Regulatory motifs involved in LRRTM2 trafficking and LRRTM2-dependent stabilisation of AMPARs at excitatory synapses

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Regulatory motifs involved in LRRTM2 trafficking and LRRTM2-dependent stabilisation of AMPARs at excitatory synapses

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ABSTRACT

Synapses are the main site of communication between neurons in the central nervous system. These specialised cell-cell contacts are initiated by cell adhesion molecules at the pre- and post-synapse, which interact with one another to form trans-synaptic complexes, and recruit molecules regulating synapse maturation, specificity and function.

Leucine Rich Repeat Transmembrane Protein 2 (LRRTM2) is a synaptic adhesion molecule that binds to pre-synaptic neurexin and is exclusively localised and enriched at excitatory synapses where it exhibits low membrane dynamics. Interestingly, LRRTM2 is involved in synaptic transmission and plasticity and regulates the surface levels of AMPARs, the main glutamatergic receptors responsible for fast neurotransmission in the brain.

In my PhD, I investigated the molecular mechanisms underlying LRRTM2 stabilisation and trafficking at excitatory synapses, as well as the interplay between LRRTM2 and AMPARs.

We demonstrated that the C-terminal domain of LRRTM2 controls its compartmentalisation in dendrites as well as its enrichment and compaction at synapses. Surprisingly, LRRTM2 synaptic confinement was found to be independent of its PDZ-like binding domain and was instead regulated by a recently identified YxxC intracellular sequence. We further confirmed that this sequence was critical for LRRTM2 trafficking and exocytosis and observed the existence of intracellular LRRTM2-containing vesicles inside spines. Regarding the interplay between LRRTM2 and AMPARs, we showed for the first time, that the recently identified neurexin-binding site in LRRTM2 (E348) is responsible for membrane stabilisation of synaptic AMPARs.

These results demonstrate that the intracellular region of LRRTM2 controls its synaptic clustering, membrane dynamics, and confinement, while extracellular binding interfaces are involved in stabilising AMPARs at the plasma membrane.

RÉSUMÉ

Les synapses constituent le principal site de communication entre les neurones du système nerveux central. Ces contacts cellule-cellule spécialisés sont initiés par des molécules d'adhésion cellulaire au niveau de pré- et post-synapses, qui interagissent entre elles pour former des complexes trans-synaptiques, recrutant à leur tour des molécules régulant la maturation, la spécificité et la fonction des synapses.

Leucine Rich Repeat Transmembrane Protein 2 (LRRTM2) est une molécule d'adhésion synaptique qui se lie à la neurexine pré-synaptique et elle est exclusivement localisée et enrichie au niveau des synapses excitatrices où elle présente une faible dynamique membranaire. LRRTM2 est impliquée dans la transmission et la plasticité synaptique et régule les niveaux de surface des récepteurs AMPA, les principaux récepteurs glutamatergiques responsables de la neurotransmission rapide dans le cerveau.

Au cours de ma thèse, j'ai étudié les mécanismes qui sous-tendent la stabilisation et le trafic membranaire de LRRTM2 au niveau des synapses excitatrices, ainsi que l'interaction entre LRRTM2 et les récepteurs AMPA.

Nous avons démontré que le domaine C-terminal de LRRTM2 contrôle sa compartimentation dans les dendrites ainsi que son enrichissement et sa compaction au niveau des synapses. De manière surprenante, le confinement synaptique de LRRTM2 s'est avéré être indépendant de son domaine de liaison PDZ-like, mais régulé par une séquence intracellulaire récemment identifiée, YxxC. Nous avons observé que cette séquence était également essentielle au trafic membranaire et à l'exocytose de LRRTM2 et caractérisé l'existence de vésicules intracellulaires contenant LRRTM2 à l'intérieur des épines. En ce qui concerne l'interaction entre LRRTM2 et les récepteurs AMPA, nous avons montré pour la première fois que le site nouvellement identifié de liaison à la neurexine (E348), est impliqué dans la stabilisation membranaire des récepteurs AMPA synaptiques.

Ces résultats démontrent que la région intracellulaire de LRRTM2 contrôle son enrichissement synaptique, sa dynamique membranaire, et son confinement aux synapses excitatrices, tandis que les domaines extracellulaires sont impliqués dans la stabilisation des récepteurs AMPA à la membrane plasmique.

INTRODUCTION

1. Neuronal connectivity in the CNS

Billions of cells in the human brain need to connect and communicate with one another in a sophisticated and complex manner. In the central nervous system, neurons and glial cells are the major cell-types exerting distinct but complementary roles. Neurons are the main cells in the brain responsible for communication transferring electrical and chemical signals from one neuron to another. Glial cells, on the other hand, are involved in immunity responses, water and ion homeostasis, blood flow and myelination of axons among others (Jäkel and Dimou 2017). In this chapter, I will first briefly discuss the major theories that contributed to our understanding regarding neuronal connectivity in the CNS in the last two centuries. I will then review the mechanisms involved in the establishment of neuronal contacts during development with particular emphasis on the organisation and function of excitatory synapses.

1.1 A historical perspective

The organisation of neural cells and their communication remained a matter of debate for several decades. In the early 18th century, the **Cell Theory** formulated by Theodor Schwann (1810-1882), Matthias Schleiden (1804-1881) and Rudolph Virchow (1821-1902) established that:

- All living organisms are composed of cells. They may be unicellular or multicellular.
- The cell is the basic unit of life.
- Cells arise from pre-existing cells.

However, scientists studying neural cells noted that neural cells did not look like other cells throughout the body under the microscope. At that time, the most common view regarding the organisation of the nervous system was the **Reticular Theory**, developed principally by Joseph von Gerlach (1820–1896). According to this theory, the nervous system consisted of a continuous

reticulum with all the cells in direct contact with each other. The structure and functioning of the nervous system truly began to be understood when Santiago Ramon y Cajal (1852-1934) started unravelling its organisation using the Golgi method, a silver staining technique developed by Camillo Golgi (1843-1926) in 1873, used to visualise nervous tissue under a light microscope. Cajal's observations led him to formulate the **Neuron Doctrine** at the end of the 1880's according to which the brain is composed of discrete cells rather than a continuous, interconnected network of appendages, as most of his contemporaries believed. In 1891, Physician Heinrich Wilhelm Gottfried von Waldeyer-Hartz (1836-1921) first proposed the term neuron using observations from previous findings, to support his claim that the neuron is the most basic structural unit of the nervous system, consisting of branched nerve cell processes (axons and dendrites) and cell bodies. Some years later, Charles Scott Sherrington (1857-1952), an English physiologist, was among the first to use the term **synapse** (σύν (= together) + ἅπτω (= to touch)) to describe the junction where two neurons communicate, which consists of the pre-synapse,

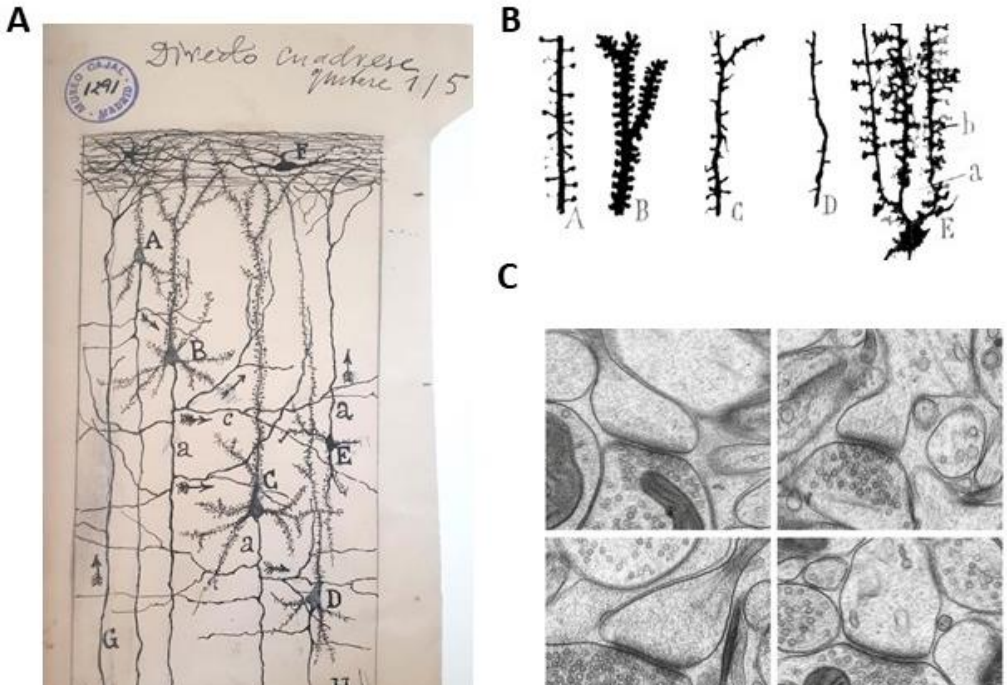


Figure 1. From 1896 to today. Original drawings of Cajal depicting **A.** neurons cerebral cortex; arrows in the drawing indicate the direction of information flow and **B.** different morphologies of dendrites, filopodia and dendritic spines (Cajal, 1896) **C.** Cryo-fixed neuropil shows synaptic contacts with large amounts of surrounding extracellular space (Korogod et al., 2015). Adapted from 'The beautiful brain' 2017, García-López et al., 2007 and Korogod et al., 2015

the post-synapse and the synaptic cleft, meaning the space in between. Yet, the exact nature of interactions between nerve cells and between nerve cells and other cell types was not really understood until much later, in the 1950s, when electron microscopy (EM) provided clear evidence regarding the discontinuity of the neuronal network by revealing for the first time a cleft separating the pre-synaptic axon from the post-synaptic dendrite (Palay and Palade 1955) (**Figure 1**).

Apart from the structural organisation of the nervous system, another puzzling question to researchers was how information was transmitted within the brain. It had been known since the time of Luigi Galvani, doctor and professor of anatomy at the University of Bologna, as early as 1791 that information is transmitted by electrical impulses, yet the precise direction of impulse activity within the neurons remained elusive. As Cajal wrote in 1917 in his autobiography Recuerdos de mi Vida: 'In what direction does the nervous impulse travel within the neuron? Does it spread in all directions, like sound or light, or does it pass constantly in one direction like water in the watermill?' The answer to those questions came about as a consequence of the Neuron Doctrine and was summarised by Cajal's theory of the **Law of Dynamic Polarisation** of nerve cells. He described how information, in the form of electrical signal, travels within individual neurons, from their dendrites to their cell bodies and finally to their axons, where it is transmitted to another cell at synapses (**Figure 2**).

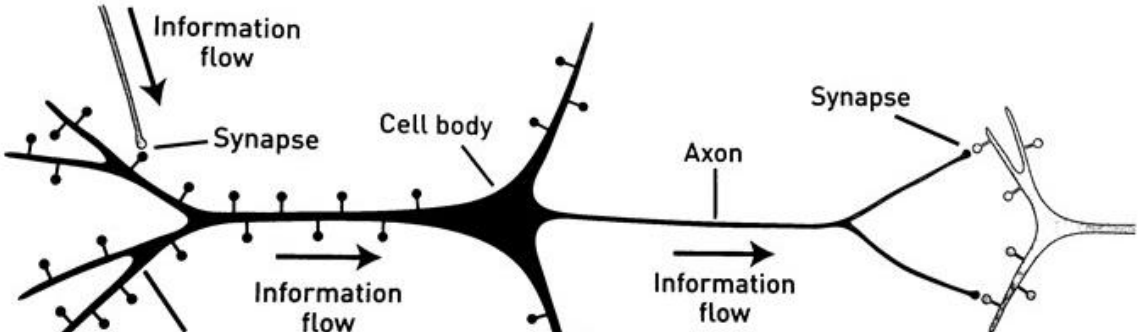


Figure 2. Information flow between neurons. Illustration of the direction of information received by dendrites, transferred across the axon and then to a different neuron. Adapted from 'The beautiful brain' 2017.

1.2 The synapse

Synaptic transmission is the biological process by which a neuron communicates with a target cell across a synapse, a specialised cell-cell contact, transferring nerve impulse information from one cell to another. Synapses in the brain are extremely heterogeneous in terms of function, morphology and localisation, however they can be categorised in two distinct types depending on the nature of their signal: **electrical** or **chemical**. Although chemical synapses are predominant in the CNS, electrical synapses also exist in some brain areas and have fundamentally different underlying mechanisms (Hinrichsen 1970; Baker and Llinás 1971).

1.2.1 Electrical synapses

Electrical synapses function like gap-junctions and contain intercellular channels located on the plasma membranes of adjacent neurons. They allow direct and bidirectional passage of signalling molecules between the cytoplasms and electrical currents to quickly propagate the signal along cells. They can be found in diverse regions of the mammalian CNS, especially in inhibitory interneurons (Nagy, Pereda, and Rash 2018) and they have the advantage of allowing almost instantaneous and bidirectional flow of information coordinating the activity of large groups of interconnected neurons (Bennett and Zukin 2004), thus increasing neuronal excitability and promoting synchronous firing (Galarreta and Hestrin 2001; Curti et al. 2012). However, their inability to modulate an excitatory signal from one neuron into an inhibitory signal in another, renders them less versatile compared to chemical synapses that can adapt their composition and response depending on environmental cues. In the developing brain there seems to be an inverse relationship between the presence of those type of synapses; in vertebrates, emergence of chemical synapses in spinal motor neurons coincides with the disappearance of gap junction coupling (Mentis et al. 2002), whereas chemical neurotransmitters have also been shown to regulate gap junctions during development ***in vitro*** (Arumugam et al. 2005). Thus, development of neural networks seems to rely on the dynamic interaction and reciprocal regulation of both electrical and chemical synapses.

1.2.2 Chemical synapses

The chemical synaptic transmission involves the release of neurotransmitters loaded in synaptic vesicles (SV) from the pre-synaptic axon terminal across the synaptic cleft, which are then recognised by the adjacent post-synaptic cell. This process is initiated by the arrival of an action potential, which is a sudden, fast, transitory and propagating change of the resting membrane potential at the pre-synaptic terminal, resulting in opening of voltage-dependent calcium channels and thus calcium influx into the neuron. Increase in calcium concentration triggers the fusion of SV with the pre-synaptic plasma membrane and allows the release of the neurotransmitters into the synaptic cleft. Finally, neurotransmitters travel across the cleft and bind to their specific post-synaptic receptors triggering changes in the membrane potential (**Figure 3**).

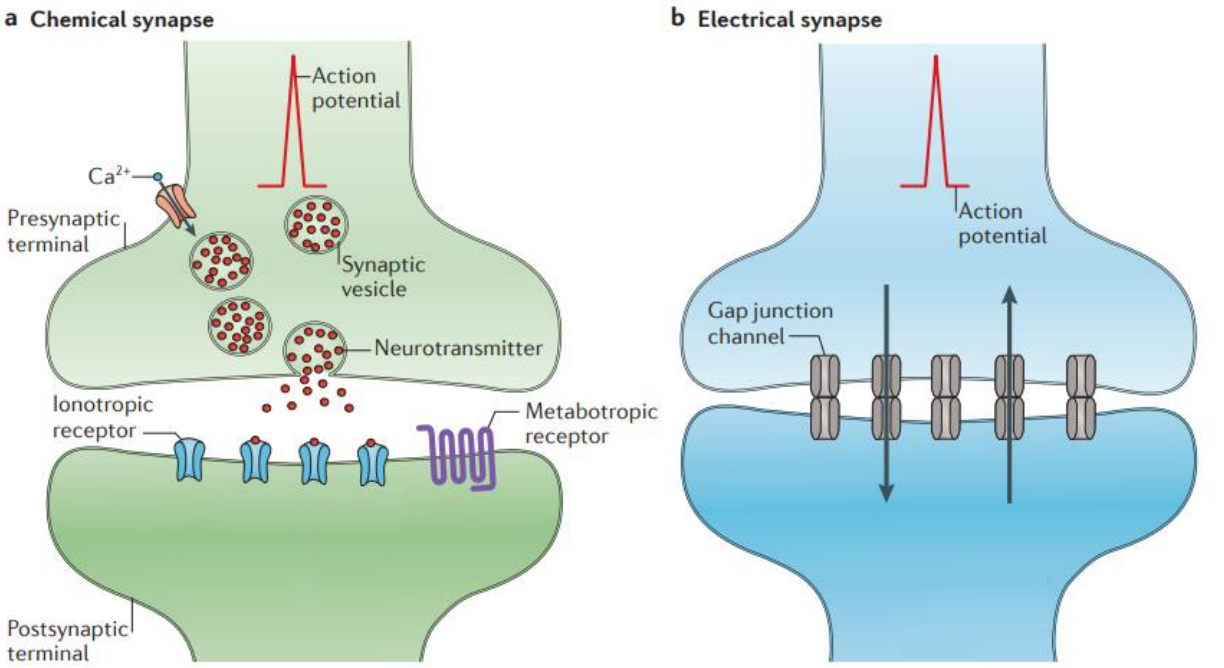


Figure 3. Main features of chemical and electrical synapses. a. Arrival of action potential at a chemical synapse leads to activation of voltage-dependent calcium channels resulting in elevation of intracellular calcium concentration at the pre-synaptic terminal. This increase triggers the fusion of neurotransmitter-containing synaptic vesicles with the plasma membrane resulting in neurotransmitter's release into the synaptic cleft. Neurotransmitter released activate specific post-synaptic receptors. **b.** At electrical synapses, gap junctions allows the bidirectional transfer of electrical signal and small molecules. Adapted from Pereda 2014.

Chemical synapses can be further classified depending on whether the neurotransmitter released promotes or inhibits the generation of an action potential in the post-synaptic cell. Back in 1959 two main types of synapses were identified in the cerebral cortex depending on their synaptic complex observed under an electron microscope: type I and type II (Gray et al., 1959). Type 1 synapses, also called asymmetric, were characterised by a prominent accumulation of high-electron density only on the post-synaptic side compared to type II, or symmetrical synapses, which exhibited comparable electron-dense zones on both sides. Correlating physiological and morphological data, Gray proposed that asymmetric synapses were **excitatory**, whereas symmetric ones were **inhibitory**. Inhibitory synapses usually contain gamma aminobutyric acid (GABA) or glycine and they are localised mostly on the dendritic shaft, but also on the axon initial segment (Villa and Nedivi 2016). Excitatory synapses can release different neurotransmitters including acetylcholine, the first neurotransmitter identified, serotonin, histamine, catecholamines and glutamate, the primary excitatory neurotransmitter in the CNS.

1.2.3 More than meets the eye

Synapses require transmembrane molecules, called **synaptic adhesion molecules**, which bridge the gap between the two cells to initiate cell-cell recognition, promote local specialisations by recruiting additional proteins via their intracellular domains and trigger further modifications in cell structure and function. More than 600 distinct proteins are estimated to be found at synapses (Collins et al. 2006); actin-cytoskeleton associated proteins provide the necessary mechanical support and flexibility to synapses, while interacting with a large variety of scaffold proteins. Those proteins in turn, anchor transporters, receptors and other essential elements of the synaptic machinery that work together to ensure efficient neurotransmitter release and synaptic response.

Of course, this is a rather simplistic view of the extremely complex connectivity in the brain and one should be cautious not to see the forest for the trees. Axons from single neurons can traverse

from one region of the brain to another, forming hundreds of synapses along the way, whereas dendrites communicate with numerous axons through synaptic connections and, like 'trees', form branches of variable length reflecting the diversity in the function and properties of different neuronal types. Synapses can also vary in their structural and functional properties (O'Rourke et al. 2012), modulating the flow of information in neural circuits, while they can also undergo dynamic changes in response to experience or injury. In addition, the term of the '**tripartite synapse**' conceptualised what was previously suggested in the '90s: that neurons are not the only excitable cells in the brain. Astrocytes, besides their supportive and homeostatic role, can also respond to neuronal activity and they can subsequently regulate neuronal activity and synaptic strength (Araque et al. 1999), revealing a bidirectional communication between neurons and glia. Astrocytes not only express a wide variety of functional receptors that can be

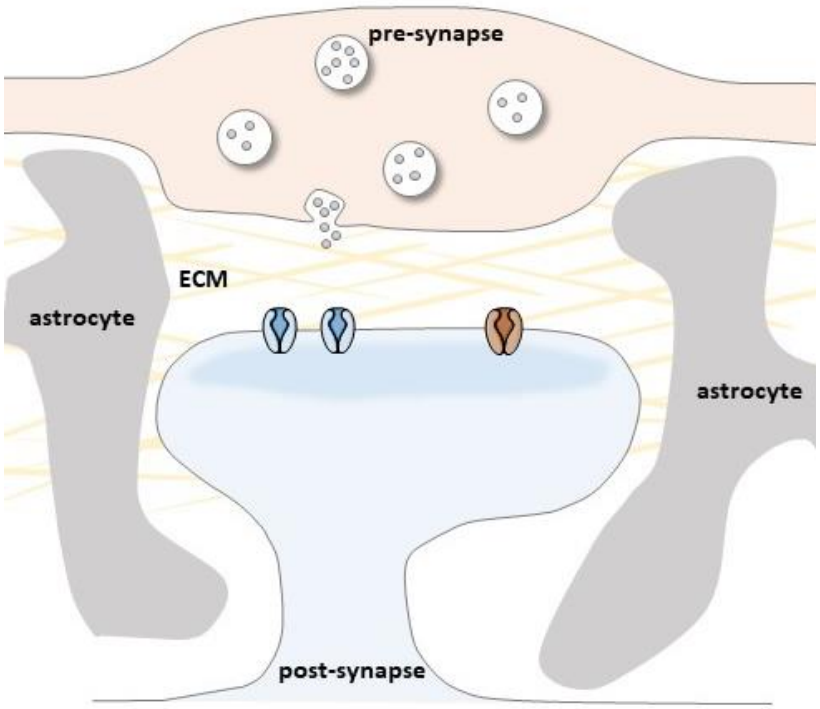


Figure 4. The tripartite synapse. Neurons are surrounded by astrocytes and are embedded in the extracellular matrix (ECM).

activated by neurotransmitters, but they can themselves release gliotransmitters to regulate synaptic transmission and plasticity (Covelo and Araque 2016). In addition, their extensive cellular branching allows a single astrocyte to be in contact with thousands of synapses (Halassa

et al. 2007), while still being connected to other astrocytes through gap-junctions, suggesting that glial cells can form a functional network capable of regulating neuronal activity (Covelo and Araque 2016). Finally, the numerous cells of the brain are embedded in the **extracellular matrix** (ECM), a dense network of macromolecules synthesised by neurons and glia (Ruoslahti 1996), consisting primarily of proteins and polysaccharides, accounting for around 20% of the total volume of the adult brain (**Figure 4**). This self-assembled matrix provides structural support to brain cells, but also acts as a physical barrier reducing the diffusion of soluble and membrane-associated molecules or even modulates synaptic activity via direct binding to adhesion molecules on the surface of neurons.

1.3 Synapse formation during neuronal development

During embryonic neural development, new-born neurons deriving from the neuroepithelium, a proliferative layer of the neural tube, migrate towards their final destination where they integrate into neuronal circuits. Subsequently, cells form neurites, membrane protrusions, which will give rise to one axon and multiple dendrites, in a process called **neuronal polarisation**. This polarisation underlies the flow of information in the CNS, so the establishment and maintenance of neuronal polarisation is crucial for correct development and function. Great progress in our understanding of how neurons establish their polarity and thus their connectivity, has been made by studying cultured neurons that constitute a simplified model to study cell-cell interactions and the formation of synapses. In the next part, I will focus on the mechanisms related to neuronal polarisation in vitro, resulting in the establishment of cell-cell contacts and in the formation of mature synapses.

1.3.1 Neuronal polarisation in vitro

Neurons are highly polarised cells, as they possess structurally and functionally different processes, **axons** and **dendrites**, which extend from the **cell body** (soma) in order to mediate information flow. An axon is typically a single long process that transmits signals to other neurons by the release of neurotransmitters. Dendrites are composed of multiple branched processes and

dendritic spines, small protrusions from the dendritic membrane, where contact with neighbouring axons is formed in order to receive synaptic input. The formation and maintenance of such distinct cellular compartments are crucial for the neuronal function.

Banker and colleagues established dissociated rodent hippocampal neurons as a basic model for neuronal polarity (Dotti, Sullivan, and Banker 1988; Craig and Banker 1994). The hippocampus, or other regions of interest, are dissected from embryonic or postnatal brain tissue and is subsequently mechanically dissociated to obtain isolated cells which are then plated in culture dishes. After plating, hippocampal neurons form round spheres that spread filopodia, dynamic thin protrusions of the membrane (stage 1). These neurons subsequently form several neurites of similar length (stage 2; 0.5-1.5 days in vitro), until one of these neurites grows rapidly to become the axon while the other pause (stage 3; 1.5-3 days in vitro). Subsequently, the remaining short neurites develop into dendrites and branch (stage 4; 4-7 days in vitro) and as they mature, dendritic spines are formed (stage 5; >7 days in vitro)(Takano et al. 2015) (**Figure 5**).

Neuronal polarisation in vitro is achieved in a stochastic manner and without external cues, unlike polarisation in vivo (Nakamuta et al. 2011) implying that intrinsic mechanisms are involved in this process. Neurotrophic signals that regulate neuronal polarisation at stage 2 are transduced through different intracellular signalling pathways, while coordinated regulation of microtubules stabilisation and actin dynamics play crucial roles in neuronal polarisation (Takano et al., 2015). A widely accepted model regulating neuronal polarity is that of 'local activation and global inhibition' (Arimura and Kaibuchi 2007; Inagaki, Toriyama, and Sakumura 2011). According to this model, '**local activation'** is believed to trigger the formation of a single axon and promote its elongation via positive regulators, while '**global inhibition'** on the other hand is thought to prevent the formation of multiple axons, therefore promoting dendritic growth and maintaining neuronal polarity. However, the molecular mechanisms of 'global inhibition' are poorly understood and additional research is needed to fully comprehend how the formation of multiple axons is prevented and how dendritic outgrowth is induced.

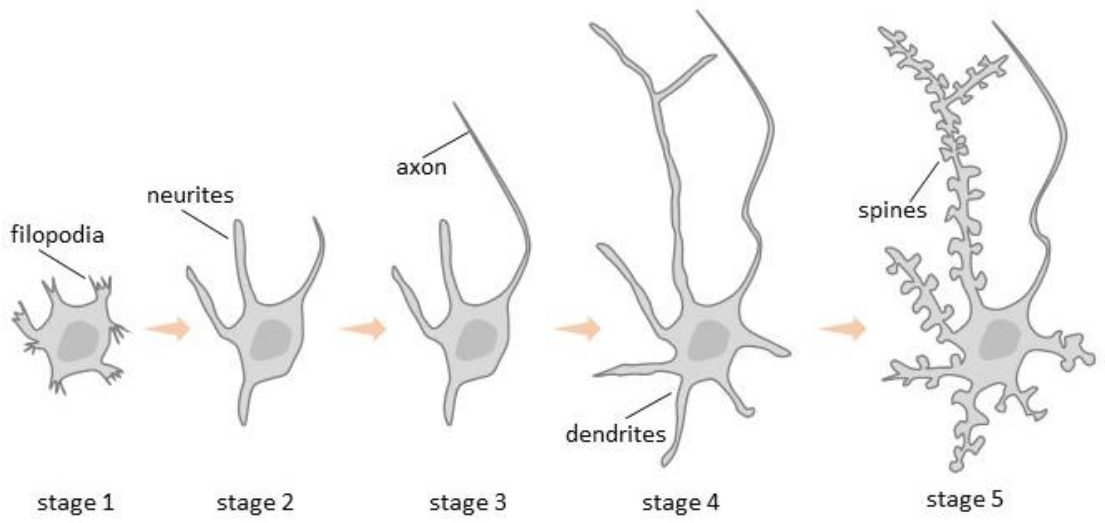


Figure 5. Stages of neuronal polarisation in vitro. Newly plated neurons extend filopodia around the cell body. Then, they form neurites; one of these neurites will give rise to the axon which starts elongating. The remaining neurites develop into dendrites and as the neuron matures, dendritic spines are formed to establish synaptic contacts with other neurons.

1.3.2 Establishment of neuronal contacts: from initial cell-cell contact to mature synapses

Once dendrites have formed and branched, protrusions on the dendritic membrane are developed, called **filopodia**. Filopodia are long, thin, actin-rich and dynamic protrusions of the plasma membrane. Early in neuronal development, they are the dominant structure on dendrites and exhibit a dynamic and transient behaviour with a life span ranging from one second to one hour (Ziv and Smith, 1996; Dailey and Smith, 1996; Zuo et al., 2005), allowing for selection of synaptic partners. It is believed that these transient structures are crucial for the initial contact between dendrites and axons in order to form a synapse. Gradually, filopodia density declines giving rise to an increase in dendritic spine density, which become the major dendritic protrusions (Takahashi et al., 2003). Once the filopodium has formed, it will probe its environment in order to meet a potential pre-synaptic partner, which will be decisive for the formation of the synapse. It should be noted, however, that although the model supporting that filopodia are the

precursors of dendritic spines has been the most commonly accepted (Ziv and Smith 1996; Yoshihara, De Roo, and Muller 2009; Hotulainen and Hoogenraad 2010), other models of synapse formation do exist and will be explained shortly.

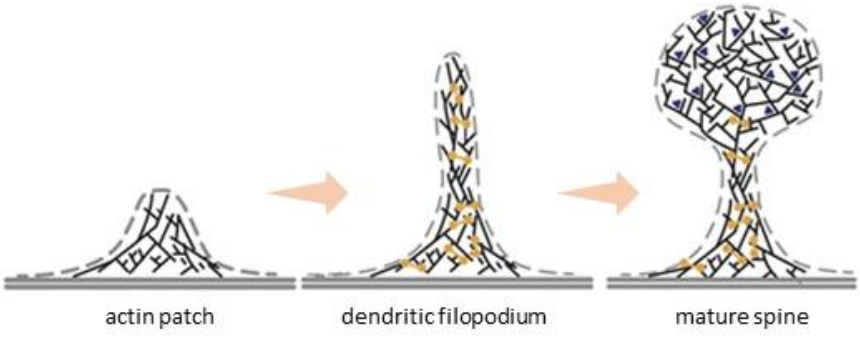


Figure 6. From filipodia to mature spines. Starting from a small protrusion of the dendritic plasma membrane, the filopodium extends to finally form a mature synapse**.** Modified from Miermans et al., 2017

The pre-synaptic **axonal growth cone**, a highly motile structure, also plays an active role in initiating cell-cell contact. It was first observed in chick embryos by Cajal and described as 'a cone-like lump with a peripheral base' (Cajal, 1890), containing actin filaments and microtubules. Actin filaments are particularly present in **filopodia**, dynamic thin protrusions at the periphery of the growth cone and in **lamellipodia** that are flat regions in between filopodia. The axonal growth cone can contact immature synapses on the dendrite and exert forces capable of inducing a protrusion that will become a dendritic spine (Yuste and Bonhoeffer 2004; Washbourne et al. 2002; Meyer 2006). During axon guidance, growth cones can be attracted or repelled by chemotropic factors and the same molecule can elicit different responses on different axon types and the same axons can respond differently to the same cue during different developmental stages. At the same time, interaction of the growth cone with the substrate mediated by cell adhesion molecules, such as the immunoglobulin superfamily or integrins and ECM proteins, such as laminin or fibronectin among others, provides the necessary mechanical support by eliciting cytoskeletal responses (Myers and Gomez 2011). The rate of F-actin polymerisation and

retrograde actin flow are tightly associated with the extension of filopodia and lamellipodia and therefore dynamically regulate the translocation of the growth cone (Mallavarapu and Mitchison 1999). Spatiotemporal external cues, including chemotropic proteins, cell-cell, or cell-substrate interactions, guide the growth cone through heterogeneous cellular environments to find its target cell.

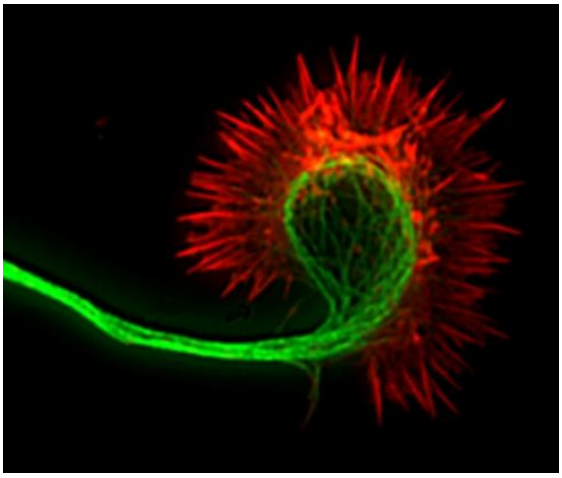


Figure 7. Fluorescence image of a cortical axon and its growth cone. Actin filaments (in red) occupy the lamellipodium and the spiky filopodia that protrude from its periphery. Microtubules (in green) occupy the axon and central region of the growth cone. Dynamic microtubules can extend from the central region into the growth cone periphery to interact with actin filaments. From Kalil et al., 2011.

Apart from dendritic filopodia, the filopodia of dendritic growth cones can also contact preferential areas of the axon (Sabo, Gomes, and McAllister 2006). It has also been shown that synapses can be created directly between the dendrite and the axon without forming a pre-existing filopodium (Washbourne et al. 2002; Gerrow et al. 2006). Finally, as the pre-synaptic axon is also capable of filopodial protrusions (Spillane et al. 2012; Greif et al. 2013) it is possible that these contact the dendrite or dendritic filopodia. The temporal and hierarchical order, if any, of these processes is still unclear, although it is highly likely that they could all contribute to initial cell-cell contact between axons and dendrites.

1.3.2.1 Recognition signals triggering initial contact

Different recognition signals are involved in initial cell-cell contact such as soluble factors and adhesion molecules. Soluble factors include diverse molecules, such as growth factors, cytokines, cleaved membrane molecules, deriving from neurons or glia. **Soluble factors** can trigger the initial

phase of synapse formation, as their function is independent of cell-cell adhesion. Members of the fibroblast growth factor (FGF) family are expressed in most tissues and have diverse roles in all stages of development but also during adulthood. In the hippocampus, FGF22 and FGF7 are first secreted from excitatory and inhibitory post-synaptic sites, respectively (Terauchi et al. 2010; 2014), and bind to distinct FGF receptor tyrosine kinases located on the surface of the growth cone, whose deletion impairs pre-synaptic differentiation (Dabrowski et al. 2015), suggesting that FGF signalling is critical for synapse initiation. Wnt signalling, a family of secreted glycoproteins, is also involved in this process. Wnt7a is secreted from cerebellar granule cells and induces presynaptic clustering of synapsin through binding to Wnt receptor, Frizzled, on pontine axons (Hall, Lucas, and Salinas 2000), whereas Wnt3a retrogradely regulates microtubule reorganisation on the growth cone (Purro et al. 2008). Despite evidence that certain soluble factors secreted from presynaptic sites are involved in synapse formation or maturation, such as semaphorins (Tran et al. 2009; Johnson-Venkatesh and Umemori 2010) and pentraxins (Bjartmar et al. 2006), they do not seem to trigger the initial cell-cell contact.

Adhesion molecules are key regulators of synapse formation, as they can initiate bidirectional signalling on both sides of the contact, by forming trans-synaptic complexes. Their diversity, trans-membrane localisation and complex pattern of interaction enables them to promote specific cell-cell interactions in a finely tuned manner. Several studies suggest that N-cadherin plays a role in initiating adhesion between the pre- and post-synaptic compartments. The cadherin family consists of more than 100 members classified into several subfamilies in mammals and their adhesive function depends on calcium, hence their name (Masatoshi Takeichi and Abe 2005). Classical cadherins are the most abundant cadherins family in vertebrates encoding for 18 members in the human genome and they are indispensable for cell-cell adhesion. Their extracellular part consists of five extracellular cadherin (EC) repeats followed by a single-pass transmembrane domain and an evolutionary conserved intracellular part. Homophilic binding of the extracellular domains mediates cadherin adhesion, while intracellular interaction with catenins bridges cadherins with actin filaments. Live-cell imaging studies suggest that cadherins are enriched at both pre-and post-synaptic compartments at nascent synapses, where

they are maintained after synapse maturation (Jontes 2004; Salinas and Price 2005). In addition, crystal structures of cadherin extracellular domain suggest that trans-synaptic cadherin-mediated complexes have the perfect length (~40 nm) to span the synaptic cleft (Boggon et al. 2002; Harrison et al. 2011), reinforcing the notion that initial cadherin-based adhesion stabilised transient contacts long enough to allow other classes of adhesion molecules to interact (Stan et al. 2010; Aiga, Levinson, and Bamji 2011; M. Yamagata, Duan, and Sanes 2018). Nectins, an immunoglobulin-like adhesion molecule, are found at the initial contact between the growth cone and the post-synaptic neuron where they homophilic trans-synaptic bridges and colocalise with cadherin complexes (Mizoguchi et al. 2002).

To avoid interrupting the flow of this chapter, a more detailed overview of adhesion molecules and their contribution to excitatory synapse formation and differentiation can be found in chapter 2 of this introduction.

1.3.2.2 Towards mature synapses

Shortly after the initial contact is established, internal membrane proteins, called scaffold **proteins**, accumulate at the pre- and post-synaptic sites, together with receptors and other essential components of the synapse. These proteins are not synthesised upon the establishment of the contact, but instead they are already present in neurons (Fletcher, De Camilli, and Banker 1994) usually in small, heterogeneous clusters of proteins, called transport packets, which are mobile in axons and dendrites (McAllister 2007). The assembly of the pre- and post-synaptic machinery and further synaptic differentiation and maturation gives rise to a mature synapse. Trans-synaptic complexes assembled by adhesion molecules guide these stages to regulate synapse number and morphology (Missler, Südhof, and Biederer 2012), but equally importantly to promote specific interactions.

Synapse maturation includes modifications in the pre-synaptic site, including recruitment of the pre-synaptic machinery and proteins into the axon terminal as well as recruitment of SV. The major cytoplasmic and membrane-associated protein precursors of the pre-synaptic release machinery are preassembled and transported along developing axons towards the axonal

terminal as discrete packets, containing distinct cargo (Ahmari, Buchanan, and Smith 2000; Shapira et al. 2003). Presynaptic scaffolding proteins are believed to play a crucial role in presynaptic assembly through their protein– protein interaction domains (Bury and Sabo 2010). It has been proposed that key scaffold proteins create a network of protein-protein interactions at the base of activated receptors that dynamically recruit several pre-synaptic elements (Bury and Sabo 2011; Siddiqui and Craig 2011). Ultrastructural studies indicate that developing synapses have few, sparsely packed synaptic vesicles and that the most reliable ultrastructural indicator of synaptic maturation is the density of synaptic vesicles.

At the post-synapse, recruitment of receptors is critical for synapse differentiation and studies on glutamatergic synapse development have contributed to a great extent to our understanding of the underlying mechanisms. The network of neurotransmitter receptors, scaffolding proteins, **adhesion molecules** and signal transduction enzymes at glutamatergic synapses is collectively referred to as the **postsynaptic density** (PSD) (Kennedy, 1997). Glutamate receptors and scaffold proteins are present in dendrites prior to synapse formation and are trafficked to axodendritic contacts in multi-molecular transport packets usually with overlapping cargo (Washbourne et al. 2002; Gerrow et al. 2006).

Synaptic transmission at newly formed synapses is mediated by the N-methyl-D-aspartate (NMDA)-type glutamate receptors (NMDARs) type of glutamate **receptors**, which require both ligand (i.e., glutamate) binding and post-synaptic depolarisation in order to permit conductance through the channel. As synapses mature, a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type ionotropic glutamate receptors (AMPARs) are recruited, rendering the synapses excitable at resting potentials. Although some glutamatergic synapses can develop without NMDARs, a sequence in which transmission at new synapses is mediated principally by NMDARs followed by the addition of functional AMPARs to synaptic sites appears to occur at the majority of glutamatergic synapses. **Scaffolding proteins** are a major group of proteins present at the PSD. Many of these molecules contain multiple domains for protein-protein interaction, such as PDZ domains, and thus mediate the recruitment of several other proteins (Kim and Sheng, 2004). The membrane-associated guanylate kinase (MAGUK) family, major scaffold proteins, as

well as the SHANK family members that interconnect several intermediate scaffold proteins, are critical for anchoring glutamate receptors at excitatory synapses (Peça et al. 2011; Scheefhals et al. 2019). Gephyrin, the main scaffold protein at inhibitory synapses, clusters glycine receptors, but also the ionotropic $GABA_A$ receptors (Tretter et al. 2008).

Changes in the **cytoskeleton** are also involved in synapse maturation. F-actin can interact with pre-synaptic active zone proteins and affect the recruitment of active zone components to synapses (Chia et al. 2014; W. Zhang and Benson 2001) or cluster synaptic vesicles around the active zone (Doussau 2000; Murthy and Camilli 2003). At the post-synaptic site, actin is involved in the morphological changes of the synapse, but also in receptors' trafficking and anchoring by interacting with scaffold proteins (Kuriu et al. 2006). Perturbing the F-actin cytoskeleton has more pronounced effects in nascent synapses than in mature synapses (W. Zhang and Benson 2001), suggesting that F-actin in crucial for the differentiation of synapses.

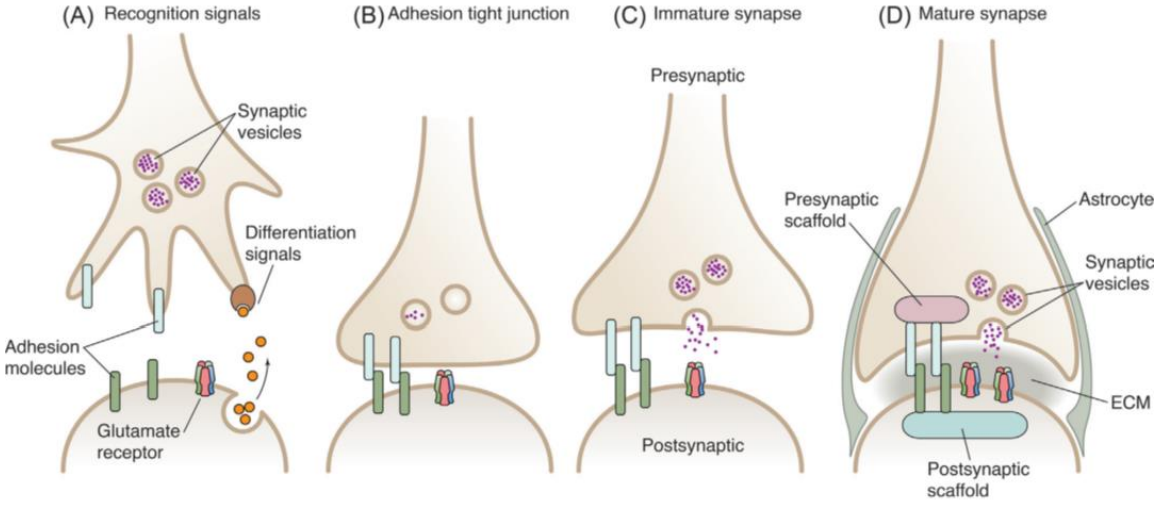


Figure 8. **Stages of synapse formation**. (A) In the model of the growth cone, the latter is induced to differentiate when its filopodia detect soluble differentiation signals from a postsynaptic target neuron. (B) The growth cone filopodia retract and the membranes become tightly apposed to one another through a cell adhesion mechanism. (C) The immature synapse may display a few vesicles pre-synaptically and a small post-synaptic density with neurotransmitter receptors. (D)The mature synapse exhibits an accumulation of presynaptic vesicles, a dense extracellular matrix (ECM) in the cleft, and both pre- and post-synaptic scaffolds. (Adapted from Development of the Nervous System 2019)

1.4 Excitatory synapse organisation and function

Glutamatergic synapses are the main excitatory synapses in the CNS and glutamate is the primary excitatory neurotransmitter in the CNS and the most abundant free amino acid in the brain; however, it was only until the early 1980s it was characterised as a neurotransmitter (Curtis and Johnston 1974). Previously, glutamate was mostly known for its role in brain metabolism (Krebs 1935), as well as for protein and peptide synthesis (Weil-Malherbe 1950). In the early '60s, electrophysiological studies demonstrated the powerful and excitatory action of glutamate on spinal cord neurons, although at the time it was difficult for the scientific community to consider it as a neurotransmitter because of its abundant expression in the brain and its extensive implication in many metabolic pathways.

Glutamate-containing synaptic vesicles at the pre-synaptic terminal are stocked and released from the **active zone**, a specialised region of the pre-synaptic plasma membrane. Subsequently, glutamate travels across the 20nm-wide cleft (Zuber et al. 2005) and binds to transmembrane glutamatergic receptors, located at the postsynaptic density (PSD), a huge complex of interlinked proteins associated with postsynaptic membranes of excitatory synapses. Influx of ions and electric depolarisation of the post-synaptic neuron, triggers signalling cascades to propagate further communication. Remaining glutamate at the synaptic cleft is removed by glutamate transporters to regulate its concentration and prevent excitotoxicity. Synaptic adhesion molecules located on both sides not only facilitate the physical proximity of the pre- and the post synaptic components, but also promote the recruitment of molecules and signalling cascades on the post-synaptic side.

1.4.1 Synaptic transmission

The first step of synaptic transmission is the generation of an **action potential**, which is a sudden, fast, transitory, and propagating change of the resting membrane potential, generated by voltage-dependent Na+ and K+ channels located in the membrane of the pre-synaptic neuro (**Figure 9**). Depolarisation of the pre-synaptic membrane results in calcium influx into the neuron

by opening of voltage-dependent calcium channels localised at a region adjacent to the synaptic vesicles that contain glutamate. The increase in Ca^{2+} triggers the exocytosis of these synaptic vesicles and thus the release of glutamate into the synaptic cleft. Spatial and temporal coordination of exocytosis and endocytosis ensures sufficient neurotransmitter release and indispensable for this coupling is a complex machinery of proteins that finely tune SV recycling. Ca^{2+} ions bind to synaptotagmin, a protein of the synaptic vesicle, considered the key Ca^{2+} sensing protein that triggers vesicle fusion, initiating synaptic transmission (Südhof 2012). Other proteins involved in the regulation of Ca^{2+}-triggered exocytosis are the SNARE (Soluble N-

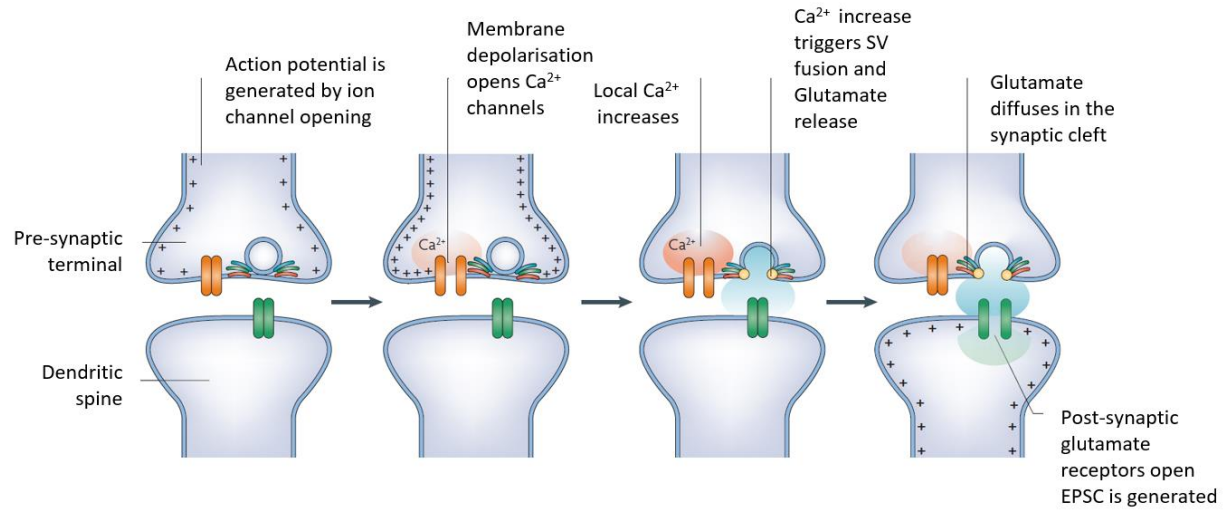


Figure 9. Synaptic transmission at glutamatergic synapses. Arrival of the action potential at pre-synaptic terminal increases local calcium concentration through opening of Ca^{2+} channels resulting in fusion of glutamate-containing synaptic vesicles with the plasma membrane. This fusion leads to the release of glutamate into the synaptic cleft which then binds to post-synaptic glutamate receptors. Activation of these receptors induces their opening and allows an ionic flow resulting in depolymerisation of the post-synaptic neuron and the transmission of the signal. Adapted from Lisman et al., 2007.

ethylmaleimide sensitive factor Attachment protein REceptor), the cytoplasmic complexins, DoC2 (Ramakrishnan, Drescher, and Drescher 2012) and the assembly/disassembly factors Munc18, Munc13, NSF and SNAP (Brunger et al. 2019). Once glutamate travels across the synaptic cleft, it binds and activates postsynaptic receptors triggering changes in the membrane potential by opening or closing of ion channels. The post-synaptic receptors can be ionotropic forming an ion channel pore, thus directly allowing or preventing ion passage depending on their

state (open/close), or metabotropic that are indirectly linked with ion channels through signal transduction mechanisms. This creates an evoked post-synaptic excitatory potential (EPSP) and leads to the activation of second messengers (**Figure 9**). Finally, remaining glutamate is removed from the synaptic cleft by glutamate transporters present in the plasma membranes of both glial cells and neurons.

