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An Essential Role for the Tetraspanin LHFPL4 in the Cell-Type-Specific Targeting and Clustering of Synaptic GABA<sub>A</sub> Receptors

**Highlights**
- LHFPL4 is a tetraspanin enriched at inhibitory synapses that complexes with GABA<sub>A</sub>Rs
- LHFPL4 is important for GABA<sub>A</sub>R clustering both in vitro and in vivo
- LHFPL4 is required for the surface clustering but not the trafficking of GABA<sub>A</sub>Rs
- GABAergic synaptic inputs on CA1 pyramidal neurons, but not interneurons, require LHFPL4

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**In Brief**
Davenport et al. identify LHFPL4 as a transmembrane protein that interacts with GABA<sub>A</sub>Rs and is essential for their synaptic clustering. Deletion of LHFPL4 results in dramatic cell-type-specific deficits in inhibitory synaptic transmission.
An Essential Role for the Tetraspanin LHFPL4 in the Cell-Type-Specific Targeting and Clustering of Synaptic GABA<sub>A</sub> Receptors

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INTRODUCTION

Synaptic inhibition mediated by GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) regulates the balance of excitation and inhibition in the brain and, thus, plays a critical role in information processing. The stabilization of synaptic GABA<sub>A</sub>Rs opposite GABA-releasing presynaptic terminals is crucial for efficient synaptic inhibition, appropriate regulation of circuit excitability, and animal behavior. Changing the number of postsynaptic GABA<sub>A</sub>Rs can rapidly control the strength of inhibitory synapses. This is achieved by the trafficking of receptors to, and their removal from, the plasma membrane and by their surface lateral diffusion into and out of synaptically stabilized clusters. Notably, TARPs and GSG1L are members of the tetraspanin superfamily of transmembrane proteins and have been shown to function as transmembrane GABA<sub>A</sub>R accessory proteins. In the case of ionotropic glutamate receptors, various membrane-spanning receptor-associated proteins have emerged as key regulators of receptor trafficking, synaptic targeting, and receptor function. These include transmembrane extracellular-membrane-protein-2, extracellular-membrane-protein-3, and extracellular-membrane-protein-4, which are known to regulate GABA<sub>A</sub>R trafficking, synaptic stability, and function. Here, using biochemical, imaging, mouse transgenic, and electrophysiological approaches, we demonstrate a critical role for the previously uncharacterized tetraspanin LHFPL4 (Lipoma HMGIC Fusion Partner-Like 4) in driving the surface clustering of GABA<sub>A</sub>Rs at inhibitory synapses. LHFPL4 is exquisitely targeted to inhibitory synapses and forms high-affinity interactions with GABA<sub>A</sub>R subunits. In the absence of LHFPL4, GABA<sub>A</sub>Rs can still reach the cell surface but are no longer synaptically anchored, leading to a loss of inhibitory postsynaptic currents. We find that LHFPL4 acts in a cell-type-specific manner to provide fresh insights into the molecular make-up of inhibitory synapses.
within the hippocampus, with excitatory pyramidal cells but not inhibitory interneurons affected by its deletion. Our identification of a new machinery for synaptic targeting of GABA<sub>A</sub>Rs opens up new avenues for understanding the construction and regulation of inhibitory synapses in the brain.

**RESULTS**

**LHFPL4 Is Targeted to Inhibitory Synapses and Interacts with GABA<sub>A</sub>Rs**

Although recent mass spectrometry studies have identified a number of candidate GABA<sub>A</sub>R-interacting proteins (Heller et al., 2012; Nakamura et al., 2016), the biochemical validation and functional role of the majority of these putative partners remain undetermined. We noted with interest the identification of LHFPL4, a predicted member of the tetraspanin superfamily of transmembrane proteins of unknown function. We initially tested the ability of LHFPL4 to interact with GABA<sub>A</sub>R subunits. Mouse turbo-GFP-tagged LHFPL4 (mLHFPL4<sub>GFP</sub>) could be readily co-immunoprecipitated with GABA<sub>A</sub>R subunits (β2, β3, and γ2) from lysates of co-transfected COS-7 cells, suggesting that other neuronally expressed synaptic proteins, such as gephyrin, are not essential for the interaction. In the reverse experiment, GFP-tagged human LHFPL4 (hLHFPL4<sub>GFP</sub>) could also readily co-immunoprecipitate the GABA<sub>A</sub>R-β1 subunit from COS-7 cell lysates (Figures 1A and 1B).

To determine whether LHFPL4 was present at synaptic sites, we examined the subcellular localization of LHFPL4<sub>GFP</sub> in cultured rat hippocampal neurons. Using laser scanning confocal microscopy (LSCM), LHFPL4<sub>GFP</sub> was observed to form discrete membrane clusters on the soma and throughout the dendrites. These robustly colocalized with the inhibitory postsynaptic marker gephyrin opposite vesicular GABA transporter (VGAT)-labeled inhibitory presynaptic terminals. Line scans through LHFPL4<sub>GFP</sub> clusters revealed peak fluorescence essentially overlapping with gephyrin and adjacent to the peak of VGAT fluorescence (Figures 1C, 1D, and S1A). Co-labeling of LHFPL4<sub>GFP</sub> with gephyrin and the excitatory synaptic postsynaptic density (PSD) protein homer revealed that LHFPL4<sub>GFP</sub> clusters were 6-fold more enriched at inhibitory compared to excitatory synapses (Figures 1E and S1B). We next explored the postsynaptic distribution of LHFPL4 at inhibitory synaptic sites, using structured illumination microscopy (SIM) to overcome the resolution limit of conventional fluorescence microscopy. SIM imaging of LHFPL4<sub>GFP</sub> co-labeled with antibodies to gephyrin and VGAT revealed LHFPL4<sub>GFP</sub> to form groups of nano-clusters overlaying gephyrin puncta (Figures 1F and S1C), further supporting an inhibitory postsynaptic localization for LHFPL4. Importantly, immunolabeling with an LHFPL4-specific antibody demonstrated that endogenous LHFPL4 is selectively enriched at gephyrin-labeled inhibitory synapses (Figure 1G). Together, these data indicate that LHFPL4 intimately associates with GABA<sub>A</sub>Rs and is specifically enriched at inhibitory postsynaptic domains.

