Role of regulatory C-terminal motifs in synaptic confinement of LRRTM2

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Abstract
Leucine Rich Repeat Transmembrane proteins (LRRTMs) are neuronal cell adhesion molecules involved in synapse development and plasticity. LRRTM2 is the most synaptogenic isofrom of the family, and its expression is strongly restricted to excitatory synapses in mature neurons. However, the mechanisms by which LRRTM2 is trafficked and stabilized at synapses remain unknown. Here, we examine the role of LRRTM2 intracellular domain on its membrane expression and stabilization at excitatory synapses, using a knock-down strategy combined to single molecule tracking and super-resolution dSTORM microscopy. We show that LRRTM2 operates an important shift in mobility after synaptogenesis in hippocampal neurons. Knock-down of LRRTM2 during synapse formation reduced excitatory synapse density in mature neurons. Deletion of LRRTM2 C-terminal domain abolished the compartmentalization of LRRTM2 in dendrites and disrupted its synaptic enrichment. Furthermore, we show that LRRTM2 diffusion is increased in the absence of its intracellular domain, and that the protein is more dispersed at synapses. Surprisingly, LRRTM2 confinement at synapses was strongly dependent on a YxxC motif in the C-terminal domain, but was independent of the PDZ-like binding motif ECEV. Finally, the nanoscale organization of LRRTM2 at excitatory synapses depended on its C-terminal domain, with involvement of both the PDZ-binding and YxxC motifs. Altogether, these results demonstrate that LRRTM2 trafficking and enrichment at excitatory synapses are dependent on its intracellular domain.

KEYWORDS
cell adhesion, light microscopy, membrane protein, synapse

INTRODUCTION

Neurons exhibit a complex morphology that requires compartmentalization to ensure the efficiency of neuronal communication (Terenzio et al., 2017). This communication takes place at specialized cell-cell contacts, synapses, and depends among other, on the proper localization and distribution of synaptic cell adhesion molecules (CAMs) that not only provide a physical bridge across the synaptic cleft, but also contribute to cell–cell recognition, specialization, and signaling (Chamma & Thoumine, 2018; Kim et al., 2021; Missler et al., 2012; Ribeiro et al., 2018). Thus, delivery and stabilization of these molecules at the plasma membrane are essential for neuronal function.

Leucine-rich-repeat transmembrane proteins (LRRTMs) are a family of neuronal cell adhesion molecules involved in synapse specification and function.
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(Schroeder et al., 2018). They are highly expressed in the brain and each isoform possesses a specific pattern of expression within different brain regions (Laurén et al., 2003). Among the four isoforms, LRRTM1-4, LRRTM2 is the most synaptogenic, inducing pre-synaptic differentiation when expressed in heterologous cells co-cultured with neurons (Linhoff et al., 2009). LRRTM2 is a post-synaptic protein exclusively localized at excitatory synapses (de Wit et al., 2009; Linhoff et al., 2009). Its overexpression was shown to increase excitatory synapse density, whereas down-regulation using different shRNAs showed contradictory results concerning the decreased synapse density (de Wit et al., 2009; Ko et al., 2011) in hippocampal neurons. LRRTM2 knock-down (KD) (de Wit et al., 2009) or double conditional knock-out of LRRTM1 and 2 (Bhouri et al., 2018) further reduces AMPAR-mediated synaptic transmission and blocks long-term potentiation in CA1 pyramidal neurons (Bhouri et al., 2018; Roppongi et al., 2017; Soler-Llavina et al., 2013).

One of the striking features of LRRTM2 is its high enrichment and exclusive localization at excitatory postsynapses (Chamma et al., 2016). To date, LRRTM2 was shown to interact with the presynaptic adhesion molecules Neurexins (Südhof, 2017) and the excitatory post-synaptic scaffold PSD-95 (de Wit et al., 2009; Linhoff et al., 2009; Won et al., 2018). Postsynaptic LRRTM2 binds to pre-synaptic adhesion proteins Neurexins 1–3, α- or β-, lacking the insert at the splice-site 4 (SS4-) (Ko et al., 2009; Siddiqui et al., 2010). A recent crystallography study of the complex formed between Nrx1β and LRRTM2 showed that the binding interface involves a critical glutamic acid residue at position 348 (E348) in the C-terminal cap of the extracellular LRR domain of LRRTM2 (Yamaga et al., 2018). Intracellularly, LRRTM2 can bind the scaffolding protein PSD-95 through a non-canonical type 1 PDZ-like binding motif, ECEV, at the very end of the C-terminal domain (CTD) (de Wit et al., 2009; Linhoff et al., 2009). Furthermore, a YxxC motif at position -16 from the C-terminus, was recently identified to be critical for LRRTM2 normal trafficking to the cell surface and efficient synaptic clustering (Minatohara et al., 2015). In previous work, we showed that LRRTM2 exhibits very low surface mobility, with 80% of LRRTM2 molecules confined at synapses and organized into compact synaptic domains (Chamma et al., 2016). However, the molecular mechanisms responsible for these effects remain unknown.

Here, we investigate the role of the intracellular domain of LRRTM2 in its membrane clustering and stabilization at excitatory synapses, using a replacement strategy in combination with single molecule tracking and super-resolution microscopy. We observed that knock-down of LRRTM2 during synapse development reduces excitatory synapse density, consistent with previous studies. We further show that the C-terminal domain is responsible for compartmentalization of LRRTM2 in dendrites and for its clustering. Using single molecule tracking, we observed that LRRTM2 was confined at excitatory synapses via its C-terminal domain and identified the YxxC motif at position -16 from the CTD as a critical sequence for synaptic confinement. Surprisingly, LRRTM2 confinement at synapses did not appear to involve the PDZ-like binding motif ECEV, known to bind PSD-95. Finally, we demonstrate that the C-terminal domain is responsible for the nanoscale organization of LRRTM2 at post-synapses, an organization that involves both the YxxC and ECEV motifs.

