; R484Q, n = 10; V511M, n = 10; E555D, n = 11; R792C, n = 10; S323N, n = 10; V589I, n = 10; V201I, n = 10; *p < 0.01, ***p < 0.001, ****p < 0.0001; ANOVA with a non-parametric Kruskal–Wallis test). See Source data for raw data values and Supplementary Table 4 for statistical details.

Fig. 5 Generation of Slitrk2-CKO mice. a Strategy used to generate Slitrk2-CKO mice. LoxP sites were introduced at positions flanking the neomycin gene, FLP recombinant target (FRT), and exon 2 of Slitrk2 gene. Black arrows indicate forward and reverse primers used for genotyping. Note that lacZ and neomycin cassettes are two separate markers. b PCR genotyping of Slitrk2-floxed mice (top) and Nestin-Cre;Slitrk2-CKO (Nestin-Slitrk2) mice (bottom). The band size for the lacZ and neomycin cassettes are two separate markers. c Quantitative RT-PCR analyses of Slitrk mRNA levels in Nestin-Slitrk2 mice. Data are mean ± SEMs (n = 4 mice; *p < 0.05; two-tailed Mann–Whitney U test). d Body weights of Nestin-Slitrk2 and Slitrk2-floxed (Ctrl) mice at P30, P40, and P50. Data are means ± SEMs (‘n’ denotes the number of mice; P30: Ctrl, n = 15; Nestin-Slitrk2, n = 10; P40: Ctrl, n = 20; Nestin-Slitrk2, n = 14; P50: Ctrl, n = 20; Nestin-Slitrk2, n = 14; *p < 0.05, ***p < 0.001; two-tailed Mann–Whitney U test). e Representative photographs of whole brains (e) and quantification of brain weights and size (f) of P42 Nestin-Slitrk2 and Slitrk2-floxed (Ctrl) mice. Data are means ± SEMs (‘n’ denotes the number of mice; Ctrl and Nestin-Slitrk2, n = 5; two-tailed Mann–Whitney U test). g Normal gross morphology of the Nestin-Slitrk2 brain at 7 wks, as revealed by Nissl staining. Scale bar: 1 mm (applies to both top and bottom). h Representative images of NeuN (a neuronal marker) staining in the Nestin-Slitrk2 brain. Normal numbers of neurons in hippocampal regions. Top, hippocampus; bottom, CA1 stratum pyramidale layer. Scale bar: 1 mm (top) and 20 μm (bottom). i Summary graphs for neuron density and cortical thickness. Data are means ± SEMs (‘n’ denotes the number of mice; neuron density: Ctrl and Nestin-Slitrk2, n = 4; cortical thickness: Ctrl and Nestin-Slitrk2, n = 6; two-tailed Mann–Whitney U test). j Representative images of GAD67 (GABAergic interneuron marker) staining in the hippocampal CA1 of Nestin-Slitrk2 mice. Scale bar: 0.1 mm. k Summary data for GAD67 staining from (j). Data are means ± SEMs (n = 3 mice each after averaging data from 3 sections/mouse; two-tailed Mann–Whitney U test). l, m Representative immunoblotting (l) of hippocampal lysates, and summary data showing synaptic protein levels (m) in crude synaptosomal fractions of P42 control and Nestin-Slitrk2 brains, analyzed by semi-quantitative immunoblotting. Data are means ± SEMs (n = 4 mice/group; **p < 0.005, ***p < 0.0001; two-tailed unpaired t-test). See Source data for raw data values and Supplementary Table 4 for statistical details.

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L74S, T312A, P374R, and R426C) exhibiting entrapment in intracellular compartments could induce an ER stress response in cultured hippocampal neurons. However, immunoblot analyses using lysates of neurons overexpressing SLITRK2 WT or the indicated SLITRK2 variants showed no difference in the levels of BiP, which are activated transcriptionally by ER stressors, between control and Nestin-Slitrk2 WT or the indicated mutant forms of SLITRK2 together with EGFP vector at DIV8–9. Scale bar: 10 μm (applies to all images). **Biological replicates**: Twelve independent experiments; Control vs. indicated SLITRK2 variant: +p < 0.05, ++p < 0.01, ***p < 0.001; ANOVA with a non-parametric Kruskal-Wallis test. See Source data for raw data values and Supplementary Table 4 for statistical details.

**Slitrk2-cKO mice exhibit memory deficits and abnormal gait.** We then tested whether Slitrk2-cKO mice exhibited the behavioral features that manifest in patients with NDD and SLITRK2 variants (see Table 1). To this end, we performed a battery of behavioral tests, including those measuring learning and memory, locomotion, anxiety, and motor coordination (Fig. 8 and Fig. S18). Ten-week-old male Nestin-Slitrk2 mice displayed comparable exploratory behavior (assessed by open-field tests) (Fig. S18a, b), sociability, and social novelty recognition memory (assessed by three-chamber tests) as littermate control mice, showing preference for the first novel mouse over an inanimate object (Fig. S18c, d) and preference for a novel stranger mouse over familiar mouse (Fig. S18e, f). However, Nestin-Slitrk2 mice exhibited reduced anxiety-like behavior, as evidenced by increased time spent in open arms (with a similar number of entries into each open arm) in the elevated plus-maze test, without a change in time spent in the light compartment in the light-dark test (Fig. S18g–i). Moreover, Nestin-Slitrk2 mice showed impaired spatial learning and memory (assessed by novel object-recognition tests), although spontaneous alternation in Y-maze tests was similar to that of littermate control mice (Fig. S18j–m). We further examined spatial reference memory, as evaluated by the Barnes maze test. Both Control and Nestin-Slitrk2 mice learned to locate the target hole during the course of the training period, as indicated by gradual reductions in the number of search errors, escape latency and total...
Correlation of impaired hippocampal CA1 excitatory synapse properties of Slitrk2-cKO mice with deficits in spatial reference memory. Because Nestin-Slitrk2 mice exhibited impairments in the Barnes maze test (Fig. 8a–f), a hippocampal CA1-dependent spatial task34, we asked whether loss of Slitrk2 in the hippocampal CA1 region could recapacitate this behavioral deficit. To this end, we stereotactically injected adeno-associated viruses (AAVs) expressing Cre or ΔCre (Ctrl) into the CA1 of adult Slitrk2-floxed mice and performed semi-quantitative immunohistochemistry and electrophysiological recordings three weeks after injections (Fig. 9a, b). We found that the frequency, but not amplitude, of mEPSCs was markedly decreased in hippocampal CA1-specific Slitrk2-cKO mice (Fig. 9c–e). Moreover, these mice exhibited a significant reduction in the integrated intensity of VGLUT1 and PSD-95 puncta in the SO and SR layers (Fig. 9f, g), without changes in VGAT puncta in any examined hippocampal CA1 layer (Fig. 9h), in keeping with the anatomical phenotype of Nestin-Slitrk2 mice (Fig. 9i). Notably, co-expression of SLITRK2 WT, but not that of the SLITRK2 T312A variant, completely rescued the decreased integrated intensity of VGLUT1 and PSD-95 puncta in hippocampal CA1-specific Slitrk2-cKO mice (Fig. 9f, g). These results reinforce the phenotype of SLITRK2 T312A in hippocampal CA1 pyramidal neurons.