**LHFPL4 Is Essential for the Clustering of GABA<sub>A</sub>Rs but Not Their Surface Delivery**

To investigate the consequences of LHFPL4 loss on the targeting of GABA<sub>A</sub>Rs to inhibitory synapses, we characterized neurons from a constitutive LHFPL4 knockout (KO) mouse (Lhfpl4<sup>−/−</sup>) (Figures 2A–2C). These animals were viable until adulthood, were fertile, and showed no obvious behavioral differences from wild-type (WT) animals. A band at the expected molecular weight for LHFPL4 (27 kDa) was detected from WT, but not Lhfpl4<sup>−/−</sup>, brain lysate by western blotting, and a further strong LHFPL4-specific band was detected at ~17 kDa, which may represent a second isoform or a cleaved product (Figure 2C). Hippocampal neurons from WT and Lhfpl4<sup>−/−</sup> mice were transfected with GFP to reveal cell morphology and were fixed and labeled with an antibody specific to a surface epitope on the GABA<sub>A</sub>R-γ2 subunit before being permeabilized and labeled with antibodies against gephyrin and VGAT (Smith et al., 2014). Quantification of LSCM images revealed a dramatic loss of both gephyrin and GABA<sub>A</sub>R-γ2 clustering and a marked decrease in VGAT-positive clusters co-labeled for gephyrin in Lhfpl4<sup>−/−</sup> neurons compared to WT (Figures 2D–2G). By contrast, clustering of VGAT alone and homer were unchanged in Lhfpl4<sup>−/−</sup> neurons, indicating that inhibitory presynaptic terminals and excitatory synapses were unaffected (Figures 2H–2J).

Importantly, the loss of gephyrin clustering in Lhfpl4<sup>−/−</sup> neurons could be robustly rescued upon overexpression of LHFPL4<sub>GFP</sub>, while LHFPL4<sub>GFP</sub> had no effect on synaptic number or area when overexpressed on the WT background (Figures 2K and 2L).

To determine whether the loss of synaptic GABA<sub>A</sub>R clusters in Lhfpl4<sup>−/−</sup> neurons was due to a disruption in GABA<sub>A</sub>R surface trafficking (Twelvetrees et al., 2010) or to altered synaptic targeting of surface-trafficked receptors (Muir et al., 2010), we next performed live imaging of a super-ecliptic pHiLuorin (SEP)-tagged GABA<sub>A</sub>R subunit (Muir and Kittler, 2014; Pathania et al., 2014). We used GABA<sub>A</sub>R-γ2<sub>SEP</sub>, as this construct is easily expressed, assembles with endogenous GABA<sub>A</sub>R subunits, and demonstrates the expected pattern of surface fluorescence (Muir et al., 2010). Thus, when transfected into WT neurons, GABA<sub>A</sub>R-γ2<sub>SEP</sub> formed bright fluorescent clusters along the dendrites with lower levels of diffuse labeling, consistent with previous reports (Eckel et al., 2015; Muir et al., 2010; Tretter et al., 2008). By contrast, in the majority of transfected Lhfpl4<sup>−/−</sup> cells, GABA<sub>A</sub>R-γ2<sub>SEP</sub> clustering was absent, with only diffuse fluorescence present throughout the soma and dendrites (Figures 3A–3C). Blind scoring of the clustered or diffuse nature of GABA<sub>A</sub>R-γ2<sub>SEP</sub> fluorescence revealed significant loss of clustering in neurons cultured from Lhfpl4<sup>−/−</sup> mice (Figure 3D).

Importantly, both in WT and Lhfpl4<sup>−/−</sup> neurons, the GABA<sub>A</sub>R-γ2<sub>SEP</sub> fluorescence was rapidly and reversibly eclipsed by transient exposure to extracellular buffer of low pH (Figures 3A and 3B), confirming that the fluorescent signal originated from cell-surface receptors. Furthermore, biotinylation experiments showed that surface levels of endogenous GABA<sub>A</sub>Rs were unchanged in Lhfpl4<sup>−/−</sup> neurons compared to WT (Figures 3E and 3F). These data suggest that loss of LHFPL4 does not interfere with the trafficking of GABA<sub>A</sub>Rs to the cell surface but, in a majority of cells, disrupts their tethering in synaptic clusters.

