Region-specific role of LRRTM1 in the organization of glutamatergic synapses in mediodorsal nucleus of the thalamus and hippocampal dorsal CA1 region

By

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Abstract

Synapse organizer proteins are essential components of chemical synapses. These proteins are fundamental for development, organization, and function of chemical synapses. Copy number variations or loss of function mutations in the genes encoding these proteins can lead to development of variety of neuropsychiatric diseases. Leucine Rich Repeat Transmembrane neuronal proteins (LRRTMs) are a family of four glutamatergic synapse organizers with distinct expression patterns in mammalian brain. LRRTM1 is a paternally imprinted gene, implicated in handedness and schizophrenia. *LRRTM1* is strongly associated with schizophrenia and is highly expressed in the thalamus, prefrontal cortex, and hippocampal CA and dentate gyrus regions. Using region-specific deletion of *Lrrtm1* in mediodorsal nucleus of the thalamus and hippocampal dorsal CA1 we have shown the importance of LRRTM1 in organization of PFC-MD synapses and integrity of MD-PFC circuit. We have also shown that LRRTM1 is essential for function of CA3-CA1 circuit and its deletion results in disruption of dorsal-CA1-associated behaviour in mice. Conditional deletion of *Lrrtm1* in the MD in adult mice reduced excitatory synaptic function and caused a parallel reduction in the afferent synaptic activity of the PFC, which was reversed by the reintroduction of LRRTM1 in the MD. Conditional deletion of *Lrrtm1* in the dorsal CA1 in adult mice reduced synaptic transmission and caused a deficit in long-term potentiation in the stratum radiatum but not stratum lacunosum moleculare. The deficits were reversed by the reintroduction of LRRTM1 or perfusion with GluR2$_{3Y}$. Our results indicate that chronic reduction of synaptic strength in the MD by targeted deletion of *Lrrtm1* functionally disengages the MD from the PFC and may account for cognitive, social, and sensorimotor gating deficits, III reminiscent of schizophrenia. Our results indicate that chronic reduction of synaptic strength in the dorsal CA1 by targeted deletion of *Lrrtm1* functionally disengages the CA3 from the CA1

and may account for contextual memory and social interaction deficits observed in several neuropsychological disorders.

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Dedication

This thesis is dedicated to those who seek the truth by sacrificing their health, their wealth and their lives. To those who may remain unknown but lay the foundations for discoveries, inventions and breakthroughs. To those who burn to light the path for science, to all who try hard but achieve less.

To all unknown soldiers of enlightenment, I salute you.

This is also dedicated to the memory of my Beloved Cousin Amir. A skillful chess player and an exceptional human being. He truly was one of the brightest minds I have ever known. He would have been much more if he did not have to struggle with his mental health, discrimination, and social intolerance towards people living with mental disorders.

Table of contents

Chapter 3

Stratum-specific role of lrrtm1 in regulation of long-term potentiation and dorsal ca1-associated behaviour

Chapter 4

Discussion

Contributions of the authors

Two manuscripts one published and one that is to be published in peer-reviewed scientific journals as listed below are presented in Chapters 2 and 3 of this thesis:

Chapter 2

Karimi, B., Silwal, P., Booth, S., Padmanabhan, N., Dhume, S.H., Zhang, D. et al. Schizophrenia-associated LRRTM1 regulates cognitive behavior through controlling synaptic function in the mediodorsal thalamus. Molecular Psychiatry (2021).

T.J.S conceived research; B.K. and T.J.S. designed research; B.K performed the bulk of the experiments and analysis (85%), P.S., S.B., N.P., S.D., D.Z. and N.Z. contributed to experiments and analysis; M.F.J., and J.W.C. advised on electrophysiology experiments; and G.K. advised on behavior experiments; J.H.K supervised FDG-PET experiments; B.K. and T.J.S. wrote the paper. All authors read and approved the manuscript.

Chapter 3

Benyamin Karimi and Tabrez J. Siddiqui Stratum-specific role of LRRTM1 in regulation of long-term potentiation and dorsal CA1-associated behaviour.

T.J.S conceived research; B.K. and T.J.S. designed research; B.K performed the experiments and analysis. All authors read and approved the manuscript.