1.4.2 Excitatory synapses onto dendritic spines

Most excitatory synapses in the mature mammalian brain occur on **dendritic spines** and a typical mature spine has a single synapse located at its head (**Figure 10**). As a result, dendritic spines constitute the main post-synaptic compartment for excitatory synapse. They are small protrusions found on the dendrites of pyramidal neurons in the cortex and hippocampus and on Purkinje neurons in the cerebellum. Spines are rarely found in lower organisms (for example, Drosophila melanogaster and Caenorhabditis elegans), suggesting that spines evolved to accommodate the more complex functions of 'advanced' nervous systems, such as the mammalian brain. Dendritic spines are composed of a multitude of proteins, adhesion proteins, scaffolding proteins, membrane receptors and channels, molecular motors, GTPases and regulators, kinases and phosphatases and their regulators, membrane trafficking proteins, cytoskeletal proteins and many others (Sheng and Hoogenraad 2007).

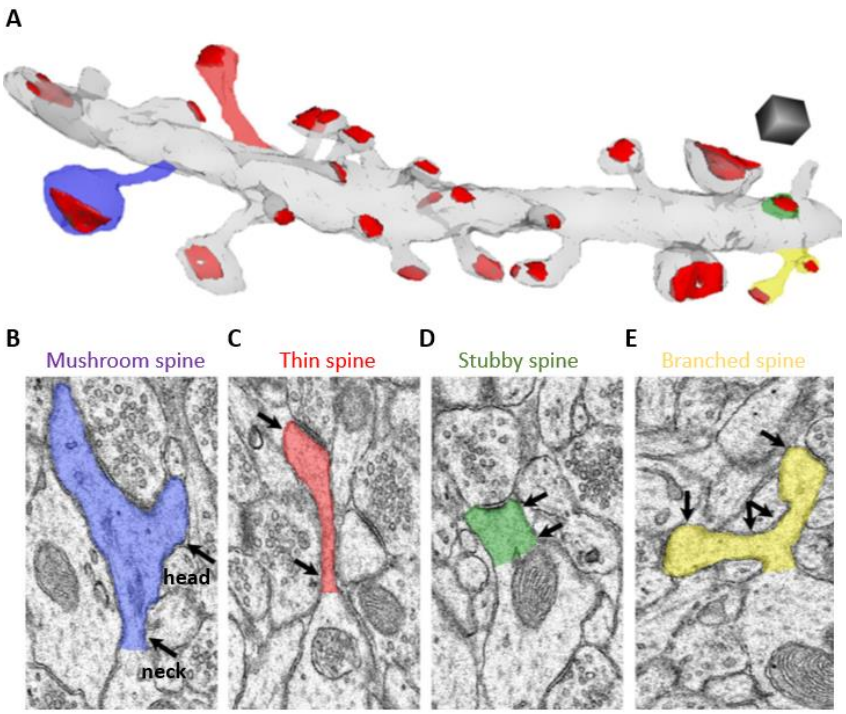


Figure 10. Morphology of dendritic spines. (A) 3D reconstruction of a hippocampal dendrite depicting the four categories of dendritic spines. From left to right: a mushroom spine in purple **(B)**, a thin spine in red **(C)**, a stubby dendritic spine in green **(D)** and a branched spine in yellow **(E)**. Arrows indicate the head and spine for the different spines. Adapted from Bourne and Harris, 2008.

They can vary in shape, size and length and can be classified into four categories: 'thin', 'mushroom', 'stubby' and 'branched' (**Figure 10**)(Diamond, Jones, and Powell 1969; Harris, Jensen, and Tsao 1992). The majority of dendritic spines fall into the first two categories and are characterised by two structures: the **spine head** with a diameter between 200nm and 1µm attached to the dendritic shaft by a narrow **spine neck** of approximately 100-200nm in diameter (**Figure 10**) (Harris, Jensen, and Tsao 1992; Nagerl et al. 2008; Izeddin et al. 2011). In addition, spines also differ in their molecular composition and of spine morphology is thought to be critical for synaptic function, as spine head size correlates with synaptic strength (Matsuzaki et al. 2001).

Spines are far from being static; they can undergo structural remodelling during development and upon neuronal activity over a timescale of seconds to minutes in cultured neurons (Fischer et al. 1998; Matus 2005). Morphological changes rely on remodelling of actin, which is highly enriched in dendritic spines, supporting their size, motility, morphological changes and consequently their function (Matus 2005; Cingolani and Goda 2008; Hotulainen and Hoogenraad

2010). The number of spines is also not stable; in the human cortex, spine density increases exponentially after birth and reaches a peak at 1-4 years depending on the region, followed by a gradual decline (**Figure 11**).

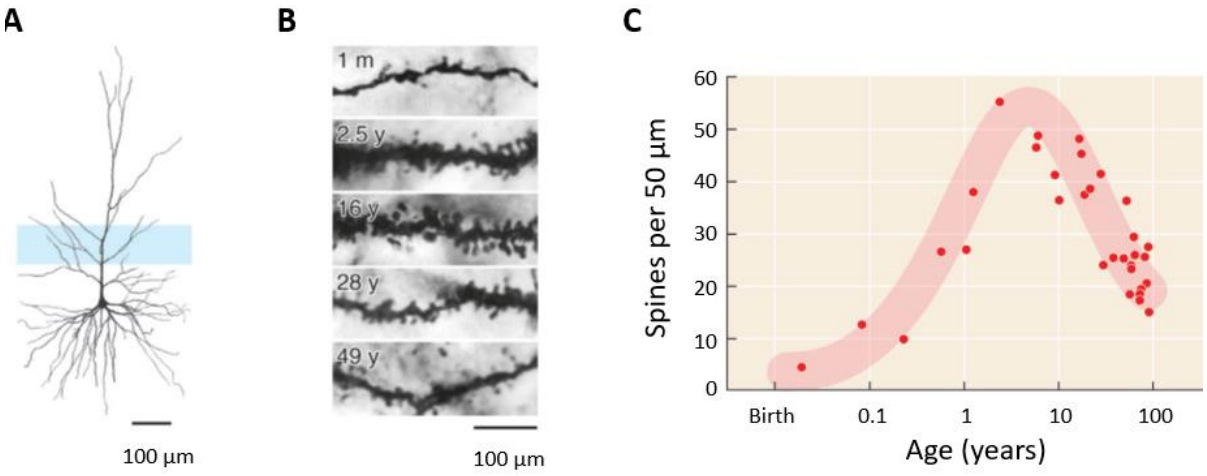


Figure 11. Spine density in the human cortex over lifetime. (A) An individual layer IIIc pyramidal neuron is shown with the analysed region shaded in blue. (B) Golgi-stained dendritic segments are shown from tissue obtained at 1 month, 2.5, 16, 28 and 49 years. (C) Density of dendritic spines in human dorsolateral prefrontal cortex as a function of postnatal age. Adapted from Development of the Nervous System 2019

Finally, the narrow necks of mushroom shaped spines are believed to act as a regulatory system forming diffusional barriers that slow down the exchange of proteins and signalling molecules between spine heads and dendrites (Müller and Connor 1991; Tønnesen et al. 2014) and impacting the kinetics and propagation of synaptic potentials in a spine-specific manner by controlling calcium influx (Hering and Sheng 2001; Grunditz et al. 2008). Altogether these observations indicate that spines are highly versatile, dynamic and specialised compartments of the dendrites that can rapidly respond to neuronal activity.

The PSD, a characteristic feature of the excitatory glutamatergic synapse, is a multi-protein complex composed by thousands of proteins in the human brain (Bayés et al. 2011) including receptors, scaffold proteins, enzymes, adhesion molecules and cytoskeleton proteins, among others. This complex group of proteins are coordinated in order to ensure the efficient reception of the pre-synaptic signal and its propagation. Depending on the brain region or the cell type,

PSD typically forms a 200-800 nm-long and 30-50 nm thick structure in mammals (Carlin et al. 1980), which can dynamically rearrange its composition during development but also in response to synaptic activity. According to proteomics studies, members of the CaMKII family are the most abundant synaptic proteins in the rat forebrain, followed by SynGAP, a postsynaptic RasGAP that also has multiple protein-protein interaction motifs and PSD-95, the most extensively studied scaffold protein of the PSD (Cheng et al. 2006). PSD-95 is also the most abundant member of the MAGUK family present at the PSD as well as the scaffold protein with the higher number of copies (X. Chen et al. 2005), rendering it a key PSD organiser. Remarkably, kinases, phosphatases and other regulators seem to constitute one of the most copious categories of molecules (Peng et al. 2004; Sheng and Hoogenraad 2007), suggesting that signalling molecules are indispensable for interactions within the PSD.

Glutamate receptors are indispensable components of synaptic transmission, they are localised at the PSD and can be either ionotropic glutamate receptors (iGluRs) or metabotropic glutamate receptors (mGluRs). iGluRs include α-Amino-3-hydroxy-5-Methyl-isoxazole-Propionic Acid Receptors (AMPARs), N-Methyl-D-Aspartate Receptors (NMDARs) and Kainate Receptors (KARs) and exhibit distinct roles in neuronal transmission. KARs are the least studied of the iGluRs, although they have both pre and postsynaptic functions. **NMDARs** are the main provider of Ca^{2+} for the postsynaptic cell, as they are permeable to calcium, which activates intracellular signalling cascades. In addition, activation of NMDARs requires the presence of a co-agonist (glycine or D-serine) apart from glutamate and the ion channel of NMDARs is also blocked by a magnesium molecule at rest. This Mg^{2+} block can be removed upon sufficient depolarisation of the postsynaptic membrane, presumably via **AMPARs** activation. These characteristics of NMDARs make them more suitable for long-term signalling, contrary to AMPARs that are believed to mediate fast synaptic transmission.

1.4.3 AMPA receptors: an overview

AMPARs are ionic channels organised in heterotetramer assemblies consisting of different subunits combination (GluA1-4) and they mediate most of the fast excitatory neurotransmission

in the CNS. Each subunit contains an extracellular domain - divided into the N-terminal and the ligand-binding domain (NTD and LBD, respectively) – is implicated in the assembly and clustering of the receptor at the synapse, four membrane-associated domains that form the channel pore and an intracellular domain that directs trafficking. The latter is variable in terms of length and composition among different subunits, providing an ideal framework for diverse protein interactions and post-translational modifications.

AMPARs preferably form heteromers, although their composition can vary depending on the brain region (Schwenk et al. 2014). GluA1/GluA2 tetramer is the most abundant at CA1 pyramidal neurons (W. Lu et al. 2009), accounting for around 80% of the entire GluA pool (Schwenk et al. 2014); although GluA1 homomers also occur in the hippocampus under specific conditions (Sans et al. 2003). The preference of AMPARs to assemble in heteromers over homomers derives from the fact they have lower affinity for themselves compared to the other subunits. Others have also reported the existence of GluA2/GluA3 tetramers in the rat hippocampus (S.-H. Shi et al. 2001), revealing that the subunit composition not only determines the biophysical properties of the receptors, but also their trafficking and function.

Central role in AMPARs trafficking and function play the **transmembrane AMPAR receptor regulatory proteins** (TARPs), which are essential components of AMPARs native macromolecular complexes. TARPs associate with AMPARs early in the synthetic pathway (Esteban 2008) and enable the latter to interact with various proteins that contain binding motifs incompatible with those of AMPARs. TARPs can interact through their PDZ sequence with the MAGUK proteins that serve as scaffolds involved in AMPARs delivery and membrane stabilisation (Elias et al. 2006). Several PDZ-domain-containing proteins have been associated with synaptic targeting of AMPARs (Garner, Nash, and Huganir 2000), while others are instead responsible for retaining them at synapses once they reach the surface (Osten et al. 2000). Apart from TARPs, other proteins are also considered as auxiliary subunits of AMPARs, including cornichon (2 and 3) (Schwenk et al. 2009), whose loss decreases surface GluA1/A2 receptors in the hippocampus (Herring et al. 2013) and Cysteine-Knot AMPA receptor Modulating Protein or CKAMPs (von Engelhardt et al. 2010; Farrow et al. 2015), that also regulate AMPARs surface levels. AMPARs

subunits have also been shown to bind to diverse proteins including the cytoskeletal 4.1N (L. Shen et al. 2000; Coleman et al. 2003), NSF protein, which is important for membrane trafficking and PICK1, involved in synaptic plasticity (Volk et al. 2010). It seems clear that all those transmembrane and intracellular interactions finely regulate the surface accumulation and trafficking of AMPARs, processes that are critical for synaptic plasticity (Kessels and Malinow 2009) (see chapter 1.5.3 of the introduction).

Apart from controlling the initial assembly of the different GluA subunits, the NTD also participates in protein-protein interactions in the extracellular domain of AMPARs. Neuronal pentraxins, secreted from the glutamatergic terminals, have been shown to interact with GluA4 subunit of AMPARs (Sia et al. 2007) and to induce post-synaptic clustering via the NTD (O'Brien et al. 1999). NTD also binds N-cadherin, although whether this interaction takes place in cis or in trans is still unclear (Nuriya and Huganir 2006; Saglietti et al. 2007). Interestingly, this interaction controls trafficking of AMPARs in neurons, suggesting that other synaptic adhesion molecules could be involved in regulating AMPARs trafficking via direct or indirect binding. The extracellular domain of AMPARs exhibits remarkable sequence diversity, flexibility, and span, which could favour interactions with multiple partners across the synaptic cleft.

1.4.4 From static to a dynamic view of synapses

Despite the notion that synapses are static, which was widely accepted for many decades, we know now that interactions between PSD-95 or other scaffold proteins and adhesion molecules or receptors are highly dynamic and finely regulated not only during development but also in mature synapses, enabling neurons to adapt their molecular composition depending on their needs.

Methodological advances in the early 2000s revolutionised the way scientists perceived the role of receptors at the synapse, unravelling lateral diffusion as an indispensable feature of membrane receptors. Glycine receptors were the first ones to be shown to juggle between an immobile state stabilised by gephyrin, the main scaffold protein at inhibitory synapses, and a

mobile state characterised by Brownian motion on the surface of dendrites (Meier et al. 2001). **Brownian motion** refers to the random and constant microscopic movements of particles in a fluid, due to thermal fluctuations. According to this notion, particles constantly move in a fluid without preferential direction, collide with each other, thus creating a chaotic random agitation that results from intrinsic thermal fluctuations of any fluid. The macroscopic result of these random trajectories of particles is a drifting displacement of the particles that is referred to as diffusion, or **lateral diffusion** in the case of a two-dimensional space (Marguet et al. 2006). Soon after this finding, AMPARs were also found to oscillate between surface diffusion and scaffold-mediated immobilisation at excitatory synapses (Borgdorff and Choquet 2002). The dogma that receptors always remain stable on the membrane was once and for all debunked. The motive force was undoubtedly the development of single-particle tracking and later on **single molecule localisation** (SML) super-resolution techniques, which accelerated research on receptor surface diffusion during the last two decades and highlighted the contribution of lateral diffusion to AMPARs trafficking.

In parallel, live-cell imaging also provided useful insights into the lateral mobility of AMPARs. **Fluorescence-Recovery-After-Photobleaching** (FRAP) experiments, which consist in measuring the rate of fluorescence recovery of a fluorescent protein (FP)-tagged molecule in a given cell area, revealed that lateral diffusion, rather than constitutive exocytosis, is responsible for SEP-GluA2 subunit of AMPARs trafficking in and out of dendritic spines, where SEP-GluA2 receptors are slowed down (Ashby et al. 2006). SEP (Super-ecliptic fluorine) is a pH sensitive GFP variant that is quenched at acidic pH (i.e., intracellular vesicles) enabling visualisation of surface proteins, when exposed to neutral pH.

Membrane mobility of receptors is highly depended on their molecular interactions, but also depends on local crowding (M. Renner et al. 2012). According to the '**diffusion-trapping**' model, receptors can freely diffuse on the plasma membrane until they encounter specific partners, so called 'traps', that can transiently stabilise them at synapses (Bressloff and Earnshaw 2007). AMPARs receptors are trapped in PSD-95 clusters for tens of seconds in pyramidal hippocampal neurons (Bats, Groc, and Choquet 2007; Mondin et al. 2011) and the resulting decreased diffusion

depends on the interaction of the auxiliary protein stargazing with PSD-95 (P. Opazo et al. 2010). Their stabilisation is reversible (Tardin 2003) and disassociation of stargazing from AMPARs upon glutamate activation, increases their mobility allowing them to diffuse out of the trapping sites at synapses (Constals et al. 2015), facilitating the removal of receptors that are desensitised outside the synaptic transmission sites only to be replaced by naïve functional receptors (Heine et al. 2008). This regulated redistribution of receptors in and out of synapses guarantees the efficiency of synaptic responses to glutamate release. However, we should mention here that that the use of specific labelling strategies and pharmacological treatments used in some of these studies to study the mobility of AMPARs, have led others to raise questions regarding the potential interference with AMPARs distribution or biophysical properties (S. H. Lee et al. 2017; Delgado and Selvin 2018). As a result, the notion that AMPARs mobility is involved in regulating fast synaptic transmission is still doubted by some scientists.

Interestingly, neuroligin1 has been shown to regulate AMPARs recruitment at synapses (Heine et al. 2008; Mondin et al. 2011; Chanda et al. 2016; Haas et al. 2018) and recently LRRTM2, a synaptic adhesion molecule exclusively localised at excitatory synapses, was found to be implicated in AMPARs synaptic positioning (Bhouri et al. 2018; Ramsey et al. 2021), suggesting that adhesion molecules could have an active role in recruiting functional receptors. However, the underlying mechanism governing LRRTM2-AMPARs interactions are only starting to be explored and a part of this thesis is dedicated to shedding light into this interaction at the molecular level.

1.4.5 A new level of organisation revealed by super-resolution microscopy

Recent advances in super-resolution techniques, including STimulated Emission Depletion (STED), PhotoActivation Localization Microscopy (PALM) (Betzig et al. 2006), direct STochastic Optical Reconstruction Microscopy (dSTORM) (Rust, Bates, and Zhuang 2006) and universal Point Accumulation for Imaging in Nanoscale Topography (uPAINT) (Gregory Giannone et al. 2010) and DNA-PAINT (Jungmann et al. 2010) have enabled the study of synaptic proteins organisation at the nanoscale. These techniques allowed us to go beyond the **diffraction limit** of 250 nm and made the visualisation of two objects that are closer than 250 nm possible. These techniques,

however, use different principles to achieve a resolution below 250 nm. In **STED**, a diffraction-limited spot is excited at one wavelength while a super-imposed, red-shifted, second laser beam, projected to a donut-shape and depletes almost all emission laterally leaving only a central focal spot with a dimension below the diffraction limit. Single Molecule Localisation Microscopy (SMLM), including dSTORM and uPAINT, relies on the stochastic switching of fluorescent molecules between a bright and a dark state. Having only a few molecules in a fluorescent state at a given time enables the location of each molecule to be individually determined with high precision. By taking a few thousand to tens of thousands of images each with a different subset of fluorescent molecules, the position information of the fluorophores can then be used to reconstruct an image with a resolution that mainly depends on the number of detected photons. **uPAINT** relies on low concentration of fluorescent ligands in the imaging medium so that a constant rate of membrane molecules is being labelled during the imaging sequence. Oblique illumination of the sample is used to excite mainly fluorescent ligands that are bound to the cell surface while not illuminating the molecules in the solution above, allowing to visualise membrane protein in living cells. **dSTORM**, on the other hand, relies on saturating staining using of certain organic dyes, like Alexa 647, that exhibit photoswitching properties. Photoswitching is achieved by using oxygen-scavenging buffers and high laser power to make dyes enter the triplet state (non-fluorescent). From this state, fluorophores can go back stochastically to their ground state, thus emitting fluorescence. **DNA-PAINT** relies on the use of DNA molecules; while one is constantly attached to the target via antibodies or chemical tags (docking strand), the other one is conjugated to an organic dye (imager strand) and can freely diffuse in the imaging solution. Transient binding of the two complementary DNA strands enables imaging of the target molecules. Using multiple complementary strands we can achieve multicolour imaging, which constitutes one of the great advantages of the DNA-PAINT technique.

Application of super-resolution microscopy has contributed to a great extend to our understanding of the **nanoscale organisation** of synaptic proteins, revealing that proteins are not uniformly distributed in the PSD. PSD-95, one of the most studied scaffold proteins at excitatory synapses, was shown to form on average two 80 nm clusters per synapse (MacGillavry et al. 2013), although different approaches have revealed clusters of around 150 nm (Fukata et al.

2013; Nair et al. 2013). Endogenous PSD-95 seems to form on average two clusters per synapse (Tang et al. 2016). Within individual synapses **AMPARs** are not uniformly distributed in the PSD either, but they are rather organised in clusters of around 70 nm, called nanodomains (MacGillavry et al. 2013; Nair et al. 2013; Fukata et al. 2013). Their composition and position are dynamically regulated in the scale of minutes and are associated with the expression levels of PSD-95 (Nair et al. 2013) and modulate synaptic efficacy. These nanodomains are believed to be aligned with the presynaptic release machinery release, giving rise to a new concept, that of the **nanocolumn** (Tang et al. 2016). In particular, pre-synaptic scaffold protein RIM nanoclusters were found in front of post-synaptic scaffold PSD-95 nanoclusters using 3D STORM imaging. As RIM is enriched at vesicle-fusion sites at the pre-synapse and PSD-95 anchors glutamate receptors at the post-synapse, their trans-synaptic alignment could contribute to efficient synaptic transmission by positioning the necessary elements in front of each other. Interestingly, this trans-synaptic alignment was found to be dynamically regulated by synaptic plasticity, suggesting that pre and post-synaptic elements can be reorganised at the nanoscale to adapt to external cues (Tang et al. 2016). Multiple mechanisms could orchestrate the trans-synaptic alignment that remain elusive, including diffusible signals, interactions between cleft proteins and pre-synaptic Ca^{2+} channels or post-synaptic receptors and trans-synaptic adhesion molecules (Biederer, Kaeser, and Blanpied 2017). **Trans-synaptic adhesion molecules** are promising candidates as they span the synaptic cleft, exhibit specific organised distribution within the synaptic cleft and are dynamically regulated. In particular, neuroligin1 has been shown to be spatially correlated with AMPARs at the nanoscale, whereas deletion of the intracellular tail of neuroligin1 disrupted the alignment of pre-synaptic RIM with post-synaptic AMPARs (Haas et al. 2018). Recently, LRRTM2 was found to be enriched in the trans-synaptic nanocolumn and its extracellular domain was involved in positioning AMPARs close to glutamate release sites (Ramsey et al. 2021) (discussed in detail in chapter 3.3 of the Discussion) suggesting that synaptic adhesion molecules could indeed organise the trans-synaptic alignment.

Despite the breakthrough of super-resolution imaging techniques, we should bear in mind that the early studies mostly used quantum dots (QD) and antibodies to visualise surface receptors. Antibodies have the advantage of labelling endogenous proteins; however, full-length antibodies

are relatively large (10- 15 nm), which raises concerns regarding potential steric hindrance, crosslinking and accessibility to the synaptic cleft, while QD, despite being photostable thus suitable for long acquisitions in live neurons, can be up to 30nm big.

The development of new imaging tools that enable full benefit from super-resolution techniques. Nanobodies, monomeric and high-affinity camelid antibodies against fluorescent proteins (FP) have been developed to deliver bright organic fluorophores to FP-tagged proteins (Rothbauer et al. 2006; Ries et al. 2012). These probes are only 3nm big and they can access the dense and confined environment of the synaptic cleft allowing precise nanoscale localisation of receptors. In addition, fluorophore-conjugated monomeric streptavidin (mSA) has the advantage of recognising a short 15-amino-acid AP tag that can be readily incorporated into extracellular protein domains, without perturbing their native function (Chamma et al. 2016), unlike FP insertion that can result in protein mislocalisation (see chapter 2.1 of the Discussion). All these tools compatible with super-resolution microscopy allow us to study the nanoscale organisation and membrane dynamics of synaptic proteins with high specificity and precision.

1.5 Synaptic plasticity

Plasticity is one of the most important and fascinating properties of the brain and can be defined as the ability of the nervous system to change its activity in response to intrinsic or extrinsic stimuli by reorganising its structure, functions or connections. **Synaptic plasticity** specifically refers to the modification of the strength or efficacy of synaptic transmission through a diverse number of activity-dependent mechanisms and it has been proposed to play a critical role in learning and memory. It is also thought to play key roles in the early development of neural circuits and impairments in synaptic plasticity mechanisms are involved in several neuropsychiatric disorders. Canadian Psychologist Donald Olding Hebb was amongst the first ones to propose that synapses could undergo dynamic changes in their activity, which led him to postulate the Hebbian theory, according to which an increase in synaptic efficacy arises from a

presynaptic cell's repeated and persistent stimulation of a postsynaptic cell (Citri and Malenka 2008; Hebb, 1949). Different forms of synaptic plasticity have been described to date, both at inhibitory and excitatory synapses, which can potentiate or attenuate synaptic transmission within a range of milliseconds, hours, day or even longer.

Short-term plasticity, lasting on the order of milliseconds to several minutes, is believed to play important roles in short-term adaptations to sensory inputs, transient changes in behavioural states and short-lasting forms of memory. The mechanism underlying most forms of short-term plasticity is triggered by short bursts of activity resulting in a transient accumulation of calcium in pre-synaptic nerve terminals. This increase in turn causes changes in the probability of neurotransmitter release by altering the exocytosis of synaptic vesicles (Citri and Malenka 2008). **Long-term plasticity** is defined as a long-lasting, activity-dependent change in synaptic efficacy and it is believed to be the major process underlying learning, memory and behavioural adaptation. Although early studies focused on post-synaptic mechanisms, it is now known that synaptic strength may be modified on either side of the synapse. Post-synaptic plasticity is usually characterised by changes in post-synaptic receptors numbers or characteristics, whereas pre-synaptic plasticity involves an increase or reduction in neurotransmitter release. The most extensively studied and therefore prototypic forms of synaptic plasticity are **Long—Term Potentiation** (LTP) and **Long—Term Depression** (LTD) observed in the CA1 region of the hippocampus, which are triggered by activation of NMDARs. However, without stabilising mechanisms neural circuits would risk of becoming hyper- or hypoactive (K. D. Miller 1996). Functional stability is maintained by **Homeostatic plasticity**, which is defined broadly as a set of neuronal changes aiming to restore activity to a setpoint following perturbation (Turrigiano and Nelson 2004).

1.5.1 Functional plasticity

The most extensively studied forms of synaptic plasticity are the LTP and LTD observed in the CA1 region of the hippocampus, which are triggered by activation of NMDARs. During LTP, glutamate binds to AMPARs resulting in K^+ and Na^+ influx and depolarisation of the post-synaptic membrane.

Once the depolarisation is strong enough, NMDARs are activated after removal of their Mg^{2+} block, resulting in increased post-synaptic calcium concentration (Malenka 1991; Malenka and Nicoll 1993). This elevation in dendritic spine calcium concentration leads to activation of intracellular signalling cascades involving a number of protein kinases, most importantly CaMKII. As a result, single-channel conductance of synaptic AMPARs is increased and AMPARs are inserted and incorporated into the PSD. The newly inserted synaptic AMPARs are stabilised through their TARP-mediated interaction with PDZ domain-containing proteins such as PSD-95. In parallel, structural rearrangement within the synapse occurs, such that the size of the PSD and dendritic spine are increased, increasing in turn the size of the pre-synaptic active zone (**Figure 12**). The maintenance of these changes for more than a few hours depends on de novo transcription as well as local dendritic protein synthesis, presumably to provide the synapses with a supply of the critical proteins necessary for maintaining synaptic strength.

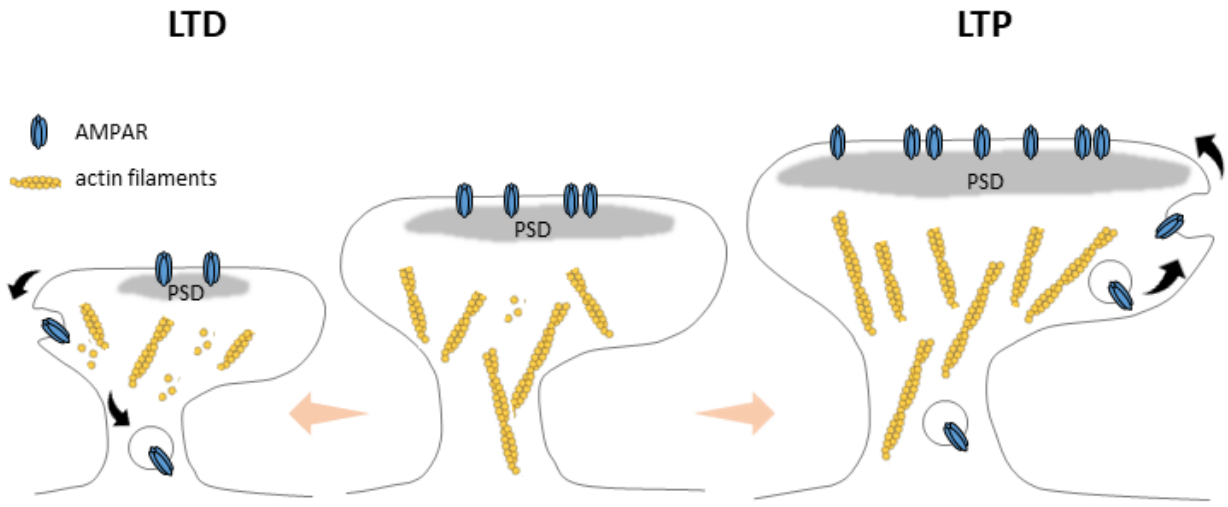


Figure 12. Simplified spine remodelling and AMPARs trafficking during LTD and LTP. During LTD AMPARs diffuse towards the periphery of the PSD and get endocytosed resulting in decrease surface AMPARs, while actin filaments depolarisation results in spine shrinkage. During LTP, increased AMPARs exocytosis and subsequent lateral diffusion results in insertion of AMPARs in the PSD, whereas polymerisation of actin filaments ensures spine increase. PSD: post-synaptic density, LTD: Long-Term Depression, LTP: Long-Term Potentiation.

In the case of the LTD, a modest increase in post-synaptic calcium concentration within dendritic spines due to modest activation of NMDARs leads to preferential activation of protein

phosphatases and key signalling proteins. As a result, AMPARs are dissociated from their scaffolds in the PSD and diffuse to endocytic zones on the periphery of the PSD, where they are endocytosed (**Figure 12**). Protein translation is probably involved in long-term maintenance expression of LTD (Pfeiffer and Huber 2006), although its mechanisms are not totally understood. It should be noted that NMDAR-independent forms of LTD do exist, including pre-synaptic LTP at synapses between the axons of dentate gyrus granule cells and the dendrites of CA3 pyramidal cells. During this form of LTD, increase in pre-synaptic calcium activates key regulators of intracellular pathways to cause a long-lasting enhancement in transmitter release (Nicoll and Malenka 1995; Nicoll and Schmitz 2005). Cerebellar mGluR-induced LTD is also linked with endocytosis of AMPARs and a decrease in their synaptic surface levels (Y. T. Wang and Linden 2000; Steinberg, Huganir, and Linden 2004), probably through dissociation of AMPARs from scaffold protein (Chung et al. 2003).

1.5.2 Structural plasticity

Apart from the functional aspects, structural rearrangements of synapses are also associated with synaptic plasticity (**Figure 12**). At excitatory synapses, dendritic spines have a unique and highly heterogeneous morphological organisation that serve as electrical and biochemical confined compartments allowing each spine to function independently, as previously discussed. The rapid alterations in spine formation and elimination are thought to be the structural substrate for memory encoding in the mammalian brain (C.-C. Chen, Lu, and Zuo 2014).

The very first evidence that morphological changes in dendritic spines are activity-dependent came from studies by Fifkova and colleagues, who showed that tetanic stimulation of the perforant path induced long-lasting enlargement of dendritic spines in the dentate granular cells (van Harreveld and Fifkova 1975), widening and shortening their spine necks (Fifková and Van Harreveld 1977; Fifková and Ander son 1981). Structural plasticity of dendritic spines is known to be correlated with circuit plasticity during learning (Trachtenberg et al. 2002; Yang, Pan, and Gan

2009; Hayashi-Takagi et al. 2015; W. Li et al. 2017), while LTP and LTD also associated with long-term enlargement and shrinkage of dendritic spines, respectively (Matsuzaki et al. 2004; Zhou, Homma, and Poo 2004).

Structural plasticity of dendritic spines is associated with cytoskeletal reorganisation. The spine cytoskeleton is mainly composed of filamentous actin (F-actin), which can polymerise and de-polymerize within seconds, allowing rapid and dynamic modulation of spine structures (**Figure 12**). The balance between actin polymerisation and depolymerisation plays a major role in structural plasticity of dendritic spines (Hotulainen and Hoogenraad 2010). 80% of actin in spines is dynamic, with a turnover that is less than a minute (Star, Kwiatkowski, and Murthy 2002), whereas remodelling of actin networks within dendritic spines has also been shown to be crucial for activity-dependent structural changes (Okamoto et al. 2004; Honkura et al. 2008; Frost et al. 2010). Chemically induced LTP in dissociated hippocampal neurons induces the rapid formation of new spines (H. Lin, Huganir, and Liao 2004; Park et al. 2006). Interestingly, glutamate uncaging-induced LTP not only forms, new spines, but also increases their stability (Hill and Zito 2013), unlike LTD, which causes a synapse-specific spine shrinkage and retraction (Oh, Hill, and Zito 2013). Spine remodelling is tightly regulated by both intracellular factors, such as transient calcium elevation triggering multiple signalling cascades (Fischer et al. 2000; Chang et al. 2017; Yasuda 2017) and extracellular factors such as autocrine signalling of brain-derived neurotrophic factor (BDNF) (Harward et al. 2016; Hedrick et al. 2016) and proteolytic cleavage of extracellular matrix and trans-synaptic cell adhesion molecules (Sonderegger and Matsumoto-Miyai 2014; Reinhard, Razak, and Ethell 2015). Finally, activity-dependent signalling such as RNA trafficking and miRNAs regulate local protein translation of β-actin and actin regulatory proteins, providing an efficient local supply of the necessary material for synaptic plasticity (Holt and Schuman 2013; Rangaraju, tom Dieck, and Schuman 2017).

1.5.3 Mechanisms of synapse potentiation: a focus on AMPARs

LTP, one of the most studied forms of synaptic potentiation, is predominantly mediated by increased function of post-synaptic AMPARs. Increased channel conductance, open probability

and receptor number on the surface have all been reported to be responsible for synaptic potentiation (Isaac, Nicoll, and Malenka 1995; Roche et al. 1996; Benke et al. 1998; S.-H. Shi et al. 1999).

In the late 90s it was found that post-synaptic vesicular fusion (Lledo et al. 1998) and an increase in synaptic AMPARs number (S.-H. Shi et al. 1999) occur during LTP, suggesting that receptors are far from being static but instead they can traffic to the surface from the intracellular recycling endosomes containing a reserve pool of AMPARs in the dendrites that are mobilised during LTP via a process that requires the small GTP-binding protein, Rab11a (Park et al. 2004). Exocytic events have been detected mostly in the dendritic shaft (D.-T. Lin et al. 2009), and more rarely at spines (Kennedy et al. 2010). Visualisation of activity-induced **exocytosis** of AMPARs in dendrites and dendritic spines is possible using the pH-sensitive superecliptic pHluorin (SEP), whose fluorescence is quenched at low pH (Miesenböck, De Angelis, and Rothman 1998). Using SEP-tagged AMPARs, the post-synaptic exocytosis of AMPARs during LTP has been directly visualised (Makino and Malinow 2009; Petrini et al. 2009; Kennedy et al. 2010). AMPARs are not directly inserted in the PSD, but are rather exocytosed at sites adjacent to PSD. Interestingly, certain populations of AMPARs can diffuse in and out of extra-synaptic and synaptic sites in order to orchestrate synaptic transmission (Borgdorff and Choquet 2002; Heine et al. 2008). This receptor exchange on the surface membrane through lateral mobility is dynamically regulated by activity, as it has been shown that extra-synaptic surface AMPARs adjacent to the PSD arrive the PSD through **lateral diffusion** upon LTP induction (Tardin 2003; P. Opazo et al. 2010; P. Opazo and Choquet 2011; Huganir and Nicoll 2013; Chater and Goda 2014; Constals et al. 2015; Penn et al. 2017). Laterally diffusing surface AMPARs are then trapped and immobilised at diffusional traps or ‘slots’ at synaptic sites via interactions with scaffold proteins (Tomita et al. 2005; P. Opazo et al. 2010). Finally, extra-synaptic AMPARs can be replenished via exocytosis of receptors from intracellular endosomes.

2. Synaptic adhesion molecules (CAMs) in neuronal connectivity

Intercellular connectivity is a fundamental characteristic of multicellular organisms and critical for development enabling the exchange of vital nutritional components between adjacent cells, but also providing the mechanical stability and flexibility that is essential for tissue formation. During adulthood, cell-cell adhesion maintains the structural and functional integrity of tissues, while allowing for dynamic reorganisation of intercellular connections.

Multiple sources highlighted the importance of **adhesion** around the beginning of the 20th century (Horwitz 2012). In 1922 Lewis suggested that adhesion underlies tissue organisation (Lewis 1922), also highlighted by studies on tissue dissociation and reassociation in invertebrates (Herbst 1900; Wilson 1907). Holtfreter accelerated adhesion research by showing that amphibian embryonic tissues could form through adhesion-based self-organisation (Tiwnes and Holtfeter, 1955), which was then generalised other organisms, resulting in the hypothesis that tissues are self-organised through the migration and arrangement of individual cells that differentially adhere to one another. On a more molecular basis, Tyler and Weis suggested that cellular adhesion arises from an antibody-antigen like interaction between surface molecules (Tyler 1947; Weiss 1947), which led Sperry to formulate the **chemoaffinity hypothesis** in the 1940s (Sperry 1954). According to his theory, neural connections arise, at least partially, from the recognition of 'identification tags' and that each axon becomes selectively attached to specific neurons depending on 'specific chemical affinities' (Sperry 1963). In 1977, a breakthrough came along with the discovery of Masatoshi Takeichi who demonstrated that cells have two adhesion systems, calcium-dependent and calcium-independent (M Takeichi 1977). His findings gave rise to a strategy for purifying adhesion molecules, leading later to the identification of E-cadherin, a founding member of the cadherin superfamily. At the same time, Edelman and colleagues isolated for the first time a neural cell adhesion molecule (N-CAM) from the neural retina of the chicken embryo (Thiery et al., 1977), laying the first stone for the discovery of other adhesion molecules in the nervous system.

In the nervous system, a complex ensemble of interconnected cells needs to be orchestrated in order to ensure its structure and functionality. The regulation of specific cellular connections emerges from different developmental events including axon guidance, target selection and synapse formation. These events critically depend on controlled spatial and temporal expression of selective molecules mediating contacts between neural cell surfaces, which are called **cell adhesion molecules**.

Synaptic adhesion molecules are adhesion molecules that are localised at synapses and are involved in their development and maintenance. They are mostly transmembrane proteins that have distinct extracellular and intracellular regions forming trans-synaptic complexes by binding to other adhesion molecules and interacting with intracellular partners, respectively. CAMs can be classified as cell–matrix or cell–cell interacting molecules based on the nature of the adhesive interfaces involved in the interaction. The first category includes receptor molecules, which mediate the binding of cell surfaces and various ligands at the extracellular matrix, with integrins being one of the main families of **cell-matrix** adhesion. The second category consists of proteins enabling **cell-cell** interactions, on which I will focus from now on. Intracellular interactions highlight the importance of synaptic CAMs as modulators of signalling cascades as they can bind to molecules like cytoskeletal proteins and cytoplasmic proteins. Extracellular regions contain numerous conserved domains that serve as diverse yet specific interfaces for interactions. These domains can be organised in repeated units, thus increasing the number of potential interactions, while spanning the synaptic cleft to provide mechanical stability. Depending on their extracellular domains, they can be classified into different superfamilies, which can either form **homophilic** trans-synaptic complexes, with both partners belonging to the same family, and/or **heterophilic** when partners come from different families. In addition, one synaptic CAM can have multiple partners and alternative splicing and post-translational modifications further increase the already highly diverse recognition selectivity and specificity creating a complex network of putative interactions. These characteristics, together with their ability to dynamically adapt to synaptic plasticity, render synaptic CAMs indispensable tools for the diverse, nonetheless targeted, connectivity in the brain. Nevertheless, when they were first discovered, synaptic

adhesion molecules were believed to be merely responsible for providing mechanical stability to synapses without having any functional role. Since then, adhesion molecules have been involved in multiple steps of synapse formation and function, as well as plasticity. In this chapter, I will give an overview of the major synaptic CAMs families, their main characteristics that differentiate them from the others, focusing on their role in synapse organisation and function. To finish, I will describe in further detail the Leucine-Rich Repeat (LRR) family and in particular the synaptogenic sub-families, by highlighting their distinct structural and functional characteristics.

2.1 Synaptogenic properties of CAMs

It was not until the early 2000s that several in vitro studies showed for the first time that synaptic CAMs are not only a physical link between the pre- and the post-synapse, but they can modulate synapse number, morphology and function. Co-culture of non-neuronal and neuronal cells showed that when expressed in non-neuronal cells, certain CAMs were capable of inducing pre-synaptic differentiation in contacting axons and they were thus called **synaptogenic** (Scheiffele et al. 2000). Neuroligin 1 and 2, post-synaptically localised CAMs, expressed in HEK293 cells were shown to induce accumulation of active zone components, morphological differentiation and cluster synaptic vesicles that undergo depolarisation-dependent exocytosis (Scheiffele et al. 2000). The term synaptogenic was later extended to pre-synaptic adhesion molecules that could induce post-synaptic differentiation in contacting dendrites in the co-culture assay. Neurexin-1, for instance, the pre-synaptic partner of neuroligin-1, has been shown to trigger post-synaptic specialisations containing Neuroligin-1, NMDARs and scaffold proteins when presented on the surface of non-neuronal cells or beads (Nam and Chen 2005; Graf et al. 2004). In the same line, overexpression of Neuroligin 1 in cultured neurons increases the number of pre-synaptic boutons (Wittenmayer et al. 2009). Overexpression of SynCAM, a member of the Immunoglobulin superfamily, in cultured neurons promotes synapse formation, whereas its overexpression in non-neuronal cells induces formation of active presynaptic terminals in contacting axons (Biederer et al. 2002). Netrin-G ligand and Leucine-Rich repeat transmembrane proteins (LRRTM), LRR-containing CAMs, were as also found to induce pre-synaptic differentiation in the

co-culture assay (S. Kim et al. 2006; J. Ko, Zhang, et al. 2009; Linhoff et al. 2009; de Wit et al. 2009). However, not all CAMs involved in synapse function and organisation are synaptogenic; cadherins, which are involved in synapse formation, are not synaptogenic, as expression of N-cadherin in non-neuronal cells is not sufficient to induce pre-synaptic differentiation in contacting axons (Scheiffele et al. 2000).

2.2 Cadherin superfamily

The cadherin family consists of more than 100 members classified into several subfamilies in mammals, including the classic cadherins, protocadherins, desmosomal, Fat and 7-pass transmembrane cadherins. Their adhesive function depends on calcium, hence their name (M Takeichi 1977). Cadherin superfamily members are conserved across species and most are expressed in the nervous system where they are involved in neural development and synapse formation and plasticity (Angst, Marcozzi, and Magee 2001). The role of classical cadherins and protocadherins in the central nervous system has been extensively studied.

Classical cadherins are the most abundant cadherins family in vertebrates encoding for 18 members in the human genome and they are indispensable for cell-cell adhesion. Their extracellular part consists of five extracellular cadherin (EC) repeats followed by a single-pass transmembrane domain and an evolutionary conserved intracellular part (**Figure 13**). Homophilic binding of the extracellular domains mediates cadherin adhesion, while intracellular interaction with catenins bridges cadherins with actin filaments and regulates actin polymerisation. Cadherins are believed to be involved in the initial cell-cell contact during development, as mentioned chapter 1.3.2.2.

Protocadherins are a group of transmembrane proteins that belong to the cadherin superfamily. They have varying numbers of the EC domains but divergent cytoplasmic domains that do not

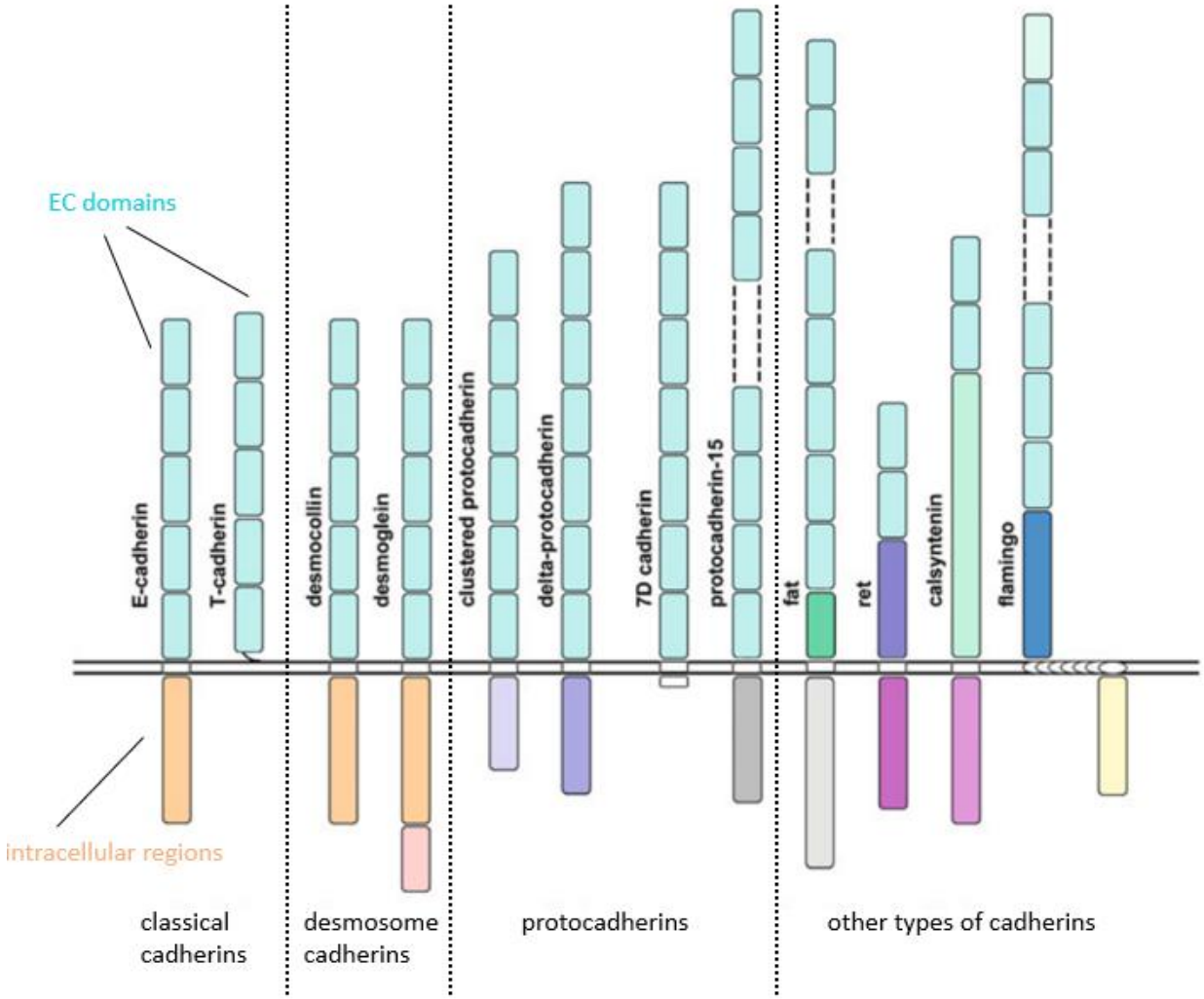


Figure 13. The Cadherin superfamily. Cadherins have repeated extracellular cadherin (EC) repeats that can vary in number in the different subfamilies. Intracellular regions are more diverse and define the interactions with intracellular binding partners. Adapted from The Cadherin Superfamily, Suzuki and Hirano, 2016.

bind to catenins (**Figure 13**). Most of the protocadherins are expressed in the nervous systems, with some of them being localised at synapses. Their complex genomic organisation and alternative splicing have led to the hypothesis that they could underlie synaptic specificity. In mammals, protocadherin genes can be non-clustered, meaning that they are scattered in the genome (N.-G. Kim et al. 2011) or clustered, because they are closely located in the genome (Q. Wu and Maniatis 1999). The latter have been associated with self-avoidance, a phenomenon during development that makes sure that sister neurites avoid forming contacts with one another

(Grueber and Sagasti 2010), promoting interactions with dendrites from different cells (Lefebvre et al. 2012).