**LHFPL4 Is Not Synaptogenic**

Many inhibitory postsynaptic transmembrane molecules, when overexpressed in non-neuronal cells maintained with dissociated neurons, can induce the formation of hemi-synapses by...
aggregating presynaptic proteins at the point of contact between the two cell types (Fuchs et al., 2013; Scheiffele et al., 2000; Takahashi et al., 2012). To test whether LHFPL4 shared these synaptogenic properties, we co-cultured COS-7 cells (overexpressing recombinant putative synaptogenic transmembrane proteins) with dissociated WT rat hippocampal neurons. After
Figure 2. Loss of Inhibitory Synapse Stability and GABAAR Clustering in Lhfpl4<sup>+/−</sup> Neurons

(A) Schematic of the LHFPL4 knockout genetic strategy showing the genotyping primer sites.
(B) Genotyping results from WT (+/+) and homozygous (−/−) animals. The product of primers aF and aR generates a 585-bp band from the WT allele, and the product of laczexF and aR generates a 230-bp band from the Lhfpl4<sup>+/−</sup> allele.
(C) Western blotting of LHFPL4 and tubulin from WT and Lhfpl4<sup>+/−</sup> mouse brain lysates. Arrowheads indicate the two LHFPL4-specific bands detected.
(D and I) Confocal images of dissociated DIV14 (14 days in vitro) WT or Lhfpl4<sup>+/−</sup> hippocampal neurons transfected with GFP and labeled with antibodies to (D) gephyrin, GABA<sub>A</sub>R-γ2, and VGAT or (I) homer.

(E–H and J) Quantification of (E) gephyrin, (F) GABA<sub>A</sub>R-γ2, (G) VGAT/gephyrin, (H) VGAT and (J) homer synaptic clusters. Cluster number and total immunolabeled area were significantly reduced in Lhfpl4<sup>+/−</sup> neurons, compared to WT neurons, when labeled for gephyrin (cluster number: from 5.7 ± 0.4 to 1.5 ± 0.2; area: from 1.8 ± 0.1 μm<sup>2</sup> to 0.4 ± 0.1 μm<sup>2</sup>; 484/166 WT/Lhfpl4<sup>+/−</sup> clusters; both p < 0.0001) and GABA<sub>A</sub>R-γ2 (cluster number: from 5.9 ± 0.9 to 2.2 ± 0.5; area: from 1.8 ± 0.3 μm<sup>2</sup> to 0.7 ± 0.1 μm<sup>2</sup>; 636/327 WT/Lhfpl4<sup>+/−</sup> clusters; p = 0.0011 and 0.0013, respectively). VGAT/gephyrin-positive clusters were significantly reduced in Lhfpl4<sup>+/−</sup> neurons (from 4.3 ± 0.5 to 1.4 ± 0.3; 294/95 WT/Lhfpl4<sup>+/−</sup> clusters; p < 0.0001). VGAT cluster number did not significantly change (from 5.5 ± 0.7 to 3.8 ± 0.7; 531/403 WT/Lhfpl4<sup>+/−</sup> clusters; p = 0.103). In each case, n = 23 WT and 23 Lhfpl4<sup>+/−</sup> cells from 3 independent preparations.

For WT and Lhfpl4<sup>+/−</sup> clusters, there was no significant change in homer cluster number (8.0 ± 1.9 and 8.2 ± 2.1, respectively; 333/345 WT/Lhfpl4<sup>+/−</sup> clusters) or area (2.5 μm<sup>2</sup> ± 0.6 μm<sup>2</sup> and 2.5 μm<sup>2</sup> ± 0.7 μm<sup>2</sup>, respectively; n = 14 WT and KO cells from 3 independent preparations; p = 0.92 and 0.98, respectively). All used Welch t tests.

(legend continued on next page)
24 hr, the cells were fixed and labeled with antibodies against VGAT and vesicular glutamate transporter (VGluT) to identify inhibitory and excitatory hemi-synapses, respectively. Consistent with previous reports (Chih et al., 2006; Scheiffele et al., 2000), COS-7 cells overexpressing neuroligin2 induced the formation of both inhibitory and excitatory hemi-synapses (Figure S2). By contrast, in cells overexpressing GFP-tagged LHFPL4, the prevalence of inhibitory or excitatory hemi-synapses did not differ from that seen in control cells expressing GFP alone (Figure S2). Thus, LHFPL4 does not have synapticogenic properties.

Loss of LHFPL4 Leads to Reduced GABA<sub>R</sub> Clustering and Aggregates of Mis-localized Gephyrin in Intact Brain

We further explored the relationship between GABA<sub>R</sub>Rs and LHFPL4 in whole brain samples. Importantly, an LHFPL4 antibody readily co-immunoprecipitated the GABA<sub>R</sub>-α1 subunit, gephyrin, and the inhibitory postsynaptic adhesion molecule neuroligin2 from WT but not Lhfpl4<sup>−/−</sup> brain lysate. In addition, LHFPL4 could be co-immunoprecipitated with a neuroligin2 antibody in the reciprocal experiment (Figures 4A and 4B). Thus, LHFPL4 can form native complexes in vivo with key components of the inhibitory scaffold in addition to GABA<sub>R</sub>Rs.

To address how loss of LHFPL4 affected GABA<sub>R</sub> clustering and inhibitory synapse integrity in the intact brain, we carried out immunohistochemistry on fixed brain sections from adult WT and Lhfpl4<sup>−/−</sup> mice. Consistent with the loss of GABA<sub>R</sub> clustering seen in cultured neurons, labeling with a GABA<sub>R</sub>-α2 antibody revealed a dramatic decrease in total GABA<sub>R</sub> cluster area in the hippocampal CA1 region of Lhfpl4<sup>−/−</sup> mice (Figures 4C and 4D). Labeling with a gephyrin antibody revealed a significant loss of total gephyrin cluster area (Figures 4E and 4F), indicating a parallel disruption of the inhibitory postsynaptic domain. The inhibitory presynaptic domain, revealed by labeling
Figure 4. LHFPL4 Is Necessary for Gephyrin and GABAAR Clustering in Intact Brain

(A) Co-immunoprecipitation (coIP) of gephyrin and GABAAR-α1 using an anti-LHFPL4 antibody from WT and Lhfpl4−/− mouse brain lysates (IP, immunoprecipitation; #, immunoglobulin G [IgG] light chain). Note that the immunoprecipitated 27-kDa band is visible just above the IgG light chain in the WT IP lane.