RESULTS

LRRTM2 operates a shift in mobility after synapse formation

In previous work, we showed that LRRTM2 is highly enriched and immobile at excitatory synapses in mature hippocampal neurons (Chamma et al., 2016). To determine whether this diffusive behavior depended on synapse formation, we performed single molecule tracking of LRRTM2 by universal Point Accumulation for Imaging in Nanoscale Topography, uPAINT (Giannone et al., 2010) at different developmental stages in culture (days in vitro DIV 7, 9, and 15) during synapse development and maturation (Grabrucker et al., 2009) (Figure 1a). To assess the presence of endogenous LRRTM2 at early developmental stages, we performed RT-qPCR, and found abundant levels of mRNA at DIV7-14, with a slight decrease at more mature stages (DIV21) (Figure S1a). In the absence of efficient antibodies to detect endogenous LRRTM2 by imaging methods, we added a 15 amino acid biotin acceptor peptide tag (AP) at the N-terminal region of LRRTM2. Neurons expressing AP-tagged LRRTM2 in combination with an ER-restricted biotin ligase that biotinylates the AP tag in the endoplasmic reticulum (ER), display selective biotinylated LRRTM2 molecules at the cell surface (Chamma et al., 2016, 2017; Howarth & Ting, 2008). Surface expressed biotinylated AP-LRRTM2 was then detected at the single molecule level using sparse labelling with fluorescently-conjugated monomeric streptavidin (mSA), as described previously (Chamma et al., 2016, 2017). We used Homer1c-GFP as a synaptic marker to label excitatory post-synapses in live neurons. Over the DIV 7–15 period, the density of Homer1c positive puncta increased gradually (Figure 1b). However, the mobility of LRRTM2 molecules was similar at DIV7 and 9 regardless of the increase in excitatory synapse density, with about half of the molecules displaying slow diffusion (Figure 1c–e). This percentage increased to ~70%
at DIV15, after synapses matured and LRRTM2 diffusion was reduced by \(~2.5\)-fold (Figure 1c–e). In parallel, the number of tracks normalized per surface was decreased and the percentage of synaptic tracks increased by more than two fold (Figure S1b,c). Thus, unlike neuroligin-1, another Neurexin binding partner at excitatory synapses whose diffusion slows down gradually during excitatory synapse development (Chamma et al., 2016), LRRTM2 displays a sudden switch in mobility after synapses have developed. Further analysis of LRRTM2 trajectories showed that at all DIVs considered, synaptic tracks displayed slower diffusion compared to extra-synaptic tracks (Figure S1d-f), suggesting a specific stabilization mechanism at synapses compared to dendritic shafts, even at early developmental stages. Furthermore, when compared to each other, synaptic tracks were even more immobilized at DIV15 compared to DIV7 and 9 (Figure S1g,h). Interestingly, the mean time spent at synapses, which reflects molecular interactions rather than steric hindrance (Renner et al., 2012) was identical at DIV7-9 and displayed an important increase at DIV15 (Figure S1i), suggesting an increase in molecular interactions. Altogether, these results show that LRRTM2 membrane expression and localization are strongly regulated during neuronal development.

**LRRTM2 membrane expression and clustering are governed by its C-terminal domain**

To investigate the molecular mechanisms regulating LRRTM2 membrane expression and localization at mature synapses, we used a replacement strategy, where LRRTM2 was knocked-down using a novel shRNA in combination with sh-resistant wild-type or mutated LRRTM2. We first validated that the shRNA was able to knock-down LRRTM2 expression, and that this knock-down was reversed using the sh-resistant form of LRRTM2 (LRRTM2-WTr). shRNA against LRRTM2 reduced protein expression observed by Western blot, by \(~50\)%, an effect that was rescued with shRNA-insensitive LRRTM2-WTr (Figure S2a,b). As previously reported using a similar knock-down strategy (de Wit et al., 2009), we observed a reduction in both the density and intensity of PSD-95 clusters in neurons, effects that were rescued with sh-resistant LRRTM2 expression (Figure S2c-f). These results show that KD of LRRTM2 during synaptogenesis significantly affects excitatory synapse development. To address the mechanisms of LRRTM2 membrane stabilization and localization, we generated mutants of the intracellular
FIGURE 2  LRRTM2 is compartmentalized to dendrites and clustered through its CTD. (a) Schematics of AP-LRRTM2-WT. LRR: Leucine-Rich-Repeat; TM: Transmembrane; CTD: C-terminal domain. Below: amino acid sequences of the intracellular region of LRRTM2 mutants, highlighting the PDZ-like binding motif ECEV; ΔCTD: 449–516a.a deleted; ΔECEV: last 4 a.a deleted; YACA: double substitution by alanine Y501A/C504A. (b) Hippocampal neurons expressing shLRRTM2 and AP-LRRTM2 constructs live-labeled with monomeric streptavidin.
C-terminal tail of the protein, a region shown to be important for the regulation of LRRTM2 membrane expression and clustering (Minatohara et al., 2015) (Figure 2a). AP-LRRTM2-ΔCTD, lacking the entire C-terminal domain, AP-LRRTM2-ΔECEV, lacking the last four amino acids (type 1 PDZ-like binding motif ECEV), and a mutant containing two mutated residues Y501A-C504A (YACA) on the YxxC motif at position -16 from the CTD (Minatohara et al., 2015). We first examined that all mutants were properly expressed. As previously reported, full truncation of the CTD or mutation of the YxxC motif both resulted in increased protein expression (Figure S3). We also assessed the ability of LRRTM2 mutants to recruit PSD-95. When co-expressed with PSD-95-GFP, AP-LRRTM2-ΔCTD, and AP-LRRTM2-YACA, induced the formation of large PSD-95-GFP clusters (Figure S4). However, AP-LRRTM2-ΔECEV and AP-LRRTM2-ΔCTD were not able to recruit PSD-95, demonstrating that these C-terminal truncations abolished the interaction of LRRTM2 with PSD-95. In mature hippocampal neurons, we expressed the C-terminal mutants on a knock-down background, and assessed their localization and distribution by live labelling with fluorophore-conjugated mSA. AP-LRRTM2-ΔCTD, and AP-LRRTM2-YACA expression was significantly increased in dendrites compared to AP-LRRTM2-ΔCTD, (Figure 2b,c). We further observed that all the mutants were mislocalized to axons, whereas AP-LRRTM2-ΔCTD, was restricted to dendrites (Figure 2b,d). However, the polarity index (dendrites/axons) was not significantly different from AP-LRRTM2-ΔCTD, for both AP-LRRTM2-ΔECEV, and AP-LRRTM2-YACA mutants, but was strongly affected upon deletion of the entire CTD (Figure 2e). The strongest effects of LRRTM2 mislocalization to axons were observed in the AP-LRRTM2-ΔCTD, and AP-LRRTM2-YACA conditions, with less impact of the ECEV truncation. These results show that the CTD of LRRTM2 is responsible for the compartmentalization of the protein, and restriction of its expression to dendrites. We also assessed the ability of LRRTM2 mutants to form clusters on the plasma membrane of dendrites. Deletion of LRRTM2 CTD, ECEV motif alone, or mutations in the YxxC motif all disrupted LRRTM2 clustering (Figure 2f,g), indicating that LRRTM2 surface expression and clustering are regulated by its CTD. Interestingly, we observed that LRRTM2 mutants seemed more clustered when they were over-expressed in the absence of the shRNA against LRRTM2 (Figure S5), potentially explaining the different results previously observed on LRRTM2 clustering with an over-expressed YFP-tagged version of LRRTM2-ΔECEV (Linhoff et al., 2009).