2.2.1 Role of cadherins in synapse function and organisation

Live-cell imaging studies suggest that cadherins are enriched at both pre-and post-synaptic compartments at nascent synapses, where they are maintained after synapse maturation (Jontes 2004; Salinas and Price 2005). In addition, crystal structures of cadherin extracellular domain suggest that trans-synaptic cadherin-mediated complexes have the perfect length (~40 nm) to span the synaptic cleft (Boggon et al. 2002; Harrison et al. 2011), reinforcing the notion that initial cadherin-based adhesion stabilise transient contacts long enough to allow other classes of adhesion molecules to interact (Stan et al. 2010; Aiga, Levinson, and Bamji 2011; M. Yamagata, Duan, and Sanes 2018). N-cadherin controls spine morphology (Togashi et al. 2002; Elia et al. 2006) and deletion of N-cadherin in hippocampal neurons increases spines' motility, while its down-regulation induces the formation of thinner spines (Mysore 2007). Deletion of β-catenin alters the localisation of axonal synaptic vesicles (Bamji et al. 2003) and induces abnormally motile spines in hippocampal cultured neurons (Abe et al. 2004).

The diversity and large number together with the adhesive properties of the cadherin superfamily implies that they could be involved in synaptic **specificity**, which is critical for regulating synaptic communication (Togashi et al. 2002; Poskanzer et al. 2003). Indeed, cadherin-9, exclusively expressed by the dentate gyrus (DG) and CA3 neurons in the hippocampus, has been shown to be required for formation of DG but not CA3 or CA1 synapses in vitro (Williams et al. 2011) and further revealed that DG-CA3 synapse development is regulated by homophilic cadherin-9-mediated interactions. In the same line, cadherin-7 has been found to be an important regulator of the pontocerebellar circuit in the developing cerebellum, where pontine nucleus axons form synapses with granule cell and not with Purkinje cells; cadherin-7 knock-down in pontine nucleus neurons forced some of their axons to wrongly migrate into the Purkinje cell layer, impeding synapse formation between pontine nucleus and granule neurons (Kuwako et al. 2014).

Even though cadherins are involved in synapse formation, they are not synaptogenic, as expression of N-cadherin in non-neuronal cells cannot induce pre-synaptic differentiation in contacting axons (Scheiffele et al. 2000). However, N-cadherin is involved in LTP-induced long-term stabilisation of synapses (Mendez et al. 2010; Chazeau et al. 2015), it binds to GluA2 subunit of AMPARs (Nuriya and Huganir 2006; Saglietti et al. 2007) and regulates their mobility on the neuronal surface (Saglietti et al. 2007), suggesting that synaptic adhesion are important for regulating AMPARs. Taken together those data, support that cadherins participate in synapse formation, specificity and function.

2.3 Immunoglobulin superfamily

Immunoglobulin (Ig) super-family CAMs are cell surface glycoproteins highly expressed in the nervous system, counting over 50 members (Gu et al. 2015) including N-CAM, SynCAMs and nectins. This family represents a highly diversified group of cell surface molecules, consisting of

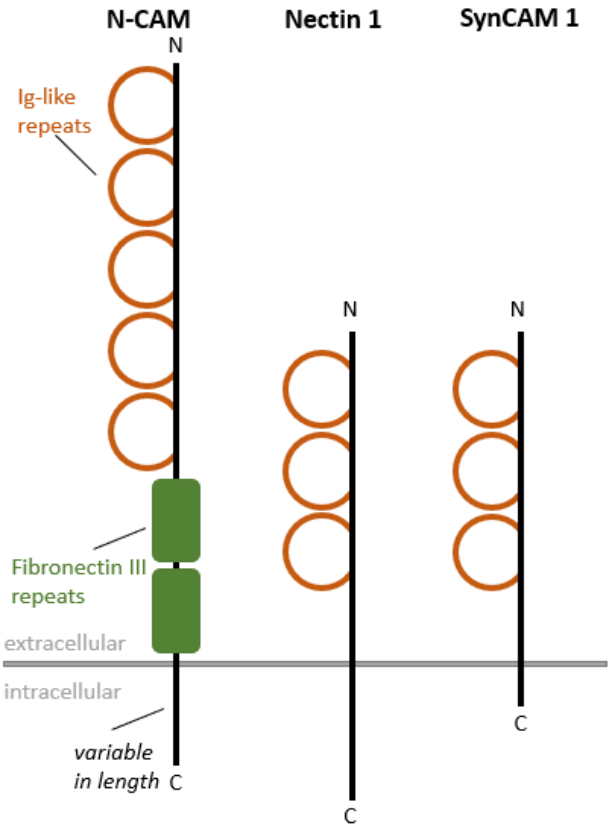


Figure 14. Representative members of the Ig superfamily. Extracellular domains contain several immunoglobulin-like (Ig) repeats. N-CAM also has Fibronectin III repeats between the Ig repeats and the transmembrane region.

a large extracellular domain containing one or several immunoglobulin-like (Ig) repeats (Shapiro, Love, and Colman 2007), which are cysteine-looped domains. One or more fibronectin type III (FNIII) repeats are present between the Ig domains and the trans-membrane domain in some member of the family. Although the majority of Ig superfamily members are single-pass transmembrane proteins with intracellular domains of various lengths, some of them are anchored to the cell surface plasma membrane via a glycosylphosphatidylinositol (GPI) anchor (**Figure 14**). Members of the family usually interact in a homophilic manner that strongly relies on the Ig domains (Zhao, Yip, and Siu 2002).

2.3.1 Role of Immunoglobulin superfamily in synapse function and organisation

Intracellularly, a number of Ig superfamily members can directly (Leshchyns'ka et al. 2003) or indirectly (Cheadle and Biederer 2012) bind to the cytoskeleton, thus actively shaping the morphology of synapses. Levels of spectrin βI, one of the major components of the post-synaptic cytoskeleton in synapses, are reduced in synapses from N-CAM-deficient mice (Sytnyk et al. 2006). Another member of the Ig superfamily, SynCAM1, intracellularly binds to Farp1, a protein that regulates synaptic actin cytoskeleton. SynCAM1 knockout mice exhibit reduced levels of Farp1 and polymerised actin indicating that SynCAM1 is required for Farp1 recruitment to synaptic membranes (Cheadle and Biederer 2012), suggesting that members of the Ig superfamily are critical for cytoskeletal architecture at synapses. Likewise, the cytoskeleton has also been shown to play an important role in regulation of the functions of Ig superfamily members. In addition, deletion of members of the SynCAM (SynCAM1-3) family decreases the size and number of synapses (Fowler et al. 2017). SynCAM1 KO mice exhibit impaired LTD (Robbins et al. 2010) and super-resolution imaging showed that SynCAM1 is localised at the periphery of synapses, close to the border of the PSD. Upon LTD SynCAM1 domains are rearranged, implying that adhesion molecules are sensitive to synaptic stimuli and can dynamically adapt their organisation (Perez de Arce et al. 2015). Inhibition of nectin in cultured rat hippocampal neurons results in a diminution of synapse size accompanied with an increase in synapse number (Mizoguchi et al. 2002). Even though nectins are not localised at the spines of mature synapses, they are found at puncta adherentia junctions (Honda et al. 2006), which constitute mechanical adhesion sites on

the dendritic shaft but in proximity with the bottom of the spine, suggesting that adhesion molecules that are not localised on the spine head are still involved in synaptic formation.

2.4 Eph family

The Eph family of proteins, consisting of Eph (erythropoietin-producing hepatocellular carcinoma) receptors, the largest known class of receptor tyrosine kinase in mammals (Tuzi and Gullick 1994), and signal bidirectionally through interaction with membrane-tethered Ephrin (Eph receptor-interacting protein) ligands. The Eph family of receptors can be divided into two subfamilies, **EphA** and **EphB**, sharing structural features and highly conserved domains yet exhibiting distinct functional outcomes depending on their interactors and downstream signalling. Structural differences between ephrin-A and ligand families, the interactors of Eph receptors, contribute to different responses upon Eph receptor activation. Ephrin-A ligands are GPI-anchored to the cell membrane, unlike ephrin-B that are transmembrane ligands with a conserved cytoplasmic tail, containing a sterile alpha motif (SAM) interaction domain and a PDZ-binding domain (**Figure 15**). They are expressed both at the pre- and/or the post-synapse (Bouvier et al. 2008) in distinct expression patterns in the hippocampus (Henderson et al., 2001) and are involved in dendritic spine stabilisation (Y. Shi et al. 2009) actin cytoskeletal remodelling (Tolias et al. 2007) and synaptic plasticity (Klein 2009).

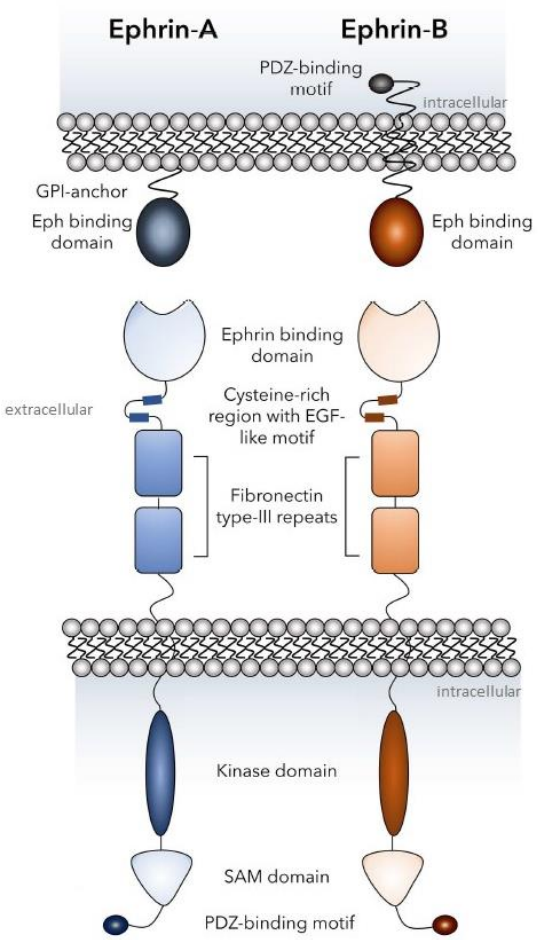


Figure 15. Eph family structural features. Eph receptors are consisting of an extracellular structure consisting of an ephrin binding domain connected to two fibronectin type-III repeats by a cysteine-rich EGF-like motif. Intracellularly, they have a kinase domain that is linked to a sterile alpha motif (SAM) domain and PDZ-binding motif. Eph receptors bind to ephrin ligands via an extracellular Eph binding domain. Ephrin-A ligands are GPI-anchored to the cell membrane and signal through co-receptors that have not yet been fully defined. Ephrin-B ligands are transmembrane with an intracellular PDZ-binding motif. Adapted from Darling and Lamb, 2019

2.4.1 Role of Eph family in synapse function and organisation

Triple knock-out mice for EphB1–3 exhibit a decrease in excitatory synapse number (Henkemeyer et al. 2003; Kayser et al. 2006). EphB2 was shown to induce pre-synaptic differentiation when co-cultured with neurons (Kayser et al. 2006), an effect that was dependent on the PDZ-binding domain in the intracellular tail of its binding partners, EphrinB1 (McClelland et al. 2009). Subsequently, EphrinB1 can recruit syntenin-1, an adaptor protein, through its PDZ-binding domain (Grembecka et al. 2006), which in turn triggers a cascade of events resulting in accumulation of neurotransmitter-loaded vesicles. At the post-synaptic site, exposure of cultured cortical neurons to EphrinB1 results in clustering of ephrin receptor, EphrinB2 and NMDA receptors clusters through direct interaction (M. B. Dalva et al. 2000). On the contrary, mice lacking the EphB2 receptors exhibit reduced LTP at hippocampal CA1 and dentate gyrus synapses

and decreased synaptic localisation of NMDA receptors (Henderson et al. 2001; Nolt et al. 2011). EphrinB2 has also been implicated in AMPARs stabilisation at the plasma membrane though indirect intracellular interactions, as ephrinB2 KO neurons show increased constitutive internalisation of AMPARs (Essmann et al. 2008), reinforcing the notion that adhesion molecules could modulate receptors' localisation.

2.5 Neurexins

Neurexins were first discovered as receptors for alpha-latrotoxin, a substance in black widow spiders' venom that triggers massive neurotransmitter release by binding to pre-synaptic nerve terminals (Ushkaryov et al. 1992). They constitute a particularly interesting family as they engage in multiple trans-synaptic complexes with structurally unrelated partners (**Figure 16**) and they also undergo extensive alternative splicing, rendering them an extremely versatile 'tool' for specifying synapse connectivity. They are localised at the pre-synapse, although post-synaptic localisation of neurexins has also been reported (Taniguchi et al. 2007; Savas et al. 2015). The neurexin family derives from three genes (*nrxn1-3*) under the control of three promoters (α, β and the recently discovered γ) (Ullrich, Ushkaryov, and Südhof 1995; Ushkaryov et al. 1992; Yan et al. 2015) and in combination with **alternative splicing**, gives rise to more than 12.000 isoforms that are expressed in different neuronal cell types (Fuccillo et al. 2015; Schreiner et al. 2014). The extracellular domain of the longer isoforms, α-neurexins, has three EGF (epidermal growth factor-like) domains interspersed amongst six LNS domains (laminin-neurexin-sex hormone binding globulin), whereas the shorter β-neurexins only have the proximal to the transmembrane region LNS domain of α-neurexins. γ-Neurexin lacks the extracellular LNS and EGF structured domains yet retains a transmembrane and intracellular tail (Yan et al. 2015).

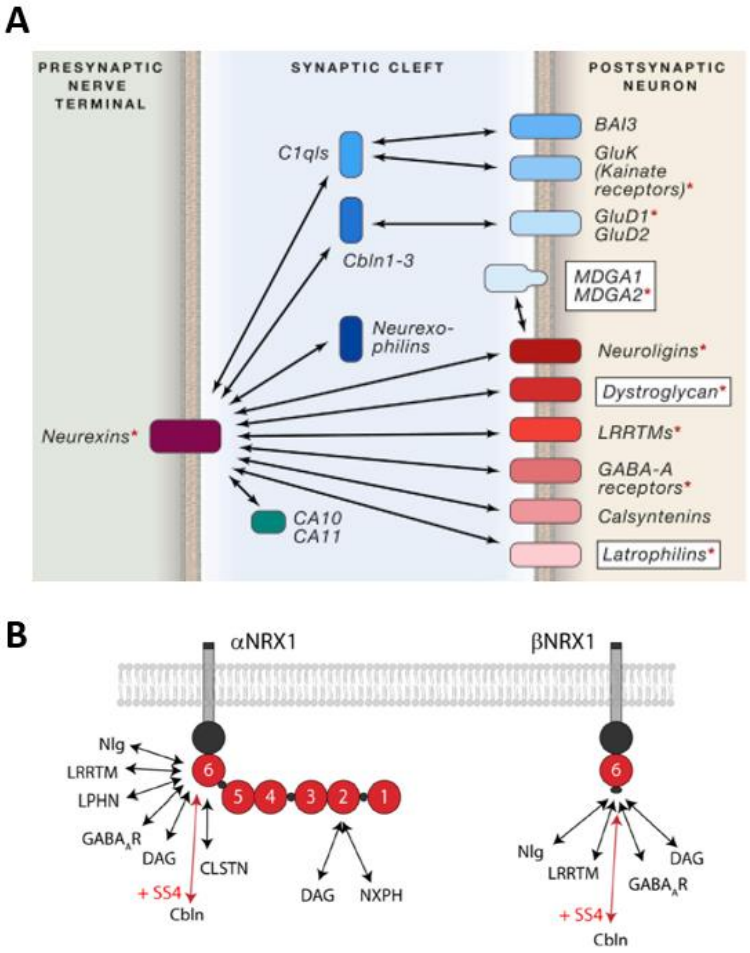


Figure 16. Extracellular interaction of Neurexins. A. Overview of neurexin trans-synaptic interactions with different partners. **B.** Binding sites of neurexin1 (α and β) extracellular interactions. Most of the proteins bind to LNS6 lacking the splice site 4 (-SS4). α NRX1 lacking the splice site 4 (-SS4) in the LNS 6 domain interacts with NLGs, LRRTMs, Latrophilins (LPHN), $GABA_A$ receptors, dystroglycans (DAG), Calsyntenins (CLSTN) and through their LNS 2 domain with DAG and Neurexophilins (NXPH). αNRX1 containing the splice site 4 (+SS4) only interacts with NLGs and cerebellins (Cblns) through their LNS 6 domain and with NXPH through their LNS 2 domain. βNRX1-SS4 interacts with the same partners as αNRX1 except for latrophilins (LPHN) through their LNS6 domain. βNRX1+SS4 interacts with NLGs and Cblns. Modified by Südhof 2017, Chamma and Thoumine 2018.

Alternative splicing of neurexins has received particular attention as it enables them to interact with multiple partners in a tightly regulated manner: neuroligin 1-4 (Ichtchenko et al. 1995), α-Dystroglycan (Sugita et al. 2001), Leucine-rich repeat transmembrane proteins (LRRTM1-4) (J. Ko, Fuccillo, et al. 2009; de Wit et al. 2009), secreted protein cerebellin 1 (Uemura et al. 2010) and calsyntenin 3 (Pettem et al. 2013) (**Figure 16**). Neurexins have five alternative splice sites in α-neurexin SS1 to SS5 and two in β-neurexin SS4 and SS5. Interestingly, the highly conserve SS2 and SS4 constitute the binding sites of all known α-neurexin binding partners, whereas SS1, SS5 and SS6 regulate the length of the linker regions (M. T. Miller et al. 2011). Those regions are very important as they modulate the exposure of the ligand binding sites and provide enough flexibility for the large extracellular domain to fit into the synaptic cleft by modulating its

configuration. Although dystroglycan, LRRTMs, cerebellin and neuroligins share the same binding site on the LNS6 of neurexin, alternative splicing at SS4 regulates their binding. Dystroglycan and LRRTM only bind to neurexins that lack the 30 a.a insert at SS4 (-SS4), while cerebellin binds to SS4+. On the other hand, neuroligin seems to bind neurexin regardless of the SS4 insert, although it preferentially binds to SS4- (Comoletti et al. 2007). In addition, even though LRRTM and neuroligin have non-homologous structures, they compete for their binding to neurexin.

2.5.1 Role of neurexins in synapse function and organisation

On the physiological level, genetic deletion of neurexin 1α decreased spontaneous activity at excitatory synapses in hippocampal pyramidal neurons (Etherton et al. 2009), whereas triple α-neurexin 1-3 KO affected both excitatory and inhibitory synapses in the neocortex (Kattenstroth et al. 2004). Neurexin is also important for neurotransmitter release, as triple knock-out of α-neurexin 1-3 reduces calcium-dependent neurotransmitter release (Missler et al. 2003) and possibly through intracellular indirect interaction with calcium channels, although there is no clear evidence for this hypothesis. Neurexin is linked with the active zone organiser, liprin-α, (LaConte et al. 2016), through PDZ-dependent binding to CASK (Mukherjee et al. 2008), a MAGUK family protein, proposing a direct link between neurexin and the pre-synaptic release machinery. Neuroligin and neurexin also participate in tripartite complexes; in the cerebellum, neurexin binds to glutamate receptor δ2 through cerebellin 1 precursor protein, a secreted glycoprotein from pre-synaptic granule cells (Uemura et al. 2010), adding another layer of complexity to the trans-synaptic interaction pattern. Tripartite complexes are not restricted to neuronal-secreted proteins. In the thalamocortical synapse formation, hevin, an astrocyte-secreted synaptogenic protein mediates trans-synaptic binding of neuroligin and neurexin isoforms, neurexin1α and neuroligin1, which cannot interact directly, reinforcing the notion that astrocytes can directly modulate the formation of synapses (Singh et al. 2016).

2.5.2 Neurexin surface mobility and nanoscale organisation

Membrane mobility studies showed that bulker alpha neurexins were unexpectedly diffusing even faster than the smaller beta neurexins, suggesting that the size of the extracellular domain

does not affect their surface dynamics (Neupert et al. 2015). However, diffusion at the pre-synaptic terminals was significantly reduced for both isoforms of neurexin 1 (Neupert et al. 2015; Chamma et al. 2016), implying that synaptic interactions are involved in confining neurexins at the pre-synaptic site. Dual-colour super-resolution imaging of neurexin 1β and neuroligin 1 revealed that the two trans-synaptic partners cluster lengths share similar frequency distribution at synapses and that NMDA treatment destabilised their trans-synaptic contacts (Chamma et al. 2016). Despite the excessive literature on neurexins and their contribution to synaptic adhesion and transmission, their precise sub-synaptic organisation has only started to be understood. Neurexin 1 was recently shown using 3D STORM in hippocampal cultures to occupy around 40% of excitatory synapses, where usually it is organised in a single nanocluster occupying a fraction of the pre-synaptic area, which seems to be dynamically regulated by ADAM10-mediated cleavage of neurexin 1 ectodomain (Trotter et al. 2019).

2.6 Neuroligins

In the mammalian brain four isoforms of neuroligin can be found (nlg1-4), with neuroligin1 being localised at excitatory synapses, neuroligin2 at inhibitory and cholinergic ones, neuroligin3 at both excitatory and inhibitory and finally neuroligin4 at cholinergic synapses (J.-Y. Song et al. 1999; Varoqueaux, Jamain, and Brose 2004; Budreck and Scheiffele 2007; Hoon et al. 2011). The assembly of either excitatory or inhibitory synapses via neuroligins is regulated by the recruitment of PSD-95 and gephyrin respectively (Graf et al. 2004; Levinson and El-Husseini 2005; Grégory Giannone et al. 2013). Through their single extracellular domain that is homologous to acetylcholinesterase, neuroligins form constitutive dimers, but also bind to pre-synaptic α- and β-neurexins, an interaction that involves the LNS domain of neurexins that is proximal to the transmembrane region (Comoletti et al. 2007). Alternative slicing of both partners regulates their binding affinities (Boucard et al. 2005), creating a sophisticated network of interactions across the synaptic cleft. Their intracellular domain is involved in interactions with class-I PDZ domains, such as PSD-95 (Irie et al. 1997) that contribute to recruiting receptors at the synapse.

2.6.1 Role of neuroligins in synapse function and organisation

Expression of neuroligin1 both in non-neuronal cells and in neurons induces pre-synaptic clustering of vesicles in contacting neurons in cultures (Scheiffele et al. 2000; Sara 2005). In young, cultured neurons, overexpression of neuroligin1 increases the number of pre-synaptic boutons, whose characteristics resemble those of mature cultures, with increased synaptic vesicles pool and exocytosis rate (Wittenmayer et al. 2009). Interestingly, the extracellular domain of neuroligin1 was proposed to enhance the number of boutons, whereas the intracellular domain was related to the structural and functional maturation, suggesting binding with the pre-synaptic partner and intracellular interaction of the protein are required in distinct pre-synaptic differentiation processes.

As neuroligin1 is the main isoform of the family in excitatory synapses, its implication in glutamatergic transmission has been extensively studied. Deletion of neuroligin1 decreases NMDAR-mediated currents (Soler-Llavina et al. 2011; Jiang et al. 2017) and the synaptogenic effect of neuroligin1 depends on the activity of NMDA receptors (Chubykin et al. 2007). In fact, neuroligin1 directly binds to NMDA receptors through its extracellular domain (Budreck et al. 2013), which is sufficient to rescue impairments in LTP and in NMDAR-mediated signalling caused by deletion of all Neuroligins (X. Wu et al. 2019). Our lab has reported that a unique intracellular tyrosine (Y782) in the intracellular tail of neuroligin1 that differentially regulates its binding to either PSD-95 or gephyrin (Grégory Giannone et al. 2013), is critical for LTP and recruitment of AMPARs (Letellier et al. 2018). The impact of neuroligin1 on AMPAR-mediated transmission is also not clear as it differs depending on the experimental design of the studies (Mondin et al. 2011; Budreck et al. 2013; Hoy et al. 2013; Jiang et al. 2017). However, using super-resolution imaging neuroligin1 has been shown to tightly colocalise with AMPARs at the nanoscale and deletion of the intracellular domain of neuroligin1 disrupts the alignment of presynaptic RIM with post-synaptic AMPARs (Haas et al. 2018), making neuroligin1 the first putative organiser of the trans-synaptic nanocolumn. Binding of pre-synaptic neurexin1β to neuroligin1 induces immobilisation of the latter and contributes to recruitment of PSD-95 (Giannone et al. 2013), while PSD-95 itself is required for neuroligin1-dependent immobilisation of GluA2 receptors

(Mondin et al. 2011). Single molecule tracking experiments have revealed that neuroligin1 is highly diffusive at extra-synaptic regions, but it is strongly confined at excitatory synapses, where it is enriched by 3-fold according to super-resolution imaging in neuronal cultures and brain slices (Chamma et al. 2016).

2.7 Leucine-Rich repeat superfamily

Leucine-Rich Repeat (LRR) is a protein-interaction motif present in more than 2000 proteins across all Kingdoms (Alder et al., 2005; Huang et al., 2008). The LRR family includes a rather diverse group of proteins such as receptors, adhesion molecules, enzymes and proteins of the extracellular matrix and their role extends from cell adhesion and signal transduction to DNA repair and apoptosis. In the human genome, more than 300 proteins contain LRR domains (Ng et al., 2011) and they are mostly known for their role in innate immunity, neuronal development and adhesion/signalling. In the nervous system LRR proteins are involved in multiple aspects of axon guidance (Long et al., 2004), myelination (Mi et al., 2005), target selection and synapse formation (de Wit et al., 2011).

2.7.1 Structural features of the LRR motif

Despite their unrelated function, LRR protein share a common feature that makes them all suitable for versatile interactions with other proteins, their curved conformation. LRRs are repetitive sequences of approximately 20 to 30 amino acids often present in a tandem manner and with a conserved sequence LxxLxLxxN/CxL (x stands for any amino acid). The ribonuclease inhibitor, a cytoplasmic protein present in a variety of mammalian tissues, was the first LRR protein to be crystallised, revealing that multiple LRR domains are organised in a curved, arc-shaped structure (Bostjan Kobe and Deisenhofer 1993), whose **concave** (inner) side is arranged as a parallel β-sheet, whereas the **convex** (outer) side can be more versatile in terms of secondary structures (**Figure 17**). The presence of both conserved amino acids, well protected in the core, and non-conserved, exposed to solvent, amino acids in the LRR offer a stable scaffold to the structure but at the same time facilitate modifications in the amino acid sequence. In addition to

their unique arrangement, the variable length and number of the LRR motifs generates an adaptable framework for interactions with various ligands (B Kobe 2001). Those interactions most commonly occur in the concave side of the LRR, although ligand-binding in the convex side has also been reported (Seewald et al. 2002; H. M. Kim et al. 2007). The hydrophobic core of the LRR structure is typically protected by N-terminal and C-terminal cap regions, which are usually rich in cysteine residues in the extracellular proteins (Kajava 1998). Interestingly, according to genomic studies from different organisms containing extracellular LRR motifs, humans were the species with the most of them, suggesting that LRR motifs, besides being evolutionary conserved, seem to be employed in high-order functions (Dolan et al. 2007).

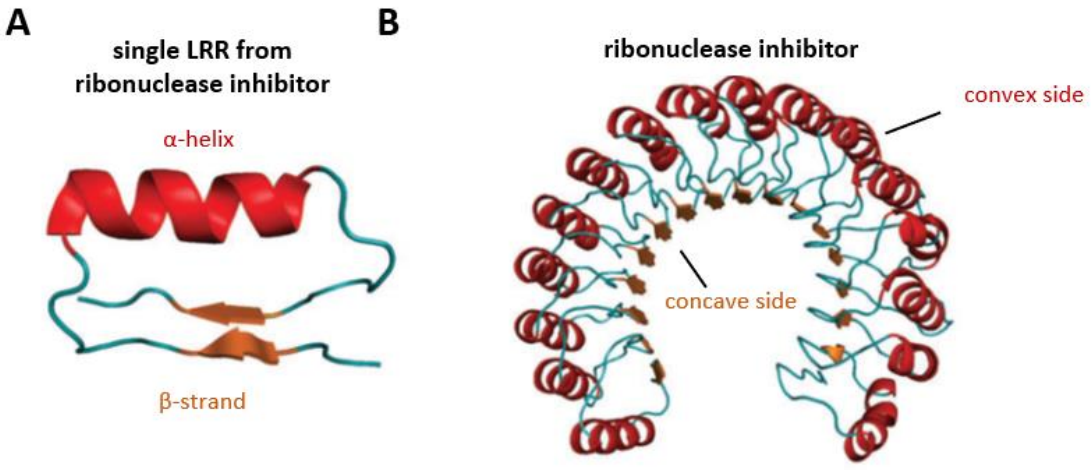


Figure 17. Structure of the LRR domain. A. Single leucine-rich repeat (domain) ribonuclease inhibitor. The LRR is composed of a right-handed β-strand (orange) connected by a loop region (teal) to an α-helix motif (red) roughly parallel to the strand. **B.** Ribbon diagram of the 3D structure of the porcine ribonuclease inhibitor (PDB ID: 2BNH). Multiple LRR domains are organised in a curved, arc-shaped structure, whose concave (orange) side is arranged as a parallel β-sheet, whereas the convex (red) side can be more versatile in terms of secondary structures. Adapted from de Wit et al., 2010.

Members of the LRR family include extracellular, membrane-anchored and intracellular proteins (i.e nucleotide-binding domain leucine-rich repeat; Jones et al., 2016), although all of the LRR proteins in the CNS known to date fall in the first two categories. In the next part, I will present in more details the synaptogenic LRR subfamilies.

2.7.2 Slitrks

Slit-like and Trk-like (Slitrk) family owes its name to homology with the axon guidance molecule, Slit, and the neurotrophin receptor, Trk (tyrosine receptor kinase) and its members were first discovered in a screen for deregulated genes in mice with neural tube deficits (Aruga and Mikoshiba 2003). Slitrks are involved in neurite growth and neuronal survival and comprise six vertebrate members (Slitrk1–6) broadly expressed in the mammalian CNS. Slitrks have also been detected in various brain tumours (Aruga, Yokota, and Mikoshiba 2003), suggesting a putative role in cancer development. Mutations in human *SLITRKs* have been associated with schizophrenia (Piton et al. 2011; Bansal et al. 2018), Tourette's syndrome (K. Zhang et al. 2015; Abelson et al. 2005) and obsessive-compulsive disorder (Shmelkov et al. 2010; M. Song et al. 2017), amongst others. Recently, missense mutations in the LRR domains of Slitrks, associated with neuropsychiatric disorders, were found to impair Slitrk trafficking and synapse formation in cultured hippocampal neurons (Kang et al. 2016).

Slitrks are single-pass transmembrane proteins localised at the postsynaptic membrane, with a divergent intracellular domain and an extracellular domain consisting of two LRR domains of 6 LRRs each (**Figure 18**) (Aruga and Mikoshiba 2003). All Slitrks, except Slitrk3, promote the formation of both excitatory and inhibitory synapses by forming trans-synaptic adhesion complexes with Protein Tyrosine Phosphatase (PTP), PTPRS and PTPRD, respectively (Yim et al. 2013), whereas Slitrk3 selectively modulates the development of inhibitory synapses via PTPRD (Takahashi et al. 2012). Insights into this trans-synaptic interaction by crystallography revealed a very interesting mechanism through which Slitrk1 promotes pre-synaptic differentiation. First, post-synaptic Slitrk1 interacts with pre-synaptic Leukocyte common Antigen-related Receptor PTP, LAR-RPTPs and subsequently, the formed complexes interact in *cis* via the convex side of Slitrk1 LRR1 and adjacent LAR-RPTPs in order to induce presynaptic differentiation (Um et al. 2014). This mechanism not only highlights the importance of synaptic clustering of adhesion molecules, but also reminds us that the convex side of the LRR domain mediates essential functional processes.

While the two extracellular LRR domains of the Slitrk family share high homology (Aruga and Mikoshiba 2003), the intracellular part varies between the members both in amino acid composition as well as in length. Those differences could account for the different neuronal functions of the Slitrks, supposedly by favouring the interactions with distinct intracellular binding partners that are yet to be identified.

2.7.3 Netrin-G ligands (NGLs)

Netrin-G ligands were first identified, as the name implies, as proteins that selectively interact with netrin-G family of cell-adhesion molecules (J. C. Lin et al. 2003; Nakashiba et al. 2000; 2002). Netrin-Gs are GPI-anchored and should not be confused with classical netrins. Netrin-G ligands are predominantly expressed in the brain (S. Kim et al. 2006), though in non-overlapping neuronal populations (Yaguchi et al. 2014), at excitatory but not inhibitory synapses in cultured neurons (S. Kim et al. 2006) and are enriched in the post-synaptic density (Sheng and Hoogenraad 2007). NGL-1 and NGL-2 bind to pre-synaptic netrin-G 1 and -2, respectively, unlike NGL-3 which interacts with tyrosine phosphatase LAR-PTP (Woo, Kwon, and Kim 2009). The extracellular part of NGLs contains nine LRRs as well as an Ig domain, followed by a single transmembrane domain and an intracellular region, ending with a PDZ domain-binding motif (**Figure 18**), through which they bind to the first two PDZ domains of PSD-95 (S. Kim et al. 2006). Even though the extracellular part of the three members of the NGL family have high homology, their cytoplasmic regions share no amino acid sequence apart from the C-terminal PDZ-binding motif, probably implying distinct roles through specific protein-protein interactions. Artificial clustering of NGL-2 and NGL-3 on dendrites induces co-clustering of NMDARs (S. Kim et al. 2006), and *ngl3*$^{-/-}$ mice exhibit moderate reduction on NMDAR-mediated synaptic transmission and LTD (Lee et al. 2019). Interestingly, NGL-3 also induces clustering of AMPARs in vitro (H. Lee et al. 2019), suggesting that interaction of NGL with different presynaptic ligands accompanied with non-homologous C-termini, can recruit distinct postsynaptic partners and thus trigger specific synaptic responses.

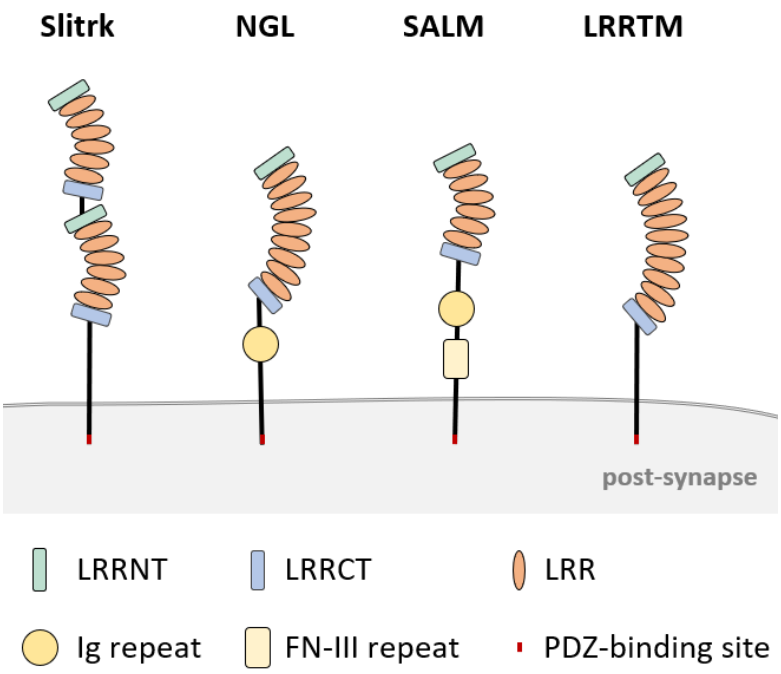


Figure 18. Synaptogenic LRR families. Netrin-G ligands (NGL) contain nine LRRs and an Ig domain, followed by a single transmembrane domain and an intracellular region, ending with a PDZ domain-binding motif. Synaptic adhesion-like molecules (SALMs) consist of a LRR domain, an immunoglobulin-like domain and a fibronectin type III domain, a transmembrane region and an intracellular domain. LRRTMs consist of 10 LRR in the LRR domain and a single transmembrane domain ending with a PDZ-like binding motif, except for the long splice variants of LRRTM3 and LRRTM4. Slit-like and Trk-like (Slitrk) family are single-pass transmembrane proteins localised at the postsynaptic membrane, with a divergent intracellular domain and an extracellular domain consisting of two LRR domains of 6 LRRs each.

The NGL/netrin-G complex has been implicated in lamina-specific segmentation of dendrites, as NGL-1 and NGL-2 are expressed in specific segments on the dendritic surface, but non-overlapping regions of the hippocampus (Nishimura-Akiyoshi et al. 2007) and in controlling synaptic plasticity in distinct excitatory circuits in the hippocampus (Matsukawa et al. 2014). On the other hand, NGL-3/LAR interaction is regulating the formation of excitatory synapses, as NGL-3 and LAR induce pre- and post-synaptic differentiation in contacting axons and dendrites, respectively (Woo, Kwon, and Kim 2009). In terms of pathophysiology, single nucleotide polymorphisms associated with schizophrenia have been detected in netrin-G genes (Aoki-Suzuki et al. 2005), whereas NGL-2 has emerged as a glioma suppressor (M. Wu et al. 2006), suggesting that NGL could have addition roles in glial cells. Even though NGL-1 is implicated in deafness

through defects in its PDZ-biding partner, whirlin (Delprat et al. 2005), the involvement of NGLs in human brain disorders still remains unclear.

2.7.4 Synaptic adhesion-like molecules (SALMs)

SALMs, or also known as Leucine-rich repeat and fibronectin III domain-containing proteins (LRFNs) were originally identified in 2006 independently by three different groups (C.-Y. Wang 2006; J. Ko et al. 2006; Morimura et al. 2006). They are expressed early in the developing brain, but distinctly expressed in brain regions, where they are particularly enriched in the postsynaptic density and are implicated in neurite outgrowth (C.-Y. Wang 2006), synapse formation and maturation (J. Ko et al. 2006). *Salm1*$^{-/-}$ mice show deficits in neural development, synaptic plasticity and autism-like behaviours (Morimura et al. 2017; Lie et al. 2018), whereas *Salm3*$^{-/-}$ mice exhibit reduced excitatory synapse number (Y. Li et al. 2015). Clinical studies have associated point mutations and copy number variation of SALMs with schizophrenic and autistic patients (Xu et al. 2009; de Bruijn et al. 2010; Farwell Hagman et al. 2017), whereas reduced levels of SALM1 have been associated with Alzheimer's disease-related dementia (Bereczki et al. 2018).

The five members of the SALM family (SALM1-5) consist of a LRR domain, an immunoglobulin-like domain and a fibronectin type III domain, a transmembrane region and an intracellular domain (**Figure 18**). Despite having similar domain organisation, SALMs exhibit distinct synaptic functions. Their extracellular regions share more than 60% amino acid sequence identity, contrary to their intracellular regions that barely have any similarities, apart from the PDZ binding motif in SALM1-3, but absent in SALM4-5 (Morimura et al. 2006). Variability in the intracellular regions is also a common feature amongst NGLs and Slitrks, as described above, suggesting that SALMs might have distinct functions mediated through binding to different intracellular partners.

The interactions in the extracellular part of SALMs have been studied in more details. SALM3 and SALM5 are the only members of the family that induce excitatory and inhibitory presynaptic differentiation (Mah et al. 2010) through binding to pre-synaptic LAR-RPTPs (Y. Li et al. 2015; Choi

et al. 2016). In addition, SALM4 and 5 can form *cis* homomeric complexes in rat brain homogenates, but also *trans* homophilic associations in heterologous cells (Seabold et al. 2008). However, the precise endogenous localisation of SALMs at synapses remains unclear. Recent crystallography studies revealed that SALM5 can form a dimer via *cis* interactions of the N-terminal LRR domain, to subsequently bridge two presynaptic PTPδ (one of the LAR-RPTPs) through both its LRR and Ig domain (Z. Lin et al. 2018). A similar mechanism has also been reported for the SALM3/PTPσ complex (Karki et al., 2020). Interestingly, the dimerisation of both SALM3 and SALM5 is critical for inducing pre-synaptic differentiation, suggesting that dimerisation is not only important for trans-synaptic adhesion but also for synaptogenic activity.

2.7.5 Leucine-Rich Repeat TransMembrane (LRRTMs) proteins

LRRTMs were first identified in 2003 while looking for proteins that shared sequence similarity with the Slit family of axon guidance molecules (Laurén et al. 2003). All four members of the family are transmembrane proteins that are exclusively present in vertebrates and predominantly expressed in the brain. The LRRTM messenger RNAs are developmentally regulated in the mouse brain, expressed from embryonic day 13 (E13) until at least postnatal day 60 (P60). In the adult brain, semi quantitative analysis of their expression in various human tissues revealed that LRRTM3 is exclusively present in the brain, at least amongst the tissues that were involved in the study, LRRTM1 and LRRTM4 have a broader distribution (present in around 40 and 70% respectively), whereas LRRRTM2 was present in almost all the tissues tested, suggesting that its role could extend beyond the CNS. In situ hybridisation in the adult mouse brain showed that LRRTM mRNAs are widely expressed yet with specific patterns for each family member (**Figure 19**). All LRRTMs are present in neuronal cells of the hippocampus with LRRTM3 and LRRTM4 being more restricted to the dentate gyrus, whereas LRRTM1 and LRRTM2 are more homogeneously expressed (Laurén et al. 2003; de Wit et al. 2009).

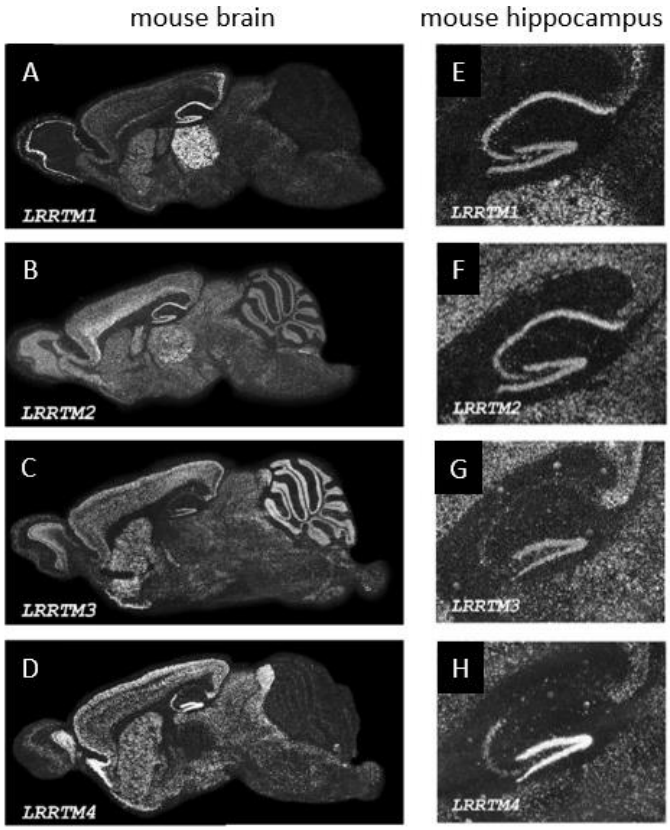


Figure 19. In situ hybridisation of LRRTM family mRNA expression in the adult mouse brain and hippocampus. On the left are shown dark-field emulsion autoradiographs of LRRTM1 (A), LRRTM2 (B), LRRTM3(C) and LRRTM4 (D) in the whole muse brain and on the right in the hippocampus (E-H). Adapted from Lauren et al., 2003.

Despite the plethora of neurexin trans-synaptic ligands, increasing evidence highlight the LRRTM family as a promising candidate in regulating synaptic organisation and function. Given its almost exclusive localisation at excitatory synapses, where it exhibits low surface dynamics and compact nanoscale organisation and its role in synaptic transmission and plasticity, during my PhD I was interested in deciphering the molecular mechanisms that govern LRRTM2 confinement at excitatory synapses and how LRRTM2 contributes to synaptic function by regulating glutamatergic AMPARs. In the next chapter, I will briefly discuss the different isoforms of the LRRTM family to finally focus on the role LRRTM2 in synapse function and organisation.

[Introduction]

3. LRRTMs: a focus on LRRTM2, a critical protein for synapse function

The LRRTM family contains 4 isoforms are located on different chromosomes: human *LRRTM1* and *LRRTM4* are in chromosome 2p12, *LRRTM2* in chromosome 5q31.2 and *LRRTM3* in chromosome 10q21.3. *LRRTM1, 2 and 3* but not *LRRTM4* reside within α-catenin genes (*ctnna)* and their protein-coding region is located in 2 exons, the first one coding for the translation initiation codon and the second one for the rest of the protein, with the exception of LRRTM1 which is encoded by a single exon (Laurén et al. 2003). According to bioinformatics and phylogenetic analysis, it is mostly likely that *lrrtm* genes emerged in early vertebrate evolution before the divergence of jawed vertebrates. They are nested in *ctnna,* presumambly due to retrotransposition and subsequent dual duplication of *lrrtm/ctnna*, resulting in four *lrrtm/ctnna* gene pairs (**Figure 20**). However, it is thought that the *ctnna* gene around *lrrtm4* was subsequently lost (Uvarov, Kajander, and Airaksinen 2014).