Finally, to determine whether CA1-specific elimination of Slitrk2 expression leads to long-term spatial memory deficits, we performed Barnes maze tests using CA1-specific Slitrk2-cKO mice. Similar to the behavioral phenotype of Nestin-Slitrk2 mice, CA1 region-specific Slitrk2-cKO mice also exhibited impaired retention of spatial reference memory (Fig. 9b–l). Collectively, these results support the conclusion that SLITRK2 synaptic functions in the hippocampus (Fig. 9l) are essential for execution of normal spatial tasks in mice.

Discussion
Although numerous genes encoding synaptic cell adhesion proteins (CAMs) have been identified as causative factors for a variety of neurodevelopmental and/or neuropsychiatric disorders, it had remained to be determined whether a subset of neurological symptoms of patients with these disorders are caused by alterations in synaptic adhesion signaling pathways that are not clearly established. As a member of a vertebrate-specific synaptic cell adhesion protein family, SLITRK2 specifically functions in mediating proper excitatory synapse development35. In particular, SLITRK2 is required for organizing trans-synaptic signaling pathways involved in excitatory presynaptic assembly and postsynaptic maintenance by binding to PTPσ and PDZ domain-containing scaffolds10,17,36. In addition, our previous study demonstrated that a subset of disease-associated SLITRK2 missense variants induce impaired dendritic targeting of Slitrk2 in neurons, leading to dysfunctions of excitatory synapse maintenance19. The SLITRK2 V89M variant reduced excitatory synaptic density, likely through its deleterious effects on surface expression and dendritic targeting19. Similarly, a series of other SLITRK variants associated with Tourette’s syndrome, trichotillomania, schizophrenia, or OCD was shown to trigger loss of...
the respective SLITRK protein functions in cultured hippocampal neurons\textsuperscript{19,26}. These results collectively suggest that pathogenic SLITRK genetic variants likely induce loss-of-function of corresponding SLITRKs, accounting for the pathogenesis of patients with neurological disorders. However, previous studies, which primarily employed knockdown (KD) and/or overexpression approaches, preclude firm conclusions about SLITRK2 functions. Previous SLITRK2 loss-of-function studies performed in cultured neurons and mice produced contradictory results\textsuperscript{10,17}, with Slitrk2 KD in cultured neurons reducing excitatory synapse density, and Slitrk2 KD in hippocampal CA1 neurons failing to affect excitatory synapse density\textsuperscript{16,17}. These results were previously attributed to differences in genetic manipulations, cellular preparation, or other unknown factor(s). Extensive analyses using Slitrk2-cKO mouse-derived cultured neurons recapitulated our previous anatomical results using Slitrk2 KD\textsuperscript{10}, in addition to exhibiting marked reduction in excitatory synaptic transmission. Moreover, Slitrk2 deletion in adult mouse hippocampal CA1 neurons similarly reduces the frequency of mEPSCs, in contrast to the absence of an effect of global Slitrk2 KD on mEPSCs\textsuperscript{17}. These results again underscore the importance of confirmatory analyses using a sophisticated system and approaches to elucidate functions of synaptic proteins.

In the current study, we identified SLITRK2 variants in NDD characterized by developmental delay and speech delay, mild to severe ID, and various neurological and behavioral comorbidities. Seven of the eight individuals with potential disease-causing variants had significant anxiety, and four patients were diagnosed with autism spectrum disorder. Other behavioral diagnoses include ADHD with executive difficulties, hyperactivity, or agitation. Several individuals had unsteady gait associated with spasticity and/or dystonia and three patients had seizures, of variable type, with the severity of ID ranging from mild to severe. Such clinical heterogeneity among individuals is often reported in NDDs associated with mutations in the same gene and even precisely the same mutation\textsuperscript{37,38}, suggesting that other genetic and/or environmental modifying factors might also be involved. SLITRK2 variants have previously been reported in two females with schizophrenia\textsuperscript{18}, but no patients affected by ID and/or NDD phenotypes and carrying SLITRK2 pathogenic variants have been reported to date. Of the seven potential disease-causing variants identified in this study, five induced prominent alterations in the properties of SLITRK2. Among these, three variants (L74S, P374R, and R426C) perturbed surface transport of SLITRK2, accompanied by impaired binding to LAR-RPTPs and synaptogenic activity, similar to most of known SLITRK1/4/5 substitutions that
represent loss-of-function mutations. One variant (E461*) in SLITRK2 was a nonsense variant, and resulted therefore in a truncated SLITRK2 protein. The fifth variant, T312A, induced normal surface expression, and normal extracellular ligand-binding and synaptogenic activity, albeit with markedly reduced excitatory synapse number and excitatory synaptic transmission. Remarkably, these five SLITRK2 variants abrogated the ability of SLITRK2 WT to negatively regulate TrkB-FL levels in neurons, revealing the significance of interactions between SLITRK2 and TrkB in terms of preventing SLITRK2 dysfunction-induced pathogenic effects. Given the well-established centrality of TrkB-mediated signaling in excitatory synapse development, it is plausible that selection of specific binding partners (i.e., LAR-RPTPs vs. TrkB) through fine regulation of SLITRK2-mediated

