

A Trans-synaptic Mechanism for the Development and Function of a Neural
Circuit

by

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Abstract

Synapses are formed by high affinity trans-synaptic interactions between pre- and post-synaptic proteins. Any disturbance in these interactions may affect synaptogenesis. We assessed the consequences of disrupted trans-synaptic interaction between presynaptic neurexins and postsynaptic LRRTM4 proteins *in vivo*. We found that neurexins bind to LRRTM4 only in the presence of heparan sulfate (HS) chains. When HS binding site on LRRTM4 was mutated, they could not bind to neurexins. Analysis of brain collected from the knock-in mice with mutation in the functional HS binding domain of LRRTM4 showed reduction in excitatory synapses at dentate gyrus (DG). The binding of neurexins and LRRTM4 via HS chains recruited PTP σ to induce optimal presynaptic differentiation. The knock-in mice also revealed reduced levels of PTP σ and neurexins in the dentate gyrus lysates. Therefore, our results provide evidence that trans-synaptic interactions between neurexins and LRRTM4 via HS chains are required for excitatory synapse formation in the DG; and any disruption in their interaction impair excitatory synapse formation and development.

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Abbreviations

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASD	Autism Spectrum Disorder
BSA	Bovine serum albumin
CA	Cornu Ammonis
CAM	Cell adhesion molecule
CH	Glycosylated region
CNS	Central Nervous System
CNV	Copy number variation
Co-IP	Co-immunoprecipitation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSPGs	Chondroitin Sulphate Proteoglycans
DG	Dentate gyrus
EC	Entorhinal Cortex
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
FNIII	Fibronectin III
GABA	Gamma aminobutyric acid

GPI	Glycophosphatidylinositol
H.M	Henry Molaison
HC	Hippocampus
HS	Heparan sulphate
HSPGs	Heparan sulphate proteoglycans
Ig	Immunoglobulin
KI	Knock in
LAR	Leukocyte common antigen receptor
LEC	Lateral entorhinal cortex
LNS	Laminin/Neurexin/Sex-hormone-binding-globulin domains
LRR	Leucine rich repeat
LRRTMs	Leucine rich repeat transmembrane proteins
MEC	Medial entorhinal cortex
NDS	Normal donkey serum
NGLs	Netrin G ligands
NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
PBS	Phosphate buffer saline

PCR	Polymorphic chain reaction
PFA	Paraformaldehyde
PI-PLC	Phosphatidylinositol-specific phospholipase C
PSD	Postsynaptic density
PTP σ	Protein tyrosine phosphatases sigma
RPTPs	Receptor protein tyrosine phosphatases
SL	Stratum lucidum
SLM	Stratum lacunosum-moleculare
SNP	Single nucleoside polymorphism
SR	Stratum radiatum
ssDNA	single stranded DNA
TBST	Tris-buffered saline-Tween20
VGlut1	Vesicular glutamate transporter 1
VGlut2	Vesicular glutamate transporter 2
WT	Wild type

1. Introduction

1.1 The hippocampus (HC) within the central nervous system (CNS)

From simple to complex activities of a multicellular organism, many functions are controlled by the central nervous system (CNS) which is made up of the brain and the spinal cord in vertebrates. The CNS plays a primary role in receiving various inputs from the environment, interpreting them and responding appropriately. Different lobes of the brain are involved in interpreting different sensory inputs and integrating with motor and visceral activity. These lobes have different layers and regions which co-ordinate and control higher functions such as memory, cognition and influence the behavior of an organism. Cortical layers represent the outermost layer of the brain anatomically. This layer in the motor cortex initiate signals for processes to happen like moving a leg. Subcortical regions are deeper within the brain relative to the cortex, and these regions are more diverse anatomically and act as information hubs of the CNS. The hippocampus, in the temporal lobe, is one of the most well studied sub-cortical regions of the brain (Anderson et al., 2007), depicted in Figure 1.1.

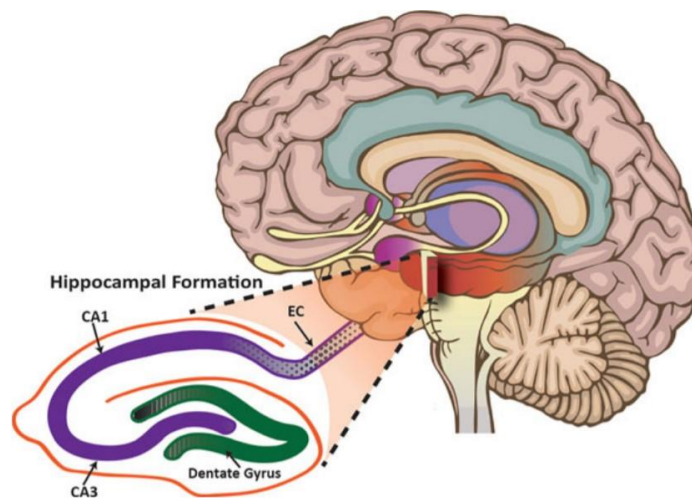


Figure 1.1. The hippocampus in the central nervous system.

Schematic illustration of the brain in a sagittal section and its deeper layer showing the hippocampus. The hippocampus is a part of the limbic system that is in the temporal lobe of the brain. Derived from Vineyard et al., © 2016 IEEE

Researchers have found the hippocampus to be interesting because of its key roles in learning and memory (DeKraker et al., 2019). The function of hippocampus in memory came into focus based on the case of Henry Molaison or patient H.M. (Scoville and Milner, 1957). H.M suffered from memory impairments following the bilateral removal of the medial temporal lobes from his brain (Milner, 1965; Milner et. al 1968). Since then, studies have focussed on the role of the hippocampus in encoding of visual and verbal information (Squire et al., 1992; Nyberg et al., 1996; Stern et al., 1996; Rombouts et al., 1997; Fernandez et al., 1998). Functional imaging studies and neuropsychological analysis showed activation of hippocampal regions while encoding declarative memory – memory of facts and events (Aggleton and Brown, 1999; Eichenbaum and Cohen, 2001; Squire et al., 2004). Discovery of “place cells” provided fundamental evidence in establishing the role of hippocampus in memory formation as several studies showed consistent firing of these cells during memory tasks (O’Keefe and Dostrovsky, 1971; O’Keefe and Nadel, 1978; Nadel, 1991).

Lesions in the hippocampus of rodents greatly impaired the spatial navigation memory. Animals with hippocampal lesion failed to recall the routes or locate submerged platforms in the Morris water maze test, or were unable to distinguish between baited and un-baited arms in radial arm maze tasks (Jarrard, 1978; Olton et al., 1978; Morris et al., 1982,1990; Maguire et al., 1997, 1998). Several studies also suggest that the hippocampus contributes to processing of social memories (Maaswinkle et al., 1996; Kogan et al., 2000). To dissect how the various regions of the hippocampus contribute to its various functions, most of the recent studies have focused on

subdivisions of the hippocampus rather than treating hippocampus as a homogenous structure (DeKraker et al., 2019).

1.2 The anatomy of the hippocampus

The hippocampus, based on histology and cytoarchitecture, is divided into distinct subfields that are called Cornu Ammonis (CA) 1 to 4, subiculum and the dentate gyrus (DG) (Duvernory et al., 2013; Witter, 2018). The Ammon's horn or simply CA, also known as hippocampus proper, is composed of layers or strata. (Fig. 1.2). The stratum pyramidale or SP layer contains cell bodies of principal or pyramidal neurons and various interneuron sub-types. The pyramidal neurons are tightly packed in the CA1 region compared to CA2 and CA3 regions (Andersen et al., 2007). The different CA regions can be clearly distinguished, based on cell packing, when stained with a nuclear marker called DAPI. The stratum lacunosum-moleculare or SLM layer consists of the distal dendrites of pyramidal neurons (Witter and Amaral, 1991; van Groen et al., 2003). The stratum lucidum or SL layer is exclusively present in CA3 region which contains mossy fibers coming from DG (Witter and Amaral, 1991; Anderson et al., 1971). The stratum radiatum or SR layer contains apical dendrites of pyramidal neurons. The majority of CA3 to CA1 connections known as Schaeffer collaterals are formed in this layer (Andersen et al., 2007). The stratum oriens or SO layer is above the pyramidal cell layer which is a relatively cell free layer. It contains the basal dendrites of pyramidal cells and some Schaeffer collateral connections (Andersen et al., 1971). The dentate gyrus will be explained in more detail in section 1.4.

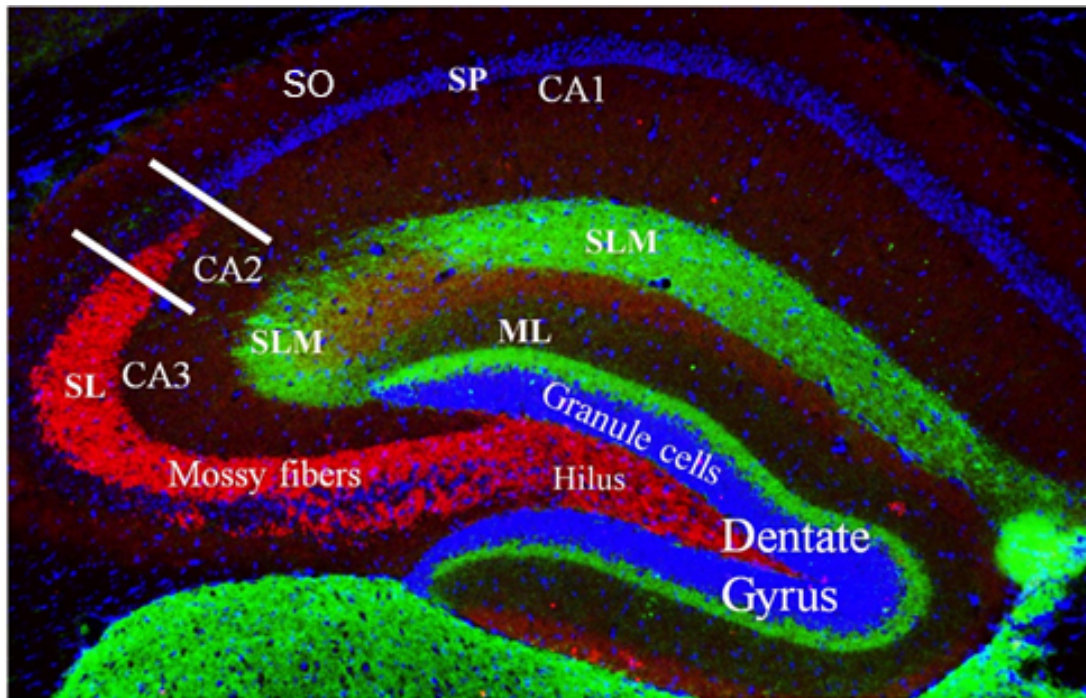


Figure 1.2. The anatomical subdivision of the dorsal hippocampus showing Cornu Ammonis and Dentate gyrus.

Fluorescence image of a sagittal mouse brain section. The blue color represents the visualization of the nuclei after staining with a nuclear marker called DAPI. These nuclei embody the pyramidal cell layer in the CA region and the granule cell layer of the dentate gyrus. The different layers are indicated by the different colors and abbreviations. ML: the molecular layer of dentate gyrus in dark greenish color above granular blue layer; SLM: stratum lacunosum-moleculare of CA1, CA2 and CA3 regions in green (green staining shows axonal terminal staining in ML and SLM); SP: stratum pyramidale layer of CA region (only labelled in CA1 but also present in CA2 and CA3) in blue; SL: stratum lucidum containing mossy fibers only present in CA3 region in red stained with a zinc transporter antibody; SO: stratum oriens layer above the SP region in dark red. This figure has been derived from Figure 2, Tzakis and Holahan, 2019.

1.3. The circuits of the hippocampus

The hippocampus receives input from various cortical projections. The conventional idea is that the flow of information in the hippocampus is unidirectional as depicted by the schematic illustration shown in Fig. 1.3. The major input to the hippocampus comes from layer II of the entorhinal cortex (EC) which sends projections mostly to the DG via the perforant pathway (synapse I). The DG sends projections to the CA3 region through mossy fibers, known as mossy fiber pathway (synapse II). The axons of CA3 terminate in the CA1 region via the Schaeffer collateral pathway (synapse III). In addition to projections to CA1, CA3 also sends collaterals to other CA3 pyramidal cells forming excitatory synapses with each other (shown in the figure). The recurrent projections from CA3 to CA3 is known as associational connections which is the characteristic feature of the CA3 network (Witter, 2018). The CA1 region projects to the subiculum and back to the entorhinal cortex completing the loop. This unidirectional pathway in the hippocampus, which connects DG and CA regions, is known as the tri-synaptic pathway. This pathway targets the basal and proximal part of apical dendrites of CA1 neurons (i.e CA1 stratum radiatum) (Kajiwar et al., 2008). A parallel projection from layer III of entorhinal cortex, known as the temporo-ammonic pathway, directly innervates the CA1 region and subiculum. (Witter et al., 2017). Unlike the diffusely spread tri-synaptic connection from layer II, the connections in the direct pathway from layer III of EC to CA1 are organized in a topographical way, forming almost one-to-one connections (Ishizuka et al., 1990; Aksel and Vaughan, 2013). This means projections from layer III lateral EC selectively innervate distal part of CA1 and proximal part of subiculum, whereas, projections from medial EC selectively innervate proximal CA1 and distal subiculum (Witter, 2018). The temporo-ammonic pathway targets the distal dendrites of CA1 pyramidal

neurons, an anatomical layer known as the stratum lacunosum moleculare (SLM) (Kajiwara et al., 2008).

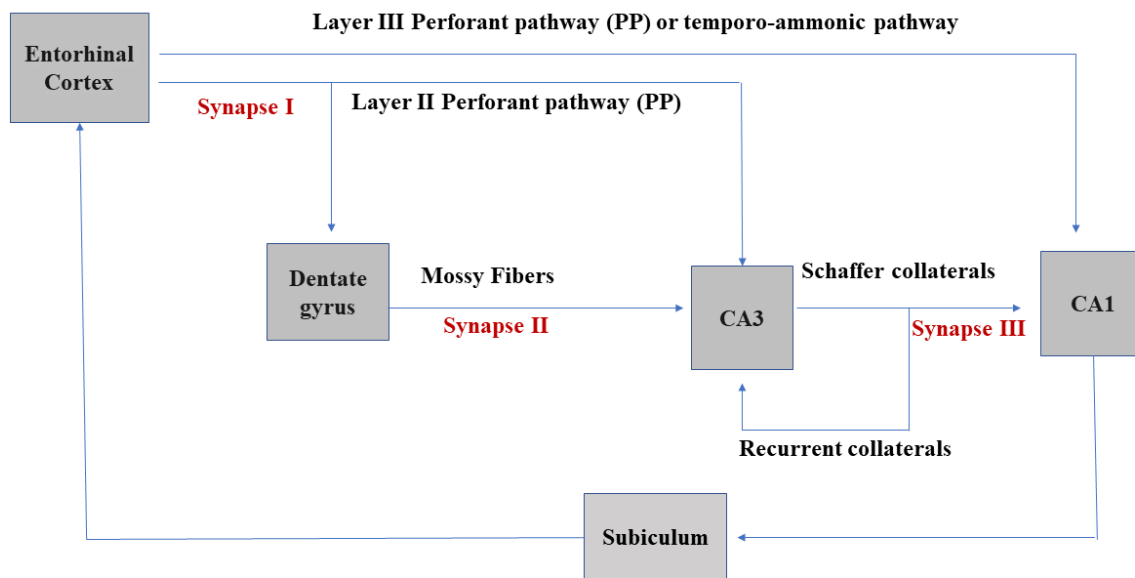


Figure 1.3. The schematic representation of the hippocampal circuit.

A simplified box diagram illustrating the direction of information flow within the hippocampus. The input from layers II and III of the entorhinal cortex is known as perforant pathway. The axons from layer II entorhinal cortex primarily form synapses with dendrites from the granule cells of the dentate gyrus. The mossy fibers from the dentate gyrus form synapses with dendrites of CA3 pyramidal neurons and this is known as the mossy fiber pathway. Axons from CA3 form synapses with CA1 dendrites via the Schaffer collateral pathway. The CA1 sends projections to subiculum. Together CA1 and subiculum send output from the hippocampus to entorhinal cortex. The axons from layer III of the entorhinal cortex form synapses with distal apical dendrites of CA1 pyramidal neurons in the CA1 stratum lacunosum-moleculare layer.

Based on input/output pathways and function, the hippocampus is arbitrarily classified into two major divisions – dorsal hippocampus and ventral hippocampus (Swanson and Cowan, 1977; Moser and Moser, 1998; Fanselow and Dong, 2010). Both divisions receive inputs from various subcortical structures channeled through EC. The EC, based on connectivity, is divided into three parallel bands – caudolateral, intermediate and rostromedial – which represent unique functional units projecting to distinct regions of the dentate gyrus (Witter et al., 1989; Dolorfo and Amaral, 1998). The dorsal hippocampus is enriched in place cells that encode spatial location (Jung et al., 1994; Muller et al., 1996). The major input to the dorsal hippocampus comes from the caudolateral band of the EC (Fig. 1.4) which sends excitatory projections to dorsal parts of the subiculum, pre-subiculum and post-subiculum (Amaral et al., 1991; Swanson and Cowan, 1977). The dorsal parts of the hippocampus and subicular complex project to retrosplenial and anterior cingulate cortices, that are regions involved in processing of visuospatial information and spatial navigation (Cenquizca and Swanson, 2007; van Groen and Wyss, 2003; Vogt and Miller, 1983; Frankland et al., 2004; Jones and Wilson, 2005). In addition, the dorsal subicular complex sends parallel projections to medial and lateral mammillary nuclei, which enables animals to behave appropriately in a familiar environment (Kishi et al., 2000; Swanson and Cowan, 1975; Muller et al., 1996; Taube et al., 1990). The dorsal hippocampus selectively projects to the caudal and the rostral part of the lateral septal nucleus which in turn projects to substantia nigra. The substantia nigra plays a critical role in voluntary locomotion (Risold and Swanson, 1996, 1997; Mogenson et al., 1983). The connections are shown in the diagram below (Fig. 1.4). Essentially, the dorsal hippocampus, with its input and output networks, mediates cognitive processes like learning, memory, navigation, exploration and locomotion (Muller et al., 1996; Jeffery, 2007).