(B) CoIP of neuroligin2 (NL2) using an anti-LHFPL4 antibody (left) and coIP of LHFPL4 using an anti-NL2 antibody (right) from WT and Lhfpl4−/− mouse brain lysates (IP, immunoprecipitation; #, IgG light chain).

(C–F) Confocal images of adult WT and Lhfpl4−/− hippocampal brain sections immunolabeled with antibodies to (C) GABAAR-α2 and (E) gephyrin, co-stained with DAPI. Normalized total cluster area quantification of (D) GABAAR-α2 and (F) gephyrin showing a loss of GABAAR-α2 (from 1.0 ± 0.2 to 0.3 ± 0.1; n = 17 WT

(legend continued on next page)
with glutamate decarboxylase (GAD6) antibody, remained intact (Figures S3A and S3B). Remarkably, we also observed the presence of large aggregates of mis-localized gephyrin within the soma and dendrites of Lhfpl4−/− neurons (Figures 4E and 4G), associated with a significant decrease in GAD6/gephyrin-positive clusters (Figure S3C). The dramatic re-distribution of gephyrin, along with the loss of GABAAR-α2 subunit clustering, is consistent with LHFPL4 playing a key role in maintaining GABAARs and their associated scaffold at inhibitory synaptic sites.

Loss of LHFPL4 Disrupts Inhibitory Postsynaptic Currents in Cultured Neurons

To determine the functional effect of LHFPL4 deletion, we initially examined hippocampal neurons in dissociated cultures and measured charge transfer mediated by miniature inhibitory post-synaptic currents (mIPSCs). When compared to neurons from WT littermates, synaptic charge in Lhfpl4−/− neurons was reduced by ~60% (Figures 5A and 5B), indicating loss of receptor/synapse number or function. Of note, we observed considerable variability in the amplitude and frequency of mIPSCs in both WT and Lhfpl4−/− cultures. This could reflect heterogeneity in the mixed hippocampal preparations and a varied contribution of LHFPL4. Indeed, immunolabeling for VGAT and GABAAR-γ2 revealed a small population of neurons cultured from Lhfpl4−/− mice that appeared to maintain their inhibitory synapses, while GABAAR-α2/SEP fluorescence also remained clustered in a proportion of cells. These observations suggested that LHFPL4 effects may be cell type specific. To test this, we compared the effect of LHFPL4 deletion on GABAAR-γ2 clustering in excitatory and inhibitory neurons, identified using antibodies against CAMKII and GAD6, respectively. Whereas CAMKII-positive cells showed a loss of GABAAR-γ2 clustering, GAD6-positive cells did not (Figures 5C and 5D). Thus, LHFPL4 appears to be essential for GABAAR clustering only in excitatory hippocampal neurons.

Cell-Type- and Synapse-Specific Effects of LHFPL4 Deletion

To further explore LHFPL4 function and its putative cell-type specificity, we made recordings in acute hippocampal slices from WT and Lhfpl4−/− mice. LHFPL4 deletion resulted in a profound loss of mIPSCs in CA1 pyramidal neurons (Figure 6A), with the mIPSC-mediated charge transfer reduced by ~80% (Figure 6B). Fast mIPSCs (median, 10%–90%; rise time, 0.4 ms; and τw_decay, 12 ms), likely originating from perisomatically projecting basket cells (Miles et al., 1996), were markedly reduced in both frequency and amplitude (Figure 6B), although their rise and decay were not changed (Figures 6C and 6D). By contrast, slow mIPSCs (Pearce, 1993) (median, 10%–90%; rise time, 9 ms; and τw_decay, 21 ms), of the type thought to originate from neurogliaform/ivy cells (Armstrong et al., 2012; Szabadics et al., 2007), were modestly increased in frequency and unaltered in amplitude (Figures 6E and 6F). Importantly, LHFPL4 deletion had no effect on AMPAR-mediated miniature excitatory post-synaptic currents (mEPSCs) (Figures 6G and 6H), confirming a selective effect on inhibitory synapses.

We next examined the effect of LHFPL4 deletion on bicuculline-sensitive tonic currents reflecting the persistent activation of extrasynaptic GABAARs (Farrant and Nusser, 2005) (Figures 7A–7C). In CA1 pyramidal cells from Lhfpl4−/− mice, the magnitude of the tonic current was slightly increased (Figure 7C), suggesting that LHFPL4 is required for the targeting of synaptic, but not extrasynaptic, GABAARs. Finally, to determine whether the functional effects of LHFPL4 were, indeed, cell type specific, we examined presumptive inhibitory interneurons. In marked contrast to the profound loss of mIPSC-mediated charge transfer seen in CA1 pyramidal cells, charge transfer in non-pyramidal cells in CA1 stratum oriens, radiatum, or lacunosum-moleculare from Lhfpl4−/− mice was unaffected (Figures 7D and 7E).

DISCUSSION

As clustering of GABAARs at synapses is essential for correct inhibitory signaling in the brain (Luscher et al., 2011b; Smith and Ktittler, 2010; Tyagarajan and Fritschy, 2014; Vithlani et al., 2011), it is vital to understand the machinery and regulatory pathways involved. Using molecular, imaging, electrophysiological, and mouse transgenic approaches, we show that the previously uncharacterized tetraspanin, LHFPL4, forms a complex with GABAARs, localizes to the inhibitory postsynaptic domain, and is critical for postsynaptic GABAAR clustering and fast GABAergic transmission in excitatory principal cells.