Fig. 9 Proper Slitrk2 function in the hippocampal CA1 is required for mediating spatial reference memory in mice. a Experimental configuration for electrophysiology and immunohistochemistry experiments (c-g). b Representative brain sections illustrating the precise targeting of AAVs for expression of Cre recombinase in the hippocampal CA1. Scale bar: 500 µm. c-e Representative traces (c) and summary graphs showing the frequency (d) and amplitude (e) of mEPSCs recorded from Slitrk2-flxed mice injected with AAVs expressing ΔCre or Cre. Data are shown as means ± SEMs (ΔCre, n = 13; Cre, n = 20; *p < 0.05; two-tailed Mann–Whitney U test). f, g Representative images (f) and summary graphs quantifying the integrated intensity (g) of VGLUT1 and PSD-95 puncta. Data are shown as means ± SEMs (ΔCre, n = 6; Cre, n = 6; Cre+WT, n = 5; and Cre+T312A, n = 5 mice; *p < 0.05, **p < 0.01, ***p < 0.001; ANOVA with Tukey’s test). Scale bar, 10 µm (applies to all images). h Experimental configuration for Barnes Maze tests (i-l). i Representative traces of locomotive behavior for Barnes Maze tests. Target box is indicated with a yellow circle. j Number of errors before first encountering the escape hole, escape latency, and total distance for Slitrk2-flxed mice injected with ΔCre (Control) or Cre during the training session. Data are presented as means ± SEMs (ΔCre, n = 12; Cre, n = 8; two-way ANOVA with Sidak’s multiple comparisons test). k, l Number of errors before first encountering the escape hole for Slitrk2-flxed mice injected with ΔCre (Control) or Cre during 1st (k) and 2nd (l) probe trials. Data are presented as means ± SEMs (ΔCre, n = 12; Cre, n = 8; *p < 0.05, **p < 0.01; two-tailed unpaired t-test). See Source data for raw data values and Supplementary Table 4 for statistical details.
extracellular interactions constitutes an important signaling pathway for excitatory synapse organization. Our preliminary analyses suggested that Slitrk2, Slitrk3, and Slitrk5, but not other Slitrks, bind to TrkB (Fig. S20). Further experiments are warranted to dissect the molecular determinants underlying Slitrk2/5-TrkB interactions.

Our extensive behavioral analyses showed that Slitrk2 loss-of-function in mice recapitulated a subset of behavioral symptoms found in patients with SLITRK2 variants. Strikingly, the current study demonstrated that selective elimination of Slitrk2 expression in the hippocampal CA1 results in impaired retention of spatial reference memory. It is likely that specific alterations in synaptic inputs on CA1 neurons, possibly from either entorhinal cortex afferents or CA3 Schaffer-collateral afferents, are responsible for abnormalities in this behavioral task. Whether SLITRK2 is involved in the assembly of specific hippocampal circuits remains to be determined. In addition, Nestin–Slitrk2 mice exhibited impaired long-term memory and altered footprint patterns. However, these mice displayed normal grip strength and somewhat enhanced motor coordination and learning. In general, the cerebellum is critical for coordinating movements, and its dysfunction often leads to uncoordinated walking or gait disturbance,40 a feature of patients harboring a subset of SLITRK2 variants, even though they were not clearly ataxic and the brain MRI did not show cerebellar anomalies (except for one patient with severe cerebral and cerebellar volume loss). Although the current study primarily correlated SLITRK2 synaptic functions in the hippocampal CA1 with spatial memory performance, future studies are warranted to identify the neuronal populations and/or neural circuits embedded in other brain areas that might underpin abnormal footprint patterns seen in Nestin–Slitrk2 mice. Moreover, most patients harboring potential disease-causing variants in SLITRK2 are anxious, whereas Nestin–Slitrk2 mice appeared to exhibit reduced anxiety-like behavior in the elevated plus-maze tests. Future studies utilizing knock-in mice expressing disease-associated SLITRK2 variants should help resolve this issue using additional behavioral paradigms that examine anxiety-like behavior. Most patients exhibit seizures and speech delay. Thus, it will be necessary to clarify whether Nestin–Slitrk2 mice exhibit seizure-like phenotypes or communication deficits by measuring electroencephalography and measuring ultrasonic vocalization, respectively. Because our various analyses did not reveal any prominent phenotypes in the case of E210K and V511M (predicted to be deleterious by in silico programs), their pathogenicity remains uncertain, although it is still possible that these variants are involved in SLITRK2-associated pathophysiology via as-yet unidentified mechanism(s) encompassing early brain developmental processes. Given that Slitrk2 is also expressed in specific astrocytic subtypes in adult mice41, it is reasonable to speculate that these SLITRK2 missense variants with no clear phenotypes in current functional assays might affect specific aspects of astrocytic functions that remain to be determined.

In conclusion, we demonstrated nonsense and missense variants in SLITRK2 in a novel form of X-linked NDD characterized by different degrees of ID associated with motor, speech, and behavioral impairments, that are caused by perturbation of SLITRK2 function. Further studies with larger cohorts are needed to confirm the involvement of this gene in a NDD and better delineate the clinical manifestations of the latter, especially at the behavioral level.