Confinement of LRRTM2 at excitatory synapses requires the YxxC motif

To examine the dynamic behavior of LRRTM2 mutants, we performed uPAINT experiments in mature neurons expressing AP-LRRTM2-WTr, ΔCTD, ΔECEV, and YACA, mutants on a LRRTM2 KD background. As we previously observed, AP-LRRTM2-WTr, was highly confined at excitatory synapses in DIV 15 neurons and displayed slow overall diffusion, with an important fraction of immobile molecules (~80%) located at synapses (Figure 3a–d). Deleting the CTD or mutating the YxxC motif (YACA) led to an increase in LRRTM2 diffusion and a reduction in the proportion of immobile trajectories (Figure 3a–d). Surprisingly, deletion of the PDZ-like binding motif ECEV did not have any significant effects on LRRTM2 mobility, proportion of immobile tracks, or mean square displacement (MSD) (Figure 3a–f), suggesting that this PDZ-like binding motif is not responsible for LRRTM2 confinement at synapses. We thus examined in more details the synaptic trajectories of LRRTM2 and found similar results (Figure 3g,h, Figure S6). Interestingly, the YACA mutant showed an intermediate synaptic MSD between the WT and ΔCTD mutant, suggesting that the YxxC motif plays a critical role in LRRTM2 confinement at excitatory synapses (Figure 3g,h). Finally, the mean time spent at synapses showed a tendency to a decrease in the ΔCTD condition but remained similar for ECEV and YxxC mutants (Figure S6e). These experiments show that LRRTM2 diffusion and trapping at excitatory synapses require its C-terminal domain, and more specifically the YxxC motif, but appear independent from its PDZ-like binding motif.

LRRTM2 nano-organization at synapses depends on its C-terminal domain

To gain insight into the nano-organization of LRRTM2 at synapses, we further explored the role of the CTD mutants on the nanoscale distribution of LRRTM2 using dSTORM (Heilemann et al., 2008; Rust et al., 2006). As previously observed with over-expressed LRRTM2 (Chamma et al., 2016), LRRTM2-WTr, formed compact domains containing a high density of molecules and a ~5-fold enrichment at excitatory post-synapses, compared to the neuronal shaft (Figure 4a,d). Full truncation of the C-terminal domain, mutation of the YxxC motif, or deletion of the PDZ-like binding motif disrupted the compact organization of LRRTM2, with molecules showing more dispersed labelling at the nanoscale.
FIGURE 3 LRRTM2 diffusion and confinement at synapses are independent from its PDZ-like binding motif. (a) Representative examples of DIV15 neurons expressing sh-LRRTM2, Homer1c-DsRed and AP-LRRTM2 (WT, ΔCTDr, ΔECEVr or YACAr) labeled with mSA-STAR635P to track individual molecules by uPAINT. Homer1c-DsRed (grey) signal is overlaid with AP-LRRTM2 trajectories (magenta); insets show individual synapses together with synaptic AP-LRRTM2 trajectories. (b) Semi-log distribution of diffusion coefficients for AP-LRRTM2-WTr, AP-LRRTM2-ΔCTDr, AP-LRRTM2-ΔECEV, and AP-LRRTM2-YACAr, and (c) median diffusion coefficient, showing an increase with the ΔCTDr and YACAr mutants (d) Percentage of immobile trajectories for the different conditions (e) Mean square displacement of the different conditions over time (f) Mean square displacement of the different conditions at t = 0.2 s (g) Synaptic mean square displacement of the different conditions over time (h) individual synaptic trajectories with the same number of frames overlaid with Homer1c-DsRed (grey). Data acquired from at least three experiments (WT: n = 31, ΔCTDr: n = 25, ΔECEV: n = 24, YACAr: n = 22 cells). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

