Distinct but overlapping roles of LRRTM1 and LRRTM2 in developing and mature hippocampal circuits

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Abstract LRRTMs are postsynaptic cell adhesion proteins that have region-restricted expression in the brain. To determine their role in the molecular organization of synapses in vivo, we studied synapse development and plasticity in hippocampal neuronal circuits in mice lacking both Lrrtm1 and Lrrtm2. We found that LRRTM1 and LRRTM2 regulate the density and morphological integrity of excitatory synapses on CA1 pyramidal neurons in the developing brain but are not essential for these roles in the mature circuit. Further, they are required for long-term potentiation in the CA3-CA1 pathway and the dentate gyrus, and for enduring fear memory in both the developing and mature brain. Our data show that LRRTM1 and LRRTM2 regulate synapse development and function in a cell-type and developmental-stage-specific manner, and thereby contribute to the fine-tuning of hippocampal circuit connectivity and plasticity.

Editor’s evaluation
This study aims to systematically investigate the role of LRRTM 1 and 2 in hippocampal circuit function. These neuronal proteins are organizing proteins in the synapse and are known to play a role...
in development and plasticity. The current literature is unclear on the exact role these proteins play due to the diverse technical approaches used in previous studies which often led to contradictory findings. The current study aims to clearly identify the roles of these proteins by using a thorough experimental design. The clear strength of the study is in the systematic and careful comparison between various approaches that aim to eliminate LRRTM1/2 from the synapses and the thorough examination of several parameters under these different conditions.

Introduction

Neurons are interconnected by specialized and highly differentiated synaptic junctions. Synapse organizing proteins coordinate the precise alignment of pre- and post-synaptic components of a synapse and impart them with distinct physiological properties necessary for neuronal circuits to function and control behavior. Presynaptic neurexins, arguably the best characterized synapse organizers, bind to multiple postsynaptic ligands, including neuroligins and leucine-rich-repeat transmembrane neuronal proteins (LRRTMs) (Südhof, 2017), and act as hubs for presynaptic development and signaling (Reissner et al., 2013; Südhof, 2017).

The LRRTMs are a family of four postsynaptic transmembrane proteins that instruct presynaptic differentiation in contacting axons, and mediate postsynaptic recruitment of scaffold proteins and transmitter receptors (Lindhoff et al., 2009; Roppongi et al., 2017). In cell culture studies, LRRTM1 and LRRTM2 exhibited similar synaptogenic activities through dual binding to the heparan sulfate modification and splice variants of neuromodulin (Roppongi et al., 2020; Zhang et al., 2018) indicating that LRRTM1 and LRRTM2 likely have overlapping functions. In contrast to neuroligins, which have broad expression profiles (Varoqueaux et al., 2006), individual LRRTMs are expressed in a region-restricted manner in the brain (Laurêns et al., 2003). Within the hippocampus, for instance, LRRTM1 and LRRTM2 are the only family members expressed in the CA1 region, whereas all LRRTMs are expressed in the dentate gyrus (DG). Accordingly, LRRTMs are thought to function as cell-type selective synapse organizers, but the exact roles of LRRTM1 and LRRTM2 in synapse development have remained unresolved as corresponding studies yielded divergent and partially contradictory results. ShRNA-based knock-down (KD) studies in cultured neurons or brain slices produced differing results (de Wit et al., 2009; Ko et al., 2011; Soler-Llavina et al., 2011), where KD of LRRTM2 alone reduced the numbers of excitatory synapses (de Wit et al., 2009) whereas combined KD of LRRTM1 and LRRTM2 did not affect synapse numbers (Ko et al., 2011). Further, KD or genetic knock-out (KO) of Lrutm1 in mice causes a~10% loss of excitatory synapses and a redistribution of synaptosomal vesicles in certain sublaminae of the hippocampal CA1 region (Lindhoff et al., 2009; Schroeder et al., 2018; Takashima et al., 2011). These subtle alterations are likely due to functional compensation by LRRTM2, which is co-expressed with LRRTM1 in the CA1 region. Finally, it is unclear whether the synapse loss caused by Lrutm1 deletion is due to a role of LRRTM1 in synapse formation or the prevention of their elimination. While the reasons for the multiple discrepant data on LRRTM1 and LRRTM2 are not known, it is likely that the well-known confounds of KD approaches, overexpression artifacts, and ectopic synaptogenic activity have played a major role in in vitro studies but less so in experiments involving in vivo models (Südhof, 2018).

Due to their role in synapse formation, maturation, and function, LRRTM1 and LRRTM2 are critical players in multiple aspects of circuit and brain function. Most notably, LRRTM1 and LRRTM2 contribute to long-term potentiation (LTP) in CA1 pyramidal neurons. This is likely due to their ability to anchor AMPA receptors at synapses (Bhouri et al., 2018; Ramsey et al., 2021), but what is not known in this context is whether LRRTMs are required for LTP in other cell-types that express them. This is an important open question because synaptic plasticity is a partially cell-type specific phenomenon (Larsen and Sjöström, 2015). In human, alterations in LRRTM genes are associated with several neuropsychiatric disorders, including autism, schizophrenia, and bipolar disorder (Francks et al., 2007; Leach et al., 2014; Ludwig et al., 2009; Malhotra et al., 2011; Pinto et al., 2010), and in partial accord with human genetics, constitutive or conditional Lrutm1 KO mice exhibit defined forms of cognitive impairment (Karimi et al., 2021; Takashima et al., 2011; Voikar et al., 2013). However, it is unclear what neural circuit origins these dysfunctions have or whether the contribution of LRRTM1 and LRRTM2 to cognitive processes stems from their role in developmental synapse organization or synaptic plasticity.
In the present study, we devised spatially and temporally controlled genetic approaches in mice to investigate the contributions of LRRTM1 and LRRTM2 to synapse organization, synaptic plasticity, and cognitive function in the developing and mature brain. We assessed the joint roles of LRRTM1 and LRRTM2 in the hippocampal CA1 region, where LRRTM3 and LRRTM4 are not expressed, and compared their role in the dentate gyrus, where all four LRRTMs are co-expressed. Our results reveal cell-type and development-stage dependent functions of these LRRTMs in synapse development and synaptic transmission, but broad roles in synaptic plasticity and cognitive function, indicating a context-dependent role for these synapse organizers in the molecular organization of neural circuits.

Results

Basic characteristics of constitutive Lrrtm1 and Lrrtm2 (LRRTM1/2) double KO (DKO) mice

To investigate the role of LRRTM1 and LRRTM2 in developing neural circuits, we generated constitutive LRRTM1/2-DKO mice. Briefly, the major coding exon 2 was excised in both Lrrtm1 and Lrrtm2 by crossing the Lrrtm1 floxed/floxed and Lrrtm2 floxed/floxed mice (Bhouri et al., 2018) with the Ella-Cre line (Lakso et al., 1996; Figure 1—figure supplement 1). The heterozygous mice thus generated were crossed to obtain homozygous KO mice. Single KOs were then crossed to obtain LRRTM1/2-DKOs. The expected complete loss of LRRTM1 and LRRTM2 was confirmed by western blotting of brain homogenates (Figure 1—figure supplement 1). The heterozygous mice thus generated were crossed to obtain homozygous KO mice. Single KOs were then crossed to obtain LRRTM1/2-DKOs. The expected complete loss of LRRTM1 and LRRTM2 was confirmed by western blotting of brain homogenates (Figure 1—figure supplement 1). Studies were performed on DKO mice and control wild-type (WT) mice, derived from the same double heterozygous parents and matched for age and sex.