Methods

Participants and genetic analysis. Individuals were referred by clinical geneticists for genetic testing as part of routine clinical care. All patients enrolled and/or their legal representative signed informed consent for research use and authorization for publication. All the institutions received local institutional review board (IRB) approval to use these data in research. The main IRB approval was obtained from the IRB committee of the Stanford University School of Medicine (IRB# 20-0020). Individuals P1 and P2 (male siblings) were followed up in the Department of Clinical Genetics of Montpellier University Hospital, and exome sequencing was performed for P1 in the Molecular Genetics Laboratory of Strasbourg University Hospital, France. The six remaining patients (P3–P10) were recruited through an international collaborative study and data-sharing through Genematcher42. All patients were followed by a referring clinical geneticist and underwent exome sequencing in the following molecular laboratories as detailed in Supplementary information. Sanger sequencing confirmed the SLITRK2 variants in all affected probands and was used for follow-up validation studies. Variants have been submitted to ClinVar database. The referencing physicians completed clinical forms and sent morphological pictures and brain MRI reports, when available. Clinical information is detailed in Table 1. All patients’ families provided written informed consent, and all procedures performed in the studies were done in accordance with the ethical standards of the local institutional research committee and the Declaration of Helsinki. Effects of SLITRK2 missense variants were predicted using PolyPhen-243, SIFT44, and CADD (combined annotation-dependent depletion) scores44.

Expression vectors. pDisplay-SLITRK2 missense variants (L74S, V201I, E210K, P311A, T312A, S323N, P374R, R426C, R484Q, V511M, E553D, V589H, and R792C) were generated by polymerase chain reaction (PCR)-based mutagenesis with PrimeSTAR HS DNA polymerase (Takara) using a pDisplay SLITRK2 wild-type (WT) vector. pDisplay-SLITRK2 E461F was generated by amplification of the indicated region of human SLITRK2 (amino acid [aa] 22–460), followed by digestion with Xmal and SacII and subcloning into a pDisplay vector (Invitrogen). pCMV-SLITRK2 missense variants (S91I and G15E) were generated by PCR-based mutagenesis with PrimeSTAR HS DNA polymerase (Takara) using a pCMV-5–WT SLITRK2–mvenus construct as a template. pCMV6-XL4-SLITRK2 variant-Fc constructs (L74S, P374R, and R426C) were generated by PCR-based mutagenesis with PrimeSTAR HS DNA polymerase (Takara) using the pCMV6-XL4-SLITRK2-Fc construct as a template. pCMV6-XL4-SLITRK2 E461F–Fc was generated by PCR amplification of the indicated region of human SLITRK2 (aa 22–460) using the following primers: SLITRK2-Fc construct as a template, followed by NolI and Xbal and subcloning into a modified pCMV6-XL4 vector in frame as a C-terminal Fc fusion protein. L313 SLITRK2 missense variants were constructed by amplifying the indicated full-length SLITRK2 variants by PCR, digesting with NotI and BsrGI, and subcloning the resulting products into the L313 vector (lentiviral expression vector) for pAAV-SLITRK2 WT and T312A. pAAV-SLITRK2 WT and T312A were amplified by PCR amplification of the indicated region of human SLITRK2, followed by digestion with Xbal and BamHI and subcloning into the pAAV-T2A-GFP vector. pDisplay-SLITRK3 and pDisplay-SLITRK5 were generated by PCR amplification of mouse Slitrk3 (aa 28–652) and human SLITRK3 (aa 41–664), respectively, followed by digestion with Xmal and SacII and subcloning into the pDisplay vector. pCAGG-His-HA-Nlg2 was generated by PCR amplification of rat Nlg2 (aa 1–678) using pCMV5–Nlg2 as a template, followed by digestion with EcoRI and NotI and subcloning into the pCAGG-His-HA vector. pCMV-SPORT6-Tbrk was purchased from Korean Human Gene Bank. The following constructs were previously described: pDisplay-SLITRK2 WT45, pDisplay-SLITRK3 WT46, and pDisplay-SLITRK5 WT46. Antibodies. A polyclonal rabbit antibody against SLITRK2 was generated using a fusion protein of glutathione S-transferase (GST) with human SLITRK2 (aa 705–784) produced in BL21 Escherichia coli and purified on a glutathione-Sepharose column (GE Healthcare). Following immunization of rabbits with GST-SLITRK2 [705–784], the SLITRK2-specific antibody, designated IJ177 (RRID: AB_2892626), was affinity-purified using a GST-SLITRK2 [705–784]–immobilized Sulfolink column (Pierce). The following commercially available antibodies were used: mouse monoclonal anti-GM130 (clone 35/GM130; BD Transduction Labs; Cat# 610822; RRID: AB_398141), rabbit monoclonal anti-CD63 (clone 1F11; BioLegend; Cat# 901501; RRID: AB_2565006), mouse monoclonal anti-HA (clone 12CA5; Roche; Cat# 11074857002), rabbit monoclonal anti-phospho-TrkB (Thermo Fisher; Cat# PA5-36695; RRID: AB_2553666), mouse monoclonal anti-TrkB (clone 80E3; Cell Signaling; Cat# 4603; RRID: AB_2155125), rabbit monoclona...
AB_310837), mouse monoclonal α-actin (clone C4; Santa Cruz; Cat# sc-47778; RRID: AB_2714189), mouse monoclonal α-neuN (clone A60; Millipore; Cat# MAB377; RRID: AB_2533962), HRP-goat anti-human IgG cross-adsorbed secondary antibody (Thermo Fisher; Cat# 62-8420; RRID: AB_2533962), Cy3-AffiniPure donkey anti-rabbit IgG antibodies (Jackson ImmunoResearch; Cat# 711-165-152; RRID: AB_2307443), Cy3-AffiniPure donkey anti-mouse IgG antibodies (Jackson ImmunoResearch; Cat# 715-165-150; RRID: AB_2340813), Cy3-donkey anti-horse IgG antibodies (Jackson ImmunoResearch; Cat# 709-165-149; RRID: AB_2340535), Cy3-donkey anti-guinea pig IgG antibodies (Jackson ImmunoResearch; Cat# 706-035-148; RRID: AB_2340447), FITC-AffiniPure donkey anti-mouse IgG antibodies (Jackson ImmunoResearch; Cat# 715-095-150; RRID: AB_2340792), FITC-AffiniPure donkey anti-goat IgG antibodies (Jackson ImmunoResearch; Cat# 705-095-147; RRID: AB_2340401), FITC-AffiniPure Donkey anti-rabbit IgG antibodies (Jackson ImmunoResearch; Cat# 711-095-152; RRID: AB_2315776), and goat anti-guinea pig IgG antibodies (Thermo Fisher; Cat# A-21450; RRID: AB_141882). The rabbit polyclonal anti-VGLUT1 (KJ11; RRID: AB_2810945) and the rabbit polyclonal anti-PSD-95 (KJ016; RRID: AB_2722693) were previously described11. Rabbit polyclonal anti-pan-Shank (1172; RRID: AB_2810261), rabbit polyclonal anti-GluA2 (1193; RRID: AB_2722772), and rabbit polyclonal anti-GluA1 (1195; RRID: AB_2722773) were gifts from Dr. Eunjoon Kim (KAIST, Korea) and previously described11. See Supplementary Table 3 for detailed information of antibody dilutions.