(Figure 4a). When analyzed using the density-based spatial clustering of applications with noise (DBSCAN) method (Deschout et al., 2014; Ester et al., 1996), we found that all mutants disrupted the formation of large compact clusters observed in the WT condition, with no cumulative effect when deleting the entire CTD (Figure 4a–c). These mutations also led to a reduced enrichment of LRRTM2 molecules at excitatory synapses, that is, the number of detections at synapses compared to molecules present on the dendritic shaft (Figure 4d). While truncation of the full CTD completely abolished synaptic LRRTM2 enrichment, deletion of the ECEV or mutation of the YxxC reduced it by two-fold. These results indicate that both the ECEV and the
LRRTM2 nano-organization at synapses depends on its C-terminal domain. (a) dSTORM imaging of hippocampal neurons expressing sh-LRRTM2-GFP (red), BirAER, Homer1c-DsRed (green) as a synaptic marker and AP-LRRTM2-WTr, ∆C, ∆ECEV or -YACAr constructs (blue) that were live labeled with Alexa647-mSA at 14 DIV. Super-resolved localization maps based on LRRTM2 single molecule detections are shown in gold. Boxes indicate the location of insets. Insets show images obtained by DBSCAN analysis using the SMAP software (Ries, 2020). Gray points represent the coordinates of the different super-resolved localizations. Colored points represent localizations inside segmented clusters. Clusters were segmented using the same parameters in all conditions (Neighborhood radius 10nm, minimum number of detections per cluster 30). (b) Corresponding semi-log plot of the numbers of detections per cluster in the different conditions, and (c)
YxxC motifs are necessary for maintaining a compact nano-organization of LRRTM2 at synapses. Finally, we measured the percentage of LRRTM2 clusters overlapping with synapses in the different mutant conditions. Deletion of the entire CTD or the ECEV motif both significantly reduced the percentage of remaining clusters localized at synapses, while mutation of the YxxC motif did not have an impact on their localization (Figure 4e). These results show that both ECEV and YxxC motifs are critical for LRRTM2 nanoscale cluster organization at synapses.

DISCUSSION

In this study, we addressed the role of the C-terminal domain of LRRTM2 in its membrane stabilization, clustering, and nanoscale organization using a knock-down strategy in combination with wide-field fluorescence imaging, single-particle tracking, and super-resolution microscopy. We show that LRRTM2 becomes highly confined at mature excitatory post-synapses during neuronal development. The C-terminal domain of the protein controls its polarization in mature neurons and is responsible for restricting its membrane expression in dendrites. Furthermore, we found that the YxxC motif at position -16 from the C-terminal part of LRRTM2 is responsible for LRRTM2 confinement at synapses, and that both this motif and the PDZ-like binding motif ECEV are important for the nanoscale organization and compaction of LRRTM2 at synapses.

Membrane diffusion during neuronal development

When we monitored LRRTM2 diffusion over synapse development, we did not observe a gradual stabilization paralleling the gradual increase in synapse number, as was seen with another synaptic adhesion protein Neuroligin1 (Chamma et al. 2016), but instead, we observed a shift in diffusion after synaptogenesis. Synapses are highly crowded macromolecular platforms densely packed with hundreds of synaptic proteins, and a rich cytoskeletal environment, which altogether constitute obstacles for protein diffusion in the plasma membrane (Renner et al., 2012). One possibility is that the composition of excitatory synapses changes during development and facilitates LRRTM2 binding only after DIV9 by providing interaction partners not expressed earlier. To date, only two binding partners of LRRTM2 have been identified, the post-synaptic scaffold protein PSD-95 and the pre-synaptic adhesion molecules Neurexins (de Wit et al., 2009; Linhoff et al., 2009). These proteins are up-regulated during neuronal development, as synapses mature (Bustos et al., 2014). Thus, it is possible that an increased availability of LRRTM2 binding partners might contribute to increased stabilization after DIV9, as suggested by an increase in the time spent at synapses at DIV15 in our experiments (Figure S1i). Interestingly, synaptic tracks were even more immobilized at DIV15 compared to DIV7 and 9, suggesting that LRRTM2 stabilization at synapses could involve a multi-step process through different and complementary interactions with proteins over development. Surprisingly, the PDZ-like binding motif ECEV which interacts with PSD-95, was not involved in LRRTM2 synaptic confinement, suggesting that other interactions or diffusion constraints might be at play in this process (Kokolaki et al., 2020; Renner et al., 2012). Considering that extra-synaptic trajectories were also further immobilized after DIV9, another possibility is that non-stabilized proteins present at the surface of neurons are selectively removed from the cell surface by endocytosis as was observed for other membrane proteins (Garrido et al., 2001; Sampo et al., 2003).

Membrane expression and compartmentalization

The shift in mobility of LRRTM2 after synaptogenesis suggests that its membrane expression is tightly regulated during neuronal maturation. Interestingly, a myc-tagged version of LRRTM2 was previously observed to be diffusively distributed in both axons and dendrites earlier in development (Minatohara et al., 2015), and mislocalization of LRRTM2 to axons has been observed upon deletion of its C-terminal domain (Linhoff et al., 2009), although these phenotypes were not further studied. The diffusive distribution at early developmental stages suggests that LRRTM2 could be first targeted to all neurites indistinctively, and be retained only in dendrites at later stages. Interestingly, full truncation of the C-terminal domain, deletion of the PDZ-like binding motif, or mutation of the YxxC motif all abolished the dendrite-restricted expression of LRRTM2 in mature neurons. Several surface proteins are compartmentalized in neurons. The adhesion molecule NgCAM has been shown to be polarized to axons via selective fusion of intracellular vesicles with the axonal membrane (Burack et al., 2000), whereas voltage-gated sodium