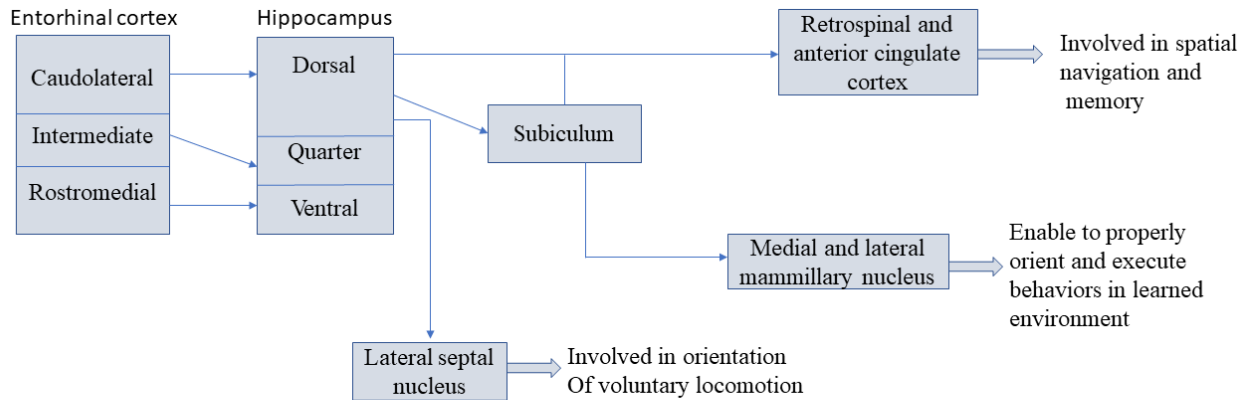


Figure 1.4. The schematic diagram of neuronal connectivity of the dorsal hippocampus.

A simplified box diagram illustrating the direction of information flow within the dorsal hippocampus. The caudolateral band of the entorhinal cortex projects to the dorsal hippocampus which sends projections to subiculum. The dorsal hippocampus also sends projections to retrosplinal and anterior cingulate cortex – both regions are involved in spatial navigation and memory. Another parallel projection from the dorsal hippocampus is the pathway to the lateral septal nucleus that plays a role in initiating voluntary movements. The dorsal hippocampus and subiculum projections reach the medial and the lateral mammillary nucleus. These nuclei are regions that enable proper orientation and execution of behaviors in learned/familiar environment.

The ventral hippocampus receives its major input from rostromedial band of the EC (Fig, 1.4). The ventral hippocampus sends direct projections to the olfactory bulb (Cenquizca and Swanson, 2007; Fanselow and Dong, 2009), the amygdala (Saunders et al., 1988; Pitkanen et al.,

2000; Cenquiza and Swanson, 2007), and the rostral hypothalamus (Canteras and Swanson, 1992). The ventral hippocampus has been reported to be involved in emotional memory processing such as anxiety and fear conditioning (Moser and Moser, 1998; Fanselow and Dong, 2010). In addition, the ventral hippocampus sends ipsilateral, unidirectional and glutamatergic projections to the prefrontal cortex (Jay et al., 1992, 1995). The ventral hippocampus – prefrontal cortex circuit has been associated with spatial short-term and working memory formation (Floresco et al., 1997; Seamans et al., 1998). (Barker et al., 2007, 2017; Chao et al., 2016). The ventral hippocampus also sends direct projections to the caudomedial nucleus accumbens and plays a role in reward processing as well as motivation-based feeding behaviour (Groenewegen et al., 1996; Naber and Witter, 1998; Wassum et al., 2009; Kelley et al., 2005). Unlike the dorsal hippocampus, the ventral hippocampus is more involved in emotional memory, episodic memory and motivated behavior (Kelly et al., Fanselow and Dong, 2010).

1.4. Dentate gyrus (DG)

The dentate gyrus (DG) is the first input region of the hippocampus (Amaral et al., 2007). The major extrinsic projections to the DG come from the EC (Deller et al., 1996). The DG is also known for its role in adult neurogenesis (Altman and Das, 1965; Eriksson et al., 1998; Gould et al., 1999; Imayoshi et al., 2008; Nakashiba et al., 2012). Anatomically, the DG consists of three layers: (1) molecular layer, (2) granule cell layer and (3) polymorphic layer (or hilus) (Fig. 1.5).

The molecular layer is a relatively cell-free layer that can be further distinguished into outer, medial and inner molecular layers. The molecular layer is occupied by dendrites of the DG cells and a few interneurons. This layer contains almost 90% of all DG synapses. Fibers from various input regions terminate here. The outer and medial molecular layers receive perforant path projections from superficial layer II of lateral and medial EC, respectively, (Amaral and Witter,

1995; Deller et al., 1996). The inner molecular layer receives projections from deep layers IV – VI of medial EC (Deller et al., 1996). The outer, medial and inner molecular layers can be distinguished by the Timm's stain method. The Timm's stain method (used to visualize heavy metals in the brain) demonstrates dense staining in the outer layer, hardly any staining in the medial layer and dark staining in the inner layer. (An example of this staining is shown in Figure 2, Amaral et al., 2007).

The primary cell layer of the DG is made up of tightly packed granule cells which are principal cells of the DG. The granule cells are bipolar, i.e. upper pole of cell body gives rise to spiny dendrites and axons originates from the base of the cell body (Seress and Pokorny, 1981; Claiborne et al., 1990; Schmidt-Hieber et al., 2007). Granule cells are morphologically diverse because new, adult-born cells are mixed with existing mature cells in the population (Cameron et al., 1993, 2001, Zhao, 2006). The granule cells receive all the excitatory synapses from stellate neurons of EC on their dendritic spines. DG granule cells, in turn, give rise to distinct unmyelinated axons, called mossy fibers. The mossy fibers innervate the mossy cells of the polymorphic layer and the pyramidal cells of the CA3 region. Basket cells are often found in the boundary between the granule cell and the polymorphic layers. These cells are GABAergic inhibitory interneurons and preferentially form inhibitory synapses on the cell body or shaft of apical dendrites of the granule cells.

The polymorphic cell layer, or hilus, is enclosed by the granule cell layer. This layer consists of various cell types, of which the mossy cells are the most prominent. The dendrites of mossy cells bifurcate and generally give rise to branches which remain within the polymorphic

layer. Occasionally these dendrites pierce granule cells and enter molecular layer. The DG tends to have more of “V” shape dorsally and more of “U” shape ventrally (see arrow on Figure 1.5).

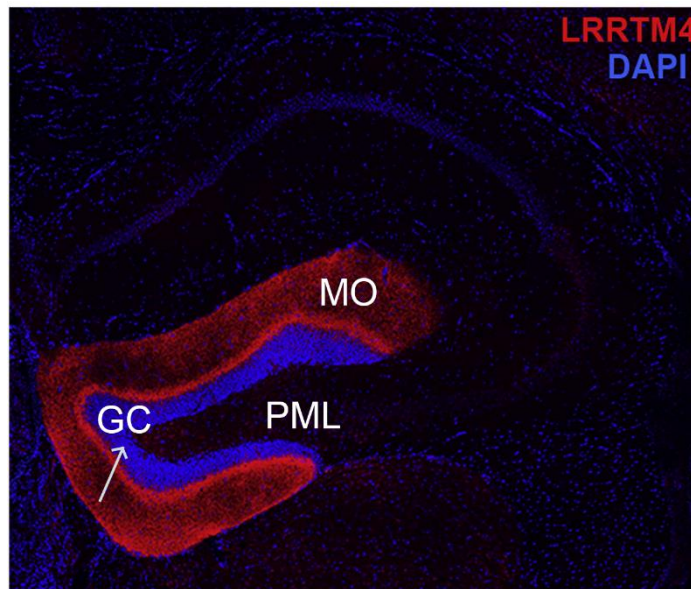


Figure 1.5. The anatomical structure of the dentate gyrus with different layers.

An image of fluorescent microscopy of a frozen mouse brain section showing the dorsal dentate gyrus. The blue layer represents the DAPI staining of granule cell nuclei (GC) of the dentate gyrus. Note the “V” shape indicating dorsal dentate gyrus on the lower part of the image (arrow). The MO represents the molecular layers of the dentate gyrus. The layer enclosed by the granule cell layer is called polymorphic layer (PML). Figure and image adopted from Figure 1F, Siddiqui et al., 2013.

The hippocampus is known for its function in learning and memory. Among the other known functions of the hippocampus is the ability to distinguish between two similar contexts (Rolls, 1996) or experiences (i.e. pattern separation) and to retrieve the information whenever necessary (i.e. pattern completion) (Marr, 1971). The DG, the first input region of the hippocampus, has been proposed to play a critical role in pattern separation (Trevis and Rolls,

1994). The input to the DG comes from layer II of EC which processes spatial and non-spatial information (Hargreaves et al., 2005; Kensler, 2013). The DG can separate two similar but distinct patterns by activating different granule cells in the same population. The new adult-born cells and old mature cells have different firing rates and when activated together permit separation of similar patterns (Deng et al., 2010). There are more granule cells than input cells that allow non-overlapping encoding of information (Chavlis et al., 2017). Pattern separation is also supported by feed-forward inhibition from interneurons onto granule cells (Jinde et al., 2012).

Pattern completion is achieved by autoassociational CA3 network i.e. the dense recurrent projections within CA3 contribute to pattern completion. Behavioural studies indicate that DG lesions or granule cell damage result in animals failing to distinguish between similar contexts (Gilbert et al., 2001; Jessberger et al., 2009; Lee and Solivan 2010). Therefore, the DG allows the brain to organize and separate similar contexts and experiences from one another.

1.5. Synapse formation and maintenance

Synapses are highly specialized neuronal junctions that allow communication between neurons. Synapses connect neurons to form neural circuits that underlie brain functions such as learning, memory and influence behavior.

Synapse formation or synaptogenesis is robust during early development. However, synapses are formed throughout life and adult synaptogenesis is believed to contribute significantly to learning and memory (Waites et al., 2005). When the previously formed synapses are not required anymore, they are eliminated. The elimination of synapses or synaptic pruning is essential for forming precise neural circuits to enhance brain functions.

Synapse formation takes place in several steps and each step must be strictly regulated to allow proper neural connections. These steps can be broadly listed as target selection, synapse

assembly, synapse maturation, synaptic plasticity and synapse elimination (Missler et al., 2012; Giagtzoglou et al., 2009). The target selection/recognition is a crucial step wherein axons and dendrites seek appropriate targets. Multiple extracellular axon guidance molecules like netrin, semaphorins and cell adhesion molecules also assist in the initial navigation process (Geng et al., 2016). The initial contact between potential synaptic partners is mediated by filopodia originating in axons and dendrites and results in axo-axonal, axo-dendritic or axo-somatic contact sites (Garner et al., 2002). The axo-dendritic contact is the most common, for instance, synapses formed by the axons of stellate neurons of entorhinal cortex and dendrites of dentate granule cells. An example of axo-somatic contact is between axons of basket cells and cell body of dentate granule cells (Amaral et al., 2007). The axo-axonal contact usually induces presynaptic inhibition and is found between chandelier cells and pyramidal cells in the piriform cortex (Wang and Sun, 2012). At the site of contact, pre- and postsynaptic proteins are recruited that lead to establishment of stable cell-to-cell contacts (Waites et al., 2005).

The molecular assembly of the presynaptic release apparatus, the synaptic cleft and the postsynaptic receptor apparatus make synapses functional (Garner et al., 2000; Waites et al., 2005). The presynaptic axon and postsynaptic dendrite undergo synaptic differentiation and recruit appropriate proteins at points of contact. The postsynaptic dendrite consists of electron-dense thickening called postsynaptic density (PSD) which clusters and anchors postsynaptic receptors and ion channels at high density (Scannevin and Huganir, 2000; Sheng, 2001).

In the maturation phase, synapses grow i.e. number of synaptic vesicles per terminal, bouton volume, docked vesicles, active zone area, PSD area and spine head volume increases (Harris and Stevens 1989, Pierce and Mendell 1993; Schikorski and Stevens 1997). The synapses are maintained or stabilized primarily by a specialized group of proteins known as cell adhesion

molecules (discussed below). The cell adhesion molecules are proteins with adhesive property that serve as anchor proteins to assemble or recruit receptors and components of signaling machinery and stabilize synapses. The most drastic changes are observed in the postsynaptic membrane during maturation. The glutamatergic synapses at their mature phase is characterized by the presence of spines in dendrites, AMPA and NMDA receptors on the postsynaptic membrane and active zone and synaptic vesicle fusion machinery in the presynaptic membrane. The mature inhibitory postsynaptic membrane consists of GABA_A receptors and less dense scaffolding protein complexes consisting of gephyrin and cell adhesion molecules (Tao et al., 2018).

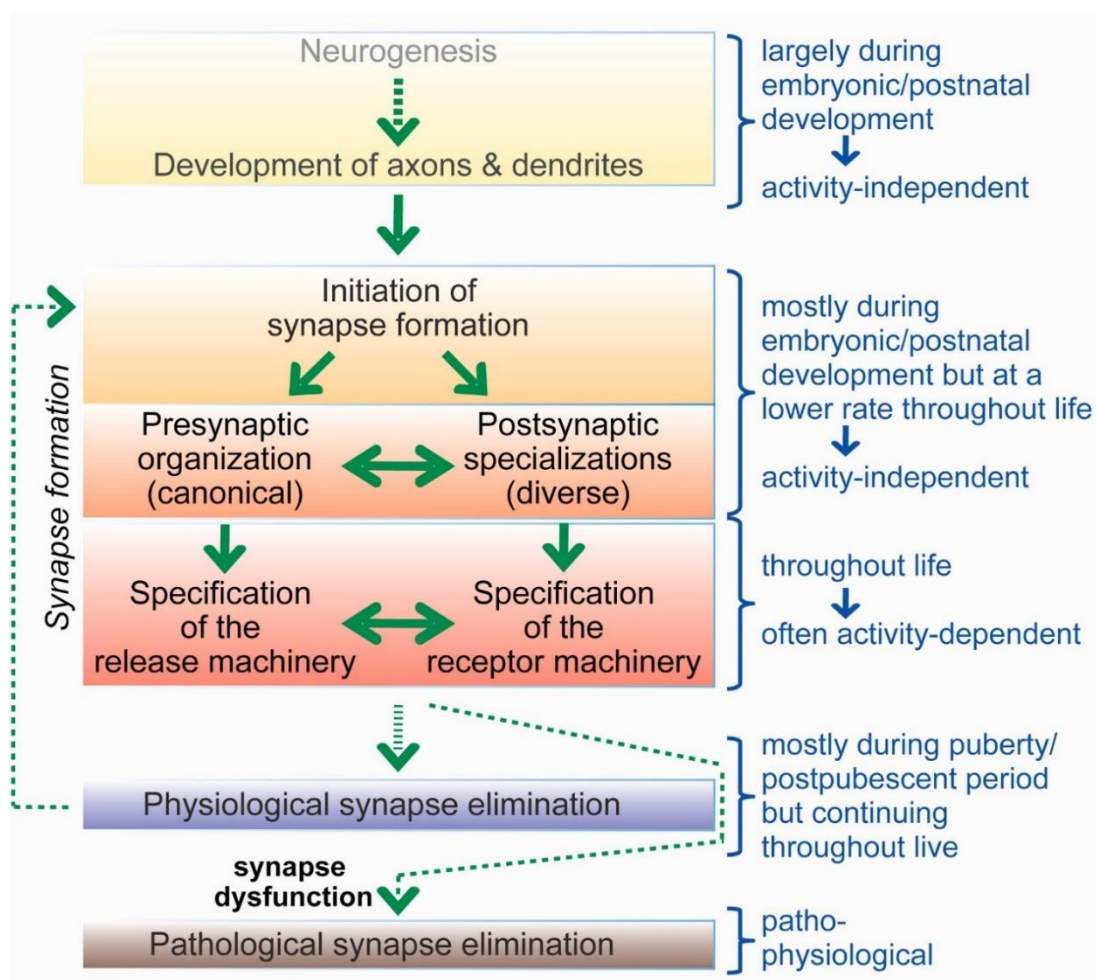


Figure 1.6. Flow diagram of synapse dynamics.

A box diagram of steps and factors involved in neurogenesis, synaptogenesis and elimination of synapses. Activity independent process mostly take place during embryonic/postnatal life when axons and dendrites are formed. With the help of cell adhesion molecules, the presynaptic organization and postsynaptic specializations take place that initiate the synapse formation process. This phase is activity independent and takes place mostly in postnatal development. Synapse maturation involves specification of release machinery in the presynaptic membrane and the specification of the receptor machinery in the postsynaptic membrane and is activity dependent phase. Elimination is the last phase of synaptogenesis where unwanted neural connections are removed naturally. This process can also occur during synaptic dysfunction or some pathological conditions. Derived from Figure 2, Sudhof, 2018 © 2018.

1.6. Excitatory versus inhibitory synapses

Based on the type of neurotransmitters released and their effect on the postsynaptic neuron; the chemical synapses are classified as excitatory and inhibitory synapses. Synapses releasing neurotransmitters that depolarize the postsynaptic membrane are excitatory while those that hyperpolarize the postsynaptic membrane are inhibitory.

Excitatory and inhibitory synapses are visualized using electron microscope or immunofluorescence imaging. Different markers for excitatory or inhibitory synapses are used for immunostaining and by viewing them at high magnification, synapses are distinguished into excitatory or inhibitory. Glutamate is the most common excitatory neurotransmitter. The commonly used markers for glutamate synapses are glutamate transporter VGluT1 (vesicular glutamate transporter 1) or VGluT2 (vesicular glutamate transporter 2) (Fremeau et al., 2002). VGluT1 and VGluT2 are glutamate transporters that transport the cytoplasmic glutamate into synaptic vesicles and are differentially expressed in the brain. The hippocampus is known to

express VGlut1 at higher levels (Kaneko and Fujiyama, 2002; Fremeau et al., 2004). Therefore, in this study, we used the VGlut1 marker. The postsynaptic density protein 95, or PSD-95 is a scaffolding protein in the postsynaptic region, and it is also often used as an indicator of excitatory synapses in the hippocampus.

Inhibitory synapses are marked by gephyrin, vesicular GABA transporter (VAT), GABA_A receptor $\gamma 2$ subunit (GABA_AR $\gamma 2$), and glutamate decarboxylase (GAD65 and GAD67) (Frederick and Craig, 2011; Gresa-arribas et al., 2014). GAD65 and GAD67 are isoforms of glutamic acid decarboxylase enzyme that catalyze the formation of GABA from glutamate. In this study, we used GAD65 to mark inhibitory synapses.