LRRTM1/2-DKOs survived and bred normally at the predicted Mendelian frequencies, had normal body weight, and exhibited no obvious behavioral abnormalities in the home cage environment. Further, LRRTM1/2-DKOs were indistinguishable from WT mice with respect to gross brain morphology and cytoarchitectural organization as assessed by confocal microscopy analysis of brain sections labeled for the nuclear marker DAPI and the synaptic markers vesicular glutamate transporter (VGlut1) and glutamic acid decarboxylase (GAD65) (Figure 1A). To determine whether embryonic loss of Lrrtm1 and Lrrtm2 alters synaptic composition globally in the brain, we prepared crude synaptosomal fractions from whole brain of LRRTM1/2-DKO and control WT mice at 6–7 weeks postnatally (Figure 1—figure supplement 2). Quantitative immunoblotting of these fractions revealed no differences in the levels of the active zone proteins bassoon and CASK, the SNARE proteins SNAP-25 and VAMP2, the synaptic vesicle markers VGLut1 and synapsin, the inhibitory synapse scaffolding protein gephyrin, the excitatory synapse scaffolding proteins PSD95, SAPAP, SHANK, and SynGAP, the ionotropic glutamate receptor subunits GluA2, GluN1, and GluN2A, and the adhesion/receptor proteins neuroligin 1, neuroligin 2, LRRTM4, and TrkC. A small but significant increase in the level of the synaptic vesicle protein synaptophysin was observed in LRRTM1/2-DKO synaptosomes. These results indicate that loss of LRRTM1 and LRRTM2 does not substantially affect global synaptic protein content.

LRRTM1 and LRRTM2 control the numbers of excitatory synapses and spines selectively in CA1

In the assays described above, changes in the protein composition of synapses in specific brain regions might have gone unnoticed due to contributions from unaffected brain regions. We therefore focused further analyses on specific circuits, comparing the hippocampal CA1 region with the dentate gyrus. To this end, we performed high-resolution confocal imaging of markers of excitatory and inhibitory synapses in CA1 and dentate gyrus dendritic layers, at 6–7 weeks postnatally. As compared to control WT mice, quantitative confocal analysis of LRRTM1/2-DKOs revealed significantly reduced VGlut1 puncta immunofluorescence in all CA1 dendritic layers, that is stratum oriens, stratum radiatum, and stratum lacunosum (Figure 1B and C). In the dentate gyrus, however, VGlut1 puncta immunofluorescence was elevated in the outer molecular layer but remained unchanged in the inner and medial molecular layers of LRRTM1/2-DKOs when compared to controls (Figure 1D and E), indicating potential compensation by other synapse organizers in the dentate gyrus of LRRTM1/2-DKOs. Puncta immunofluorescence of GAD65 in the same co-labeled regions in both the CA1 and dentate gyrus was comparable between LRRTM1/2-DKO and control mice (Figure 1—figure supplement 3). Thus,
**Figure 1.** LRRTM1 and LRRTM2 control excitatory presynapse development in the hippocampus. (A) Confocal immunofluorescence images for VGlut1, GAD65, and the nuclear marker DAPI revealed normal hippocampal morphology and large-scale synaptic organization in LRRTM1/2-DKO mice compared with wild-type (WT) mice. Scale bar is 500 µm. (B and D) High-resolution confocal images revealed a reduction in punctate immunofluorescence for the excitatory presynapse marker VGlut1 in the CA1 dendritic layers oriens, radiatum and lacunosum, and increase in outer molecular layer of dentate gyrus (DG) in LRRTM1/2-DKO mice as compared with WT mice at 6 weeks postnatal. Scale bar is 10 µm for (B) and 20 µm for (D). (C and E) Quantitation of VGlut1 punctate integrated intensity per tissue area (Multiple t-test, *p<0.05, **p<0.01 comparing LRRTM1/2-DKO and wild-type mice for CA1 and DG, n=3–5 mice each after averaging data from 6 sections per mouse). Data presented as mean ± SEM.

The online version of this article includes the following source data and figure supplement(s) for figure 1:

**Source data 1.** Source data related to Figure 1B, C.

**Source data 2.** Source data related to Figure 1D, E.

**Figure supplement 1.** LRRTM1/2-DKO generation and confirmation of loss of LRRTM1 and LRRTM2.

**Figure supplement 1—source data 1.** Figure 1—figure supplement 1 source blots.

**Figure supplement 2.** Levels of synaptic proteins in crude synaptosomal brain fractions of LRRTM1/2-DKO mice.

**Figure supplement 2—source data 1.** Figure 1—figure supplement 2 source blots.

**Figure supplement 3.** Inhibitory presynapse development is unaltered in CA1 and DG of LRRTM1/2-DKO.

**Figure supplement 3—source data 1.** Figure 1—figure supplement 3 source data related to 1B.

**Figure supplement 3—source data 2.** Figure 1—figure supplement 3 source data related to 1D.
LRRTM1 and LRRTM2 contribute to the development of excitatory presynapses differentially in hippocampal regions.

In the hippocampus, prominent populations of excitatory synapses are formed on dendritic spines of CA1 pyramidal neurons and on dentate gyrus granule cells (Harris and Kater, 1994; Trommald and Hulleberg, 1997). We measured the density of spines on CA1 pyramidal neurons in Golgi-stained brain sections, focusing on CA1 stratum radiatum, which predominantly contains inputs from CA3 Schaffer collaterals. Spine density was significantly reduced in CA1 pyramidal neurons of LRRTM1/2-DKOs as compared to WT cells (Figure 2A and B). In contrast, spine density in dentate gyrus granule cell medial molecular layer was unchanged in LRRTM1/2-DKOs (Figure 2A and B). Thus, LRRTM1 and LRRTM2 are required for the generation of normal numbers of dendritic spines in CA1 pyramidal neurons but are dispensable in this context in dentate gyrus granule cells.

Reductions in VGlut1 puncta immunofluorescence and spine density in the CA1 region of LRRTM1/2-DKOs indicate overall reductions in the number of excitatory synapses in this region. To assess synapse numbers by a more rigorous method, we used transmission electron microscopy. The density of asymmetric synapses, representing excitatory synapses, measured on dendritic spines was significantly reduced (27% decrease) in CA1 stratum radiatum of LRRTM1/2-DKOs as compared to WT controls (Figure 2C and D). As LRRTM1 and LRRTM2 are postsynaptic proteins, we also tested whether spine morphology was altered in LRRTM1/2-DKO mice. However, spine width in the CA1 stratum radiatum was indistinguishable between LRRTM1/2-DKO and WT mice (Figure 2E). Overall, our data indicate that LRRTM1 and LRRTM2 are required for the normal development of excitatory synapses and spines in the CA1 region.

**Excitatory neurotransmission is differentially altered in the CA1 and dentate gyrus of LRRTM1/2-DKOs**

We next tested whether the function of excitatory synapses is compromised in LRRTM1/2-DKOs. We performed whole-cell voltage clamp recordings from CA1 pyramidal neurons and dentate gyrus granule cells in hippocampal slices from LRRTM1/2-DKO and control WT mice. Miniature excitatory postsynaptic current (mEPSC) recordings from LRRTM1/2-DKO CA1 pyramidal neurons revealed a significant reduction in mEPSC frequency compared with control WT neurons (Figure 3A and B). mEPSC amplitudes were not significantly different between the groups (Figure 3A and C). In contrast, mEPSC frequency but not amplitude was increased in the LRRTM1/2-DKO dentate gyrus granule cells when compared to data from control WT mice (Figure 3D–F). Thus, LRRTM1 and LRRTM2 contribute to the development of functional excitatory synapses in CA1 pyramidal neurons but their loss in dentate gyrus during early development may be compensated by other synapse organizers. The changes in mEPSC frequency but not amplitude in the CA1 pyramidal neurons are consistent with...
corresponding changes in imaging and electron microscopy data, which indicated roles for LRRTM1 and LRRTM2 in controlling the number of excitatory synapses in CA1 pyramidal neurons.