Cell culture. HEK293T cells (ATCC; CRL-3216) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Welgene) supplemented with 10% fetal bovine serum (FBS; Tissue Culture Biologicals) and 1% penicillin-streptomycin (Thermo Fisher Scientific). HEK293T cells were transfected with a PTPδ-IgC construct or pCMV-IgC empty vector. After 72 h, the media from transfected cells were collected, and 50 mM HEPES (pH 7.4) and 0.5 mM EDTA were added. Soluble PTPδ-fusion proteins were purified using protein A-Sepharose beads (GE Healthcare). Pulled-down proteins were eluted with 0.1 M glycine (pH 2.2) and then neutralized with 1 M Tris-HCl (pH 8.0). HEK293T cells expressing HA-SLITRK2 and its variants were incubated with 10 µg/ml of the indicated PTPδ-fusion proteins. Images were acquired using a confocal microscope (LSM800; Zeiss).

Biotinylation assays. Recombinant PTPδ-fusion proteins were produced in HEK293T cells. HEK293T cells were transfected with a PTPδ-IgC construct or pCMV-IgC empty vector. After 72 h, the media from transfected cells were collected, and 50 mM HEPES (pH 7.4) and 0.5 mM EDTA were added. Soluble PTPδ-fusion proteins were purified using protein A-Sepharose beads (GE Healthcare). Pulled-down proteins were eluted with 0.1 M glycine (pH 2.2) and then neutralized with 1 M Tris-HCl (pH 8.0). HEK293T cells expressing HA-SLITRK2 and its variants were incubated with 10 µg/ml of the indicated PTPδ-fusion proteins. Images were acquired using a confocal microscope (LSM800; Zeiss).

Staining for surface/intracellular protein levels. HEK293T cells were transfected with expression vectors for HA-SLITRK2 WT or the indicated variants. After 48 h, cells were washed twice with ice-cold phosphate-buffered saline (PBS), incubated with 1 mg/ml SulfoNHS-LC-biotin (Pierce) in ice-cold PBS for 30 min on ice, rinsed briefly once with 0.1 M glycine in PBS, and incubated with 0.1 M glycine/PBS for 10 min at room temperature to completely quench biotin reactions. Cells were lysed with lysis buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris pH 7.4, 150 mM NaCl, and protease inhibitors) and incubated for 20 min on ice. After removal of cell debris by centrifugation, 250 µl of lysates was incubated with 30 µl streptavidin agarose beads (Pierce) for 4 h at 4 °C. After washing beads three times with lysis buffer, surface-labeled proteins were eluted with 2X sample buffer and analyzed by immuno- blotting using the indicated antibodies. Anti-transferrin receptor (TIR) and β-actin antibodies were used as surface and total protein loading controls, respectively.

Production of recombinant viruses

Lentiviruses. Lentiviruses were produced by transfecting HEK293T cells with three plasmids—lentivirus vectors, pPAX2, and pMD2.G—at a 2:2:1 ratio. After 72 h, lentiviruses were harvested by collecting the media from transfected HEK293T cells and centrifuging at 1000 × g to remove cellular debris, as previously described31. Briefly, HEK293T cells were co-transfected with pHelper and pAAV10.2/9 vectors together with pAAV-hsyn-SLITRK2-T2A-EGFP, pAAV-hsyn-SLITRK2-T2A-EGFP (T312A), pAAV-hsyn-Cre-EGFP, or pAAV-hsyn-Cre-EGFP. Cells were harvested 72 h later, lysed, mixed with 40% polyethylene glycol and 2.5 mM NaCl, and centrifuged at 2000 × g for 30 min. The resulting pellets were resuspended in HEPEs buffer (20 mM HEPES, 115 mM NaCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 2.4 mM KH2PO4), mixed with an equal volume of chloroform, and centrifuged at 400 × g for 5 min. The supernatants were concentrated three times with a Centriprep centrifugal filter (15 ml, 3 K MWCO; Millipore) at 16,000 × g for 10 min. The infectious titers of viruses was assessed by qRT-PCR detection of GFP sequences with subsequent reference to a standard curve generated using the pAAV-E6-EGFP plasmid.