1.7. Cell adhesion molecules

The cell adhesion molecules or CAMs are surface proteins that structurally hold presynaptic and postsynaptic neurons together at synaptic junctions (Chamma and Thoumine, 2018). The CAMs are present on both axonal and dendritic cell surfaces and allow them to “stick” with one another or to their extracellular environment i.e. onto the extracellular matrix (ECM) (Horstkorte and Fuss, 2012). CAMs generally have three different domains: an intracellular domain, a transmembrane domain and an extracellular domain. The intracellular domain interacts with the cellular cytoskeleton and activates intracellular signalling, the transmembrane domain lies within the membrane bilayer and the extracellular domain interacts with other proteins or the ECM. The extracellular domain of CAMs can mediate either homophilic or heterophilic interactions, a property identified for stabilizing initial contact between neurons (Ozkan et al., 2014).

During synaptogenesis, CAMs play role in cell-cell recognition and stability in the initial phase and mediate signalling mechanisms for synapse maintenance, maturation and plasticity in later phases (Chamma and Thoumine, 2018). Several classes of CAMs like cadherins and

protocadherins contribute to target recognition (Shapiro and Colman, 1999; Takai et al., 2003). In addition, binding of these CAMs across the synaptic cleft provide structural stability to the synapse.

The presynaptic CAMs consist of neuroligins and LAR-type receptor tyrosine phosphatases that interact with multiple classes of postsynaptic CAMs such as neuroligins, leucine-rich-transmembrane neuronal proteins (LRRTMs) and netrin G ligands (NGLs) (Dalva et al., 2007; Shapiro and Colman, 1999; Linhoff et al., 2009; de Wit and Ghosh, 2015; Horstkorte and Fuss, 2012). The postsynaptic CAMs like neuroligins and LRRTMs induce presynaptic differentiation by aggregating neuroligins on the axon surface. The aggregation of neuroligins facilitates a network of high-affinity protein-protein interactions to recruit synaptic vesicles and active zone machinery. The postsynaptic NGLs act on presynaptic LAR family proteins to induce presynaptic differentiation by recruiting liprins. The aggregation of neuroligins, LRRTMs and other postsynaptic proteins on dendrites by presynaptic CAMs mediate postsynaptic differentiation. The high-affinity protein-protein interactions between postsynaptic proteins and scaffolding proteins such as PSD-95, PSD-93, Shank, homer, SynGap, and NMDA receptors induce postsynaptic differentiation. Therefore, cell adhesion molecules play a critical role in guiding, establishing and maintaining synapses. Mutations in the genes encoding the CAMs are associated with neurodevelopmental and psychiatric disorders. Table 1 shows a list of selected CAMs and disorders associated with their mutation.

Gene	Mutation	Disease	References
<i>LRRTM1</i>	Single nucleotide polymorphism (SNP)	Schizophrenia	Brucato et al., 2014; Francks 2009
<i>LRRTM2</i>	Copy number variation (CNV) SNP	Intellectual disability Bipolar disorder	Kleffmann et al., 2012 Malholtra et al., 2011
<i>LRRTM3</i>	SNP SNP SNP	Autism Spectrum disorder Alzheimer's disease Late onset Alzheimer's disease	Sousa et al., 2010 Majercak et al., 2006; Reitz et al., 2012; Thornton-Wells et al., 2008
<i>LRRTM4</i>	CNV, SNP	Autism spectrum disorder	Michaelson et al., 2012 and Pinto et al., 2010
<i>GPC1</i>	SNP	Schizophrenia Multiple sclerosis	Potkin et al., 2009; Baranzini et al., 2009; Gourraud et al., 2011; Sellebjerg et al., 2014
<i>GPC6</i>	SNP SNP SNP	Neuroticism Attention deficit hyperactivity disorder Alzheimer's disease	Calboli et al., 2010; Lesh et al., 2008, Sherva et al., 2014
<i>NRXN1</i>	CNV, SNV, truncation CNV, SNV, interstitial deletion Deletion, CNV, SNP Deletion, SNV	Schizophrenia Autism spectrum disorder Language development disorder Intellectual disability/mental retardation Nicotine dependence	Gauthier et al., 2011; Margi et al., 2010; Mozhui et al., 2011; Calahorra et al., 2009; Imitola et al., 2014; Wisniowiecka-Kowalik et al., 2010; Duong et al., 2012; Bierut et al., 2007; Ching et al., 2010; Rose et al., 2010
<i>NRXN2</i>	Truncation	Autism spectrum disorder	Gauthier et al., 2011

<i>NRXN3</i>	Deletion, SNP	Substance related disorder Schizophrenia Nicotine dependence	Hishimoto et al., 2007; Hu et al., 2013; Novak et al., 2009; Rose et al., 2010
<i>PTPRS</i>	de novo CNV (deletion, multigenic)	Autism spectrum disorder	Pinto et al., 2010
<i>PTPRD</i>	de novo CNV (deletion, duplication, multigenic)	Autism spectrum disorder Bipolar disorder Attention deficient hyperactivity disorder	Pinto et al., 2010; Elia et al., 2010; Malhotra et al., 2011

Table 1. Diseases associated mutations in the genes of cell adhesion molecules.

Listing of diseases associated with genes of cell adhesion molecules in neurons. The name of the genes (column on the left), the mutation(s) (middle column) and the associated diseases (column on the right) are listed. It is not an exhaustive list though. Data compiled from Table 2 in Roppongi et al., 2017 and from a Table 1 in Takahashi and Craig, 2013.

1.8. Synaptic organizing proteins

A subset of CAMs such as neurexins, neuroligins and LRRTMs induce synaptic differentiation in addition to adhesion (Scheiffele et al., 2000; Graf et al., 2004; Linhoff et al., 2009). These specialized CAMs are known as *synaptic organizing proteins* (Siddiqui and Craig, 2011). The synaptogenic adhesion complex comprises presynaptic and postsynaptic CAMs that bind to each other with high affinity in trans across the synaptic cleft. Some examples of such complexes are neurexin-neuroligin, neurexin-LRRTMs and RPTPs-NGLs (Ichtchenko et al., 1995, 1996; Ko et al., 2009; de Wit et al., 2009; Siddiqui et al., 2010; Woo et al., 2009; Kwon et al., 2010). Some secreted growth or neurotropic factors, derived from axons, dendrites, adjacent neurons or astrocytes that promote different aspects of synaptic differentiation are also considered

synaptic organizing proteins. These secreted factors induce synaptic differentiation by directly activating signal transduction cascades involving kinases and GTPases (Siddiqui and Craig, 2011).

1.8.1. Neurexins

Neurexins are presynaptic synapse organizing proteins that are transcribed by three genes (Nr α 1-3 in mice) each expressing α - and β - neurexins from alternate promoters (Ushkaryov et al., 1992,1993,1994; Sterky et al., 2017). The extracellular domain of α -neurexin contains six LNS (Laminin/ Neurexin/Sex-hormone- binding- globulin domains) and three interspersed EGF domains (Epidermal Growth Factor domains) (Ichtchenko et al., 1996). The shorter β -neurexin shares only the sixth LNS domain of α -neurexin. Both α and β neurexins have common transmembrane and 55-56 residues cytoplasmic domains (Ullrich et al., 1995; Missler et al., 1998). Recently discovered γ -neurexins, transcribed from Nr α 1 gene, contains threonine-rich and cysteine loop sequences only as extracellular domain followed by the transmembrane and cytoplasmic domains (Sterky et al., 2017).

In the brain, neurexins are localized to synapses and function in synaptogenesis from initial contact to maturation and plasticity (Reissner et al., 2013; Sudhof, 2018). Neurexins exist in thousands of isoforms due to extensive alternative splicing at six canonical sites referred to as SS1 to SS6 (Ullrich et al., 1995). The alternate splicing regulates trans-synaptic interaction of neurexins with their ligands such neuroligins, LRRTMs and Cbln1-Glu δ 2 (Ichtchenko et al., 1995, 1996; Ko et al., 2009; de Wit et al., 2009; Siddiqui et al., 2010; Woo et al., 2009; Kwon et al., 2010; Krueger et al., 2012; Uemura et al., 2010; Roppongi et al., 2017). The interactions of neurexins with their postsynaptic ligands were thought to be mediated purely by protein domain, especially LNS domains 2 and 6, until the recent discovery of neurexins as evolutionarily conserved heparan sulfate proteoglycans (HSPGs) (Chamma and Thoumine, 2018; Peng et al., 2018). The discovery

of post-translational heparan sulfate (HS) modification of neurexins has uncovered their key regulatory mechanisms in synapse development. The HS of neurexin and LNS domain are both important for presynaptic differentiation induced by LRRTM2 and neuroligins (Zhang et al., 2018). However, the role of neurexin in trans-synaptic interactions with LRRTM4 has not been explored.

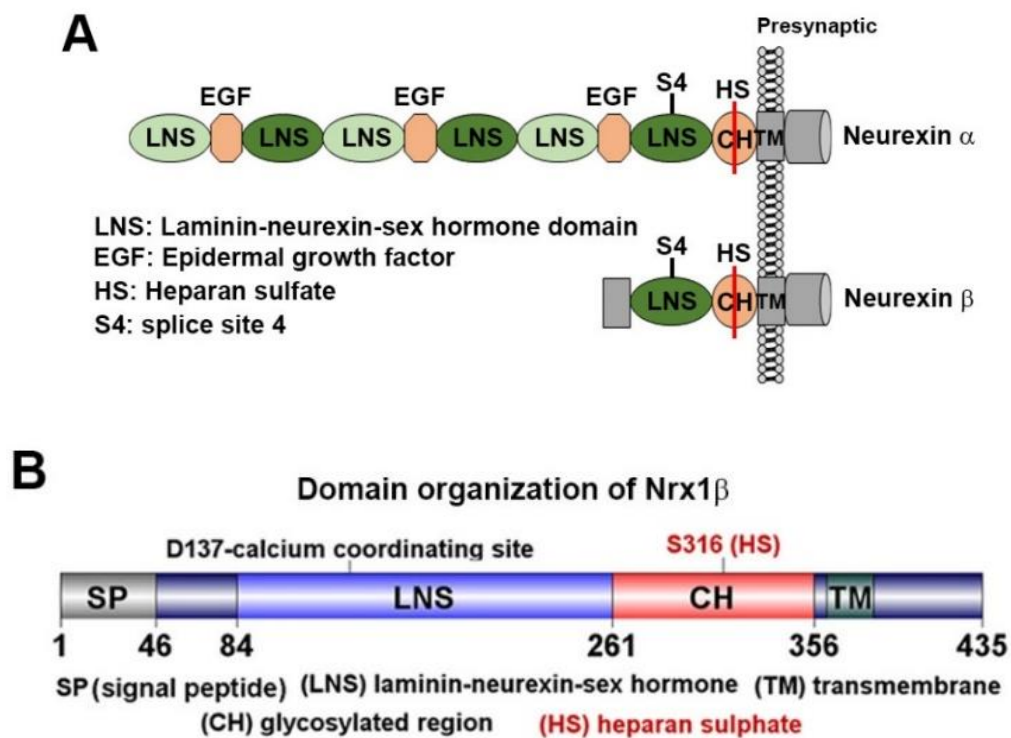


Figure 1.7. Schematic representation of Neurexin structure and domain organization.

A) Schematic representation of α and β neurexins. The extracellular region of α neurexin comprises six Laminin/ Neurexin/Sex-hormone- binding- globulin (LNS) domains with interspersed three epidermal growth factor (EGF) domains. The β neurexins contain just the sixth LNS domain as their extracellular domain. The transmembrane and cytoplasmic domains are same for both neurexins. The heparan sulfate (HS) chains are present in both forms of neurexin. The functionally important splice site SS4 is represented in the figure. B) Schematic representation of

domain organization of β neurexins showing the only LNS domain, with calcium coordinating site D137. The glycosylated region (CH) has the S316 HS binding site. SP represents single peptide and TM represents transmembrane region. (Figure taken from Roppongi et al, 2020).

1.8.2. Leucine rich repeat transmembrane proteins (LRRTMs)

LRRTMs are a family of four postsynaptic proteins (LRRTM1-4) exclusively present in vertebrates (Lauren et al., 2003). The LRRTMs are composed of ten extracellular leucine rich repeat (LRR) domains capped by LRR C-terminal and LRR N-terminal domains, a transmembrane domain and cytoplasmic a C-terminal tail (de Wit et al., 2009; Lauren et al., 2003; Roppongi et al., 2017). LRRTMs may bind to postsynaptic scaffolding proteins such as PSD-95 and other yet unidentified signaling proteins through their C- terminal tail which ends in a type I PDZ-binding motif (Minatahara et al., 2015; Roppongi et al., 2017). Alternate splicing of LRRTM3 and LRRTM4 at C-terminal may not end in PDZ binding motifs (Lauren et al., 2003; Roppongi et al., 2017). The LRR domains of LRRTMs are required for interactions with their trans-synaptic partners. These domains are rich in positively charged residues like arginine (R) and lysine (K).

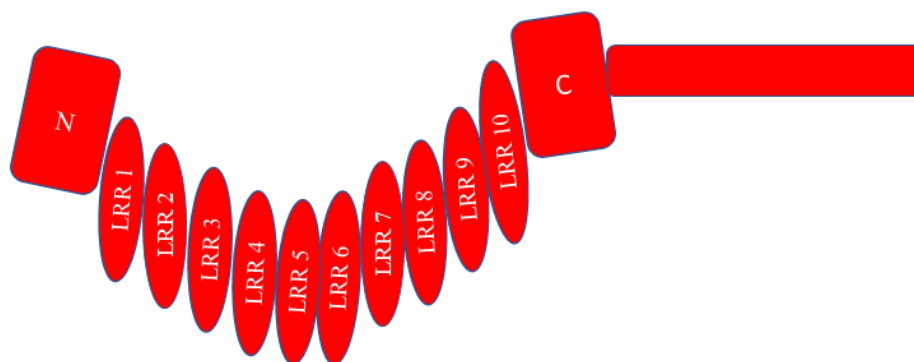


Figure 1.8. The structural representation of LRRTM4 protein.

Diagram of the LRRTM4 protein that has extracellularly ten leucine rich repeat (LRR) domains (ovals) capped by LRR C-terminal (C) and LRR N-terminal (N) domains followed by a transmembrane and short cytoplasmic tail (rectangle). The curved structure of the protein is due to LRR domains, which are the functional domains of LRRTM4.

The LRRTMs are expressed in discrete brain regions with partial overlapping distribution (Lauren et al., 2003). LRRTM1 and LRRTM2 are closely related to each other than to LRRTM3 and LRRTM4, which in turn are more evolutionarily conserved (Lauren et al., 2003; Uvarov et al., 2014). LRRTM4 expression is high in the DG assessed by in situ hybridization and immunostaining of fixed brain sections (de Wit et al., 2013; Siddiqui et al., 2013). As LRRTM4 is enriched in the DG, we have focused our study in the DG. LRRTM4 is not expressed in CA1 so CA1 stratum oriens, was a control region in this study.

LRRTMs were identified as synaptogenic proteins in a screen for proteins promoting presynaptic differentiation in an artificial synapse formation assay (Linhoff et al., 2009). All LRRTMs are localized to postsynaptic density and were reported to mediate excitatory synaptogenesis (de Wit et al., 2009; Linhoff et al., 2009; Siddiqui et al., 2013; Um et al., 2016). However, a recent study reported the role of LRRTM4 in organizing inhibitory synapses on the retinol rod bipolar axon terminals (Sinha et al., 2020). This novel finding suggests the need for further studies to explore the role of LRRTM4 in synaptogenesis.

Among the LRRTMs, LRRTM2 has the strongest synaptogenic activity followed by LRRTM1 and LRRTM4 (Linhoff et al., 2009). LRRTM1 and LRRTM2 induce presynaptic differentiation through trans-synaptic binding to neurexins (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010). The presynaptic partners for LRRTM3 and LRRTM4 were not identified

until two independent studies, using unbiased proteomics screens, reported heparan sulfate proteoglycans (HSPGs) as the presynaptic partner of LRRTM4 (Siddiqui et al., 2013; de Wit et al., 2013).

In a fibroblast-neuron co-culture assay (an assay where synaptogenic proteins are expressed on non-neuronal fibroblast cells and cultured together with neurons), when all neuroligins were knocked down, none of the LRRTMs could induce presynaptic differentiation (Um et al., 2016; Roppongi et al., 2020). This result showed neuroligins – recently discovered HSPGs – as presynaptic partners for LRRTM3 and LRRTM4 (Roppongi et al., 2020). When neurons in coculture were treated with heparinases (enzyme that cleaves all heparan sulfate chains from their core proteins), presynaptic differentiation induced by LRRTM4 was abolished (Siddiqui et al., 2013). This finding suggested that HS chain in the contacting neuron is required for LRRTM4 induced presynaptic differentiation. Moreover, LRRTM4 knock-out mice showed a 36% reduction in excitatory synapse density in the DG as compared to wild-type littermates (Siddiqui et al., 2013) suggesting that LRRTM4 is an important synapse organizing protein in the DG. In this study, we examined the role of trans-synaptic interaction between LRRTM4 and its presynaptic partner neuroligins in synapse development in the DG.

1.8.3. Receptor type protein tyrosine phosphatases (RPTPs)

Receptor protein tyrosine phosphatases (RPTPs) are a large protein family consisting of intracellular and transmembrane proteins. They are named so for removing phosphate groups from phosphotyrosine residues inside the cells (Schaapveld et al., 1997). The most well characterized subfamily of RPTPs is type IIa RPTPs (Johnson and Vactor, 2003). In vertebrates, type IIa RPTPs are composed of three cell-adhesion molecules like members named as leucocyte common antigen-related (LAR), PTP σ and PTP δ (Johnson and Vactor, 2003; Takahashi and Craig, 2013;

Pulido et al., 1995). Structurally, RPTPs are made up of three extracellular immunoglobulin (Ig)-like domains followed by four to eight fibronectin III (FNIII) domains and two intracellular domains, membrane proximal catalytic D1 domain and membrane distal non-catalytic D2 domain (Johnson and Vactor, 2003; Schaapveld et al., 1997; Chagnon et al., 2004). RPTPs undergo alternative splicing at various sites resulting in multiple isoforms in the brain. The binding of RPTPs to their postsynaptic ligands is splice-selective (Takahashi and Craig, 2013).