**LRRTM1 and LRRTM2 regulate hippocampal synaptic plasticity and memory**

We assessed presynaptic function in CA1 pyramidal neurons of LRRTM1/2-DKOs. We observed modest changes in paired-pulse facilitation at more delayed inter-stimulus intervals (ISIs) in LRRTM1/2-DKO compared to WT controls (Figure 4—figure supplement 1). Next, we assessed LTP at CA3-CA1 synapses after chronic deletion of LRRTM1 and LRRTM2. LTP was induced with a single train of high frequency stimulation (HFS; 1 s at 100 Hz). Both WT and LRRTM1/2-DKO slices exhibited an

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**Figure 3.** Excitatory synapse function is differentially altered in the CA1 and DG of LRRTM1/2-DKO mice. (A and D) Representative mEPSC recordings from wild-type (WT) and LRRTM1/2-DKO hippocampus CA1 pyramidal neurons and DG granule cells. (B and C) Cumulative distributions of mEPSC inter-event intervals (B) and amplitudes (C) in wild-type and LRRTM1/2-DKO neurons. Insets display mean ± SEM for mEPSC frequency (B) and amplitude (C) in CA1 pyramidal cells, respectively. Double knockout of Lrrtm1 and Lrrtm2 decreased mEPSC frequency, Student’s t-test, **p=0.0090, without affecting mEPSC amplitude, p=0.6873, n=10–11 neurons per group, three mice each. (E and F) Cumulative distributions of mEPSC inter-event intervals (E) and amplitudes (F) in wild-type and LRRTM1/2-DKO DG granule cells. Insets display mean ± SEM for mEPSC frequency (E) and amplitude (F) in DG granule cells, respectively. Double knockout of Lrrtm1 and Lrrtm2 increased mEPSC frequency, Student’s t-test, ***p=0.0004, without affecting mEPSC amplitude, p=0.4731, n=11–15 neurons per group, three mice each.

The online version of this article includes the following source data for figure 3:

**Source data 1.** Source data for Figure 3B, C.

**Source data 2.** Source data for Figure 3E, F.
initial potentiation of the average field excitatory postsynaptic potential (fEPSP) (Figure 4A). However, whereas fEPSP values recorded 40–60 min after HFS revealed potentiation to 126% ± 4% of baseline in slices from WT mice, a significant loss of potentiation, with fEPSP values at 106% ± 6% of baseline, was observed in LRRTM1/2-DKO slices (Figure 4A and B). This deficit at later but not early phases following LTP induction is consistent with a role of LRRTM1 and LRRTM2 in the postsynaptic maintenance of synaptic potentiation. These results also indicate that the LTP deficits caused by the loss of LRRTM1 and LRRTM2 in CA1 cannot be compensated by other proteins.

Impaired hippocampal LTP has been linked to reduced performance in behavioral memory tasks (Nabavi et al., 2014; Orsini and Maren, 2012; Schafe et al., 2001). Moreover, reduced synapse numbers in the CA1 and altered synaptic transmission in the CA1 and dentate gyrus of LRRTM1/2-DKOs likely influence hippocampal network function. To determine if hippocampus-dependent memory is compromised in LRRTM1/2-DKO mice, we used a classical fear conditioning paradigm to assess contextual memory (Figure 4C). Mice were trained to associate a foot shock with a particular context, and learning and memory was assessed as extent of freezing in response to subsequent exposure to the context only. At 24 hr post-training, LRRTM1/2-DKO mice exhibited significantly reduced levels of freezing compared to WT controls (Figure 4D). These data indicate that LRRTM1 and LRRTM2 are required for the maintenance of long-term contextual memories.

**Local deletion of LRRTM1/2 in the CA1 of developing brain impairs excitatory neurotransmission**

Although excitatory synaptic transmission in the CA1 of mice lacking LRRTM1 and LRRTM2 globally (LRRTM1/2-DKO) is impaired, the global deletion approach does not allow one to distinguish whether the observed deficits are due to presynaptic or postsynaptic changes. To resolve this issue, we deleted Lrtn1 and Lrtn2 selectively in the CA1 of postnatal 0 (P0) mice. To do so, we delivered AAV8-hSyn-Cre-eGFP (P0-LRRTM1/2-cDKO) or AAV8-hSyn-eGFP (control) to the dorsal CA1 of P0 Lrtn1flxed/
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floxed/Lrrtm2floxed/floxed mice (**Figure 5—figure supplement 1**). All analyses were done at 6–7 weeks of age, the same age as for analyses of the global LRRTM1/2-DKO. We performed whole-cell voltage clamp recordings from CA1 pyramidal neurons of P0-LRRTM1/2-cDKO and control mice. Similar to the LRRTM1/2-DKO mice, mEPSC frequency was significantly reduced in the CA1 pyramidal neurons of P0-LRRTM1/2-cDKO as compared to control mice (**Figure 5A and B**). A modest reduction in mEPSC amplitude was also observed in the P0-LRRTM1/2-cDKOs (**Figure 5C**). Thus, reduced synaptic transmission in the CA1 of P0-LRRTM1/2-cDKO are due to postsynaptic deficits.

**LRRTM1 and LRRTM2 are not required for maintaining synapse numbers in the mature brain**

Next, we investigated the role of LRRTM1 and LRRTM2 in synapse development and plasticity in the mature brain. We therefore generated mice in which Lrrtm1 and Lrrtm2 were acutely deleted in the adult hippocampus. To do so, we delivered AAV8-hSyn-Cre-eGFP (LRRTM1/2-cDKO) or AAV8-hSyn-eGFP (control) to the dorsal hippocampus of 3 weeks old Lrrtm1floxed/floxed/Lrrtm2floxed/floxed mice (**Figure 6A and B**). Given that the half-lifes of LRRTM1 and LRRTM2 are approximately 3 and 7 days, respectively, in mouse cortical synapses (Fornasiero et al., 2018), all analyses were done 3 weeks after virus delivery which should allow for the effective expression of Cre recombinase and deletion of the targeted genes. Thus, all analyses were done at 6–7 weeks of age, the same age as for the Global DKO and Local early cDKO. We obtained ~98% (dorsal CA1) and ~90% (dorsal dentate gyrus) maximal infection efficiency at the site of injection (average efficiency of infection in dorsal hippocampus was ~75%), as assessed by GFP+ DAPI cells (**Figure 6C**). Both LRRTM1 and LRRTM2 were markedly depleted in the CA1 of LRRTM1/2-cDKO when compared to that in controls (**Figure 6—figure supplement 1**). All analyses for synapse numbers and plasticity were done near the sites of maximal infection. Virus spread into the ventral CA1 and dentate gyrus was significantly lower (~10%), thus allowing us to selectively assess the functions of LRRTM1 and LRRTM2 in the mature dorsal CA1 and dentate gyrus.

We first performed apotome-enabled optical sectioning and imaging of VGlut1 and GAD65 in CA1 and DG dendritic layers. Quantitative analysis revealed that puncta immunofluorescence for VGlut1...
Figure 6. Excitatory presynapse development is unaltered in CA1 dendritic layers and DG molecular layers of LRRTM1/2-cDKO. (A) Representative image of dorsal hippocampus (injection site) with infected CA1 and dentate gyrus neurons (green) from AAV8-hSYN-Cre-eGFP. Below, zoomed in images of the dorsal CA1 and dentate gyrus. Scale bar is 20 µm. (B) Representative images of whole brain (modified from Allen Brain Institute) showing bregma points −1.34, −2.06, and −2.80, with 2.06 AP as the injection site in LRRTM1/2-cDKO mice and controls. (C) Percent infected cells (Number of GFP+ neurons / Number of DAPI+ neurons) across the dorso-ventral axis for CA1 and dentate gyrus. (D and F) High-resolution immunofluorescence of VGlut1 punctate was unaltered in the dendritic layers of CA1 and DG molecular layers in LRRTM1/2-cDKO mice as compared with control mice. Scale bar is 5 µm. (E and G) Quantitation of VGlut1 punctate integrated intensity per tissue area, (Multiple t-test, n=3 mice each after averaging data from 6 sections per mouse). Data presented as mean ± SEM.

The online version of this article includes the following source data and figure supplement(s) for figure 6:

Source data 1. Figure 6 source data for 6C.
Source data 2. Figure 6 source data for 6DG.
Figure supplement 1. LRRTM1 and LRRTM2 knockout confirmation.
Figure supplement 2. Inhibitory presynapse development is unaltered in CA1 and DG of LRRTM1/2-cDKO mice.
Figure supplement 2—source data 1. Figure 6—figure supplement 2 source data.
was comparable between the LRRTM1/2-cDKO and control mice in all CA1 (stratum oriens, stratum radiatum, stratum lacunosum moleculare) and dentate gyrus layers (inner, medial, outer molecular layer) (Figure 6D–G). Likewise, GAD65 puncta immunofluorescence was comparable between LRRTM1/2-cDKO and control mice in all CA1 and DG dendritic layers (Figure 6—figure supplement 2). Thus, while required for synapse formation in the developing brain, LRRTM1 and LRRTM2 are dispensable for maintaining synapse numbers in the mature brain.