Animals. All mice were on a C57BL/6j background, produced by back-crossing with C57BL/6j wild-type (WT) mice (purchased from Jackson Research Laboratory). The mice were kept and produced in the animal facility of Daegu Gyeongbuk Institute of Science and Technology under standard, temperature-controlled laboratory settings, including a 12-h light/dark cycle (lights on at 7 am) and free access to water and food. Pregnant Sprague-Dawley rats (Daehan Biolink) were used to prepare in vitro cultures of dissociated hippocampal neurons. All procedures were carried out in compliance with the guidelines and protocols for rodent experimentation in accordance with protocols (DGIST-IACUC-19052109-00) approved by the Institutional Animal Care and Use Committee of Daegu Gyeongbuk Institute of Science and Technology.

Generation of Slitrk2-floxed mice. The C57BL/6j mouse strain was used to produce our Slitrk2−/− KO mice. All mice were housed in a specific pathogen-free facility.

Microinjection. The targeting vector was linearized and electroporated into C57BL/6j mESCs. After G418 selection, a total of 1000 clones were selected in three separate rounds. Selected cell clones were verified by Southern blotting, karyotyping, and sequence validation of PCR products. Chimeric mice were generated by injecting positively selected C57BL/6j mESCs into a total of 80 (two rounds of 30 and 50) pre-implantation BALB/c mouse embryos (blastocysts).

Southern blot analysis. Chimeric offspring were crossed with WT C57BL/6j mice to produce the F1 generation. Germline transmission was then confirmed by Southern blotting and PCR analysis of tail DNA from F1 generation mice. The restriction enzymes EcoRI (5′-Probe-A), HindIII (Neo Probe) (3′), and SacI (3′Probe-A) (all from New England Biolabs) were used for Southern blotting of genomic DNA. After digestion, genomic DNA extracted from mouse tails was separated on a 1% agarose gel and transferred to a positively charged nylon membrane (Hybond N+; Amer sham International plc). The membrane was hybridized using DIG Easy Hyb Granules (Roche Applied Science Inc.) at 42 °C overnight in a mixture containing a PCR-generated probe labeled using the PCR DIG probe synthesis kit (Roche Applied Science Inc.). Hybridization signals were detected using the DIG Luminescent Detection Kit (Roche Applied Science Inc.).

Quantitative RT-PCR in cultured neurons. Cultured hippocampal and cortical neurons from Slitrk2-floxed mice were infected with ACre or Cre lentivirus at DIV4. Infected neurons were homogenized at DIV12 in TRIzol Reagent (Invitrogen), and total RNA was extracted according to the manufacturer’s protocol. cDNA was synthesized from total RNA using a cDNA synthesis kit (Takara Bio), qRT-PCR was performed on a CFX96 Real-Time PCR system (Bio-Rad) with TB protocol. cDNA was used to prepare in vitro cultures of dissociated hippocampal neurons. All procedures were carried out in compliance with the guidelines and protocols for rodent experimentation in accordance with protocols (DGIST-IACUC-19052109-00) approved by the Institutional Animal Care and Use Committee of Daegu Gyeongbuk Institute of Science and Technology.

Structural modeling. The domain structures of human SLITRK2 (LRR1, aa C33-P287; LRR2, aa P341-P579) were modeled with AlphaFold222,23. LRR1 and LRR2 domains of human SLITRK2 were presumed to be connected by a flexible linker based on previous negative-stain electron microscopy images of the full
ectodomain of human SLITRK1. Protein stability of domain structures of human SLITRK2 and variants was calculated using FoldX 5 suite.

**Nissl staining.** Mice underwent intracardiac perfusion with PBS and then with 4% paraformaldehyde. Fixed brain tissue was isolated, post-fixed for 12 h at 4 °C, and dehydrated in 30% sucrose for 6–8 d. Then, brain tissue was embedded in OCT (Optimal Cutting Temperature) compound, frozen, cryo-sectioned at 20-μm thicknesses using a cryostat (Leica CM1500). Slices were mounted on glass slides, rinsed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Permeabilized samples were washed twice with PBS (5 min each), then incubated for 20 min in 200 μl of NeuroTrace 500/525 Green Fluorescent Nissl Stain (Molecular Probes), diluted 1:100 in PBS before use. Following three more washes with PBS, the specimens were dried and mounted using Vectashield Mounting Medium containing 4-6-diamidino-2-phenylindole (DAPI; Vector Laboratories). A slide scanner (Axio Scan.Z1; Carl Zeiss) was used to capture green fluorescence.

**Measurement of mouse ventricle volumes.** Acute brain slices (50 μm thickness) were obtained from 7-wk-old mice using a vibratome. Every sixth brain section (300 μm between sections) was collected starting from bregma +0.86 mm and continuing to the dorsal end at bregma −0.82 mm. Sections were immunostained with anti-NeuN (clone A60; Millipore), and whole-brain slice images were obtained using a slide scanner (Axio Scan.Z1; Carl Zeiss) with a ×20 objective lens. All image settings were kept constant. Lateral ventricle areas were manually sketched and calculated using MetaMorph software (Molecular Devices Corp.). Lateral ventricle volumes were calculated as the sum of sectional areas of lateral ventricles times the layer thickness.

**Heterologous synapse-formation assays.** Forty-eight hours after transfecting with the indicated expression vectors, HEK293T cells were trypsinized, seeded onto cultured hippocampal neurons at days in vitro (DIV)10, and cocultured for 48 h, as indicated. Cocultured neurons were co-immunostained with antibodies against the indicated proteins. Fluorescence images were acquired by confocal microscopy (LSM780, Carl Zeiss), and results were quantified by measuring the fluorescence intensities of synaptic marker puncta in randomly selected transfected HEK293 cells (region of interest), normalized with respect to the area of each cell. Results were quantified for both red and green channels using MetaMorph software (Molecular Devices Corp.; RRID: SCR_002285).