At inhibitory synapses, the canonical scaffold gephyrin, in complex with collybistin and the trans-synaptic adhesion molecule neuroligin2, plays a key role in GABAAR clustering and anchoring (reviewed in Tyagarajan and Fritschy, 2014; Varoqueaux et al., 2004). Nevertheless, in the absence of gephyrin, subsets of inhibitory synapses remain (Kneussel et al., 1999; O’Sullivan et al., 2009; Panzanelli et al., 2011). Moreover, the role for collybistin in inhibitory synapse formation appears to be region specific (Papadopoulos et al., 2007). Thus, there has been great interest in identifying other molecules that drive GABAAR clustering in a cell-type- or synapse-specific manner.

We found LHFPL4 to be enriched at inhibitory postsynaptic sites, and SIM imaging revealed clusters of LHFPL4 to overlay gephyrin clusters, placing it in the exact location required to scaffold GABAARs at the synapse. Importantly, we did not find LHFPL4 at excitatory synapses, suggesting that it is not a general synaptic organizer but rather an exclusive regulator of the inhibitory synaptic domain. This is supported by our finding that LHFPL4 and GABAARs interact with high affinity and that deletion of LHFPL4 leads to a dramatic loss of GABAAR and gephyrin clustering, while excitatory synapses are unaffected. It is
currently thought that GABA<sub>RS</sub> are initially trafficked extrasynaptically and subsequently diffuse to, and are trapped at, synaptic sites (Bannai et al., 2009; Bogdanov et al., 2006; Luscher et al., 2011b). In Lhfpl4<sup>–/–</sup> neurons, GABA<sub>RS</sub> were still found at the neuronal surface. This suggests that, in the absence of LHFPL4, the cell-surface GABA<sub>R</sub> trafficking pathways are preserved but that, once at the cell surface, receptors can no longer be trapped at synapses.

It is well established that synaptic GABA<sub>RS</sub> are reciprocally required for the clustering of gephyrin (Tyagarajan and Fritschy, 2014). Thus, it is difficult to determine whether the loss of gephyrin clustering we observed upon LHFPL4 deletion is a cause or a consequence of the LHFPL4-dependent disruption of GABA<sub>R</sub> clustering. In this regard, it has previously been described that, in hippocampal and thalamic relay neurons, subunit-specific knockout of GABA<sub>RS</sub> leads to the accumulation of large intracellular gephyrin aggregates (Studer et al., 2006). This phenomenon was attributed to the need for synaptic GABA<sub>RS</sub> to maintain gephyrin clustering (Essrich et al., 1998). Interestingly, we found that loss of LHFPL4 in vivo leads to a similar accumulation of intracellular gephyrin aggregates in the cell-body layer of the hippocampus.

Accompanying the loss of GABA<sub>R</sub> clusters, in CA1 pyramidal neurons from Lhfpl4<sup>–/–</sup> mice, we observed a dramatic decrease in both the amplitude and frequency of fast mIPSCs. The reduced amplitude of mIPSCs would, most straightforwardly, reflect loss of postsynaptic receptors. The primarily postsynaptic action of LHFPL4 is supported by the fact that, in our rescue experiments, sparse re-expression of LHFPL4 cDNA in Lhfpl4<sup>–/–</sup> neurons readily rescued postsynaptic GABA<sub>R</sub> clustering. The reduced frequency of mIPSCs could reflect both direct and indirect effects of such receptor loss; namely, a reduction of mIPSC amplitude below the threshold for detection and a possible loss of inhibitory synapses. Indeed, GABA<sub>R</sub> clusters; p = 0.0058, Wilcoxon rank-sum test). Box-and-whisker plots indicate median (line), 25th-75th percentiles (box), the range of data within 1.5 x interquartile range (IQR) of box (whiskers), and mean (open circles).

(C and D) Confocal images and cluster quantification of dissociated DIV14 WT or Lhfpl4<sup>–/–</sup> hippocampal neurons transfected with GFP and labeled with antibodies to GABA<sub>R</sub>γ2 and either (C) CAMKIIα or (D) GAD6 to label excitatory neurons and inhibitory neurons, respectively. GABA<sub>R</sub> cluster number and area were significantly reduced in Lhfpl4<sup>–/–</sup> CAMKIIα-positive cells (cluster number: from 8.9 ± 1.0 to 3.1 ± 0.9; area: from 3.3 μm<sup>2</sup> ± 0.4 μm<sup>2</sup> to 1.0 μm<sup>2</sup> ± 0.2 μm<sup>2</sup>; 362/131 WT/Lhfpl4<sup>–/–</sup> clusters; p = 0.00032 and 0.00013, respectively), but not GAD6-positive cells, compared to WT (cluster number: from 7.9 ± 1.2 to 8.6 ± 1.3; area: from 2.9 μm<sup>2</sup> ± 0.4 μm<sup>2</sup> to 3.0 μm<sup>2</sup> ± 0.4 μm<sup>2</sup>; 376/362 WT/Lhfpl4<sup>–/–</sup> clusters; p = 0.69 and 0.81, respectively). Error bars indicate mean ± SEM. For all conditions, n = 14 cells from 3 independent preparations. All used the Welch t test. **p < 0.01, ***p < 0.001. Scale, 2 μm.
extrasympathetic trapping of δ5-subunit-containing GABAARs (Lobrich et al., 2008).