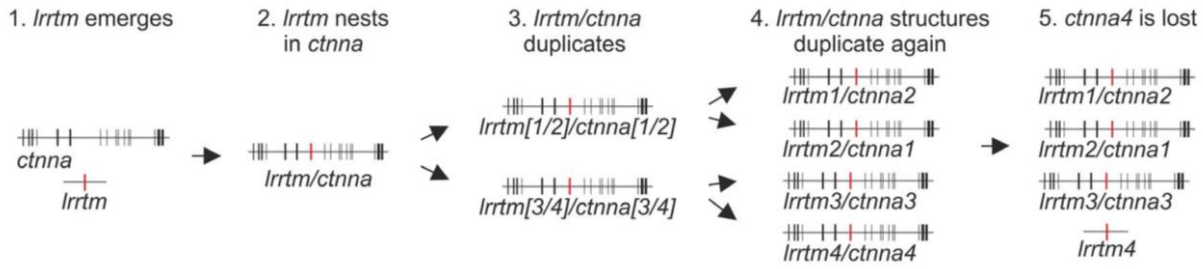


Figure 20. Hypothetical model of the nested lrrtm/ctnna gene structure evolution. 1. Emergence of the first *lrrtm* gene in early jawless vertebrates. 2. Translocation of the *lrrtm* gene into a *ctnna* intron. 3–4. Double duplication of the nested *lrrtm/ctnna* gene structure. 5. Loss of one *ctnna* host resulted in *lrrtm4* that is not nested and three nested *lrrtm/ctnna* genes. Uranov et al., 2014

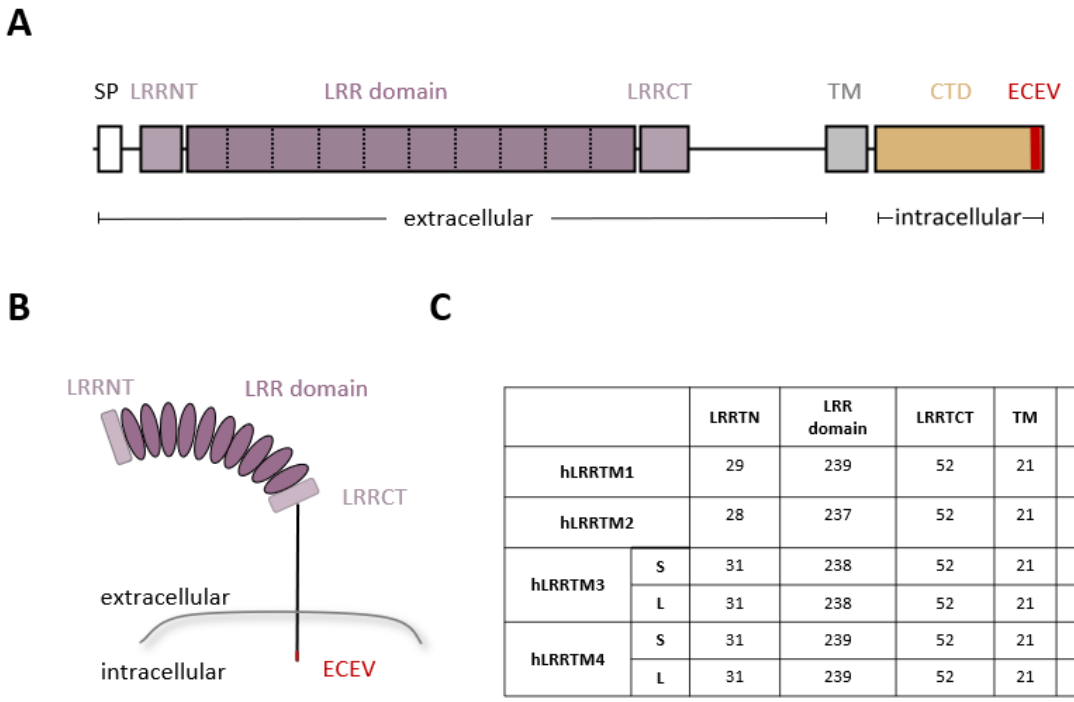
3.1 Structure

LRRTMs share similar domain architectures, with the extracellular domain of LRRTMs consisting of 10 Leucine-Rich Repeats (LRR) flanked by cysteine-rich domains, followed by a single

transmembrane domain and then by a short intracellular tail of around 70 a.a (**Figure 21**) The latter is conserved with 22% of the amino acids being identical and 50% highly similar in all LRRTMs and surprisingly it has no clear homology with any other protein, rendering the intracellular tail of LRRTMs a very unique partner for protein-protein interactions. The last four amino acids of the intracellular tail constitute, **E-C-E-V**, constitute a non-canonical PDZ-like binding motif, as it does not fall in any of the three classes of PDZ motifs (Hung and Sheng 2002). Last but not least, the intracellular tail of LRRTMs also contains conserved tyrosine, serine and threonine residues that could potentially be phosphorylated according to bioinformatics analysis (Laurén et al. 2003), although this hasn't been studied experimentally yet. The cysteine-rich domains are believed to protect the hydrophobic core of the LRRs from the solvent (Gangloff et al. 2013).

3.2 Synaptogenic properties of LRRTMs

All four members of the LRRTM family exert synaptogenic properties as when expressed in non-neuronal cells they induce clustering of presynaptic synapsin in co-cultured neurons (J. Ko, Fuccillo, et al. 2009; Linhoff et al. 2009; de Wit et al. 2009). LRRTM2 is the most potent of the family (Linhoff et al., 2009), a property that depends on its LRR domain (Linhoff et al. 2009; de Wit et al. 2009). In particular, shRNA against neurexin1 (de Wit et al. 2009) or incubation with soluble Ig-Neurexin (J. Ko, Fuccillo, et al. 2009) decreased the synaptogenic activity of LRRTM2 in the co-culture assay (de Wit et al. 2009), suggesting that the binding of LRRTM2 to its presynaptic partner neurexin is indispensable for its synaptogenic activity. Triple KD of neurexins 1, 2 and 3 also abolished the synaptogenic effect of LRRTM2, LRRTM3 and LRRTM4 in HEK cells (Um et al. 2016). Overexpression of LRRTM2 in cultured hippocampal neurons increases excitatory synapse density (VGluT1/PSD-95 puncta/length of dendrite), a capacity that is lost upon deletion of the extracellular domain but is maintained upon deletion of the intracellular domain (de Wit et al. 2009), suggesting that the extracellular part of LRRTM2 is involved in regulating VGluT1/PSD-95 density. Overexpression of LRRTM3 or LRRTM4 also increases excitatory synapse numbers in the DG granule and hippocampal neurons, respectively (Um et al. 2016).



		LRRTN	LRR domain	LRRTCT	TM	CTD	ECEV
hLRRTM1		29	239	52	21	74	4
hLRRTM2		28	237	52	21	73	4
hLRRTM3	S	31	238	52	21	73	4
	L	31	238	52	21	141	-
hLRRTM4	S	31	239	52	21	73	4
	L	31	239	52	21	145	-

Figure 21. Structure of the LRRTM family members. A and **B**. Schematics of human LRRTM1, 2, 3S and 4S proteins. Extracellular part consists of the signal peptide (SP), cysteine rich domain at the N-terminus (LRRNT), cysteine rich domain at the C-terminus (LRRCT), 10 Leucine-Rich-Repeats (LRR domain), a single transmembrane domain (TM), and the C-terminal domain (CTD) ending with the PDZ-like binding motif (ECEV). Dotted lines in the LRR domain separate the individual LRR repeats. **C**. Length of amino acid sequence for the LRRTM proteins. Organisation and length of the different domains is similar amongst members of the family. However, LRRTM3 and LRRTM4 can be alternatively spliced resulting in a small (S) and a long (L) that have a longer CTD that does not contain the PDZ-like binding motif. hLRRTM: human LRRTM.

3.3 LRRTM1

Little is known on the subcellular localisation of LRRTM1, as previous studies have reported that LRRTM1 constructs traffic poorly to the plasma membrane and are largely accumulating in the endoplasmic reticulum (Francks et al. 2007; de Wit et al. 2009; Linhoff et al. 2009). However, the

role of LRRTM1 in behaviour, physiology and disease has been extensively studied using loss-of-function approaches in rodents and genetic studies in humans.

3.3.1 Role in physiology and disease

LRRTM1$^{-/-}$ mice were shown to have defective responses to stressful stimuli and novel objects, spatial memory and social discrimination deficiencies (Takashima et al. 2011) and avoidance of small enclosures (Voikar et al. 2013), although a more recent study did not fully agree with those findings (Voikar et al. 2013). Some of those behaviours could be associated to morphological abnormalities observed in the Lrrtm1 KO mouse brain, even though they are not very robust (Takashima et al. 2011). LRRTM1$^{-/-}$ mice exhibit normal brain morphology, although they have an increase in the size of VGluT1 puncta in some hippocampal sub-regions, without affecting bassoon (Linhoff et al. 2009). Interestingly, those effects were observed in regions where LRRTM2 levels are relatively low. In addition, genetic deletion of LRRTM1 reduced retinal convergence in visual thalamus, the sharing of a single nerve fibre by several rods (Monavarfeshani et al. 2018).

LRRTM1 was the first gene to be associated with human handedness in as sample of reading-disabled siblings, as identified by a genetic association mapping and gene-functional analysis (Francks et al. 2007). In fact, LRRTM1 expression is down-regulated maternally (imprinted gene). Imprinted genes can be epigenetically regulated in order to be differentially active depending on the parent-of-origin. The initial evidence that the LRRTM family is associated with neurological disorders came as early as 2007, when a paternal effect of the LRRTM1 locus in familial schizophrenia was found, with the same associated haplotype as implicated in handedness. The 2p12-11 region, where LRRTM1 resides, was previously suggested to be linked with hand skill (Francks 2003) and with schizophrenia (Lewis et al. 2003).Association between LRRTM1 and schizophrenia was also supported by other studies carried out on larger independent sample of schizophrenic patients (Ludwig et al. 2009) and healthy individuals (Leach et al. 2014).

3.4 LRRTM3

LRRTM3, like all LRRTMs, is localised at excitatory synapses, where it interacts with the excitatory scaffold molecule PSD-95 (Laurén et al. 2003; Linhoff et al. 2009) and pre-synaptic neurexins (1-3, α or β) (Roppongi et al. 2020). LRRTM3 has also been identified as a binding partner of the glypican GPC4 by mass spectrometry (de Wit et al. 2013), although the functional outcome of this interaction has not been further studied. However, alternative splicing of LRRTM3 mRNA produces longer protein variants, LRRTM3L, which does not have a PDZ-like binding motif at the very end of the C-terminal, thus no longer interacts with PSD-95 as shown by co-IP and co-clustering assays (Um et al. 2016).

3.4.1 Role in physiology and disease

On the functional level, loss-of-function studies have unravelled the roles of LRRTM3 in synaptic transmission. Knock-down of LRRTM3 reduces excitatory but not inhibitory synapse density in cultured DG granule neurons. Even though LRRTM3 KO mice display normal gross morphology, they exhibit altered excitatory synapse density, excitatory synaptic transmission and excitability that are specific to the DG region of the hippocampus (Um et al. 2016). LRRTM3 KO does not alter the expression levels of either GluA1 or GluA2 in adult mice; however, it prevents the increase in surface GluA1 receptors density upon cLTP in DG granule cultured neurons, an effect that can be rescued with the short but not the long splice variant of LRRTM3 (Um et al. 2016), suggesting that interactions with PSD-95 or other MAGUK proteins are involved in LRRTM3-dependent maintenance of AMPARs on the surface. LRRTM3 has been proposed as a susceptibility factor for late-onset Alzheimer's disease (Majercak et al. 2006), as high-throughput siRNA screening of 15.200 genes revealed LRRTM3 as a neuronal gene that promotes the processing of amyloid precursor protein (APP). Interestingly, LRRTM3 gene is also located in a region of chromosome 10 that has been linked to Alzheimer's disease (Liang et al. 2007) and polymorphisms in LRRTM3 were linked to autism-spectrum disorder (Sousa et al. 2010).

3.5 LRRTM4

LRRTM4 has unique features that are not common to the other members of the LRRTM family, including the fact that it is the only one of the family whose gene is not located within an α-catenin gene (Laurén et al. 2003; Uvarov, Kajander, and Airaksinen 2014). LRRTM4 is localised at excitatory synapses, where it interacts with the excitatory scaffold molecule PSD-95 (Laurén et al. 2003; Linhoff et al. 2009) and pre-synaptic neurexin and glypicans (discussed in 3.5.1). Alternative splicing of LRRTM4 mRNA produces a longer protein variant, LRRTM4L, which no longer interacts with PSD-95 as shown by co-IP and co-clustering assays as it does not possess a PDZ-like binding motif (Um et al. 2016).

3.5.1 Presynaptic binding partners

Using proteomics screens, two groups discovered in parallel heparan sulphate proteoglycans (HSPGs) as pre-synaptic partners of LRRTM4 (de Wit et al. 2013; Siddiqui et al. 2013). However, there is still discrepancy as to whether LRRTM4 can bind neurexin. Even though LRRTM4 has been shown to bind neurexin (de Wit et al. 2013), it preferably binds presynaptic glypicans (GPCs), through which it induces pre-synaptic differentiation at excitatory but not inhibitory synapses (de Wit et al. 2013; Siddiqui et al. 2013). Interestingly, glypican-4, interacts in *cis* with PTPσ, a member of the LAR-RPTPs, in a heparan sulphate (HS)-dependent manner in order to form a trans-synaptic complex with LRRTM4 in the rat brain. This interaction is important for the synaptogenic effect of LRRTM4 in the co-culture assay (J. S. Ko et al. 2015), making PTPσ a pre-synaptic receptor for the glypican-4/LRRTM4 complex. PTPσ also interacts with neurexin1β in an HS-dependent manner in order to recruit post-synaptic LRRTM4. The PTPσ/glypican-4 – LRRTM4 complex exists independently of the PTPσ/neurexin – LRRTM4 (Roppongi et al. 2020), suggesting that LRRTM4 actively engages in distinct trans-synaptic complexes. Glypicans and other HSPGs have long been implicated in various aspects of neuronal development, yet their role in the function of mature synapses has been mostly studied in non-mammalian models. LRRTM4 is one of the first synaptic adhesion molecules in mammalian synapses to interact with glypicans.

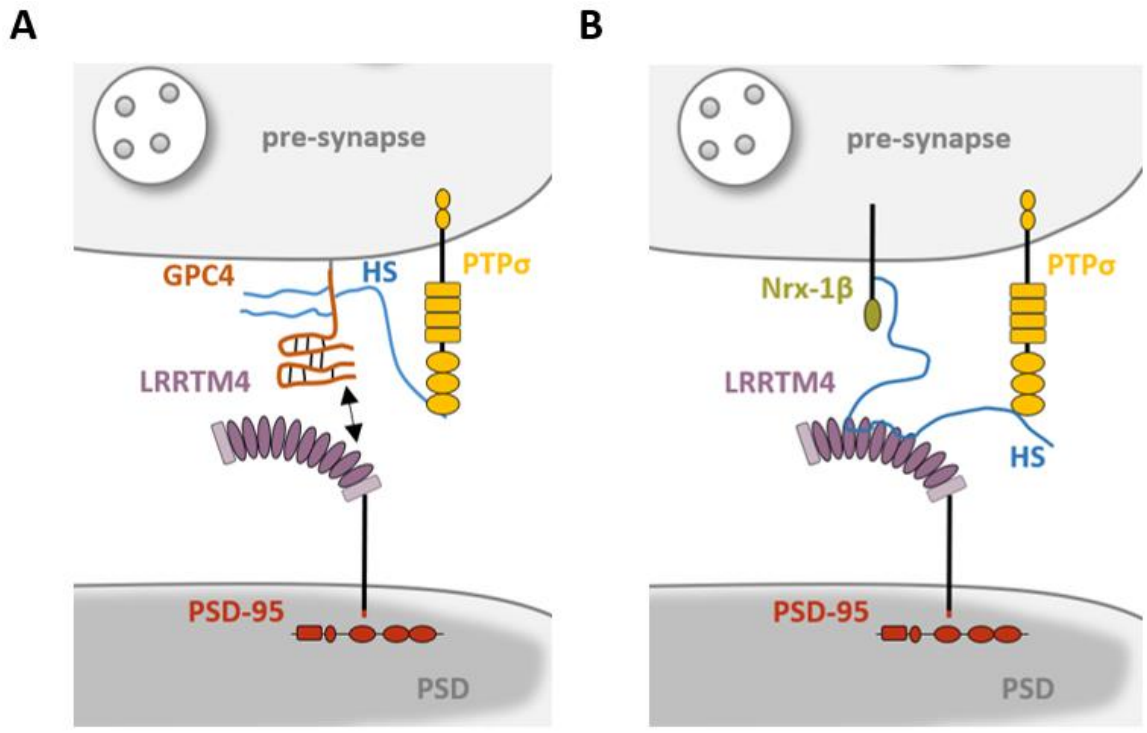


Figure 22. Presynaptic binding partners of LRRTM4. A. Glypican-4 (GPC4) interacts in cis with PTPσ a member of the LAR-RPTPs, in a heparan sulphate (HS)-dependent manner in order to form a trans-synaptic complex with postsynaptic LRRTM4. **B.** PTPσ interacts with neurexin1β (Nrx-1 β) in an HS-dependent manner in order to recruit post-synaptic LRRTM4.

3.5.2 Role in physiology and disease

Overexpression of LRRTM4 in hippocampal cultured neurons increases excitatory synapse density, whereas shRNA-mediated knock-down of LRRTM4 decreases excitatory synapse density and impairs synaptic transmission (de Wit et al., 2013). In vivo knock-down of LRRTM4 in cortical layer 2/3 during embryonic development, impeded excitatory synaptic transmission (de Wit et al. 2013) and LRRTM$^{-/-}$ mice exhibit alterations in synaptic composition and transmission that are specific to the DG (Siddiqui et al. 2013). In addition, LRRTM4 was shown to co-immunoprecipitate with endogenous GluA1 receptors in synaptosomal brain homogenates, although GluA1 and GluA2 protein levels were unaltered in DG synaptosomes from LRRTM4$^{-/-}$ mice compared to their

WT littermates (Siddiqui et al. 2013). In addition, multiepitope affinity purifications (Bildl et al. 2012) and mass spectrometry revealed LRRTM4 as a native component of the GluA2 subunit of AMPARs in the adult rat brain (Schwenk et al. 2012).

Most of the studies on synaptic adhesion molecules and LRRTMs have been focused on the hippocampus as it is a well-established model and can also undergo plasticity regulations. Surprisingly, LRRTM4 was recently found to be localised at GABAergic synapses at rod bipolar cells of the retina, where it regulates GABAergic transmission (Sinha et al. 2020) and interacts with pikachurin (Agosto and Wensel 2021), a HSPG expressed by photoreceptors and secreted into the synaptic cleft (Sato et al. 2008). LRRTM4-C538Y gene mutation has been associated with hereditary macular degeneration in the retina, a rare dystrophy with central visual loss and atrophy of the macula and surrounding retina (Kawamura et al. 2018).

3.6 LRRTM2

LRRTM2 is the most synaptogenic isoform of the LRRTM family and its role in synaptic transmission and plasticity has been revealed by several studies since its discovery. However, little is known about the mechanisms that regulate LRRTM2 itself. In the upcoming pages I will focus on the known literature on LRRTM2, its binding partners, role in synaptic function and interplay with AMPARs at excitatory synapses.

3.6.1 Binding partners

Despite the discovery of LRRTMs as synaptic organisers more than 10 years ago (Linhoff et al. 2009; J. Ko, Fuccillo, et al. 2009), there are only few known binding partners, presumably due to lack of efficient antibodies against LRRTM2. Pre-synaptic neurexins, post-synaptic PSD-95, AMPARs and HSPGs constitute the known interactors for the LRRTM family.

3.6.1.1 LRRTM2 binds to PSD-95

The only intracellular binding partner of LRRTM2 to date is PSD-95. LRRTM2 not only colocalises with PSD-95 at excitatory synapses (de Wit et al. 2009), but recombinant PSD-95 can also co-immunoprecipitate with recombinant LRRTM2 in heterologous cells, an interaction that depends on the non-canonical PDZ-like binding motif on the C-terminal of LRRTM2 (E-C-E-V) (Linhoff et al. 2009; de Wit et al. 2009). Recombinant LRRTM2 colocalises with endogenous PSD-95 but not gephyrin in cultured hippocampal neurons (Linhoff et al. 2009; de Wit et al. 2009).

3.6.1.2 LRRTM2 binds to neurexin

In 2009 two papers revealed at the same time the family of pre-synaptic molecules neurexin as binding partners of LRRTMs (de Wit et al. 2009; J. Ko, Fuccillo, et al. 2009). Using mass spectrometry on purified synaptosomal membrane proteins from P18 rat brains, de Wit and colleagues found LRRTMs as potential ligands of neurexin. To further identify specific neurexin family proteins, the authors assessed the ability of LRRTM-ecto-Fc to bind to different neurexin isoforms expressed on the surface of HEK cells and highlighted Neurexin 1β as the strongest binding partner. However, after more thorough analysis it seemed that the binding affinity of LRRTM2 to Neurexin 1α was stronger compared to Neurexin 1β. Finally, affinity chromatography with LRRTM2-ecto-Fc immobilised on protein A beads showed a direct interaction between LRRTM2 and Neurexin 1α and 1β. Using similar techniques, Ko and colleagues also identified LRRTM2 as Neurexin 1α and 1β ligands and further showed that their interaction is calcium-dependent. In addition, they showed that LRRTM2 can bind to Neurexins (1-3), either α or β, as long as they lack the insert at splice-site 4 (SS4-), results that were later confirmed by other groups (Siddiqui et al. 2010). In a recent crystallography study, Surface Plasma Resonance (SPR) analysis showed that the 30aa insert at SS4 of neurexin 1β decreases its binding affinity to LRRTM2 by >20-fold (A. Yamagata et al. 2018). The critical position of SS4 (Ala204 and Gly205 in human Nrxn1β) next to LRRTM2-binding region of Neurexin 1β and its secondary structure in mammalian cells, could explain why the presence of the insert at SS4 decreases the binding

affinity to LRRTM2. Other members of the LRRTM family were also pulled-down together with IgNrx1β^{SS4-} in HEK cells, suggesting that they also serve as neurexin ligands (J. Ko, Fuccillo, et al. 2009). A very interesting finding of this study was that recombinant Ig-LRRTM2 competes with FLAG-tagged Neuroligin 1, another partner of Neurexin, for its binding to HA-tagged Neurexin 1β, as shown by co-immunoprecipitation, suggesting that those two adhesion molecules cannot bind neurexin simultaneously (J. Ko, Fuccillo, et al. 2009). Similar results were also reported by others as pre-incubation with LRRTM2 prevented neuroligin expressed in cos cells from binding to neurexin 1β^{SS4-} and vice versa (Siddiqui et al. 2010).We could speculate that this could be a fine-tuning mechanism to promote specific interactions between the pre- and the post-synapse that could thus regulate synapse connectivity or maintenance.

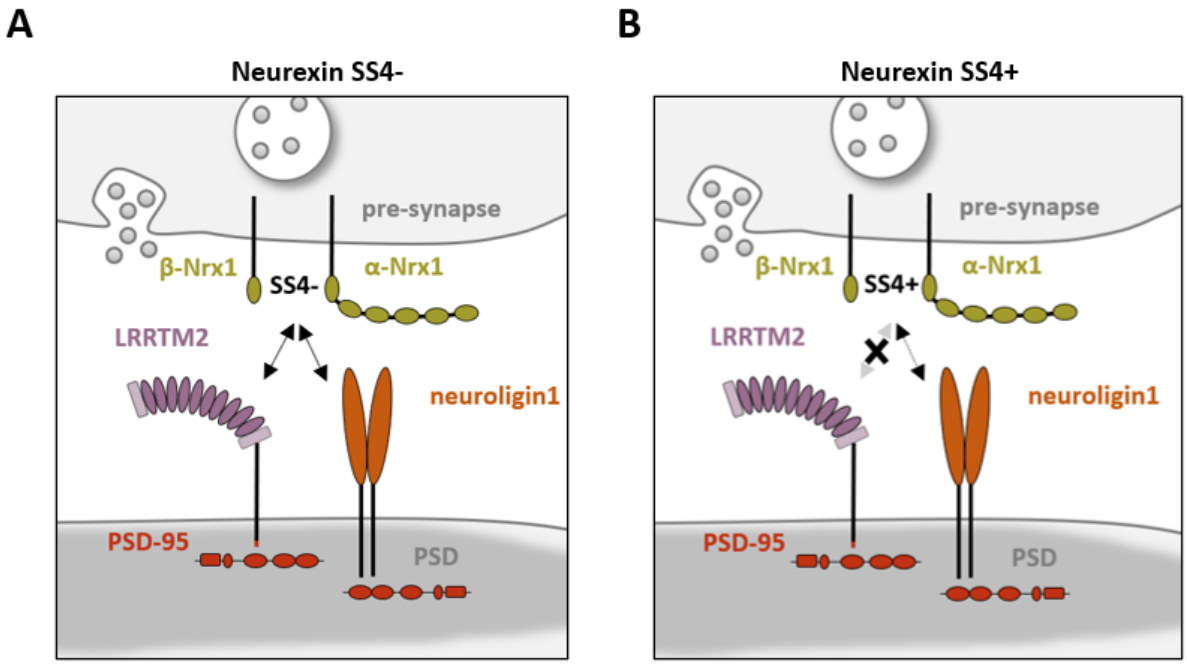


Figure 23. Binding partners of LRRTM2. A. In the absence of the 30 a.a insert at the splice site 4 of α-neurexin1 or β-neurexin1 (SS4-), LRRTM2 competes with neuroligin1 for the binding to neurexin. B. When the insert at the SS4 is present (SS4+) LRRTM2 can no longer bind neurexin, whereas neuroligin1 can. In both cases, LRRTM2 binds to PSD-95 through its ECEV motif at the very end of the intracellular tail.

3.6.1.2.1 Binding site of neurexin on LRRTM2

The first evidence for the binding site of neurexin on LRRTM2 came from the group of A.M Craig. After testing several mutations in the extracellular part of recombinant LRRTM2, they identified the D260A/T262A in the concave side of the 9th LRR repeat as a mutation that abolishes the binding of LRRTM2 to Neurexin-1β(SS4-)-Fc in non-neuronal cells (Siddiqui et al. 2010). Some years later, Paatero and colleagues engineered for the first time the crystal structure of mouse LRRTM2 extracellular domain and proposed that the interaction between LRRTM2 and Neurexin 1β takes place in the concave surface formed by LRR1-LRR5 and partially the N-terminal cap of LRRTM2 (Paatero et al. 2016). Even though this study provided useful insights into the structure of LRRTM2, 33% of the residues were mutated to ensure the thermostability of the protein, which resulted in a 40-fold decrease in the binding affinity of the protein to Neurexin 1β. This was surprising as the authors tried to keep the highly-conserved core of the LRR repeats intact and only carried out consensus mutations in the convex part of the LRR domain, which mostly contains variable amino acids. However, a subsequent study reported a new crystal structure of the Nrx1β-LRRTM2 complex and localised the interaction site in the C-terminal cap of the LRR domain (Yamagata et al. 2018). The residue responsible for binding neurexin was shown to be E348 which interacts with a calcium ion coordinated by the neurexin side chains and this interaction is mediated by a water molecule. Mutating this residue (E348Q) abolished the binding to Nrx1β in vitro and disrupted the synaptogenic effect of LRRTM2. The differences between those two studies could be attributed to distinct experimental design and mutations introduced in the first study to ensure the thermostability of the protein, among other factors.

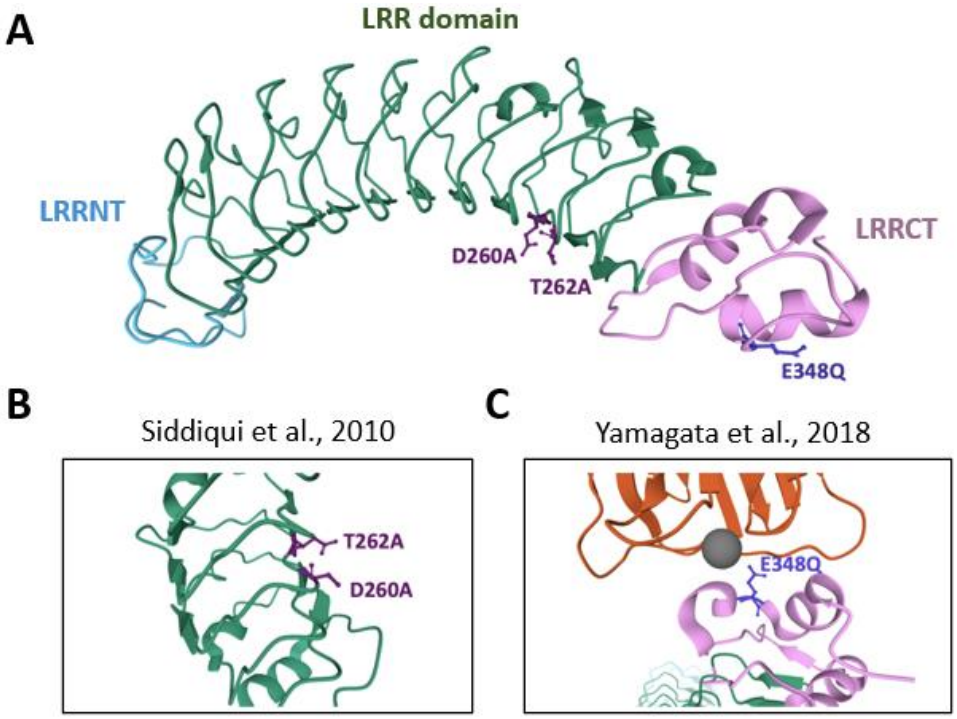


Figure 24. Crystal structure of LRRTM2 extracellular domain. A. LRRTM2 extracellular domain depicting the LRRNT (blue), the LRR domain consisting of 10 LRR repeats (green) and LRRCT (pink) domains. Previously reported binding sites to neurexins are shown as sticks: D260A/T262A (purple, from Siddiqui et al., 2010) and E348 (dark blue, from Yamagata et al. 2018). B. Close-up view of the Nrx1b-binding residues D260A/T262A on the concave side. C. Close-up view of Nrxn1β (orange)–LRRTM2 interaction. Adapted from PDB, structures 5Z8X and 5Z8Y.

3.6.1.2.2 Heparan sulphate modification of neurexin

A recent study revealed that LRRTM2 does not only bind to the LNS domain of neurexin, but also interacts with the heparan sulphate modification of neurexin and proposed that this interaction is mediated through specific residues in its LRR5-LRR7 domain. Even though 5-point mutations in those domains did not disrupt the binding of LRRTM2 to presynaptic neurexin, they dramatically decreased neurexin and synapsin clustering in the contacting axons in the co-culture assay. In addition, treatment with heparanase or the presence of neurexin with a point mutation (Ser316) abolishing HS modification, also abrogated the synaptogenic effect of LRRTM2 (P. Zhang et al. 2018), while the latter also reduced the binding affinity to LRRTM2. In addition, deletion of the LNS domain of neurexin, where LRRTM2 directly binds, or deletion of the highly glycosylated (CH)

domain on neurexin both impaired the synaptogenic activity of LRRTM2 (Roppongi et al. 2020), suggesting that both protein-protein and glycan-protein interactions between LRRTM2 and neurexin are required for formation of this synaptic complex.

The physiological role of LRRTM2 binding to presynaptic neurexin has been extensively studied by different groups, and it is described in detail in 3.2 and 3.6.3.4 of this introduction.

3.6.1.3 LRRTM2 binds to AMPARs?

LRRTM2 has been shown to interact with the GluA1 and GluA2 subunits of AMPARs through its extracellular domain, as recombinant GluA1 or GluA2 co-imminoprecipitated with recombinant LRRTM2 in HEK cells (de Wit et al. 2009). This interaction was independent of the intracellular domain of LRRTM2, as LRRTM2 lacking this domain could still co-imminoprecipitate the receptors. However, evidence is lacking for this interaction in neuronal cells, despite the importance of LRRTM2 in synaptic transmission (see 3.6.3.3), LTP (see 3.6.3.4) and in positioning AMPARs at the synapse (see 3.6.5). This point is further discussed in the discussion part 3.2.1 of the discussion.

3.6.2 Subcellular distribution

A very characteristic and striking feature of LRRTMs, and especially LRRTM2 which has been mostly studied, is its almost exclusive synaptic localisation and its absence from the dendritic shaft. Unlike other families of synaptic adhesion molecules where some members can be found at excitatory and some at inhibitory synapses, LRRTMs are specific for excitatory synapses, where they are colocalised with PSD-95. In the hippocampus of mouse brain fixed slices, LRRTM2 is

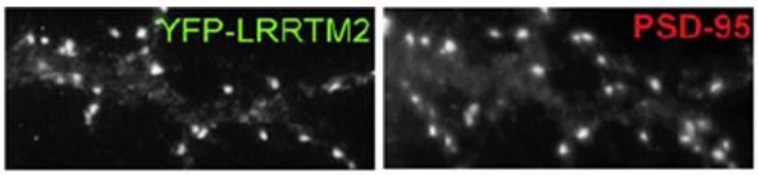


Figure 25. LRRTM2 is exclusively localised at excitatory synapses. Hippocampal neurons expressing YFP-LRRTM2 and immunostained for endogenous PSD-95, showing extremely high colocalization of YFP-LRRTM2 puncta with puncta of PSD-95. Adapted from Linhoff et al., 2009.

mostly detected in CA3 mossy fibers synapses (Linhoff et al. 2009), where it colocalises with the pre-synaptic excitatory marker VGluT1.

One could imagine that a synaptic adhesion molecule that such a unique localisation would play a central role in synapse organisation, thus deciphering the molecular mechanisms underlying LRRTMs synaptic targeting and distribution could contribute to a better understanding of synapse assembly and function.

3.6.2.1 Role of the C-terminal

Early in the discovery of LRRTMs, it had been observed that deletion of the intracellular domain of LRRTM2 disrupted the clustered organisation of the protein in hippocampal cultured neurons (Linhoff et al. 2009). However, it was not until 2015 that Minatohara and colleagues conducted the first detailed study highlighting the importance of the intracellular domain of the protein for governing LRRTMs synaptic localisation and clustering. First of all, deletion of the C-terminal tail of LRRTM1-4 (~55a.a ~75a.a) diminished the synaptic clustering of all isoforms by more than 3-fold in cultured hippocampal neurons (Minatohara et al. 2015) and increased the overall expression of LRRTM1 and LRRTM2, but not LRRTM3 and LRRTM4, as shown by immunoblot of cultured neuron lysates. Interestingly, using a biotinylation assay they showed that this increase was specific to the surface pool of those isoforms, suggesting that their C-terminal domains regulate membrane trafficking. Then, the authors focused on LRRTM2 in order to identify the region responsible for its membrane targeting and for this purpose conducted serial deletions in the C-terminal domain of LRRTM2 that were then expressed in HEK cells. They found that deletion of the last four amino acids (E-C-E-V: Δ4), which correspond to the PDZ-like binding motif, did not alter the overall of surface expression of LRRTM2 in HEK cells or in neurons, whereas deletion of 6 residues from Y500 to C505 yielded the maximum increase in protein expression compared to control. Finally, to associate this increase to specific residues within this region they did a series of point mutations in conserved amino acids between LRRTM1 and LRRTM2 and identified Y500 and C503 as critical residues for the expression of LRRTM2. Double mutations to alanine (Y500A/C503A: YACA mutant from now on) displayed significantly increased overall and surface expression not only in HEK cells, but also in neuronal lysates, revealing YxxC

sequence at the -13 to -16 amino acids from the C-terminal region as a novel motif governing LRRTM2 level of expression in heterologous and neuronal cells. It should be noted that single substitutions of Y500 or C503 to alanine also increased the expression of LRRTM2 in HEK cells, but to a lesser extent than the double mutation. To assess whether the increased surface expression of the YACA mutant was due to defective internalisation, the authors measured the internalised protein in HEK cells by using sulfo-NHS-SS-biotin and found that the rate of internalisation between the WT and the YACA was similar, suggesting that accumulation of LRRTM2-YACA at the surface is not due to impaired internalisation. Interestingly, LRRTM2 lacking its intracellular domain showed an increase only in the population passed through the medial Golgi, suggesting that the intracellular trafficking of this mutant could be altered. It was not investigated whether the exocytosis of LRRTM2 is altered due to YACA mutations.

When recombinant LRRTM2 proteins carrying the intracellular mutations validated by immunoblot were introduced in neurons, they all elicited a decrease in the synaptic clustering of the protein, although Δ16, Δ22 and YACA had the maximum decrease of around 50%. So, the YxxC sequence is not only important for targeting LRRTM2 to the surface, but also for its synaptic localisation and clustering. It is noteworthy that the Δ4 mutant, lacking the PDZ-like binding domain, was less clustered compared to the WT. This result is not in accordance with one of the first studies on LRRTM2 that showed that the same deletion does not impair the clustering of the protein, although only depicted with representative images (Linhoff et al. 2009).

3.6.3 Role of LRRTM2 in synapse function

3.6.3.1 Synapse formation

The physiological role of LRRTM2 has been studied mostly by loss-of-function studies that have revealed subtle anatomical and robust functional and behavioural deficits. Knock-down of LRRTM2 using sh-RNAs reduces the excitatory synapse density in cultured neurons, without affecting the inhibitory synapse density (de Wit et al. 2009), an effect that can be rescued by re-expressing LRRTM2. However, in another study a different sh-RNA against LRRTM2, no effect on VGluT1 density was observed, thus the authors claimed that the decrease in excitatory synapse

density observed earlier was due to off-target effects (J. Ko et al. 2011). Nonetheless, we should mention that both the culture type (hippocampal vs cortical) and the measurements (VGluT1/PSD-95 vs VGluT1 density) in the two studies were different. Surprisingly, Ko and colleagues could only report synapse loss under the condition that LRRTM1, LRRTM2 and neuroligin 3 were knocked-out in neuroligin 1 KO hippocampal neurons. The synapse loss could be rescued upon over-expression of either Neuroligin1, LRRTM2 or just the ectodomains of those proteins (J. Ko et al. 2011), suggesting that LRRTMs and Neuroligins redundantly maintain excitatory synapse density.

3.6.3.2 Pathophysiology

Genome-wide analysis of de novo copy-number variants (CNVs), which are associated with increased risk for neuropsychiatric diseases (Levy et al. 2011), highlighted LRRTM2 as one of the genes that have de novo CNVs in bipolar patients (Malhotra et al. 2011). In addition, microdeletions of 5q31.1q31.2, the locus of *LRRTM2*, have been reported in patients with intellectual disability and/or developmental delay of varying degrees (see Kleffmann et al. 2012 for details). Despite the involvement of LRRTMs in neurological diseases, the underlying mechanisms are still poorly understood. Insights into the localisation and regulation of LRRTMs could contribute to our poor understanding regarding the pathophysiological role of those proteins in the human brain.

3.6.3.3 Synaptic transmission

The role of LRRTMs has been mainly focused on two isoforms of the family: LRRTM1 and LRRTM2. Even though there is a relation between the LRRTM family and excitatory synapse transmission, the exact contribution of each isoform is still under question. Most of the studies that investigated the functional outcome of LRRTMs manipulation have used either shRNA against LRRTM2 or double knock-out of both LRRTM1 and LRRTM2, the main isoforms of the CA1 region of the hippocampus (Laurén et al. 2003) as an experimental model.

Knock-down of LRRTM2 using sh-RNA decreases the amplitude of both AMPA- and NMDA-mediated EPSCs around 50% in acute hippocampal slices, causing a slight reduction in the

AMPA/NMDA ratio, without affecting the paired-pulse ratio (de Wit et al. 2009). In addition, LRRTM2 over-expression rescues the diminution of AMPA- and NMDA-mediated EPSCs in TKD (LRRTM1, LRRTM2, Neuroligin3)/Neuroligin 1 KO cultured neurons (Ko et al., 2011). The in-vivo continuation of Ko et al 2011 was published the same year by the groups of Sudhof and Malenka. In this study the authors down-regulated in different combinations the expression of LRRTM1, LRRTM2 and neuroligin 3 by injecting shRNAs against LRRTM1 and LRRTM2 (DKD) and neuroligin 1 (TKD) in the hippocampus of WT or neuroligin1 KO mice. TKD before synaptogenesis decreased AMPAR-mediated amplitude, but also that of NMDA-mediated. Unlike their in vitro results DKD at P0 on a WT background was sufficient to impair AMPAR-mediated transmission, an effect that could be rescued by expression of LRRTM2 or its extracellular domain attached to a GPI anchor (Soler-Llavina et al. 2011). Surprisingly, over-expression in this context of LRRTM2 did not affect synaptic transmission. DKD after synaptogenesis (P21) had no effect synaptic transmission, yet in a more recent study cKO of LRRRTM1 and LRRTM2 impaired LTP by decreasing the amplitude of evoked of miniature AMPAR-mediated EPSCs (Bhouri et al. 2018) without affecting that of NMDARs at mature synapses on CA1 pyramidal neurons. These discrepancies could be attributed to imperfect knock-down of LRRTM1 and LRRTM2 in the first study resulting in remaining endogenous LRRTMs that could still contribute to synaptic transmission or to a developmental effect of LRRTM DKD.

Finally, acute cleavage of the extracellular domain of LRRTM2 upon thrombin application in rat hippocampal neurons, decreased the amplitude of the evoked but not spontaneous EPSCs, suggesting that even a few minutes without the extracellular domain of LRRTM2 can be proven detrimental for synaptic transmission (Ramsey et al. 2021).

3.6.3.4 Synaptic plasticity: Long Term Potentiation

The groups of Malenka and Südhof, having already demonstrated the role of LRRTMs in efficient AMPAR-mediated synaptic transmission, carried out a thorough study regarding the involvement of LRRTM1 and LRRTM2 in synaptic plasticity *in vivo* and specifically in LTP. Their study combined cellular imaging together with slice electrophysiology and demonstrated that LRRTMs are

essential for maintaining LTP in acute hippocampal slices (**Figure 26**) (Soler-Llavina et al. 2013). They first showed that DKD of both LRRTM1 and LRRTM2 using a lentivirus expressing shRNA impairs LTP in CA1 pyramidal neurons, whereas it does not affect LTD. A closer look to their data reveals that DKD neurons are able to induce LTP at the beginning, only to start losing this capacity minutes later, resulting in impaired LTP, suggesting that LRRTMs are important for maintaining LTP. Afterwards, they showed that expression of recombinant LRRTM2 or its extracellular domain GPI-anchored could compensate the decrease in EPSCs currents caused by the DKD, suggesting that this domain is sufficient for its function in LTP. Interestingly, DKD neurons expressing a recombinant form of LRRTM2 where two mutations had been introduced to the LRR5 (D260A, T262A, see chapter 3.1 of the Discussion) showed reduced LTP compared to the control, to levels that resembled those of the DKD. The authors attributed this effect to the fact that this mutant was unable to bind to the presynaptic partner of LRRTM2, neurexin, as at the time of this publication this mutant had been reported to abolish binding of LRRTM2 to neurexin 1β (Siddiqui et al. 2010).

Bhouri and colleagues used the first double conditional knock-out mouse model of both LRRTM1 and LRRTM2 in order to investigate the role of LRRTMs in synaptic plasticity using Cre-recombinase viruses. Genetic deletion of LRRTM1 and 2 in vivo in CA1 hippocampal neurons impaired LTP, in line with previous studies, a deficit that could be rescued by solely the expression

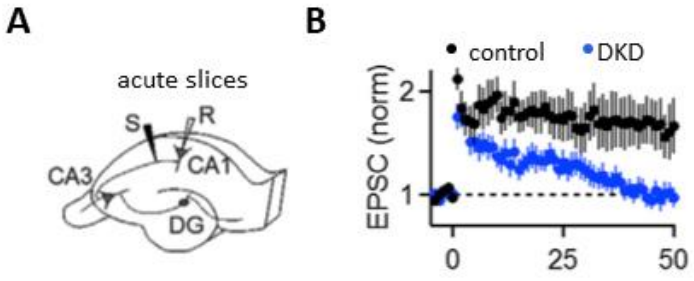


Figure 26. Effect of LRRTM1 and LRRTM2 double knock-down (DKD) on LTP. A. Schematics of experimental approach for LTP experiments in young mice. **B.** LTP magnitude for DKD cells and controls showing that in the absence of LRRTM1 and LRRTM2 LTP can be induced but not maintained. Adapted from Soler-Llavina et al., 2013.

of recombinant LRRTM2, suggesting that LRRTM2 is sufficient to restore LTP under these conditions. Apart from the well-established high-frequency stimulation protocol to trigger LTP, the authors also repetitively activated L-type Ca^{2+} channels via voltage pulses during NMDAR blockade and without presynaptic stimulation, a form of LTP that was also attenuated in the absence of LRRTM1 and LRRTM2. Expression of GPI-anchored LRRTM2 or recombinant LRRTM2 with its PDZ-like domain replaced by that of the human beta-2 adrenergic receptor also rescued LTP, meaning that the PDZ-like domain of LRRTM2 does not regulate LTP. On the contrary, introduction of two-point mutations in the extracellular domain of LRRTM2 (D260A and T262A), a mutation that was believed to abolish the binding to neurexin (Siddiqui et al. 2010), failed to rescue LTP, an effect similar to that reported by shRNA-mediated LRRTM DKD (Soler-Llavina et al. 2013). However, the new crystal structure of the LRRTM/Neurexin interaction involving the E348 residue of LRRTM2 in the C-terminal cap of the extracellular domain, and not the D260/D262 residue (see chapter 3.1 of the Discussion), questions the dependency to neurexin binding in these experiments.

3.6.4 LRRTM2 dynamics and nano-scale organisation

The development of super-resolution microscopy and novel imaging probes has enabled researchers to study synaptic molecules at the nano-scale with a resolution that goes beyond the diffraction limit of 250nm, as explained before (see chapter 1.4.5). Using dSTORM and monomeric-streptavidin, our group showed for the first time in 2016 that recombinant LRRTM2 is not only highly enriched at excitatory synapses in hippocampal rat neurons, but even within individual synapses LRRTM2 is organised in compact nano-clusters of around 107 nm (Chamma et al. 2016). Another remarkable feature of the protein that was unveiled in the same study was that LRRTM2 exhibits an astonishingly low lateral mobility on the membrane surface, as measured by uPAINT, compared to its competitor for neurexin binding, neuroligin1, with almost 80% of LRRTM2 trajectories localised at the synapse (**Figure 27**). At the population level, FRAP experiments revealed a very slow recovery of LRRTM2 compared to neuroligin1, in accordance with the single-molecule tracking experiments, reinforcing the idea that LRRTM2 and neuroligin1

exert different properties and presumably functions at the synapse besides sharing the same pre- and post- synaptic partners.

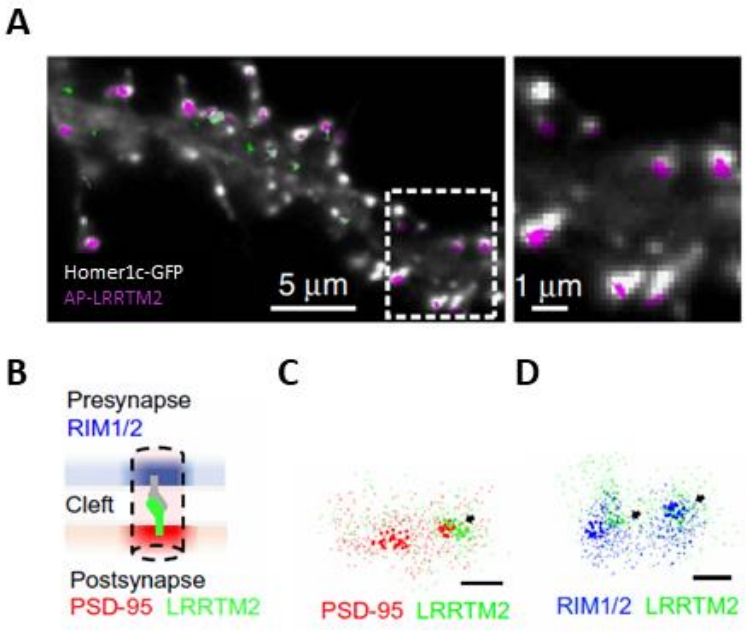


Figure 27. LRRTM2 dynamics and nanoscale organisation. A. Trajectories of surface LRRTM2 (magenta) in rat hippocampal neurons obtained with uPAINT and overlaid with Homec1c-GFP as an excitatory synaptic marker. Note the confinement of LRRTM2 at synapses and the absence of diffusion on dendritic shaft. Adapted from Chamma et al., 2016. B. Schematic showing the trans-synaptic nanoscale organisation of LRRTM2 relative to presynaptic RIM and postsynaptic PSD-95. C. En face view of the localised positions of PSD-95 (red) and LRRTM2 (green) after 3D STORM reconstruction. D. En face view of the localised positions of RIM1/2 (blue) and LRRTM2 (green) after 3D STORM reconstruction. Detected nanoclusters indicated in bold Scale bar: 100 nm. Adapted from Ramsey et al., 2021.