Next, we investigated the role of LRRTM1 and LRRTM2 in synaptic transmission in the mature CA1 and dentate gyrus. Consistent with our previous report (Bhouri et al., 2018), whereas LRRTM1 and LRRTM2 are required for normal mEPSC frequency in the CA1 of developing brain without affecting mEPSC amplitudes (Figure 3A–C), the opposite is the case in the mature circuit, where LRRTM1 and LRRTM2 loss in both CA1 and DG affects the amplitude but not the frequency of mEPSCs.
Thus, LRRTM1 and LRRTM2 regulate synaptic transmission differentially in the developing and the mature brain.

**LRRTM1 and LRRTM2 are required for LTP in multiple neural circuits in the mature brain**

Knockout of Lrmt1 and Lrmt2 in sparsely targeted adult hippocampal CA1 impaired LTP in individual pyramidal neurons (Bhouri et al., 2018). As we achieved an average of ~85% virus spread across the dorsal CA1 (dorsoventral axis ~1.54 to ~2.30 from bregma), we investigated LTP upon widespread KO of LRRTM1 and LRRTM2. We assessed LTP using field recordings of evoked fEPSPs. LTP was induced by high frequency stimulation of Schaffer collaterals (2 × 100 Hz, 20 s interval) and fEPSPs were recorded. Whereas fEPSPs after stimulation increased to 228% ± 16.57% in control mice, LTP was significantly impaired in LRRTM1/2-cDKO mice (144.4% ± 17.5%) (Figure 8A–C). To assess whether pre-synaptic function may be compromised in the CA1 of LRRTM1/2-cDKO mice, we examined paired-pulse facilitation (Figure 8—figure supplement 1). There was no difference between control and LRRTM1/2-cDKO mice suggesting that mechanisms underlying short-term facilitation are not impacted by local deletion of Lrmt1 and Lrmt2 in the mature CA1.
Next, we assessed whether acute deletion of Lrmt1 and Lrmt2 alters LTP in the dentate gyrus, a region with strong co-expression of LRRTM3 and LRRTM4. We achieved an average of ~65% virus spread across the dorsal dentate gyrus (dorsoventral axis −1.54 to −2.30 from bregma). We induced LTP in the inner molecular layer of the dentate gyrus with a high frequency stimulation (4 × 100 Hz, 5 min interval). Whereas fEPSPs after stimulation increased to 203.1% ± 24% in control mice, LTP was significantly impaired in LRRTM1/2-cDKO mice (126% ± 13.84%) (Figure 8D–F). These results show that LRRTM3 and LRRTM4 cannot functionally compensate the effect of the loss of LRRTM1 and LRRTM2 on LTP, indicating that LRRTM1 and LRRTM2 control LTP by a distinct mechanism.

**LRRTM1 and LRRTM2 are required for dorsal hippocampus-dependent cognitive function in the mature brain**

The effective loss of LRRTM1 and LRRTM2 in vivo significantly reduced both basal synaptic strength and LTP in the dorsal CA1 region and the DG. These deficits could alter network function and consequently alter cognitive functions associated with the dorsal hippocampus (Fanselow and Dong, 2010).

A prerequisite for assessing cognitive function is normal locomotor activity and motor coordination. To assess gross motor skills, we used an accelerating rotarod with an acceleration rate of 20 rpm/min. The latency to fall was comparable between the LRRTM1/2-cDKO and control groups (Figure 9—figure supplement 1A). We then assessed locomotor performance using the open field test. Average speed and distance travelled were again comparable between LRRTM1/2-cDKOs and control animals (Figure 9—figure supplement 1C and D), indicating that locomotion is not affected by acute deletion of Lrmt1 and Lrmt2 in the dorsal hippocampus. The open-field test is frequently used to infer risk-associated anxiety levels in mice (Seibenhener and Wooten, 2015) as mice with high anxiety levels spend considerably less time in the inner zone of the open field. Time spent in the inner and outer zones of the open field was equivalent between the genotypes (Figure 9—figure supplement 1B), indicating that deletion of Lrmt1 and Lrmt2 in the dorsal hippocampus does not increase risk-associated anxiety behavior. We then used the more sensitive elevated plus maze (EPM) test to assess avoidance-related anxiety behavior, where mice are allowed to explore open and closed arms of the EPM. The EPM test utilizes the innate behavior of mice to prefer enclosed spaces and their tendency to avoid heights and open areas; the open arms of the EPM are more anxiogenic than the closed arms (Walf and Frye, 2007). The time spent in and the number of entries into the open arms were comparable between LRRTM1/2-cDKO and control mice (Figure 9—figure supplement 1E and F). The total distance travelled and the average speed in the EPM were also comparable between the two groups (Figure 9—figure supplement 1G and H). Thus, conditional deletion of Lrmt1 and Lrmt2 in dorsal hippocampus of adult mice does not alter avoidance-related anxiety behavior in mice.

Next, we assessed social memory in the LRRTM1/2-cDKO and control groups using Crawley’s three chambered sociability test (Kaidanovich-Beilin et al., 2011; Figure 9A). Social memory is primarily associated with the ventral hippocampus along with amygdala and hypothalamus (Okuyama et al., 2016). In Crawley’s sociability and preference test, mice can freely choose to interact with familiar and nonfamiliar conspecifics. The first block of the test included 10 min habituation for the test mice in all three chambers of the test. In the second block, Stranger I was introduced in one of the chambers and interaction of control and LRRTM1/2-cDKO mice with Stranger I was assessed. Both groups spent more time interacting with Stranger I as compared to the middle and empty chambers (Figure 9B). In the third block, a second mouse (Stranger II) was introduced in the third chamber. Both control and LRRTM1/2-cDKO mice interacted more with Stranger II as compared to Stranger I (Figure 9C). Thus, Lrmt1/2-cDKO do not differ from control mice in sociability or social novelty preference behavior.

Context acquisition through the hippocampus requires integration of various visuo-spatial, auditory, and olfactory cues (Fanselow, 2000; Sanders et al., 2003; Wiltgen et al., 2006). These cues are acquired by exploratory behavior encoded in cortical structures, such as subiculum, and nucleus accumbens which send projections to the dorsal hippocampus and are consolidated in the prefrontal cortex (Fanselow, 2000). In both, LRRTM1/2-DKO and -cDKO mice, LTP is impaired in the hippocampus (Figures 4A and 8B). In hippocampus, LTP maintenance is required for coding integral representation of contexts and its association with unconditional stimuli (Nicoll, 2017; Penn et al., 2017; Takeuchi et al., 2014). Therefore, we examined hippocampus-dependent contextual fear conditioning to assess long term memory encoding in LRRTM1/2-cDKO and control mice. Mice were pre-exposed to a context 24 hr before a mild foot shock, and were then trained to associate mild foot shocks with a
particular context. Learning and memory were assessed as percent freezing and rearing in response to subsequent exposure to the context alone (Figure 9D). LRRTM1/2-cDKO mice exhibited significantly reduced levels of freezing and rearing as compared to controls (Figure 9E and F), but their average speed was comparable to control values (Figure 9G). Thus, conditional deletion of Lrrtm1 and Lrrtm2 in dorsal hippocampus causes deficits in hippocampus-dependent long-term contextual memory.

