Assessing the Role of LRRTMs in Synapse Development and Function

by

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Abstract

The leucine rich repeat transmembrane neuronal (LRRTM) proteins are a family of four synaptogenic cell adhesion molecules that instruct excitatory presynaptic differentiation and mediate postsynaptic differentiation. LRRTM1 and LRRTM2 are most potent at inducing presynaptic differentiation and have been shown to interact with neurexins at glutamatergic synapses. LRRTM4 has been recently identified as a major component of native AMPA-type glutamate receptor complexes, and is expressed at very high levels in dentate gyrus granule cells. Similar to neurexins, neuroligins, and several other synapse organizing proteins, LRRTMs are linked to psychiatric disorders such as autism spectrum disorders. LRRTM4 is also linked to risk of attempted suicide in females based on a recent genome-wide association study of over 2500 patients with bipolar disorder. Our project on LRRTM1 and LRRTM2 involved determining the role of these proteins in synapse development and function using LRRTM1 and 2 double knockout mice. Our results indicated that LRRTM1 and 2 are essential for normal excitatory synapse development and function in CA1 region but not the dentate gyrus. Our project on LRRTM4 assessed the role of this protein in synapse development. Using targeted deletion in mice, our results revealed that LRRTM4 is essential for normal excitatory synapse development and function in dentate gyrus granule cells but not in CA1 hippocampal pyramidal neurons. In addition, it was shown that LRRTM4 differentiates from LRRTM1 and 2 in terms of binding partners as it binds heparan sulfate proteoglycans (HSPGs). Experiments indicated that HSPGs are essential to mediate the synaptogenic activity of LRRTM4. Overall, our results from LRRTM1 and 2 and LRRTM4 projects indicate that members of the LRRTM family function in a cell-type
specific manner through different presynaptic molecular pathways. This emphasizes the complexity of synapse-organizing protein networks and the importance of studying region specific roles of these synaptic proteins.
Preface

Unless otherwise stated, the author conducted the work presented in this thesis; including design of the research program, performance of experiments and the analysis of research data. Dr. Tabrez J. Siddiqui performed the design and production of LRRTM1 and 2 DKO and LRRTM4 KO mice, LRRTM4 antibody, as well as fusion proteins and binding assays. Electron Microscopy experiments for LRRTM1 and 2 project were performed by Fergil Mills at Dr. Shernaz Bamji’s laboratory. Dr. Steven A. Connor at Dr. Yu Tian Wang laboratory performed the electrophysiological studies for both LRRTM1 and 2 and LRRTM4 projects.

The work in chapter 4 on LRRTM4 has been published as:

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<table>
<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADHD</td>
<td>Attention Deficit Hyperactivity Disorder</td>
</tr>
<tr>
<td>AMPA</td>
<td>A-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ASD</td>
<td>Autism Spectrum Disorder</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Centigrade</td>
</tr>
<tr>
<td>CA</td>
<td>Cornu Ammonis</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis Region Superior</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornu Ammonis Region Inferior</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell Adhesion Molecules</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan Fluorescent Protein</td>
</tr>
<tr>
<td>COS</td>
<td>CV-1 (simian) in Origin carrying SV40</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate Gyrus</td>
</tr>
<tr>
<td>DHet</td>
<td>Double Heterozygous</td>
</tr>
<tr>
<td>DKO</td>
<td>Double Knockout</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
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GABA: *gamma*-Aminobutyric acid
GAD65: Glutamic Acid Decarboxylase 65
GAG: Glycosaminoglycan
GFP: Green Fluorescent Protein
GPC: Glypican
HA: Human Influenza Hemagglutinin
HEK: Human Embryonic Kidney
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HET: Heterozygous
HS: Heparan Sulfate
HSPG: Heparan Sulfate Proteoglycan
IgG: Immunoglobulin G
KD: Knockdown
kDA: Kilo Dalton
KO: Knockout
LAR: Liprin-alpha-1
LNS: laminin-neurexin-sex hormone binding globulin
LRR: Leucine-Rich Repeat
LTD: Long Term Depression
LTP: Long Term Potentiation
µg: Micrograms
µl: Microlitres
µM: Micromolar
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitres</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MAGUK</td>
<td>Membrane-Associated Guanylate Kinases</td>
</tr>
<tr>
<td>mEPSC</td>
<td>Miniature Excitatory Postsynaptic Current</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NLG</td>
<td>Neuroligin</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-Aspartic Acid</td>
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<tr>
<td>NRX</td>
<td>Neurexin</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PDZ</td>
<td>PSD-95/Disc-Large/ZO-1</td>
</tr>
<tr>
<td>PSD</td>
<td>Post Synaptic Density</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SAP</td>
<td>Synapse-Associated Protein</td>
</tr>
<tr>
<td>SDC</td>
<td>Syndecan</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Of The Mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Small Hairpin RNA</td>
</tr>
<tr>
<td>SV</td>
<td>Synaptic Vesicle</td>
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<tr>
<td>TKD</td>
<td>Triple Knockdown</td>
</tr>
<tr>
<td>VGlut1</td>
<td>Vesicular glutamate transporter-1</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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</table>
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I would like to thank my beloved family, and my inspiring mother in particular, without whom I would not be the person I am today. Although far away, their love and support constantly motivate and inspire me.
To the Loving Memory of My Dear Uncles,

Farhad and Faramarz
Brain function relies on communication between neurons through highly specialized junctions known as synapses. A synapse is the point of contact between neurons that allows neurons to transmit electrical and chemical signals from one to another. The majority of synapses in humans form during early prenatal and postnatal development until about 1 year after birth. By the end of this period, the 100 billion neurons in the brain have each formed thousands of synapses. The formation of these contacts is not a random process; strongly regulated mechanisms are thought to dictate the location, number and type of synapses during development, leading to the formation of highly reproducible synaptic networks (Lisé & El-Husseini, 2006). Recent studies indicate that synapses consist of complex, yet distinct networks of proteins on the pre- and postsynaptic sides, which play a role in synapse formation and specificity.

1.1 The CNS Synapse

Synapses are generally characterized by the neurotransmitters that they utilize to propagate signals. Neurotransmitters that are commonly found include glutamate, GABA, glycine, acetylcholine, dopamine and serotonin. Excitatory synaptic transmission in the brain is predominantly mediated by the neurotransmitter glutamate, while inhibitory transmission is mediated mainly by the neurotransmitter gamma-amino butyric acid (GABA). Synapses that contain each respective neurotransmitter differ in their composition and structure. The majority of excitatory signals in the brain are transmitted
by glutamatergic synapses, which are often referred to as excitatory synapses. The GABAergic synapses transmit the majority of inhibitory signals and are usually referred to as inhibitory synapses. Excitatory synapses are formed between axons and dendritic shafts or dendritic spines. They generate excitatory postsynaptic potentials, which increase the possibility of the postsynaptic neuron reaching action potential threshold (Levinson & El-Husseini, 2005). In contrast, inhibitory synapses are formed on the cell bodies of neurons, axon initial segments or the shaft of dendrites; and they generate inhibitory postsynaptic potentials that decrease the probability of the postsynaptic neuron reaching action potential threshold. In addition to the neurotransmitters and neurotransmitter receptors utilized at the excitatory and inhibitory synapses, they also differ in morphology and protein composition. A proper balance between excitatory and inhibitory synapses is essential for the normal function of networks for sensory information and cognitive processing. In fact, imbalance between excitatory and inhibitory synapses has been related to neuropsychiatric disorders such as autism, schizophrenia and Tourette’s syndrome (Cline, 2005).

1.1.1 Synapse Structure

Synapses consist of several specialized domains, including the presynaptic bouton, the synaptic cleft and the postsynaptic reception apparatus (Garner et al., 2000). Presynaptic compartments are typically formed within small varicosities that stud the length of axons and include hundreds of synaptic vesicles carrying neurotransmitter
molecules. A few synaptic vesicles are physically in contact with the plasma membrane at release sites within specialized domain called the active zone. Ultrastructurally, active zones are characterized by the presence of a dense matrix of proteins, the cytomatrix, that extends from the plasma membrane deep into the bouton (Garner et al., 2002). Once the presynaptic membranes depolarize, some of the synaptic vesicles fuse with the presynaptic membrane and release neurotransmitters into the synaptic cleft, a narrow gap between the pre- and postsynaptic neurons. The released neurotransmitters diffuse across the cleft and bind to their receptors in the postsynaptic density (PSD), an electron-dense network of proteins at the postsynaptic membrane, attributable to the high density of neurotransmitter receptors and associated molecules at these sites. Filamentous proteins extending across the synaptic cleft are thought to work to hold the active zone and PSD in register (Waites, et al., 2005). Although inhibitory postsynaptic sites lack PSDs, they also contain complex protein matrices (Keith & El-Husseini, 2008).

1.1.2 **Dendritic Spines**

The majority of glutamatergic synapses are typically formed onto bulbous protrusions known as dendritic spines. Dendritic spines are actin rich protrusions that are involved in synapse development and function as the primary recipients of excitatory input in the central nervous system (Bourne & Harris, 2008). These protrusions vary in length from 0.5-2 µm and have diverse morphologies (Svitkina et al., 2010). Several lines of evidence have shown that spines are important in synapse development and function, with changes in shape and size of spines being correlated with the strength of
glutamatergic synaptic connections. Structural plasticity of hippocampal spines correlates with physiological changes in synaptic efficacy underlying learning and memory (Bourne & Harris, 2008). Dendritic spines exhibit three main morphologies: filopodia, thin spines, and mushroom spines. The filopodia contributes to synaptogenesis through initiation of contact with axons of other neurons. The dynamism of spines allows for the conversion of filopodia into the mushroom spines that are the primary sites of glutamate receptors and synaptic transmission (Toni et al., 2007). In addition, alteration in spine morphology has been found in patients with mental retardation and schizophrenia (Ramakers, 2002).

1.2 Hippocampus

The hippocampus is a part of the limbic system in the brain, located in the medial temporal lobes, surrounded by the entorhinal, parahippocampal and perirhinal cortices (Bird & Burgess, 2008). Main roles of the hippocampus include the consolidation of information from short-term memory to long-term memory, encoding contextual information and spatial navigation (Bird & Burgess, 2008; Smith & Bulkin, 2014). In diseases such as Alzheimer's disease, the hippocampus is one of the first regions of the brain to be damaged and this leads to memory loss and disorientation associated with the condition (Hampel et al., 2008).

The hippocampus contains two main interlocking parts, the Ammon’s horn and the dentate gyrus (DG). The Ammos’s horn or Cornu Ammonis (CA), also known as the
hippocampus proper, consists primarily of pyramidal neurons, while the dentate gyrus consists primarily of granule cells. While the hippocampal subregions must act cooperatively to produce a functional hippocampus, strong evidence demonstrates that each subregion provides unique contributions to specific processes associated with intrinsic information processing (Kesner et al., 2004). The hippocampus coordinates information from a variety of sources. Most hippocampal functions are performed in collaboration with several of its partners, of which the most prominent is the entorhinal cortex, and strongly influenced by subcortical neuromodulators (Andersen et al., 2006). A major flow of information through the hippocampus is a one-way circuit. The CA region and dentate gyrus are interconnected to each other and to neighboring entorhinal cortex through the trisynaptic circuit. The main input pathway or the perforant path of entorhinal cortex axons comes from the surface layers of entorhinal cortex, which project densely to the granule cells in the dentate gyrus, less densely to the apical dendrites of CA3 and even more sparsely to CA1. Thus, the perforant path establishes the entorhinal cortex as the main interface between the hippocampus and other parts of the cerebral cortex. DG granule cells then project to pyramidal neurons in the CA3 region via the mossy fiber pathway. CA3 pyramidal neurons project and synapse onto CA1 pyramidal neurons via the Schaffer collateral pathway.
Figure 1-1 The Hippocampal Network

Information flows into and through the hippocampus by three principal pathways: the perforant pathway from the entorhinal cortex to granule cells of the dentate gyrus, the mossy fiber pathway from the granule cell of the dentate gyrus to the pyramidal cells of the CA3 region of the hippocampus; and the Schaffer collateral pathway from the CA3 region of the hippocampus to the CA1 region of the hippocampus (Diagram from http://neuroscience.uth.tmc.edu/s4/chapter05.html).
1.3 Synaptogenesis

The establishment of synapses in the vertebrate central nervous system occurs over a protracted period of time, from embryonic development to postnatal life. Although the majority of synaptogenesis happens during early postnatal development, synapse formation can also occur in the mature brain, which is thought to contribute greatly to learning and memory. The formation of synapses is a multi-step process, which begins with cell-cell contact mediated by filopodia that originate from axons and dendrites, providing axo-dendritic, axo-somatic or axo-axonal contact sites (Garner et al., 2002). It is followed by the recruitment of pre- and postsynaptic proteins to these points of contact, leading to the establishment of stable sites of cell-cell contact and subsequently pre- and postsynaptic differentiation (Garner et al., 2002; Waites et al., 2005). On both sides of the synapse, the actin cytoskeleton supports the architecture and function of specialized synaptic domains by strengthening filopodial protrusions, binding transmembrane adhesion molecules and scaffolding synaptic proteins (Gallo, 2013). With an increase in the volume of the pre- and postsynaptic terminals, the number of synaptic vesicles and the area of the active zone and post-synaptic density, these emerging synapses expand in size (Waites et al., 2005). The appearance of spines and AMPA-mediated postsynaptic currents marks the maturation of synapses morphologically and functionally (Waites et al., 2005).
1.4 Cell Adhesion Molecules