The postsynaptic single-pass membrane-spanning molecules neuropilin2, calyxin3, and slitk3 promote the development and stabilization of mammalian GABAergic synapses through transsympathetic interactions with presynaptic proteins—neurexins and PTPS, respectively. Calyxin3 and slitk3 loss of function leads primarily to a reduction in mIPSC frequency, while disruption of neuropilin2 causes both frequency and amplitude reductions in several cell types (Pettem et al., 2013; Pouloupoulos et al., 2009; Takahashi et al., 2012). Although we identified LHFPL4 as a membrane-spanning inhibitory synaptic protein, we found no evidence that it could drive the formation of synapses onto trans- fected non-neuronal cells. Moreover, overexpression of LHFL4 did not increase synapse formation in neurons. This suggests that LHFL4 does not mediate GABAAR clustering through syn- apse specification and trans-synaptic interaction with presynap- tic terminals but, rather, acts to stabilize postsynaptic GABAAR clusters, possibly through enhancing interactions between GABAARs and synaptogenic molecules such as neuropilin2.

Understanding the processes that mediate cell- and synapse- specific GABAAR clustering is key to a better understanding of the underlying logic of inhibitory control in the brain. The homol- phic adhesion molecule IgSF9b was recently demonstrated to promote inhibitory synapse development in interneurons by coupling to neuropilin2 and S-SCAM (Woo et al., 2013). By contrast, we show here that the impact of LHFL4 deletion on GABAAR clustering and synaptic inhibition in the hippocampus is specific to CamKIIα-positive excitatory principal cells. As we found LHFL4 to interact tightly with neuropilin2, it is likely that neuropilins work in concert with an array of clustering molecules to drive cell-specific regulation of postsynaptic receptor clus- tering. In this regard, further studies of identified interneuron types will be necessary to determine whether resistance to LHFL4 deletion is a property of all interneurons or specific classes.

As the strength of inhibitory synaptic transmission directly correlates with the number of surface GABAARs at synaptic sites, modulation of GABAAR synaptic accumulation is a key mecha- nism underlying inhibitory synaptic plasticity. It will be interesting to determine whether LHFL4 plays a role in previously reported mechanisms of activity-dependent tuning of synaptic GABAAR number (Bannai et al., 2009; Muir et al., 2010; Petrin et al., 2014). Deficits in GABAergic neurotransmission can result in alter- ations in information processing at the network level and have been implicated in multiple neuropsychiatric disorders (Blundell et al., 2009; Charych et al., 2009; Crestani et al., 1999; Luscher et al., 2011a; Yizhar et al., 2011). Thus, identification of the molecular mechanisms by which LHFL4 regulates inhibitory transmis- sion may be critical to understanding both normal and disordered states. In line with other knockout mouse models that result in dis- rupted GABAAR and gephyrin clustering, such as the GABAAR-ζ1 or β2 subunit knockouts, we saw no obvious home cage behavioral deficits in Lhfpl4 knockout mice (Kratic et al., 2006; Sur et al., 2001; Vicini et al., 2001). Further detailed characterization will be necessary to identify any disease-associated behavioral deficits, such as the anxiety- and schizophrenia-related sensorimotor def- icits observed in neuropilin2 and GABAAR-ζ3 knockout mice, respectively (Blundell et al., 2009; Yee et al., 2005).

Very recently, a paper describing LHFL4 as a GABAAR regulatory Lhfpl (GARLH) protein was published (Yamasaki et al., 2017). Although not the first to establish LHFL4 as a GABAAR-interacting protein (Heller et al., 2012; Nakamura et al., 2016), these authors identified a tripartite interaction between GABAARs, neuropilin2, and LHFL4. Using the compli- mentary approaches of short hairpin RNA (shRNA)-mediated LHFL4 knockdown in culture and virally mediated CRISPR knockout in Cre-dependent Cas9 knockin mice, they also re- ported a marked reduction in mIPSC frequency in hippocampal CA1 neurons. Of note, here we further demonstrate that LHFL4 is essential for inhibitory synapse stability in CA1 pyramidal cells.