**Primary neuronal culture, transfection, immunocytochemistry, and image acquisition and analysis.** Hippocampal and cortical mouse neuron cultures were prepared from embryonic day 17 (E17) Slitk2-floxed mouse embryos, as previously described. In brief, neurons were seeded on 18-mm poly-D-lysine (0.1 mg/ml)-coated coverslips and cultured in Neurobasal media (Gibco) containing penicillin-streptomycin and 0.5 mM GlutaMax (Thermo Fisher) supplemented with 2% B-27 (Thermo Fisher) and 0.5% FBS (HyClone). Cultured hippocampal neurons were infected with lentiviruses expressing Cre or inactive Cre (ACre) recombinase at DIV5, and immunostained at DIV12 or DIV14. For rescue experiments, hippocampal neurons were transfected at DIV8–9 with SLITRK2 WT or the indicated point variants using a CalPhos Kit (Clontech) and incubated at DIV13–14; controls were transfected with EGFP. For immunocytochemistry, cultured rat neurons were fixed with 4% paraformaldehyde/hyde/4% sucrose in PBS for 30 min at 4 °C and permeabilized with 0.2% Triton X-100 in PBS for 30 min at 4 °C. Neurons were then blocked by incubating with 3% horse serum/0.1% RSA in PBS for 15 min at room temperature, then stained with the indicated primary and secondary antibodies for 70 min at room temperature. Z-stack images of randomly selected neurons were acquired using a confocal microscope (LSM780; Carl Zeiss) with a ×63 objective lens. Obtained Z-stack images were converted to maximal projections, and colocalized puncta densities were analyzed in a blinded manner using MetaMorph software (Molecular Devices Corp.).

**Sholl analysis.** Rat cultured hippocampal neurons were co-transfected with EGFP and SLITRK2 WT or the indicated SLITRK2 variants for 5 d at DIV9. Transfected neurons were stained with anti-EGFP and anti-HA antibodies, and costained neurons were used for quantitative analysis. Dendritic branching was analyzed by performing a Sholl analysis using the Scoll Analysis plugin installed in ImageJ software (Fiji; RRID: SCR_002285). The number of branches at increasing distances from the soma was quantified.

**Stereotactic surgery.** Mice were anesthetized with isoflurane (3–4%) and then placed in a stereotactic device. Viral solutions (titers ≥1 × 10^{11} viral genomes/ml) were injected bilaterally at a rate of 0.2 μl/min with a NanoFlit syringe and a Nanoliter 1000 Injector (World Precision Instruments). The AP = 2.5 mm, ML ± 1.7 mm, and DV = –1.3 mm (from the dura) coordinates were used for hippocampal CA1 regions. Mice were used 23 weeks after stereotactic surgeries.

**Immunohistochemistry.** Mice were transcardially perfused first with PBS for 3 min and then with 4% paraformaldehyde in PBS for 5 min. Post-fixation overnight, mouse brains were slowly sectioned at a thickness of 40 μm using a vibratome (VT1200S; Leica). Brain sections were permeabilized by incubation with 0.2% Triton X-100 in PBS containing 10% horse serum and 0.2% RSA for 1 h at room temperature. For immunostaining, sections were incubated overnight at 4 °C with primary anti-VGLUT1 (1:500), anti-PSD-95 (1:500), or anti-VGAT (1:500) antibodies diluted in the same blocking solution. After washing three times, sections were incubated with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch) for 1 h at room temperature. Sections were then washed extensively and mounted on glass slides with Vectashield Mounting Medium (Vector Laboratories). Images were acquired by confocal microscopy (LSM780; Zeiss). Synaptic puncta were quantified using MetaMorph software (Molecular Devices), and their density and average area were measured.

**Mouse behavioral tests.** Male mice at the age of 6–10 weeks were used for all behavioral tests. Tests were conducted in a sound-proof room under dim lighting (∼5 lux).

**Open-field test.** Each mouse was placed into an open-field apparatus (40 × 40 × 40 cm) and allowed to freely explore the field for 30 min. The total distance traveled and time spent in the center area were recorded by a top-view infrared camera and analyzed using EthoVision XT 10.5 (Noldus).

**Three-chamber test.** The testing apparatus consisted of a white acrylic box divided into three chambers (20 × 40 × 22 cm each) with small openings on the dividing walls. Wire cages were employed to enclose social conspecifics in the corners of both sides of the chamber. A mouse was placed in the central chamber for a 10-min habituation. Following habituation, an age-matched social conspecific was placed into the wire cup on the left side-chamber, and the sociability of the subjects was assessed by measuring subjects’ exploration times for the enclosed conspecific and the empty cup during the second 10-min session. An exploration was counted when the subject deflected their nose into the vicinity of the wire cups. In the last 10-min session, a new social conspecific was placed into the empty wire cup on the right side-chamber, and social recognition was assessed by measuring subjects’ exploration times for the familiar conspecific and the novel conspecific in a wire cup.

**Novel object-recognition test.** Object recognition test was performed in the open-field box. On the first day, mice were habituated to the maze for more than 10 min. On the subsequent day, mice were allowed to explore two identical objects twice for 10 min. Exploration time for each object was measured. Twelve hours later, one of two objects was replaced with a new distinct object and mice were returned to the field and allowed to explore for 10 min to test novel-object recognition. Exploration time for each object was measured.

**Barnes maze test.** The Barnes maze test was conducted on a white circular platform, 95 cm in diameter, with 20 holes equally spaced around the perimeter. The hole above the escape box represented the target. The location of the target was consistent for a given mouse but randomized across mice. The maze was rotated daily, with the spatial location of the target unchanged with respect to the distal visual room cues, to prevent a bias based on olfactory or proximal cues within the maze. Three trials per day were conducted for 7 successive days. On day 10, the first probe trial was conducted without the escape box to confirm that the spatial task was acquired based on navigation by distal environment room cues. On day 17, the second probe trial was conducted. During acquisition and probe trials, the following parameters were measured by a top-view infrared camera and analyzed using EthoVision XT 10.5 (Noldus): velocity, error score, total distance, first visit time to escape box, and total latency.
Grip strength test. A grip strength meter (BioSEB) was used to measure forelimb grip strength. A mouse was allowed to grasp the steel bar mounted on the force gauge. The gauge was reset after stabilization, and the mouse’s tail was slowly pulled back by an experimenter. Maximal grip force was automatically recorded as newtons by the gauge. We performed 3 consecutive measurements per day for 3 consecutive days.