**Discussion**

We demonstrate here, using global and region-restricted genetic approaches combined with multiple independent analyses, that LRRTM1 and LRRTM2 have differential and context-dependent functions in the organization and plasticity of synapses and in cognition. For comprehensive analysis, we generated and analyzed three models: (1) Global early deletion model (LRRTM1/2-DKO); (2) Local early deletion (P0-LRRTM1/2-cDKO) and; (3) Local late deletion (LRRTM1/2-cDKO).
Our investigations led us to the following conclusions. First, LRRTM1 and LRRTM2 are important for the normal development of excitatory synapses, selectively in the hippocampal CA1 region. Second, inhibitory synapses throughout the hippocampus and are not affected by the loss of LRRTM1 and LRRTM2. Third, LRRTM1 and LRRTM2 are dispensable for the maintenance of normal numbers of excitatory synapses in mature neurons. Fourth, synaptic deficits due to LRRTM1 and LRRTM2 deletion are postsynaptic in origin. Fifth, LRRTM1 and LRRTM2 are required for LTP at CA3-CA1 synapses in both developing and mature neural circuits. Surprisingly, in this context, LRRTM1 and LRRTM2 are also required for LTP at dentate gyrus synapses. These results indicate that the roles of LRRTM1 and LRRTM2 in synapse development and plasticity are functionally dissociable. Finally, our results show that LRRTM1 and LRRTM2 are essential for enduring hippocampus-dependent long-term contextual memory.

There is substantial discord in the LRRTM literature due to conflicting data obtained in LRRTM KD experiments (de Wit et al., 2009; Ko et al., 2011; Soler-Llavina et al., 2011). Whereas in one study, shRNA-mediated KD of LRRTM2 in hippocampal neuron cultures was shown to significantly reduce synapse numbers (de Wit et al., 2009), a different study indicated that shRNA-mediated KD of LRRTM1 and LRRTM2 in hippocampal cultures does not alter synapse numbers (Ko et al., 2011). When combined with neuroligin-3 KD in neuroligin-1 KO neurons, however, shRNA-mediated KD of LRRTM1 and LRRTM2 caused reductions in the number of excitatory synapses in hippocampal cultures but did not reduce spine density in CA1 neurons in vivo (Soler-Llavina et al., 2011). Our findings provide clarity and resolve some of these discrepancies. In contrast to previous studies, we found that constitutive deletion of Lrtrim1 and Lrtrim2 causes significant reductions in the numbers of asymmetric synapses, VGlut1 puncta fluorescence, spine density, and mEPSC frequency in CA1 neurons in vivo. Likely reasons for these discrepancies include differences between in vitro and in vivo settings and problems with the specificity, efficacy, and timing of KD approaches. As opposed to our constitutive deletion approach, acute deletion of Lrtrim1 and Lrtrim2 in mature neurons did not alter the number of synapses but led to significantly reduced mEPSC amplitudes (Figure 7; Bhouri et al., 2018). However, consistent with the constitutive deletion approach, local early deletion of Lrtrim1 and Lrtrim2 in the CA1 (P0-LRRTM1/2-cDKO) significantly reduced mEPSC frequency, and was accompanied with modest decrease in mEPSC amplitude (Figure 5). These results indicate that LRRTM1 and LRRTM2 mediate distinct functions in developing and mature neurons. Specifically, during development, LRRTM1 and LRRTM2 contribute to synapse development whereas in the mature brain, consistent with previous reports (Bhouri et al., 2018; Karimi et al., 2021; Ramsey et al., 2021), they contribute to maintaining the complement of AMPARs at synapses.

A key open question in our understanding of neural circuit formation concerns the molecular factors that determine the regional, or cell-type specificity of synapse formation. We show that the constitutive loss of Lrtrim1 and Lrtrim2 leads to reduced synapse numbers selectively in the CA1 region. Our results are in agreement with previous studies showing dispersed synaptic vesicles or slightly reduced synapse numbers in specific laminae of CA1 in mice lacking Lrtrim1 (Linhoff et al., 2009; Schroeder et al., 2018; Takashima et al., 2011). Loss of Lrtrim3 or Lrtrim4, or a knock-in mutation in Lrtrim4 that blocks its interaction with heparan sulfate, selectively reduce synapse numbers in the dentate gyrus but not in the CA1 region (de Wit et al., 2013; Roppongi et al., 2020; Siddiqui et al., 2013; Um et al., 2016). In contrast, deletions of several other organizers of excitatory synapses have either no or much more widespread effects on synapse numbers. Synapse numbers in mice lacking neuroligin 1 or neuroligin 3 are not altered (Blundell et al., 2010; Varoqueaux et al., 2006), and even in cultured neurons and the brain of newborn neuroligin 1–3 triple KO mice, synapse numbers are unchanged (Varoqueaux et al., 2006). Hence, neuroligins are thought to function in synapse maturation and plasticity but not in initial synapse formation (Südhof, 2017). On the other hand, mice lacking IL1RacP, for instance, have reduced numbers of excitatory synapses and reduced spine density in all brain regions analyzed (Yoshida et al., 2012), and EphB1-3 triple KO mice exhibit widespread reductions in the numbers of excitatory synapse numbers and dendritic spines, for example in cortex and hippocampus (Henkemeyer et al., 2003; Kayser et al., 2006). These more widespread functions of IL1RacP and EphB1-3 contrast with the cell selective functions of LRRTMs.

LRRTMs are co-expressed with multiple other synapse organizers in both pyramidal neurons and granule cells. In the developing hippocampus (Global DKO), we observed a 25–30% loss of excitatory synapses in the CA1 indicating that in pyramidal neurons, LRRTM1/2 are important drivers of
synapse development. However, CA1 pyramidal neurons express multiple other postsynaptic organizers, including neuroligins, which likely account for significant functional compensation for the loss of LRRTMs in these cell types.

LRRTMs have discrete expression profiles, and levels of individual LRRTMs in different brain regions and cell types vary substantially. In the hippocampus, LRRTM1 and LRRTM2 are the only LRRTM family members expressed in CA1, whereas all LRRTMs are expressed in the dentate gyrus, with LRRTM4 expression exhibiting a particularly high level (Laurén et al., 2003). Given their sequence homology and common associations with AMPA receptors and PSD-95 family scaffold proteins (de Wit et al., 2009; Linhoff et al., 2009; Siddiqui et al., 2013; Soler-Llavina et al., 2013), it is likely that in developing dentate gyrus neuronal circuits, LRRTM3 and LRRTM4 can compensate for the loss of LRRTM1 and LRRTM2. Indeed, loss of LRRTM3 or LRRTM4 substantially reduces synapse numbers in the dentate gyrus, without affecting synapse development in CA1 pyramidal neurons (de Wit et al., 2013; Rappongi et al., 2020; Siddiqui et al., 2013; Um et al., 2016). Interestingly, we discovered now that loss of LRRTM1 and LRRTM2 in the CA1 pyramidal neurons and dentate gyrus granule cells have differential effects on synapse numbers and synaptic transmission. Outside the hippocampus, LRRTM1 is important for complex retinogeniculate synapse development and retinal convergence in visual thalamus (Monavarfeshani et al., 2018) and for controlling excitatory synaptic function in the mediodorsal thalamus (Karimi et al., 2021). Thus, LRRTMs are necessary cell-type selective mediators of synapse development and function. Additionally, NGL-2 and Latrophilin2 regulate synapse development specifically in the stratum radiatum and stratum lacunosum moleculare of CA1, respectively (DeNardo et al., 2012; Sando et al., 2019). In more general terms, synaptic specificity in the mammalian central nervous system may be conferred not by a single synapse organizer but by a set of factors that define a combinatorial code such as those contributed by cell types, subcellular compartments, and dendritic domains. Careful parsing of the roles of individual proteins or subsets of proteins will help us to devise a comprehensive catalog of the molecular factors that contribute to the development of synaptic connectivity in specific brain circuits.

Interestingly, both constitutive and adult deletion of Lrutm1 and Lrutm2 led to a robust impairment of LTP at CA3-CAsynapses in the stratum radiatum, accompanied by a deficit in hippocampus-dependent long-term contextual memory. These results are consistent with previous KD studies (Soler-Llavina et al., 2013), with behavioral studies on Lrutm1 KO mice (Karimi et al., 2021; Takashima et al., 2011), and with the growing evidence of a key role of synapse organizing proteins in synaptic plasticity (Bhouri et al., 2018; Wu et al., 2019). Furthermore, we found that acute deletion of Lrutm1 and Lrutm2 in the dentate gyrus caused a similar LTP impairment as in the CA1 region. Although the role of LRRTM3 and LRRTM4 in synaptic plasticity remains untested, our results demonstrate that LRRTM1 and LRRTM2 contribute to LTP through mechanisms that do not require other LRRTMs.