The pre- and postsynaptic membranes are held in apposition by transsynaptic cell-surface adhesion molecules. Cell adhesion molecules (CAM), such as Neuroligins, Neurexins, SynCAMs, NGLs, Slitrks, and LRRTMs (Linhoff et al., 2009; Togashi et al., 2009) are present at synaptic sites throughout the lifetime of a synapse and are involved in the formation, function and plasticity of synaptic connections. CAMs have been shown to be crucial candidates to trigger synaptogenesis since many of these molecules are present early in synapse development and are recruited to contact sites before synapse formation and have been shown to be capable of inducing presynaptic differentiation in co-culture assay (Gerrow et al., 2006). Additionally, CAMs are expressed trans-synaptically and can coordinate synapse differentiation bi-directionally (Siddiqui & Craig, 2011).
Figure 1-2 Cell Adhesion Molecules

Synaptic cell adhesion molecules, including neurexins, neuroligins and LRRTMs, are thought to organize synapse assembly and specify synapse function. (Diagram from Brose, 2009)

1.4.1 Network Of Neuroligins, Neurexins And LRRTMs

One of the most well studied groups of CAMs, neuroligins, are a family of postsynaptic cell adhesion molecules that are sufficient to induce both excitatory and inhibitory synapse development. Neuroligins (NLG 1–3) are expressed predominantly in
the brain and bind neurexins (NRX), which are presynaptic transmembrane proteins, via an extracellular esterase-like domain. Neuroligins undergo alternative splicing, which promotes binding to either α- or β-neurexin (Lisé & El-Husseini, 2006). So, based on structure and splicing, neurexins and neuroligins are equipped to take part in determining the specificity and differentiation of synaptic contacts. The trans-synaptic adhesion between neuroligins and neurexins induces both pre- and postsynaptic differentiation. Furthermore, cis-expression of neurexin-1β with neuroligin1 inhibits trans-binding to recombinant neurexins, blocks the synaptogenic activity of neuroligin1 and reduces the density of presynaptic terminals (Taniguchi et al., 2007). Remarkably, neuroligins alone can induce the formation of fully functional presynaptic terminals in contacting axons (Scheiffele et al., 2000). On the other hand, neurexins alone can induce postsynaptic differentiation and clustering of receptors in dendrites (Graf et al., 2004). Therefore, the neuroligin–neurexin interaction has the unique ability to act as a bi-directional trigger of synapse formation.

Neurexins comprise a family of three genes that undergo extensive alternative splicing, giving rise to over 1000 different products that are believed to play fundamental roles in defining synaptic diversity (Garner et al., 2002). Neurexins mainly function at the cell surface of neurons and are critical for Ca\(^{2+}\)-dependent transmission at various types of excitatory and inhibitory synapses as well as synapse formation and differentiation (Reissner, et al., 2013). Although the most studied interacting partner of neurexins are postsynaptic neuroligins, a number of other molecules associated with the synaptic cleft have been identified as binding partners including LRRTMs, neurexophilin, dystroglycan
and cerebellin (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010; Uemura et al., 2010). α-Neurexins contain six LNS (laminin-neurexin-sex hormone binding globulin) domains as well as three epidermal growth factor-like (EGF) domains interspersed. The shorter β-neurexins are identical to the carboxyl terminus of α-neurexins starting from αLNS6; However they have a unique amino-terminal stretch of 37 histidine-rich residues (Reissner, et al., 2013). The cytoplasmic domains contain a potential endoplasmatic retention signal, a cytoskeleton integrating protein 4.1, and a PDZ-binding motif that is required for trafficking of neurexins and binding to some interacting proteins (Garner, et al., 2002; Reissner, et al., 2013). Interestingly, despite having non-homologous structures, neuroligin and LRRTM, compete for the same Ca$^{2+}$-binding epitope on αLNS6 (Siddiqui et al., 2010).

The LRRTMs (leucine-rich repeat transmembrane neuronal proteins) are of particular interest because LRRTM isoforms are differentially expressed by neuronal populations in the CNS (Laurén et al., 2003) suggesting that they may contribute to the development of specific synaptic connections. Functional studies of neuroligins and LRRTMs using overexpression in non-neuronal cells or cultured neurons have shown that an increase in the levels of these proteins generally increase the number of synapses (Linhoff et al., 2009). On the other hand, loss of function experiments aiming to address the requirement for neuroligins and LRRTMs in synapse formation and mature synaptic function have yielded inconsistent results depending on whether knockout (KO) or knockdown (KD) approaches were used (Linhoff et al., 2009; Soler-Llavina, Fuccillo, Ko, Südhof, & Malenka, 2011). Nonetheless, even though LRRTMs and neuroligins
perform divergent functions after synaptogenesis, they are part of a functionally dynamic cell adhesion network that regulates excitatory synaptic transmission. The trans-synaptic connections NRX–NLG and NRX–LRRTM are both sensitive to extracellular Ca\(^{2+}\) concentrations which appear to trigger post-synaptic differentiation and control the balance of inhibitory GABA-ergic and excitatory glutamatergic inputs (Clarke & Eapen, 2014).

Overall, complexes of postsynaptic neuroligins and LRRTMs with presynaptic neurexins contribute greatly to the development of excitatory synapses, and mutations in these gene families have been shown to increase the risk of developing psychiatric disorders. Association studies have implicated NLG, NRX, and LRRTM genes with autism spectrum disorder (ASD), tourett’s syndrome and schizophrenia (Clarke & Eapen, 2014; Voikar et al., 2013).

1.5 LRRTMs

An unbiased expression screen for synaptogenic proteins in co-culture assay, revealed a prevalence of leucine-rich repeat (LRR) synaptogenic proteins (Linhoff et al., 2009). Leucine-rich repeats (LRR) are protein modules that are commonly involved in protein–protein interactions and have critical roles in regulating numerous cellular events during nervous system development and maintenance. In particular, LRRs are common motifs in the extracellular region of transmembrane proteins and also in secreted proteins that are involved in ligand–receptor interactions or in cell adhesion (Kobe & Kajava,
While all LRRs share the same overall structure, high specificity in signaling is attained by direct interaction of individual amino acid side chains within the pocket formed by LRRs and the binding partner. Mutations in genes encoding neuronal LRR proteins have been reported in various diseases, including one hereditary form of epilepsy and the X-linked congenital stationary night blindness (Laurén et al., 2003).

The leucine rich repeat transmembrane neuronal (LRRTM) proteins are a family of four synaptogenic cell adhesion molecules that are found in human and mouse. There is a high level of sequence conservation between human and mouse LRRTMs, which indicates that functional requirements have not allowed for the sequences to diverge at a fast pace. Human LRRTM1, LRRTM2, and LRRTM3 but not LRRTM4 are located within introns of α-catenin genes, which are involved in mediating the adhesive properties of the homophilic adhesion molecules cadherins (Laurén et al., 2003). LRRTMs encode LRR-containing type I transmembrane proteins and share an identical overall structure containing 10 extracellular LRRs as well as a short intracellular part. LRRTMs are distant homologs of virtually all LRR proteins, including Slit family members and Nogo receptor, with approximately 30% amino acid identity within the LRR regions and no homology in other regions (Laurén et al., 2003). The LRR domain of LRRTMs has been found to be necessary and sufficient for synaptogenic activity (Linhoff et al., 2009). The LRRTMs contain a relatively short intracellular tail that may be involved in the binding of signal-transducing molecules or structural intracellular elements. In addition, all the LRRTM proteins contain the C-terminal sequence ECEV, which resembles the consensus docking site sequences for PDZ domain proteins (Hung &
Sheng, 2002). Proteins containing PDZ domains are involved in protein complex assembly, protein targeting and synaptic signaling (Laurén et al., 2003). LRRTMs localize to excitatory synapses, and are able to instruct excitatory presynaptic differentiation and mediate postsynaptic differentiation (Linhoff et al., 2009). As synaptic cell-adhesion molecules, LRRTMs could be involved in one or several steps during synapse formation, from their initial establishment to their maturation and remodeling. As reported by Soler-Llavina et al. (2011), knockdown of LRRTMs during the first two weeks of postnatal development leads to a specific decrease in AMPAR-mediated transmission. Experiments on recombinant LRRTM2 have shown that its extracellular LRR domain, probably via interactions with neurexins, is critical for LRRTM function in long term potentiation (LTP) (Soler-Llavina et al., 2013). Overall, a functional paradigm can be proposed that LRRTMs are required for maintaining or trapping AMPARs at synapses during the initial phase of LTP.

*In situ* hybridization, RT-PCR and immunofluorescence analysis have established that LRRTMs are predominantly expressed in the central nervous system and that each LRRTM presents a specific, partially overlapping expression pattern; and their expression is developmentally regulated (Haines & Rigby, 2007; Laurén et al., 2003; Linhoff et al., 2009). The four LRRTMs are expressed in neurons of the hippocampus, cerebral cortex and in the striatum. LRRTM1 and LRRTM2 are also prominently expressed in the thalamus. In contrast, neither LRRTM3 nor LRRTM4 are expressed in these structures. In the mouse hippocampus, LRRTM1 and LRRTM2 are expressed in CA1–CA3 pyramidal and dentate gyrus granular cells; while LRRTM3 is expressed mainly in
dentate gyrus granular neurons only and LRRTM4 is expressed in CA3 pyramidal and dentate gyrus granular neurons (Laurén et al., 2003). LRRTM1 is not expressed in the cerebellum, whereas LRRTM2 and LRRTM3 are expressed in the granular neurons of this region; and LRRTM4 is expressed at low levels in the Purkinje cell layer only (Figure 1-3 C) (Laurén et al., 2003). This partially non-overlapping neuronal expression patterns of LRRTMs indicate a possible functional division between members of the LRRTM family. The embryonic and postnatal differential expression of LRRTM mRNAs implies that the proteins they encode may have distinct roles in the formation of the central nervous system as well as the maintenance of CNS structure and function.

All four LRRTMs family members are postsynaptically localized and when expressed in non-neuronal cells co-cultured with hippocampal neurons they can induce presynaptic differentiation in contacting axons (Linhoff et al., 2009). When overexpressed, their extracellular domains are necessary and sufficient for their ability to promote synaptogenesis both in nonneuronal cells and cultured neurons (de Wit et al., 2009 and Linhoff et al., 2009). LRRTM1 and LRRTM2 promote excitatory, but not inhibitory presynaptic differentiation (Linhoff et al., 2009). In addition, Linhoff et al (2009) and de Wit et al (2009) demonstrated that LRRTM2 interacts with the postsynaptic protein PSD-95 and regulates surface expression of AMPA receptors. Independent studies have shown that postsynaptic LRRTM1 and LRRTM2 bind specifically to presynaptic neurexins lacking an insert at S4 (de Wit et al., 2009; Ko et al., 2009; Siddiqui et al., 2010). Thus, whereas neuroligins bind neurexins containing or lacking an insert in splice site S4, LRRTMs bind only neurexins lacking an insert in this
splicing site (de Wit et al., 2009; Ko et al., 2009). The ability to regulate interactions of NRX-NLG and NRX-LRRTM provides an intriguing mechanism for regulating synaptic specificity.