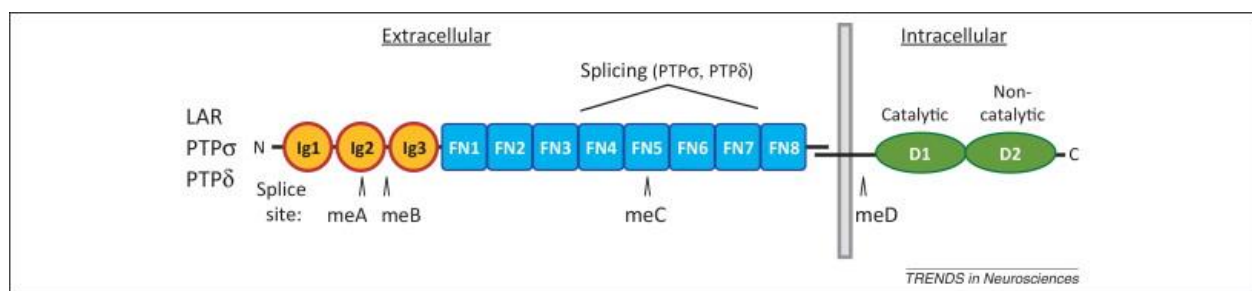


Figure 1.9. The schematic representation of structure of $PTP\sigma$.

Diagram of the $PTP\sigma$. Extracellularly three Ig-Immunoglobulin-like domains (Ig2, Ig2, Ig3) followed by FN-Fibronectin III domain (four to eight depending on alternate splicing, FN1-8), a transmembrane region and intracellular D1-membrane proximal domain that has catalytic activity and D2-membrane distal domain that do not have catalytic activity. The extracellular domains mediate the cell adhesion property of $PTP\sigma$ and gives the flexibility for both cis-interaction and trans-interaction with their partners. (The schematic structure of $PTP\sigma$ from Takahashi and Craig, © 2013).

The role of RPTPs in mediating neurite outgrowth and regeneration has been extensively studied. These functions may be accomplished by their binding to HSPGs and chondroitin sulfate proteoglycans (CSPGs) (Chen et al., 2013; Takahashi and Craig, 2013). However, the recent work

has illuminated their extensive role in synapse development (Johnson and Vactor, 2003; Schaapveld et al., 1997; Chagnon et al., 2004).

RPTPs are presynaptic proteins that mediate synaptic adhesion and synapse organization by trans-synaptic interaction with multiple postsynaptic partners (Woo et al., 2009; Kwon et al., 2010; Takahashi et al., 2011; Yoshida et al., 2011; Valnegri et al., 2011). The pathways involving RPTPs synaptic complexes may co-operate with other synaptic complexes during synapse development. Previously, LRRTM4 was proposed to induce presynaptic differentiation by interacting with glypican4-PTP σ complex (Ko et al., 2015). However, in the absence of RPTPs, LRRTM4 induced moderate presynaptic differentiation which was observed in an *in vitro* coculture assay (Roppongi et al., 2020). This result suggested that RPTPs were not critical for LRRTM4 mediated presynaptic differentiation. Also, *in vitro* neuron binding assays disclosed direct binding of neurexins and PTP σ via the HS modification of the neurexins. Based on these *in vitro* results, we investigated role of PTP σ in presynaptic differentiation induced by LRRTM4 in the DG *in vivo*.

1.8.4. Glypicans

Glypicans are HSPGs that adhere to the cell surface via glycosphosphatidylinositol (GPI) anchors (Fig 1.10) (Filmus et al., 2008). Vertebrates express six members of glypicans which, based on their sequence similarity, are classified into two groups: i) Glypicans 1/2/4/6 and ii) Glypicans 3/5. Glypicans have a protein core which is covalently conjugated to HS glycosaminoglycan chains. The attachment sites for HS chains are restricted to the last 50 extracellular membrane-proximal amino acids. These HS chains may mediate the interaction of glypicans with other cell surface proteins and growth factor receptors.

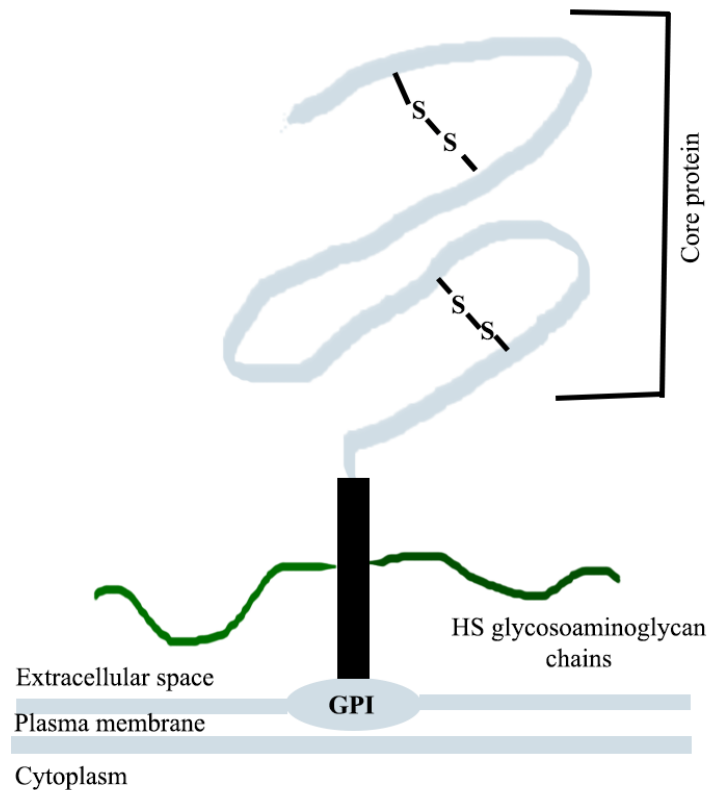


Fig. 1.10. Schematic of glypican protein structure at the cell surface.

The glypican protein is held in the plasma membrane by a GPI anchor. Numerous HS glycosaminoglycan chains (in green) are attached to the core protein (curved structure in blue) that allow glypicans to interact with other proteins and growth factor receptors.

Glypicans are expressed by neurons and glia in the CNS. Neuronal glypicans are proposed to play role in synapse development. Siddiqui et al, 2013 reported that LRRTM4 binds to glypicans via their HS chains. They also reported that LRRTM4 requires HSPGs to enhance presynaptic differentiation. Later, Ko et al. proposed that glypican 4-LRRTM4 postsynaptic complex mediates excitatory synaptic transmission by interacting with presynaptic PTP σ (Ko et al., 2015). However, in a recent study, Roppongi et al presented that LRRTM4 can induce presynaptic differentiation in absence of glypicans and PTP σ (Roppongi et al., 2020). This finding suggests that glypicans

may not be required by LRRTM4 to induce presynaptic differentiation. Therefore, we did not focus on glypicans in this section of the project.

2. Rationale, Objectives and Hypothesis

The HSPGs are reported as presynaptic ligands of LRRTM4 proteins (Siddiqui et al., 2013; de Wit et al., 2013). Presynaptic differentiation induced by LRRTM4 is mediated by their interaction with HSPGs. Neurexins, a recently discovered HSPG (Zhang et al., 2018) and their HS glycans are required by all LRRTMs to induce presynaptic differentiation in fibroblast-neuron co-culture assay *in vitro* (Roppongi et al., 2020) When neurexins were depleted in cultured neurons using the knockdown (KD) approach, LRRTM4 could not induce pre-synaptic differentiation in contacting axons. Molecular replacement with a KD resistant wild-type neurexin but not a neurexin lacking the HS glycan rescued synapse development deficit in those neurons indicating the requirement of neurexin-HS modification for LRRTM4 to induce presynaptic differentiation (Roppongi et al., 2020).

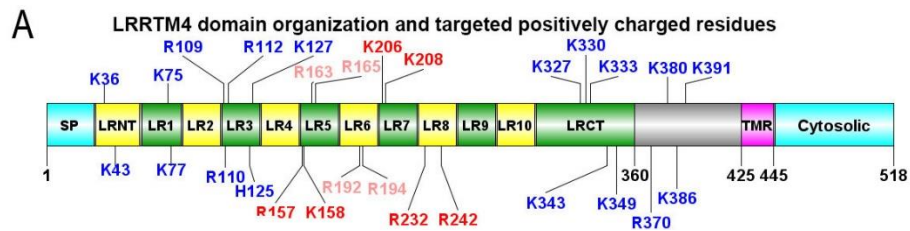


Figure 2.1. Schematic representation of leucine rich repeats of LRRTM4 with its leucine rich repeat (LRR) domains (derived from Roppongi et al., 2020).

The LRR domains of LRRTM4 and positions of the mutations in the LRR domains to test binding to neurexins. The red highlight represents the residues required for neurexin binding which were replaced by neutral amino acid alanine.

LR represents leucine rich repeat domains, R represents arginine residue, K represents lysine residue.

In order to better understand the mechanisms allowing the interactions involved in synapse formation and to identify the binding site of neurexin-HS in LRRTM4; a series of mutations were generated where alanine (A) replaced positively charged residues (arginine-R and lysine-K) in LRRTM4 as illustrated in Fig 2.1. The binding of recombinant neurexin to LRRTM4 with positively charged residues, arginine and lysine, mutated to alanine within LRR 5-8 was markedly reduced. The mutation in the LRR domain 5 was used to generate an LRRTM4 knock-in mouse line to test the consequences of altered interactions. The key question addressed here was what consequences would be observed *in vivo* in the DG when LRRTM4 lost its interaction with neurexins.

The first objective was to examine LRRTM4 and neurexin interaction in the DG of the knock-in mutant mice. As the LRRTM4 in these knock-in mice have a mutation in their neurexin binding site, we hypothesized that neurexin-LRRTM4 interactions would be lost in these mice and less synaptogenesis would be expected in the DG.

The second objective was to elucidate the role of PTP σ in LRRTM4-induced presynaptic differentiation *in vivo*. We hypothesized that for optimal presynaptic differentiation induced by LRRTM4, it requires the co-operation of PTP σ and neurexins. These hypotheses were tested in the new knock-in mouse model.

3. Methods

All the animal procedures used in the project complied with guidelines set by the Canadian Council on Animal Care (CCAC, <http://www.ccac.ca>) and were approved by the Animal Care Committee of the University of Manitoba. Mice from the local colony were used in the project.

3.1 Generation of *Lrrtm4*^{RK-KI} knock-in mice

The *Lrrtm4*^{RK-KI} mice for the experiments were generated using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 technology. Briefly, the region of exon 2 containing R157 and K158 encoding sequence was selected for guide sequence targeting. We chose this region because it is one of the binding sites for HS chain of neurexin. The guide sequence (5' ctgagcagtttaaaggcctc-3') was synthesized by Integrated DNA Technologies (IDT; Coralville, IA, USA) to cleave the DNA at the selected target site. To introduce R157A and K158A substitutions, a single stranded DNA (ssDNA) donor 5' tctcctacaacaaacttcagaccttacagtctgagcagtttaaaggcctcgcggccactcatcatttgcacttgcgatctaattctctcaagaccg tgcccatacgggtttt 3' was designed and synthesized. The ssDNA was provided after desalting by IDT to incorporate changes in DNA. The recombination of ssDNA also destroyed the CRISPR Pam site stopping further modification of the region (Fig 3.1). These changes were introduced into the mouse genome using *in vitro* fertilized embryos (for more detailed information see Roppongi et.al., 2020).

The confirmation of the knock-in sequence in the *Lrrtm4*^{RK-KI} mice was performed by Polymerase Chain Reaction (PCR). First the DNA from ear samples of wild type and knock in mice were extracted. The sequence was amplified using forward primer 5' -attagctcagtggaactaatatg-3' and reverse primer 5' atggatcgaggttgaagag-3. When DNA incorporated an alanine substitution, it created a sequence that was recognized by restriction digestion enzyme BstUI. The amplified

DNA sequence was digested with BstUI for 2.5 hours at 60°C and run in 8% polyacrylamide gel. The wild type sequence gave fragments of 312bp and 100bp while the mutant sequence gave fragments of 182bp, 130bp and 100bp (Fig. 3.1).

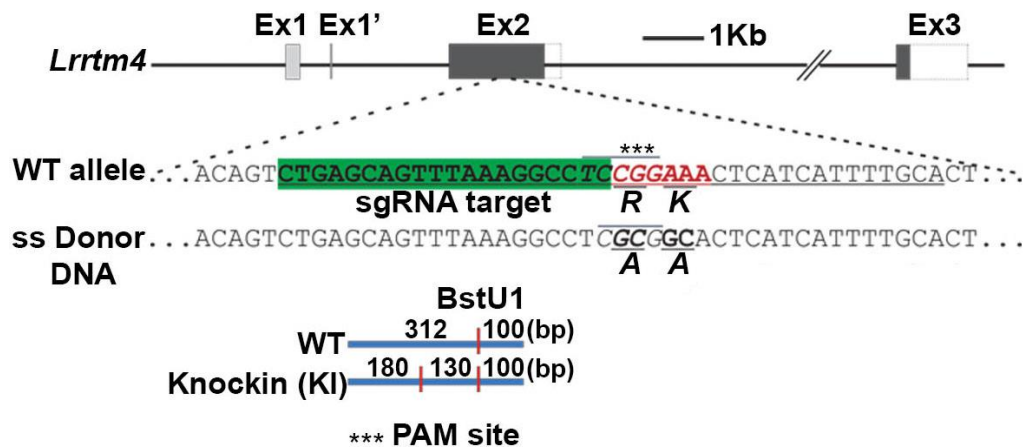


Figure 3.1. Schematic of the knock-in mouse gene organization.

The gene encoding the *Lrrtm4* protein is shown and the detailed sequence of Exon2 (Ex2) that contains the PAM site that was modified (***) is shown as the wild-type (WT allele). A single-stranded donor DNA (ssDonor DNA) with CRISPR-based targeting was used to substitute the indicated arginine (R) and lysine (K) residues to alanine (A) while introducing a BstUI restriction site. The single guide RNA (sgRNA) targets the sequence before PAM site for cutting the DNA at that site. The WT shows 312bp and 100bp fragments while the KI shows 180bp, 130bp and 100bp fragments. The figure is derived from Roppongi et al., 2020.

3.2 Histology

3.2.1 Preparation of brain sections and processing

The brain sections from 5-6 weeks old *LRRTM4^{RK-KI}* and *LRRTM4^{+/+}* littermates were used for most of the immunostaining experiments, *LRRTM4^{-/-}* mice were used as a control for LRRTM4 localization. The mice were anesthetized by intraperitoneal injection of chloral hydrate (dosage:600mg/kg). The transcardial perfusion was performed by passing ice-cold phosphate buffer saline (1X PBS) followed by paraformaldehyde (PFA). The concentration of PFA used for fixation was determined by optimal working condition for each antibody. Different antibody worked in different fixation methods, thus, brain sections for staining were prepared accordingly.

For staining the brain sections with presynaptic excitatory and inhibitory markers, VGlut1 and GAD65 respectively, mice were fixed with 4% paraformaldehyde and 4% sucrose solution. The brains were carefully removed and immersed in the same fixative solution overnight at 4°C. For cryoprotection, brains were initially transferred to 20% sucrose made in PBS and after 10-12 hours they were transferred to 30% sucrose (in PBS) overnight at 4°C. The brains were then embedded in OCT (Tissue Tek) compound and snap froze using dry ice and stored at -80°C. After 24 hours, the brains were sectioned into 20µm thick sections in a cryostat (Leica) while maintaining the temperature of cryostat between -20 to -25°C. The thin brain sections were collected onto gelatin coated SuperFrost Plus slides for better adhesion. The slides were air-dried for 30-60 minutes before staining. The slides that were not used for staining immediately were stored at -80°C.

For PTPσ labelling and its co-labelling with other presynaptic markers (VGlut1, Synapsin, and Bassoon), mice were anesthetized and perfused with ice cold pre-fixative solution comprising 50mM phosphate buffer, 0.1% sodium nitrite and 1unit/ml heparin followed by chilled fixative

solution containing 0.16M sodium phosphate buffer, pH 7.1, 0.2% picric acid and 1% paraformaldehyde and finally flushed with 10% sucrose made in 25mM phosphate buffer, pH 7.4. The brains were removed with caution and immersed in cryoprotectant (25mM phosphate buffer, pH 7.4, 10% sucrose, 0.04% sodium azide) and stored at 4°C until the cryo-sectioning was performed. Before cryo-sectioning, brains were embedded in OCT and dry ice was used for immediate freezing. The frozen brains were stored at -80°C for at least 24 hours. The cryostat temperature was set between -15 and -18°C for cutting 20µm thick brain sections. If the sections were not used for staining the same day, they were stored at -80°C.

The LRRTM4 immunostaining was performed without perfusion because anti-LRRTM4 only worked on fresh frozen tissues. The 5-6 weeks old wild-type, knock-in and knock-out mice were used for staining. The knock-out mice originated from a local colony previously developed by Dr. Siddiqui (Siddiqui et al., 2013). The mice were anaesthetized and sacrificed immediately. The brains were carefully removed, rinsed with ice cold PBS and submerged in OCT compound. For rapid freezing, 2-methylbutane (Sigma) in dry ice/ ethanol was used. Fresh frozen brains were stored at -80°C before sectioning. For obtaining decent brain sections, the cryostat temperature was maintained between -15 and -18°C. The brain sections were collected in SuperFrost Plus slides and stored at -80°C.

3.2.2 Immunostaining of brain sections

(i) Presynaptic markers VGlut1 and GAD65 immunostaining – The 4% PFA fixed brain sections were completely dried and a hydrophobic barrier using PAP pen was drawn around the tissue. The blocking solution (5% bovine serum albumin (BSA) + 5% normal goat serum (NGS) + 0.25% Triton X100 in PBS) was prepared and used for blocking the brain sections. Blocking is important to avoid non-specific interaction between other proteins and the antibody. It was ensured that

blocking solution covered every section on the slide. A humid chamber was used for the process to avoid drying of brain tissue, blocking was done for an hour at room temperature. The slides were incubated overnight at 4°C with primary antibodies anti-VGlu1 (rabbit, 1:1000; Synaptic Systems; 135 302) and anti-GAD65 (mouse IgG2a, 1:100, GAD-6-c, Developmental Hybridoma) diluted in the blocking solution. Next day the slides were washed three times with PBS, fifteen minutes each wash. The slides were then incubated with appropriate secondary antibodies conjugated to Alexa 488 (1:500 for VGlu1; Invitrogen) and Alexa 568 (1:500 for GAD65; Invitrogen) for an hour at room temperature. The slides were washed three times, fifteen minutes each wash, with PBS. After final wash, excess PBS was soaked using Kim wipes and slides were mounted using Fluoromount-G with DAPI (4',6 diamidino-2-phenylindole) (Southern Biotech 0100-20), avoiding air bubbles. The slides were dried in the dark at room temperature then imaged using fluorescence microscope. For gross hippocampal morphology, 4% PFA fixed brain sections from *Lrrtm4^{RK-KI}* and wild-type littermate were stained and mounted in the similar manner with primary antibody anti-synapsin I (rabbit, 1:2000; Millipore, AB1543P).