Our results provide a causal link between the role of LRRTM1 and LRRTM2 in the control of synapse function in the dorsal hippocampus and long-term contextual memory. The region selective functions of LRRTM1 and LRRTM2 are further demonstrated by the lack of effect of Lrutm1 and Lrutm2 deletion in the dorsal hippocampus on social interaction or avoidance-related anxiety, which have been linked to the ventral hippocampus (Fanselow and Dong, 2010). Future studies could address the functional contributions of the LRRTMs in the ventral hippocampus and other brain regions where they are expressed.

In conclusion, the present study emphasizes the importance of studying brain-region-specific and developmental-stage-specific roles of synaptic adhesion molecules in synapse development and function. As is the case with their ligands of the neurexin family, alterations in LRRTM genes increase the risk for neurodevelopmental and psychiatric disorders, including autism, schizophrenia, and bipolar disorder (Francks et al., 2007; Malhotra et al., 2011; Pinto et al., 2010). Based on the present data, we propose that regional and temporal variations in synaptic development, transmission, and plasticity due to alterations in LRRTM1 or LRRTM2 contribute to these disorders.

Materials and methods

Generation of LRRTM1/2-DKO mice

All animal experiments complied with government and institutional guidelines. The conditional Lrutm-1\(^{1}\)\text{lox/lox} and Lrutm2\(^{2}\)\text{lox/lox} mouse lines were described previously (Bhouri et al., 2018). These mice were
crossed with the Ella-Cre line that expresses Cre recombinase in early embryonic stages (Lakso et al., 1996) to generate Lrmt1\(^{−/−}\) and Lrmt2\(^{−/−}\) mice. The heterozygotes were crossed to obtain single KOs. Finally, Lrmt1\(^{−/+}\) and Lrmt2\(^{−/+}\) mice were crossed to obtain LRRTM1/2-DKO mice.

**Animals**

All animal experiments complied with government and institutional requirements of the University of Manitoba and conformed to ethical and procedural guidelines of the Canadian Council on Animal Care (CCAC, http://www.ccac.ca), and were approved under Protocols 15–042 and 19–054. The experimental analyses for each mice group was conducted between 6 and 7 weeks. For all experiments and analyses, the experimenter was blind to genotype.

**Tissue immunofluorescence and image analysis**

Immunofluorescence studies were performed on brain tissue from 6 to 7 weeks old LRRTM1/2-DKO, P0-LRRTM1/2-cDKO and LRRTM1/2-cDKO. Mice were anaesthetized with 20% urethane and transcardially perfused with cold 0.1 M phosphate buffered saline (PBS) followed by 4% formaldehyde/4% sucrose in PBS (pH 7.4). The brains were then extracted, post-fixed in 4% formaldehyde overnight, and cryoprotected in 20% sucrose followed by 30% sucrose in PBS at 4 °C. For sectioning, the brains were frozen in dry ice in OCT compound (Tissue-Tek). Twenty-µm-thick coronal sections at the hippocampal level were cut in a cryostat and mounted on Superfrost Plus slides. Slides were incubated in blocking solution (5% BSA +5% normal goat serum +0.25% Triton X100 in PBS) for 1 hr, incubated at 4 °C overnight with primary antibodies (VGlut1 rabbit, 1:1000; Synaptic Systems; 135 302) and (GAD65 mouse IgG2a, 1:100, GAD-6-c, Developmental Hybridoma) diluted in the same blocking solution, followed by incubation with the appropriate secondary antibodies conjugated to Alexa 488, 568 or 647 (Invitrogen) for 1 hr at room temperature. For LRRTM1/2-DKO and P0-LRRTM1/2-cDKO analysis, DAPI (4′,6 diamidino-2-phenylindole) was added to the final wash of the sections with PBS and sections were coverslipped using Immuno Mount (Thermo Scientific). For LRRTM1/2-cDKO, after final wash with PBS, sections were mounted using Fluoromount-G with DAPI (4′,6 diamidino-2-phenylindole) (Southern Biotech 0100–20). In order to obtain images for synaptic density quantification, mice sections were fixed and stained simultaneously. At least three to six animals were used for each genotype.

Imaging and image analysis was carried out blind to the experimental condition. Images were captured on a confocal microscope (Zeiss LSM 700) which is equipped for automated tiling or 63 x/1.4 NA oil objective and custom filters on a Zeiss Observer Z.1 wide-field microscope equipped with Apotome 2.0 for optical sectioning through structured illumination. For quantitative analysis, 15–20 images per mouse were acquired with a 40 x/1.4 NA oil objective at an additional x2.5 magnification and sequential scanning with individual lasers with optimized filters. For quantification of excitatory (VGLu1) and inhibitory (GAD65) synaptic markers in brain sections, images were thresholded manually to define puncta and total integrated intensity of puncta per image area were measured. The same regions were measured for both VGLu1 and GAD65. Analysis was performed using Metamorph (Molecular Devices), Excel (Microsoft) and GraphPad Prism (GraphPad Software). Statistical comparisons were made with multiple t-tests. All data are reported as the mean ± SEM.

**Golgi staining**

To determine the density of dendritic spines, Golgi stainings of different hippocampal regions were done using the FD Rapid GolgiStain Kit (FD NeuroTechnologies), essentially as recommended by the manufacturers. Brains from euthanized 6–7 weeks old mice were rapidly removed, rinsed in PBS and
their olfactory bulbs and cerebellum excised. The rest of the brain was incubated in the rapid Golgi solutions (Glaser and Van der Loos, 1981) for a total of 3 weeks. The brains were then snap frozen and cut into 100-µm-thick sections at the level of the hippocampus using a cryostat. Sections were mounted on slides pre-treated with 3% gelatin. The slides were subsequently dried, dehydrated, cleared with xylene and mounted with Permount (Fisher Scientific). Imaging and image analysis was carried out blind to the experimental condition. Morphologically comparable slices from wild-type and LRRTM1/2-DKO mice were analyzed in bright field on a Zeiss Axioplan2 microscope with a 63 × 1.4 NA oil objective. Spines were counted manually on specific dendrites while altering the focal plane to account for all spines. Images of the dendrites were acquired to determine their length. Three to 4 mice were used in each group.

**Electron microscopy**

Three to 4 male mice from each group (6–7 weeks old) were deeply anesthetized by intraperitoneal injection of 150 mg/kg ketamine and 15 mg/kg xylazine, and transcardially perfused with PBS followed by fixative solution (4% formaldehyde, 1.25% glutaraldehyde in PBS). The brain was removed and fixed overnight at 4 °C. A 1 mm³ section of hippocampus including the CA1 region and stratum radiatum was dissected. Tissue was dehydrated in an ethanol series and embedded in JEMBED/Spurr’s resin. Ultrathin sections (70 nm) were cut and stained with 2% uranylacetate and Reynold’s lead and imaged on a Hitachi H7600 TEM (Hitachi). For each mouse, an equivalent region of the stratum radiatum directly underlying the CA1 pyramidal cell layer was imaged to analyze synapse density (×15,000 magnification, 50+images per mouse) or morphology (×60,000 magnification, 100+images per mouse, >500 synapses per condition). All images were acquired and analyzed blind to the genotype of each mouse.