A potential role of LRRTM2 that has long been implied is that of a nano-column organiser (A.-H. Tang et al. 2016). LRRTM2 role in synaptic transmission, its localisation at excitatory synapses, its low surface dynamics, its organisation in compact nano-clusters and its interaction with AMPARs in heterologous cells, render LRRTM2 an ideal candidate to govern the alignment of the pre- and post-synaptic components, thus facilitating efficient synaptic transmission. To assess the involvement of LRRTM2 in trans-synaptic alignment, Ramsey and colleagues profited from the advances in super-resolution microscopy and found that LRRTM2 is enriched within the trans-synaptic nanocolumn, as its nanoclusters are enriched within PSD-95 nanodomains and are

aligned with those of RIM1/2 across the synaptic cleft (**Figure 27**) (Ramsey et al. 2021). Cross-correlation between LRRTM2 and RIM1/2 or PDF-95 density distribution also demonstrated high similarities. However, it is still unknown whether the absence of LRRTM2 could impair the alignment of the pre- with the post-synapse.

3.6.5 Interplay between LRRTM and AMPARs

A number of studies described in the previous chapters have highlighted the importance of LRRTM2 in maintaining LTP in vivo. LTP constitutes one of the most thoroughly explored model of synaptic plasticity in the last decades and AMPARs are indispensable for this process. However, increasing evidence support the regulation of AMPARs by LRRTM2 in basal transmission. Indeed, KD of LRRTM2 in cultured hippocampal neurons results in decreased overall surface AMPA receptors, as well as synaptic ones, suggesting that LRRTM2 is involved in maintaining AMPARs at the synaptic level (de Wit et al., 2009). In addition, recombinant LRRTM2 has been shown to co-immunoprecipitate with subunits GluA1 and GluA2 of AMPARs in heterologous cells (de Wit et al. 2009). Deletion of the extracellular domain of LRRTM2 abolished the interaction whereas deletion of the intracellular domain had no effect, suggesting that LRRTM2 could directly bind to AMPARs via its extracellular domain

Surprisingly, Soler-Llavina and colleagues showed that the surface levels of GluA1 receptors are increased in DKD cultured neurons, a finding that was initially incompatible with the previous results of the group demonstrating a reduced AMPAR-mediated synaptic currents *in vivo* under the same conditions (Soler-Llavina et al. 2011) and of others (de Wit et al. 2009). To explain this increase in surface AMPARs the authors hypothesised that it could be attributed to an excess of extrasynaptic AMPARs and not concern the synaptic ones. Indeed, even though LRRTM DKD resulted in an increased intensity of the overall surface population of AMPARs, it decreased the intensity of the synaptic receptors. Electrophysiological experiments further supported this hypothesis as DKD increased the amplitude of AMPAR-mediated currents evoked by fast glutamate application in somatic, outside-out patches. A constitutive genetic inclusion of splice

site 4 in Neurexin3 (Nrx3) which prevents, among others, the binding of LRRTM to neurexin, diminished the puncta size of both subunits GluA1 and GluA2 of AMPARs without affecting their density in cultured hippocampal neurons and increased the intracellular pool of GluA1 receptors in accordance with others (Soler-Llavina et al. 2013). All in all, these results suggest that LRRTM regulates postsynaptic AMPAR levels. A possible mechanism could be by stabilising AMPARs at the synapse. A recent study coupling photo-activatable GFP-tagged GluA1 (paGFP-GluA1) and live-cell imaging demonstrated that after activation the fluorescent intensity of paGFP-GluA1 in spines lacking LRRTM1 and LRRTM2 (cKO) was decreased faster compared to spines containing the LRRTM isoforms, suggesting that paGFP-GluA1 could not be stabilised at spines in the absence of LRRTM1 and 2 (Bhouri et al. 2018).

Apart from stabilising AMPARs on the surface of neurons, there is increasing evidence supporting the role of LRRTM in the trafficking of AMPARs to and from the surface. LRRTM DKD (Soler-Llavina et al., 2013) or constitutive genetic inclusion of splice site 4 in Nrx3 (Aoto et al. 2013) in hippocampal neurons increases the intracellular pool of endogenous AMPARs without affecting the overall pool. However, cKO of LRRTM1 and 2 had no effect on the surface/intracellular ratio of paGFP-GluA1 (Bhouri et al. 2018), which could be due to non-specific localisation of the recombinant GluA1 protein. Live-cell imaging could provide dynamic information to shed more light into the regulation of AMPARs trafficking by LRRTM.

A recent study by the group of Blanpied investigated the subsynaptic positioning of AMPARs by LRRTM2 using super-resolution microscopy and a recombinant LRRTM2 whose extracellular domain could be acutely cleaved upon thrombin application. Unlike previous studies, they were interested in the role of LRRTM2 in synapse organisation and function of already established excitatory synapses, independently of synaptogenesis and genetic compensation. Live-cell imaging revealed that acute cleavage of the extracellular domain of LRRTM2 had no effect on the intensity measurements of recombinant GluA1/2 receptors for the first 30 minutes; however, 2 hours upon LRRTM2 cleavage a 23% decrease was found. Interestingly, this decrease was even more drastic after 24 hours, as shown on fixed samples, resulting in almost 3-fold diminution of AMPARs intensity (Ramsey et al. 2021). Therefore, even though the extracellular domain of

LRRTM2 does not seem to play a role in maintaining AMPARs at LRRTM2-enriched spots acutely, it is implicated in stabilising them in the long-term. In addition, deletion of the extracellular domain of LRRTM2 decreased AMPARs density directly across from RIM1/2 nanodomains, without affecting RIM1/2 density in front of AMPARs nanodomains, suggesting that AMPARs are redistributed within the synapse away from glutamate release sites shortly after LRRTM2 ECD cleavage. LRRTM2 is important for positioning and stabilising AMPARs at excitatory synapses, however conditional knock-out models avoiding overexpression could advance our understanding regarding the domains involved in this process.

In the context of LTP, LRRTM DKD increased the levels of synaptic GluA1 receptors when the neurons were fixed 10 minutes after cLTP, but decreased when the fixation was carried out 20 minutes after the cLTP, suggesting that the absence of LRRTM1 and LRRTM2 does not prevent GluA1 receptors from being inserted at synapses, but rather fails to stabilise them in the long term. This could also explain why in vivo LTP can be induced but not maintained in LRRTM DKD CA1 pyramidal neurons. LRRTM4 has also been suggested to be involved in cLTP, as surface synaptic GluA1 intensity is not increased upon cLTP in LRRTM4$^{-/-}$ (KO) hippocampal cultures (Siddiqui et al. 2013).

MATERIALS AND METHODS

1. Plasmids

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: CAATGAGTGTGGTATTAAA. 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 sequencefor the LRRTM2 shRNA (LRRTM2, CAATGAGTGTGGTATTAAA changed to CAATGAGCGTTGTCTTAAA). 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-□CTD and AP-LRRTM2-□ECEV were derived from previously described myc-LRRTM2-□CTD and myc-LRRTM2-□ECEV (de Wit et al., 2009). The myc tag (EQKLISEEDL) was replaced by the biotin AP tag (GLNDIFEAQKIEWHE). 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).

2. Antibodies

The following primary antibodies were used: mouse α-PSD-95 (Thermo Fischer Scientific, clone 7E-1B8, 1:400), guinea-pig α-VGluT-1 (AB5905, Merck Chemicals, 1:1000), mouse α-GluA1/2 (Synaptic Systems, 182411, 1:100). The following secondary antibodies were used: goat anti-mouse Alexa568 (Thermo Fischer Scientific, 1:800), DyLight 405 (Jackson ImmunoResearch, 1:800).

3. Heterologous cells and transfection

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-1 penicillin and 100 mgml-1 streptomycin at 37°C with 5% of CO2 atmosphere. 2-3 hours after plating, transfections were done using the X-treme GENE™ HP DNA Transfection Reagent (Roche) following the manufacturer's recommendations. 1µg 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 minutes. 75 µl 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.

4. Western Blot

48 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 minutes at 4°C on a rotating device. Lysates were centrifuged at 8 000 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 minutes 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 (Merck, 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.

To detect endogenous LRRTM2, neurons were lysed 7 days post infection using the same lysis buffer described above for 2h at 4°C on a rotating device. Lysates were centrifuged at 8 000 x g during 15 min at 4°C and the supernatant was collected for Western blotting. Protein concentrations were quantified using the Direct Detect™ Infrared Spectrophotometer (Merck-Millipore), and protein amounts were adjusted for concentrations. Samples were warmed 10 minutes at 70°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 antibody sheep anti-LRRTM2 (R&D Systems, AF5589, 1:200) overnight at 4°C and with horseradish peroxidase (HRP)-conjugated donkey anti-secondary antibody (Jackson Immunoresearch; 713-035-003). Target proteins were detected by chemiluminescence using Clarity MAX Western ECL Substrate (BioRad) on the ChemiDoc Touch system (Bio-Rad). Average intensity values were calculated using Image Lab 5.0 software (Bio-Rad). The intensity of LRRTM2 signal was normalized to beta-actin.

5. 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 calculated using the comparative method (2-ΔΔCt) (Livak et al., 2001). The following set of primers was used: LRRTM2 Forward: 5'GCTCCTGCATAAGCCT 3' Reverse: 5'GAAATGTAAGCCCATTCTTCTGAG 3' and SDHA Forward: 5' TGCGGAAGCACGGAAGGAGT 3' Reverse: 5' CTTCTGCTGGCCCTCGATGG 3'. Add mice primers

6. Primary hippocampal culture

Rat cultures. Rat cultures were only used for the first paper of the results entitled 'Role of regulatory C-terminal motifs in synaptic confinement of LRRTM2'. Dissociated hippocampal neurons from E18 Sprague-Dawley rats embryos of either sex were prepared as described previously (Kaech and 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 Fischer Scientific). Coverslips were flipped on a 60mm dish containing a glia monolayer 2 hours after plating and 2µM Ara-C was added after 72 hours. Astrocytes feeder layers were prepared from the same embryos, plated between 20,000 to 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 4.5g/l Glucose, 2mM GlutaMAX and 10% horse serum heat inactivated (Thermo Fischer Scientific, GIBCO) for 14 days. Cytosine arabinoside (3.4mM) was added at DIV 3 to control glial growth.

Mice cultures. Mice cultures were used for the second paper and the rest of the results. Hippocampal neurons were dissociated from P0 LRRTM2-floxed mice embryos as previously described (Kaech and Banker, 2006). Cells were plated on 18 mm coverslips previously coated with 30 µ g/ml poly-D-lysine overnight and subsequently with 2 µ g/ml laminin overnight at a concentration of 150,000 cells per coverslip. Coverslips were already placed in 60 mm dishes containing Neurobasal™-A medium (Thermo Fisher Scientific), supplemented with NeuroCult™ SM1(STEMCELL), 2mM L-glutamine glutamine (NB-A) and 10% Horse Serum. 30 minutes after plating, the medium was replaced by NB-A without serum to disadvantage glial growth on the coverslips. 2 hours later the coverslips were flipped onto 60 mm dishes containing a glial cell layer in NB-A medium and cultured for 2 weeks at 37 °C and 5% CO2. Cytosine arabinoside (3.4mM) was added at DIV 3 to control glial growth.

For biochemistry experiments, hippocampal neurons dissociated from P0 LRRTM2-floxed mice embryos were plated at a concentration of 500k cells/well on a 6-well plate previously coated

with poly-L-lysine containing Neurobasal™-A medium (Thermo Fisher Scientific), supplemented with NeuroCult™ SM1(STEMCELL), 2mM L-glutamine glutamine (NB-A) and 10% Horse Serum. 30 minutes after plating cell media was replaced with supplemented Neurobasal™-A- 3% Horse Serum to remove any cellular debris. After 3 days media was again partially replaced by supplemented Neurobasal™ without serum to prevent glial growth. Cytosine arabinoside (3.4mM) was added at DIV 3 to control glial growth.

Transfection. Calcium-phosphate transfection was used in the majority of the experiments to introduce the plasmids of interest in the neurons, unless stated otherwise. Briefly, this method of transfection relies on the formation of calcium phosphate-DNA precipitates to facilitate the entry of the DNA into the cells via endocytosis (Graham et al., 1973). Neurons were transfected at DIV 7 using 1.5-1.8 µg of plasmidic DNA and the following solutions: TE (1M Tris-HCl pH 7.5, 250 mM EDTA), CaCl2 (2.5 M CaCl2 in 10mM HEPES - pH 7.2) and 2xHEPES-buffered saline (274mM NaCl, 10mM KCl, 1.4mM Na2HPO4, 12mM glucose, 42mM HEPES - pH 7.2. Prior to transfection, 2mL of the appropriate equilibrated media was added to each dish. The combination of different plasmids was diluted in TE, then CaCl2 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 200µl/well of cultured medium and 50µl of 5x kynurenic acid (10mM stock solution). 50µl of the precipitate solution was added to each well and neurons were incubated for 30min at 37°C. Afterwards, the cells were washed for 15min at 37°C with fresh equilibrated medium containing 2mM kynurenic acid and were then returned to their original culture dish until imaging.

Viral Infections. Viruses were used in the appropriate combination and concentration to infect primary hippocampal neurons plated in 6-well plates at DIV6. 1mL was discarded from each well resulting in 1mL final volume. Viruses were diluted in cell media from each well to ensure even distribution and were added on the cells. The plate was placed back at 37 °C and 5% CO2 and after 6 hours 1mL of fresh equilibrated media was added to each well.

Electroporation. Electroporation was only for Fig.1 of Chapter 1 of the Results. 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.

7. Electrophysiology

Electrophysiological recordings were carried out at room temperature on primary hippocampal neurons from LRRTM2 floxed mice transfected with an empty vector (control), a plasmid encoding Cre + mcherry reporter alone (cKO) or together with AP-LRRTM2 construct (rescue). Neurons were observed with an upright microscope (Nikon Eclipse FN1) equipped with a motorized 2D stage and micromanipulators (Scientifica). Whole-cell patch-clamp was performed using micropipettes pulled from borosilicate glass capillaries using a micropipette puller (Narishige). Pipettes had a resistance in the range of 5–6 MΩ. The recording chamber was continuously perfused with aCSF containing (in mM): 130 NaCl, 2.5 KCl, 2.2 CaCl2, 1.5 MgCl2, 10 D-glucose, 10 HEPES, and 0.02 bicuculline (pH 7.35, osmolarity adjusted to 300 mOsm), while the internal solution contained (in mM): 135 Cs-MeSO4, 8 CsCl, 10 HEPES, 0.3 EGTA, 4 MgATP, 0.3 NaGTP, and 5 QX-314. Salts were purchased from Sigma-Aldrich and drugs from Tocris. Neurons were voltage-clamped at a membrane potential of −70 mV and AMPA receptor-mediated mEPSCs were recorded in the presence of 0.5 µM TTX using Clampex software (Axon Instruments). The series resistance Rs was left uncompensated. Recordings with Rs higher than 30 MΩ were discarded. mEPSCs were detected and analysed using MiniAnalysis software (Synaptosoft).

8. Immunocytochemistry

To visualize surface AP-tagged LRRTM2 and endogenous proteins (PSD-95, VGluT1, GluA receptors), live neurons were incubated with STAR635P-conjugated monomeric streptavidin in Tyrode or ACSF solution (Tyrode 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-4% 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-1B8, 1:400) for 1 hour followed by Alexa568 goat anti-mouse antibody (Thermo Fischer Scientific, 1:800) for 1 hour. Coverslips were then mounted in Mowiol (Calbiochem).

9. Epifluorescence microscopy

Immunostained neurons were visualized using an inverted epifluorescence microscope (Nikon Eclipse TiE) equipped with a 60x/1.40 NA objective and filter sets for EGFP (Excitation: FF01-472/30; Dichroic: FF-495Di02; Emission: FF01-525/30); Alexa568 (Excitation: FF01-543/22; Dichroic: FF562Di02; Emission: FF01-593/40); and Alexa647 (Excitation: FF02-628/40; Dichroic: FF-660Di02; Emission: FF01-692/40) (SemRock). Images were acquired with a Prime 95BTM sCMOS camera (Phototometrics®), using Metamorph® software (Molecular Devices). Transfected cells were identified with the GFP reporter and images for AP-LRRTM2 and PSD-95 were acquired. To measure the effect of sh-LRRTM2 on PSD-95 (Supplementary figure 2c-f), a region around a GFP positive dendrite was created and the area, average intensity and density (number of clusters per unit dendrite length) of PSD-95 clusters were measured after segmentation of each image using a custom program written in Metamorph. For the replacement conditions, only cells with AP-LRRTM2 mutants staining were considered. For the first paper of the results, to assess the distribution of LRRTM2 in dendrites and in axons, a region around a GFP positive dendrite (presence of Homer1c signal) or axon (absence of Homer1c signal) was created and the total fluorescence of AP-LRRTM2 was measured. The polarity index was calculated by dividing the intensity on the dendrite by that of the axon for each cell (Fig. 2e). To determine the density of AP-LRRTM2 clusters (Fig. 3f), a region around a GFP positive dendrite was created and the number of clusters per unit dendrite length of AP-LRRTM2 clusters were measured after segmentation of each image using a custom program written in Metamorph.

10. Fluorescence Recovery After Photobleaching (FRAP) and analysis

For GluA receptors' experiments, neurons transfected with SEP-GluA1 and Homer-BFP (ctrl), SEP-GluA1, Homer-BFP and Cre-mCherry (Cre) or SEP-GluA1, Homer-BFP, Cre-recombinase and different AP-LRRTM2 constructs (Cre + AP-LRRTM2 constructs) were mounted in ACSF solution and observed under the same set-up used for the super-resolution experiments. For LRRTM2 experiments, neurons were transfected with the Cre-mCherry, Homer-BFP and SEP-LRRTM2-WT or SEP-LRRTM2-YACA. The laser bench (comprising 488, 561 and 642 nm lasers, 100 mW each, Roper Scientific) has a second optical fibre output connected to an illumination device containing galvanometric scanning mirrors (ILAS, Roper Instrument) controlled by MetaMorph software. Using this system we were able to precisely photobleach regions of interest, either on spines or on the shaft of dendrites. After acquiring a 10-s baseline at 1 Hz frame rate with the fluorescent lamp, photobleaching of 5 synaptic and 2 dendritic regions (shaft) was achieved at higher laser power. Fluorescence recovery was then recorded immediately after the bleach sequence for approximately 14 min at a 0.5–1 Hz frame rate. A perfusion pump was used for all experiments to ensure stable imaging medium in the ludin chamber.

Fluorescence intensity of all regions was then background-subtracted and bleach-corrected. In parallel, the intensities for each frame before photobleaching (10 frames/region) were normalised to their mean intensity value and to 1, whereas the intensity of the first frame after the photobleaching was normalised to 0. The percentage of bleaching depth was calculated for each region: bleaching depth=(intensity after/intensity before)*100. Regions with negative values after normalisation or regions with bleaching depth inferior to 55% were excluded from the analysis.

Big FRAP. In order to study the exocytosis of with SEP-GluA1, SEP-LRRTM2 or SEP-LRRTM2 YACA in COS-7 cells we adapted a protocol previously used by others. The whole image plane was bleached using the same laser configuration as described above for the classic FRAP experiments, aiming to quench all the surface SEP-tagged proteins. Non-fluorescent SEP-tag proteins (localised

in acidic compartments) are protected from the bleach a recovery of fluorescence was monitored with at 0.5Hz.

11. pH change/NH4Cl protocol

To visualise the intracellular pool of LRRTM2 we combined SEP-tagged proteins with a pH change protocol. Frame rate was set at 0.1Hz and transfected neurons were mounted in imaging chamber perfused with ACSF (pH7.4) (baseline 1). Acidic ACSF (pH5.5) was then perfused to quench surface SEP-tagged proteins, followed by ACSF pH7.4 (baseline 2). Subsequently, ACSF containing 50mM ammonium chloride was perfused to reveal both surface and intracellular SEP-tagged proteins and finally ACSF pH7.4 was again perfused (baseline 3). Experiments were carried out in a thermostatic box providing air 37 °C and all imaging media were kept warm throughout acquisitions. Surface proteins were calculated as following: mean baseline fluorescence – mean fluorescence at pH5.5, whereas intracellular proteins were calculated as following: mean NH4Cl fluorescence - mean baseline fluorescence.

12. Single Particle Tracking

Cells were mounted in Tyrode or ACSF solution containing 0.1% Albumin Fraction V, biotin-free (Roth) in an open Inox observation chamber (Life Imaging Services, Basel, Switzerland). The chamber was placed on an inverted microscope (Nikon Ti-E Eclipse) equipped with an EMCCD camera (Evolve, Roper Scientific, Evry, France), a thermostatic box (Life Imaging Services) providing air at 37 °C and an APO total internal reflection fluorescence (TIRF) 100 × /1.49 NA oil objective. GFP-expressing cells were detected using a mercury lamp (Nikon Xcite) and appropriate filter sets from described above (SemRock). To track biotinylated AP-tagged LRRTM2 molecules a 1nM dilution of STAR635P-conjugated monomeric streptavidin was used. A four-color laser bench (405; 488; 561; and 642 nm, 100 mW each; Roper Scientific) is connected through an optical fiber to the TIRF illumination arm of the microscope. Laser powers were controlled through acousto-optical tunable filters driven by Metamorph. STAR635P was excited with the 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.

13. dSTORM acquisition and analysis

Primary hippocampal neurons co-expressing shLRRTM2, Homer1c-DsRed, BirAER and AP-LRRTM2 constructs were live labelled with Alexa647-conjugated monomeric streptavidin in Tyrode (100mM) 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.

RESULTS

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

Synaptic cell adhesion molecules (CAMs) are no longer merely considered as physical bridges connecting the pre- and post-synapse. The discovery that some of them exhibit synaptogenic properties, having the ability to induce pre- or post- synaptic differentiation when presented on the surface of non-neuronal cells (Biederer et al., 2000), established them as crucial functional components of the synapse. They contribute to synaptic differentiation and transmission by recruiting neurotransmitter receptors and signalling molecules and they are dynamically regulated in response to plasticity. However, their localisation at the narrow synaptic cleft of around 20 nm, together with the commonly used bulky antibodies, were hindering their precise visualisation within individual synapses.

The development of super-resolution techniques (Betzing et al., 2006; Rust et al., 2006) allowed us to go beyond the diffraction limit of 250 nm and combined with small monovalent probes (Rothbauer et al., 206; Ries et al., 2012; Chamma et al., 2016) gave us access to the crowded environment of the synaptic cleft, enabling imaging of synaptic molecules at the sub-synaptic level. Visualising synaptic CAMs surface dynamics and nanoscale organisation profiting from advances in super-resolution techniques is starting to shed light into their complex trans-synaptic interaction patterns at synapses. Understanding how those molecules engage in multiple trans-synaptic complexes to promote synapse diversity, while being tightly regulated to ensure synapse specificity, would be a major step into understanding neuronal connectivity.

When I first arrived in the lab, the team was mostly focusing on neurexin-neuroligin trans-synaptic complex and how they contribute to synapse assembly and function. However, post-synaptic adhesion molecule LRRTM2, the most synaptogenic of the LRRTM family, was a partner of pre-synaptic neurexin of particular interest. First, it is highly enriched and exclusively localised at excitatory synapses (de Wit et al., 2009; Ko et al., 2009). Second, in the team we had previously

shown using super-resolution imaging that almost 80% of LRRTM2 molecules were confined at synapses and organised in compact clusters (Chamma et al., 2016). Finally, LRRTM2 is involved in synaptic transmission and plasticity (Sloler-Llavina et al., 2013; Bhouri et al., 2018), however the mechanisms regulating LRRTM2 itself remain purely unknown. Intracellularly, LRRTM2 can bind to scaffold protein PSD-95 through its PDZ-like binding motif, ECEV, at the very end of its intracellular region (de Wit et al., 2009; Linhoff et al., 2009), whereas a YxxC intracellular motif was recently identified to be critical for LRRTM2 surface expression and clustering (Minatohara et al., 2015).

Thus, the goal of this project was to further decipher the molecular mechanisms underlying LRRTM2 clustering and confinement at excitatory synapses. For this purpose, during the beginning of my PhD I implemented a replacement strategy using shRNA to knock-down endogenous LRRTM2 in rat hippocampal neurons and replaced it with mutated counterparts of the intracellular region.

Knock-down of LRRTM2 during synapse formation reduced excitatory synapse density in mature neurons, whereas deletion of LRRTM2 C-terminal domain abolished the compartmentalization of LRRTM2 in dendrites and disrupted its synaptic enrichment. Furthermore, we found 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.

In conclusion, this paper sheds light for the first time on the molecular mechanisms governing LRRTM2 dynamics and organisation at the nanoscale opening questions regarding novel intracellular partners involved in LRRTM2 stabilisation and membrane targeting, which are yet to be identified.

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RESEARCH ARTICLE

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 isoform 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. Furtheremore, 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

(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 post-synapses (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). Post-synaptic 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 (Yamagata 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%

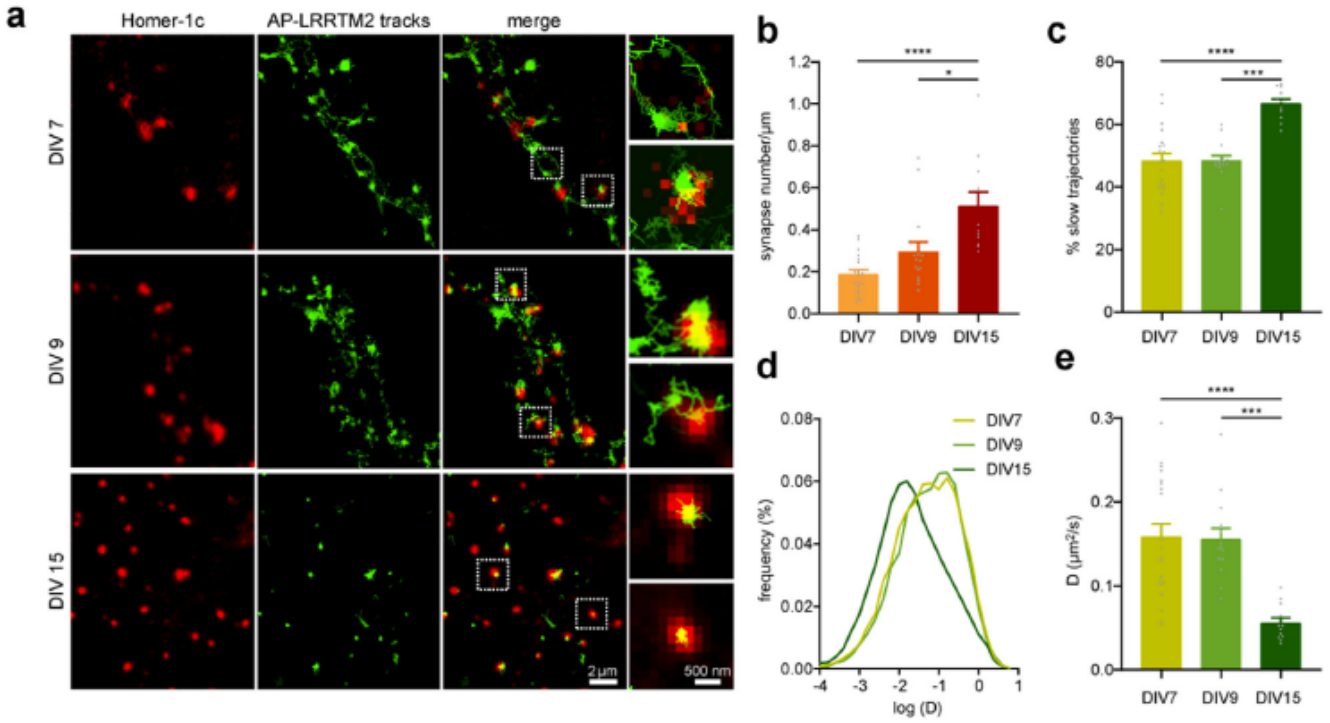

FIGURE 1 A switch in LRRTM2 mobility occurs after synaptogenesis. (a) Representative example of DIV 7 to 15 neurons expressing the excitatory synapse marker Homer1c-GFP and biotinylated wild type AP-LRRTM2 labeled with monomeric streptavidin-ATTO647N and tracked by uPAINT. Left, Homer1c signal (red); middle, AP-LRRTM2 trajectories reconstructed from 4000 frames acquired at 50 Hz (green); right, merge and insets showing LRRTM2 trajectories (green) overlaid with synaptic marker signal (Homer1c, red). (b) Quantification of synapse number (Homer1c-positive puncta) per dendrite unit length (μm) showing gradual synapse formation across neuron development. (c) % of slow LRRTM2 trajectories in the different conditions. (d) Frequency distribution of Log(D) and (e) mean diffusion coefficient D at different developmental stages, showing drastic decrease in diffusion between DIV9 and DIV15. Data are from 2 independent experiments (DIV7, n = 20; DIV9, n = 15; DIV15, n = 12). *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$

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-WT_r). shRNA against LRRTM2 reduced protein expression observed by Western blot, by ~50%, an effect that was rescued with shRNA-insensitive LRRTM2-WT_r (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

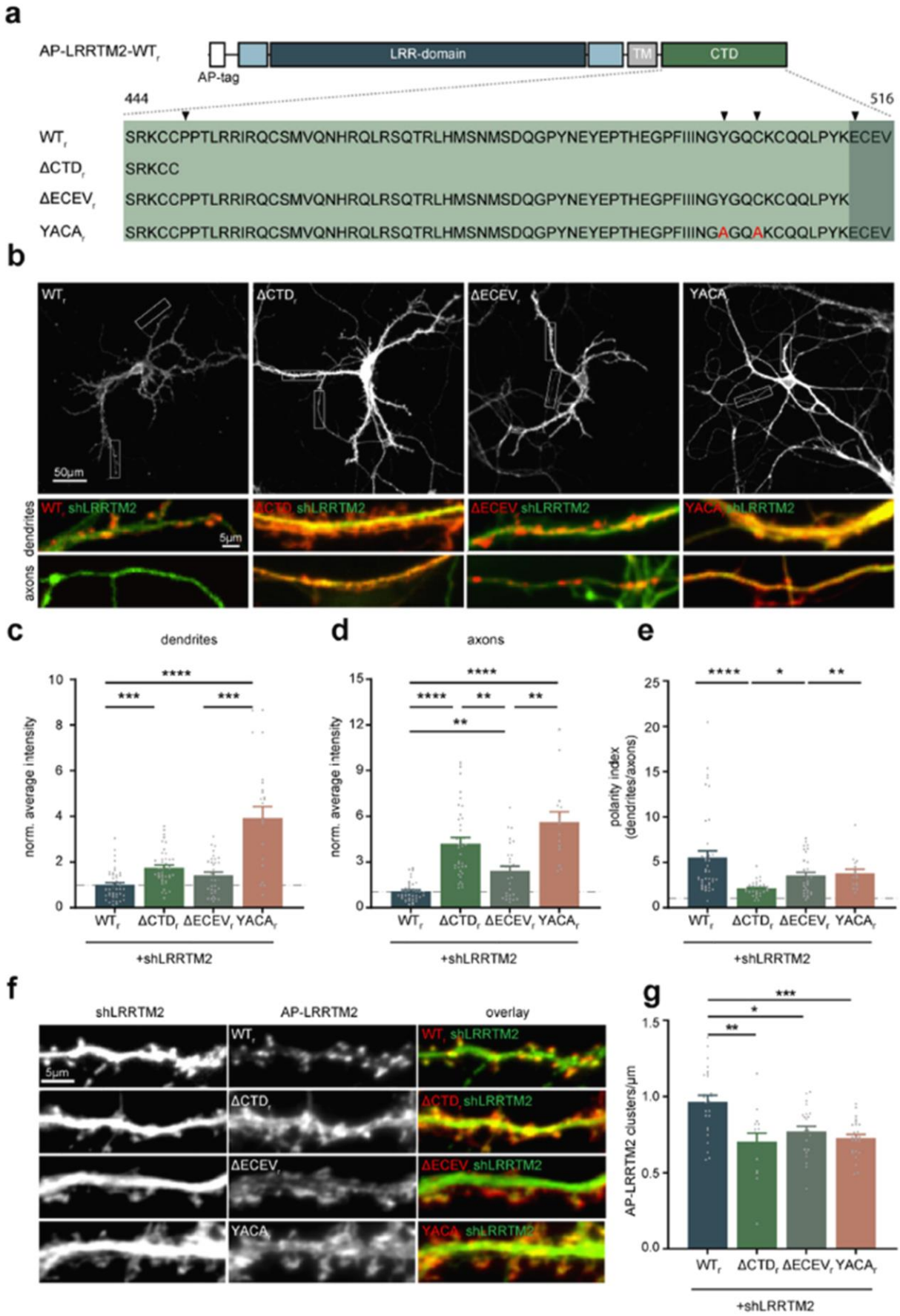

FIGURE 2 LRRTM2 is compartmentalized to dendrites and clustered through its CTD. (a) Schematics of AP-LRRTM2-WTr. LRR: Leucine-Rich-Repeat; TM: Transmembrane; CTD: C-terminal domain. Below: amino acid sequences of the intracellular region of LRRTM2 mutants, highlighting the PDZ-like binding motif ECEV; ΔCTD_r: 449–516a.a deleted; $\Delta ECEV_r$: last 4 a.a deleted; $YACA_r$: 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$_r$, lacking the entire C-terminal domain, AP-LRRTM2-ΔECEV$_r$, 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-WT$_r$ and AP-LRRTM2-YACA$_r$ induced the formation of large PSD-95-GFP clusters (Figure S4). However, AP-LRRTM2-ΔECEV$_r$ and AP-LRRTM2-ΔCTD$_r$ 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$_r$ and AP-LRRTM2-YACA$_r$ expression was significantly increased in dendrites compared to AP-LRRTM2-WT$_r$ (Figure 2b,c). We further observed that all the mutants were mislocalized to axons, whereas AP-LRRTM2-WT$_r$ was restricted to dendrites (Figure 2b,d). However, the polarity index (dendrites/axons) was not significantly different from AP-LRRTM2-WT$_r$ for both AP-LRRTM2-ΔECEV$_r$ and AP-LRRTM2-YACA$_r$ 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$_r$ and AP-LRRTM2-YACA$_r$ 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-WT$_r$, ΔCTD$_r$, ΔECEV$_r$, and YACA$_r$ mutants on a LRRTM2 KD background. As we previously observed, AP-LRRTM2-WT$_r$ 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-WT$_r$ 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

White boxes indicate locations of dendrites and axons shown in the index below (red) and overlaid with sh-LRRTM2 (green). (c) Normalized surface expression of AP-LRRTM2 mutants in dendrites and (d) in axons. (e) Polarity index of AP-LRRTM2 constructs. (f) Hippocampal neurons expressing shLRRTM2 and AP-LRRTM2 mutants live-labeled with monomeric streptavidin showing a loss of clustering with the mutants. (g) Quantification of AP-LRRTM2 mutant cluster density. Data from 3 independent experiments (c, d and e: WT^r, n = 46; ΔCTD^r, n = 43; ΔECEV^r, n = 33, YACA^r, n = 19; g: WT^r, n = 26; ΔCTD^r, n = 16; ΔECEV^r, n = 23, YACA^r, n = 24). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

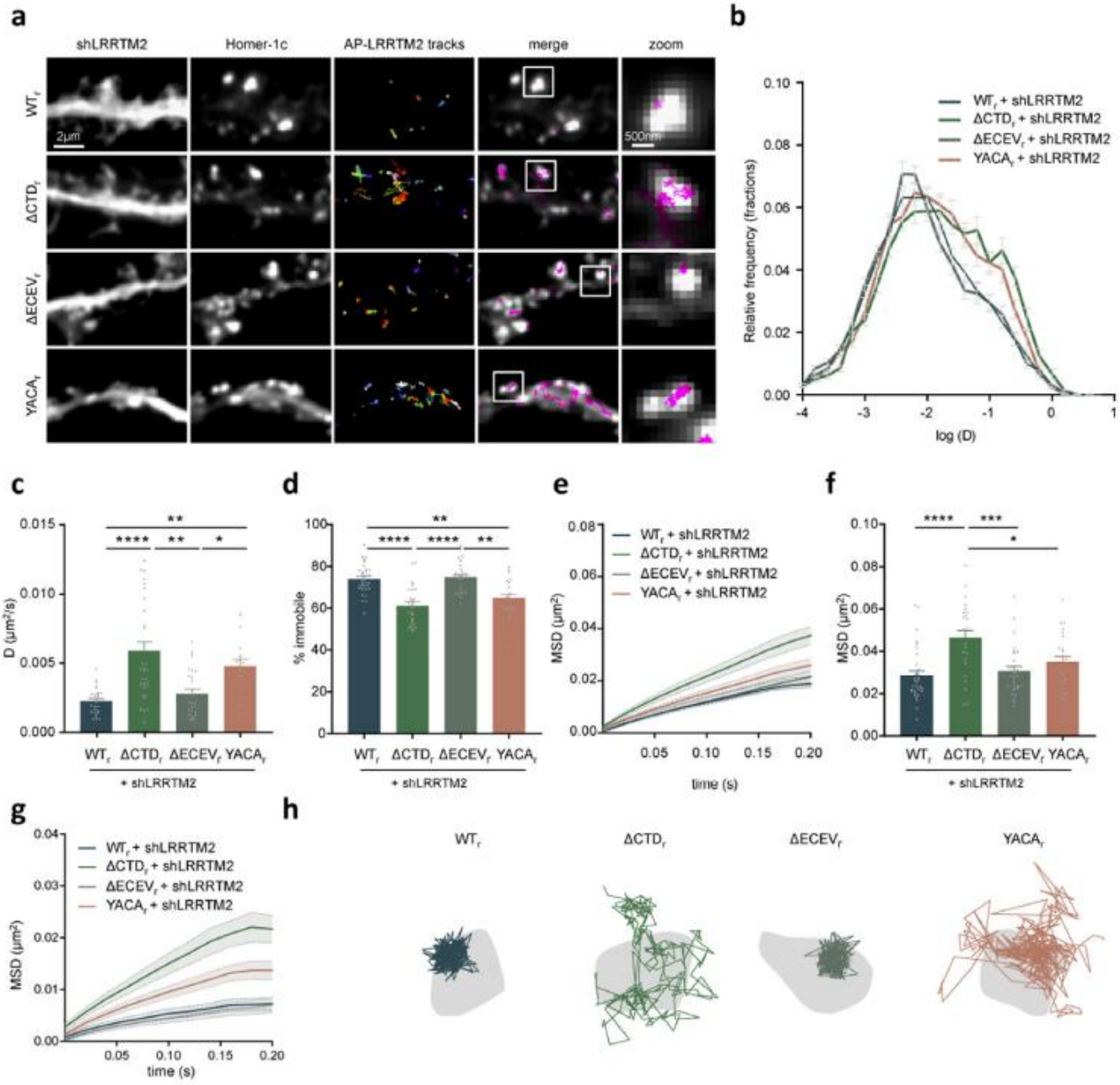

FIGURE 3 LRRTM2 diffusion and confinement at synapses are independent from its PDZ-like binding motif. (a) Representative examples of DIV15 neurons expressing sh-LRRTM2, Homer1c-DsRed and AP-LRRTM2 (WT_r, ΔCTD_r, $\Delta ECEV_r$ or $YACA_r$) labeled with mSA-STAR635P to track individual molecules by 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-WT_r, AP-LRRTM2-ΔCTD_r, AP-LRRTM2-$\Delta ECEV_r$ and AP-LRRTM2-$YACA_r$ and (c) median diffusion coefficient, showing an increase with the ΔCTD_r and $YACA_r$ 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_r: n = 31, ΔCTD_r: n = 25, $\Delta ECEV_r$: n = 24, $YACA_r$: 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

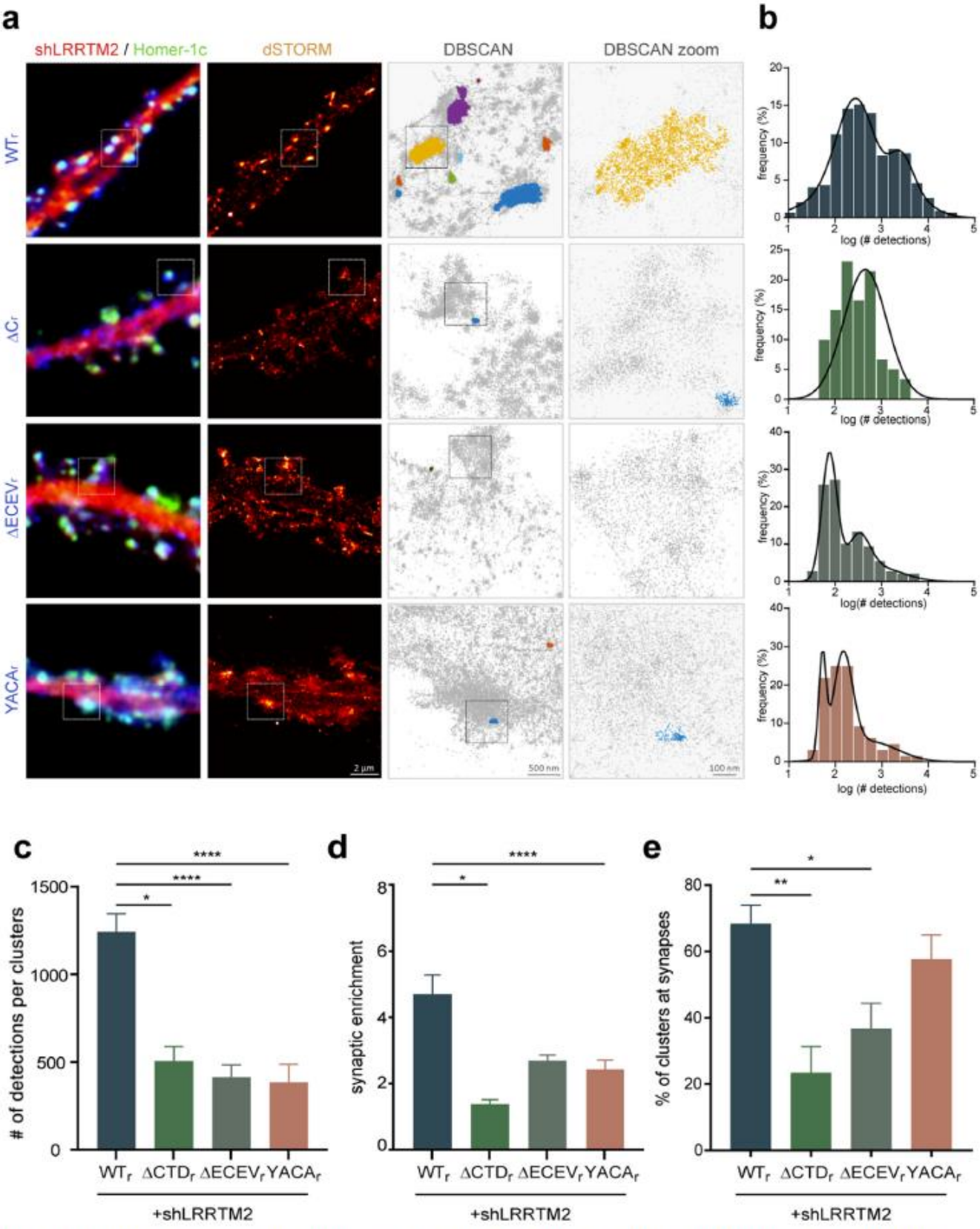

FIGURE 4 LRRTM2 nano-organization at synapses depends on its C-terminal domain. (a) dSTORM imaging of hippocampal neurons expressing sh-LRRTM2-GFP (red), BirA^{ER}, Homer1c-DsRed (green) as a synaptic marker and AP-LRRTM2-WT$_r$, -ΔC$_r$, -ΔECEV$_r$ or -YACA$_r$ constructs (blue) that were live labeled 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_r: 301 clusters; ΔC_r: 62 clusters; $\Delta ECEV_r$: 152 clusters; $YACA_r$: 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^r n = 9; ΔC^r n = 6; $\Delta ECEV^r$ n = 7, $YACA^r$ 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 neurexin-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 (Farías 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., 2018). 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 (Minatohara 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; Neupert 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 cysteins 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: CAATGAGTGTGGTATTAAA. 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, CAATGAGTGTGGTATTAAA changed to CAATGAGCGTTG TCTTAAA). 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 (EQKLISEEDL) was replaced by the biotin AP tag (GLNDIFEAQKIEWHE). 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 $BirA^{ER}$ 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 (Abberior), 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 CO^2 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 $BirA^{ER}$ ± 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% biotin-free 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, 1× 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 (Merck, 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 (Kaech & Banker, 2006) at a density of 200,000 cells per 60-mm dish on poly-l-lysine precoated 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 Fischer 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; $BirA^{ER}$, 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_2HPO_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 5× kynurenic acid (10 mM stock solution). Fifty microliter 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 + $BirA^{ER}$ (0.3:0.6:0.6:0 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 calculated using the comparative method ($2^{-\Delta\Delta Ct}$) (Livak et al., 2001). The following set of primers was used: LRRTM2 Forward: 5'GCTCCTGCATAAGCCT 3' Reverse: 5'GAAATGTAAGCCCATTCTTCTGAG 3' and SDHA Forward: 5′ TGCGGAAGCACGGAAGGAGT 3′ Reverse: 5′ CTTCTGCTGGCCCTCGATGG 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-1B8, 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/1.40 NA objective and filter sets for EGFP (Excitation: FF01-472/30; Dichroic: FF-495Di02; Emission: FF01-525/30); Alexa568 (Excitation: FF01-543/22; Dichroic: FF562Di02; Emission: FF01-593/40); and Alexa647 (Excitation: FF02-628/40; Dichroic: FF-660Di02; Emission: FF01-692/40) (SemRock). Images were acquired with a Prime 95B sCMOS camera (Phototometrics), using Metamorph software (Molecular Devices). Transfected cells were identified with the GFP reporter and images for AP-LRRTM2 and PSD-95 were acquired. To measure the effect of sh-LRRTM2 on PSD-95 (Figure S2c–f), a region around a GFP positive dendrite was created and the area, average intensity and density (number of clusters per unit dendrite length) of PSD-95 clusters were measured after segmentation of each image using a custom program written in Metamorph. For the Rescue condition, only cells with AP-LRRTM2 staining were taken into account. To assess the distribution of LRRTM2 in dendrites and in axons (Figure 2b–d), a region around a GFP positive dendrite (presence of Homer1c signal) or axon (absence of Homer1c signal) was created and the total fluorescence of AP-LRRTM2 was measured. The polarity index was calculated by dividing the intensity on the dendrite by that of the axon for each cell (Figure 2e). To determine the density of AP-LRRTM2 clusters (Figure 3f), a region around a GFP positive dendrite was created and the number of clusters per unit dendrite length of AP-LRRTM2 clusters were measured after segmentation of each image using a custom program written in Metamorph.