Consistent with the effects of LRRTMs on neuronal connectivity, deletion of LRRTM1 in mice reveals altered distribution of the excitatory presynaptic vesicular glutamate transporter vGlut1 \textit{in vivo} (Ko et al., 2009; Linhoff et al., 2009). Furthermore, it was demonstrated that lentivirus-mediated knockdown of LRRTM2 \textit{in vivo} decreases the strength of glutamatergic synaptic transmission. Conversely, LRRTM2 overexpression resulted in an increase of excitatory synapses (de Wit et al., 2009; Linhoff et al., 2009). Despite a few apparent differences between the studies on two independent LRRTM1 KO mice, behavioral experiments on these mice have shown behavioral abnormalities, impaired cognitive function and altered responses to environmental change in several tests (Takashima et al., 2011; Voikar et al., 2013). Interestingly, LRRTM1 KO mice exhibit some similarities in behaviour as NRX-1α KO mice, consistent with the reported interaction of LRRTM1 and neurexin1 (Voikar et al., 2013). LRRTM1 function \textit{in vivo} is likely to be compensated by LRRTM2 since they have overlapping expression pattern in the brain, both bind to neurexin and have similar synaptogenic activity levels. Based on previous results, LTP deficit in LRRTM1 and LRRTM2 DKD \textit{in vivo} in neonatal CA1 pyramidal neurons, can be rescued by wild-type LRRTM2, with its extracellular domain which binds to neurexin being essential for the rescue (Soler-Llavina et al., 2013).
Figure 1-3 Comparison Of LRRTMs Synaptogenic Activity, Binding And Expression Patterns

(A) LRRTM1 and 2 have similar synaptogenic strength. LRRTM4 possesses similar synaptogenic activity strength as neuroligin 1. LRRTM3 has the lowest synaptogenic activity of the LRRTM family. (B) LRRTM1 and LRRTM2 both bind presynaptic neurexins. (C) In situ hybridization analysis of LRRTM family mRNA expression in the adult mouse brain. Shown are dark-field emulsion autoradiographs: LRRTM1, LRRTM2, LRRTM3, and LRRTM4. (Laurén et al., 2003)

1.5.1 LRRTM1 And 2

LRRTM1 and LRRTM2 are most potent members of the LRRTM family at inducing presynaptic differentiation. LRRTM1 and LRRTM2, together with neuroligins
and Cbln-GluRδ, are postsynaptic binding partners of presynaptic neurexins at glutamatergic synapses (Siddiqui and Craig, 2011). Although LRRTM1 and LRRTM2 bind neurexins with a similar affinity as neuroligins, they follow a different and unique binding code which could be of significant importance for binding of neurexins to other postsynaptic partners (Siddiqui et al., 2010). While neuroligin-1 binds to neurexins 1, 2, and 3 β but not α variants, regardless of insert at splice site 4, LRRTM1 and LRRTM2 bind to neurexins 1, 2, and 3 α and β variants specifically lacking an insert at splice site 4 (Siddiqui et al., 2010). Binding of LRRTMs to presynaptic neurexins is critical for their maintenance and perhaps function at synapses; specifically prevention of NRX binding to LRRTMs by constitutive genetic inclusion of splice site 4 in NRX-3 results in decreases in basal AMPA receptor synaptic content, a block of LTP, an enhancement of constitutive AMPA receptor endocytosis, and an ~45% decrease in surface levels of LRRTM2 (Aoto et al., 2013). Interestingly, these synaptic deficits caused by inclusion of splice site 4 in NRX3 are somewhat similar to those caused by LRRTM 1 and LRRTM2 DKD (double knockdown) (Soler-Llavina et al., 2013, 2011), suggesting that a critical trans-synaptic protein complex required for maintaining AMPARs at synapses may involve presynaptic neurexin interactions with postsynaptic LRRTMs. Nevertheless, binding of postsynaptic LRRTMs and neuroligins with presynaptic neurexins greatly contribute to the excitatory synapse development.

In rodents, LRRTM1 contributes to synaptic organization in vivo with targeted deletion resulting in altered distribution of the vesicular glutamate transporter in hippocampus (Linhoff et al., 2009). Similar to neuroligin-3 R451C knock-in mice,
LRRTM1 KO mice exhibit an increase in the immunoreactivity for the vesicular glutamate transporter VGLUT1, confirming an *in vivo* synaptic function for LRRTM1 (Linhoff et al., 2009; Tabuchi et al., 2007). Correspondingly, endogenous LRRTM2 concentrates at multiple classes of glutamate synapses. Double knockdown (DKD) of LRRTM1 and LRRTM2 significantly affects AMPAR-mediated transmission in CA1 pyramidal cells during early postnatal development, suggesting that LRRTMs are involved in recruitment or maintenance of AMPAR at synapses (Soler-Llavina et al., 2011). In addition, assays of AMPAR surface expression in cultured hippocampal neurons showed that LRRTMs are required for the stabilization of AMPARs at synapses after LTP induction (Soler-Llavina et al., 2013). In fact, viral-mediated double knockdown of LRRTM1 and LRRTM2 blocks long term potentiation (LTP) in neonatal (P14–P18) CA1 pyramidal neurons and dramatically impaired LTP in mature (P35–P39) CA1 pyramidal neurons (Soler-Llavina et al., 2013); revealing an unexpected role for LRRTMs in LTP at both young and mature synapses. Thus, like neuroligins, these proteins are potent synaptic organizing molecules that contribute functionally to specific synaptic circuits.

LRRTM1 is a maternally suppressed gene on chromosome 2p12 and is associated paternally with handedness and schizophrenia (Francks et al., 2007; Ludwig et al., 2009). Findings of LRRTM1 contribution to synaptic organization *in vivo*, suggest a prevalence of LRR domain proteins in trans-synaptic signaling and provide a cellular basis for the reported linkage of LRRTM1 to handedness and schizophrenia (Linhoff et al., 2009). Involvement of both LRRTM1 and neurexin-1 in schizophrenia may delineate a common
mechanism explaining the similarities between LRRTM1 KO mice and the NRX-1α KO mice (Voikar et al., 2013). Takashima et al. (2011) reported impaired cognitive function for LRRTM1 KO mice, which could be related to the cognitive dysfunction seen in schizophrenia patients. In addition, the reduction of hippocampal volume in these LRRTM1 KO mice is morphologically parallel to that seen in first-episode schizophrenia patients (Takashima et al., 2011).

LRRTM2, on the other hand, has been shown to have associations with bipolar disorder (Malhotra et al., 2011). LRRTM2, has a very similar synaptogenic effect as LRRTM1 (Figure 1-3 A). It has been shown that LRRTM2 increases the abundance of excitatory but not inhibitory synapses in artifical synapse-formation assay as well as in transfected neurons (Ko et al., 2009; Linhoff et al., 2009). Knockdown of LRRTM2 in vivo decreases the strength of glutamatergic synaptic transmission, while LRRTM2 overexpression results in an increase of excitatory synapses (de Wit et al., 2009; Ko et al., 2009). Binding of LRRTM2 to neurexin is calcium-dependent and mutually exclusive with binding of neuroligin1 (Siddiqui et al., 2010). Point-mutation studies have revealed that despite the disparate nature of the LRR domain of LRRTM2 and the acetylcholinesterase-homologous domain of neuroligin1, these partners bind to a highly overlapping face of neurexin-1β (Siddiqui et al., 2010). Moreover, LRRTM2 is more potent than neuroligin-1 at recruiting postsynaptic PSD-95 and at recruiting presynaptic synaptophysin and bassoon (Siddiqui et al., 2010).
The individual LRRTM1 KO mice demonstrate no significant synapse loss and a subtle phenotype with altered VGlut1 immunofluorescence in hippocampal subfields expressing the lowest levels of LRRTM2 (Linhoff et al., 2009). This subtle phenotype can be explained by the overlap of LRRTM1 and LRRTM2 in expression pattern, synaptogenic activity levels and binding partners that could lead to compensation of one for the other in vivo (Figure 1-3). Although single short hairpin RNA (shRNA)-dependant knockdown of LRRTM2 was reported to cause significant synapse loss (de Wit et al., 2009), later studies of individual or combined KDs of the two LRRTMs by Ko et al. (2011) did not decrease synapse numbers in wild type neurons. Nevertheless, the combined triple KD (TKD) of LRRTM1, LRRTM2, and NLG3 in NLG1 KO neurons caused a robust decline in excitatory synapse density (measured using VGlut1 and other postsynaptic markers), suggesting the cooperation of neuroligins and LRRTMs to maintain normal levels of excitatory synapses (Ko et al., 2011). An independent study by Soler-Llavina et al. (2011) also found a substantially large decrease in excitatory transmission with TKD of LRRTM1, LRRTM2 and NLG3 in NLG1 KO mice, with both the AMPAR- and NMDAR-mediated components being affected; however no change in the density of synapses defined by the density of postsynaptic spines on dendrites was observed (Soler-Llavina et al., 2011).

Overall, previous knockout and knockdown studies to clarify the role and function of the LRRTM1 and LRRTM2 have led to many contradicting results (de Wit & Verhaagen, 2007; Ko, Fuccillo, et al., 2009; Ko, Zhang, et al., 2009; Soler-Llavina et al.,
2013, 2011); necessitating a more comprehensive approach. In our project, we use a double knockout model of LRRTM 1 and 2 as a powerful approach to study the role of these proteins in vivo. Our results indicate that LRRTM1 and 2 are essential for normal excitatory synapse development and function in CA1 hippocampal neurons but not in dentate gyrus granule cells.

1.5.2 LRRTM4

LRRTM4 regulates excitatory synapse development and has been found to induce pre-synapse differentiation with a similar potency to LRRTM2 (Linhoff et al., 2009). However, the expression of LRRTM4 in brain is more restricted. LRRTM4 mRNA is expressed at very high levels in dentate gyrus granule cells and in the anterior olfactory nucleus and at low or moderate levels in other brain regions such as cortex and striatum (Figure 1-3 C) (Siddiqui et al., 2013). LRRTM4 has been recently identified as a major component of native AMPA-type glutamate receptor complexes (Schwenk et al., 2012). Similar to neurexins, neuroligins, and several other synapse organizing proteins, LRRTM4 is linked to autism spectrum disorders (Pinto et al., 2010). LRRTM4 is also linked to risk of attempted suicide in females based on a recent genome-wide association study of over 2500 patients with bipolar disorder (Willour et al., 2012).

Despite the relatively extensive studies on LRRTM1 and LRRTM2, molecular interactions and roles of LRRTM4 in synapse development have not been previously well studied. Our project on LRRTM4 involved characterization of synaptic protein LRRTM4
and assessment of its role in synapse development. Our experiments using targeted deletion in mice showed that LRRTM4 is essential for normal excitatory synapse development and function in dentate gyrus granule cells but not in CA1 hippocampal pyramidal neurons. In addition, it was shown that LRRTM4 differentiates from LRRTM1 and 2 in terms of binding partners as it binds a family of new extracellular binding partners, heparan sulfate proteoglycans (HSPGs). Experiments indicated that HSPGs are essential to mediate the synaptogenic activity of LRRTM4.

1.6 HSPGs

Heparan sulfate proteoglycans (HSPGs) are another class of cell surface molecules that have a critical role in organizing neuronal connectivity. Proteoglycans are cell surface and extracellular matrix constituents that consist of a core protein and covalently attached glycosaminoglycan (GAG) chains, which are made of repeating disaccharide units. The GAG chains are enzymatically modified to contain highly sulfated domains that are negatively charged and serve as protein binding sites (Bernfield et al., 1999; Esko et al., 2009). The extracellular domains of these proteoglycans can be shed from the cell surface, generating soluble heparan sulfate proteoglycans that can inhibit interactions at the cell surface (Bernfield et al., 1999). Exostosin Ext1 is an essential enzyme for the synthesis of the heparin sulfate (HS) chains of HSPGs, and conditional knockout of EXT1 in late postnatal neurons leads to deficits in excitatory synaptic transmission and a reduction in surface AMPA receptors (Irie et al., 2012).
Acute cleavage of HS chains does not cause any changes to the basal transmission in hippocampal slices but prevents long-term potentiation (Lauri et al., 1999).

The function of HSPGs in the development of neuronal connectivity has been best described for axon pathfinding, where they regulate axon guidance cue distribution, availability, and function (de Wit and Verhaagen, 2007). The major HSPGs in the brain are the cell surface GPI-anchored glypicans, the transmembrane syndecans, and the secreted proteins agrin and perlecan. Agrin is a well-known synapse-organizing protein at the mammalian neuromuscular junction, and syndecan and the glypican Dally-like regulate synapse development in different ways at the Drosophila neuromuscular junction (Johnson et al., 2006). In contrast to their role in postsynapse maturation, a function of HSPGs in central neuron presynapse maturation and the mechanisms of actions for these proteins at central synapses are not yet well studied.

1.6.1 Glypicans

Glypicans (GPC) are a family of six, cell-surface glycoposphatidylinositol (GPI)-anchored HSPGs being expressed in the CNS (Fransson et al., 2004). Glypicans constitute one of the two major families of heparan sulfate proteoglycans, with the other major family being syndecans. They are widely present throughout the body and bind many secreted and surface-bound proteins (de Wit et al., 2013). Glypicans are critically involved in developmental morphogenesis, and have been implicated as regulators in several cell signaling pathways (Filmus & Selleck, 2001). Like other HSPGs, glypicans
contain protein backbones that are covalently conjugated to heparan sulfate (HS) glycosaminoglycan chains. Glypican-4 and glypican-6 released from glia cells after phospholipase cleavage were recently shown to promote GluA1-containing AMPA receptor surface insertion and functional synapse development in isolated retinal ganglion cells (Allen et al., 2012). GPC4-deficient mice show a reduction in synaptic strength, which has been attributed to decreased recruitment of the AMPAR subunit GluR1 to synaptic sites (Allen et al., 2012). All glypicans are expressed by neurons, thus neuronal glypicans in their cell surface GPI-anchored or cleaved soluble forms may also contribute to synapse development. Genome-wide association studies have linked GPC1 and GPC6 to ADHD, neuroticism, and schizophrenia (Calboli et al., 2010; Lesch et al., 2008; Potkin et al., 2009). The association of glypicans with these nervous system disorders indicates that glypicans may be important for proper brain function.