(ii) PTP σ labelling – The 1% PFA fixed brain sections were air dried and hydrophobic barrier was drawn around the tissue. The slides were incubated in blocking solution, 5% BSA +5% NGS +0.25% Triton X100 in PBS, for an hour at room temperature in a humid chamber. The primary antibody anti-PTP σ (mouse, 1:1000, 17G7.2, MM-0020, Medimabs) was diluted in blocking solution and added to the brain sections and stored at 4°C overnight. The slides were washed with PBS and incubated with appropriate secondary antibody conjugated to Alexa 488 (1:500; Invitrogen) for an hour at room temperature. The slides were washed thrice with PBS and after the last wash they were mounted using Fluoromount-G with DAPI. Until the mounting media dried, slides were kept in dark at room temperature and then used for imaging.

(iii) PTP σ co-labelling with presynaptic markers VGlut1, Bassoon and Synapsin – The 1% PFA fixed brain sections were air dried completely and soaked in 1.5X TBST buffer solution for twenty minutes. One litre of 1.5X TBST, pH 7.6, was prepared by dissolving 6.05gms of Trizma Base (Sigma) and 15gms of NaCl (Fisher Chemical) in distilled water and adding 3ml of Triton X-100 (Sigma). The primary antibody was prepared in blocking solution comprised of 10% Normal Donkey Serum (NDS; Jackson Laboratory) in 1.5X TBST. Three sets of primary antibodies were prepared, (i) anti-PTP σ (mouse, 1:1000, 17G7.2, MM-0020, Medimabs) and anti-VGlut1 (rabbit, 1:1000; Synaptic Systems; 135 302), (ii) anti-PTP σ (mouse, 1:1000, 17G7.2, MM-0020, Medimabs) and anti- bassoon (mouse IgG2A, 1:1000; Stressgen; VAM-PS003) and (iii) anti-PTP σ (mouse, 1:1000, 17G7.2, MM-0020, Medimabs) and anti- synapsin I (rabbit, 1:2000; Millipore, AB1543P). The brain sections were incubated overnight with either of the primary antibody set at 4°C in a humid chamber. The next day, primary antibody was washed three times, each wash 20 minutes in 1.5X TBST. Appropriate secondary antibody (Life Technologies; 1:600) was prepared in 10% NDS +1.5X TBST solution. The slides were incubated with secondary antibody at room temperature for 1.5 hours. After the incubation, slides were washed three times, first two washes were with 1.5X TBST and the last wash was with 50mM Tris HCL solution (25mL of 1M Tris-HCL + 475mL dd water). The excess solution was wiped out and Fluoromount-G with DAPI was used to mount the slides. The slides were kept at room temperature in the dark and imaged using fluorescence microscope.

(iv) Localization of LRRTM4 protein in the hippocampus – The fresh-frozen brain section slides were kept in 37°C incubator for drying for 10 minutes. The dried slides were fixed by submerging in cold methanol (-20°C) for 10 minutes. The slides were blocked with blocking solution (5% bovine serum albumin (BSA) + 5% normal goat serum (NGS) + 0.25% Triton X100 in PBS) for

an hour at room temperature. The primary antibody anti-LRRTM4 BC262 (rabbit, 1:500), (Siddiqui et al., 2013) was diluted in blocking solution to block the brain sections. After blocking, slides were incubated overnight in the primary antibody at 4°C. The slides were washed off primary antibody, three washes in PBS with each wash 15 minutes. Appropriate secondary antibody conjugated to Alexa 488 (Invitrogen; 1:500) was diluted in blocking solution and the slides were incubated for an hour at room temperature. The slides were washed three times with PBS and after the last wash, slides were mounted using Fluoromount-G and stored in the dark for drying before imaging.

Primary Antibody (Name)	Source	Concentration	Secondary Antibody (Name)	Source	Concentration
Rabbit-anti-VGlut1	Synaptic Systems 135 302	1:1000	Goat anti-rabbit-Alexa568 Or Alexa488	Invitrogen	1:500
MouseIgG2a-anti-GAD65	Developmental Hybridoma GAD-6-c	1:100	Goat anti-mouse-Alexa488	Invitrogen	1:500
Rabbit-anti-SynapsinI	Millipore AB1543P	1:2000	Goat-anti-rabbit-Alexa488	Invitrogen	1:500

Mouse-anti-PTP σ	Medimabs 17G7.2 MM-0020	1:1000	Goat-anti-rabbit-Alexa488	Invitrogen	1:500
MouseIgG2A-anti-Bassoon	Stressgen VAM-PS003	1:1000	Donkey-anti-mouse-Alexa568	Invitrogen	1:500
Rabbit-anti-LRRTM4	Siddiqui et al., 2013	1:500	Goat-anti-rabbit - Alexa568	Invitrogen	1:500

Table 2. Comprehensive list of all antibodies used.

As indicated in the column headers, all primary and secondary antibodies (same row indicates subsequent applications); the source and the catalogue number or the publication where it has been described as well as the concentration used for the immunohistological tests described in this thesis.

3.3 Fluorescence imaging

Imaging of all the brain sections was performed blind to the experimental conditions. Wide-field immunofluorescence was conducted for quantitation of VGlut1, GAD65 and PTP σ with a 63x/1.4 NA oil objective and custom filters on a Zeiss Observer Z.1 wide-field microscope with Apotome 2.0 for optical sectioning through structural illumination. Images were taken from outer, middle, and inner molecular layers of the dorsal DG taking CA1 oriens as control. CA1 oriens was used as a control because LRRTM4 protein is not present in that region. For quantitative analysis, random samples were used (3 mice per genotype, 8 sections per mouse, 8 images per hippocampus).

The 20X objective was used to capture tiled images for localization of LRRTM4 in the hippocampus to compare morphology of hippocampus between wild type and knock-in mice. The tiled images for co-labelling of PTP σ with presynaptic markers (VGlu1, Bassoon and Synapsin) were captured with 20X objective on a Zeiss Axioskop2 fluorescence microscope (Carl Zeiss Canada, Toronto, ON, Canada).

3.4. Data analysis

All the data analysis was performed blind to the experimental conditions. Quantification of presynaptic excitatory and inhibitory markers, VGlu1 and GAD65 respectively, as well as the PTP σ marker, was performed using Metamorph (Molecular Devices) software. Threshold-based detection methods were used on all images that were manually set to define puncta and total area. The total integrated intensity of puncta per tissue area was calculated in Excel (Microsoft). The graphs were prepared in GraphPad Prism (GraphPad Software) with 50-75 images per mouse used for analysis. Statistical comparisons were made by Kruskal-Wallis and Dunn's multiple comparison tests for VGlu1 and GAD65 markers whereas one-way ANOVA and post hoc Bonferroni's multiple comparison tests were used for the PTP σ marker.

3.5. Preparation of P2' synaptosomal fraction

P2' synaptosomal fractions were prepared to perform western blot (immunoblot) and co-immunoprecipitation experiments. Brains were removed following decapitation of anesthetized mice (n=3/4, 5-6 weeks old, *Lrrtm4*^{+/+}, *Lrrtm4*^{-/-}, *Lrrtm4*^{RK-KI}) and homogenized using ice cold homogenization buffer (320mM sucrose, 4mM Hepes-NaOH, pH 7.3, protease inhibitors). The brain tissue was homogenized in a glass Teflon homogenizer applying nine strokes at 1200 rpm. The homogenizer was rinsed with 70% ethanol and distilled water after each homogenization. The homogenate was centrifuged at 1000g for 10 minutes, the pellet P1 containing nuclei and cell

debris was discarded. The supernatant S1 was carefully transferred to different centrifuge tube and centrifuged at 12,000g for 15 minutes. The cytosolic supernatant S2 was carefully removed, the pellet P2 was resuspended in homogenization buffer and centrifuged again at 11,000g for 15 minutes. The supernatant was discarded and the resulting pellet P2' or synaptosomal fraction was resuspended in proper lysis buffer (complexiolite-48 buffer (Logopharm) or cold 50mM Tris pH 7.4, 100mM NaCl, 0.5% n-dodecyl- β -D-maltoside (DDM, Avanti Polar Lipids) containing protease inhibitors. The resuspended P2' synaptosomal fraction was incubated for 30-60 minutes at 4°C and centrifuged at 12,000g for 15 minutes to remove insoluble fraction. The solubilization of synaptosomal fraction was approximately 95%. For preparing synaptosomal lysates from DG, hippocampi were removed and sliced using a vibratome. DG was carefully dissected from 200-300 μ m thick hippocampal slices.

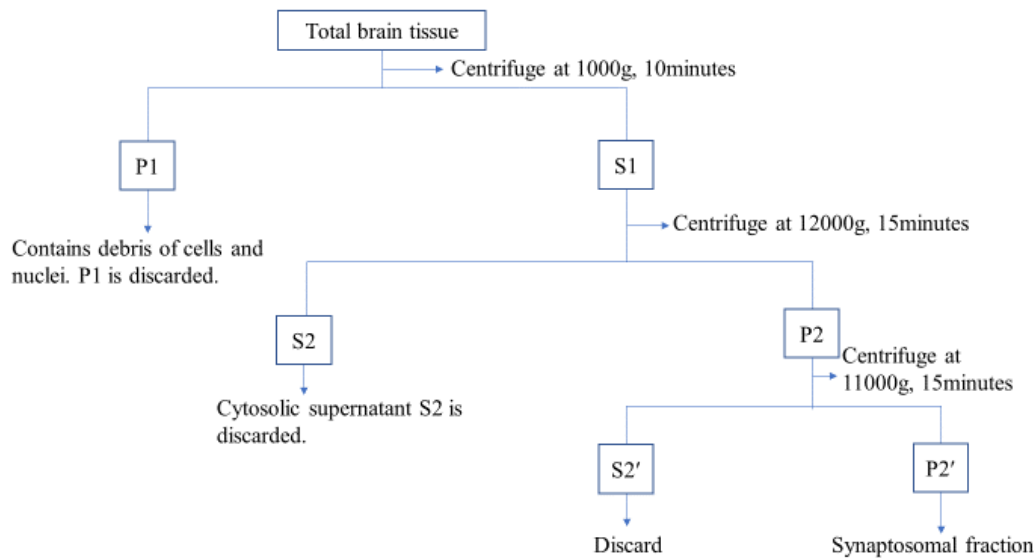


Figure 3.2. Workflow of P2' synaptosomal fraction preparation from brain tissue.

The P2' synaptosomal fraction from whole brain tissue is prepared by first centrifuging the homogenized brain tissue at low speed to remove cell debris and nuclei that form the pellet (P1). The supernatant (S1) is centrifuged at high speed to remove the supernatant (S2) containing cytosolic compositions. The pellet (P2) is resuspended in buffer and centrifuged at high speed to finally get the synaptosomal fraction P2'.

3.6. Co- Immunoprecipitation (co-IP)

Co-IP was performed using synaptosomal fraction that were prepared as described above. To assess integration of LRRTM4-neurexin interaction in *Lrrtm4*^{RK-KI} mice, the protein G sepharose beads were conjugated with anti-pan-neurexin (Millipore, ABN161-I) overnight at 4°C in three microfuge tubes (Eppendorf plastic, 1.5 mL). The crude P2' synaptosomal fractions from 5-6 weeks old *Lrrtm4*^{+/+}, *Lrrtm4*^{RK-KI} and *Lrrtm4*^{-/-} mice were prepared and incubated overnight with the antibody conjugated beads at 4°C. All immunoprecipitation assays were performed at 4°C to minimize proteolytic degradation of target protein. The next day, the beads were washed 3 to 4 times with buffer, proteins were eluted by boiling in 4X Laemmli buffer for 10 minutes at 95°C.

To assess interaction between neurexin and LRRTM4 or neurexin and PTPσ – The protein G sepharose beads were washed with binding buffer and conjugated with anti-pan-neurexin (Millipore, ABN161-I) antibody for at least 12 hours at 4°C in two microfuge tubes. The crude synaptosomal P2' fraction from whole mouse brain (1.0-1.2 mg/ml protein concentration) was added to the antibody conjugated tubes and rotated using a rotator at 4°C overnight. After overnight incubation, the beads were washed carefully (to avoid bead loss) 3 to 4 times with buffer. One of the tubes with lysate-antibody conjugated beads was treated with a cocktail of 1 U/ml each of Heparinases I, II, III for 2 hours at 37°C in heparinase digestion buffer (20mM Tris-HCl, pH 7,

100mM NaCl, 1.5mM CaCl₂, protease inhibitors). The proteins were eluted from samples by boiling in 4X Laemmli buffer for 10 minutes at 95°C.

3.7. Immunoblotting

The protein samples from co-IP assays were normalized using Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific). For LRRTM4 levels, the lysates from whole mouse brain from *Lrrtm4*^{+/+}, *Lrrtm4*^{RK-KI} and *Lrrtm4*^{-/-} were normalized. The samples were run in 10% SDS-polyacrylamide gels. Proteins on gels were transferred onto Immobilon P membranes (Millipore), setting at 35mA constant overnight at 4°C for better transfer. The membranes were blocked in 5% skim milk prepared in Tris-buffered saline/0.001% Tween-20 (TBST) for 60 minutes at room temperature. After blocking, the membranes were incubated with either anti-LRRTM4 (mouse, 1:1000, N205B/22, Neuromab) or anti-PTPσ (mouse, 17G7.2, MM-0020, Medimabs) or anti-β-actin (rabbit, 1:5000, ab8227, Abcam) overnight at 4°C. The membranes were washed 4-5 times with 1X TBST and incubated with secondary antibody (goat anti-mouse HRP conjugate from Southern Biotech) for chemiluminescence (1 hour at room temperature on a shaker). The membranes were washed 4-5 times with TBST then detected either using Clarity Western ECL or Clarity Max Western ECL substrate (Bio-rad) and a Bio-Rad gel documentation system.

4. Results

The morphological and biochemical studies were performed to examine the consequences of disrupted trans-synaptic interaction between LRRTM4 and neuexin in a unique mouse model, the *Lrrtm4*^{RK-KI} model. This mouse line is a knock-in model that allowed for the first time to examine trans-synaptic interactions in the DG. I performed PCR validation of our knock-in mice, followed by investigating levels and localization of LRRTM4 in the DG. Then I assessed the number of synapses in *Lrrtm4*^{RK-KI} young adult mice. Interestingly, we found that LRRTM4 requires co-operation of two presynaptic proteins, neuexins and PTPσ, to induce optimal presynaptic differentiation. These findings are also reported in Roppongi et al., 2020. However, one outstanding question before starting the *in vivo* experiments was: **Is *Lrrtm4*^{RK-KI} mouse suitable model to study role of trans-synaptic interaction in LRRTM4 induced presynaptic differentiation in the dentate gyrus?**

4.1. PCR technique confirms genotype of the knock-in mice

I confirmed the genotype of the *Lrrtm4*^{RK-KI} mice by PCR from ear biopsies. The identified bands are illustrated from a litter as an example in Fig. 4.1. There were two bands of 312bp and 100bp for *Lrrtm4*^{+/+} for the wild-type gene and there were three bands of 180bp, 130bp and 100bp for the modified gene in the *Lrrtm4*^{RK-KI}. Similar results were obtained for all the knock-in mice used in the project.

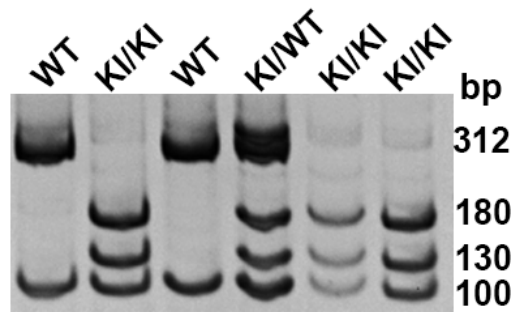


Figure 4.1. Example of the genotyping results of the *Lrrtm4*^{RK-KI} mice.

A photograph image of a gel showing bands at various base pairs after amplification of extracted DNA from ear samples. The wild-type gene is localizing at 310 and 100 bp, while the knock-in gene is localized at 180, 130 and 100 bp. The presence or absence of these bands were used to assign knock-in (KI/KI), wild-type (WT) or both genotypes (KI/WT).

4.2. Immunofluorescence imaging shows comparable gross morphology of hippocampus in *Lrrtm4*^{+/+} and *Lrrtm4*^{RK-KI} mice.

I stained fixed brain sections from *Lrrtm4*^{+/+} and *Lrrtm4*^{RK-KI} mice with synapsin and nuclear marker DAPI to examine gross hippocampal morphology. As shown by a representative example from each strain of mice in Fig. 4.2, I observed densely packed pyramidal neurons in the Cornu Ammonis (CA) region and densely packed granule cells in the dentate gyrus in both strains. The distribution of laminar layers of the CA region and molecular layers of the DG were also comparable in both genotypes. Overall, the hippocampal morphology of *Lrrtm4*^{RK-KI} mice was similar to *Lrrtm4*^{+/+} mice. To assess immunostaining for different proteins (see later in Results) the brains from six *Lrrtm4*^{RK-KI} mice and six *Lrrtm4*^{+/+} mice were stained. In all those mice, distribution of laminar layers of the CA region and molecular layers of the DG were similar as shown in Fig. 4.2.