Single Particle Tracking

Cells were mounted in Tyrode solution containing 0.1% Albumin Fraction V, biotin-free (Roth) in an open Inox observation chamber (Life Imaging Services, Basel, Switzerland). The chamber was placed on an inverted microscope (Nikon Ti-E Eclipse) equipped with an EMCCD camera (Evolve, Roper Scientific, Evry, France), a thermostatic box (Life Imaging Services) providing air at 37°C and an APO total internal reflection fluorescence (TIRF) 100 × /1.49 NA oil objective. GFP-expressing cells were detected using a mercury lamp (Nikon Xcite) and appropriate filter sets from described above (SemRock). To track biotinylated AP-tagged LRRTM2 molecules a 1 nM dilution of STAR635P-conjugated monomeric streptavidin was used. A four-color laser bench (405; 488; 561; 100 mW each; and 642 nm, 1000 mW Roper Scientific) is connected through an optical fiber to the TIRF illumination arm of the microscope. Laser powers were controlled through acousto-optical tunable filters driven by Metamorph. STAR635P was excited with the 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 shLRRTM2, Homer1c-DsRed, BirA^{ER} 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 super-resolved 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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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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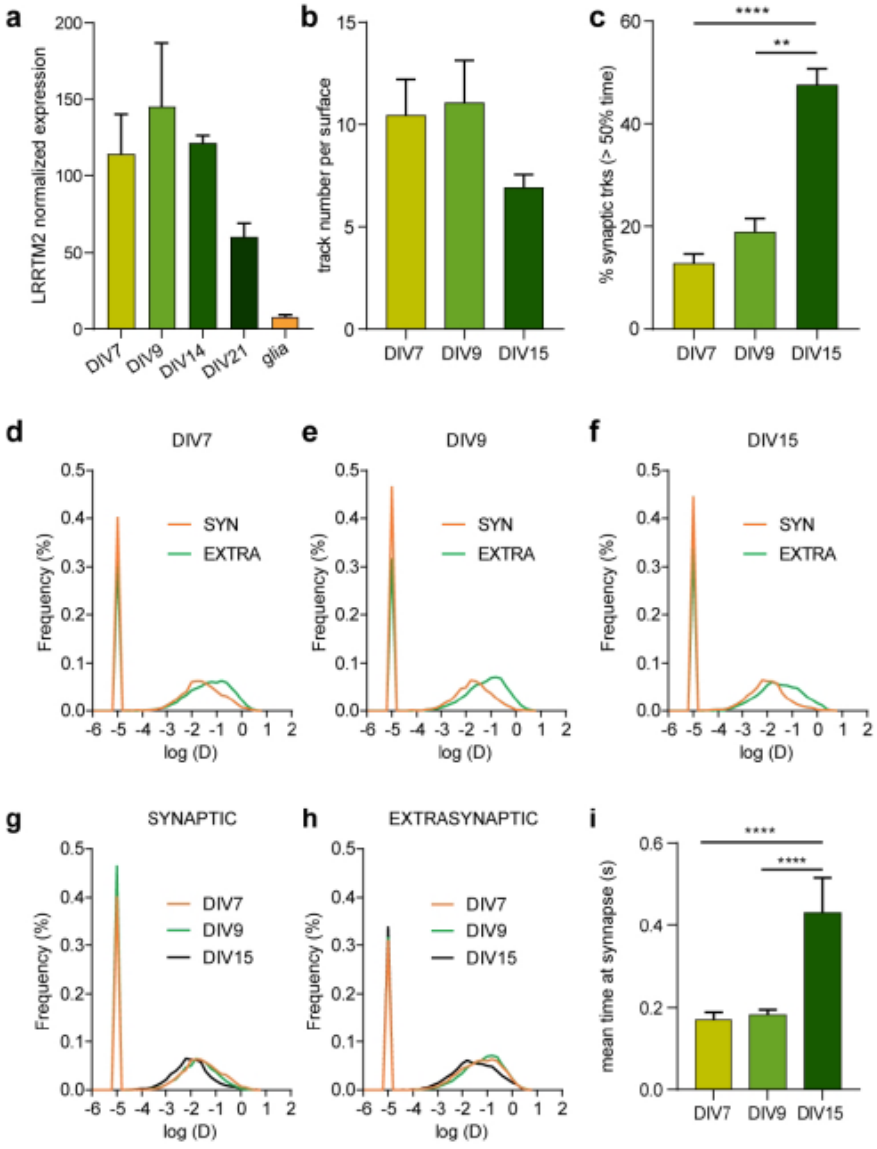
Supplementary Figure1



Supplementary Figure 1. LRRTM2 expression and diffusion during neuronal development. (a) Normalised expression of LRRTM2 in developing hippocampal neurons at different days in vitro (DIV), assessed by RT-qPCR using specific rat primers. Data are represented as mean ± SEM of at least 3 independent cultures per condi-tion. (b) Number of trajectories obtained by uPAINT normalized to the cell area and (c) percentage of synaptic trajectories (> 50% track length inside synapses) for the different DIVs. (d-h) Frequency distribution of log(D) comparing synaptic and extrasynaptic trajectories at DIV7 (d), 9 (e) and 15 (f). Frequency distribution of log(D) of synaptic (g) and extrasynaptic (h) trajectories at different developmental stages. (i) Mean residency time of AP-LRRTM2 at synapses at different developmental stages. (DIV7, n=20; DIV9, n=15; DIV15, n=12).

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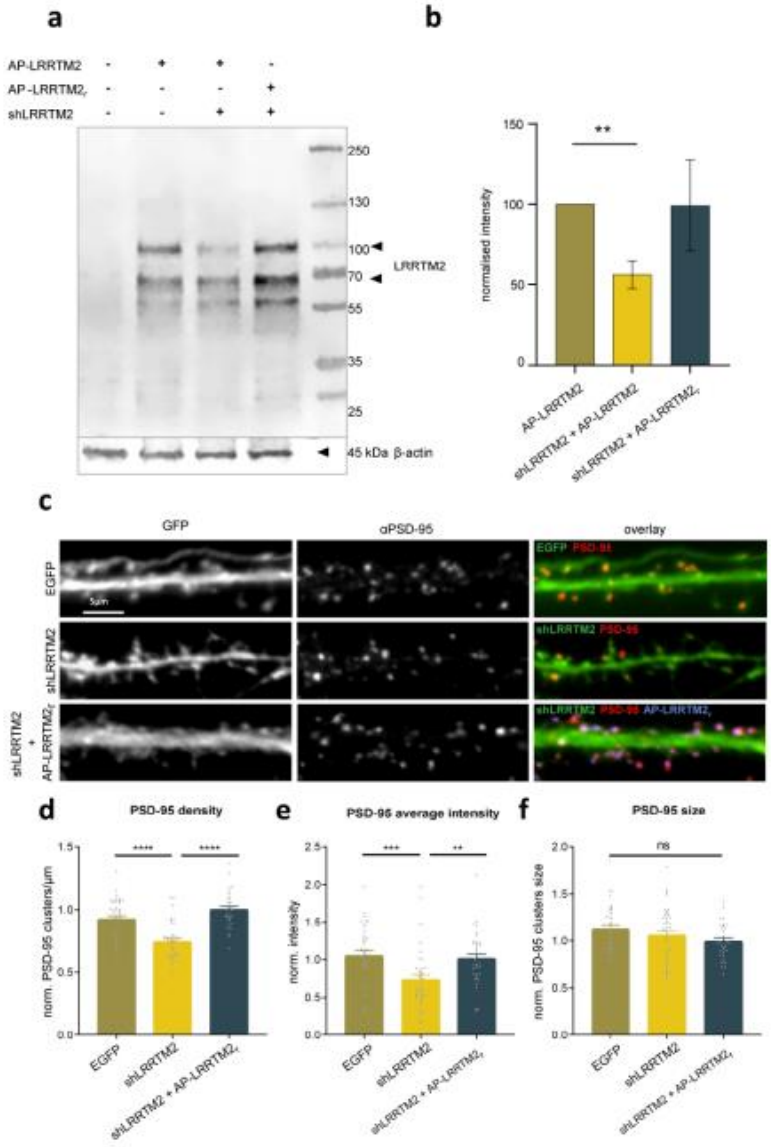
Supplementary Figure 2



Supplementary Figure 2. LRRTM2 knockdown by shRNA disrupts excitatory synapse density. (a) Representative Western blot performed on protein extracts from COS-7 cells expressing AP-LRRTM2 or AP-LRRTM2r in the presence or absence of shLRRTM2. (b) Quantification of protein expression for LRRTM2 detected in the different conditions and normalized to β-actin levels, and expressed as a percentage of the control condition. Data are from 3 independent experiments. (c) Hippocampal neurons expressing soluble EGFP, or shLRRTM2 or shLRRTM2+AP-LRRTM2r+BirAER and immunostained for endogenous PSD-95 at DIV 14 (d) Quantifications of endogenous PSD-95 cluster density, average intensity, and size. Data are from 4 independent experiments, represented as mean ± SEM, and individual values (normalized for the average intensity).

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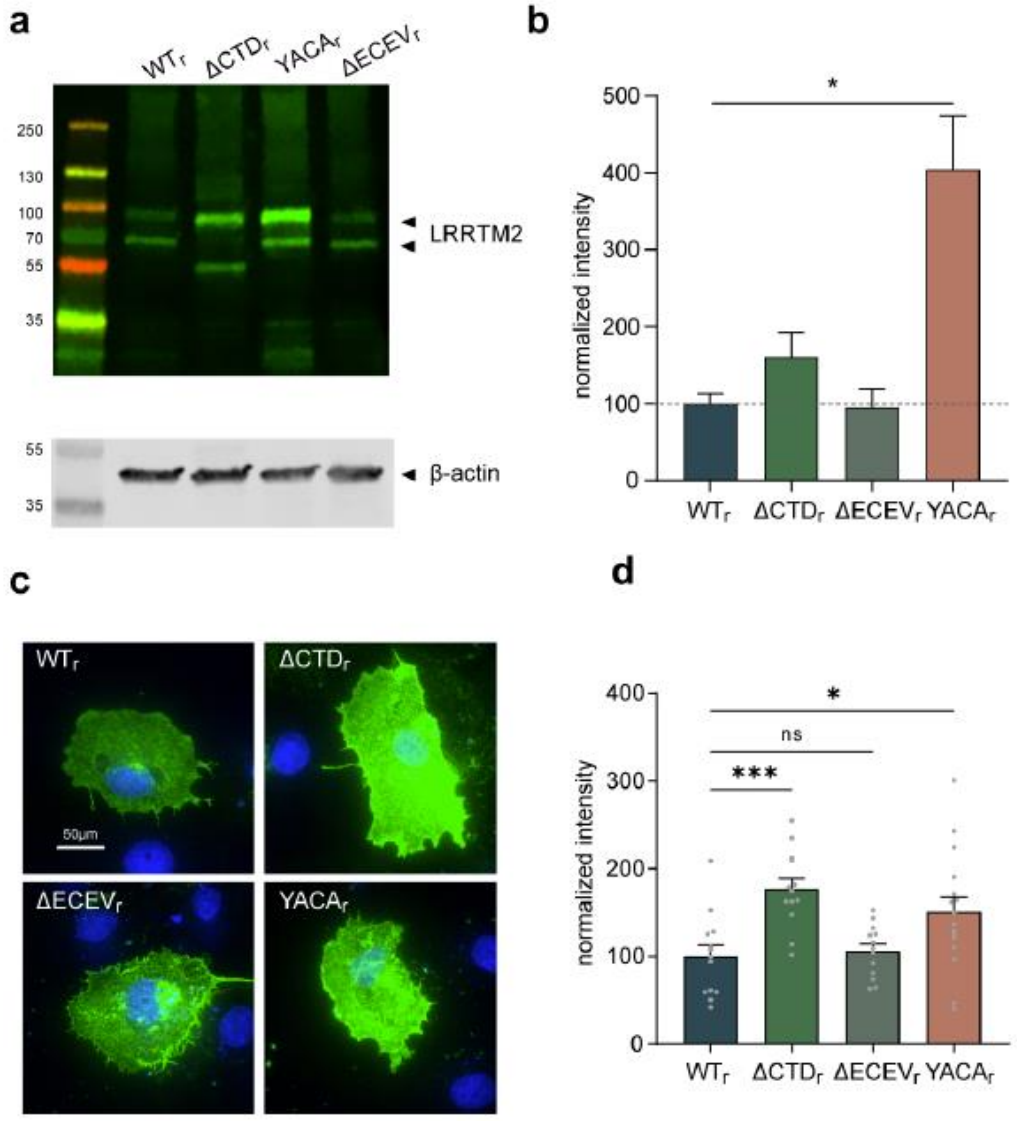
Supplementary Figure 3



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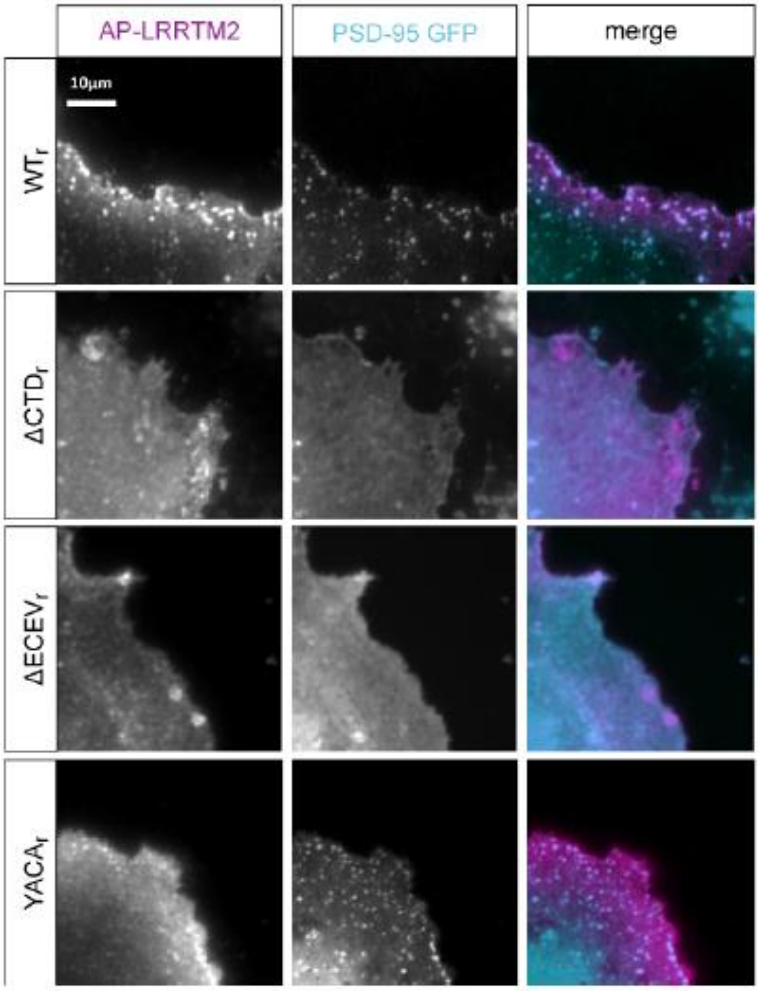
Supplementary Figure 4



Supplementary Figure 4. ECEV deletion but not YxxC mutation disrupt LRRTM2-PSD-95 interaction. COS-7 cells co-expressing mutants of AP-LRRTM2, BirAER and GFP-tagged PSD-95 and surface labeled with Alexa647-conjugated mSA. Notice that AP-LRRTM2-YACAr can still cluster PSD-95 like AP-LRRTM2-WTr, whereas AP-LRRTM2-ΔCTDr and AP-LRRTM2-ΔECEVr cannot.

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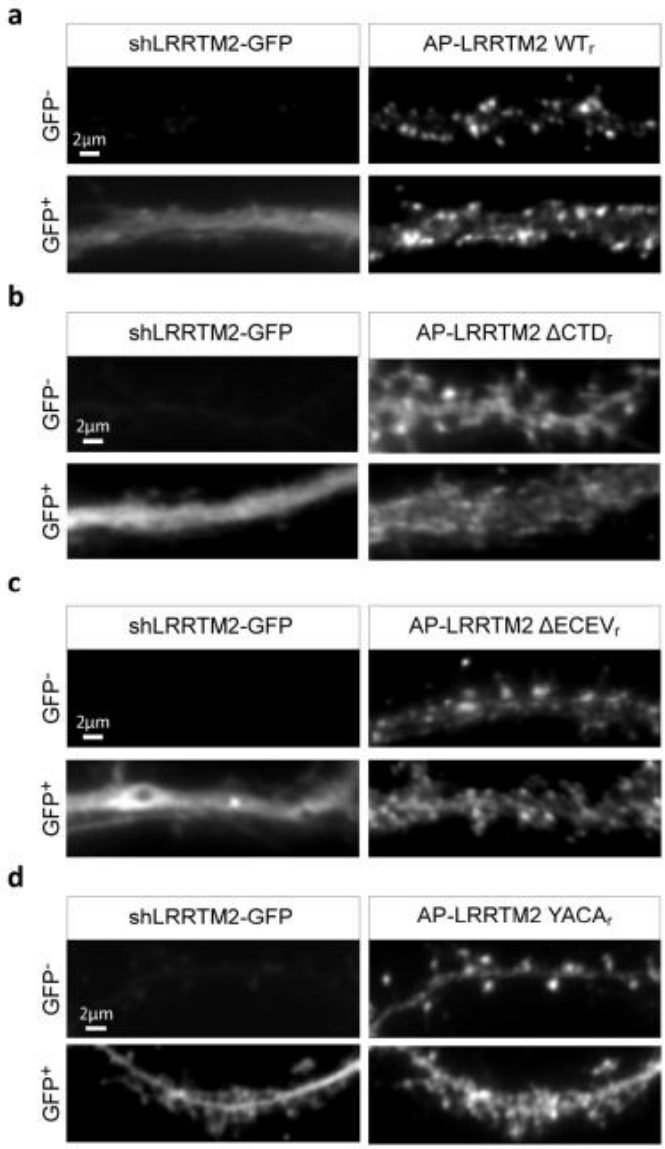
Supplementary Figure 5



Supplementary Figure 5. Clustering of AP-LRRTM2 in the absence of shLRRTM2. Surface labelling of AP-LRRTM2 WTr (a) ΔCr (b) ΔECEVr (c) and YACAr (d) in the absence (GFP-) or presence (GFP+) of shLRRTM2. In the absence of shLRRTM2 all mutants appear more clustered. Images are scaled individually for each panel.

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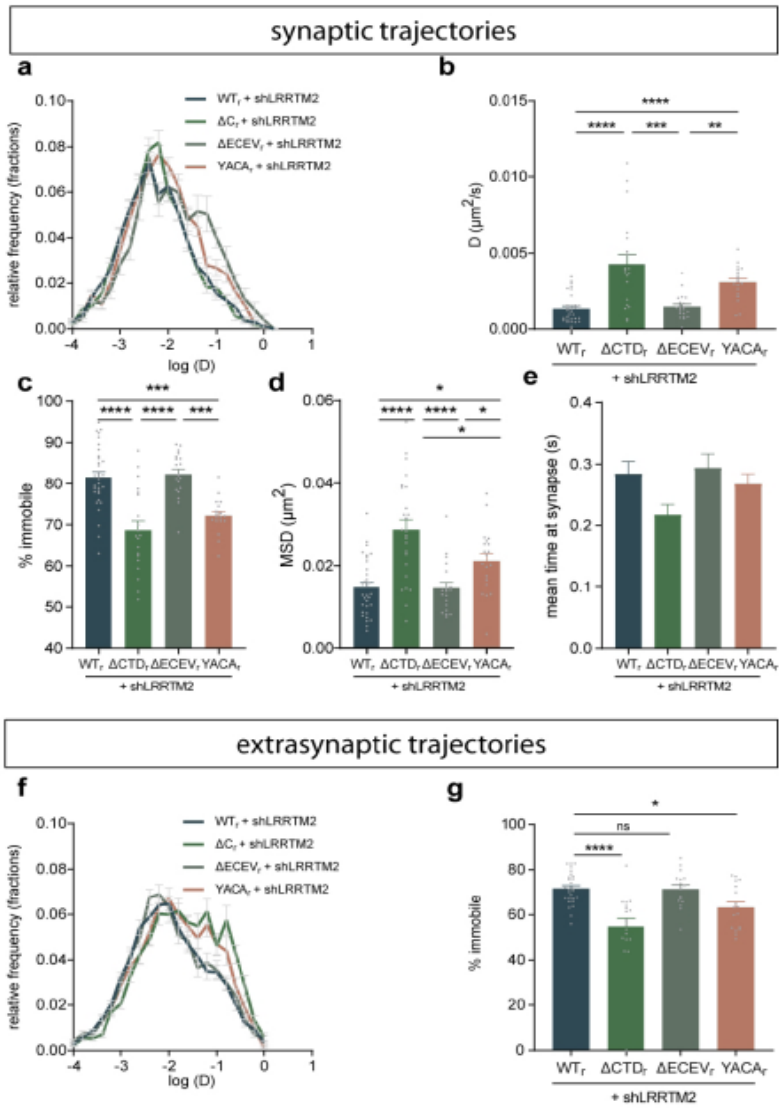
Supplementary Figure 6



Supplementary Figure 6. Synaptic diffusion of AP-LRRTM2 mutants. (a) Semi-log plot of diffusion coefficient distributions for synaptic AP-LRRTM2-WTr, AP-LRRTM2-ΔCTDr, AP-LRRTM2-ΔECEVr and AP-LRRTM2-YACAr (b) median diffusion coefficient showing an increase in diffusion in the ΔCTDr and YACAr mutant conditions (c) Percentage of synaptic immobile trajectories and (d) Mean squared displacement at t = 0.2sec. (e) Mean tra-jectory time spent at synapses for each LRRTM2 mutant (f) Semi-log distribution of diffusion coefficients for extra-synaptic AP-LRRTM2-WTr, AP-LRRTM2-ΔCTDr, AP-LRRTM2-ΔECEVr and AP-LRRTM2-YACAr (g) Percentage of synaptic immobile trajectories for the different conditions.

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2. Trafficking of LRRTM2 and LRRTM2-dependent stabilisation of AMPARs at excitatory synapses

Synaptic transmission relies on the molecular composition and sub-synaptic organisation of synaptic elements. Synaptic adhesion molecules are key organisers of synapse organisation and function, yet our knowledge on their precise and specific regulation within synapses is still incomplete. Most of the studies investigating the role of LRRTM2 in synaptic transmission have generated double knock-out of both LRRTM1 and LRRTM2, the main isoforms of the CA1 region of the hippocampus (Laurén et al. 2003). As a result, the exact contribution of each isoform to synaptic function is not clear. In the previous chapter, we demonstrated the role of the intracellular region of LRRTM2 in its membrane dynamics. However, how does LRRTM2 reach the surface of synapses in the first place? Synaptic adhesion molecules have also been shown to contribute to synaptic organisation and transmission by recruiting glutamate receptors (Mondin et al. 2011; Xiaobing Chen et al. 2015). At the nanoscale, synaptic adhesion molecule neuroligin1 has been shown to regulate AMPARs localisation in front of the pre-synaptic release machinery (Haas et al. 2018). Down-regulation of LRRTM2 has been shown to decrease surface AMPARs resulting in impaired AMPAR-mediated currents (Soler-Llavina et al. 2013). In addition, LRRTM2 was suggested to stabilise AMPARs (Bhouri et al. 2018) and to regulate synaptic strength by positioning AMPARs at the sub-synaptic level (Ramsey et al. 2021).

Here, we provide further insights into the trafficking of LRRTM2 to the surface and its interplay with synaptic AMPARs, using a unique cKO model of LRRTM2 in combination with live-cell imaging and single-molecule tracking.

Provided that most of the previously used models to study the role of LRRTM2 in synapse organisation and function were knocking-out/down both LRRTM1 and LRRTM2, we developed a novel mouse model to specifically knock-out LRRTM2 using the Cre-Lox system and replaced the endogenous protein with mutated counterparts of either the intracellular or the extracellular region.

We showed that conditional knock-out (cKO) of LRRTM2 during synaptogenesis impairs excitatory synapse development and function, confirming the results obtained with the KD strategy. We then showed that the C-terminal, but not the LRR domain, is responsible for synaptic clustering and confirmed using super-resolution imaging that LRRTM2 diffusion and confinement at synapses is independent from its PDZ-like binding motif, as previously found with the shRNA approach (see results section, chapter 1). To further study the trafficking of LRRTM2 to the membrane, we combined live-cell imaging with a pH-change protocol and revealed a substantial intracellular pool of LRRTM2 localised in spines, although its role remains unclear. Interestingly, we found that the YxxC involved in LRRTM2 synaptic confinement, also regulates its trafficking and exocytosis.

Initially, we had hypothesised that LRRTM2 and AMPARs could traffic together to the plasma membrane; however, a series of observations appeared against this scenario. We thus focused on how LRRTM2 could regulate AMPARs directly at the plasma membrane. LRRTM2 has been suggested to interact with AMPARs in heterologous cells via its extracellular domain (de Wit et al. 2009). Although we could reproduce these findings in heterologous cells using biochemistry, we did not succeed in mapping the extracellular regions involved in this putative interaction and we could not co-precipitate AMPARs and LRRTM2 in neuronal preparations. To examine whether LRRTM2 stabilises AMPARs at the plasma membrane, we used FRAP imaging and confirmed the role of LRRTM2 in synaptic AMPARs stabilisation, as previously suggested (Bhouri et al. 2018), and we showed for the first time that this stabilisation depends on both the recently identified neurexin-binding interface (E348) and the 9th LRR domain of LRRTM2, previously thought to mediate Neurexin binding (Siddiqui et al. 2010). Note that in this project we generated a LRRTM2 mutant to abolish neurexin binding based on the recent crystallised complex of neurexin1β-LRRTM2 (A. Yamagata et al. 2018) and found that mutation of E348 completely abolishes Neurexin binding, as expected from Yamagata et al., whereas mutation of D260 and T262 to Alanines, corresponding to the mutant initially described by Siddiqui et al., and used in subsequent papers to assess the role of Neurexin binding interface on LRRTM2, did not abolish Neurexin binding in our experiments. Our results, in agreement with Yamagata et al., question the dependence of LRRTM2 function at synapses on Neurexin binding found in previous studies

(Siddiqui et al. 2010; Soler-Llavina et al. 2013; Bhouri et al. 2018) and show that trans-synaptic interactions between Neurexin and LRRTM2 involving the E348 residue are important for stabilising AMPARs at the synapse, yet the interface containing D260 and T262 residues is equally involved in this stabilisation process possibly through different interactions yet to be identified.

2.1 Generation and characterisation of LRRTM2 Floxed mice

To specifically study the role of LRRTM2 in synapse development and function, we generated a conditional knock-out (cKO) mouse model, where exon 2 was flanked by two loxP sites on a C57BL/6 background (**Figure 28a**). This design was chosen because mouse LRRTM2 gene consists of only 2 exons, with the first one covering part of the 5' UTR, the ATG translation initiation codon and an additional nucleotide (guanine), while the protein-coding region resides in exon 2 (Lauren et al., 2003). As expected, LRRTM2$^{Flox/Flox}$ mice developed normally and the mutation had no impact on their fertility. To verify that LRRTM2 expression was impaired in the presence of Cre recombinase, we performed quantitative reverse transcriptase PCR (RT-qPCR) on DIV15 hippocampal cultures from LRRTM2 $^{Flox/Flox}$ mice to measure the RNA levels of LRRTM2. Infection with Cre expressing lentiviruses dramatically reduced LRRTM2 mRNA levels in a dose-dependent manner (5k: 61.45 ± 0.32%, n=2; 10k: 45.75 ± 5.8%, n=3; 50k: 17.94 ± 2%, n=2, 70k: 17.67 ± 0.8%, n=2 normalised to control, n=3 independent experiments; $p < 0.001$) (**Figure 28b, c**). We further assessed protein levels upon infection with Cre recombinase in organotypic hippocampal slices using Western Blot and found an 82% decrease in LRRTM2 protein levels compared to non-infected control slices **(Figure 28d, e**).

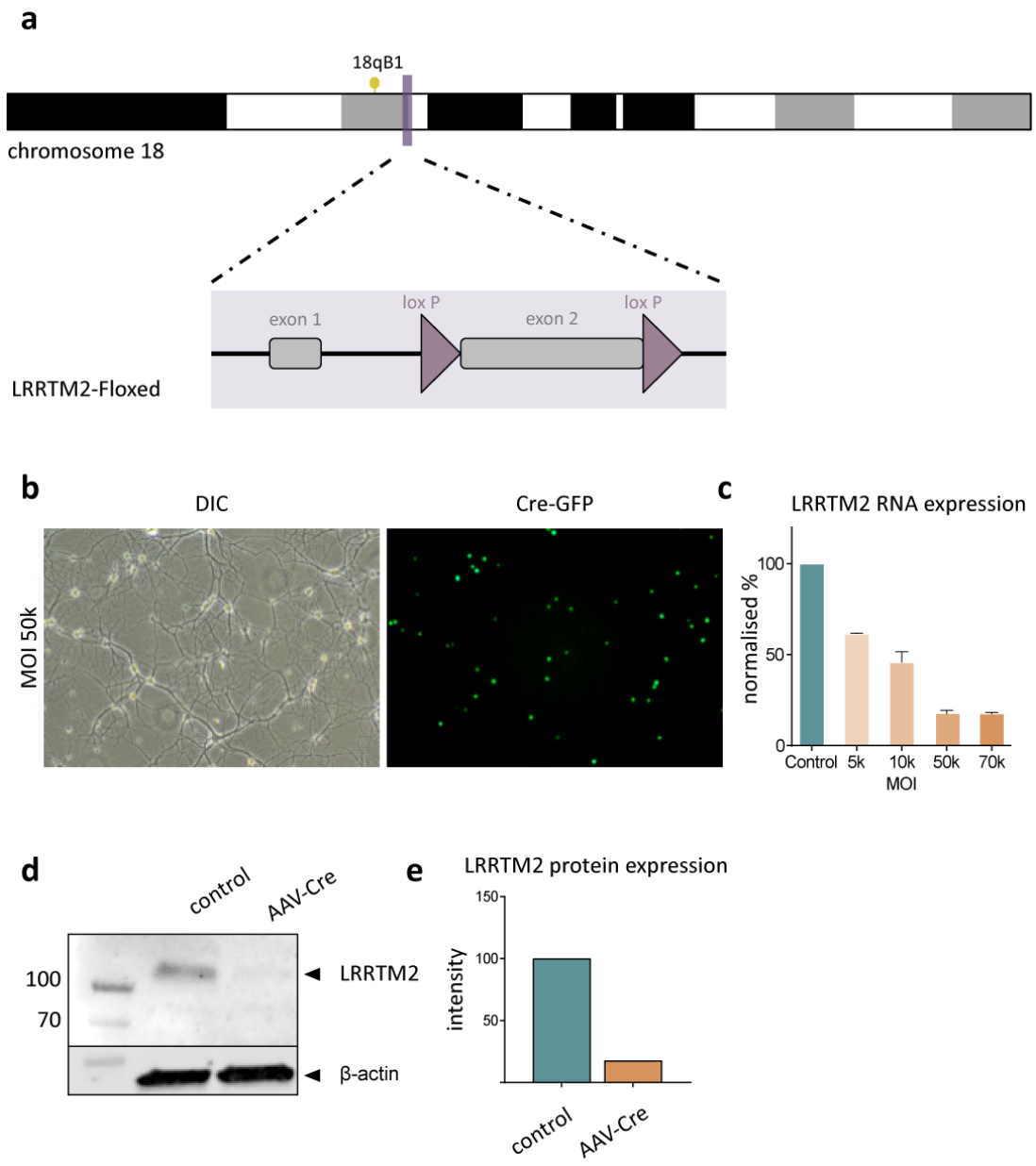

Figure 28. Characterisation of LRRTM2-Floxed mouse model. (a) Schematic representation of the location of LRRTM2 gene locus in mouse chromosome 18. Purple inset shows the 2 exons of LRRTM2 gene and the insertion of loxP sites upstream and downstream of exon 2. (b) Representative images of cultured hippocampal neurons from LRRTM2-Floxed mice infected with a nuclear Cre-GFP-encoding virus. (c) RT-qPCR quantification of LRRTM2 RNA expression in control neurons (control) or in neurons infected with an increasing dose of Cre-GFP-encoding virus (MOI: 5k, 10k, 20k, 50k and 70k). Data acquired from at least 2 experiments for each condition. MOI: multiplicity of infection. (d) LRRTM2 and β-actin immunoblots of proteins extracted from organotypic hippocampal slices not infected (control) or infected with an AAV-Cre virus (AAV-Cre). Data acquired from at 1 experiment.

2.2 Conditional knock-out of LRRTM2 specifically impairs excitatory synapse development and function

To specifically address the role of LRRTM2 in synapse formation and function, we conditionally knocked-out (cKO) LRRTM2 during synaptogenesis. Cre recombinase expression in DIV7 dissociated LRRTM2$^{Flox/Flox}$ hippocampal neurons reduced excitatory synapse density in mature (DIV15) neurons by ~ 30%, assessed by co-labelling endogenous PSD-95 and VGlut1 (control: 0.36 ± 0.01, n=50; Cre: 0.25 ± 0.15, n=39) (**Figure 29a, b**). Furthermore, surface AMPA receptor density was substantially reduced as assessed by the labelling of endogenous GluA1/2 subunits (normalised control: 1 ± 0.04, n=31; cKO: 0.76 ± 0.05, n=16) (**Figure 29c, d**) and AMPAR mEPSC frequency was impaired in Cre-expressing cells (control: 19.5 ± 0.04, n=17; Cre: 11.5 ± 0.05, n=13) (**Figure 29e, f**). These effects were all rescued by re-expressing a WT biotin-acceptor-tagged form of LRRTM2 (Cre+LRRTM2-WT) that allowed for labelling of the protein (**Figure 29a-d**). The Biotin-acceptor-tagged LRRTM2 was described in previous work (Chamma et al. 2016). Briefly, a biotin acceptor peptide tag (AP) is inserted at the N-terminal of LRRTM2 co-expressed with an ER-restricted biotin-ligase enzyme that covalently adds biotin on the AP tag, in the ER. Biotinylated LRRTM2 can thus be labelled at the cell surface using monomeric forms of streptavidin for imaging purposes and to validate the presence of the rescue construct (Lim et al. 2011; Chamma et al. 2017). Re-expression of WT-LRRTM2 rescued excitatory synapse density, surface AMPAR density and mEPSC frequency (**Figure 29e, f**). Thus, these results indicate that disruption of LRRTM2 during synapse development impairs excitatory synapse formation and function. Finally, genetic invalidation of LRRTM2 expression did not affect inhibitory synapse development (**Figure 30**), as previously shown using a knock-down approach (de Wit et al. 2009), confirming a specific effect of LRRTM2 on excitatory but not inhibitory synaptic transmission.

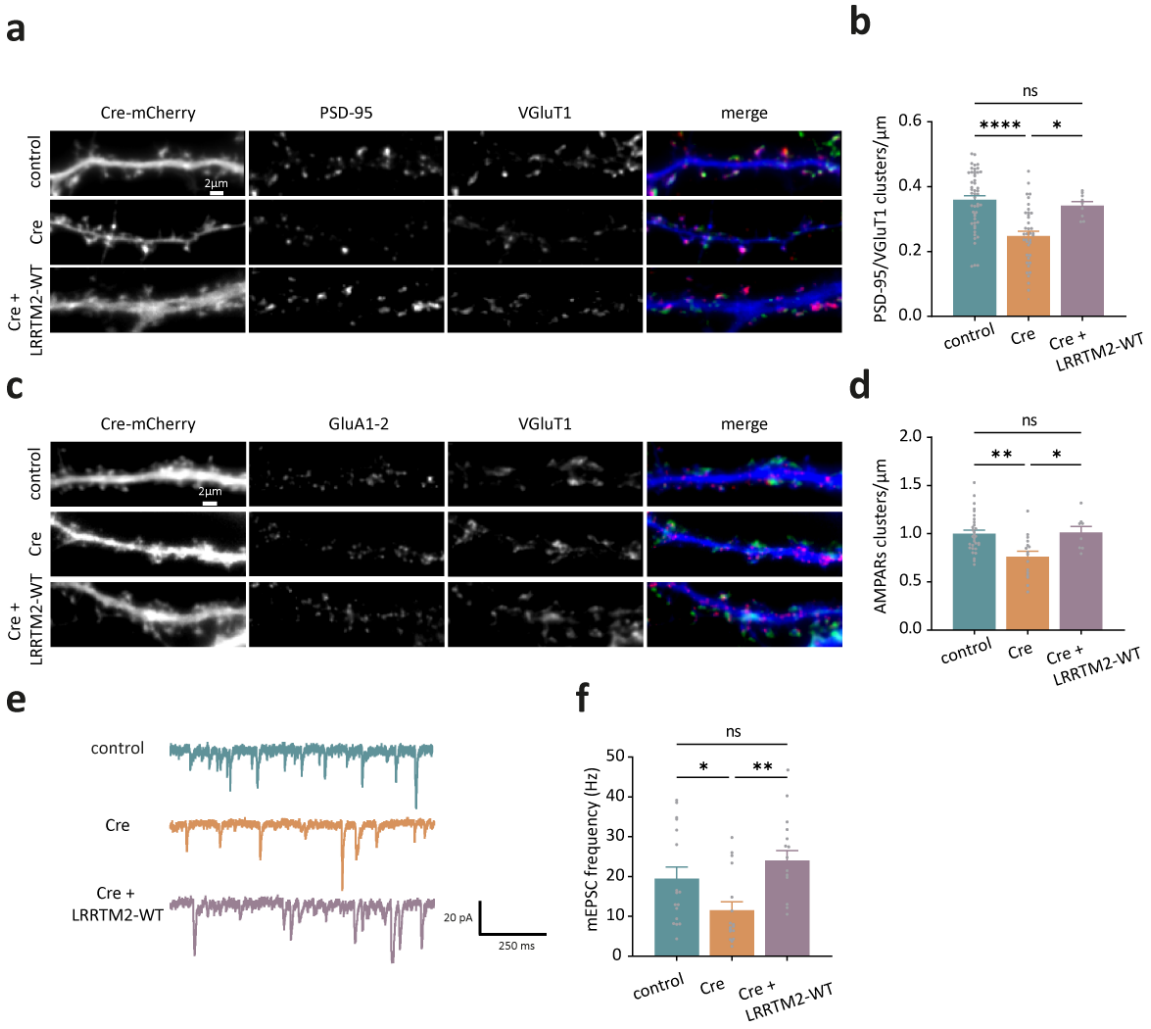

Figure 29. Conditional knock-out of LRRTM2 impairs excitatory synapse development and function. (a) DIV15 hippocampal neurons expressing mCherry (control), Cre-mCherry (Cre) or Cre-mCherry and biotinylated AP-LRRTM2 (Cre+LRRTM2-WT) were immunostained for endogenous PSD-95 and VGlut1 as a pre- and post-synaptic marker respectively. On the right, cre-mCherry (blue) signal is overlaid with PSD-95 (red) signal and VGluT1 (green) signal. (b) Quantification of synaptic density measured as the apposition of PSD-95 and VGluT1 puncta. Data acquired from at least 2 independent experiments (control: n=49, Cre: n=38, Cre+LRRTM2-WT=9 cells) **** $p<0.0001$. (c) DIV15 hippocampal neurons expressing mCherry (control), Cre-mCherry (Cre) or Cre-mCherry and biotinylated AP-LRRTM2 (Cre+LRRTM2-WT) were immunostained for endogenous GluA1/2 subunits of AMPARs and VGluT1. On the right, Cre-mCherry (blue) signal is overlaid with GluA1/2 (red) signal and VGluT1 (green) signal. Data acquired from 3 independent experiments (control: n=31, Cre: n=16, Cre+LRRTM2-WT=8 cells) ** $p<0.005$. (d) Quantification of normalised AMPARs density. (e) Representative mEPSC traces recorded from DIV15 hippocampal neurons expressing mCherry (control), Cre-mCherry (Cre) or Cre-mCherry and biotinylated AP-LRRTM2 (Cre+LRRTM2-WT). (f) mEPSC mean frequency for control, Cre and Cre+LRRTM2-WT showing a decrease in the frequency in the cKO. Data acquired from 2 independent experiments (control: n=17, Cre: n=17, Cre+LRRTM2-WT=13 cells) ** $p<0.005$

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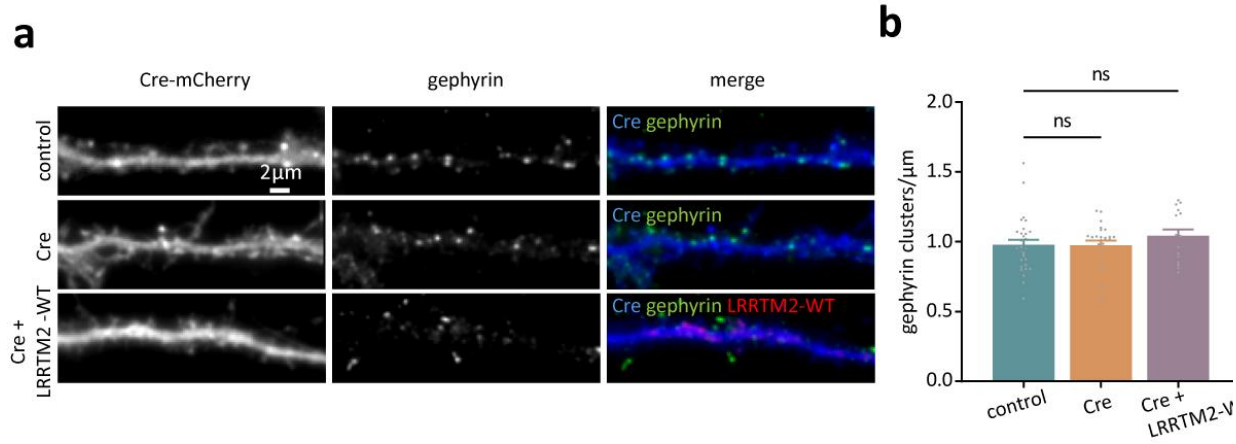

Figure 30. LRRTM2 cKO does not affect inhibitory synapse density. (a) DIV15 hippocampal neurons expressing mCherry (control), Cre-mCherry (Cre) or Cre-mCherry and biotinylated AP-LRRTM2 (Cre+LRRTM2-WT) were immunostained for endogenous gephyrin as marker for inhibitory synapses. On the right, Cre-mCherry (blue) signal is overlaid with gephyrin (green) signal and for the Cre+LRRTM2-WT condition, with AP-LRRTM2 (red) signal. (b) Quantification of inhibitory synaptic density measured as the number of gephyrin clusters/µm. Data acquired from at least 2 independent experiments (control: n=30, Cre: n=26, Cre+LRRTM2-WT=16 cells).

2.3 The C-terminal, but not the extracellular LRR domain is responsible for synaptic clustering of LRRTM2

To examine the mechanisms of LRRTM2 regulation at excitatory synapses, we generated AP-tagged mutants of the C- and N- terminal domains of the protein (AP-LRRTM2-ΔC, AP-LRRTM2-ΔLRR) and immunostained endogenous PSD-95 as a post-synaptic marker (**Figure 31a**). In a previous study (Liouta et al. 2021), we had shown that LRRTM2 C-terminal domain was important for membrane stabilisation and clustering using a knock-down strategy, but we had not explored the role of the extracellular LRR domain. Here, we confirm using a conditional knock-out approach, that deletion of the C-terminal domain, but not that of the extracellular LRR domain, impairs LRRTM2 synaptic localisation and clustering (**Figure 31**). Thus, our results

indicate that LRRTM2 clustering and localisation at synapses depend on the C-terminal domain and are independent of interactions in the extracellular LRR domain. Furthermore, we examined the influence of the two previously described motifs in LRRTM2 C-terminal domain, the PDZ-like binding motif ECEV (ΔECEV) and the YxxC motif (YACA) shown to regulate LRRTM2 membrane expression and diffusion (Minatohara et al. 2015; Liouta et al. 2021). Both mutants exhibited decreased synaptic clustering to similar levels as deletion of the entire CTD (ΔC), however this decrease was not significant compared to the WT condition and remains to be confirmed (**Figure 32**). We believe that this could come from a lower number of cells in these conditions, as we had previously observed that these mutants significantly increase LRRTM2 density (Liouta et al. 2021). Thus, these results are hard to interpret given the low number of cells and the heterogeneity between them.

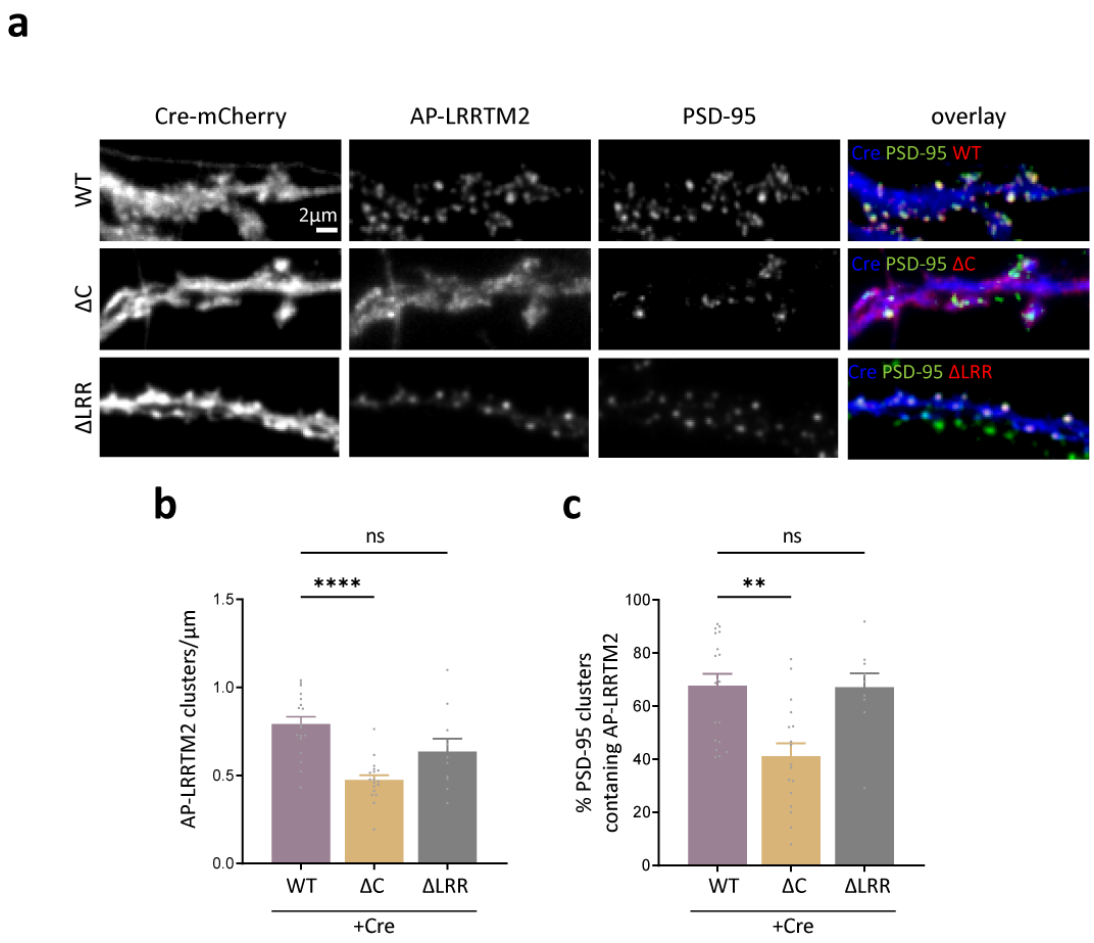

Figure 31. The C-terminal, but not the LRR domain, is responsible for synaptic LRRTM2 clustering. (a) DIV15 hippocampal neurons expressing Cre-mCherry, biotinylated AP-LRRTM2 (WT, ΔC or ΔLRR) and immunostained for endogenous PSD-95 as a synaptic marker. On the right, Cre-mCherry (blue) signal is overlaid with PSD-95 (green) signal and AP-LRRTM2 mutants' (red) signal showing increased synaptic localisation for the WT and ΔLRR. (b) Quantification of AP-LRRTM2 mutants' cluster density. (c) Percentage of PSD-95 positive clusters colocalised with AP-LRRTM2 mutants clusters. Data acquired from 3 experiments (WT: n=18, ΔC: n=18, ΔLRR=10 cells). **** p<0.0001

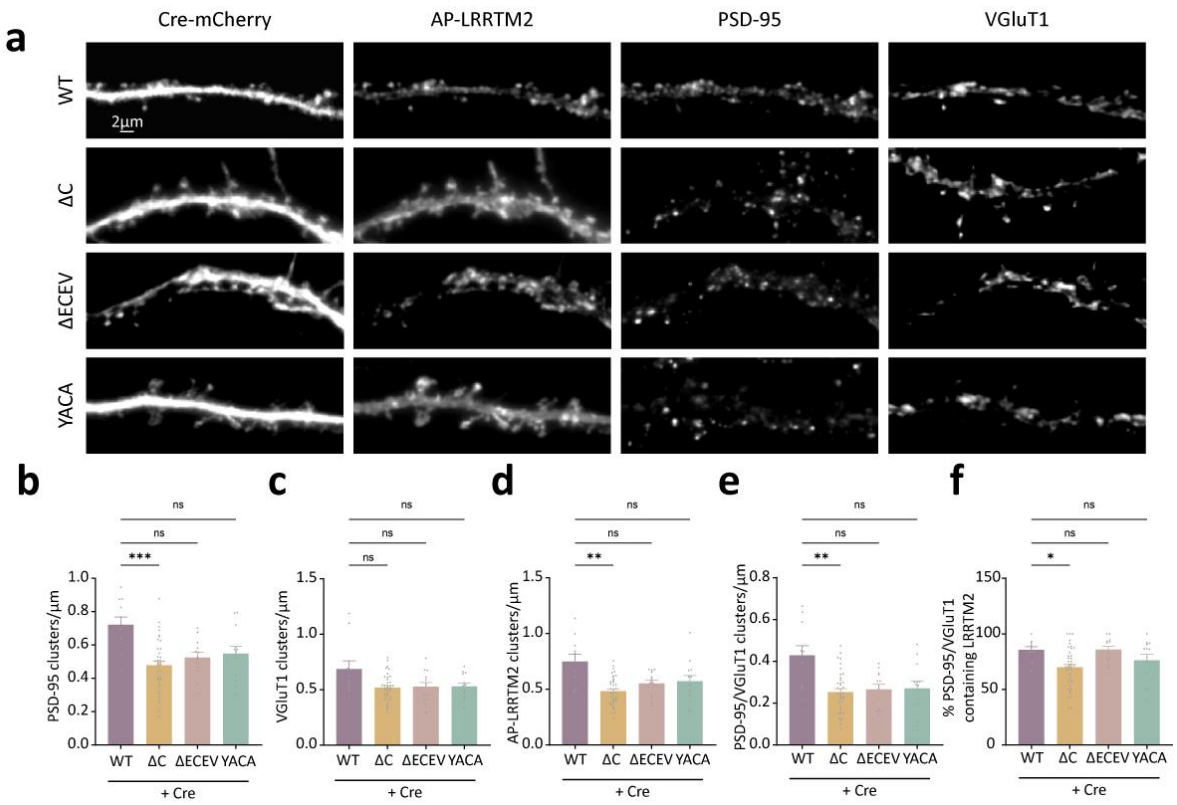

Figure 32. C-terminal motifs responsible for synaptic LRRTM2 clustering. (a) DIV15 hippocampal neurons expressing Cre-mCherry, biotinylated AP-LRRTM2 (WT, ΔC, ΔECEV or YACA) and immunostained for endogenous PSD-95 and VGluT1 as synaptic markers. Quantification of (b) PSD-95 density, (c) VGluT1 density, (d) AP-LRRTM2 density, (e) PSD-95/VGluT1 density and (f) percentage of synaptic LRRTM2 mutants. (c) Percentage of PSD-95 positive clusters colocalised with AP-LRRTM2 mutants clusters. Data acquired from at least 2 experiments (WT: n=1, ΔC: n=38, ΔECEV: n=13, YACA: n=13 cells). *** p<0.0005

2.4 The YxxC, but not the PSD-95-binding motif regulates LRRTM2 confinement

In a previous study, we had shown that the YxxC motif in the intracellular domain is responsible for confinement of LRRTM2 (Liouta et al. 2021). However, due to incomplete knock-down of the endogenous protein with the shRNA against LRRTM2, the effects that we observed could have been biased by the presence of endogenous proteins. Thus, we re-examined this observation

here, using a more rigorous cKO model, where LRRTM2 is genetically invalidated and replaced with mutated counterparts. We tracked single molecules of LRRTM2 using uPAINT and fluorophore-conjugated monomeric streptavidin (Chamma et al. 2016; Gregory Giannone et al. 2010) in live hippocampal neurons from LRRTM2$^{Flox/Flox}$ mice expressing Cre recombinase, Homer-1c-DsRed as a synaptic marker and WT, ΔC, ΔECEV, or YACA -LRRTM2 (**Figure 33a**). Deletion of the entire C-terminal domain resulted in increased overall diffusion, reduction of immobile trajectories and increased confinement (**Figure 33b-e**). Similar effects were observed with mutation of the YxxC motif, suggesting that this motif in the C-terminal domain is, at least partially, responsible for LRRTM2 confinement. However, deleting the PDZ-like binding motif ECEV had no effect on the analysed diffusion parameters (**Figure 33b-e**), indicating, as we had previously observed, that the ECEV motif is not involved in LRRTM2 confinement. Further analysis of the synaptic diffusion (**Figure 34a-d**) showed that ~ 80% of the WT and ΔECEV trajectories are synaptic, whereas a decrease of ~15% was found for the ΔC and YACA condition (**Figure 34g**), suggesting that deletion of the CTD or mutations in the YxxC motif shift LRRTM2 trajectories towards the extrasynaptic sites. Analysis of extrasynaptic diffusion showed a clear increase in the diffusion coefficient for the ΔC and YACA condition, whereas the ΔECEV behaved similar to the WT (**Figure 34e**). However, analysis of the synaptic diffusion showed a clear decrease in diffusion coefficient for the ΔC mutant (**Figure 34a**), although the MSD was increased for both the ΔC and YACA condition (**Figure 34c, d**).