Figure 6. Loss of Fast mIPSCs in CA1 Pyramidal Neurons from Lhfpl4−/− Mice
(A) Representative recordings of mIPSCs (−70 mV) in CA1 pyramidal cells from a WT mouse (left) and an Lhfpl4−/− mouse (right). Lower panels are representative sections of recordings (contiguous 1 s segments) showing a loss of fast mIPSCs (red) but a maintained presence of slow mIPSCs (orange). Records are digitally filtered at 2 kHz for illustration purposes.
(B) Pooled data showing reduction in mean mIPSC charge transfer (from 1.59 pC ± 0.21 pC to 0.31 pC ± 0.11 pC; n = 18 WT and 18 Lhfpl4−/− cells), frequency (from 2.86 Hz ± 0.54 Hz to 1.0 Hz ± 0.03 Hz; n = 18 WT and 18 Lhfpl4−/− cells), and amplitude (from 141.9 pA ± 3.8 pA to 22.3 pA ± 1.1 pA; n = 18 WT and 14 Lhfpl4−/− cells). All p < 0.0001, Wilcoxon rank-sum test.
(C) Top: images illustrating the alignment and amplitudes of selected mIPSCs with uncontaminated rise and decay from representative WT and Lhfpl4−/− recordings. Bottom: average mIPSC waveforms (black), SEM (gray), and fitted sum of exponentials (red). The 10%–90% rise times and weighted time constant of decay (τw) are shown for each representative record.
(D) Pooled data showing the lack of effect on mIPSC rise time (0.38 ms ± 0.01 ms and 0.42 ms ± 0.04 ms; n = 18 WT and 11 Lhfpl4−/− cells; p = 0.86, Wilcoxon rank-sum test) and decay (12.0 ms ± 0.5 ms and 14.0 ms ± 1.4 ms; n = 18 WT and 11 Lhfpl4−/− cells; p = 0.20, Welch t-test).
(E) Representative slow mIPSCs (−70 mV) from CA1 pyramidal neurons (three different WT mice). Orange lines indicate fits of an empirical equation (see the Supplemental Experimental Procedures) from which 10%–90% rise time and 63% decay time measures were taken.
(F) Pooled data showing the modest increase in frequency of slow mIPSCs in Lhfpl4−/− mice (from 0.021 Hz ± 0.007 Hz to 0.051 Hz ± 0.01 Hz; n = 18 WT and 18 Lhfpl4−/− cells; p = 0.014, Wilcoxon rank-sum test) but a lack of change in amplitude (36.0 pA ± 8.4 pA and 24.8 pA ± 2.4 pA; n = 12 WT and 16 Lhfpl4−/− cells; p = 0.37 Wilcoxon rank-sum test), rise time (9.5 ms ± 1.1 ms and 9.4 ms ± 0.4 ms; n = 12 WT and 16 Lhfpl4−/− cells; p = 0.77, Welch t-test), and decay (21.9 ms ± 1.7 ms and 22.5 ms ± 0.9 ms; n = 12 WT and 16 Lhfpl4−/− cells; p = 0.93, Welch t-test). Box-and-whisker plots indicate median (line), 25th–75th percentiles (box), the range of data within 1.5 × IQR of the box (whiskers), and mean (open circles).
(G) Representative recordings of mEPSCs (−70 mV) in CA1 pyramidal cells from a WT mouse (left) and a Lhfpl4−/− mouse (right). Lower panels are representative sections of recordings (as in A), with mEPSCs in blue.
(H) Pooled data showing no change in mean mEPSC charge transfer (0.44 pC ± 0.07 pC and 0.33 pC ± 0.03 pC), frequency (2.01 Hz ± 0.35 Hz and 2.15 Hz ± 0.27 Hz), and amplitude (23.7 pA ± 2.1 pA and 23.2 pA ± 2.4 pA). In each case, n = 6 WT and 4 Lhfpl4−/− cells (p = 0.21, 0.75, and 0.87, respectively; Welch t-test). See also the Supplemental Experimental Procedures.
Figure 7. GABA-Mediated Tonic Current in Pyramidal Cells and mIPSCs in Interneurons Are Not Disrupted by LHFPL4 Deletion

(A) Representative record (−70 mV) from a WT CA1 pyramidal neuron showing the block of mIPSCs and the shift in holding current produced by bath application of bicuculline. Lower panels show the time course of the synaptic charge transfer, mean holding current, and its SD (see Supplemental Experimental Procedures).

(B) Representative all-point amplitude histograms from 1-s segments in the control period (upper) and in the presence of bicuculline (lower). Red lines indicate fits of a single-sided Gaussians to the most positive current values yielding the mean and the SD. The mean was taken as the baseline current for that segment and subtracted from the record. The integral of the subtracted current provided the charge carried by the synaptic events.

(C) Pooled data showing increased tonic GABA-mediated current in CA1 pyramidal neurons (from 6.2 pA ± 1.3 pA to 11.0 pA ± 1.7 pA; n = 22 WT and 15 Lhfpl4−/− cells; p = 0.037, Welch t test).

(D) Representative recordings of mIPSCs (−70 mV) in non-pyramidal cells (INs, presumptive interneurons) from a WT mouse (left) and an Lhfpl4−/− mouse (right). Lower panels are representative sections of recordings (contiguous 1-s segments) showing no change in fast mIPSCs (red). Records digitally filtered at 2 kHz for illustration purposes.

(E) Pooled data showing the lack of effect of LHFPL4 deletion on mIPSC-mediated charge transfer in presumptive interneurons (1.24 pC ± 0.39 pC and 1.34 pC ± 0.34 pC; n = 6 WT and 6 Lhfpl4−/− cells; p = 0.84, Welch t test).

Box-and-whisker plots indicate median (line), 25th–75th percentiles (box), the range of data within 1.5*IQR of the box (whiskers), and mean (open circles).

*p < 0.05.

See also the Supplemental Experimental Procedures.

EXPERIMENTAL PROCEDURES

Details regarding animals, antibodies, immunocytochemistry and immunohistochemistry, co-immunoprecipitations, biotinylations, cDNA cloning, and data analysis are included in the Supplemental Experimental Procedures.

Cell Culture and Transfections

Hippocampal cultures were obtained from E16 mouse or E18 rat embryos of either sex, as previously described (López-Doménech et al., 2016; Vaccaro et al., 2017). All procedures for the care and treatment of animals were in accordance with the Animals (Scientific Procedures) Act 1986. Neurons were transfected using Lipofectamine 2000 (Invitrogen). COS-7 cells were maintained in DMEM supplemented with fetal calf serum and antibiotics and were transfected using the Amaxa Nucleofector device (Lonza) following the manufacturer’s protocol.

Microscopy

Confocal images were acquired on a Zeiss LSM700 upright confocal microscope using a 63× oil objective (NA: 1.4) and digitally captured using LSM software. For cultured neurons, a whole-cell single-plane image was captured using a 0.5× zoom. From this, 3 sections of dendrite, ~100 μm from the soma, were imaged with a 3× zoom (equating to a 30-μm length of dendrite). For brain sections from adult male and female fixed brains, a low-magnification region of the hippocampus was captured using a 63× objective and a 0.5× zoom. From this, 2–3 zoom regions were imaged within each hippocampal strata with a 2× zoom for analysis. Acquisition settings and laser power were kept constant within experiments. For details of

but not in hippocampal interneurons. Moreover, we additionally observed that fast, but not slow, mIPSCs are reduced in Lhfpl4−/− mice, suggesting that LHFPL4 effects may be not only cell type specific but also synapse specific. It will be interesting to determine whether the modestly increased frequency of slow mIPSCs and increased tonic inhibition we identified in Lhfpl4−/− mice are adaptive changes due to prolonged disruption of synaptic inhibition (Brickley et al., 2001).