### 1.6.2 Syndecans

Syndecans (SDC), another family of HSPGs, are single transmembrane domain proteins that are thought to act as co-receptors, especially for G protein-coupled receptors. These core proteins carry three to five heparan sulfate and chondroitin sulfate chains, which allow for interaction with a large variety of ligands. The syndecan protein family has four members (SDC1-4); all of which have an N-terminal signal peptide, an ectodomain, a single hydrophobic transmembrane domain, and a short C-terminal cytoplasmic domain (Tkachenko et al., 2005). All syndecans are anchored to plasma membrane via a 24-25 amino acid long hydrophobic transmembrane domain (Tkachenko
et al., 2005). Syndecans have been shown to have an essential role in mediating cell signaling, cytoskeletal organization, neural patterning and axon guidance (Lauri et al., 1999). Drosophila Syndecans have been reported to promote the growth of presynaptic terminals and bind at high affinity to the protein tyrosine phosphatase LAR, a conserved receptor that controls both neuromuscular junction growth and active zone morphogenesis (Johnson et al., 2006). Syndecan-2 is present at both presynaptic and postsynaptic sites of glutamatergic synapses (Hsueh et al., 1998), and postsynaptic syndecan-2 regulates dendritic spine development (Ethell et al., 2001).
Chapter 2. Materials And Methods

2.1 DNA Constructs

LRRTM4-Fc contains the signal sequence from neurexin1β, then amino acids 31-362 of LRRTM4, then human IgG Fc in the pc4-sp-Fc1 vector (Takahashi et al., 2011). Myc-LRRTM4, HA-LRRTM4 and Myc-Glypican-5 contain the signal sequence from rat neuroligin 1, then the Myc or HA tag, then their mature coding sequence and express from the CMV promoter in the Clontech EGFP-N1 vector (lacking EGFP). HA-Glypican-5 and HA-SDC2 contain the signal sequence from rat neuroligin 1, then the HA tag, then the mature HSPG coding sequence in the pLentiLox3.7 synapsin promoter vector. Myc-GPC5ΔGAG expressing from the CMV promoter and HA-GPC5ΔGAG expressing from the synapsin promoter were generated by substituting serine at amino acid positions 441,486,495,507,509 to alanine (Li, et al., 2011) by the overlapping PCR method.

2.2 Neuron Culture And Transfection

Dissociated rat primary hippocampal neuron cultures were prepared from embryonic day 18 rat embryos essentially as described before in Kaech and Banker (2006) and She and Craig (2011). Rat neurons were plated at a final density of 300,000 cells per dish on poly-L-lysine-coated coverslips in 60 mm culture dishes and inverted over a glial feeder layer. Cytosine arabinoside (5 μM) was added to neuron culture dishes.
two days after plating to avoid overgrowth of glial cells. In order to limit excitotoxicity during transfection or co-culture, the neuron media was supplemented with 100 µM APV (Research Biochemicals). For neuron-COS7 cell co-culture assays, neurons were transfected with 4–5 µg DNA at DIV0 using nucleofection (AMAXA Biosystems) and seeded at a density of one million per 60 mm dish. Co-culture assays were set up as previously described in Graf et al. (2004).

2.3 Cell Culture And Transfection

HEK293 and COS7 cells were grown in 10% fetal bovine serum (FBS) in DMEM (GIBCO) at 37 °C and 5% CO₂. TransIT-LT1 Transfection Reagent (Mirus) was used to perform transfections of COS7 cells. Co-cultures of primary hippocampal neurons with COS7 cells were performed as described previously by Graf et al. (2004). Transfected COS7 cells were trypsinized on the day after transfection and resuspended in conditioned neuron culture media. Neurons grown on poly-L-lysine-coated coverslips for 9–10 DIV were inverted in a 12-well plate, and COS7 cells were added. After 2 hours, the neuron coverslips were flipped back over on their home dish so the neurons and COS7 cells were facing the glial feeder layer. After 20–24 hours of co-culture, cells were fixed for analysis. For testing the effect of heparinases on COS7-neuron cocultures, coverslips were incubated with or without the glial feeder layers in conditioned media for 20–24 hours with or without 0.2 to 0.4 U/ml each of Heparinases I (H2519), II (H6512), III (H8891) all from Sigma.
2.4 Soluble Fc Fusion Protein Production

LRRTM4-Fc protein was generated by transient transfection of encoding plasmid into HEK293 cells which were cultured in DMEM with 10% FBS and selected for 28 days with 0.5 mg/ml Zeocin (Invitrogen). Subsequent to selection with zeocin, medium was replaced with serum-free AIM V synthetic medium (Invitrogen). A total of 400-500 mL of the conditioned medium was collected every alternate day for four weeks. LRRTM4-Fc were purified using protein-G sepharose 4 fastflow columns (GE Healthcare) and concentrated in PBS with Centricon filters (Millipore). Purified Fc or LRRTM4-Fc fusion proteins were immunoblotted, imaged by chemiluminescence using a Bio-Rad image documentation system and quantified by densitometry relative to a human IgG standard curve using ImageJ (National Institutes of Health).

2.5 Cell Binding Assays

Cell binding assays were performed in EGB buffer (containing 168 mM NaCl, 2.6 mM KCl, 10 mM HEPES [pH 7.2], 2 mM CaCl$_2$, 2 mM MgCl$_2$, 10 mM D-glucose, and 100 mg/ml BSA) at 4 °C essentially as described in Siddiqui et al. (2010). To assess LRRTM4-Fc binding to Myc-Glypicans, Myc-Glypican5ΔGAG, SDC2-CFP or CFP, cDNAs encoding these constructs were transfected into COS7 cells and were incubated for 1 hour at 4 °C with LRRTM4-Fc fusion protein in EGB buffer, sometimes together with with anti-Myc antibody (1:500). Cells were subsequently fixed in pre-warmed 4%
formaldehyde / 4% sucrose, blocked in 10% BSA in PBS, and incubated with FITC-conjugated anti-human IgG to detect bound LRRTM4-Fc and with Alexa-568-conjugated anti-mouse antibody to detect surface Myc. To detect whether Heparinase treatment affected LRRTM4-Fc binding to Myc-Glypican5 or SDC2-CFP, transfected COS7 cells were incubated with or without a cocktail of Heparinases I, II and III at 0.2 U/mL.

2.6 Immunocytochemistry

Neurons, COS7 cells, and co-cultures were fixed using warm 4% formaldehyde and 4% sucrose in PBS (pH 7.4) for 12-15 minutes at room temperature and then permeabilized with 0.25% Triton X-100 for 8 minutes, except where live staining or staining of unpermeabilized fixed cultures was used. Live stainings were followed by fixation with warm 4% formaldehyde / 4% sucrose in PBS (pH 7.4). Fixed cultures were blocked with 10% BSA in PBS for 30 minutes at 37 °C. Primary antibodies were applied in 3% BSA in PBS and incubated overnight at 4 °C. The coverslips were then washed 3 times with PBS for 10 minutes each and incubated in secondary antibodies in 3% BSA in PBS for 1 hour at 37 °C. After washing the coverslips again in PBS for 3 times, they were mounted in elvanol (Tris-HCl, glycerol, and polyvinyl alcohol, with 2% 1,4-diazabicyclo[2,2,2] octane).
2.7 Immunohistochemistry

Brain tissue samples from 6 weeks old LRRTM4 KO and littermate wild-type male mice were used for immunofluorescence studies. The mice were anaesthetized by 20% urethane before transcardial perfusion was carried out with cold 0.1 M phosphate buffered saline (PBS) followed by 4% formaldehyde / 4% sucrose in PBS (pH 7.4). After the brains were extracted, they were post-fixed in 4% formaldehyde overnight, and cryoprotected in 30% sucrose in PBS at 4 °C. Using OCT compound (Tissue-Tek), the brains were frozen in dry ice and then kept at -80 °C until sectioning. Coronal cryostat sections 20 µm thick at hippocampal level were cut at -20 °C. Sections were then mounted on Superfrost Plus slides and stored at -80 °C until staining. For fresh-frozen brains, extracted brains were frozen in OCT compound using 2-methylbutane (Sigma) in dry ice/ethanol and then sectioned. Fresh frozen sections to be stained with LRRTM4 BC262 and PSD-95 antibodies were fixed by incubating in cold methanol for 10 min and sections to be stained with LRRTM4 BC262 and bassoon were fixed for 7 minutes in 4% formaldehyde/4% sucrose, and then blocked for 1 hour. For double staining of VGlut1 and GAD65, the 4% PFA fixed slides were incubated in blocking solution (5% BSA + 5% normal goat serum + 0.25 % Triton X100 in PBS) for 1 hour at room temperature. Sections were incubated with primary antibodies diluted in the same blocking solution at 4 °C overnight, followed by 3 washes with PBS and incubation with the appropriate secondary antibodies conjugated to Alexa 488, 568 or 647 (Invitrogen) for 1 hour at room temperature. This procedure was the same for all immunostainings performed. DAPI (4’,6 diamidino-2-phenylindole) was added to the final wash of the sections with PBS.
and were mounted using Immuno Mount (Thermo Scientific). In order to obtain images for synaptic density quantification, 5-6 mice for LRRTM4 wild type, HET and knockouts were fixed and stained simultaneously.

### 2.8 Antibodies

The C-terminal antibody against LRRTM4, BC262, was raised in rabbits as a GST fusion protein spanning amino acids 447 to 518 (SWKRY...GSRECE) of mouse LRRTM4 cDNA, BC037216 (Open Biosystems), expressed from the pGEX-4T-1 vector and purified from bacteria. Additional primary antibodies used in immunocytochemistry and immunohistochemistry experiments are as followed: anti-synapsin I (rabbit, 1:2000; Millipore; AB1543P), anti-PSD-95 family (mouse IgG2a; 1:500; clone 6G6-1C9; Thermo Scientific and Millipore, recognizes PSD-95, PSD-93, SAP102 and SAP97), anti-bassoon (mouse IgG2A; 1:1000; Stressgen; VAM-PS003), anti-VGlut1 (guinea pig, 1:2000, AB5905, Millipore), anti-VGlut1 (rabbit, 1:1000; Synaptic Systems; 135 302), anti-VGAT (rabbit, 1:1000; Synaptic Systems; 131 003) anti-gephyrin (mouse IgG1, 1:500, mAb7a, Synaptic Systems), anti-GAD65 (mouse IgG2a, 1:100, GAD-6-c, Developmental Hybridoma), anti-Myc (mouse IgG1, 1:500, 9E10, Millipore), anti-Myc (rabbit,1:4000, C3956, Sigma), anti-HA (rat IgG1, 1:1000, 11867431001, Roche). Dendrites were identified using anti-MAP2 (chicken polyclonal IgY; 1:2000; Abcam; ab5392) and axons were identified using anti-Tau (mouse mlgG2a 1:2000, clone PC1C6; Millipore; MAB3420; recognizes dephosphorylated tau). Secondary antibodies used were raised in goat against the appropriate species and monoclonal isotype, stringently cross-
adsorbed, and conjugated to Alexa-488, Alexa-568, and Alexa-647 dyes (1:500; Invitrogen).

2.9 Fluorescence Imaging

All imaging was performed blind to the experimental conditions. Images of brain sections were acquired on a Zeiss LSM 700 confocal microscope equipped for automated tiling. For quantitative analysis, 6-8 images per mouse were captured with a 40x / 1.4 NA oil objective, an additional 2.5x magnification and sequential scanning with individual lasers and optimized filters. For immunohistochemistry, 4 slices on one slide for each mouse were imaged, with 5-6 mice for each group of WT, Het and KO. Images of cultured cells were acquired on a Zeiss Axioplan 2 microscope with 63x/1.4 NA, 40x/1.3 NA and 25x /1.25 NA oil objectives, a Sensys cooled CCD camera, and custom filters. For neuronal overexpression experiments, neurons were chosen for imaging based on YFP or CFP signal, as well as healthy morphology under phase contrast and MAP2 channels. Neighbouring cells for overexpression analysis were chosen based on similar MAP2 staining.

2.10 Image And Data Analysis

Image and data analysis for all experiments was performed blind to the experimental conditions.
To measure the binding of Fc fusion proteins to surface-expressed HA- or Myc-tagged proteins, regions were drawn around the perimeter of each COS7 cell, and the average intensity values of bound protein and expressed protein were measured within the region and corrected for average off-cell background. For co-culture experiments, transfected COS7 cells were selected for scoring based on similar moderate surface expression, normal morphology, and contact with neurites, determined by Tau staining or phase contrast. HA-tagged cDNAs expressed in axons were analyzed for recruitment by LRRTM4-CFP expressed in COS7 cells. When an HA expressing axon intersected an LRRTM4-CFP expressing COS7 cell, only if the relative level of HA overlapping the COS7 cell was significantly higher than in the adjacent isolated axon, a contact was scored positive. More than 300 cells for each condition were scored across three independent experiments.

For quantification of excitatory (VGlut1) and inhibitory (GAD65) synaptic markers in brain sections, images were manually thresholded to define puncta and total area and total integrated intensity of puncta per tissue area measured. Measures were performed on a minimum of six images per animal for each region and a total of five to six animals for each genotype, measuring the same regions for both VGlut1 and GAD65.