Quantification of the number of detections per cluster. WT: 301 clusters; ΔC: 62 clusters; ΔECEV: 152 clusters; YACAR: 66 clusters. (d) Quantification of the synaptic enrichment of AP-LRRTM2 mutants (normalized number of detections inside synapses divided by normalized number of detections outside synapses) and (e) percentage of clusters localized at excitatory synapses. Data are from three independent experiments (WT’ n = 9; ΔC’ n = 6; ΔECEV’ n = 7; YACAR’ n = 8). *p < 0.05, ** p < 0.01, *** p < 0.0001
channel chimera and VAMP2 are distributed to all neurites but are then selectively removed from the somatodendritic domain via endocytosis (Garrido et al., 2001; Sampo et al., 2003). Interestingly, redistribution of surface proteins between axons and dendrites during development has also been shown for other synaptic adhesion proteins, such as neurolin-1α (Ribeiro et al., 2019), a presynaptic partner of LRRTM2. Deletions or mutations in the CTD of LRRTM2 could alter its dendritic polarization by impairing potential interactions with proteins involved in membrane regulation, such as clathrin adaptor-protein 2, which selects cargo from the plasma membrane for clathrin-mediated endocytosis. Interestingly, AP-2 has been associated with selective endocytosis of surface proteins with intracellular YxxØ motifs, Ø being a hydrophobic amino acid (Ohno et al., 1995; Traub, 2009) and we showed that point-mutations in this sequence mislocalize LRRTM2 and increase its surface expression. YxxØ motifs also serve as sorting motifs at the trans-Golgi network and bind to different adaptor proteins (Li et al., 2016) in order to address cargo proteins to specific compartments (Farias et al., 2012). Thus, it is possible that mutations in the YxxC motif could mislocalize LRRTM2 to axons by impeding or favoring interactions with specific adaptor proteins in the secretory pathway.

### Synaptic clustering

In accordance with previous studies, we showed that clustering of LRRTM2 critically depends on its CTD. Deletion of the PDZ-like binding motif or mutation of the YxxC motif reduced the clustering of the protein to the same extent as deletion of the entire CTD, suggesting that both motifs are involved in LRRTM2 clustering, without additive effects. This is not in agreement with one of the first studies on LRRTMs using overexpression of a YFP-tagged LRRTM2-ΔECEV that had found that deletion of the PDZ-like binding motif did not abolish LRRTM2 clustering (Linhoff et al., 2009). These differences might be due to overexpression, as we have observed in our experiments that when overexpressed in the absence of the shRNA, all the mutants seemed more clustered. This might be explained by oligomerization with endogenous forms of LRRTM2 (Paatero et al., 2016), although it is not clear whether LRRTM2 can form oligomers, or whether these are physiologically relevant (Yamagata et al., 2016). Compensation mechanisms involving other LRRTM family members upon LRRTM2 knock down cannot be excluded, in particular for LRRTM1, although these are not known to co-cluster with LRRTM2. 20% of the amino acids in the intracellular region are identical among LRRTMs, whereas 50% are highly similar (Laurén et al., 2003), suggesting that they could interact with the same binding partners. However, LRRTM4 is not expressed in hippocampal pyramidal neurons (Siddiqui et al., 2013), the principal cell type in hippocampal cultures (Kaech & Banker, 2006), and LRRTM3 is expressed only in the granule cells of the dentate gyrus (Laurén et al., 2003), reducing the potential influence of these LRRTMs. In an extensive study on clustering of exogenously expressed LRRTM2 in cultured neurons, deletion of the PDZ-like binding motif modestly but significantly decreased the synaptic cluster index compared to WT-LRRTM2, whereas mutations in the YxxC motif resulted in a 50% reduction (Mina-tohara et al., 2015). Interestingly, truncation of a larger intracellular sequence including both the ECEV and the YxxC motifs did not have any further impact on the synaptic cluster index, suggesting that the YxxC motif is the main CTD sequence governing LRRTM2 clustering in neurons. Using super-resolution dSTORM imaging, we observed similar results: WT-LRRTM2 formed large compact clusters at synapses, that were disrupted in all mutant conditions. Paradoxically, we found that the ECEV motif was important for LRRTM2 clustering, but its deletion did not affect synaptic confinement. These result indicate that LRRTM2 is immobilized at mature synapses independently from its PDZ-like binding motif, as reflected by diffusion measurements but local enrichment of the protein at synapses requires the ECEV domain. Further studies will be necessary to better understand these regulatory mechanisms.

#### YxxC at position -16 from the CTD is a critical motif for LRRTM2 confinement at excitatory synapses

We showed for the first time that the CTD of LRRTM2 governs its membrane diffusion. Surprisingly, confinement of LRRTM2 at excitatory post-synapses was independent from its PDZ-like binding motif which binds the major post-synaptic scaffolding protein PSD-95 (de Wit et al., 2009; Linhoff et al., 2009). This finding was very surprising as binding to scaffolding proteins via PDZ motifs is one of the key elements that govern trapping and stabilization of molecules at synapses (Chamma et al., 2019; Opazo, 2012; Sheng & Hoogenraad, 2007). For example, a point mutation of a critical tyrosine residue in the gephyrin binding motif of neuroligin-1 (Y782F) that weakens the interaction with PSD-95, increases neuroligin-1 diffusion (Letellier et al., 2018). Here, mutation of the YxxC motif disrupted LRRTM2 diffusion to the same extent as deletion of the entire CTD and strongly affected the confinement of LRRTM2 at synapses. Thus, LRRTM2 does not seem to follow the canonical model of diffusion-trapping, where mobile synaptic proteins such as other adhesion molecules (Neurexins and Neuroligins) (Chamma et al., 2016; Neu-pert et al., 2015; Schneider et al., 2015) or neurotransmitter receptors can diffuse at the plasma membrane and get trapped at synapses as a result of binding to stable
elements such as scaffolding proteins (Choquet & Triller, 2013; Czöndör et al., 2012). In the case of LRRTM2, unknown molecular interactions could explain trapping at excitatory post-synapses, but interaction with PSD-95 through the ECEV motif does not seem to be the dominant interaction in the context of synaptic stabilization. This might be explained by the fact that the ECEV motif is not a canonical PDZ-binding motif, as cysteines have not been reported at the -2 position for PDZ domain ligands (Linhoff et al., 2009; Tonikian et al., 2008). LRRTM2 YxxC motif could also be involved in specific interactions important in its membrane stabilization, independently from the ECEV motif. Further studies are necessary to clarify this point.