Gait test. The forepaws and hind paws of a mouse were coated with different colored nontoxic acrylic paints. The mice were trained to run down the runway (30-cm long and 5-cm wide) in a straight line before the test. The footprints were traced on the white paper covering a runway. The footprints were analyzed for overlap, each side of stride length, each side of stance length, and each side of sway length. All parameters were analyzed by MetaMorph software (Molecular Devices Corp.).

Electrophysiology
Cultured neuron electrophysiology. Hippocampal cultured neurons obtained from Slitrk2–cKO mice were infected on DIV4 with lentiviruses encoding Cre-EGFP or ΔCre-EGFP, followed by analysis at DIV13–16. Similarly, hippocampal cultured neurons obtained from Nestin–Slitrk2 mice were analyzed at DIV13–16. Pipettes were pulled from borosilicate glass (o.d. 1.5 mm, i.d. 0.86 mm; Sutter Instruments) using a Model P-97 pipette puller (Sutter Instruments). The resistance of patch pipettes filled with internal solution varied between 3 and 8 MΩ. The internal solution contained 145 mM CsCl, 5 mM NaCl, 10 mM HEPES, 10 mM EGTA, 0.3 mM MgATP and 4 mM MgCl2, with pH adjusted to 7.2–7.4 with CsOH and an osmolality of 290–295 mOsmol/l. The external solution consisted of 130 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM D-glucose, with pH adjusted to 7.2–7.4 with NaOH and an osmolality of 300–305 mOsmol/l. The whole-cell configuration was obtained at room temperature using µl-TSC manipulators (SENSAPEX). Electrophysiological data were acquired with a Multiclamp 700B amplifier (Axon instrument) and pCLAMP software, and were digitized using an Axon Digidata 1550B data acquisition board (Axon Instruments). mEPSCs were recorded at a holding potential of −70 mV. Synaptic currents were analyzed offline using Clampfit 10.8 software (Molecular Devices). For mEPSC recordings, the external solution contained 1 mM TTX and 50 µM picrotoxin to block GABA receptor and Na+ currents, respectively. For mIPSC recordings, the external solution contained 1 mM TTX, 10 µM CNQX, and 50 µM D-AP5 to block Na+ currents, AMPA receptors, and NMDA receptors, respectively.

Hippocampal CA1 pyramidal neuron electrophysiology. Whole-cell voltage-clamp recordings were obtained from acute brain slices. Brain slices were transferred to a recording chamber and perfused with a bath solution of aerated (O2 95%/CO2 5%) artificial cerebrospinal fluid (aCSF) consisting of 124 mM NaCl, 3.3 mM KCl, 1.3 mM NaH2PO4, 26 mM NaHCO3, 11 mM D-glucose, 2.5 mM CaCl2, and 0.3 mM Na–GTP. Whole-cell recordings of mEPSCs were performed on CA1 pyramidal neurons, voltage clamped at −70 mV, and currents were pharmacologically isolated by bath applications of 50 µM picrotoxin (Tocris), 1 µM tetrodotoxin (Tocris). Electrophysiological data were acquired using pCLAMP software and a MultiClamp 700B (Axon Instruments) and were digitized using an Axon Digitax 1550B data acquisition board (Axon Instruments). mEPSCs were recorded at a holding potential of −70 mV. Synaptic currents were analyzed offline using Clampfit 10.8 software (Molecular Devices). For mEPSC recordings, the external solution contained 1 mM TTX and 50 µM picrotoxin to block GABA receptor and Na+ currents, respectively. For mIPSC recordings, the external solution contained 1 mM TTX, 10 µM CNQX, and 50 µM D-AP5 to block Na+ currents, AMPA receptors, and NMDA receptors, respectively.

Data analyses. Data analysis and statistical tests were performed using GraphPad Prism® software (RRID: SCR_002798). Heterologous synapse-formation assays and surface-bounding assays were quantified by randomly selecting transfected HEK293T cells as the region of interest. The fluorescence intensities of synaptic marker puncta or Fc-fusion proteins were normalized to transfected protein signal intensities using MetaMorph software (Molecular Devices Corp.). All data are expressed as means ± standard error of the mean (SEM) unless stated otherwise, and significance is indicated with an asterisk. All experiments were performed using at least three independent mice, cultures, and/or cohorts of grouped mice, and the normality of data distributions was evaluated using the Shapiro–Wilk test. Data were compared using Student’s t-test or one-way analysis of variance (ANOVA) using a non-parametric Kruskal–Wallis test, followed by Dunn’s multiple comparison test for post hoc group comparisons, t-test, Mann–Whitney U test, or Fisher’s least significance difference; ‘n’ values used are indicated in the figure legends, and numbers shown indicate replicates. Tests used to determine statistical significance are stated in the text and legends of figures depicting the results of the respective experiments. A p-value < 0.05 was considered statistically significant, and individual p-values are indicated in the respective figure legend. All experiments were performed and analyzed in a blind manner by the experimenter.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Data sets presented in this study are included in full wherever possible, including the display of individual data points. All relevant data supporting the findings of this study are available from the corresponding authors upon reasonable request. Biological materials, including mutant mice and custom antibodies generated for this study, will be shared upon request within the limits of respective material transfer agreements for as long as they are available in the laboratory. Source data are provided with this paper.

Code availability
All relevant code supporting the findings of this study is available from the corresponding authors upon request.

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Competing interests

The authors declare no competing interests.

Additional information

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