Analysis was performed using Metamorph (Molecular Devices), Excel (Microsoft) and GraphPad Prism (GraphPad Software). Statistical comparisons were made with student t tests and one-way ANOVA with post hoc tests as mentioned in the figure legends. All data are reported as the mean ± SEM.
2.11 Preparation Of Crude Synaptosomal Fraction From Dentate Gyrus

Brains were rapidly removed and placed in oxygenated ice-cold slicing solution (in mM: 124 NaCl, 3 KCl, 1.25 NaH$_2$PO$_4$, 26 NaHOC$_3$, 10 MgSO$_4$, 1 CaCL$_2$ and 15 D-glucose, pH 7.3) following cervical dislocation. The hippocampus was then removed and 400 µm transverse slices were cut using a manual chopper (Stoelting, Wood Dale, IL, USA). After one hour of recovery time at room temperature in oxygenated artificial cerebrospinal fluid (ACSF) composed of (in mM): 124 NaCl, 3 KCl, 1.25 NaH$_2$PO$_4$, 26 NaHOC$_3$, 1 MgSO$_4$, 2 CaCL$_2$ and 15 D-glucose, pH 7.3, the dentate gyri were rapidly dissected in ice-cold ACSF. Homogenization of the micro-dissected dentate gyri (in 320 mM sucrose, 4 mM Hepes-NaOH, pH 7.3, protease inhibitors) was performed in a glass teflon homogenizer by nine strokes at 1200 rpm. The homogenate was centrifuged for 10 min at 1000g and the resulting pellet P1 was discarded. The supernatant S1 was then centrifuged for 15 min at 12000g. Homogenization buffer was used to re-suspend the pellet P2. It was then centrifuged for 15 min at 14500g and the resulting pellet P2’ or crude synaptosomal fraction was re-suspended in buffer B (in mM: 6 Tris, pH 8.1, 320 sucrose, 1 EDTA, 1 EGTA, 1 DTT, protease inhibitors).

2.12 Immunoblotting

Subcellular fractionation of whole brain was performed essentially as previously described by Linhoff et al. (2009). For all samples, protein concentrations were
normalized, samples were eluted into SDS sample buffer by heating at 95 °C for 5 minutes and run on 10% polyacrylamide gels. Gels were transferred onto Immobilon P membranes (Millipore) which were blocked in 5% skim milk in Tris-buffered saline/0.05% Tween-20 or 5% BSA in PBS overnight at 4 °C. Membranes were then incubated with one of the following primary antibodies overnight at 4 °C: anti-LRRTM4 (mouse, 1:1000, N205B/22, Neuromab), anti-PSD-95 family (mouse IgG2a; 1:2000; clone 6G6-1C9; Millipore), anti-GluA1 (rabbit; 1:1000, Upstate (Millipore)), anti-GluA2 (mouse; 1:1000, Millipore), anti-glypican-2 (mouse; 1:200, Zymed laboratories), anti-syndecan-4 (rabbit, 1:200, SantaCruz), anti-heparan sulfate stub region following heparinase treatment (mouse IgG2b; 3G10, 1:3000, Seikagaku), anti-gephyrin (mouse IgG1; mab7, 1:1000, Synaptic Systems) and anti-actin (rabbit, 1:5000, ab8227, Abcam). After washing 3 times with PBS-T, membranes were incubated with the following secondary antibodies for 1 hr at room temperature on a rotator: goat anti-mouse or goat anti-rabbit HRP conjugate from Millipore for chemiluminiscence, or anti-rabbit IRD800 Rockland or anti-mouse Alexa Fluor 680 from Invitrogen for Li-COR fluorescence infrared system. Immunoblots were detected using the SuperSignal Chemiluminescent kit (Thermo Scientific) and a Bio-Rad gel documentation system or the Odyssey Li-COR fluorescence infrared system (Li-COR Bioscience) and normalized to the actin levels.

2.13 Golgi Staining

The FD Rapid GolgiStain Kit (FD NeuroTechnologies) was used for Golgi stainings to determine the spine density of different hippocampal regions, essentially as
recommended by the manufacturers. 6 weeks old mice were euthanized and their brains were rapidly removed. After rinsing in PBS, their olfactory bulbs and cerebellum were excised and the remaining portion of the brain containing the hippocampus was incubated in the rapid Golgi solutions (Glaser & Van der Loos, 1981). After treatment with the rapid Golgi solutions for a total of three weeks, the brains were snap frozen and stored at -80 °C until sectioning. Using a cryostat, the brains were cut into 100 µm thick sections at the level of the hippocampus. Sections were mounted on slides pre-treated with 3% gelatin. The slides were left to dry overnight at room temperature, followed by being dehydrated, cleared with xylene and mounted with Permoun (Fisher Scientific).

Image acquisitions and analysis were all done blind to the experimental condition. Morphologically comparable slices from wild type and KO mice for both LRRTM4 and LRRTM1 and 2 projects were analyzed in bright field on a Zeiss Axioplan2 microscope with a 63X 1.4 NA oil objective. Spines were counted manually on a specific dendrite all the while altering the focal plane, and an image of the dendrite was acquired to determine its length.

### 2.14 DiI Labeling

Wild-type and knockout Mice were perfused with 1.5% formaldehyde, followed by PBS and the brains were rapidly removed. Brains were then postfixied in 1.5% formaldehyde for 1 hour and rinsed in PBS. 200 µm thick brain slices at the level of hippocampus were prepared using a vibrotome and placed on slides. Solid DiI crystals
(1,1’-dioctadecyl-3,3,3’3’-tetramethylindocarbocyanine perchlorate, Molecular Probe) were applied to the slices under a dissecting microscope with a borosilicate glass micropipette. Slices were then incubated in PBS at room temperature for 12 hours and further fixed in 4% formaldehyde for 30 minutes and mounted. Images of morphologically comparable slices from LRRTM4 wild-type and KO mice from medial molecular layer of dentate gyrus were acquired on a Zeiss LSM 700 confocal microscope and spines were analyzed using Metamorph (Molecular Devices).

2.15 Electron Microscopy

LRRTM1 and 2 DKO and wild-type littermate mice were perfused transcardially with 4% paraformaldehyde and then PBS. Brain tissue was collected and postfixed with 4% paraformaldehyde for 24 hours. Hippocampal CA1 region was blocked and fixed with osmium oxide, and then dehydrated. The brain tissue was then embedded in a JEMBED/Spurr’s mixture, and 70 nm sections were generated. Sections were stained with 2% uranyl acetate and Reynold’s lead for imaging. Images of stratum radiatum (>50) were obtained on a Hitachi H7600 transmission electron microscope. Symmetric and asymmetric synapses were visually identified and counted.
2.16 Electrophysiological Studies

Hippocampal slices were prepared from LRRTM4 KO and LRRTM1 and 2 DKO mice and wild-type littermates (6 to 10 weeks old). Following cervical dislocation, brains were rapidly removed and placed in ice-cold slicing solution consisting of (in mM): 120 NMDG, 2.5 KCl, 1.2 NaH$_2$PO$_4$, 25 NaHCO$_3$, 1.0 CaCl$_2$, 7.0 MgCl$_2$, 2.4 Na-pyruvate, 1.3 Na-ascorbate, 20 D-glucose with pH adjusted to 7.35 using HCl acid (unless stated, all chemicals and drugs were purchased from Sigma or BioShop, Canada). The hippocampus was removed and sliced in the transverse plane (400 µm thickness) using a manual tissue chopper (Stoelting, Wood Dale, IL, USA). Slices were subsequently transferred to a heated (30 °C) incubating chamber for 1 hr which contained ACSF composed of (in mM): 124 NaCl, 3 KCl, 1.25 NaH$_2$PO$_4$, 1 MgSO$_4$, 7 H$_2$O, 2 CaCl$_2$, 26 NaHCO$_3$ and 15 D-glucose which was bubbled continuously with carbogen (95% O$_2$ / 5% CO$_2$) to adjust the pH to 7.3. Slices were then maintained at room temperature for 30 minutes prior to being transferred to a submerged recording chamber where slices were perfused continuously with carbogenated ACSF (2-3 ml/min).

Mini-excitatory postsynaptic current (mEPSC) recordings were performed in cells that were voltage clamped at -60 mV. Recording pipettes were filled with solution containing (in mM): 122.5 Cs-methanesulfonate, 17.5 CsCl, 2 MgCl$_2$, 10 EGTA, 10 HEPES, 4 ATP (K), and 5 QX-314, with pH adjusted to 7.2 by CsOH. Tetrodotoxin (TTX; 500 nM; Ascent Scientific) and bicuculline methiodide (10 µM; Abcam) were added prior to recordings to block action potentials and GABA receptor-mediated inhibitory synaptic currents respectively. CNQX (10 µM; Abcam) and DL-AP5 (50 µM;
Abcam) were added towards the conclusion of some experiments to verify that remaining responses were mEPSCs. Frequency and amplitude were analyzed using MiniAnalysis software. GraphPad InStat and SigmaPlot were used for statistical analyses. Extracellular recordings to measure paired-pulsed facilitation and input-output responses were conducted in dentate gyrus molecular layer while stimulating perforant path fibers.
Chapter 3. Results I: LRRTM1 And 2

3.1 LRRTM 1 And 2 Double Knockout (DKO) Mice

Considering the similar strength of synaptogenic activity, common binding partner and similar expression pattern in the brain for LRRTM1 and LRRTM2, it is likely that these two proteins could compensate for each other’s functions in vivo. Therefore a powerful approach to study the role of these proteins in vivo is to use a double knockout model. The LRRTM1 and 2 double knockout mice were generated in our lab with targeted deletion in LRRTM 1 and LRRTM2 by deleting exon 2, which encodes a large portion of each of these proteins. In order to validate the loss of these two proteins, western blots were performed against both of these proteins using brain lysates from DKO and wild type mice (Figure 3-1 A and B). The LRRTM 1 and 2 DKO mice were viable and fertile and indistinguishable from wild-type mice with respect to gross brain morphology and cytoarchitectural organization. Confocal microscopy analysis of brain sections was performed to assess the normal brain characteristics of these mice by labeling for the nuclear marker DAPI, as well as the synaptic markers VGlut1 and GAD65 (Figure 3-1 C).
Figure 3-1 LRRTM1 And 2 DKO Mice Show Normal Brain Morphology

(A) Western blot confirmation of loss of LRRTM1 protein in LRRTM1 and 2 DKO mouse brain homogenate. (B) Western blot confirmation of loss of LRRTM2 protein in LRRTM1 and 2 DKO mouse brain homogenate. Het mice show an intermediate expression levels between WT and KO mice. (C) Confocal immunofluorescence images for synaptic markers VGlu1 and GAD65, and the nuclear marker DAPI revealed normal hippocampal morphology and large-scale synaptic organization in LRRTM1 and 2 DKO
and DHet mice as compared with wild-type (WT) mice. Data contributed by Dr. Tabrez J. Siddiqui.

3.2 LRRTM 1 And 2 Are Essential For Normal Excitatory Synapse Development In CA1

In order to examine the effect from loss of LRRTM1 and LRRTM2 in vivo, confocal analysis was performed on LRRTM1 and 2 DKO, DHet and wild type mice at 6 week postnatal using synaptic markers VGlut1 and GAD65 to compare excitatory and inhibitory synapses respectively. Considering the expression pattern for LRRTM1 and LRRTM2, we focused our analysis on CA1 layers of the hippocampus as well as the dentate gyrus region as the control (Figure 3-2 A and B). High resolution confocal images revealed a reduction in punctate immunofluorescence for VGlut1 in Oriens, Lacunosum and Radiatum layers of CA1 with no difference being observed for the inhibitory presynaptic marker GAD65 by double-labeling of the same regions (Figure 3-3 A-D). This pattern was not observed in the CA1 pyramidale layer as no significant difference was detected for VGlut1 punctate immunofluorescence between LRTM1 and 2 DKO versus wild type. The lack of an observed change in the CA1 pyramidale layer could most likely be due to the limitation in number of puncta in the region which could significantly affect accurate measurements from this area. VGlut1 measurements were not affected in dentate gyrus medial molecular layer in LRRTM1/2 DKO mice as compared with wild type (Figure 3-3 A and C). Similarly, no difference was observed for GAD65 measurements of the same region. Same trend of results were observed when
measuring total area and total integrated intensity of the markers in all mentioned regions. DHet mice showed an intermediate results for VGlut1 staining in all regions with no significant change from wild type littermates being recorded (Figure 3-3). Overall these results indicate that LRRTM1 and 2 are essential for normal excitatory synapse development and function in CA1 region but not the dentate gyrus.

Figure 3-2 LRRTM 1 And 2 Mice Confocal Microscopy Images
(A) High-resolution confocal images revealed a reduction in punctate immunofluorescence for VGlut1 in the Oriens, radiatum and lacunosum layers of CA1. No such change was observed for the dentate gyrus medial molecular layer but not in LRRTM1 and 2 DKO mice as compared with wild-type at 6 weeks postnatal. Pyramidal layer of CA1 did not have enough puncta staining to show any change. DHet mice showed an intermediate immunofluorescence between the WT and DKO mice. (B) No difference was observed in the inhibitory presynapse marker GAD65 by double labeling of the same regions. Scale bars represent 10 µm in (A) and (B).