To conclude, we show for the first time the involvement of the C-terminal domain, and in particular the YxxC motif, in LRRTM2 membrane stabilization, synaptic confinement, and nanoscale organization at mature excitatory synapses. These results bring new insights into the molecular regulation of a major synaptic adhesion protein involved in synapse specification and plasticity. Further studies will be necessary to examine precisely the molecular interactions that regulate LRRTM2 trafficking at the plasma membrane during neuronal maturation, and to identify novel intracellular partners and/or post-translational modifications that regulate its membrane polarization and stabilization.

MATERIALS AND METHODS

DNA constructs

LRRTM2 shRNA was designed and ligated into pSuper.retro.neo+GFP (OligoEngine) according to the vendor’s directions. shRNA targeting the following sequence in mouse LRRTM2 (100% homologous between mouse and rat LRRTM2) was generated: CAATGAGTGTGGTATTTAAA. The H1 promoter-driven shRNA cassette was cloned into the lentiviral plasmid pFUGW. shRNA-resistant constructs were made by introducing three silent mutations into the target sequence for the LRRTM2 shRNA (LRRTM2, CAATGAGTGTGGTATTTAAA changed to CAATGAGCGTTGCTTTA). In vitro mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis Kit from Agilent. Primers for the QuikChange Lightning PCR were designed with the QuikChange primer design program and used on p-EF-BOS vectors containing mouse LRRTM2 cDNA. AP-LRRTM2 was previously described (Chamma et al., 2016). AP-LRRTM2-ΔCTD and AP-LRRTM2-ΔECEV were derived from previously described myc-LRRTM2-ΔCTD and myc-LRRTM2-ΔECEV (de Wit et al., 2009). The myc tag (EQK- LISEEDL) was replaced by the biotin AP tag (GLN- DIFEAQQKIEWHE). For the YACA mutant, WT LRRTM2 Y501/C504 sequence was mutated to A501/A504 (generated by Eurofins) at the NdeI site. Homer1c-DsRed was described previously (Mondin et al., 2011). Biotin ligase BirAER was a gift from A. Ting (Stanford University, CA). PSD-95-GFP containing an EGFP inserted at position 253 on PSD-95 (rat, UniprotKB/Swiss-Prot P31016) was described previously (Sainlos et al., 2011). pET-IG-mSA plasmid (Addgene, cat. no. 80706) was described previously (Chamma et al., 2016, 2017).

Protein expression, purification, and conjugation

Monomeric streptavidin mSA was produced, purified, and conjugated to fluorophores for fluorescence imaging as previously described (Chamma et al., 2017). Briefly, pET-IG-mSA (Addgene, cat. no. 80706) was produced in E. coli BL21 codon plus (DE3)-RIL, purified using a Ni-NTA affinity column, concentrated to ~1 mg/ml using Amicon Ultra centrifugal filters with a 10 kDa cutoff, and coupled to ATTO-647N (ATTO-TEC), STAR635P (Abbe rior), and Alexa Fluor 647 (Life Technologies) with the corresponding NHS ester derivatives for each dye.

Heterologous cell transfection and labelling

COS-7 cells were plated at a density of 100,000 cells per well into 6-well plates for biochemistry or 50,000 cells per well into 12-well plates containing sterile glass coverslips for live imaging, cultured in DMEM (GIBCO/BRL) supplemented with 10% fetal bovine serum (Eurobio), 100 units/ml penicillin and 100 mg/ml streptomycin at 37°C with 5% of CO2 atmosphere. 2–3 h after plating, transfections were done using the X-treme GENE HP DNA Transfection Reagent (Roche) following the manufacturer’s recommendations. One microgram of DNA (0.4 μg AP-LRRTM2-WTr or mutants + 0.4 μg BirAER ± 0.2 μg PSD-95-GFP) were mixed with 2 μl X-treme gene reagent in 100 μl using DNA Transfection Reagent (Roche) following the manufacturer’s recommendations. One microgram of DNA (0.4 μg AP-LRRTM2-WTr or mutants + 0.4 μg BirAER ± 0.2 μg PSD-95-GFP) were mixed with 2 μl X-treme gene reagent in 100 μl PBS, and incubated at room temperature for 30 min. Seventy five microliter of this solution was added per well for a total volume of 2.5 ml (6-well plates), or 30 μl for a total volume of 1 ml (12-well plates) and incubated at 37°C. For labelling, cells plated on glass coverslips were washed in PBS, fixed for 20 min in 4% paraformaldehyde - 20% sucrose, and permeabilized for 7 min with 0.1% Triton X-100. Non-specific binding was blocked using PBS containing 1% bovine Serum Albumin (carlroth) for 45 min at room temperature. Cells were then immunostained for LRRTM2 using a sheep monoclonal anti-LRRTM2 (Thermo Fischer Scientific, clone 7E-1B8, 1:100) for 1 h followed by Alexa488 goat anti-sheep antibody (Thermo Fischer Scientific, 1:800) for 1 h. Coverslips were then mounted in DAPI Fluoromount-G (SouthernBiotech).
Western blot