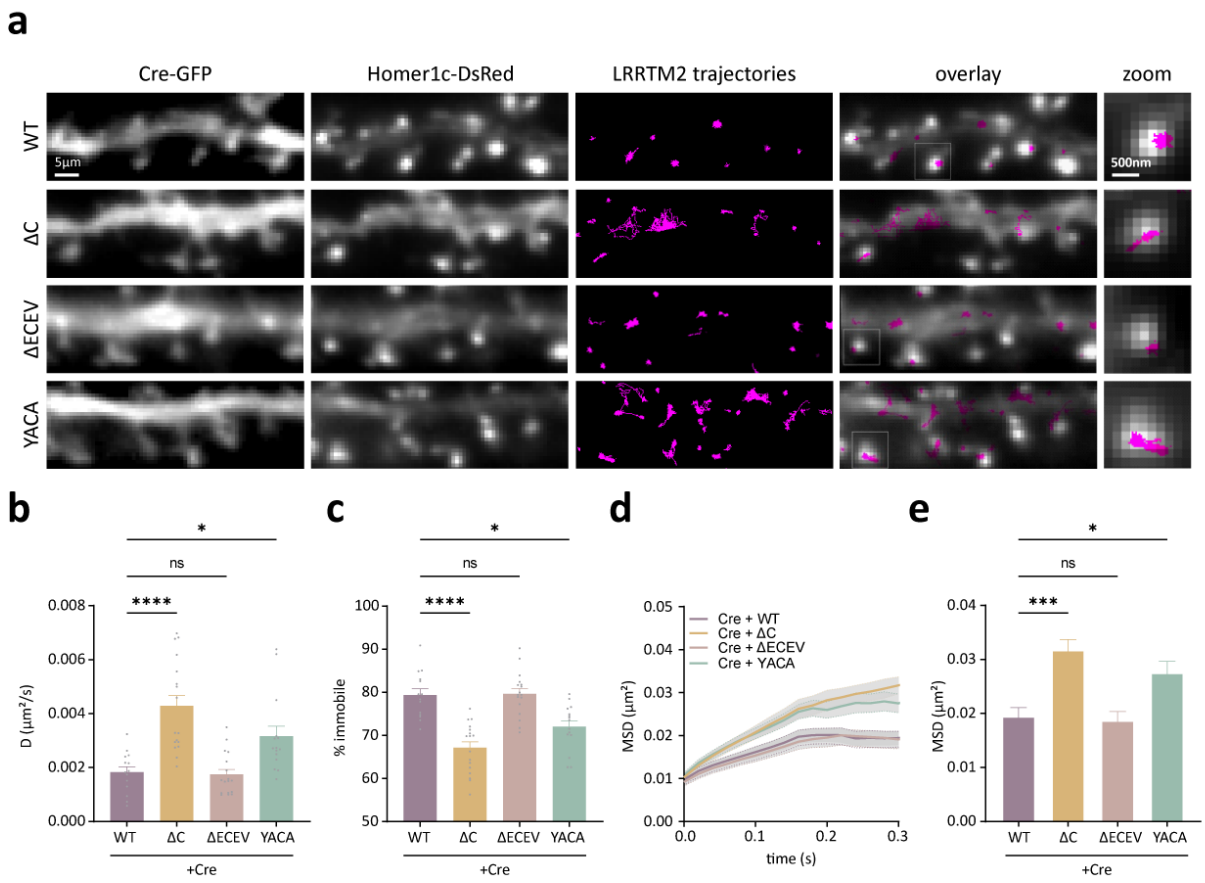

Figure 33. LRRTM2 diffusion and confinement at synapses is independent from its PDZ-like binding motif. (a) Representative examples of DIV15 neurons expressing Cre-GFP, Homer1c-DsRed and AP-LRRTM2 (WT, ΔC, ΔECEV or YACA) labelled with mSA-STAR635P to track individual molecules by uPAINT. Homer1c-DsRed (grey) signal is overlaid with AP-LRRTM2 trajectories (magenta); inserts show individual synapses together with synaptic AP-LRRTM2 trajectories. (b) Median diffusion coefficient of AP-LRRTM2-WT, AP-LRRTM2-ΔC, AP-LRRTM2-ΔECEV and AP-LRRTM2-YACA, showing an increase with the ΔC and YACA mutants. (c) Percentage of immobile trajectories for the different conditions. (d) Mean square displacement of the different conditions over time (e) Mean square displacement of the different conditions at t=0.2sec. Data acquired from at least 3 experiments (WT: n=14, ΔC: n=17, ΔECEV: n=17, YACA: n=15 cells). **** p<0.0001

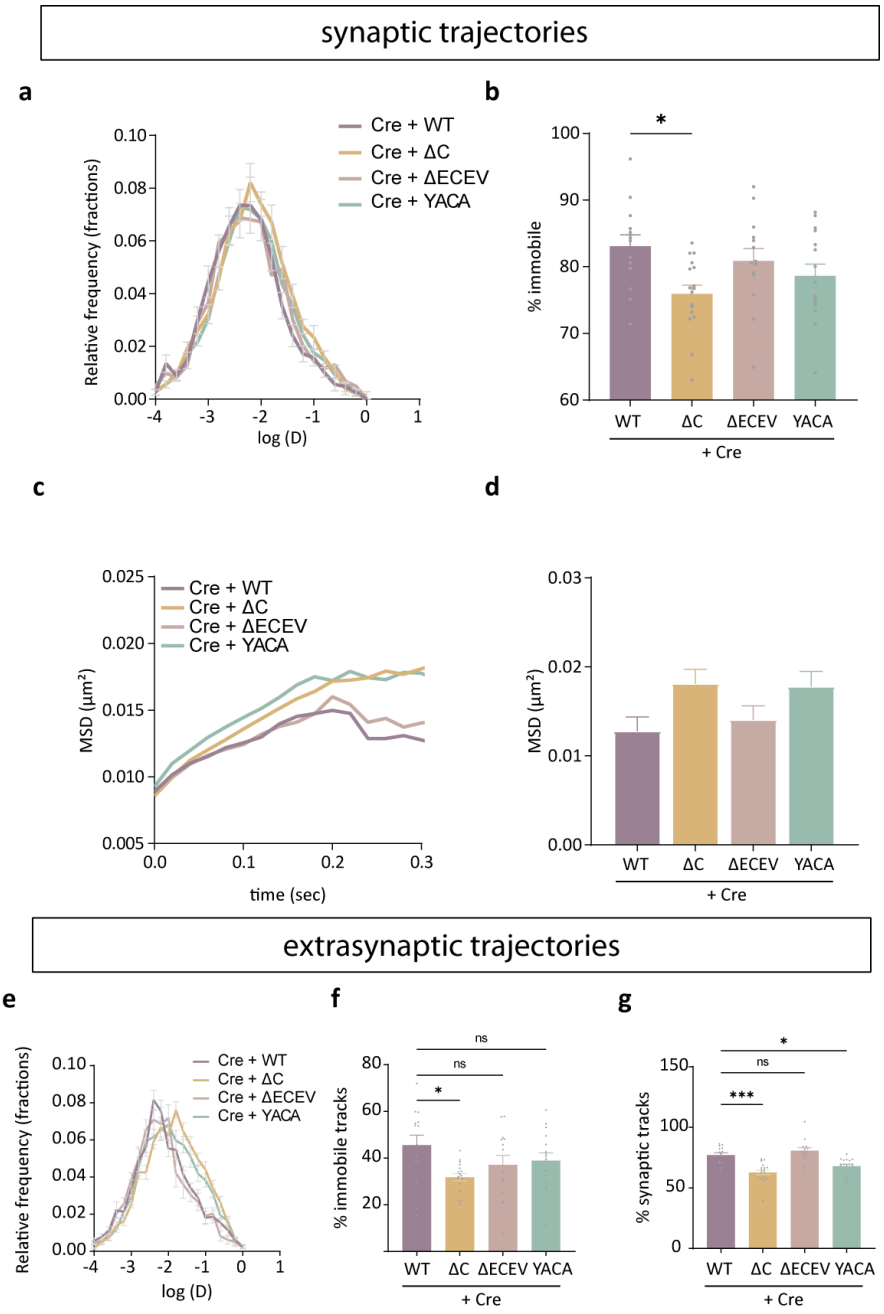

Figure 34. Synaptic and extrasynaptic LRRTM2 diffusion. (a) Semi-log distribution of synaptic diffusion coefficients for Cre+WT, Cre+ΔC, Cre+ΔECEV and Cre+YACA, showing an increase with the ΔC mutant. (b) Percentage of immobile trajectories for the different conditions. (c) Mean square displacement of the different conditions over time (d) Mean square displacement of the different conditions at t=0.2sec. Data acquired from at least 3 experiments (WT: n=14, ΔC: n=17, ΔECEV: n=17, YACA: n=15 cells).* p<0.05 (e) Semi-log distribution of extrasynaptic diffusion coefficients for Cre+WT, Cre+ΔC, Cre+ΔECEV and Cre+YACA. (f) Percentage of immobile tracks for the different conditions. (g) Percentage of synaptic tracks for the different conditions. WT: n=14, ΔC: n=17, ΔECEV: n=17, YACA: n=15 cells). * p<0.05

2.5 The YxxC motif regulates LRRTM2 membrane turnover in spines

The YxxC motif was initially identified as a critical sequence regulating LRRTM2 surface expression (Minatohara et al. 2015). In this study, the internalisation ratio of LRRTM2-YACA, assessed by biotinylation assay in HEK cells, was found to be comparable to that of LRRTM2-WT, suggesting that LRRTM2-YACA was normally accessible to the internalisation trafficking pathway (Minatohara et al. 2015). To determine whether the YxxC motif had an effect on LRRTM2 exocytosis, we generated pH-sensitive phluorin-tagged SEP-LRRTM2-WT and SEP-LRRTM2-YACA and expressed them on a LRRTM2 cKO background in dissociated neurons.

To determine the impact of the YxxC motif on membrane turnover of LRRTM2 at synapses, we performed Fluorescence Recovery After Photobleaching (FRAP) of SEP-tagged LRRTMs localised at synapses (**Figure 35a-c**). Interestingly, the recovery of SEP-LRRTM2-WT was comparable to that of surface labelled AP-LRRTM2-WT (**Figure 35**; Chamma et al., 2016), suggesting that exocytosis of WT LRRTM2 at synapses within this timeframe is negligible and consistent with our results showing that the majority of LRRTM2 is localised at the surface (**Figure 37d**). However, when we performed the same FRAP experiments using SEP-LRRTM2-YACA, the fluorescence recovery was drastically increased at synapses and on the shaft, indicating an increase in exocytosis rate and/or an increase in diffusion (**Figure 35c, d**).

To isolate the exocytic component from the membrane diffusion behaviour and characterise in more detail the exocytosis of SEP-LRRTM2-YACA without the crowded synaptic environment, we performed whole FRAP experiments in COS-7 cells. In this paradigm, the whole field of view is frapped to bleach surface molecules. The intracellular SEP-tagged molecules are preserved from bleaching as they remain in a pH-dependent fluorescence-quenched form inside intracellular vesicles (**Figure 36a, b**). Using this strategy, we found that the frequency of exocytic events in this experimental paradigm was increased more than 3-fold in the YxxC mutant compared to WT-LRRTM2, whereas the amplitude of events was doubled (**Figure 36c, d**), suggesting that more LRRTM2 is released faster upon mutation of the YxxC motif. These results suggest for the first time that the YxxC motif regulates LRRTM2 exocytosis. In addition, SEP-WT exocytic events

remained stable for at least 100 seconds, unlike most of SEP-YACA events that disappeared within 20 seconds after appearance on the surface (**Figure 36e, f**), suggesting that mutations in the YxxC motif impair the membrane stabilisation of newly-exocytosed LRRTM2 molecules.

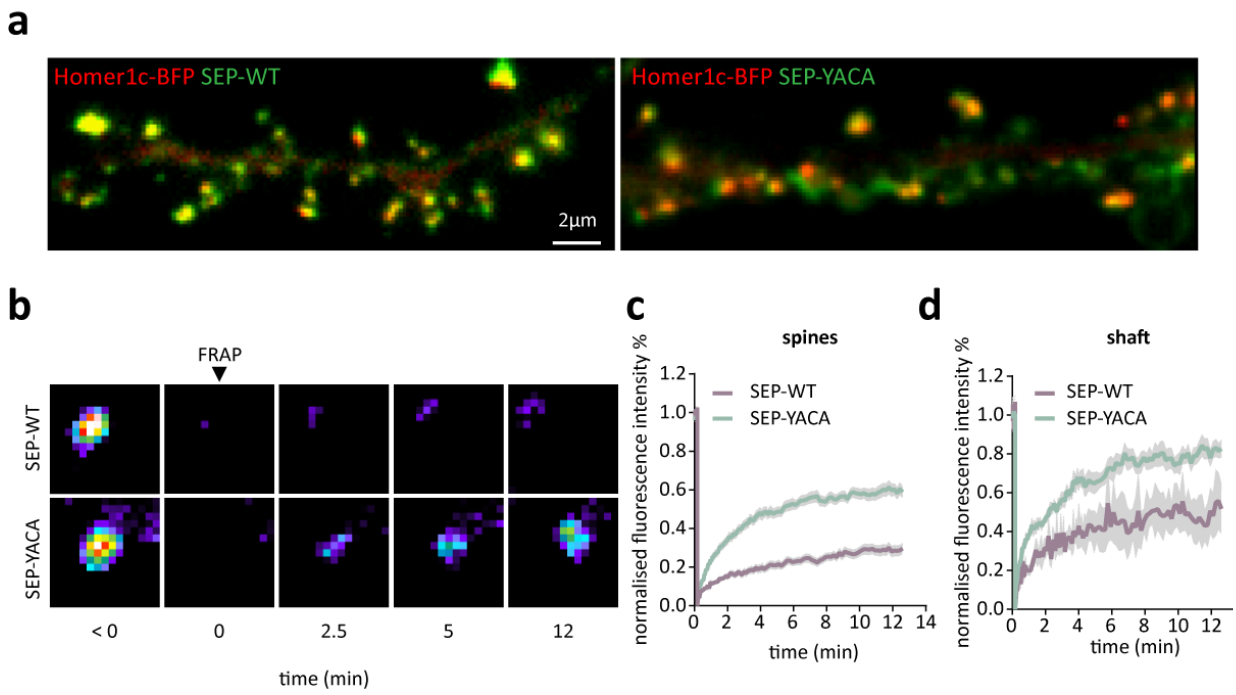

Figure 35. The C-terminal motif YxxC regulates LRRTM2 membrane turnover in spines. (a) Representative images of DIV15 hippocampal neurons expressing Cre-mCherry, Homer1c-BFP (red) and SEP-WT or -YACA (green). (b) FRAP experiments performed on synaptic SEP-LRRTM2-WT or SEP-LRRTM2-YACA expressing Cre-mCherry and SEP-LRRTM2 mutants showing a faster recovery for the YACA mutant. (c) Corresponding normalised fluorescence recovery curves in spines and (d) in the shaft. Data obtained from at least 2 different experiments (spines: SEP-WT: n=30 regions, SEP-YACA: n=27 regions; shaft: SEP-WT: n=3 regions, SEP-YACA: n=9 regions).

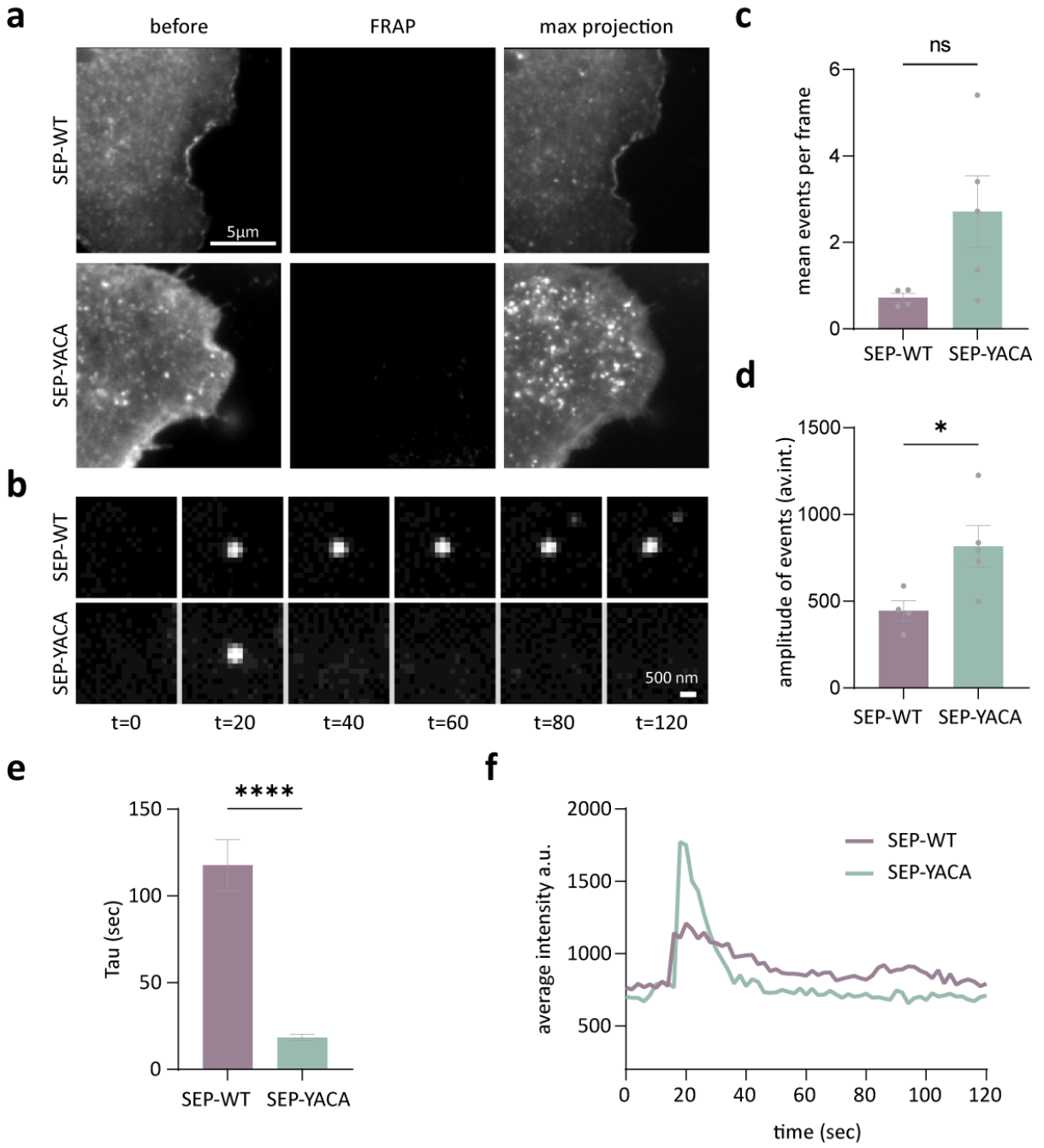

Figure 36. The C-terminal YxxC motif regulates LRRTM2 exocytosis in cos cells. (a) Representative images of cos cells expressing SEP-WT or SEP-YACA. After photobleaching of surface proteins, emergence exocytic events was monitored with time-lapse imaging. Maximum projection of the frames acquired is depicted in the right panel, showing increased exocytic events in the SEP-YACA condition. (b) Examples of individual exocytic events of SEP-WT and SEP-YACA. (c) Quantification of the mean exocytic events per frame. (d) Quantification of the amplitude of exocytic events. (e) Rate of fluorescence decay of exocytic events. (f) Example of individual exocytic events over time. Data acquired from 1 experiment. SEP-WT: n= 4 cells, SEP-YACA: n= 5 cells.

Finally, because LRRTM2-WT exhibits almost no diffusion at the plasma membrane, we reasoned that it could be released directly in close proximity to synapses by local exocytosis. To examine this hypothesis, we examined whether intracellular pools of LRRTM2 were present inside spines, where excitatory synapses form. To this aim, we applied a pH 5.5 external solution, leading to complete quenching of the SEP fluorescence (85%) followed by a solution of ammonium chloride to disrupt transmembrane proton gradients and deacidify intracellular vesicles, leading to increased SEP fluorescence (43%) and thus revealing intracellular protein pools (36% of the total pool) (**Figure 37a-d**). By subtracting the surface population from the total pool, we could access intracellular LRRTM2 pools. Using this assay, we observed that the majority of LRRTM2 is localised at the cell surface (64% ± 0.03%, n=6). However, we found a substantial pool of protein in the intracellular compartments (36% ± 0.03%, n=6). 30% of these proteins were located inside spines (neck, heads) and 70% trafficked on dendrites (**Figure 37e, f**), with synaptic vesicles being more stable compared to the ones in the dendritic shaft that seemed to be more mobile.

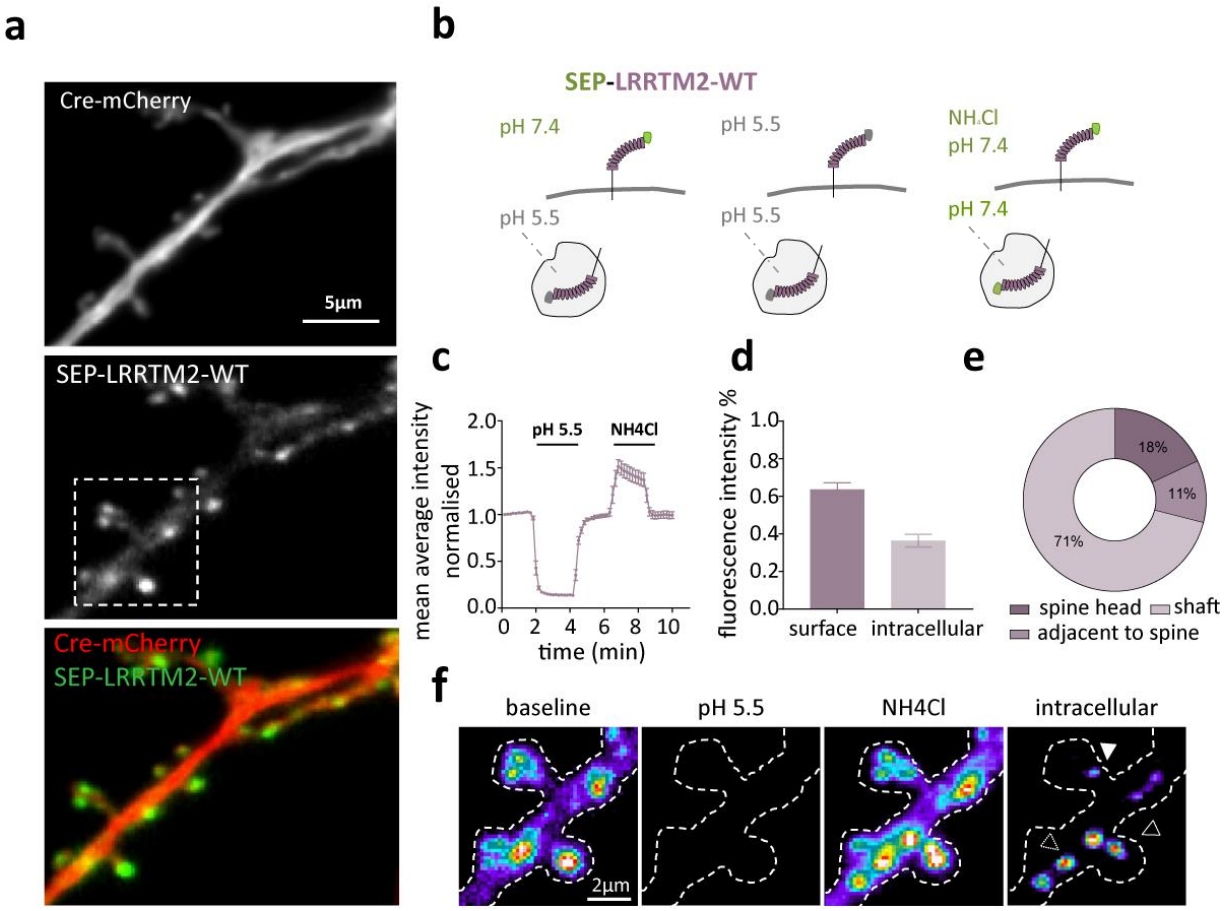

Figure 37. An intracellular pool of SEP-LRRTM2-WT in dendritic spines and shaft. (a) Representative images of DIV15 hippocampal neurons expressing Cre-mCherry (red) and SEP-LRRTM2-WT (green) Dotted square indicates the position of panel e. (b) Schematic representation of SEP-LRRTM2-WT fluorescent properties. At pH 7.4 SEP-LRRTM2 is fluorescent, unlike at pH 5.5. Bath application of pH 5.5 renders the surface SEP-tagged proteins non-fluorescent and NH4Cl de-acidifies the intracellular vesicles, rendering the SEP-tagged proteins located there fluorescent. (c) Normalised mean average intensity of SEP-LRRTM2-WT over time. (d) Percentage of fluorescent intensity for surface and intracellular SEP-LRRTM2-WT. (e) Percentage of localisation of intracellular SEP-LRRTM2-WT vesicles. Data acquired from 1 experiment and 6 cells. (f) Inset of SEP-LRRTM2-WT showing the existence of LRRTM2-containing intracellular vesicles in the shaft and in spines.

2.6 LRRTM2 controls AMPAR surface expression and synaptic stabilisation through its extracellular domain

Double knock-out of LRRTM1 and 2 has been shown to stabilise spine AMPARs (Bhouri et al. 2018), although it was not examined whether these were surface AMPARs or intracellular pools. Thus, to assess the role of LRRTM2 in stabilisation of surface synaptic AMPARs, we conditionally knocked-out LRRTM2 and performed FRAP experiments on synaptic SEP-GluA1 in the absence of LRRTM2. cKO of LRRTM2 led to an increased turnover of surface GluA1 at synapses, an effect that was rescued by re-expression of AP-LRRTM2-WT, assessed by monomeric streptavidin labelling (**Figure 38**).

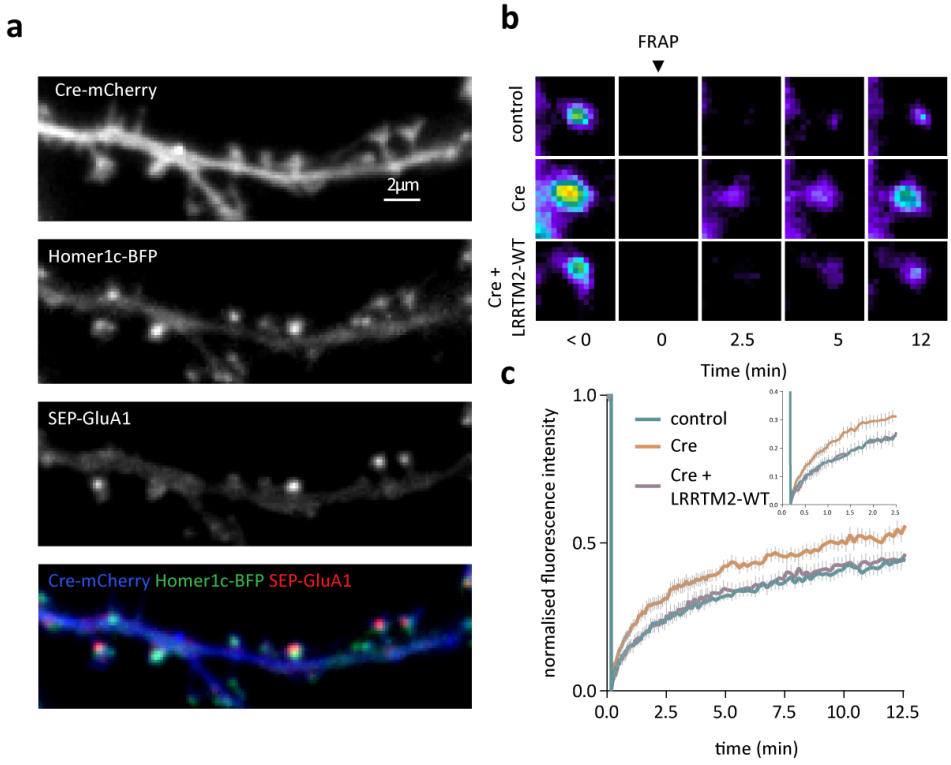

Figure 38. AP-LRRTM2 controls AMPARs stabilisation at excitatory synapses. (a) Example of DIV15 hippocampal neurons expressing Cre-mCherry (blue), Homer1c-BFP (green) and SEP-GluA1 subunit of AMPARs (red). (b) FRAP experiments performed on synaptic SEP-GluA1 expressing soluble mCherry (control), Cre-mCherry (Cre) or Cre-mCherry and biotinylated AP-LRRTM2 (Cre+LRRTM2-WT) showing rapid recovery in the absence of LRRTM2. (c) Corresponding normalised fluorescence recovery curves. Inset depicts the curves between 0 and 2.5min. Data obtained from 3 different experiments (control: n=51 regions, Cre: n= 35 regions, Cre+LRRTM2-WT: n= 19 regions).

To determine which domains of LRRTM2 are involved in AMPAR stabilisation, we used mutants of the intra- and extra-cellular domains (AP-LRRTM2-ΔC and AP-ΔLRR-LRRTM2 respectively) (**Figure 39a**) and assessed synaptic GluA1 membrane turnover by FRAP (**Figure 39b**). Consistent with previous work showing that the LRR domain, but not the C-terminal domain, is important for AMPAR stabilisation by LRRTM2 (de Wit et al. 2009; J. Ko et al. 2011; Aoto et al. 2013), deletion of the LRR domain led to the strongest destabilisation of synaptic AMPARs (50% increase in the turnover), whereas C-terminal domain deletion only led to a mild destabilization (20%) (**Figure 39c**), presumably due to the reduction observed in PSD-95 scaffolds upon LRRTM2 cKO (**Figure 30**), which are important for AMPARs stabilisation at synapses (Bats, Groc, and Choquet 2007; Xiaobing Chen et al. 2015). This effect was specific to synapses, as the turnover of dendritic SEP-GluA1 was comparable in all conditions (**Figure 39d**).

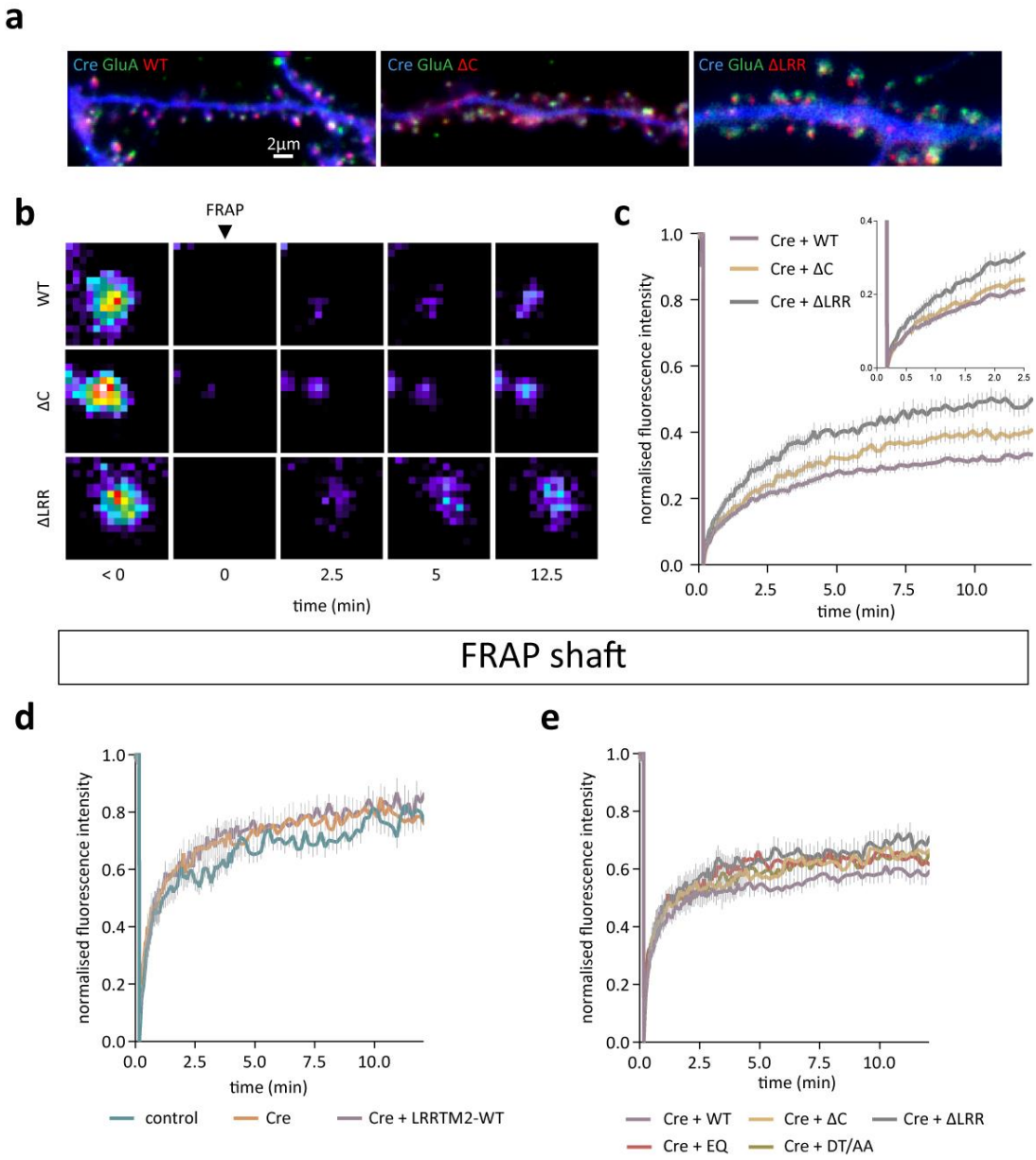

Figure 39. AP-LRRTM2 controls AMPARs stabilisation via its extracellular region. (a) Example of DIV15 hippocampal neurons expressing Cre-mCherry (blue), SEP-GluA1 subunit of AMPARs (green) and AP-LRRTM2 (WT, ΔC or ΔLRR) (red). (b) FRAP experiments performed on SEP-GluA1 expressing Cre-mCherry and AP-LRRTM2 mutants showing a faster recovery in the ΔLRR condition. (c) Corresponding normalised fluorescence recovery curves showing an intermediate recovery for the ΔC. Inset depicts the curves between 0 and 2.5min. Data obtained from 3 different experiments. WT and ΔLRR are the same datasets presented in the main figures (Cre+WT: n=52 regions, Cre+ΔC: n= 28, Cre+ΔLRR: n= 27 regions). (d) Related to figure 6: Corresponding normalised fluorescence recovery curves of SEP-GluA1 in regions located on the shaft (control: n=10 regions, Cre: n= 9 regions, Cre+LRRTM2-WT: n= 11 regions). (e) Related to figure 7: Corresponding normalised fluorescence recovery curves of SEP-GluA1 in regions located on the shaft (Cre+WT: n=23 regions, Cre+ΔC : n= 12 regions, Cre+ΔLRR : n= 16 regions, Cre+EQ: n=17 regions, Cre+DT/AA: n=27 regions).

AMPAR destabilisation upon deletion of the LRR domain was previously attributed to the binding of Neurexins (Nrx) to LRRTM2, since point mutations designed to impair Nrx binding in the 9th LRR motif (D260, T262) (Siddiqui et al. 2010), affected AMPAR transmission (Soler-Llavina et al. 2013; Bhouri et al. 2018). However, a recent crystallography study identified a critical residue for the Neurexin-1β/LRRTM2 interaction, located in the C-terminal cap of the extracellular LRR domain (A. Yamagata et al. 2018), questioning previous results. To examine which of these residues are important for Neurexin binding and AMPAR stabilisation, we generated mutants of the extracellular domain:AP-E348Q-LRRTM2 (EQ), containing a mutation in the C-terminal cap of the extracellular domain to disrupt Nrx-binding according to the recent work of Yamagata and colleagues (Yamagata et al. 2018) and AP-D260A/T262A-LRRTM2 (DT/AA), containing the double mutation in the 9th LRR domain widely used in previous studies (Siddiqui et al. 2010; Soler-Llavina et al. 2013; Bhouri et al. 2018). To clarify the discrepancies around the Nrx-binding site, we performed Nrx-binding assays in COS-7 cells expressing these mutants, using purified Nrx1β (-SS4) (**Figure 40a-c**). We first assessed the expression levels of the different mutants, by performing a surface labelling using mSA. In COS-7 cells, we found that the EQ mutant was properly expressed at the cell surface similarly to the WT, unlike DT/AA mutant whose surface expression was decreased by 50% (**Figure 40b**). In the WT condition, purified Nrx was detected on all cells expressing AP-LRRTM2-WT. Interestingly, the E348Q mutation completely abolished Nrx binding (98% decrease), while the D260A/T262A mutation only did so by 50%, presumably due to decreased level of expression (**Figure 40c**). These results confirm that Nrx binding on LRRTM2 involves the critical E348 residue identified by Yamagata and colleagues. However, mutation of D260 and T262 residues in the 9th LRR motif, did not impair Nrx binding. We thus colclude that E348 and not D260/T262 of LRRTM2 mediate Nrx binding.

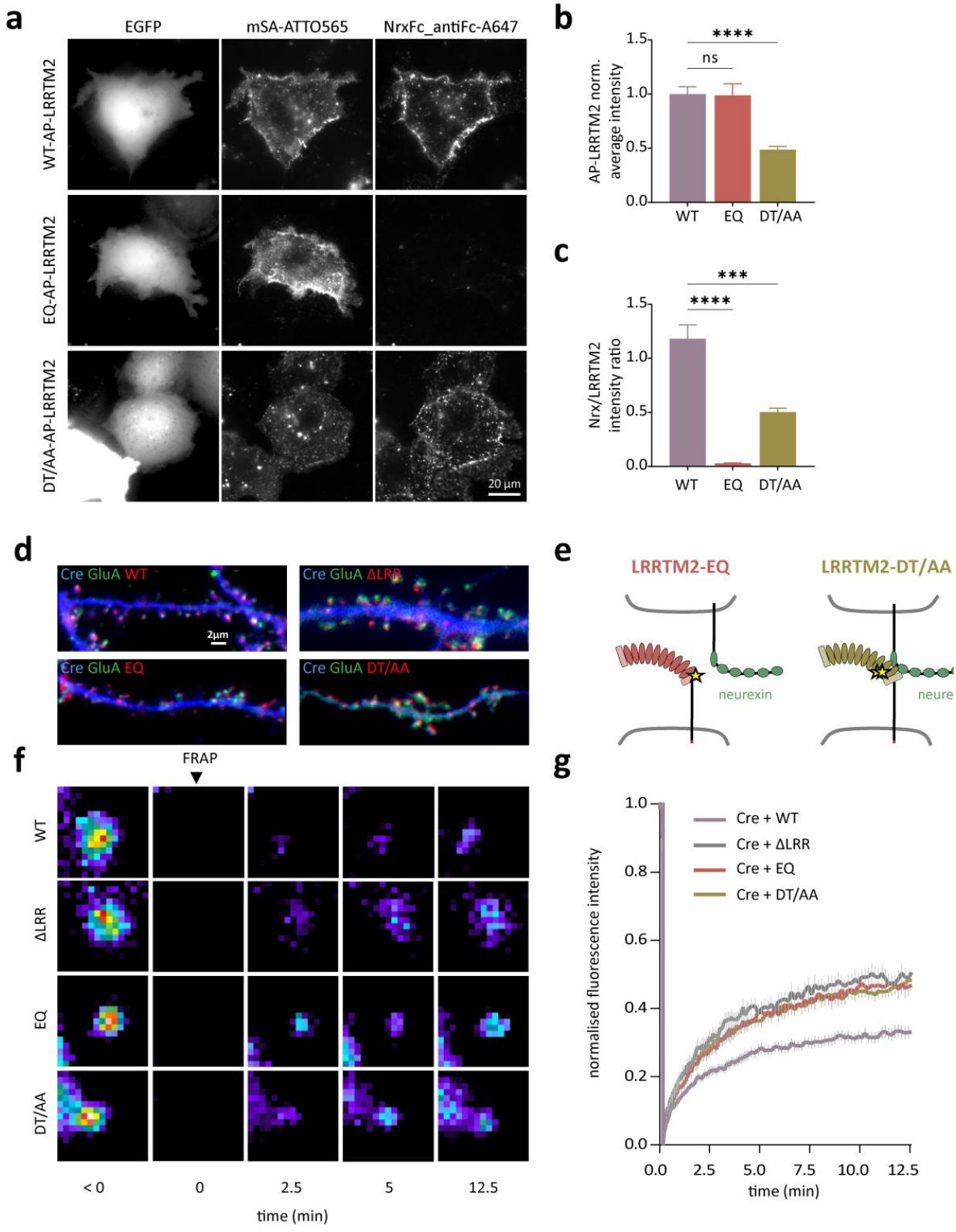
a
EGFP
mSA-ATTO565
NrxFc_antiFc-A647
WT-AP-LRRTM2
EQ-AP-LRRTM2
DT/AA-AP-LRRTM2
20 µm
b
AP-LRRTM2 norm. average intensity
****
ns
WT
EQ
DT/AA
c
Nrx/LRRTM2 intensity ratio
***
****
d
Cre GluA WT
Cre GluA ΔLRR
Cre GluA EQ
Cre GluA DT/AA
2µm
e
LRRTM2-EQ
LRRTM2-DT/AA
neurexin
f
FRAP
WT
ΔLRR
EQ
DT/AA
< 0
0
2.5
5
12.5
time (min)
g
normalised fluorescence intensity
Cre + WT
Cre + ΔLRR
Cre + EQ
Cre + DT/AA
time (min)

Figure 40. E348Q mutation on the C-terminal cap of LRRTM2 extracellular domain abolishes binding to neurexin. (a) Representative images of COS-7 cells expressing soluble EGFP and biotinylated WT-AP-LRRTM2, EQ-AP-LRRTM2 or DT/AA-AP-LRRTM2 labelled with mSA-ATTO565 and incubated with purified Nrx1-Fc cross-linked with antiFc-A647 antibodies. (b) Normalised average intensity of LRRTM2 mutants. (c) Ratio of Nrx/LRRTM2 intensity. Data acquired form 2 independent experiments WT: n=46 cells, EQ: n=63 cells, DT/AA: n=44 cells. **** p<0.0001 (d) Example of DIV15 hippocampal neurons expressing Cre-mCherry (blue), SEP-GluA1 subunit of AMPARs (green) and AP-LRRTM2 (WT, ΔLRR, EQ, DT/AA) (red). (e) Schematic representation showing that the E348Q mutation on the C-terminal cap of the extracellular region of LRRTM2 abolishes its binding to neurexin. DT/AA mutation on the 9th LRR maintains neurexin binding. (f) FRAP experiments performed on synaptic SEP-GluA1 expressing Cre-mCherry and AP-LRRTM2 mutants showing a similar recovery. (g) Corresponding normalised fluorescence recovery curves. Data obtained from at least 3 different experiments (Cre+WT: n=52 regions, Cre+ΔLRR: n= 27 regions, Cre+EQ: n= 31 regions, Cre+DT/AA: n=63 regions).