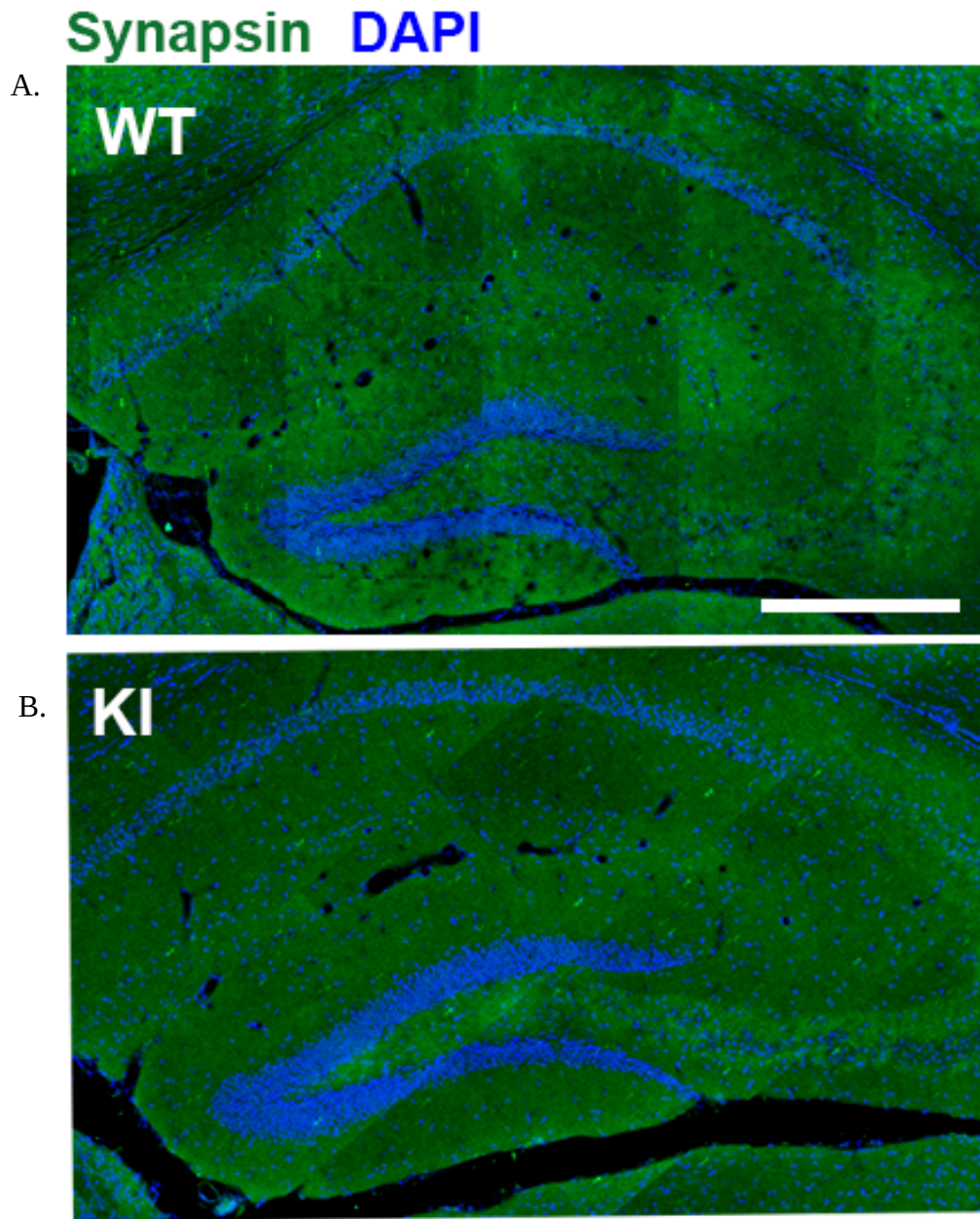


Figure 4.2. Images of sagittal brain sections from *LRRTM4* wild-type and knock-in mice. Images of thin brain sections from *Lrrtm4*^{+/+} mice (A) and *Lrrtm4*^{RK-KI} (B). Immunostaining with Synapsin (green) and nuclear marker DAPI (blue) showed normal gross morphology of hippocampus in *Lrrtm4*^{+/+} and *Lrrtm4*^{RK-KI} mice. The scale bar is 500 μ m.

4.3. Immunofluorescence-based detection of LRRTM4 shows normal LRRTM4 distribution in *Lrrtm4*^{RK-KI} mice compared to *Lrrtm4*^{+/+} mice

To investigate whether mutation in LRRTM4 gene has altered the localization of the protein in our knock-in mice, I stained fresh-frozen brain sections from *Lrrtm4*^{RK-KI}, *Lrrtm4*^{+/+} and *Lrrtm4*^{-/-} mice with anti-LRRTM4 antibody (BC262). I observed comparable distribution of LRRTM4 between *Lrrtm4*^{RK-KI} mice and *Lrrtm4*^{+/+} mice (Fig. 4A and 4C), LRRTM4 was not detectable in *Lrrtm4*^{-/-} mice (Fig.4.3B).

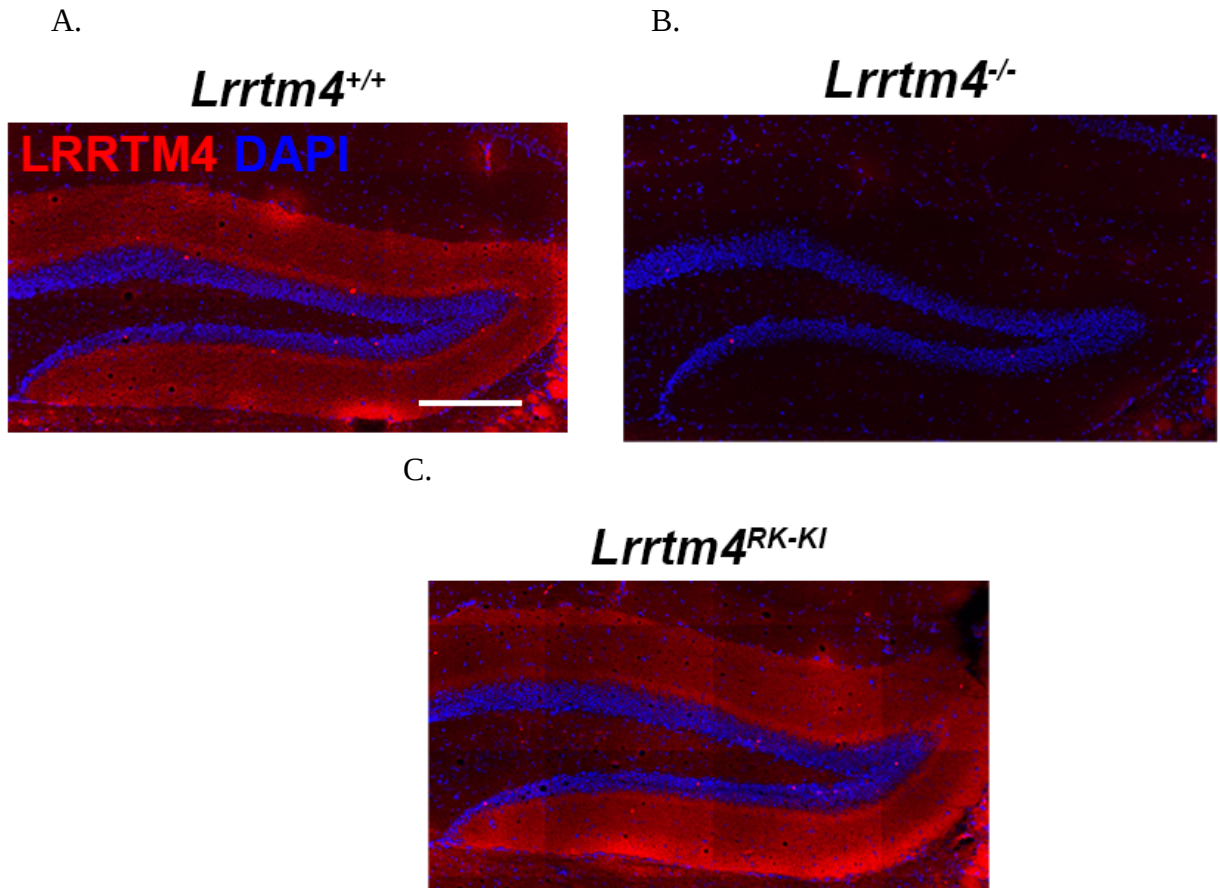


Figure 4.3 Images of sagittal dentate gyrus sections from three strains of mice. Immunofluorescence images showing the distribution of LRRTM4 (red) in the DG and the granule cell somas (blue) in a wild-type mouse (A, *Lrrtm4*^{+/+}), in a knock-out mouse (B, *Lrrtm4*^{-/-}) and in

a knock-in mouse (C, *Lrrtm4*^{RK-KI}). Normal localization of LRRTM4 was observed in all but the knock-out strain. The scale bar represents 500µm.

4.4. Levels of LRRTM4 protein in *Lrrtm4*^{+/+} and *Lrrtm4*^{RK-KI} mice is comparable but absent in *Lrrtm4*^{-/-} mice

To test the protein levels of the mutated form of LRRTM4 in *Lrrtm4*^{RK-KI} mice when compared to *Lrrtm4*^{+/+} mice, Dr. Padmanabhan prepared P2' synaptosomal lysates from each genotype. As shown in Fig. 4.4 below, the level of LRRTM4 was comparable between *Lrrtm4*^{RK-KI} and *Lrrtm4*^{+/+} and absent in *Lrrtm4*^{-/-} mice.

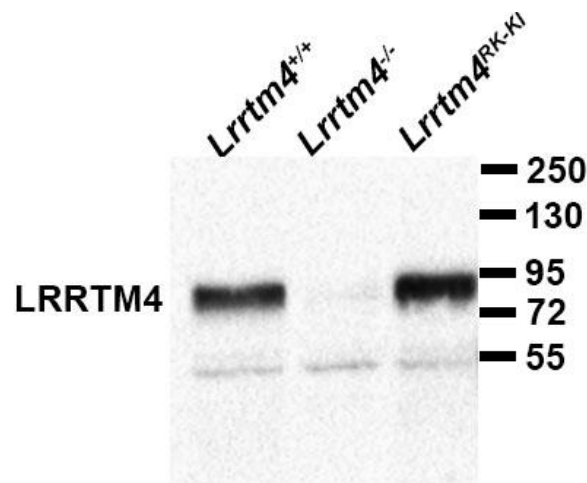


Figure 4.4. Representative samples of immunoblots of P2' synaptosomal lysates prepared from three strains of mice.

Image of a Western blot of whole mouse brain P2' synaptosome lysates from *Lrrtm4*^{+/+}, *Lrrtm4*^{-/-} and *Lrrtm4*^{RK-KI} mice show comparable levels of LRRTM4 protein in *Lrrtm4*^{+/+} and *Lrrtm4*^{RK-KI} mice as represented by similar thickness bands at 85kd in the blot in these samples. The middle column does not contain the band around 85kd showing the absence of the LRRTM4 protein in *Lrrtm4*^{-/-} mice. This figure was contributed by Dr. Nirmala Padmanabhan.

The immunofluorescence-based analysis and the biochemical assays indicated that the RK-AA mutation in the LRRTM4 gene does not interfere with the protein's distribution and

expression. Therefore, this mouse model is suited for studying trans-synaptic interactions involving LRRTM4. As mentioned above, the LRRTM4 binds neurexins. Their binding is mediated by the HS of neurexin. Guided by the *in vitro* experimental results (Roppongi et al., 2020), we were particularly interested in elucidating co-operation of the two presynaptic hubs – neurexins and PTPs in synapse development. We therefore examined whether the HS of neurexin is required for trans-interaction with LRRTM4 and cis interaction with PTP σ *in vivo*.

4.5. LRRTM4 and PTP σ require HS of neurexin for interaction *in vivo*

To assess the requirement of neurexin HS modification for its interaction with LRRTM4 or PTP σ , I performed co-immunoprecipitation (co-IP) experiments in collaboration with Dr. Padmanabhan. P2' synaptosomal lysates were prepared from whole brain of wild-type mice. We compared conditions when the lysates were incubated with heparinases, to remove all HS chains, before adding lysates to the beads conjugated with anti-pan-neurexin antibody to conditions when there was no heparinase treatment. As represented in Fig. 4.5, we observed clear band on heparinase untreated lysate (third lane) when blotted with anti-LRRTM4 (Fig. 4.5A) or anti-PTP σ (Fig. 4.5B) antibodies i.e. in the presence of HS, neurexins interact with their partners – LRRTM4 and PTP σ . The lysate in the left lane in Fig. 4.5 A and B is an input control, ensuring the presence of the protein of interest and the efficiency of the co-IP test. The middle lane in Fig. 4.5A shows no band indicating loss of interaction between neurexin and LRRTM4 after heparinase treatment. Similarly, the absence of band in the middle lane in Fig. 4.5B represents loss of interactions between neurexin and PTP σ after heparinase treatment.

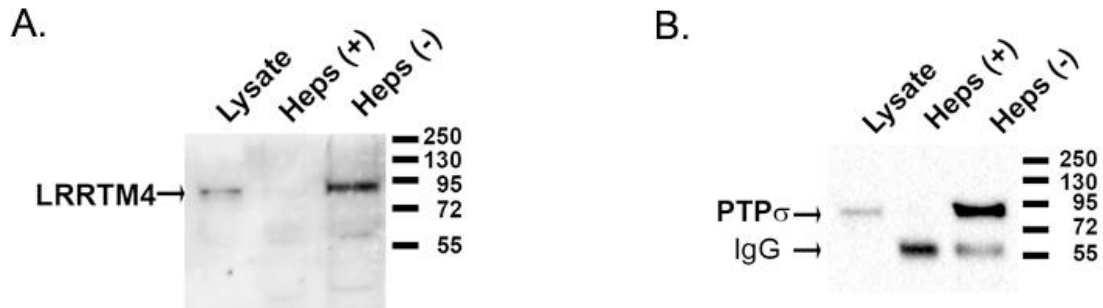


Figure 4.5. Representative co-immunoprecipitation immunoblots of whole mouse brain P2' synaptosomes.

Images of Western blots of Co-IP from heparinase treated and untreated mouse brain P2' synaptosomal lysates. In A), anti-pan-neurexin antibody co-immunoprecipitated LRRTM4 from heparinase untreated (Heps (-), 90 kd) lysates but not from heparinase treated (Heps (+)) lysates. In B), anti-pan-neurexin antibody co-immunoprecipitated PTP σ from heparinase untreated (Heps (-), 95kd) lysates but not from heparinase treated (Heps (+)) lysates. Lysate was used as input (1%) (in first lane on both blots). The IgG band (55 kB) seen in B is the heavy chain of the primary antibody. Similar results were found after comparisons made in 4 mice indicating that neurexin can not interact with LRRTM4 or PTP σ without its HS chains. These experiments were done collaboratively with Dr. Nirmala Padmanabhan and the figure was prepared by her.

4.6. LRRTM4 and neurexin trans-synaptic interaction is disrupted in *Lrrtm4*^{RK-KI} mice

The *Lrrtm4*^{RK-KI} mouse model, as mentioned before, has mutation in the neurexin HS binding site in its LRR domain. Whether neurexins can interact with the mutant form of the LRRTM4 is what was tested next.

Dr. Padmanabhan conducted co-IP experiments from P2' synaptosomes prepared from *Lrrtm4*^{+/+}, *Lrrtm4*^{-/-} and *Lrrtm4*^{RK-KI} mice. As seen in Fig. 4.6, the anti-pan-neurexin antibody co-immunoprecipitated LRRTM4 only from *Lrrtm4*^{+/+} mice. We did not observe clear bands in the

middle or the right lanes which indicated loss of neurexin-HS-LRRTM4 interactions in *Lrrtm4*^{-/-} and *Lrrtm4*^{RK-KI} mice. The presence of the band represents protein-protein interactions and its absence implies the loss of interactions between neurexin and LRRTM4 in *Lrrtm4*^{-/-} and *Lrrtm4*^{RK-KI} mice.

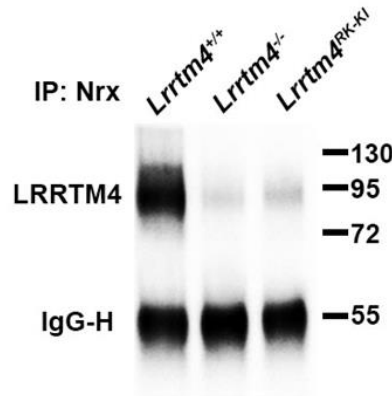


Figure 4.6. Representative immunoblot of Neurexin IP blotted with anti-LRRTM4 antibody from three different strains of mice.

Images of Western blot of immunoprecipitation from solubilized whole mouse brain P2' synaptosomal lysates prepared from *Lrrtm4*^{+/+}, *Lrrtm4*^{-/-} and *Lrrtm4*^{RK-KI} mice. The anti-pan-neurexin antibody (IP:Nrx) was used in beads which co-immunoprecipitated LRRTM4 from *Lrrtm4*^{+/+} mice as indicated by the presence of thick band (90kD) in *Lrrtm4*^{+/+} lane. This band was absent in *Lrrtm4*^{-/-} and *Lrrtm4*^{RK-KI} lanes. IgG-H band (55kd) represents the heavy chain of the anti-pan-neurexin antibody. Similar results were found from samples prepared from 4 mice each. This figure was contributed by Dr. Nirmala Padmanabhan.

4.7. Excitatory presynaptic inputs to the dentate gyrus molecular layers are reduced in *Lrrtm4*^{RK-KI} mice

To investigate if any changes occur in synapse levels because of disrupted interaction between LRRTM4 and neuexins, I performed quantitative immunofluorescence imaging assays. Considering the expression pattern of LRRTM4, the study was focused on DG of hippocampus and the CA1 stratum oriens was a control region. I stained the fixed brain sections with a presynaptic excitatory synapse marker, VGlut1. VGlut1 punctate immunofluorescence integrated intensity per tissue area was reduced in all the DG molecular layers, by 10-30%, in *Lrrtm4*^{RK-KI} mice compared to *Lrrtm4*^{+/+} littermate (at 5-6 weeks postnatal age) (Fig. 4.7A and B). The VGlut1 punctate immunofluorescence in CA1 oriens was comparable in *Lrrtm4*^{RK-KI} mice compared to the *Lrrtm4*^{+/+} littermate mice. These results indicate disrupted neuexin-LRRTM4 complex in *Lrrtm4*^{RK-KI} mice reduces the excitatory inputs to the DG.