Forty eight hours after transfection, COS-7 cells were rinsed twice in ice-cold PBS, and lysed in 85 μl lysis buffer per well (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% TritonX-100, 1x protease inhibitor cocktail (Sigma-Aldrich, #P2714)) for 45 min at 4°C on a rotating device. Lysates were centrifuged at 8000 x g during 15 min at 4°C and the supernatant was collected for Western blotting. Protein concentrations were quantified using the BCA assay (Thermo Fisher Scientific, 10750985), and protein amounts were adjusted for concentrations. Samples were boiled 5 min at 95°C, before loading on a 4–20% SDS-PAGE gel (Biorad) to allow protein separation. Proteins were transferred onto a nitrocellulose membrane, and incubated in blocking solution (LI-COR) for 1h at room temperature (RT), before incubation with primary and secondary antibodies: sheep anti-LRRTM2 (R&D Systems, AF5589, 1/200); Alexa Fluor 790-conjugated AffiniPure Donkey Anti-Sheep (Jackson Immunoresearch, 713-655-147); mouse anti-β-actin (Merk, A5316, 1/1 000); IRDye 800CW Goat anti-Mouse IgG (H + L) (LI-COR, 926–32210, 1/15 000). Positive bands were visualized using the Odyssey FC imaging system (LI-COR). Average intensity values were calculated using Image Studio 5.2 software (LI-COR). The intensity of LRRTM2 signal was normalized to beta-actin.

Primary neuronal cultures, electroporation, and transfection

Dissociated hippocampal neurons from E18 Sprague-Dawley rats embryos of either sex were prepared as described previously (Kaec & Banker, 2006) at a density of 200,000 cells per 60-mm dish on poly-l-lysine pre-coated 1.5H coverslips (Marienfeld, cat. no. 117 580). Neurons cultures were maintained in Neurobasal Plus medium supplemented with 0.5 mM GlutaMAX and 1X B-27 Plus supplement (Thermo Fisher Scientific). Coverslips were flipped on a 60 mm dish containing a glia monolayer 2 h after plating and 2 μM Ara-C was added after 72 h. Astrocytes feeder layers were prepared from the same embryos, plated between 20,000 and 40,000 cells per 60-mm dish (according to the Horse Serum batch used) and cultured in MEM (Fisher Scientific, cat. no. 21090-022) containing 4.5 g/L Glucose, 2 mM GlutaMAX and 10% horse serum heat inactivated (Thermo Fischer Scientific, GIBCO) for 14 days. For Figure 1, neurons were electroporated prior to plating on pre-coated coverslips with the Amaxa system (Lonza) using 500,000 cells per cuvette, and the following plasmid combination: Homer1c-GFP, 1.5 μg; BirAER, 1.5 μg; AP-LRRTM2, 1.5 μg. For the remaining experiments, neurons were transfected at DIV 7 with the calcium-phosphate method using 1.5-1.8 μg of plasmidic DNA and the following solutions: TE (1 M Tris-HCl pH 7.5, 250 mM EDTA), CaCl\(_2\) (2.5 M CaCl\(_2\) in 10 mM HEPES - pH 7.2) and 2XHEPES-buffered saline (274 mM NaCl, 10 mM KCl, 1.4 mM Na\(_2\)HPO\(_4\), 12 mM glucose, 42 mM HEPES - pH 7.2). Briefly, the plasmids were diluted in TE, then CaCl\(_2\) was added drop-wise to form the precipitates and this solution was transferred to the HEPES solution. The coverslips containing the neurons were then transferred to 12-well plates containing 200 μl/well of cultured medium and 50 μl of 5x kynurenic acid (10 mM stock solution). Fifty micro-liter of the precipitate solution was added to each well and neurons were incubated for 30 min at 37°C. Afterwards, the cells were washed for 15 min at 37°C with fresh equilibrated medium containing 2mM kynurenic acid and were then returned to their original culture dish until imaging. Combinations of plasmids used for the different experiments: immunocytochemistry: sh-LRRTM2-GFP + AP-LRRTM2-WT, -ΔC, -ΔECEV or -YACA + BirA\(_{ER}\) (0.3:0:6:0.6 μg DNA/coverslip) or EGFP + EV (0.3:1.2 μg DNA/coverslip) or sh-LRRTM2-GFP + EV (0.3:1.2 μg DNA/coverslip); Super Resolution Imaging: sh-LRRTM2-GFP + AP-LRRTM2-WT, -ΔC, -ΔECEV or -YACA + BirA\(_{ER}\) + Homer-DsRed (0.3:0:6:0.6:0.3 μg DNA/coverslip).

RT-qPCR

Cultures were lysed at different days in vitro (DIV 3, 7, 9, 14, 21) using QIAzol Lysis Reagent (Qiagen) and RNA was isolated using the Direct-Zol RNA microprep (Zymo Research) according to manufacturer’s instructions. cDNA was synthesized using the Maxima First Strand cDNA synthesis kit (ThermoFischer Scientific). At least two neuronal cultures were analyzed per condition and triplicate qPCR reactions were made for each sample. Transcript-specific primers were used at 6 μM and cDNA at 5 ng in a final volume of 10 μl. The LightCycler 480 ONEGreen Fast qPCR Premix kit (Ozyme) was used according to manufacturer’s instructions. The Ct value for each gene was normalized against that of Succinate Dehydrogenase Complex Flavoprotein Subunit A (SDHA). The relative level of expression was used according to manufacturer’s instructions. The ΔΔCt value for each gene was normalized against that of Succinate Dehydrogenase Complex Flavoprotein Subunit A (SDHA). The relative level of expression was calculated using the comparative method (2−ΔΔCt) (Livak et al., 2001). The following set of primers was used: LRRTM2 Forward: 5’GCTCCTGCATAAGCCT 3’ and Reverse: 5’TGCGGAAGCACGGAAGGAGT 3’. Immunocytochemistry

To visualize surface AP-tagged LRRTM2 and endogenous PSD-95, live neurons were incubated with STAR635P-conjugated monomeric streptavidin in
Tyrode solution (in mM: 15 d-glucose, 108 NaCl, 5 KCl, 2 MgCl2, 2 CaCl2, and 25 HEPES, pH 7.4) for 10 min at room temperature and subsequently fixed for 10 min in 4% paraformaldehyde-20% sucrose and permeabilized for 7 min with 0.1% Triton X-100 in PBS. Non-specific binding was blocked using PBS containing 1% biotin-free Bovine Serum Albumin (carlroth) for 45 min. Neurons were then immunostained for endogenous PSD-95 using a mouse monoclonal anti-PSD-95 (Thermo Fischer Scientific, clone 7E-1BB, 1:400) for 1 h followed by Alexa568 goat anti-mouse antibody (Thermo Fischer Scientific, 1:800) for 1 h. Coverslips were then mounted in Mowiol (Calbiochem).