**Immunoblotting**

Three to 10 mice from each group were used for subcellular fractionation of whole brain to obtain the crude synaptosomal fraction was done essentially as described (Roppongi et al., 2020). For all samples, protein concentrations were normalized and run on 10% polyacrylamide gels. Gels were transferred onto Immobilon P membranes (Millipore) which were blocked in 5% skim milk in Tris-buffered saline/0.05% Tween-20 or 5% BSA in PBS and incubated with one of the following primary antibodies: Anti-LRRTM1 antibody (BC267) Bhouri et al., 2018; anti-LRRTM2 antibody (512KSCN) (Bhouri et al., 2018), anti-bassoon (mouse IgG2A; 1:1000; Stressgen; VAM-PS003), anti-CASK (mouse IgG1, 1:1000, K56A/50, Neuromab), anti-neuroligin 1 (mouse IgG, 1:1000, 4C12, Synaptic Systems), anti-neuroligin 1,3,4 (mouse IgG2a, 1:1000, 4C12, Synaptic Systems), anti-neuroligin 1,3,4 (mouse IgG2a, 1:1000, 4C12, Synaptic Systems), anti-neuroligin 3 (mouse IgG, 1:1000, N110/29, Neuromab), anti-GluN1 (mouse, 1:1000, Millipore), anti-GluN2A (rabbit, 1:1000, Chemicon), anti-pan-SAPAP (mouse IgG2b, 1:1000, N127/31, Neuromab), anti-NEA-25 (mouse IgG1, 1:1000, CI71.1, Synaptic Systems), anti-Synapsin (mouse IgG1, 1:1000, Synaptic Systems), anti-Synaptophysin (mouse IgG1, 1:1000, B.D. Phamigen), anti-Synaptotubulin (mouse, 1:1000, Cl69.1, Synaptic Systems), anti-VGluT1 (mouse, 1:1000, N29/9, Neuromab), anti-SynGAP (rabbit, 1:1000, Affinity Bioreagents), anti-pan-Shank (mouse IgG1, 1:1000, N23B/49), anti-TrkC (rabbit, 1:1000, C44H5, Cell Signaling), anti-LRRTM4 (mouse, 1:1000, N205B/22, Neuromab), anti-PSD-95 family (mouse IgG2a; 1:2000; clone 6G6-1C9; Millipore), anti-GluA2 (mouse, 1:1000, Millipore), anti-gephyrin (mouse IgG1; mab7, 1:1000, Synaptic Systems) and anti-β-actin (rabbit, 1:5000, ab8227, Abcam) and secondary antibodies (goat anti-mouse or goat anti-rabbit HRP conjugate from Millipore). Immunoblot signals were detected using the SuperSignal Chemiluminescent kit (Thermo Scientific) and a Bio-Rad gel documentation system and normalized to the β-actin levels.

**Stereotaxic injections**

Animals were anesthetized with isoflurane (500–1000 ml/min flowrate) and were maintained at the surgical plane (100–200 ml/min flowrate) for the whole surgical process. After deep plane of anesthesia is obtained, the mice were administered 0.5% Bupivacaine (8 mg/kg) to intended area of incision subcutaneously and Meloxicam (2 mg/kg) subcutaneously. The mice were kept immobilized using Kopf stereotaxic apparatus. Bilateral craniotomy was made in the skull using co-ordinates for dorsal hippocampus (AP –2.1; ML ±1.4; DV –2.0). Glass pipette containing viral solution was inserted and the
virus was delivered at a flow rate of 0.1 μl/min through a picospritzer. The skin was then sutured, and the mice were allowed to recover for 3 weeks.

For P0 injections, the virus was mixed with Trypan blue to obtain a final concentration of 0.1% Trypan blue. The virus mixture was then loaded in a 5 μl Hamilton syringe. For injections, a stage was made manually according to the dimensions used previously (Mathon et al., 2015). The stage was then placed in a petri dish filled with ice on the stereotaxic frame. LRRTM1/2\textsuperscript{floxed/floxed} P0 pups were anesthetized by placing the pups on ice for 2 min. Anesthesia was confirmed by gently squeezing the paws or tail of the pup and monitored lack of movement. The pup was then placed on the stage and 1 μl of the viral mixture was used to inject in both sides of the dorsal CA1 (AP –1.5; ML ±1.5; DV –1.25–1.35 from lambda). The pup’s head remained held by the surgeon using an index finger for the duration of the injection. After the injection, the pups were recovered using a warming pad and returned to the home cage with the mother after the skin color returned to pink and the pup regained movement. The pups were monitored after the injection for 72 hr to check for health and visible milk spot.

**Electrophysiology**

For preparation of mouse hippocampal slices, 6- to 7-week-old LRRTM1/2-DKO, wild-type littermates, LRRTM1/2-cDKO, P0 LRRTM1/2-cDKO and control injected mice underwent cervical dislocation and decapitation. Four to 5 mice were used in each group with 3–4 slice recordings from each animal.

**Field recordings**

For LRRTM1/2-DKO LTP analysis, whole brains were transferred to ice-cold slicing solution consisting of (in mM): 120 NMDG, 2.5 KCl, 1.2 NaH2PO4, 25 NaHCO3, 1.0 CaCl2, 7.0 MgCl2, 2.4 Na-pyruvate, 1.3 Na-ascorbate, 20 D-glucose with pH adjusted to 7.35 using HCl acid (unless stated, all chemicals and drugs were purchased from Sigma or BioShop, Canada). The hippocampus was removed and sliced in the transverse plane using a manual tissue chopper (Stoelting, Wood Dale, IL, USA). Slices were maintained in a recovery chamber at 30 °C for 1 hr in ACSF composed of the following: (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 1 MgSO47H2O, 2 CaCl2, 26 NaHCO3 and 15 D-glucose which was bubbled continuously with carbogen (95% O2/5% CO2) to adjust the pH to 7.3. After an additional 30 min at room temperature, slices were transferred to a submerged recording chamber and were continually perfused with ACSF (2–3 ml/min) at room temperature. Extracellular field EPSPs (fEPSPs) were elicited by using two bipolar nickel–chromium electrodes placed in stratum radiatum of area CA1 and recorded with a glass microelectrode filled with ACSF (resistances, 2–3 MΩ). Baseline responses were set at 40% of maximum and taken for 20 min prior to inducing LTP. High-frequency stimulation consisting of 1 × 100 Hz, 1 s duration was used to induce LTP. Extracellular recordings were acquired using WinLTP. The initial slope of the fEPSP was measured to quantify synaptic strength (Johnston, 1995). Student’s t-test was used for statistical comparisons of mean fEPSP slopes between groups. All values shown are mean ± SEM with n=number of slices.

For LRRTM1/2-cDKO slice electrophysiology, mice were anesthetized and the brain was rapidly removed and placed in ice-cold standard ACSF composed of (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 1.3 MgSO47H2O, 2.6 CaCl2, 26 NaHCO3 and 10 D-glucose saturated with 95% O2 and 5% CO2. The hippocampus was removed and sliced in the transverse plane (350 μm thickness) using vibratome (ThermoFisher vibrating microtome 650 V). Slices were transferred in ACSF and incubated for 1 hr at room temperature to equilibrate before placing in a recording chamber continuously perfused with 95% O2; and 5% CO2 at a flow rate of 3 ml/minute (for CA1) and 1.5 ml/minute (for dentate gyrus). For CA1, 2 μM bicuculline methiodide was added to standard ACSF. Recording electrodes consisted of borosilicate pipettes (TW150-F3; WPI, Sarasota, FL, USA) pulled with a two-stage puller (PP-83; Narishige, Greenvale, NY, USA). Extracellular field EPSPs (fEPSPs) were elicited by using two electrodes placed in stratum radiatum of area CA1 and recorded with a glass microelectrode filled with ACSF (resistances, 2–3 MΩ). Baseline responses were set at 30–40% of maximum and taken for 20 min prior to inducing LTP. High-frequency stimulation consisting of 2 × 100 Hz, 1 s duration was used to induce LTP in CA1. Student’s t-test was used for statistical comparisons of mean fEPSP slopes between groups. All values shown are mean ± SEM, with n=number of slices.

For DG LTP, 10 μM bicuculline methiodide was added to standard ACSF. For recording fEPSPs, the stimulating electrode was positioned on dentate gyrus molecular layer in 10–12 μm away from
granular layer (inner molecular layer) and responses were recorded using a borosilicate glass capillary pipette filled with ACSF with a tip resistance of 2–3 MΩ. High-frequency stimulation consisting of 4 × 100 Hz, 1 s duration was used to induce LTP in dentate gyrus. Student's t-test was used for statistical comparisons of mean fEPSP slopes between groups. All values shown are mean ± SEM, with n=number of slices.