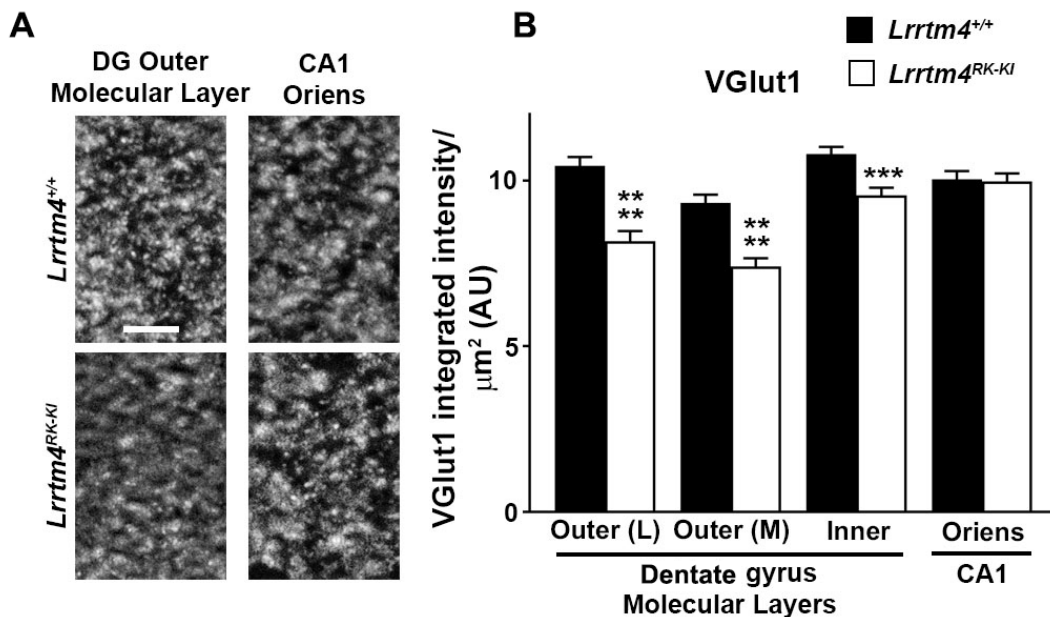


Figure 4.7. Comparison of excitatory synapses (by VGlut1 immunostaining) in dentate gyrus molecular layers and in the Cornu Ammonis 1 (CA1) regions in wild-type and knock-in mice.

A) High-resolution, representative fluorescence images showing reduction in Vesicular Glutamate Transporter 1 (VGlut1) punctate immunofluorescence in outer molecular layer of DG of *Lrrtm4^{RK-KI}* mice compared to *Lrrtm4^{+/+}* littermate. Similar intensity of VGlut1 puncta were evident in the CA1 (right top and bottom panels) while less intense labeling was evident in the DG of the knock-in mouse (left bottom) than in the wild-type mouse (left top panel). B) Quantitation of VGlut1 punctate integrated intensity (AU) per tissue area (μm^2) from different molecular layers of DG and of the CA1 oriens. Significant reduction in all molecular layers in *Lrrtm4^{RK-KI}* mice (no fill) compared to *Lrrtm4^{+/+}* littermate (solid fill). Outer L represents region receiving inputs from lateral entorhinal cortex and Outer M represents region receiving inputs from medial entorhinal cortex and Inner represents the third layer examined here. *** $p < 0.001$, **** $p < 0.0001$ when using Kruskal-Wallis and Dunn's multiple comparison tests, $n = 246-465$ fields from 8 sections from 3 mice in each genotype. The error bars represent standard error of the mean. Scale bar represents $5\mu\text{m}$. The perfusion of mice for this experiment was performed by Benjamin Karimi.

4.8. Inhibitory inputs to the dentate gyrus molecular layers are unaffected in *Lrrtm4^{RK-KI}* mice

The brain sections used to examine excitatory synaptic changes were co-stained with the presynaptic inhibitory synapse marker, GAD65. GAD65 punctate immunofluorescence integrated intensity per tissue area was unaffected in all the DG molecular layers in *Lrrtm4^{RK-KI}* mice compared to *Lrrtm4^{+/+}* littermate (at 5-6 weeks postnatal age) (Fig. 4.8A and B). Similarly, the GAD65 punctate immunofluorescence in CA1 oriens was comparable in *Lrrtm4^{RK-KI}* compared to the *Lrrtm4^{+/+}* littermate mice. These results indicate disrupted neurexin-LRRTM4 complex in *Lrrtm4^{RK-KI}* mice does not affect inhibitory inputs to the DG.

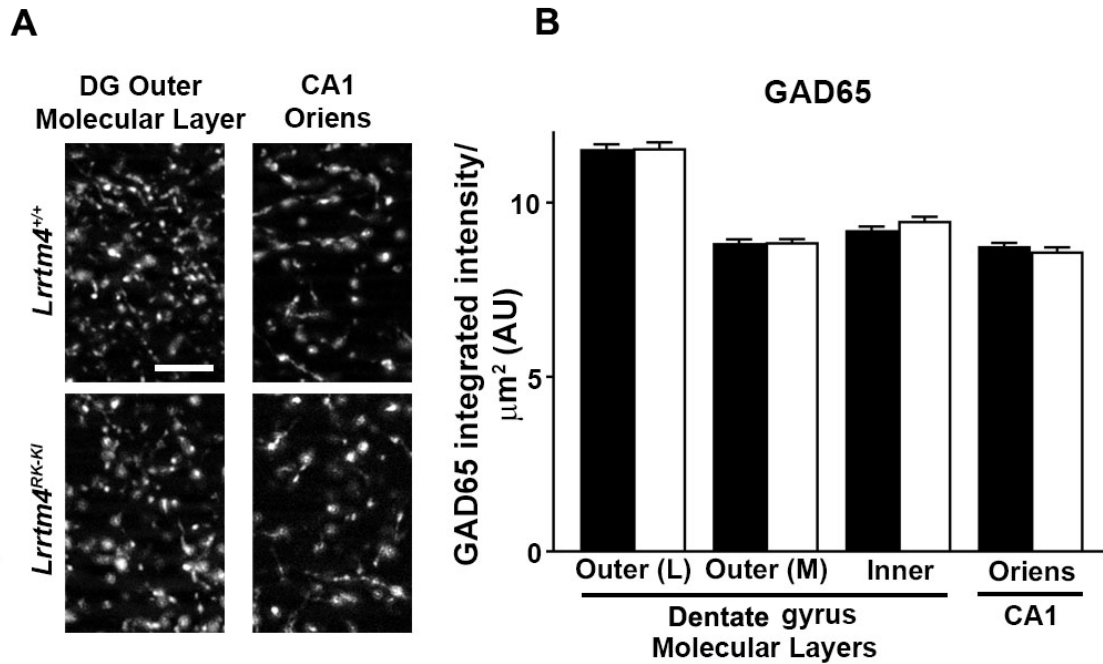


Figure 4.8. Comparison of inhibitory synapses (by GAD65 immunostaining) in dentate gyrus molecular layers and Cornu Ammonis 1 (CA1) regions in wild-type and knock-in mice.

A) High-resolution, representative fluorescence images showing similar GAD65 punctate immunofluorescence in outer molecular layer of DG (left panels) and CA1 oriens (right panels) of *Lrrtm4*^{RK-KI} mice (bottom panels) compared to *Lrrtm4*^{+/+} littermate (top panels). B) Quantitation of GAD65 punctate integrated intensity (AU) per tissue area (μm²) from different molecular layers of DG and of the CA1 oriens. No significant differences in all molecular layers in *Lrrtm4*^{RK-KI} mice (no fill) compared to *Lrrtm4*^{+/+} littermate (solid fill). Outer L represents region receiving inputs from lateral entorhinal cortex and Outer M represents region receiving inputs from medial entorhinal cortex and Inner represents the third layer examined here. n= 246-465 fields from 8 sections from 3 mice in each genotype. The error bars represent standard error of the mean. Scale bar represents 5μm. The perfusion of mice for this experiment was performed by Benyamin Karimi.

PTP σ is recruited on axonal membrane by neurexin-HS-LRRTM4 interaction *in vitro*. In absence of PTP σ , there was inadequate presynaptic differentiation induced by LRRTM4 in cocultured neurons (Roppongi et al., 2020). Based on the results from *in vitro* experiments, we wanted to assess changes, if any, in the levels of PTP σ in the DG.

4.9. Optimization and validation of PTP σ antibody in brain sections

The anti- PTP σ antibody was tested in three different concentration of fixation (1%, 2% and 4% PFA) and three different dilutions (0.2%, 0.1%, 0.05%). The anticipated punctate staining of PTP σ was best observed on 1% fixed brain section at 0.1% dilution (Figure 4.9 shows images from this condition only). To the best of our knowledge, PTP σ immunostaining on brain sections had not been reported before. Co-staining of the brain sections with three presynaptic markers – bassoon, VGlut1 and synapsin showed them to be co-localized to PTP σ . Thus, PTP σ is localized to presynapses, as expected.

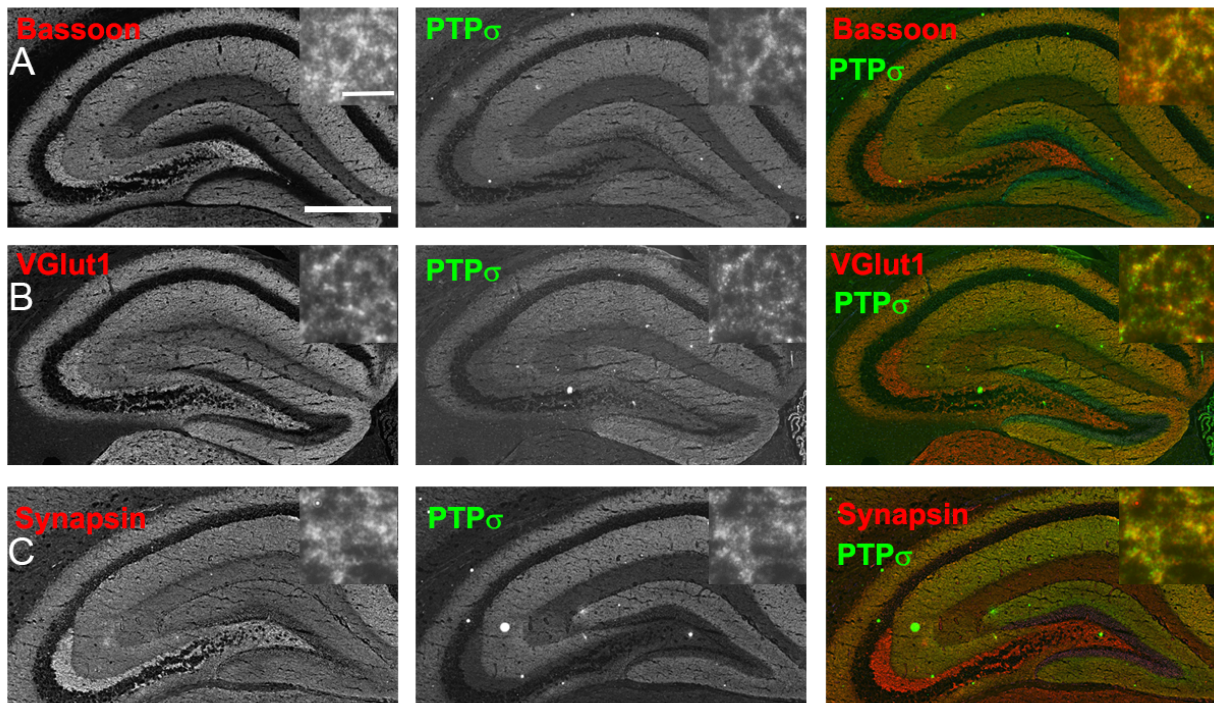


Figure 4.9. Immunofluorescence images showing co-localization of PTP σ with various presynaptic markers.

Representative immunofluorescence images showing PTP σ staining with different presynaptic markers – bassoon, VGlut1 and synapsin. A) Representative images of similar staining pattern of Bassoon (left panel) and PTP σ (middle panel). The right panel image shows co-localization of bassoon and PTP σ . High resolution images are shown in a square box (inset) in each panel. B) Representative image of similar staining pattern of VGlut1 (left panel) and PTP σ (middle panel). The right panel image illustrates co-localization of PTP σ and VGlut1. C) Representative images of similar staining pattern of Synapsin (left panel) and PTP σ (middle panel). The right panel image displays co-localization of bassoon and PTP σ . Scale bars represents 500 μ m in images and 5 μ m in insets (top right corner of each image).

4.10. PTP σ recruitment in the molecular layers of the dentate gyrus is reduced in *Lrrtm4*^{RK-KI} mice

We then compared the levels of PTP σ between *Lrrtm4*^{RK-KI} mice and *Lrrtm4*^{+/+} littermates. I stained the 1% fixed brain sections with anti-PTP σ antibody. PTP σ punctate immunofluorescence integrated intensity per tissue area was reduced in all the DG molecular layers in *Lrrtm4*^{RK-KI} mice compared to *Lrrtm4*^{+/+} littermate (at 5-6 weeks postnatal age) (Fig. 4.10A and B). The PTP σ punctate immunofluorescence in CA1 oriens was comparable in *Lrrtm4*^{RK-KI} compared to the *Lrrtm4*^{+/+} littermate mice. These results indicate disrupted neurexin-LRRTM4 complex in *Lrrtm4*^{RK-KI} mice reduces the recruitment of PTP σ in the DG.

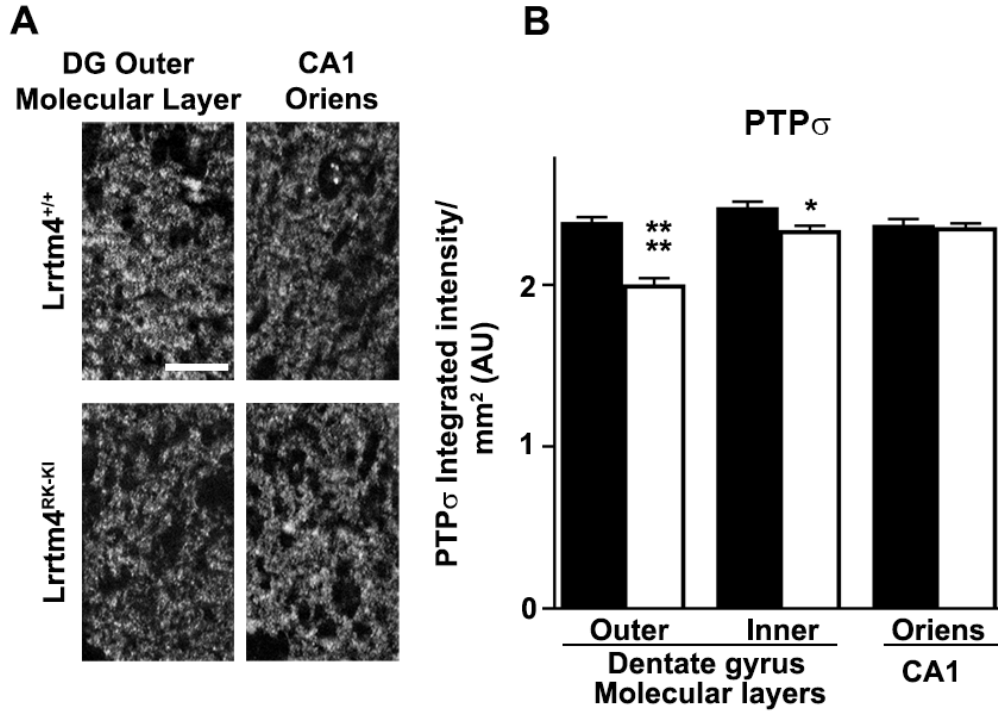


Figure 4.10. Comparison of PTP σ recruitment (by PTP σ immunostaining) in dentate gyrus molecular layers and Cornu Ammonis 1 (CA1) regions in wild-type and knock-in mice.

A) High-resolution, representative fluorescence images showing reduction in Protein Tyrosine Phosphatase sigma (PTP σ) punctate immunofluorescence in the outer molecular layer of DG of *Lrrtm4*^{RK-KI} mice (top) and in *Lrrtm4*^{+/+} littermate (bottom). Similar intensity of PTP σ puncta were evident in the CA1 (right top and bottom panels) while less intense labeling was evident in the DG of the knock-in mouse (left bottom) than in the wild-type mouse (left top panel). B) Quantitation of PTP σ punctate integrated intensity (AU) per tissue area (μm^2) from different molecular layers of DG and of the CA1 oriens. Significant reduction in molecular layers in *Lrrtm4*^{RK-KI} mice (no fill) compared to *Lrrtm4*^{+/+} littermate (solid fill). $p < 0.05$, **** $p < 0.0001$ comparing *Lrrtm4*^{RK-KI} and *Lrrtm4*^{+/+} littermate by one-way ANOVA and post hoc Bonferroni's multiple comparison test. $n = 252\text{--}352$ fields from 4-5 sections from 3 mice in each genotype. The error bars represent standard error of the mean. Scale bar represents $5\mu\text{m}$.

4.11. Neurexin and PTP σ levels are reduced in dentate gyrus synaptosomal lysates

To assess if the levels of PTP σ and neurexin are altered in the DG of the knock-in mice, together with Dr. Padmanabhan, synaptosomal lysates from the DG of 5-6 weeks old animals were prepared. A representative image of Western blot results is shown in Fig. 4.11A, indicating a reduction of neurexin (upper bands) and PTP σ (lower bands) as represented by fainter and thinner Nrxa and PTP σ bands in *Lrrtm4*^{RK-KI} mice lysates. Quantitation from combined data collected from seven mice in Fig. 4.11B and C revealed a significant reduction of neurexin and PTP σ in *Lrrtm4*^{RK-KI} mice. The synaptic neurexin levels reduced by nearly 50% in *Lrrtm4*^{RK-KI} mice compared to *Lrrtm4*^{+/+} mice. Similarly, the synaptic PTP σ levels reduced by 46% in *Lrrtm4*^{RK-KI} mice compared to *Lrrtm4*^{+/+} littermate.

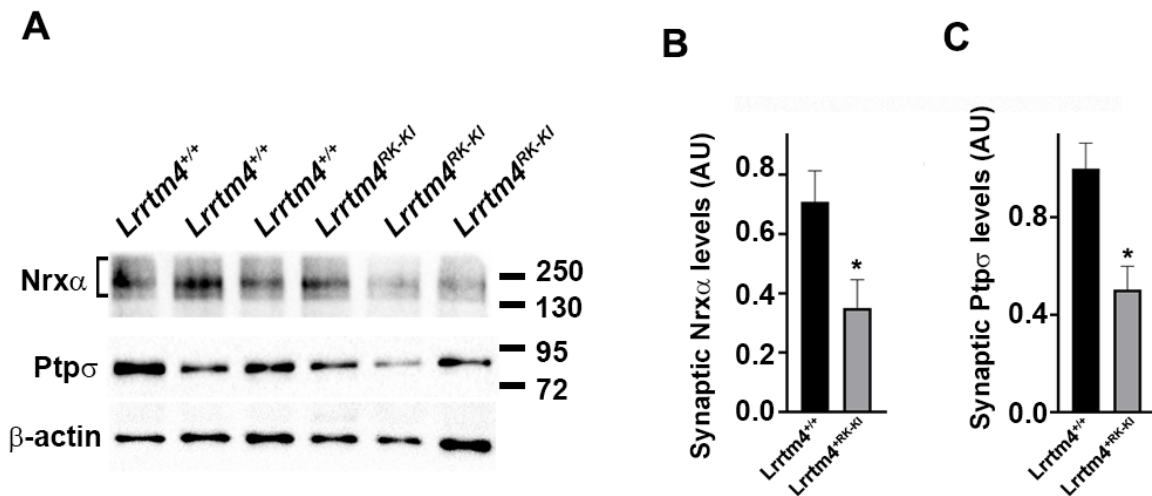


Figure 4.11. Representative image of western blot and quantitation graph.