Epifluorescence microscopy and image analysis

Immunostained neurons were visualized using an inverted epifluorescence microscope (Nikon Eclipse TiE) equipped with a 60x/1.4 NA objective and filter sets for EGFP (Excitation: FF01-472/30; Dichroic: FF-495Di02; Emission: FF01-525/30); Alexa647 (Excitation: FF01-543/22; Dichroic: FF562Di02; Emission: FF01-676/29); and Alexa568 (Excitation: FF01-593/40); and Alexa488 (Excitation: FF01-628/40; Dichroic: FF-642-nm laser line through a four-band beam splitter (R405/488/561/635, SemRock). Samples were imaged by oblique laser illumination, allowing the excitation of individual STAR-conjugated ligands bound to the cell surface, without illuminating ligands in solution. Fluorescence was collected using a FF01-676/29 nm emission filter (SemRock) placed on a filter wheel (Sutter Instruments). Stacks of 2000 consecutive frames were obtained from each cell, with an integration time of 20ms.

Trajectory analysis and Image reconstruction

Image stacks were analyzed using a custom program running on Metamorph based on wavelet segmentation for localization and simulated annealing algorithms for tracking, described earlier (Izeddin et al., 2012; Kechkar et al., 2013), allowing both the reconstruction of the super-resolution image by summing the positions of localized single molecules into a single image, and tracking of localized molecules through successive images. The instantaneous diffusion coefficient, D, was calculated for each trajectory from linear fits of the first four points of the mean square displacement (MSD) function versus time. For trajectory analysis, synapses were identified by thresholding the Homer1c-GFP image, used as a postsynaptic marker. A trajectory was considered as synaptic when spending more than 50% of its duration inside the regions defined by Homer1c-GFP signal. The mean time spent at synapses was calculated as the number of frames a molecule was detected inside a synaptic area multiplied by the integration time (20 ms) for each trajectory crossing a synapse, using a custom program developed on MATLAB (MathWorks). Masks of synapses were generated based on the Homer-1c labelling using the Integrated Morphometry Analysis on MetaMorph (Molecular Devices).
dSTORM acquisition and image analysis

Primary hippocampal neurons co-expressing shLR-RTM2, Homer1c-DsRed, BirAER and AP-LRRTM2 constructs were live labelled with Alexa647-conjugated monomeric streptavidin in Tyrode (100 mM) for 10 min at room temperature and subsequently fixed for 10 min in 4% paraformaldehyde-20% sucrose in the presence of 0.2% glutaraldehyde for 10 min in room temperature and kept in PBS at 4°C until imaging. The coverslips were mounted in an open Inox observation chamber (Ludin) in an oxygen-scavenging imaging buffer (Tris-HCl buffer pH 7.5 containing 10% glycerol, 10% glucose, 0.5 mg/ml glucose oxidase (Sigma), 40 mg/ml catalase (Sigma C100-0.1% w/v), and 50 mM β-mercaptoethylamine (MEA) (Sigma M6500)) (Heilemann et al., 2008) and sealed using a second glass coverslip. The same microscope described for single particle tracking was used. A high-laser 642nm laser was used to induce the triplet state of Alexa647 dye and subsequently the same laser but with less power was used for acquisitions using the same optics and detector as described above for uPAINT. 100-nm nano-diamonds (Adamas Nanotechnologies) were used to register long-term acquisitions and correct for lateral drift. 10–20 streams of 4000 frames each were acquired at 50 Hz using Metamorph.

dSTORM analysis

The super-resolution microscopy analysis platform SMAP (2020) was used for reconstruction, registration, and analysis of the dSTORM data. Analysis by DBSCAN was performed on SMAP, using a neighborhood radius of 10 nm and a minimum number of objects in neighborhood k = 30, allowing to segment synaptic LRRTM2 clusters. The same parameters were applied to all conditions. The distributions of number of detections per cluster were extracted and plotted using MATLAB. Synaptic enrichment was calculated as a ratio of surface-normalized number of detections at synapses by surface-normalized number of detections outside synapses. The percentage of clusters localized at synapses was determined by segmenting superresolved images overlapped with synaptic masks using the Integrated Morphometry Analysis on MetaMorph (Molecular Devices).

Statistics

All data are represented as mean ± SEM unless otherwise stated. The number of cells and experiments are indicated in corresponding figures. Non parametric Kruskal-Wallis tests were performed to assess significant differences in datasets containing more than two conditions, followed by a post-hoc Dunn’s multiple comparisons tests using GraphPad Prism 8.0. Statistical significance is indicated in corresponding figures.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Konstantina Liouta and Ingrid Chamma designed research and wrote the manuscript. Konstantina Liouta, Julia Chabbert, and Ingrid Chamma performed experiments and analysed data. Sebastien Benquet and Béatrice Tessier designed and created plasmids. Joris De Wit designed and provided the shRNA and shRNA-resistant constructs. Olivier Thoumine provided scientific and financial support. Matthieu Sainlos and Ingrid Chamma produced and characterized monomeric streptavidin preparations. Vincent Studer contributed to image analysis. All authors discussed the results and manuscript.

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