Members of the tetraspanin superfAMILY of integral membrane proteins have emerged as key regulators of excitatory synaptic function. Notably, TARPs and GSG1L have been shown to associate with AMPARs to regulate their trafficking and functional properties (Jackson and Nicoll, 2011; McGee et al., 2015). Our findings provide new insights into the molecular make-up of the inhibitory PSD and reveal a key role for an inhibitory synapse-specific tetraspanin. LHFPL4 is one of a subfamily of five tetraspanins that includes the homologous LHFPL3 and LHFPL5 and the more distantly related LHPL1 and LHPL2. Interestingly, LHFPL5 has been implicated in the mechanotransduction pathway of the inner hair cell, and its dysfunction contributes to hearing loss in humans and mice (György et al., 2017; Kalay et al., 2006; Longo-Guess et al., 2005; Xiong et al., 2012). Whether the other family members also have roles in synaptic function remains to be determined.
antibody labeling and image analysis, see Supplemental Experimental Procedures.

SIM was performed on a commercial Zeiss ELYRA PS.1 inverted microscope. Images were acquired with a 63X oil objective lens (NA: 1.4) using a pco.edge sCMOS camera and ZEN Black (v.11.0.2.190) software (2,430 x 2,430 pixels, 78.32-μm image size, 16 bit). Typically, images were acquired with 34-μm grating and three rotations by exciting fluorophores with 1%-3% laser intensity and 120-ms to 150-ms exposure time. Images were processed with ZEN Black using the SIM reconstruction module with default settings; drift corrections between the channels were performed with respect to 100-nm Tetraspec fluorescent microspheres (Molecular Probes).

Electrophysiology
Standard whole-cell voltage-clamp techniques were used to record mIPSCs in cultured neurons. For details, see the Supplemental Experimental Procedures. For slice electrophysiology, hippocampal slices from male and female mice (P30–P45) were perfused at room temperature with external solution containing (in millimolar): 125 NaCl, 2.5 KCl, 1.25 NaH2PO4, 2 CaCl2, 38 glucose, and 26 NaHCO3 saturated with 95% O2/5% CO2 (pH 7.4). Cells were visualized using oblique illumination. Currents were recorded using a MultiClamp 700B amplifier (Molecular Devices), filtered at 4 kHz, and digitized at 50 kHz using WinWCP and WinEDR (Strathclyde Electrophysiology Software) and an IntrustEC TITCH-1C2-2 interface (HEKA Elektronik). Series resistance was typically compensated by 50%-80%, and data were discarded if the series resistance varied by >20%.

For mIPSCs and tonic current measurement, recording pipettes were filled with an internal solution containing (in millimolar): 128 CsCl, 10 EGTA-Cs, 10 HEPES, 1 QX-314 (Tocris Bioscience), and 5 TEA-Cl (adjusted to pH 7.3 with CsOH). In some cases, this solution also contained 0.2% biocytin (Molecular Probes). D-AP5 (20 μM, Abcam) and NMDX (10 μM, Abcam) were added to the external solution to block NMDA receptors (NMDARs) and AMPARs. All mIPSCs were blocked by biocytin (20 μM Tocris) or gabazine (20 μM, Abcam). mEPSCs were recorded at 32.5°C using an internal solution containing (in millimolar) 135 Cs-gluconate, 10 HEPES, 10 Na-phosphocreatine, 4 MgATP, 0.4 NaGTP, 2 QX-314, and 10 TEA-Cl (adjusted to pH 7.3 with CsOH). The external solution contained an additional 5 mM KCl and biocytin (20 μM) or gabazine (20 μM). All mEPSCs were blocked with NMDX (10 μM; Abcam or Tocris). Interneurons were identified under infrared video microscopy by their relatively small and rounded or ovoid cell bodies, compared to the large triangular somata of pyramidal cells, and by the absence of conspicuous apical dendrites. Their cell bodies were located in CA1 stratum oriens, radiatum, or lacunomus-moleculare. Although reconstruction was not performed, post hoc examination of each biocytin-filled putative interneuron confirmed its location outside the pyramidal cell layer and its non-pyramidal cell morphology.

Statistics
All data were obtained from at least three different cell preparations or animals. Data are reported as mean ± SEM. Repeated for experiments are for the figure legends as n numbers. No statistical test was used to pre-determine sample sizes; these were based on standards of the field. Statistical analyses were carried out using GraphPad Prism (GraphPad Software, La Jolla CA, USA), Microsoft Excel or R (v3.2.3; the R Foundation for Statistical Computing: http://www.r-project.org/), and R Studio (v.0.99.893; RStudio). Data were tested for normality (D’Agostino-Pearson test or Shapiro-Wilk test) and compared using either parametric (unpaired Welch t test) or non-parametric tests (Wilcoxon rank-sum test or Kruskal-Wallis one-way ANOVA). Exact p values are presented to two significant figures, except when p < 0.0001. Differences were considered significant at p < 0.05. No blinding or randomization was used.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.09.025.

AUTHOR CONTRIBUTIONS
This study was conceived by J.T.K. Experiments were designed by J.T.K., E.C.D., and M.F. Experiments were performed by E.C.D., V.P., G.K., T.P.M., D.F.S., and G.L.-D. Data were analyzed by E.C.D., V.P., G.K., M.F., and J.T.K., and the manuscript was written by E.C.D., M.F., and J.T.K.

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