Paired pulse ratio was recorded from CA1 of Global DKO (LRRTM1/2-DKO) and Local late cDKO (LRRTM1/2-cDKO) CA1 at stimulation intensities that resulted in response-amplitudes 1/3 of maximum. Inter-stimulus intervals were as follows:, 20ms, 50ms, 100ms, 250ms, and 500ms.

Patch clamp recordings
Slices for whole-cell recordings of LRRTM1/2-DKO CA1, brain slices were prepared as described above. Neurons were recorded using the “blind” method with a MultiClamp 700B amplifier with cells voltage clamped at −70 mV and continually perfused with ACSF (same as above). Recording pipettes were filled with solution containing (in mM): 122.5 Cs-methanesulfonate, 17.5 CsCl, 10 EGTA, 10 HEPES, 4 ATP(K), and 5 QX-314, with pH adjusted to 7.2 by CsOH. Prior to recording mEPSCs, bicuculline methiodide (10 μM; Abcam) and tetrodotoxin (TTX; 500 nM; Ascent Scientific) were added to block GABA receptor-mediated inhibitory synaptic currents, and action potentials respectively. CNQX (10 μM; Abcam) and DL-AP5 (50 μM; Abcam) were added toward the conclusion of some experiments to confirm that remaining currents were mEPSCs. mEPSCs were recorded using WinLTP in continuous acquisition mode. Analyses for frequency and amplitude were conducted using MiniAnalysis software. Statistical analyses were completed using GraphPad InStat and SigmaPlot. Student’s t-tests were conducted to test for differences between groups with statistical significance set at p<0.05 with n=number of cells. Data are presented as mean ± SEM.

For all other whole-cell recordings (LRRTM1/2-DKO DG, LRRTM1/2-cDKO and P0 LRRTM1/2-cDKO), mice were anesthetized and the brain was rapidly removed and placed in ice-cold standard ACSF composed of (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 1.3 MgSO47H2O, 2.6 CaCl2, 26 NaHCO3, 10 D-glucose and 3 Na-Pyruvate saturated with 95% O2 and 5% CO2. The hippocampus was removed and sliced in the transverse plane (250 μm thickness) using vibratome (Campden model 7,000smz-2). Slices were then recovered in NMDG ACSF for 5 min at 32 °C. NMDG ACSF was composed of (in mM): 94 NMDG, 3 KCl, 1.25 NaH2PO4, 30 NaHCO3, 0.5 CaCl2, 5 MgCl2, 3 Na-pyruvate, 5 Na-ascorbate, 25 D-glucose with pH adjusted to 7.35 using HCl acid. The slices were transferred to HEPES ACSF and recovered for 1 hr at room temperature before recording. HEPES ACSF was composed of (in mM): 95 NaCl, 3 KCl, 1.25 NaH2PO4, 30 NaHCO3, 0.5 CaCl2, 5 MgCl2, 3 Na-pyruvate, 5 Na-ascorbate, 25 D-glucose with pH adjusted to 7.35. Recording pipettes were filled with solution containing (in mM): 142 cesium gluconate, 10 HEPES, 8 NaCl, and 2 MgCl2, pH 7.2 (adjusted with 1M-CsOH). Prior to recording mEPSCs, tetrodotoxin (TTX; 100 nM; Alomone lab) were added to block action potentials. mEPSCs were recorded using Clampex software. The cells were voltage clamped at −60 mV and mEPSCs were recorded for a minimum period of 2–5 min. Student’s t-tests were conducted to test for differences between groups with statistical significance set at p<0.05 with n=number of cells. Data are presented as mean ± SEM.

Contextual fear conditioning
Contextual fear conditioning (CFC) for LRRTM1/2-DKO mice was performed in a black Plexiglas chamber with a camera mounted above for recording and offline behavioral analysis. Mice were gently handled for 3 days prior to experiments. The mice were habituated to the context by placing them in the chamber for 2 min prior to administering a single electrical foot shock (0.7 mA foot shock, 2 s duration) administered through a stainless-steel grid floor. Following conditioning, mice remained in the chamber for an additional 3 min to allow for encoding of the context shock association. Mice were subsequently returned to their home cage after completing the conditioning procedure. CFC was then assessed 24 hr after the conditioning session by placing mice in the conditioning chamber for 5 min. Absence of movement except respiration constituted freezing time which was converted to freezing percentage (total amount of freezing time/5 min) to assess contextual memory for each mouse. Student’s t-tests was performed to compare the amount of freezing between groups.

For LRRTM1/2-cDKO, the mice were habituated to the context by placing them in the chamber for 10 min on the first day. Twenty-four hr later, three electrical foot shocks (1 mA foot shock, 30 s
interval) were administered through a stainless-steel grid floor after placing the mice in chamber for 2 min. Following conditioning, mice remained in the chamber for an additional 1 min to allow for encoding of the context + shock association. Mice were subsequently returned to their home cage after completing the conditioning procedure. CFC was then assessed 24 hr after the conditioning session by placing mice in the conditioning chamber for 5 min. Absence of movement except respiration constituted freezing time which was converted to freezing percentage (total amount of freezing time/5 min) to assess contextual memory for each mouse. Rearing was analyzed as the time assessed when each mouse would keep two front paws on the wall. Analysis were done on AnyMaze software. Student's t-tests was performed to compare the amount of freezing between groups. Ten to –11 mice were used in each group.

**Accelerating rotarod**

An accelerating rotarod (Harvard Apparatus, USA) was used to assess gross motor skills and motor coordination of mice. Mice were brought to the behavior room 10 min before starting the tests. The rotarod was set to 4 rpm, accelerating rate 20 rpm/min. The mice were placed on the rotarod opposite from the direction of acceleration. Three trials were given to each mouse. Time to fall from the rotarod (latency to fall) was measured for each mouse. Average of the three trials were included in analysis. Student’s t-tests was performed to compare between groups. Ten to 11 mice were used in each group.

**Open-field maze**

The open-field maze was made of 50 cm (length) x 50 cm (width) x 38 cm (height) non-porous hard plastic. The mice were brought to the behavior room 20 min before the start of the test. Each mouse was placed in the center of the maze and allowed to explore the maze for 10 min. After 10 min, the mice were returned to their home cages. The behavior was then analyzed using AnyMaze software. Student’s t-tests was performed to compare between groups. Ten to 11 mice were used in each group.

**Elevated plus maze**

The maze is made of hard plastic of four arms with dimensions 30 cm long and 5 cm wide (two open without walls and two enclosed by 15.25 cm high walls). The maze is elevated 40 cm off the table with legs. The mice were brought 15 min before the behavioral test for acclimatization. Each test mouse is placed in the center of the maze with its head facing the closed arms and opposite to the experimenter. The mice were allowed to explore the maze for 10 min before putting back in their home cages. The behavior was then analyzed using AnyMaze software. Student’s t-tests was performed to compare between groups. 10–11 mice were used in each group.

**Crawley’s three-chambered social approach**

The mice were brought to the behavior room 20 min before the start of the test to acclimate. The sociability chamber comprised of a rectangular box with three chambers of equal size (20 × 40 x 22 cm) made from clear Plexiglas. The test mouse was introduced in the middle chamber of three-chambered apparatus for 10 min with doors closed to both chambers for habituation. After 10 min, a Stranger I mouse (the placement of Stranger I in the left or right side of the chamber was randomly altered between trials) was placed in one of the inverted wire cups. The doors of the chambers were opened, and the test mouse is allowed to explore both the chambers (stranger I and empty wire cup). Ten min later, Stranger II mouse is placed in the empty wire cup for another 10 min. Time spent by the test mice in each chamber was measured and analyzed manually with experimenter being blind to groups. The apparatus was cleaned with 10% ethanol after every session. Note: All Stranger mice were of the same gender as the subject mice and had no prior interaction with them. Ten to 11 mice were used in each group.

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• Transparent reporting form

Data availability
All data generated during this study are included in the manuscript and supporting files. Source data files have been provided for all main Figures (Figures 1-9), and for Figure 1- Figure supplements 1,2,3, Figure 4- Figure Supplement 1, Figure 6- Figure supplement 1, Figure 8 - Figure supplement 1, and Figure 9- Figure supplement 1.

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