Figure 3-3 Quantitation Of VGlut1 And GAD65 Punctate Immunofluorescence
(A and C) Quantitation of VGlut1 and GAD65 punctate total integrated intensity per tissue area. Within the CA1 region, Oriens, radiatum and lacuniform show a significant reduction for VGlut1 puncta immunofluorescence. Medial molecular layer of dentate gyrus shows no such change for LRRTM1 and 2 DKO mice as compared with WT at 6 weeks postnatal. DHet mice showed an intermediate immunofluorescence between the WT and DKO mice. Values for Pyramidal layer of CA1 are too low to show any change. (B and D) No difference was observed in total integrated intensity or the total area of the inhibitory presynapse marker GAD65 by double labeling of the same regions. ANOVA p < 0.0001, #p < 0.001 comparing LRRTM1 and 2 DKO and littermate wild-type mice for each region by Tukey’s multiple comparison test, n = 5–6 mice each after averaging data from 6 sections per mouse. Data are presented as mean ± SEM.

3.3 LRRTM1 And 2 Contribute To Excitatory Postsynapse Development In CA1 Radiatum

Dendritic spines are critical in excitatory synapse development and function and can be visualized clearly for analysis by Golgi staining. We examined the role of LRRTM1 and LRRTM2 on excitatory synapse development by counting spine density in Golgi-stained brain sections from LRRTM1 and 2 DKO as well as wild type mice (Figure 3-4 A). Spine density on CA1 radiatum dendrites was significantly reduced in LRRTM1 and 2 DKO mice as compared to wild type littermates. However, no difference was observed between spine density of dentate gyrus granule cell dendrites in the outer molecular layer (Figure 3-4 B). This reduction in the dendritic spine density of CA1
indicates the importance of LRRTM1 and 2 in excitatory postsynaptic development and function in this region.

**Figure 3-4 Excitatory Postsynapse Development In CA1 Is Affected By LRRTM1 And 2**

(A) Golgi staining revealed a reduced density of dendritic spines along CA1 radiatum in LRRTM1 and 2 DKO mice as compared with wild-type (WT) mice at 6 weeks postnatal. No differences were observed between genotypes along dentate granule cells in the outer molecular layer. (B) Quantitation of dendritic spine density along CA1 radiatum cell dendrites and along dentate granule cell dendrites in the outer molecular layer. ***p < 0.0001 comparing WT and KO by Student’s t test, n = 60–109 dendrites each from 3 mice.
3.4 Synapse Density Is Affected In LRRTM1 And 2 DKO Mice

The effect of LRRTM1 and 2 on synapses of CA1 region was also further confirmed by electron microscopy (Figure 3-5). Using LRRTM1 and 2 DKO, electron microscopy on hippocampal brain slices revealed a significant reduction in synapse density in CA1 radiatum when compared to wild-type littermates (Figure 3-5 B). DHET mice showed an intermediate results for synapse density with no significant change from the wild type mice. In order for further examine the effect of LRRTM1 and 2 on synapse morphology, the active zone length and spine width were also measured by electron microscopy in the CA1 radiatum region. Interestingly, the presynaptic active zone length was elongated significantly in LRRTM1 and 2 DKO as compared to the wile type (Figure 3-5 C). A possible explanation for this elongation in Synapse length could be that synapses in LRRTM1 and 2 DKO mice are less compact. Measurements of spine width led to no significant change between the LRRTM1 and 3 DKO, DHET and wild-type littermates (Figure 3-5 D).

Overall, results from the electron microscopy analysis confirm the importance of LRRTM1 and 2 in normal synapse development in the CA1 region of the brain.
Figure 3-5 LRRTM 1 And 2 Contribute To Changes In Synapse Density And Morphology
(A) Electron microscopy images showing synapses, the spine width and the active zone region. (B) Ultrastructure analysis showed synapse loss in hippocampal CA1 region of LRRTM1 and 2 DKO mice. (C) Active zone length is increased for LRRTM1 and 2 DKO mice compared with the wild type littermates. DHet mice exhibit intermediate values between the WT and DKO mice. (D) No Change in spine width was observed between WT, DHet and DKO mice. ANOVA, p < 0.0001 (n = 4 mice each after averaging data from more than 50 images per mouse per region), age P50–P60; *p < 0.01 by post hoc Bonferroni multiple comparison test comparing LRRTM1 and 2 DKO and WT for each region. Data are expressed as mean ± SEM. Contributed by Fergil Mills and Dr. Shernaz Bamji.

3.5 LRRTM1 And 2 DKO Mice Exhibit Impaired Excitatory Transmission In CA1

Deficits in CA1 radiatum of LRRTM1 and 2 DKO was also observed by electrophysiology experiments (Figure 3-6). We performed whole-cell recordings from CA1 radiatum cells in hippocampal slices of LRRTM1 and 2 DKO and wild-type littermate mice. CA1 radiatum cells in LRRTM1 and 2 DKO were shown to be deficient in excitatory synapse transmission as the miniature excitatory postsynaptic current (mEPSC) frequency was significantly reduced when compared to cells from wild type mice (Figure 3-6 B). mEPSC amplitude remained unchanged for the CA1 radiatum cells between the LRRTM1 and 2 DKO and wild type mice (Figure 3-6 C).
These results are consistent with the previously discussed data, indicating a role for LRRTM1 and 2 in controlling excitatory synapse density specifically in the CA1 region.

Figure 3-6 LRRTM1 and 2 DKO CA1 Radiatum Cells Are Deficient In Excitatory Synaptic Transmission

(A) Representative mEPSC recordings from CA1 radiatum cells in acute hippocampal slices from LRRTM1 and 2 DKO and littermate wild-type (WT) mice. (B and C) Cumulative distributions of mEPSC interevent intervals (B) and amplitudes (C) in LRRTM1 and 2 DKO and littermate wild-type CA1 radiatum neurons (wild-type, represented in black; LRRTM1 and 2 DKO, represented in red). Insets display mean ± SEM for mEPSC frequency (B) and amplitude (C), respectively. mEPSC frequency was significantly reduced in LRRTM1 and 2 DKO neurons, whereas no differences in amplitude were observed. *p < 0.01 by Student’s t test, n = 8 cells each. Data contributed by Dr. Steven Connor.
Chapter 4. Results II: LRRTM4

4.1 LRRTM4 Expression Parallels Time Course Of Synaptogenesis And Localizes To Excitatory Postsynaptic Sites On Dentate Gyrus Granule Cells

Considering the possible synaptogenic role of LRRTM4, a western blot experiment was designed to follow the expression of LRRTM4 during different stages of development using mouse whole-brain homogenates (Figure 4-1 A). LRRTM4 was easily detected at postnatal day 6; The protein levels increased in later stages until postnatal day 30 at which point it remains at relatively same high level a month later (Figure 4-1 B). Results indicate that LRRTM4 expression parallels the time course of synaptogenesis as it increases consistently during the peak phase of synaptogenesis and then reaches a plateau.

In order to observe the cellular and subcellular distribution of LRRTM4 in vivo, an antibody against the intracellular domain of LRRTM4 was generated. Immunostaining of coronal sections from adult mouse brain showed a distinct and strong pattern for LRRTM4 in the dentate gyrus molecular layers consistent with previously observed localization of this protein (Laurén, et al., 2003). LRRTM4 was present throughout the dentate gyrus and slightly more concentrated in the inner molecular layer (Figure 4-1 C). Within the dentate gyrus molecular layer, LRRTM4 was present in a punctate pattern overlapping with the localization of PSD-95 and the active zone molecule bassoon.
Overall, the subcellular localization and expression profile of LRRTM4 indicate that it operates at excitatory postsynaptic sites in dentate gyrus granule cells.

Figure 4-1 LRRTM4 Localizes To Excitatory Postsynaptic Sites On Dentate Gyrus Granule Cells

(A and B) Western blot analysis of LRRTM4 expression levels in mouse brain homogenates from postnatal day 6 (P6) to P60 and quantitation of the relative expression level of LRRTM4 in mouse brain homogenate during postnatal development. Data are presented as mean ± SEM of three to five experiments. (C and D) LRRTM4 immunoreactivity in coronal sections of adult mouse brain was observed at highest levels in the dentate gyrus molecular layer and was not observed elsewhere within the hippocampus (C). Within the dentate gyrus, LRRTM4 was present in a punctate pattern...
showing overlap with PSD-95 and bassoon (D). This antibody was validated using tissue lacking LRRTM4. Scale bars represent 500 µm in (C), 250 µm in D (top), and 5 µm in (D) (bottom). LRRTM4 antibody validation and microscopy images were performed by Dr. Tabrez J. Siddiqui.

4.2 The LRRTM4 Ectodomain Binds To HSPGs

For LRRTM4 to mediate its synaptogenic effect, it must directly or indirectly interact with presynaptic ligands. It had been previously shown that other members of the LRRTM family, LRRTM1 and 2 bind to the LNS domain of neurexins and induce presynaptic differentiation through this interaction. However, our lab showed that LRRTM4 does not share the same binding pattern and partners as LRRTM1 and 2 (Siddiqui et al., 2013).

A recombinant protein containing the ectodomain of LRRTM4, LRRTM4-Fc was generated and used for affinity purification of ligands from a solubilized crude rat brain synaptosomal fraction. Through an unbiased search for binding partners of LRRTM4 followed by mass spectrometry analysis, 4 members of the glypican family (glypican-1, glypican-3, glypican-4 and glypican-5) were isolated. As a representative of the glypican family, glypican-5 was used to show the direct binding of LRRTM4 to this protein in COS7 cells (Figure 4-2 A and B). Other members of the Glypican family were also tested to investigate their interaction with LRRTM4 and were shown to directly bind LRRTM4.
Myc-tagged glypican-5 was expressed in COS7 cells and incubated with LRRTM4-Fc. COS7 cells expressing glypican-5 showed strong binding of LRRTM4-Fc. However, LRRTM4-Fc did not bind to the surface of COS7 cells expressing a mutant of GPC5 that lacks the five serine residues involved in glycosaminoglycan linkage and cannot be glycanated (GPC5ΔGAG) (Figure 4-2 A and B). The binding of LRRTM4-Fc to GPC5 was abolished when cells were treated with heparinases, which cleaves the HS chains (Figure 4-2 A and B). Therefore, it became apparent that the HS chains on HSPGs are critical for their interaction with LRRTM4.

Additionally it was shown that glypicans are not the only family of HSPGs that interact with LRRTM4. The syndecan family members were also shown to bind LRRTM4 through their HS chain, another interaction that could be abolished by heparinases treatment. These results clearly indicate glypicans and syndecans as direct binding partners of LRRTM4 with the interacting taking place via their HS chains.

Figure 4-2 LRRTM4 Binds HSPGs
(A) Myc-GPC5 and Myc-GPC5ΔGAG proteins were expressed in COS7 cells and tested for binding of the LRRTM4-Fc fusion protein with or without heparinases. Expressed myc-tagged proteins were visualized by surface labeling. Upon expression in COS7 cells, GPC5 mediated strong binding of LRRTM4-Fc. The HS-deficient mutant GPC5ΔGAG did not mediate binding of LRRTM4-Fc. Treatment of the cultures with heparinases to cleave the HS chains eliminated the binding of LRRTM4-Fc to COS7 cells expressing GPC5. Scale bars represent 50 µm. (B) Quantitation of LRRTM4-Fc binding to COS7 cells expressing the indicated constructs. ANOVA p < 0.0001, *p < 0.05 compared with Myc-GPC5ΔGAG by Tukey’s multiple comparison test, n = 15 cells each. Data contributed by Dr. Tabrez J. Siddiqui.

4.3 LRRTM4 in Trans Recruits Axonal HSPGs to Cell Contact Sites

To determine whether LRRTM4 and HSPGs interact in trans on cellular surfaces and recruit each other to developing contact sites, an experiment was designed to coculture COS7 cells expressing LRRTM4 with neurons expressing HSPGs. LRRTM4-CFP expressed in COS cells was able to recruit neuronally expressed HA-GPC5 or HA-SDC2 but not the HS-deficient mutant HA-GPC5ΔGAG to contact sites (Figure 4-3 A and B). The absence of recruitment activity for HA-GPC5ΔGAG indicates that the HS chains are required for the mutual recruitment of LRRTM4 and HSPGs to cell contact sites. In order to further examine the interaction between LRRTM4 and HSPGs a reverse experiment with LRRTM4 expressed in neurons and HSPGs in COS cells was performed.
This experiment led to consistent findings as GPC-5 and SDC-2 in COS cells were able to recruit neuronally expressed LRRTM4, which the mutant form of GPC5 (GPC5∆GAG) was not (Results not shown). Additionally, HA-GPC5 targeted selectively to axons in cultured hippocampal neurons, both in pure neuron cultures and in the COS7 co-cultures (Figure 4-3 C). HA-SDC2 targeted to both axons and dendrites, although LRRTM4-expressing COS7 cells induced local aggregation of both recombinant HSPGs mainly along contacting axons in co-culture (Figure 4-3 D). These results indicate that LRRTM4 on dendrites could recruit axonal HSPGs to contact sites.
Figure 4-3 LRRTM4 In trans Recruits Axonal HSPGs To Cell Contact Sites

(A) Cultured hippocampal neurons were transfected at plating to express the indicated HA tagged glypican (GPC) or syndecan (SDC) and were cocultured at 11 DIV with COS7 cells expressing LRRTM4-CFP. LRRTM4-CFP on COS7 cells recruited HA-GPC5 and HA-SDC2, but not the HS-deficient mutant HA-GPC5ΔGAG, to high local density at cell contact sites along dephospho-tau-positive axons. (B) Quantitation of the percentage of COS7 cells expressing LRRTM4-CFP and in contact with HA-HSPG
expressing axons that induced local recruitment of the HA-HSPG. ANOVA p < 0.0001, *p < 0.001 compared with HA-GPC5ΔGAG by Tukey’s multiple comparison test, n = 3 experiments. Data are presented as mean ± SEM. Scale bar represents 10 µm. (C) HA-tagged glypican-5 (HA-GPC5) expressed in cultured hippocampal neurons targeted selectively to axons (arrowhead) lacking the dendritic marker MAP2, whereas transfected dendrites showed little signal for HA-GPC5. Co-cultured COS7 cells expressing LRRTM4-CFP recruited axonal HA-GPC-5. (D) HA-tagged syndecan-2 (HA-SDC2) expressed in cultured hippocampal neurons targeted to both axons (arrowhead) and to MAP2-positive dendrites of transfected cells. Co-cultured COS7 cells expressing LRRTM4-CFP recruited primarily axonal HA-SDC2. Scale bar represents 50 µm.