A) Image of a Western blot of heparinase-treated synaptosomal lysates from dentate gyrus of *Lrrtm4*^{RK-KI} and *Lrrtm4*^{+/+} littermates at 5-6 weeks postnatal revealed reduction of neurexin (Nrxa) and PTP σ (Ptp σ) levels in *Lrrtm4*^{RK-KI} mice compared to *Lrrtm4*^{+/+} mice. β -actin was used as loading control. B) Summary graph of quantitation for neurexin showing significant reduction of

neurexin levels in *Lrrtm4*^{RK-KI} mice compared to *Lrrtm4*^{+/+} littermate (unpaired t-test, **p*<0.05, *n*=8 for *Lrrtm4*^{+/+} and *n*=7 for *Lrrtm4*^{RK-KI}). C) Summary graph of quantitation for PTP σ showing significant reduction of PTP σ levels in *Lrrtm4*^{RK-KI} mice compared to *Lrrtm4*^{+/+} littermate (unpaired t-test, **p*<0.05, *n*=11 for *Lrrtm4*^{+/+} and *Lrrtm4*^{RK-KI} mice). Protein levels were estimated relative to β -actin loading control. These experiments were done collaboratively with Dr. Nirmala Padmanabhan. The dentate gyrus was dissected by Shreya Dhume.

5. Discussion

Overview of the objectives and results

In this project we have examined the role of trans-synaptic interaction of synapse organizing proteins in synapse development. We focused on the DG to study the transsynaptic interaction between postsynaptic LRRTM4 and a presynaptic partner neurexins and how their interactions influence synaptogenesis. In the absence of LRRTM4, the excitatory synapse number and function reduced by nearly 30% in the DG (Siddiqui et al., 2013) suggesting that LRRTM4 is one of the key synapse organizing proteins in the DG. We hypothesized in the first objective that trans-synaptic interaction of LRRTM4 and neurexin controls synapse development in the DG. We generated a knock-in mouse model with a point mutation in the heparan sulfate binding site of LRRTM4. Thus, neurexins cannot bind to LRRTM4 in these mice. We observed significant reduction in the excitatory synaptic marker VGlut1 staining in the knock-in mice which supported our hypothesis that trans-synaptic interaction of LRRTM4 and neurexins are necessary for excitatory synapse development in the DG.

As the trans-synaptic binding of neurexin-HS-LRRTM4 complexes also recruit PTP σ for optimal synaptic differentiation *in vitro* (Roppongi et al., 2020) we hypothesized (in the second objective) that in the knock-in mice, the synaptic levels of PTP σ would be reduced. We observed reduction in PTP σ recruitment in the DG molecular layers which supported our hypothesis.

5.1. Validation of a unique model to study trans-synaptic interactions of synapse organizing proteins

Within the field of synapse organizing proteins, the highest-level studies are those which put effort into validating mechanisms not only *in vitro* but also *in vivo*. Based on the *in vitro* work, a knock-in mouse model, *Lrrtm4*^{RK-KI}, with a mutation in one of the heparan sulfate binding sites of

LRRTM4 was generated. Because of this mutation, trans-synaptic interaction between LRRTM4 and neuexins was disrupted. We examined the consequences of this putative disruption on excitatory synapse development in the DG.

Mouse models lacking pre or postsynaptic proteins have been extensively used in research to elucidate their role in synaptogenesis. However, these mouse models may not be ideal to test the hypothesis that trans-synaptic interactions between pre-and post-synaptic proteins mediate synapse development and maturation. One of the limitations of such *in vivo* knock-out animal studies is that there could be developmental compensation from other proteins (Sudhof, 2017). To avoid that and to better understand the role of trans-synaptic interactions between proteins on synaptogenesis, we generated a *Lrrtm4*^{RK-KI} knock-in mouse model. The *Lrrtm4*^{RK-KI} is a unique mouse model to study trans-synaptic interaction in the development and function of synapses.

The *Lrrtm4*^{RK-KI} mice were created by using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 technology. This technology is widely used for site-specific genetic manipulation and it allows researchers to elucidate links between genetic variations and biological phenotypes. CRISPR/Cas9 is an RNA-programmable technology that provides exceptional efficiency and accuracy to knock-out or knock-in genes using DNA cleavage and cellular DNA repair mechanism. As mentioned in the Methods section, the knock-in mouse model was generated by substituting the neutral alanine (A) with positively charged residues of arginine (R157) and lysine (K158) in the coding exon of LRRTM4 protein. Since the HS is highly negatively charged, it has high affinity towards positively charged residues such as R and K. The binding affinity of neuexin-HS to LRRTM4 was reduced when R and K were substituted by neutral A in an *in vitro* neuron binding assay (Roppongi et al., 2020). However, we did not know how the knock-in modifications would affect LRRTM4 localization, protein levels and protein interactions *in vivo*.

Therefore, in the *in vivo* studies, we examined whether the mutated form of LRRTM4 was correctly localized to the DG.

As shown in Fig. 4.2, the comparison of the hippocampus region between knock-in and wild-type mice suggested that the overall hippocampal morphology was not changed in the knock-in mice. This implied that having the genetic mutation in LRRTM4 has no effect on the cellular and laminar arrangement of the hippocampus – at least the organization addressed by the markers we chose for this study. The LRRTM4 protein in *Lrrtm4^{RK-KI}* mice showed similar localization in the DG as compared to a wildtype littermate (Fig. 4.3). In addition, the protein levels measured by the Western blot image from whole brain lysate in Fig. 4.4 revealed that translation of the LRRTM4 protein is normal despite the point mutation in the gene. These results together attest that there was no change in localization or expression levels of LRRTM4 in the hippocampus of *Lrrtm4^{RK-KI}* mice.

The three key observations described above in the DG of *Lrrtm4^{RK-KI}* mice (normal hippocampal morphology, comparable levels and similar localization of LRRTM4 protein) allow us to conclude that *Lrrtm4^{RK-KI}* mouse model is suitable to study trans-synaptic interactions between LRRTM4 and neuroligins in the DG. Hence, the *Lrrtm4^{RK-KI}* mouse model was used to study the role of neuroligin-LRRTM4 trans-synaptic interaction on the excitatory synaptogenesis in the DG.

DG is not the only region where LRRTM4 is expressed. LRRTM4 mRNA is highly expressed in the olfactory bulb (Lauren et al., 2003). We could have examined our hypothesis on the olfactory bulb as well. The changes due to LRRTM4 mutation in the olfactory bulb would be predicted to be similar to those observed in the DG. However, we did not use olfactory bulb samples because the anti-LRRTM4 antibody was not distinctly labelling the protein in that region and we did not

put in enough resources and effort to optimize protocols for this region. Supporting evidence from other brain regions like the olfactory bulb describing the consequences of alanine substitution on excitatory synaptogenesis would be, however, important for demonstrating that the process we uncovered is similar in different brain regions.

5.2. Neurexins require HS modification for selective interactions *in vivo*

Neurexins are one of the major presynaptic proteins that contribute to the synapse property and regulate synaptic functions. By binding to their postsynaptic ligands, neurexins recruit proteins that stabilize synapses and aid in synapse maturation and plasticity (Reissner et al., 2013; Sudhof, 2017). For a long time, the binding and the interactions of neurexins with their ligands were thought to be mediated by LNS protein domains only. However, the recent discovery of neurexins as HSPGs (Zhang et al., 2018) opened a new window of research into possible interactions between neurexins and their ligands irrespective of protein domains. They reported enhanced ligand binding due to HS glycans of neurexins. Despite LNS domain is essential for binding between LRRTM2 and neurexins, LRRTM4 does not bind to LNS domain of neurexins. (Siddiqui et al., 2010; Roppongi et al., 2020). However, it has been previously reported that the ectodomain of LRRTM4 binds to HSPGs like glypicans and syndecans via HS glycans (de Wit et al., 2013; Siddiqui et al., 2013).

In the 2018 study, Zhang et al. demonstrated both HS and protein domains of neurexins were required for functional interactions with their ligands (namely neuroligin1/2 and LRRTM2). Later, an *in vitro* neuron binding assay reported that neurexins bind to LRRTM4 solely via their HS glycans (Roppongi et al., 2020). These results support the finding of our co-immunoprecipitation experiment from the mouse brain where we observed that interactions between LRRTM4 and

neurexins is mediated by HS (Fig. 4.5A). This finding led us to conclude that interactions between LRRTM4 and neurexins is HS dependent.

In the absence of HS chains, neurexins could not bind to LRRTM4 (Fig. 4.5A) resulting in disruption of LRRTM4-neurexins transsynaptic complex. The interaction between neurexins and PTP σ was also disrupted *in vivo* (Fig. 4.5 B) when all the HS chains of neurexins were removed. This result suggests that binding of neurexins and PTP σ is HS dependent as well. This is supported by the finding that PTP σ binds to glypicans (one of the HSPGs) via HS glycans (Ko et al., 2015). In addition, the binding site for HS has been identified and reported in avian orthologue of PTP σ (Aricescu et al., 2002). The *in vitro* neuron binding assay also reported HS dependent interaction between neurexin and PTP σ (Roppongi et al., 2020). Though HS binding to PTP σ has been reported before, binding of PTP σ to neurexins via HS is novel.

In conclusion, neurexins require HS glycan chains to interact with their ligands, LRRTM4 and PTP σ . The finding that HS of neurexin is required for its binding with LRRTM4 and PTP σ was first confirmed in the *in vitro* studies (Roppongi et al., 2020). As this project is a portion of a much larger study, most of the *in vitro* work was already performed and laid the foundation for this work. A recent study reported that in the absence of HS of neurexins, presynaptic differentiation induced by neuroligins and LRRTM2 was abolished (Zhang et al., 2018). Also, neuron binding and co-culture assays disclosed the abolishment of binding and presynaptic induction mediated by LRRTM4 in the absence of HS chains of neurexins. (Roppongi et al., 2020). The results of these studies highlight the functional significance of the HS of neurexin.

5.3. Transsynaptic interactions of cell adhesion proteins that mediate synapse formation

LRRTM4 can bind to all isoforms of neurexins (Roppongi et al., 2020). However, the mutant LRRTM4 in the knock-in mice lost its ability to interact with neurexins. In this project, our primary

interest was to study the consequence of disrupted transsynaptic interaction between neurexins and LRRTM4 on synaptogenesis in the DG. A LRRTM4 knock-out mouse study reported almost 36% reduction in the excitatory synapse number and function in the DG of knock-out mice compared to wildtype littermate (Siddiqui et al., 2013). This suggested that LRRTM4 is a key synapse organizing protein in the DG. The study also proposed that the LRRTM4-HSPG complex contributes to the synapse development and function in the DG. However, the specific HSPG required to induce presynaptic differentiation by LRRTM4 was not identified. Later it was shown in the neuron co-culture assay that LRRTM4 induces presynaptic differentiation by transsynaptic interaction with neurexins (recently discovered as evolutionarily conserved HSPG) via their HS chains (Roppongi et al., 2020). Therefore, the excitatory synaptic inputs to the DG were significantly reduced in the *Lrrtm4*^{RK-KI} mice. The similar results from our study and LRRTM4 knock-out study (Siddiqui et al., 2013) strengthen the fact that LRRTM4 is the key synapse organizing protein in the DG.

In the hippocampus, LRRTM4 has been reported to exclusively promote excitatory presynaptic differentiation (Linhoff et al., 2009; Siddiqui et al., 2013). In line with previous reports, we did not observe changes in the inhibitory synaptic density (Fig. 4.8) in the DG. Therefore, our results reinforce LRRTM4 as an excitatory synapse organizer in the DG.

5.4. Interaction between LRRTM4 and neurexins recruit PTP σ for optimal synapse development

Synapse development is a complex process that does not result in a single end-product (Sudholf, 2017). The initial contacts between synaptic proteins initiate synapse formation. The interaction between pre and postsynaptic proteins stabilize synapses by recruiting more proteins on the synaptic membranes. The interaction between neurexins and neuroligins recruit proteins such as PSD-95, AMPAR, and NMDAR in the post synaptic membranes (Mondin et al., 2011).

Similarly, on the presynaptic membranes, this interaction recruits various presynaptic active zone proteins that promote exocytosis of the neurotransmitters. Though the recruitment of additional proteins on pre and postsynaptic membranes for synapse maturation is not a new concept, the cooperation of two presynaptic CAMs, PTP σ and neuexin, to promote synapse development was a novel finding of this study.

Roppongi et al. (2020) reported that PTP σ binds directly to HS of neuexins but not to LRRTM4. The binding of neuexins and LRRTM4 recruited PTP σ which is important for downstream signaling. The *in vitro* neuron co-culture experiments showed that in the absence of PTP σ , LRRTM4 could not induce presynaptic differentiation with full strength (Roppongi et al., 2020). The immunostaining assessment revealed a significant reduction in the recruitment of PTP σ in the *Lrrtm4*^{RK-KI} mice compared to *Lrrtm4*^{+/+} mice. We interpret these results as indicating that PTP σ recruitment to the presynaptic membrane is reduced when neuexin-LRRTM4 interaction is lost. This finding suggests that PTP σ recruitment may assist in inducing optimal excitatory synaptogenesis by LRRTM4 in the DG. Therefore, PTP σ may have functional significance in the DG synaptogenesis.

The biochemical assessment of DG synaptosomal lysates revealed reduced levels of PTP σ in the *Lrrtm4*^{RK-KI} mice compared to wildtype littermate (Fig. 4.11) supporting the previous immunostaining result. Neuexin levels were also reduced in the DG synaptosomal lysates. On performing quantitative analysis, the reduction was significant for both PTP σ and neuexins. This result suggests that loss of interaction between neuexins and LRRTM4 affects recruitment of both neuexins and PTP σ on the presynaptic membrane. Therefore, PTP σ and neuexins are both required by LRRTM4 to induce optimal synaptogenesis in the DG.

5.5. Summary of my contributions to the research discussed in the thesis

The project was designed by Dr. Tabrez J. Siddiqui. Dr. Nirmala Padmanabhan developed the idea and led the process of generating the *Lrrtm4*^{RR-KI} mice. I was responsible for genotype all the mice that were used in the project. Benyamin Karimi perfused mice and I collected brain tissue from animals used in VGlut1 and GAD65 staining. For the other staining studies, I performed mouse perfusions independently. I prepared all the brain sections used for immunostaining. I performed immunostaining, immunofluorescence imaging, and analyzed the data for all immunostaining experiments. The biochemical assays were planned and supervised by Dr. Padmanabhan and they were performed in collaboration with her. The synaptosomal lysates for western blot and immunoprecipitation experiments were prepared by me together with Dr. Padmanabhan. Shreya Dhume helped with the dissection of the dentate gyrus to prepare synaptosomal lysates. The biochemical analysis and immunoblot imaging were performed by Dr. Padmanabhan.

6. Future directions and limitation

The interaction of LRRTM4 and neuexin only influence excitatory synaptogenesis, at least in the DG. *Lrrtm4^{RK-KI}* is a unique mouse model that provides an exceptional opportunity to explore role of trans-synaptic interaction between LRRTM4 and neuexins in synapse formation. In future, these mice along with *Lrrtm4^{-/-}* and *Lrrtm4^{+/+}* mice can be used to investigate additional molecular pathways that contribute to excitatory synapse development. These mice could also be used to perform behavioural experiments, like pattern separation and spatial memory and allow comparative analysis between the performance of *Lrrtm4^{RK-KI}*, *Lrrtm4^{-/-}* and *Lrrtm4^{+/+}* mice. It would also provide an opportunity to study the consequence of disrupted trans-synaptic protein complex on spatial learning, memory, and pattern separation in the DG if these mice.

In this study, we focused on DG even though LRRTM4 is highly expressed in the olfactory bulb as well. Would the mutation introduced in LRRTM4 be without consequences in the olfactory bulb? This could have been another question to answer here by collecting and analysing tissue from olfactory bulb in our mice. However, the antibodies had different optimal working conditions in the DG and the olfactory bulb that made immunostaining assays difficult to perform for both regions simultaneously. In future, optimizing the staining procedures and examining both brain regions in the same animals would allow the generalization of this specific interaction.

7. Significance and clinical relevance

Multiple genetic studies have reported genes encoding neurexins as one of the strongly associated risk genes in autism spectrum disorder (ASD) (Sudhof, 2008, 2017; Roppongi et al., 2017; Takahashi et al., 2013; Wang et al., 2009; Gauthier et al., 2011; Haung et al., 2017; Pinto et al., 2010). Furthermore, disruption of the neural circuits due to mutation in genes encoding RPTPs, LRRTMs and the components of HSPG further increase the probability of ASD. An imbalance in excitatory and inhibitory synapses is one of the underlying reasons for ASD. This study has 1) provided a unique opportunity to study the importance of the trans-synaptic interaction of LRRTM4 with their presynaptic partner neurexins in excitatory synapse development (unlike the *Lrrtm4*^{-/-} mice where LRRTM4 is completely absent) and 2) uncovered a component of the molecular pathways utilized by autism-linked genes in the regulation of development of excitatory synapses. Mechanistic details such as those unveiled by this study are critical for identifying novel drug targets and eventual therapeutic interventions for certain forms of autism that link to the excitatory and inhibitory synapse imbalances. The results of this study point to the direction of developing drugs that facilitate PTP σ recruitment and link formation via the HS chains between neurexins and LRRTM4 proteins in order to maintain optimal numbers of excitatory synapses.

8. Conclusion

Numerous research studies are focused on synapse formation, yet, the molecular mechanism underlying synaptogenesis remains unclear. Advanced scientific technology has made it possible to view each synapse and witness various activities occurring on the synaptic membrane. However, the molecular pathway involved in the process is poorly understood. Solving the synaptic enigma is a challenge because of synaptic variability. This variability is created by copious CAMs and their multifarious interactions. Understanding when, where and how these CAMs engage in the formation and plasticity of synapses is essential to unfold the synaptic mystery. We believe that our study contributes to the understanding of synaptic conundrum by shedding light on trans-synaptic interaction between neurexins and LRRTM4 and co-operation of PTP σ on the development of excitatory synapses in the DG.

9. References

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