In light of these new findings, and because most of the work previously done attributed the LRRTM2-dependent AMPAR stabilisation to the binding of Nrx through D260/T262 residues, we examined the role of the Nrx binding site (E348) versus D260/T262 in AMPAR stabilisation. In neurons, unlike in COS-7 cells, both mutants were expressed similarly to the WT (**Figure 41**). When we performed FRAP experiments on synaptic SEP-GluA1 receptors in

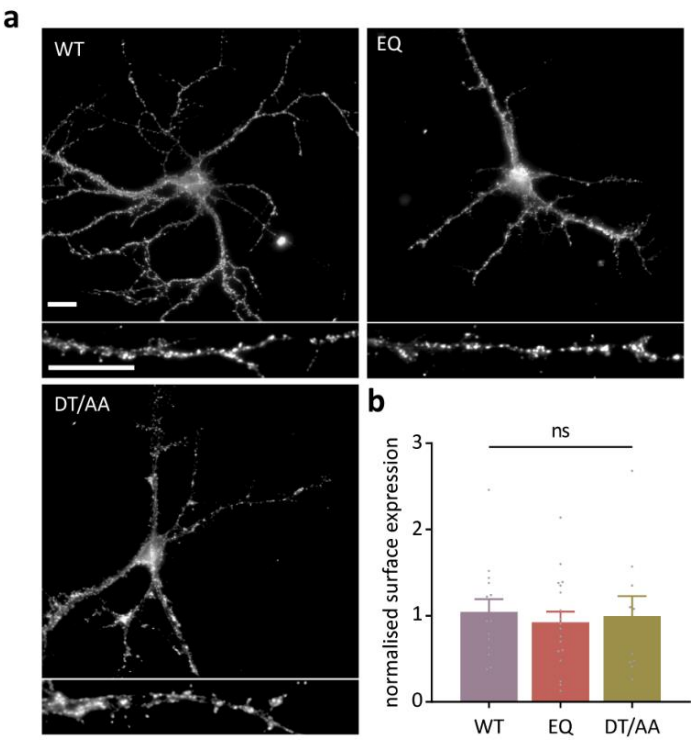

Figure 41. Levels of expression of LRRTM2 extracellular mutants. (a) Representative images of DIV15 hippocampal neurons expressing Cre-mCherry and biotinylated AP-LRRTM2 (WT, EQ or DT/AA). Insets depict labelling on individual dendrites. (b) Normalised fluorescence intensity of the mutants. Data obtained from 2 different experiments (WT: 14 cells, EQ: n= 18 cells, DT/AA: n= 10 cells).

hippocampal LRRTM2$^{Flox/Flox}$ neurons in the presence of different AP-LRRTM2 extracellular mutants (**Figure 40d**), we found that both E348Q and D260A/T262A mutations (**Figure 40e**) equally destabilised surface AMPARs at synapses and that this destabilisation was similar to that caused by deletion of the whole LRR domain of LRRTM2 (ΔLRR) (**Figure 40f, g**). Recovery of dendritic SEP-GluA1 receptors was not affected by the different mutations, supporting our findings that LRRTM2 stabilises AMPARs at synapses (**Figure 39e**). Our results thus show that although only the E348 residue is responsible for Nrx binding, both E348 and D260/T262 residues of LRRTM2 are involved in AMPARs stabilisation at excitatory synapses without additive effects and that mutations in either of these domains are sufficient to destabilise AMPARs from synaptic sites.

DISCUSSION

1. Role of LRRTM2 in synapse development and function

Different models have been previously used to study the role of LRRTM2 in synapse function using loss-of-function approaches (**Table1**). Most of these models deleted both LRRTM1 and 2 or combined with neuroligin3 deletion on a neuroligin1 KO background (J. Ko et al. 2011; Soler-Llavina et al. 2011; Bhouri et al. 2018). LRRTM1 and 2 are the main isoforms of the LRRTM family in the hippocampus (Laurén et al. 2003) and neuroligin1 the main isoform of the neuroligin family at excitatory synapses. However, possibly due to experimental designs and down-regulation of the adhesion molecules at different developmental stages, these studies have yielded controversial results regarding the contribution of LRRTM2 to synapse function. Some studies showed that deletion of LRRTM2 alone could impair synapse transmission, whereas others have reported that deletion of multiple adhesion molecules was necessary to have the same effect.

During my PhD I investigated the specific role of LRRTM2 in synapse development, aiming to clarify the contribution of LRRTM2 to synapse development and function. For this reason, I implemented two different strategies to study the role of LRRTM2 in synapse development and function: a shRNA and a conditional knock-out approach and showed that LRRTM2 invalidation alone is sufficient to reduce excitatory synapse density and impair basal synaptic transmission.

1.1 Knock-down approach (shRNA)

By using sh-mediated LRRTM2 knock-down in hippocampal cultures, de Wit and colleagues showed that in the absence of LRRTM2 the amplitude of both AMPAR- and NMDAR-mediated evoked EPSCs was reduced together with excitatory spine density (de Wit et al. 2009). However, the shRNA used in this study was criticised for having off-target effects by Ko and colleagues, who found that two different shRNA against LRRTM2 did not affect synapse number in cortical or

hippocampal cultures (J. Ko et al. 2011). Instead, they showed that triple knock-down (TKD) of LRRTM1,2 and neuroligin3 in neuroligin1 KO cultures could reduce spine density, rescued by overexpression of LRRTM2 alone or neuroligin1, and impair AMPAR- and NMDAR-mediated currents (J. Ko et al. 2011). This effect on NMDARs has not been reported by others, even though LRRTM2 has been suggested to interact with NMDARs (de Wit et al. 2009). In vivo, double knock-down (DKD) of both LRRTM1 and 2 during synaptogenesis selectively impaired AMPAR- mediated transmission, but DKD after synaptogenesis had no effect on synaptic transmission (Soler-Llavina et al. 2011).

We initially used a novel shRNA against LRRTM2 designed by our collaborator, Joris de Wit, to knock-down endogenous LRRTM2 during development in hippocampal rat cultures and replaced it with shRNA-resistant recombinant AP-LRRTM2 (further discussed in 2.1 section of the discussion 'labelling strategy'). This approach allowed us to avoid overexpression and specifically label LRRTM2. In this project, we showed that single KD of LRRTM2 was sufficient to reduce excitatory synapse density and that this effect could be restored by expression of recombinant LRRTM2 (Liouta et al. 2021). We believe that the effect of LRRTM2 on synapse density could be underestimated, given that our shRNA down-regulated by 50% recombinant LRRTM2 sensitive to this shRNA.

1.2 Conditional knock-out approach (LRRTM2-Floxed mice)

However, shRNAs potentially have off-target effects and they accomplice only partial knock-down of the protein of interest, as we observed in our experimental conditions. As a result, we cannot exclude that in neurons, remaining endogenous LRRTM2 could still be present and contribute to synaptic function. We thus developed in parallel a LRRTM2-Floxed mouse model in collaboration with Joris de Wit. We characterised and used this model to control LRRTM2 knock-out temporally and specifically in mouse hippocampal cultures. To our knowledge, this is the first LRRTM2-Flox mouse model extensively used in a study to specifically target LRRTM2, although a

[Discussion]

LRRTM2 cKO model was recently developed and crossed with a LRRTM1 cKO model to achieve LRRTM1/2 cKO (Bhouri et al., 2018).

Here, we showed that conditional knock-out (cKO) of LRRTM2 was sufficient to reduce excitatory synapse density and that this effect could be restored by expression of recombinant LRRTM2, in line with our knock-down approach (**Figure 29**). In addition, we showed using the cKO model that deletion of LRRTM2 results in decreased frequency of mEPSCs in accordance with decreased synapse density and decreased surface levels of AMPARs (**Figure 29**).

In our models, even though we expressed recombinant LRRTM2 in low levels and validated the rescue effect with biochemistry in heterologous cells (KD model), we cannot be certain that LRRTM2 levels in transfected neurons are not above endogenous levels resulting in mild overexpression. In addition, we cannot exclude that other member of the LRRTM family could compensate for the down-regulation of LRRTM2, in particular LRRTM1. The intracellular region of LRRTMs is highly conserved compared to other LRR families, with 20% of the amino acids being identical among LRRTMs and 50% 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 and 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 our models. Furthermore, the conditional nature of the genetic invalidation we used to address LRRTM2 function should reduce compensatory mechanisms compared to a full KO.

paper	KD/KO	Model	Synapse density	AMPARs-mediated currents	NMDARs-mediated currents
De Wit et al., 2009	shRNA LRRTM2	Hippocampal cultures	↓	↓ amplitude	↓amplitude
Ko et al., 2011	shRNA LRRTM2	Cortical and hippocampal cultures	no effect	not measured	*not* measured
Ko et al., 2011	shRNA LRRTM1,2 & Nlg3	Nlg1 KO cultures	↓	↓amplitude	↓amplitude
Soler-Llavina et al., 2011	shRNA KD LRRTM1&2 P0	WT mice (acute hippocampal slices)	*not measured*	↓amplitude	=
Soler-Llavina et al., 2011	shRNA TKD LRRTM1,2 & Nlg3 P0	Nlg1 KO mice (acute hippocampal slices)	= (compared to Ngl1 KO)	↓ amplitude	↓amplitude
Soler-Llavina et al., 2011	shRNA TKD LRRTM1,2 & Nlg3 P21	Nlg1 KO mice (acute hippocampal slices)	= (compared to Ngl1 KO)	=	=
Bhouri et al., 2018	cKO LRRTM1&2	LRRTM1&2-Floxed mice	*not measured*	↓amplitude	=

Table 1. Different experimental models to study the role of LRRTM2 is synapse function.

2. Regulation of LRRTM2 expression at the plasma membrane

The crucial role of LRRTM2 in synaptic transmission and especially in synaptic plasticity has been extensively studied. However, little is known regarding the regulation of this key player in synapse function. Thus, a major part of my PhD was to investigate the mechanisms that control LRRTM2 expression at the plasma membrane. Because of the remarkably low lateral diffusion of LRRTM2 (Chamma et al., 2016; Liouta et al., 2021; results **Figure 33**), we were particularly interested in studying the molecular mechanisms involved in the surface stabilisation of LRRTM2 at synapses. Using live and super-resolution imaging we showed that LRRTM2 membrane expression is highly regulated during neuronal development and synapse formation, we identified synaptic pools of LRRTM2 in spines, and show for the first time that its synaptic confinement and nano-scale organisation are regulated by specific motifs in the CTD.

2.1 Labelling strategies

AP-tagged LRRTM2

To investigate the mechanisms of LRRTM2 surface stabilisation, we needed to target and label the protein in situ. However, the available antibodies against LRRTM2 that we tested were not suitable to target the endogenous form of LRRTM2 in our imaging experiments. In order to visualise LRRTM2 in neurons, we generated and tested several tags fused to the N-terminus of LRRTM2 and chose the small 15-amino-acid acceptor peptide (AP) tag (Howarth et al. 2008) that can be enzymatically biotinylated and then recognised by fluorophore-conjugated streptavidin monomers (mSA) of 12.5kDa (Lim et al. 2011; Demonte et al. 2013). The small size of the AP tag minimises any possible conformational changes of the protein due to tag fusion, whereas mSA constitutes a stable 3-nm labelling probe that can efficiently penetrate the 20nm cleft and label synaptic proteins with fluorophores compatible with super-resolution microscopy and without inducing protein cross-linking (Chamma et al., 2016). Thanks to this labelling strategy, we could

study the surface expression, lateral diffusion and the molecular mechanisms involved in these processes (i.e. protein domains) for the first time.

One of the main advantages of the AP/mSA labelling strategy is that it allows us to monitor surface proteins in live neurons without cross-linking LRRTM2. Cross-linking of surface proteins can link to decreased mobility, increased internalisation and artificial protein clustering (Mammen, Huganir, and O'Brien 1997; Ashby et al. 2006; Heine et al. 2008), as it has been previously shown for antibodies, which becomes particularly problematic in the context of live labelling and investigation of dynamics of surface proteins, like LRRTM2. Primary and secondary antibodies have been extensively used in classical and super-resolution imaging to label endogenous proteins. However, antibody accessibility to the narrow synaptic cleft is questionable due to their bulky size (150kDa and 12nm), which can also create localisation artefacts thus misplacing fluorophores from their target proteins (Deschout et al. 2014). The presence of multiple fluorophores on a single antibody also renders intensity measurements relatively unreliable when it comes to quantifications. Other labelling strategies have also been proven useful especially for super-resolution imaging, including nanobodies, small antigen-binding fragments (15kDa) from heavy chain only antibodies usually present in camelids, suitable for super-resolution imaging (Ries et al. 2012). However, their use requires tagged proteins, with all the drawbacks that this implies. Nanobodies have also been generated against a variety of proteins, including tubulin (Mikhaylova et al. 2015), allowing direct labelling of microtubules and have been used successfully in SMLM approaches, including DNA PAINT (Fabricius et al. 2018; Sograte-Idrissi et al. 2019). Aptamers, single-stranded DNA or RNA oligonucleotides, and affimers, small proteins (10-12k Da and 3nm in size), have also been used in SMLM studies (F. Opazo et al. 2012; Tiede et al. 2017). Overall, sophisticated imaging probes are needed to accompany the advances in super-resolution microscopy in order to unravel the nanoscale organisation of highly-crowded environments like synapses with higher precision and reliability.

SEP-tagged LRRTM2

As we had previously observed that LRRTM2 trafficks substantially a lot in heterologous cells and is not stabilised at the plasma membrane, we were interested in studying the trafficking of the protein in neuronal cells and for this purpose we used the pH-sensitive SEP-tag for live-cell imaging. Although the SEP-tag (~25 kDa) is bigger that organic fluorophores and might oligomerise, increasing the risk of impaired protein localisation, trafficking and/or function (Cranfill et al. 2016), it is particularly bright, which makes it ideal for time-lapse imaging (Sankaranarayanan et al. 2000). To make sure that in our experimental conditions the SEP-tag did not alter LRRTM2 localisation and function, we compared SEP-LRRTM2-WT localisation to AP-LRRTM2-WT and found that both constructs could reach the plasma membrane and were localised at synapses. In addition, SEP-LRRTM2-WT had similar kinetics with the AP-LRRTM2-WT in the FRAP experiments (**Figure 35** compared to Chamma et al., 2016), suggesting that the SEP tag does not influence the surface dynamics of the protein. Finally, we also assessed the ability of SEP-LRRTM2-WT to bind pre-synaptic neurexin and found no impairment in the binding due to the SEP tag.

2.2 During development

LRRTM2 is a synaptogenic molecule that can induce pre-synaptic differentiation in contacting neurons when expressed in non-neuronal cells, and its overexpression has also been shown to increase the density of excitatory synapses in cultured neurons (de Wit et al., 2009), suggesting that LRRTM2 is also present in earlier stages of neuronal development. Even though LRRTM2 is the most synaptogenic isoform of the LRRTM family, no studies have addressed the behaviour of LRRTM2 during synapse formation. A previous study, though, had reported that overexpressed recombinant myc-tagged LRRTM2 in young rat cultured neurons was homogeneously distributed in dendrites and axons (Minatohara et al., 2015). We thus wondered whether the expression of LRRTM2 could be developmentally regulated.

[Discussion]

We first assessed LRRTM2 expression levels during development. Our results show that LRRTM2 is highly expressed throughout synaptogenesis (DIV7-DIV14) and its expression levels are reduced at more mature stages in cultured hippocampal neurons (DIV21). Here, we showed that LRRTM2 is highly diffusive in early stages of neuronal development and operates a shift in mobility after synaptogenesis when it is confined at excitatory synapses. It is possible that remodelling of the composition of the plasma membrane (Tulodziecka et al. 2016) could affect the mobility of transmembrane proteins, although in cultured neurons this is not clear. Macromolecular crowding and physical barriers at synapses could also slow down protein diffusion in the plasma membrane (see discussion 2.3.2). However, the diffusion of LRRTM2 remained high despite the gradual increase in excitatory synapses during DIV7 to 9, unlike Neuroligin1, a competitor for Neurexin and PSD-95 binding whose diffusion gradually decreased upon increase in synapse density (Chamma et al 2016), suggesting that the mechanisms regulating it surface mobility in early development are independent of synapse density increase. Instead, we observed a shift in diffusion after synaptogenesis (DIV14). A possibility is that the molecular composition of excitatory synapses changes during development and promotes LRRTM2 confinement only at later stages by providing interaction partners not expressed earlier. This hypothesis is supported by the drastic increase in the dwell time index - reflecting the time spent at excitatory synapses - observed between DIV9 and DIV15, which specifically reports on protein trapping and accumulation at synapses (M. Renner et al. 2012). To date, only two binding partners of LRRTM 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). PSD-95 is up-regulated during neuronal development in cultured neurons, as synapses mature (Bustos et al. 2014), but is present before DIV15, suggesting that it might not be the principal partner governing LRRTM2 trapping at excitatory synapses. Hippocampal Neurexin1a was also shown to gradually increase by 3-fold between DIV5 and DIV15 in HA-neurexin1a KI mice (Trotter et al. 2019). Neurexin1 nanoclusters in culture grow in size and content during synapse development, with only 20% of synapses containing neurexin1 in immature neurons, as opposed to 40% in mature synapses (Trotter et al. 2019) AMPARs are also upregulated during development in cultured pyramidal neurons (Tsuzuki et al. 2001). Finally. In human tissue, neurexin1 mRNA levels were

found to be increasing with age in 8-12 postconceptional-week, same as LRRTM2 (Harkin et al. 2016). Thus, it is possible that an increased availability of LRRTM2 binding partners might contribute to increased stabilisation after DIV9, as suggested by the increase in dwell time at DIV15 (Liouta et al., 2021). Interestingly, synaptic tracks were even more immobilised at DIV15 compared to DIV7 and 9, suggesting that LRRTM2 stabilisation at synapses could involve a multi-step process through different and complementary interactions with proteins over development. Identifying novel partners for LRRTM2 at synapses will be necessary to better explain this mechanism. Considering that extra-synaptic trajectories were also further immobilised after DIV9, another possibility is that non-stabilised 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. 2003; Sampo et al. 2003).

To conclude, we showed that LRRTM2 expression is tightly regulated during synapse development and that LRRTM2 becomes confined at excitatory synapses after synaptogenesis. Further studies identifying the interactome of LRRTM2 throughout synapse formation are necessary to shed light into the molecular interactions of LRRTM2 in early developmental stages.

2.3 In mature synapses

Targeting and maintenance of proteins on specific compartments of membranes is essential for efficient neuronal function, as compartmentalisation provides the optimal microenvironmental conditions for specialised processes. In particular, the neuronal plasma membrane contributes to compartmentalisation of neurons by separating the extracellular space from the cytoplasm, while allowing regulated insertion and elimination of membrane molecules. Neurotransmitter receptors were among the first molecules whose distribution at synapses was studied as they are directly related to synaptic transmission. Indeed, the number of receptors across neurotransmitter release sites is crucial for efficient synaptic transmission. As a result, understanding the mechanisms regulating receptors accumulation at synapses has received high scientific attention throughout the years. Initially, the endocytic and exocytic pathways were in

the spotlight of the processes regulating trafficking of receptors at synapses (Carroll and Zukin 2002; Shepherd and Huganir 2007). However, insertion and removal of receptors from synapses involves multiple mechanisms; trafficking to and from intracellular pools via endo/exocytosis, lateral diffusion at the plasma membrane. Nowadays, the same mechanisms of surface expression have been demonstrated for several synaptic proteins, including adhesion molecules.

2.3.1 Synaptic clustering of LRRTM2

Using the shRNA approach, we showed that clustering of LRRTM2 critically depends on its CTD (Liouta et al., 2021 Figure2), as previously demonstrated using overexpression (Linhoff et al., 2009; Minatohara et al., 2015). Deletion of the PDZ-like binding motif (ΔECEV) or mutation of the YxxC motif (YACA) reduced the clustering of the protein to the same extent as deletion of the entire CTD in the shRNA model (Liouta et al., 2021 Figure2), suggesting that both motifs are involved in LRRTM2 clustering, without additive effects. Using the cKO model, we tempted to investigate whether these mutations disrupt the synaptic clustering of LRRTM2. Deletion of the entire intracellular domain of LRRTM2 (ΔC) significantly decreased synaptic clustering, suggesting that this domain is involved in maintaining LRRTM2 at excitatory synapses (**Figure 30 and 31**). To further identify the contribution of the known intracellular motifs to LRRTM2 synaptic clustering, ΔECEV and YACA and found a tendency for decrease, which was though not significant possibly due to lower number of cells for these conditions. However, YFP-tagged LRRTM2-ΔECEV was previously shown not to impair LRRTM2 clustering (Linhoff et al., 2009), even though this could be attributed to overexpression, as we have observed in our experiments that overexpression all mutants in the absence of the shRNA resulted in increased clustering (Liouta et al., 2021 supplementary figure 5). Oligomerisation with endogenous forms of LRRTM2 could contribute to this phenomenon (Paatero et al., 2016), although the existence or physiological relevance of LRRTM2 oligomers is not known (Yamagata et al., 2018). 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 (Minatohara 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 in our shRNA model, we observed similar results: WT-LRRTM2 formed large compact clusters at synapses, which were disrupted in all mutant conditions (Liouta et al., 2021 **Figure 4**). Clustering of surface proteins has been proved important for their physiological role. Aggregation of surface NGL-2 or NGL-3 induces NMDAR clustering, whereas NGL-3 also induces AMPAR clustering (S. Kim et al. 2006; Woo, Kwon, and Kim 2009). In hippocampal neurons, clustering of LRRTM1 or LRRTM2 results in co-clustering of NMDAR subunit GluN1 and AMPAR subunit GluA1, but also PSD-95 and SynGAP, a PSD protein (Linhoff et al., 2009). SALM2 and SALM3 clustering has also been shown to induce co-clustering of PSD-95 in cultured neurons (J. Ko et al. 2006; Mah et al. 2010). Impairment of neuroligin1 clustering has also been suggested to decrease the surface mobility of GluA2 AMPARs (Moretto et al. 2019).

2.3.2 Stabilisation of LRRTM2 at the plasma membrane

Using uPAINT in both the KD and cKO models, we showed for the first time that the CTD of LRRTM2 governs its membrane diffusion (**Figure 33**). To our surprise, confinement of LRRTM2 at excitatory post-synapses was independent from its PDZ-like binding motif which binds the major post-synaptic scaffold protein PSD-95 (de Wit et al., 2009; Linhoff et al., 2009). This was quite unexpected, as binding to scaffolding proteins via PDZ motifs is one of the key elements that control trapping and stabilisation of synaptic molecules (Chamma et al., 2019; Opazo, 2012; Sheng & Hoogenraad, 2007). For instance, 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. As a result, 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; Neupert 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 and Triller 2013; Czöndör et al. 2012).

In the case of LRRTM2, unknown molecular interactions could explain trapping at excitatory post-synapses, but the ECEV motif does not seem to be involved in dominant interactions in the context of synaptic stabilisation. 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). The role of the YxxC motif is not clear. It could also be involved in specific interactions important in LRRTM2 membrane stabilisation, independently from the ECEV motif. Further studies are necessary to clarify this point. One hypothesis is that the PDZ-binding domain could be important for LRRTM2 interactions earlier in development or synapse formation. Once stabilised at the plasma membrane LRRTM2 could associate with other proteins that bind to its YxxΦ motif. Tyrosine phosphorylation of the YxxΦ motif could alter the interaction with scaffolds or unknown intracellular partners of LRRTM2; however, whether this residue can be phosphorylated still remains unknown.

Further analysis of the synaptic and extrasynaptic diffusion of intracellular LRRTM2 mutants revealed some differences between our two experimental models regarding the contribution of the YxxC motif. In the case of the shRNA project, synaptic diffusion was decreased to the same extend with the ΔC and the YACA mutant, suggesting that this motif governs to a great extent the confinement of LRRTM2 at excitatory synapses in this context. However, in the cKO model, the YACA mutant decreased, but not significantly, the synaptic diffusion and immobile trajectories. Yet, the MSD of the YACA mutant was similar to that of the ΔC and those mutants were the ones that exhibited a decrease in the percentage of synaptic trajectories (**Figure 34**), suggesting that intracellular interaction at the YxxC motif control LRRTM2 confinement at the synapse.

Restriction of diffusion by physical barriers and macromolecular crowding

Fluidity of the plasma membrane and thermal agitation makes molecules freely move on the plasma due to thermal agitation. Yet, synaptic molecules are inhomogeneously distributed and organised in specific domains. Neurotransmitter receptors were the first ones to be found to juggle between a mobile and an immobile state on the plasma membrane, resulting in

inhomogeneous distribution outside and inside synapses. Transient immobilisation of receptors is believed to be, at least partially, the result of reversible interactions with scaffolds presents at the cytoplasm of neurons, which act like diffusion traps (Triller and Choquet 2008). Receptors can freely diffuse on the plasma membrane until they are transiently immobilised by intracellular interactions. We showed that immobilisation of LRRTM2 is independent of its PDZ-like binding motif and instead it is regulated by the intracellular YxxC motif, possibly through interaction with unidentified binding partners. This is supported by a decrease in the time spent at the synapse for the ΔC and the YACA mutants, a reliable measurement to assess LRRTM2-scaffold interaction (M. Renner et al. 2012). However, these interactions are not the only factors capable of restricting the free movement of proteins and lipids on the plasma membrane. Physical barriers can also hinder the free movement of molecules at the plasma membrane. The mesh formed by the cytoskeleton tethered directly or indirectly to transmembrane proteins, can form fences, which are physical intracellular obstacles. Multiple pickets can delimit confinement zones or 'corrals' wherein molecules can still diffuse. In addition, transmembrane proteins can interact with components of the extracellular matrix, precluding the free movements of themselves or acting as barriers to other proteins (Trimble and Grinstein 2015). In addition, synapses are extremely crowded and packed with numerous proteins that can generate a confined environment limiting Brownian motion of proteins (Melo and Martins 2006; M. L. Renner et al. 2009; Santamaria et al. 2010).

2.4 Trafficking from intracellular compartments

2.4.1 Regulation of LRRTM2 exocytosis by the YxxC motif

Others and our group have shown that the YACA mutant exhibits increased surface expression in rat hippocampal neurons (Minatohara et al., 2015; Liouta et al., 2021 **Figure 2**) and it was previously shown that WT and YACA are similarly accessible to the endocytic machinery (Minatohara et al., 2015), suggesting that the increased surface expression of LRRTM2 YACA could be attributed to altered exocytosis. To further characterise the trafficking of LRRTM2 at

synapses we used live-cell and FRAP imaging to assess the effect of the YACA mutant on LRRTM2 recovery at excitatory synapses. To begin with, we found that WT LRRTM2 has a slow recovery as previously observed with LRRTM2 mild overexpression (Chamma et al., 2016), in accordance with the low lateral diffusion of the protein at the plasma membrane. We then found that recovery of SEP-tagged LRRTM2 was increased by 2-fold for the YACA mutant, suggesting that the YxxC intracellular motif not only governs the surface diffusion of the protein, but also the trafficking from the intracellular compartments (**Figure 35**). Given the experimental design of this experiment (12.5 minutes of acquisition), the recovery of fluorescence represents a mixture of membrane diffusion and exocytosis, so we cannot decipher the exact contribution of LRRTM2 exocytosis with this type of experiment.

In order to further characterise LRRTM2 exocytic events in a more simplified model, we transfected COS-7 cells with SEP-LRRTM2-WT or SEP-LRRTM2-YACA and photobleached the entire cell to quench surface fluorescence. SEP-LRRTM2 localised in intracellular vesicles non-fluorescent because of the acidic pH of the vesicles. However, as those vesicles fuse with the plasma membrane during exocytosis, SEP-LRRTM2 is exposed to the neutral pH of the imaging medium and becomes fluorescent. As all surface molecules were previously bleached, the appearance of fluorescence can only come from the intracellular of SEP-LRRTM2-containing vesicles that are exocytosed to the surface. Time-lapse imaging after FRAP showed that SEP-YACA exhibited increased number of exocytic events that seemed to be brighter (average intensity) and less stable once on the surface (**Figure 36**). Vesicles containing SEP-YACA seem to contain more LRRTM2 molecules (increased average intensity), suggesting that the YxxC could regulate the loading of LRRTM2 in vesicles. Interestingly, YXXΦ has been shown to serve as sorting motif at the trans-Golgi network and can bind to various adaptor proteins (P. Li et al. 2016) in order to address cargo proteins to specific compartment (Farías et al. 2012). In addition, SEP-WT exocytic events that were found to be stable for at least 100 seconds, unlike most of SEP-YACA events that disappeared within 20 seconds after appearance on the surface, suggesting that mutations in the YxxC motif impair the membrane stabilisation of newly-exocytosed LRRTM2 molecules (**Figure 36**). However, these experiments should be treated with cautious as they were only

performed once, thus additional experiments will be needed to confirm these results. In addition, we cannot be sure whether after fusion with the membrane SEP-YACA rapidly diffuses on the membrane or whether it gets endocytosed quickly. It is possible that SEP-WT can be stabilised on the plasma membrane through YxxC-mediated interactions and mutations in this motif impair interactions of LRRTM2 with intracellular binding partners that are yet to be identified. YXXΦ has also been associated with clathrin adaptor-protein 2 (AP-2), a protein selecting cargo from the plasma membrane for clathrin-mediated endocytosis (Ohno et al. 1995; Traub 2009). This motif has also been found to be involved in glutamatergic receptors internalisation. The GluN2B subunit of NMDARs possesses an AP-2 binding motif, YXXΦ, in the far C-terminus close to the PDZ-binding motif (Roche et al. 2001). Phosphorylation of Tyr1472 within this motif by Fyn kinase prevents the internalisation and as a result increases synaptic NMDAR currents (Prybylowski et al. 2005). However, in our experiments, mutations in the YXXΦ motif seem to be involved in the exocytic pathway, even though we did not directly assess endocytosis. Pharmacological treatment with dynasore, a dynamin inhibitor that blocks clathrin-dependent endocytosis (Preta, Cronin, and Sheldon 2015), could clarify whether the YXXΦ is also involved in LRRTM2 endocytosis. In that case, we could imagine that once LRRTM2 is inserted to the plasma membrane it gets stabilised through YxxC-mediated interactions. However, we showed that mutations in this motif impair the membrane stabilisation of the protein. If we imagine a possible model in neurons, we can hypothesise that once LRRTM2 YACA fails to get stabilised, it is internalised from the surface and is recycled at extrasynaptic sites, from where it can leave the synapse, or at the shaft. Endocytosis of LRRTM2 could take place at endocytic zones are sites of endocytosis at the post-synapse (Rosendale et al. 2017), consisting of stable clathrin assemblies coupled to the PSD through interactions with Homer1b/c and Dynamin 3 (Blanpied, Scott, and Ehlers 2002; J. Lu et al. 2007). Alternatively, LRRTM2 YACA could directly leave the synapse through lateral diffusion. At the same time, increase in the exocytic rate could replenish LRRTM2 YACA at the surface of the synapse, resulting in a vicious circle of altered trafficking.

However, the mechanism involved in LRRTM2 trafficking in neurons might be different compared to heterologous cells and similar experiments should be performed to characterise the exocytosis of LRRTM2 in hippocampal neurons.

2.4.2 Existence of an intracellular pool of LRRTM2

Could LRRTM2 be directly exocytosed at dendritic spines? Even though exocytosis has been mostly studied in the context of neurotransmitter release from the presynaptic terminals, dendrites possess all the necessary organelles of the secretory pathway (Kennedy and Ehlers 2011) as it has been mostly shown for AMPARs during LTP. In hippocampal neurons from adult rat or mouse brains, EM images show the ER extending as far as the dendritic spines (Cooney et al. 2002; Y. Wu et al. 2017). In addition, the smooth ER can invade a subset of dendritic spines, known as the spine apparatus (Gray 1959; Spacek and Harris, 1997), serving as an internal calcium store in spines (Fifkova et al., 1983) and contributing to local protein synthesis and trafficking (Pierce, Mayer, and McCarthy 2001). Nonetheless, direct experimental evidence for the role of the spine apparatus is still missing.

Here we revealed the existence of an intracellular pool of LRRTM2, with almost 30% of this pool localised in the spine head or adjacent to spines (18% and 11% respectively) (**Figure 37**). Interestingly, the vesicles localised at synapses seemed to be more stable compared to the ones localised in the shaft that were trafficking along the dendrites. We could thus imagine that the vesicles localised at spines could constitute a reserve of readily-releasable LRRTM2 molecules upon stimuli. Increased synaptic activity has been shown to spatially restrict the trafficking of the secretory pathway compartments after endoplasmic reticulum exit, suggesting that synaptic activity could favour exocytosis by restricting the cargo proteins to specific sites in the dendrites. Given the role of LRRTM2 in synaptic plasticity (Introduction 3.6.3.4) it is possible that LTP could trigger the exocytosis of these vesicles to increase the surface expression of LRRTM2 and increase synaptic strength by reinforcing the physical interaction with the pre-synapse through neurexin binding or by further stabilising AMPARs at the synapse surface (see 3.1 of the Discussion and 3.6.5 of the Introduction), although our experiments addressing this point yielded unclear results.

In the absence of LRRTM1 and LRRTM2 GluA1 receptors have been shown to be inserted in the plasma membrane upon LTP, but failed to be stabilised in the long term (Soler-Llavina et al., 2013). Experiments in this direction are necessary to identify the physiological relevance of these intracellular pools.

In addition, given the role of the YxxC intracellular motif in exocytosis and the fact that YxxC is also a sorting motif, it would be interesting to see whether mutations in this domain could alter the localisation of the intracellular pool of LRRTM2 and what would be their consequences on synapse function.

3. LRRTM2 stabilises AMPARs at synapses

Regulation of the synaptic number of AMPARs is associated with efficient synaptic transmission. Several adhesion molecules have been shown to modulate the surface expression of AMPARs at the synapse either by direct, like N-cadherin, or indirect binding, like neuroligin 1. Neuroligin 1 has been shown to recruit AMPARs via a unique intracellular tyrosine (Y782), which differentially regulates its binding to PSD-95 and gephyrin. Mutations in this residue impaired LTP, suggesting a critical role in retaining AMPARs at the synapse (Letellier et al., 2018). A mutation in a cytoplasmic arginine residue conserved in all neuroligins, R704C, associated to autism, has also been linked with altered surface levels of AMPARs and synaptic responses (Chanda et al., 2016), suggesting that modulating the interaction between synaptic adhesion molecules and receptors could be involved in the pathophysiology of neurodevelopmental diseases. Down-regulation of LRRTM2 was associated with impaired AMPAR-mediated transmission (de Wit et al., 2009) and with decreased surface levels of AMPARs (de Wit et al., 2009; Soler-Llavina et al., 2013). In addition, recombinant LRRTM2 has been shown to co-immunoprecipitate in non-neuronal cells with GluA1 and GluA2 subunits of the AMPARs, suggesting that LRRTM2 and AMPARs could directly interact, although this was never shown in neurons. A recent study coupling photo-activatable GFP-tagged GluA1 (paGFP-GluA1) and live-cell imaging showed that after activation

of paGFP-GluA1 its fluorescent intensity was decreased faster in spines lacking LRRTM1 and LRRTM2 (cKO) compared to spines containing these LRRTM isoforms, suggesting that paGFP-GluA1 could not be stabilised at the surface of spines in the absence of LRRTM1 and 2 (Bhouri et al., 2018). However, the contribution of each isoform to stabilising AMPARs on the surface was still unclear. Meanwhile, LRRTM DKD (Soler-Llavina et al., 2013) in hippocampal neurons was shown to increase the intracellular pool of endogenous AMPARs, suggesting that LRRTM2 could be involved in AMPARs trafficking, and we have identified the existence of an intracellular pool of LRRTM2 in spines; thus, initially we hypothesised that LRRTM2 and AMPARs could co-traffic together to the plasma membrane.

To test this hypothesis, we implemented a protocol similar to the one we used in COS-7 cells to monitor the exocytosis of SEP-LRRTM2. We transfected neurons with either SEP-LRRTM2 on a KO background or SEP-GluA1 and photobleached using a high-laser power the entire field of view in order to quench the surface fluorescence and monitored over 40 minutes the recovery of fluorescence. Fusion of intracellular vesicles containing non-fluorescent SEP-tagged proteins with the plasma membrane, exposes the SEP-tag to the neutral pH of the imaging medium which becomes fluorescent. However, we observed that the recovery curve of LRRTM2 was different from that of GluA1 receptors, suggesting that the two proteins do not co-traffic together. Of course, this is only an indication and does not directly show that LRRTM2 and AMPARs are not trafficking in the same vesicles. Newly formed endocytic vesicles fuse with each other to form the sorting endosome (Maxfield and McGraw 2004) (SE) or with pre-existing SE, where the fate of endocytosed cargoes is determined. Cargo proteins can be returned to the plasma membrane through endosomal recycling and AMPARs are constitutively internalised and recycled from the surface (Passafaro, Piëch, and Sheng 2001). Sorting nexin 27 (SNX27), a cargo sorting endosomal protein, has been shown to be enriched in the recycling endosomes and to be associated with AMPARs trafficking to the plasma membrane (Loo et al. 2014). Recently, the synaptic adhesion molecule leucine-rich repeat and fibronectin type-III domain containing protein 2, LRFN2, was found to associated with SNX27 and AMPARs, bridging the interaction of these proteins and regulating AMPARs recycling in primary rat cortical neurons (McMillan et al. 2020). However, it

is known whether LRRTM2 can interact with SNX27 or other cargo sorting proteins. Pairing the properties of the SEP-tag with a pH-sensitive fluorescent protein of a different colour, i.e pHuji (Y. Shen et al. 2014), could also allow the visualisation of LRRM2 and AMPARs in the same cells and clarify whether these proteins can co-traffic to the plasma membrane.

These results, together with those of Bhouri and colleagues described earlier, led us to hypothesise that LRRTM2 might stabilise AMPARs once they are inserted in the plasma membrane. To test this hypothesis, we photo-bleached SEP-GluA1 receptors in the presence or absence of LRRTM2 and found that cKO of LRRTM2 increases the recovery of SEP-GluA1 receptors by 20%, implying that in the absence of LRRTM2 SEP-GluA1 receptors are less stable. Re-expression of AP-LRRTM2 rescued this effect, which was specific for synaptic SEP-GluA1 receptors, as we found no difference in the recovery on the shaft, suggesting that LRRTM2 stabilises synaptic AMPARs on the plasma membrane (**Figure 38 and 39**). Our results show that cKO of LRRTM2 alone destabilises AMPARs at synapses and shed light into the specific contribution of the LRRTM2 isoform to synapse organisation.

We should bear in mind that we used an overexpression of SEP-GluA1. Apart from the drawbacks of overexpression, previous studies have raised concerns regarding the influence of SEP or GFP tags on the membrane targeting of AMPARs. Overexpressed GFP-GluA1 has been shown to be unable to reach the synaptic membrane without stimulation or over-expression of PSD-95 (Hayashi et al., 2000; Shi et al., 2001) and others have shown that exogenous GluA1 lacking a GFP tag can be incorporated into synapse without any kind of stimulation (Granger et al., 2012). Others have reported that the presence of a GFP tag has no (Nabavi et al., 2014) or little (Watson et al., 2017) effect on GluA1 trafficking. In our experiments, SEP-GluA1, which was previously used in multiple studies (Petrini et al. 2009; Makino and Malinow 2009; Bakr et al. 2021), was properly trafficked targeted to the surface and was enriched at synapses, suggesting that the tag did not impact its localisation (**Figure 38**).

An early study on LRRTM2 had shown that LRRTM2 interacts with GluA1 and GluA2 subunits of the AMPARs via its extracellular domain in non-neuronal cells (de Wit et al., 2009). We showed

that deletion of the extracellular domain of LRRTM2 (ΔLRR) increases the recovery of SEP-GluA1 receptors after FRAP (**Figure 41**). Deletion of the intracellular domain (ΔC) resulted in a mild destabilisation of the receptors. Previously we showed that deletion of the intracellular part of LRRTM2 decreases the density of PSD-95 (**Figure 31**) and PSD-95 is known to act as a diffusion trap for AMPARs. Thus, we believe that this increase in SEP-GluA1 surface mobility in the presence of LRRTM2-ΔC is most likely due to an indirect effect from less available scaffolds to stabilise the receptors. As a result, we focused on the role of the extracellular domain of LRRTM2 in stabilising AMPARs at excitatory synapses.

3.1 Where does LRRTM2 bind neurexin?

Through the extracellular domain LRRTM2 binds to presynaptic neurexin (1-3, α or β), however there is some controversy regarding the interaction site between LRRTM2 and neurexin 1β. We thus generated a mutant (E348Q) according to the recent study that reported the crystal structure of LRRTM2 with neurexin1β (Yamagata et al., 2018). To carefully characterise this mutant before using it in our FRAP experiment, we expressed it in heterologous cells. We showed for the first time that this mutation completely abolishes the binding of LRRTM2 to neurexin in heterologous cells (**Figure 41a-c**). However, previous studies investigating the physiological significance of LRRTM2 binding to neurexin, have reported a different site of interaction between the two proteins. Siddiqui and colleagues generated several mutants in the extracellular part of recombinant LRRTM2 and identified the D260A/T262A (DT/AA) in the concave side of the 9th LRR repeat as the mutation that abolishes the binding of LRRTM2 to Neurexin-1β(SS4-)-Fc in non-neuronal cells (Siddiqui et al., 2010). Based on this finding, subsequent studies used this mutant to study the role of LRRTM2 binding to neurexin especially in the context of LTP. Double knock-out of LRRTM1 and LRRTM2 has been shown to block LTP in acute hippocampal slices and LRRTM2-DT/AA mutation is sufficient to impair LTP, suggesting that these residues and thus binding of LRRTM2 to neurexin, are important for LTP (Bhouri et al., 2018). When we assessed the ability of this mutant to bind neurexin-1β-Fc, we found that neurexin binding was decreased but not abolished, unlike the EQ mutant (Figure 41b). We also observed a 40% decrease in the

level of expression of the DT/AA mutant in heterologous cells (**Figure 41c**), so to be sure that the decrease in neurexin binding was not due to decreased expression of LRRTM2-DT/AA mutant, we normalised to its levels of expression and found that DT/AA was still binding to neurexin to a lesser extent than the WT. However, this mutation did not affect the level of the protein in neurons (**Figure 40**), suggesting that neuron specific post-translational modifications could be important for targeting LRRTM2 to the plasma membrane. Our results confirm the newly described Nrx-binding site E348, and question the importance of LRRTM2 binding to neurexin in previous work involving the DT/AA mutant. Thus, to what extent neurexin binding is responsible for the physiological effects of LRRTM2 in synaptic plasticity remains an open question. Future studies will be necessary to investigate the involvement of the newly identified neurexin binding site in synapse function.

3.2 Important LRRTM2 motifs for stabilising AMPARs

We then wondered whether these extracellular residues, E348 and D260/T262, could be involved in AMPARs stabilisation at synapses. FRAP imaging revealed that mutations in any of these residues, DT/AA or EQ, equally increased the recovery of SEP-GluA1 receptors to levels comparable with the deletion of the entire LRR domain (ΔLRR) (**Figure 41f, g**). The fact that deletions in these residues yield the maximum effect on AMPARs recovery after photo-bleaching, makes us think that these are the major motifs of LRRTM2 involved in stabilising synaptic AMPARs. Deletions in any of these residues was sufficient to increase the recovery, suggesting that both motifs are needed equally to stabilise the receptors. Thus, stabilisation of synaptic AMPARs seems to involve both the neurexin-binding site (E348) and the concave interface of LRRTM2 containing the D260/T262 residues, suggesting that these synaptic proteins could form a tripartite complex in order to ensure AMPARs positioning and thus efficient synaptic transmission.

3.2.1 Can LRRTM2 directly bind AMPARs?

If the D260/T262 residues in the 9th LRR are not responsible for LRRTM2 binding to neurexin, how do we explain the findings from previous studies showing that mutations in these residues impair LTP? The concave side of the LRR domain is known to favour protein-protein interactions, so if neurexin does not bind there, this interface remains free for other putative interactions. Could AMPARs directly bind LRRTM2 at the D260/T262 residues? As previously mentioned, LRRTM2 has been shown to bind AMPARs in heterologous cells in 2009, but this has never been shown in neurons and LRRTM2 was not identified in proteomics studies on AMPAR subunits (Schwenk et al., 2012 and 2015). Aiming at identifying whether AMPARs could directly bind LRRTM2 via the D260/T262 residues in the 9th LRR repeat, we also tried to co-immunoprecipitate LRRTM2 and AMPAR in neuronal cultures, without success. This might be explained by the transient and/or low affinity of such an interaction. Thus, we further performed co-clustering assays and micropatterning experiments to examine whether AMPARs and LRRTM2 might be co-recruited at Neurexin-enriched sites. However, all these experiments did not yield clear results and we could not conclude clearly on an interaction between LRRTM2 and AMPARs.

In the same line, a recent study investigating the effect of acute cleavage of LRRTM2 ectodomain (ECD) in the alignment of the pre-and post-synapse (discussed in 3.3 of the discussion), showed that despite acute cleavage of LRRTM2 ECD, overexpressed SEP-GluA1,2 remained stable on the plasma membrane for 30 minutes. If LRRTM2 directly binds AMPARs through its extracellular domain, we would have expected at least a slight decrease in the intensity of SEP-GluA1, 2 within 30 minutes. Altogether, these results suggest that LRRTM2 and AMPARs might not directly interact and that more complex interactions might be at play, possibly involving other molecular partners. Interestingly, LRRTM2 and neurexin have been shown to bind in an heparan-sulfate dependent manner (Roppongi et al., 2020) and the heparan sulfate proteoglycan (HSPG) glypican 4 (GPC4), has been shown to stimulate the formation of functionally active synapses by inducing the clustering of the GluA1 subunit of AMPARs in cultured retinal ganglion cells, although the underlying mechanisms remain unclear (Allen, Halverson-Tamboli, and Rasenick 2007). In addition, AMPAR clustering at interneuron synapses is mediated by interactions of the NTD with

neuronal pentraxins (NPs) (Sia et al. 2007), glycoproteins secreted from presynaptic terminals (O'Brien et al., 1999). It would be exciting to investigate whether glycoproteins could be involved in the binding of LRRTM2 with AMPARs in hippocampal neurons.

3.3 Does LRRTM2 control the pre-/post-synaptic alignment?

The potential role of LRRTM2 as organiser of the alignment of the pre- and post-synaptic machinery has long been hypothesised. (Tang et al., 2016). LRRTM2 role in synaptic transmission, its localisation at excitatory synapses, its low surface dynamics, its organisation in compact nano-clusters and its interaction with AMPARs in heterologous cells, render LRRTM2 an ideal candidate to govern the alignment of the pre- and post-synaptic components, thus facilitating efficient synaptic transmission. Recently, LRRTM2 was found to be enriched in the synaptic nanocolumn and acute cleavage of LRRTM2 ECD resulted in long-term disorganisation of AMPARs at the nanoscale (Ramsey et al., 2021). Here, we showed using STORM imaging that LRRTM2 is organised in compact clusters within synapses and that LRRTM2 compaction and synaptic enrichment is disrupted upon deletion of its intracellular domain. On the one hand, we showed that the intracellular domain of LRRTM2 is involved in its compartmentalisation, targeting and confinement at synapses and exocytosis. On the other hand, LRRTM2 extracellular domain is important for stabilising AMPARs at synapses, although the direct interaction of these proteins seems unlikely. We showed that deletions in the intracellular domain of LRRTM2 disrupt its nanoscale organisation, but could they also affect the alignment of the pre- and post-synapse? We also showed that mutations in the neurexin binding site and in the 9th LRR domain of LRRTM2 destabilise AMPARs, however could they also alter the nanoscale organisation of the receptors? We could imagine a tripartite complex between neurexin-LRRTM2 and AMPARs, where neurexin binds to E348 of LRRTM2 and interaction via the D260/T262 residues in the 9th residue further stabilises this contact. Extracellular interaction between LRRTM2 and AMPARs, direct or indirect, could be reinforced upon binding of LRRTM2 to neurexin, thus creating a trans-synaptic complex that positions AMPARs in front of the presynaptic release machinery to ensure efficient synaptic transmission. Multi-colour super-resolution imaging could shed more light into the putative

complex interactions between neurexin-LRRTM2 and AMPARs aiming to decipher the mechanisms underlying the formation of nanocolumns and contributing to our understanding about neuronal connectivity.

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