4.4 LRRTM4 Knock Out Mice

In order to study the role of LRRTM4 in vivo, knockout mice were generated with a targeted deletion in LRRTM4 by deleting exon 2, which encodes a large portion of the LRRTM4 protein. Western blot analysis of whole brain homogenate of these mice showed the loss of LRRTM4 protein (Figure 4-4 A). This result was further confirmed by confocal microscopy analysis of brain sections with an anti-LRRTM4 antibody showing loss of LRRTM4 staining in dentate gyrus molecular layers in the knock out mice (Figure 4-4 B). Overall, the LRRTM4 KO mice were viable and fertile and indistinguishable from the wild type littermates. In addition, confocal immunofluorescence for the synaptic marker synapsin and nuclear marker DAPI revealed normal hippocampal morphology.
and large-scale synaptic organization in LRRTM4 KO mice compared to wild type (Figure 4-4 C).
(A) Western blot confirmation of loss of LRRTM4 protein in LRRTM4 KO mouse brain homogenate. (B and C) Immunofluorescence for LRRTM4 confirmed loss of protein in dentate gyrus molecular layers in LRRTM4 KO mice. Confocal immunofluorescence images for synapsin and the nuclear marker DAPI revealed normal hippocampal morphology and large-scale synaptic organization in LRRTM4 KO as compared with wild-type (WT) mice. (D) Western blotting of crude synaptosomal fractions from dentate gyrus of 6- to 7-week-old LRRTM4 KO and wild-type control mice reveal that excitatory postsynaptic scaffolding PSD-95 family molecules (using an antibody that recognizes PSD-95, PSD-93, SAP102, and SAP97) but not inhibitory scaffolding molecule gephyrin are reduced in LRRTM4 KO mice. Overall HSPGs revealed with an antibody against the glycosaminoglycan stub region after heparinase treatment, as well as the glypican GPC2 and syndecan SDC4, are also reduced in LRRTM4 KO mice. (E) Quantitation of western blots in (D), normalized to beta-actin loading controls. *p < 0.05, **p < 0.01, t test, n = 4 mice per genotype. LRRTM4 antibody validation and microscopy images were performed by Dr. Tabrez J. Siddiqui.

4.5 Levels Of HSPGs And Key Postsynaptic Molecules Are Altered In LRRTM4 KO Mice Dentate Gyrus

Considering the high levels of LRRTM4 in the molecular layers of dentate gyrus, it is very important to test whether levels of HSPGs and key postsynaptic molecules may be altered in the dentate gyrus of LRRTM4 KO mice. In order to do so, we prepared crude
synaptosomal fractions specifically from isolated dentate gyri from LRRTM4 KO and control wild-type mice at 6–7 weeks postnatally, a time when LRRTM4 expression reaches a plateau (Figure 4-1 A). Western blot analysis was carried out using these samples to determine various protein levels (Figure 4-4 D and E). Quantitative immunoblotting of these fractions revealed no difference between LRRTM4 KO and wild-type mice for the level of AMPA receptor subunits GluA1 and GluA2 (Figures 4-4 D and E). While the level of the inhibitory synapse scaffolding molecule gephyrin remained unchanged, the level of PSD-95 family proteins was significantly reduced in LRRTM4 KO mice compared to wild type, indicating that LRRTM4 is an important component of excitatory postsynapses in the dentate gyrus.

In order to determine whether the level of HSPGs may be affected by the loss of LRRTM4 in KO mice, we tested various antibodies against glypicans and syndecans. Representatives of glypicans and syndecans, GPC2 and SDC4 were both significantly reduced in the crude synaptosomal fractions of LRRTM4 KO mice dentate gyri compared to wild type samples. In addition, using an antibody that recognizes the glycosaminoglycan stub region after heparinase treatment, we found that the total level of all HSPGs in crude synaptosomal fractions of LRRTM4 KO mice dentate gyri was significantly reduced, indicating that LRRTM4 is an important functional partner of HSPGs. Overall these results indicate the importance of LRRTM4 in maintaining normal levels of HSPGs and other key synaptic proteins in the dentate gyrus.
4.6 *LRRM4* KO Mice Show Impaired Excitatory Presynapse Development In Dentate Gyrus

We next attempted to further analyze the effect of LRRM4 loss on synapse development by performing confocal microscopy on LRRM4 KO, Het and wild type littermates 6-7 weeks postnatal. We investigated confocal imaging of excitatory and inhibitory synaptic markers in LRRM4 KO dentate gyrus molecular layers (Lateral and Medial outer molecular layer and inner molecular layer), in comparison with CA1 stratum oriens, a region where LRRM4 is not expressed (Figure 4-5 A and B) (Laureñ et al., 2003; Lein et al., 2007). Quantitative confocal analysis revealed reduced punctate VGlut1 immunofluorescence in all dentate gyrus molecular layer regions of LRRM4 KO mice. No such change was observed for VGlut1 immunofluorescence in CA1 stratum oriens of LRRM4 KO mice as compared to wild type littermates (Figure 4-6 A and C). In contrast, punctate immunofluorescence of the GABA synthetic enzyme glutamic acid decarboxylase GAD65 in the same colabeled regions was indistinguishable between LRRM4 KO and wild-type littermate mice (Figure 4-6 B and D). Same trend of results were observed when measuring total area and total integrated intensity of the markers in all mentioned regions. Het mice showed an intermediate results for VGlut1 staining in all regions with no significant change from wild type littermates being recorded (Figure 4-6). Therefore, these results indicate that LRRM4 is required for the development of excitatory presynapses in specific brain regions.
Figure 4-5 LRRTM4 Mice Confocal Microscopy Images

(A) High-resolution confocal images revealed a reduction in punctate immunofluorescence for VGlut1 in the dentate gyrus molecular layer but not in CA1 stratum oriens in LRRTM4 −/− (KO) mice as compared with wild-type (WT) at 6 weeks postnatal. (B) No difference was observed in the inhibitory presynapse marker GAD65 by double labeling of the same regions between KO, Het and WT mice. Scale bars represent 10 µm in (A) and (B).
Figure 4-6 Quantitation Of VGlut1 And GAD65 Punctate Immunofluorescence In LRRTM4 Mice

(A and C) Quantitation of VGlut1 and GAD65 punctate total integrated intensity per tissue area. Within the dentate gyrus outer molecular layer, L and M indicate the more distal or proximal regions that receive inputs from the later or medial entorhinal cortex, respectively. The dentate gyrus molecular layers show a significant reduction for VGlut1 puncta immunofluorescence. CA1 stratum oriens shows no such change for LRRTM4 KO mice as compared with WT at 6 weeks postnatal. Het mice showed an intermediate immunofluorescence between the WT and KO mice. Values for Pyramidal layer of CA1
are too low to show any change. (B and D) No difference was observed in total integrated intensity or the total area of the inhibitory presynapse marker GAD65 by double labeling of the same regions. ANOVA p < 0.0001, #p<0.001 comparing LRRTM4 KO and littermate wild-type mice for each region by Tukey’s multiple comparison test, n = 5–6 mice each after averaging data from 6 sections per mouse. Data are presented as mean ± SEM.

4.7 LRRTM4 Contributes To Excitatory Postsynapse Development In Dentate Gyrus

The vast majority of excitatory synapses on dentate gyrus granule cells and CA1 pyramidal neurons form on dendritic spines (Harris and Kater, 1994; Trommald and Hulleberg, 1997). In our next experiment we examined changes in spine density using Golgi-staining as well DiI labeling of brain sections. Analysis from Golgi staining revealed that the spine density on dentate gyrus granule cell dendrites in the outer molecular layer (the region receiving inputs from the medial entorhinal cortex) was significantly reduced in LRRTM4 KO mice as compared with wild-type littermates. However, CA1 pyramidal neuron dendrites in stratum oriens showed no such difference (Golgi data not shown). To rule out any potential artifacts caused by the slow fixation in Golgi-stained tissue, we also tested the effect of LRRTM4 on spine density using carbocyanine dye diI labeling of perfused tissue. DiI staining results complemented that of Golgi by showing a significant reduction of spine density in the dentate gyrus of
LRRTM4 KO compared to wild type mice (Figure 4-7 A and B). This reduction in the dendritic spine density of the dentate gyrus indicates the importance of LRRTM4 in excitatory postsynaptic development and function in this region.

Figure 4-7 Spine Density Is Reduced In Dentate Gyrus Of Lrrtm4 KO Mice

(A) Labeling of dentate gyrus cells of lightly perfused tissue by lipophilic carbocyanine dye DiI revealed reduced density of dendritic spines along dentate granule cells in the outer molecular layer in LRRTM4 -/- (KO) mice as compared with wild type (WT) mice at 6 weeks postnatal.  (B) Quantitation of dendritic spine density along dentate granule cell dendrites in the outer molecular layer input region from the medial entorhinal cortex.  *p< 0.01 comparing wild type and LRRTM4 KO by student’s t-test, n = 54 dendrites each from 3 mice. Scale bar represents 3 µm.
4.8 Excitatory Transmission Is Impaired In LRRTM4 KO Dentate Gyrus Granule Cells

To investigate the changes in excitatory synapse function as a consequence of the loss of LRRTM4, we performed whole-cell recordings from dentate gyrus granule cells in hippocampal slices of LRRTM4 KO and wild-type littermate mice (Figure 4-8). Miniature excitatory postsynaptic current (mEPSC) recordings from LRRTM4 KO neurons revealed a 35% reduction in mEPSC frequency as compared to wild-type control neurons (Figure 4-8 B). mEPSC amplitude remained unchanged for the dentate gyrus granule cells between the LRRTM4 KO and wild type mice (Figure 4-8 C). In order to determine whether changes in mEPSC frequency were specific to dentate gyrus granule cells, we recorded mEPSCs in CA1 pyramidal cells as the control. There was no significant difference observed in mEPSC frequency or amplitude was detected between LRRTM4 KO and wild-type littermate CA1 neurons (data not shown). Thus, LRRTM4 contributes to development of functional excitatory synapses selectively in dentate gyrus granule neurons. The observed reduction in mEPSC frequency but not amplitude is consistent with the imaging data, indicating a role for LRRTM4 in controlling excitatory synapse density specifically on dentate gyrus granule neurons.
Figure 4-8 LRRTM4 KO Dentate Gyrus Granule Cells Are Deficient In Excitatory Synaptic Transmission

(A) Representative mEPSC recordings from dentate granule cells in acute hippocampal slices from LRRTM4 KO and littermate wild-type (WT) mice. (B and C) Cumulative distributions of mEPSC interevent intervals (B) and amplitudes (C) in LRRTM4 KO and littermate wild-type dentate gyrus granule neurons (wild-type, represented in black; LRRTM4 KO, represented in red). Insets display mean ± SEM. mEPSC frequency was significantly reduced in LRRTM4 KO neurons compared to WT, whereas no differences in amplitude were observed. **p < 0.01 by Student’s t test, n = 8 cells each. Data contributed by Dr. Steven Connor.
Chapter 5. Discussion

5.1 LRRTM1 And 2

LRRTM1 and LRRTM2 have been shown to possess similar strength of synaptogenic activity and similar expression patterns in the brain. LRRTM1 and LRRTM2 selectively promote excitatory presynaptic differentiation (Linhoff et al., 2009). They also have common binding partners as both postsynaptic LRRTM1 and LRRTM2 bind specifically the LNS (Laminin A G domain, Neurexin, and Sex hormone-binding globulin) domain of neurexins, lacking an insert at splice site 4 (Siddiqui et al., 2010). Considering the similarities between LRRTM1 and LRRTM2 expression pattern, synaptogenic activity levels and binding partner choice, it is likely that they may compensate for each other’s functions in knock out studies in vivo. In fact, previous loss-of-function studies to investigate the functional role of these proteins in synapse development have led to inconclusive findings. While the KD of LRRTM1 and/or LRRTM2 in vitro does not cause a change in synapse numbers (Ko et al., 2011), and LRRTM KDs in vitro and in vivo have yielded somewhat inconsistent results, decreases in AMPAR surface expression in vitro and AMPAR-mediated synaptic transmission in vivo have been observed (de Wit et al., 2009; Soler-Llavina et al., 2011). The individual LRRTM1 KO mice have been reported to demonstrate no significant synapse loss and a subtle phenotype with altered VGluT1 immunofluorescence in hippocampal subfields expressing LRRTM2 (Linhoff et al., 2009).
In order to better clarify the functional role of LRRTM1 and LRRTM2, we generated the LRRTM1 and 2 double knock out as an innovative approach to study the role of these two proteins \textit{in vivo} and investigate their effect on synapse development and function. We focused our analysis mainly on CA1 region of the hippocampus based on the expression pattern of LRRTM1 and 2. However, considering the cell-type specific function of LRRTM1 and 2, it is likely that they also contribute to synapse development and function in multiple discrete regions.

In LRRTM1 and 2 DKO mice, CA1 but not dentate gyrus shows reduction in excitatory but not inhibitory synapse inputs and spine density. Moreover, loss of LRRTM1 and 2 cause a deficit in excitatory synaptic transmission specifically in CA1 radiatum in acute brain slice. Overall, our results indicate a clear role for LRRTM1 and 2 specifically in the excitatory synapse development in hippocampal CA1.

The reductions in VGlut1 input puncta immunofluorescence in LRRTM1 and 2 DKO CA1 region but not the dentate gyrus \textit{in vivo} indicates that LRRTM1 and 2 are essential for cell-type specific excitatory synapse development. These results complement previous findings of LRRTM1 and LRRTM2 selectively promoting excitatory but not inhibitory presynaptic differentiation (Linhoff et al., 2009). Further strengthening these results, we found a significant reduction in dendritic spine density and synapse density in the CA1 radiatum region in LRRTM1 and 2 DKO mice confirming that the loss of LRRTM1 and LRRTM2 affects excitatory synapse development in the CA1 region of hippocampus. While the spine width were not affected in LRRTM1 and 2 DKO mice, an
observed elongation of presynaptic active zone length could point to further unstability of synapses formed as they are less compact. A corresponding functional reduction in excitatory synaptic transmission is indicated by the reductions in mEPSC frequency in LRRTM1 and 2 DKO CA1 radiatum cells compared to wild type mice. These results complement previous finding that double knockdown (DKD) of LRRTM1 and LRRTM2 significantly affects AMPAR-mediated transmission in CA1 pyramidal cells during early postnatal development (Soler-Llavina et al., 2011, 2013).

5.2 LRRTM4

LRRTM4 has a distinct expression pattern as compared to LRRTM1 and 2 in that it is highly expressed in the dentate. The abundance of LRRTM4 in dentate gyrus molecular layers is consistent with the high-level expression of LRRTM4 mRNA in dentate gyrus granule cells (Laure´n et al., 2003; Lein et al., 2007). Using cultured neurons and genetic knockout approaches, we demonstrated that LRRTM4 promotes excitatory synapse development. LRRTM4 is localized at excitatory postsynaptic sites in all dendritic laminae, and is associated with excitatory postsynaptic scaffold proteins of the PSD-95 family and AMPA receptors. In LRRTM4 KO mice, dentate gyrus granule cells but not CA1 neurons show reductions in excitatory but not inhibitory synapse inputs and spine density. In addition, loss of LRRTM4 leads to a deficit in excitatory synaptic transmission specifically in dentate gyrus granule cells but not CA1 neurons in acute brain slices. Loss of LRRTM4 also results in a reduced level of PSD-95 family proteins.
as well as some other key proteins in dentate gyrus crude synaptosomes. Overall, our results indicate a clear role for LRRTM4 in excitatory presynapse and postsynapse development in the dentate gyrus. These findings were further complemented by a concurrent study (de Wit et al., 2013) demonstrating that LRRTM4 regulates excitatory synapse development \textit{in vitro} and \textit{in vivo}. Their results stated that overexpression, knockdown, and competition experiments with soluble LRRTM4 ectodomains show that LRRTM4 regulates excitatory synapse development in cultured hippocampal neurons. In addition, by knocking down LRRTM4 \textit{in vivo} they observed a significant decrease in the density of dendritic spines, which are the predominant sites of excitatory synapses in the CNS (Bourne & Harris, 2008).

Cell-surface interactions play important roles in establishing functional neural circuits. We further identified a difference between LRRTM4 and LRRTM1 and 2 in their binding partners. While LRRTM1 and LRRTM2 are known to bind the LNS domain of neurexins, we isolated a new family of LRRTM4 ligands, HSPGs as direct interacting partners. LRRTM4 can directly bind to all the glypicans and syndecans tested. Their interaction requires the HS chains and appears to be relatively independent of the glypican or syndecan backbone. An independent study by de Wit et al. (2013) also identifies glypican as an LRRTM4 receptor using a proteomics-based approach. LRRTM4 and GPC4 were shown to localize to opposing membranes of glutamatergic synapses, with their interaction being dependant on HS chain (de Wit et al., 2013).
The reductions in dendritic spine density and in VGlut1 input puncta immunofluorescence in LRRTM4 KO dentate gyrus granule cells in vivo indicate that loss of LRRTM4 results in a reduction in excitatory synapse density in the dentate gyrus. Consistently, no change was observed in GAD65 input puncta immunofluorescence in the LRRTM4 KO mice. A corresponding functional reduction in excitatory synaptic transmission is indicated by the reductions in evoked transmission and in mEPSC frequency in LRRTM4 KO dentate gyrus granule cells (Figure 8).

Additional studies will be required to determine whether specific glypicans or syndecans or other HSPGs mediate presynapse induction by LRRTM4, and what downstream mechanisms are involved. HSPGs have previously been implicated in synapse development and function (Van Vactor et al., 2006; Yamaguchi, 2001). However, the mechanisms of action of HSPGs at central synapses are less well understood. Our findings showed that HSPGs are essential mediators of presynapse induction via their interaction with the native synapse-organizing protein LRRTM4. Axonal surface HSPGs were recruited by and are necessary for presynapse induction by LRRTM4. De Wit et al (2013) also reported similar findings that GPC4 and LRRTM4 expressed on the surface of nonneuronal cells induce clustering of their respective binding partners in co-cultured neurons, supporting a trans-synaptic interaction of presynaptic glypican and postsynaptic LRRTM4. In addition, overall levels of HSPGs as well as individual glypicans and syndecans are reduced in the dentate gyrus of LRRTM4 KO mice. Therefore, different postsynaptic LRRTM family members function in synapse organization through different presynaptic mechanisms, and the LRRTM4-HSPG
complex is particularly important for proper development of glutamatergic synapses on dentate gyrus granule cells.

All our evidence indicates a role for LRRTM4 exclusively at excitatory postsynaptic sites in a cell-type-specific manner. LRRTM4 promotes only excitatory and not inhibitory synapse differentiation in the dentate gyrus. LRRTM4 is also expressed outside the hippocampus, including in the anterior olfactory nucleus, superficial cortical layers, and striatum. Thus, the LRRTM4-HSPG complex is likely to contribute to synapse development and function in multiple discrete regions, which could be further studied.

5.3 Future Directions

The discovery of alternative trans-synaptic binding partners for different LRRTMs, in combination with their differential expression pattern, gives rise to a much larger spectrum of trans synaptic interactions than was originally thought. Based on this, the main challenge now will be to elucidate the complex code by which the trans-synaptic cell adhesion systems participate in the different steps of synapse formation, maturation and plasticity and to understand the importance of the different combinations of trans-synaptic partners in specific circuits. It will be important to understand how the combination of multiple synaptogenic systems may contribute to synaptic specificity, controlling exactly where and when synapses form. The fact that multiple partners function at the same synapses opens the possibility that they cooperate in the recruitment
of the same components to the synapse. Cooperation between different adhesion systems may help to stabilize interactions across the cleft by recruiting the pre and postsynaptic machinery at multiple points. However the existence of mechanisms that can modulate and modify these interactions should be important, especially for synaptic plasticity.

Therefore, further understanding of the molecular pathways and circuit events downstream these cell adhesion organizing systems will be extremely important in light of the role of transsynaptic cell adhesion molecules in neurodevelopmental and cognitive diseases.

5.3.1 LRRTM1 And 2

The role of LRRTM1 and 2 in excitatory synapse development in vivo was determined using the LRRTM1 and 2 double knockout mice. A further step into understanding the function of these proteins at the synapse is to reveal more about their structure and the mechanism in which they work in. The NLG-NRX complex structure have previously been studied showing an asymmetric tetramer consisting of a neuroligin dimer and two neruexin molecules (Araç et al., 2007). In fact, dimerization of neuroligin has been shown to be required for the synaptogenic properties of this protein; likely serving to induce presynaptic differentiation via a transsynaptic clustering of neurexin (Shipman & Nicoll, 2012). Further understanding of LRRTM1 and LRRTM2 structures can be achieved by studying whether or not they dimerize. Co-immunoprecipitation experiments of LRRTM1-LRRTM1, LRRTM2-LRRTM2 and LRRTM1-LRRTM2 could
be used to show their dimerization state. This could additionally be validated by surface staining of transfected cultured cells, testing for antibody aggregation. If some evidence of dimerization is observed, further studies could be planned to identify the dimerization domain of these proteins by point mutation experiments.

Reflecting on all the similarities between LRRTM1 and LRRTM2 it is intriguing to know how these two proteins function together in the synapse and whether they assist in each other’s roles. Furthermore, considering the similarities of LRRTM 1 and 2 with neuroligins in terms of their synaptogenic activity and common presynaptic ligand neurexin binding, it is important to design studies to reveal how these synaptic proteins cooperate or compete at the synapse.

5.3.2 LRRTM4

LRRTM4-HSPG complex was shown to be particularly important for proper development of glutamatergic synapses on dentate gyrus granule cells. While axonal HSPGs are required for presynaptic differentiation induced by LRRTM4, dendritic syndecans might also interact with LRRTM4 in cis at postsynaptic sites. Further coculture assays can be performed to determine how postsynaptic HSPGs might modulate LRRTM4 function.

Loss of LRRTM4 results in a reduced level of PSD-95 family proteins in dentate gyrus crude synaptosomes. Considering that MAGUKs (membrane-associated guanylate

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kinases) are key scaffolding proteins in many complexes containing receptors, adhesion proteins and signaling molecules, it is important to learn more about how their association with LRRTM4 affects synapse plasticity and development. Western blots of individual proteins in this family such as PSD-95, SAP-102, SAP-97 and PSD-93 can be performed to pinpoint which ones are affected by the loss of LRRTM4 in KO versus wild type mice. In addition, LRRTM4 has been identified as a component of AMPAR complex (Schwenk et al., 2012), and disruption of the LRRTM4-HSPG interaction affects recruitment or stabilization of AMPARs at the synapse (de Wit et al., 2013; Siddiqui et al., 2013). On the other hand, PSD-95 family proteins are involved in regulating AMPA receptor function and trafficking (Elias et al., 2008), therefore the reduction in PSD-95 family proteins may be involved in mediating AMPA receptor trafficking upon loss of LRRTM4. Further electrophysiology experiments can be performed to determine the role of LRRTM4-HSPG complex on recruitment and stabilization of AMPARs at the synapse and on synapse plasticity in general by assessing major forms of long term synaptic plasticity, long term potentiation (LTP) and long term depression (LTD). Additional studies examining the kinetics of AMPA receptors using biotinynation experiments can also shed more light on the interactions and role of LRRTM4-HSPG complex with AMPARs.

Moreover, considering the distinct involvement of LRRTM4 on synapse development and function in the dentate gyrus, our knockout model can be used for various behavioural experiments to determine the effect of dentate gyrus specific deficits on behaviour. Dentate gyrus provides unique contributions to specific hippocampal
functions (Kesner et al., 2004). It contributes to formation of new episodic memories and spontaneous exploration of novel environment and together with CA3, underlies spatial pattern separation (Kesner et al., 2004). Specific behavioural tests can be performed to study short-term and long-term spatial memory and learning, context memory and recognition memory in these mice (Jessberger et al., 2009). Results from these experiments can lead to a better understanding on involvement of LRRTM4 in nervous system disorders.
Chapter 6. Conclusions

In this dissertation two projects have been discussed studying the effect of members of LRRTM family on synapse development and function. Our studies on LRRTM4 and LRRTM 1 and 2 indicate that members of the LRRTM family function in a cell-type specific manner through different presynaptic molecular pathways. LRRTM1 and 2 as well as LRRTM4 were shown to contribute to excitatory synapse development. However the specific region in the hippocampus where these proteins affect vary. LRRTM1 and 2 modulate excitatory presynapse and postsynapse development in CA1, while LRRTM4 affects the dentate gyrus. In addition, LRRTM1 and 2 and LRRTM4 work through distinct binding partners to induce presynaptic differentiation; LRRTM1 and 2 have previously been shown to bind the LNS domain of neurexins lacking an insert at splice site 4 while LRRTM4 was shown here to interact with a novel binding partner, the HSPG family.

Considering our findings, changes in synapse development and function in response to alterations in the members of LRRTM family could contribute to neurological disorders. The revelation of an unexpected complexity in the design of synapse-organizing protein networks undeniably emphasizes the importance of studying region-specific roles of individual gene products in brain function and dysfunction.
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