List of Tables

List of figures

List of abbreviations

AChE: Acetylcholinesterase

ADAR: Adenosine deaminase

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor

ACC: Anterior Cingulate Cortex

aEPSC: Asynchronous EPSC

BDZ: Benzodiazepines

CAM: Cell Adhesion Molecules

CA: Cornus Ammonis

CSTC: Cortico-Striatal-Thalamo-Cortical Pathway

cAMP: Cyclic AMP

dpc: Days *Post Coitum*

DG: Dentate Gyrus

LSD: D-Lysergic Acid Diethylamide

DA: Dopamine

DRN: Dorsal Raphe Nuclei

dlPFC: Dorsao-Lateral Prefrontal Cortex

EC: Entorhinal Cortex

FNIII: Fibronectin III

GABA: Gamma amino butyric acid

GA: Golgi apparatus

GPCR: G-Protein Coupled Receptors

HSPG: Heparan Sulfate ProteoGlycan

HS: Heparan Sulfates

Ig: Immunoglobulin-Like

iGluR: Ionotropic Glutamate Receptor

LRRTM/Lrrtm: Leucine Rich Repeat Transmembrane Neuronal Protein

LRR: Leucine-Rich Repeats

LAR: Leukocyte Antigen-Related

LTD: Long-Term Depression

LTP: Long-Term Potentiation

mPFC: Medial Prefrontal Cortex

MD: Mediodorsal Nucleus Of The Thalamus

mGluR: Metabotropic Glutamate Receptors

mEPSC: Miniature Excitatory Postsynaptic Current

mIPSC: Miniature Inhibitory Postsynaptic Current

mEPSP: Miniature Excitatory Postsynaptic Potential

mIPSP: Miniature Inhibitory Postsynaptic Potential

DMT: N, N, Dimethyltryptamine

NA: Nucleus Accumbens

NGL-3: Netrin-G like-3 protein

NMDAR: N-Methyl-D-Aspartate receptors

NO: Nitric Oxide

OCD: Obsessive-Compulsive Disorder

OFC: Orbitofrontal Cortex

PPF: Paired-Pulse Facilitation

PPD: Paired-Pulse Depression

PCP: Phencyclidine

PLC: Phospholipase C

PANSS: Positive and Negative Syndrome Scale

PSD: Post Synaptic Density

PFC: Prefrontal Cortex

PPI: Pre-Pulse Inhibition

PKA: Protein Kinase A

RPTP: Receptor-Type Protein Tyrosine Phosphatases

RTN: Reticular Thalamic Nucleus

RER: Rough Endoplasmic Reticulum

SN: Salience Network

STP: Short-Term Synaptic Plasticity

SNP: Single Nucleotide Polymorphisms

SER: Smooth Endoplasmic Reticulum

sEPSCs: Spontaneous Excitatory Post-Synaptic Current

SLM: Stratum Lacunosum Moleculare

SP: Stratum Pyramidale

SR: Stratum Radiatum

SUD: Substance Use Disorder

SAM: Synaptic Adhesion Molecule

TAAR: Trace Amine Associated receptors

TARP: Transmembrane AMPA Receptor Regulatory Protein

IP3: Triphosphoric Inositol

VTA: Ventral Tegmental Area

vlPFC: Ventro-Lateral Prefrontal Cortex

VGCC: Voltage-Gated Calcium Channel

VGSC: Voltage-Gated Sodium Channel

Chapter 1

Introduction

1.1 Synapses

Information flow between neurons occurs mainly by fast point-to-point relay through synapses[2,3]. The term synapse originates from the Greek word “synapsis,” which means conjunction and was coined in 1897 by Sir Charles Scott Sherrington, an English neurophysiologist. Synapses are comprised of specialized membrane processes positioned proximally to each other. This arrangement helps information, in electrical or chemical form, to travel from the presynaptic neuron, the sender of the signal to the post synaptic cell or the receiver of the signal. The formation and accurate positioning of these processes require coordinated assembly of a vast and diverse number of proteins including but not limited to channels, synaptic adhesion molecules (SAMs) and synaptic organizers. The space between two synapse-forming neurons, the synaptic cleft, is therefore, a highly specialized continuum designed for accurate information processing[4,5].

1.1.1 Evolution and origin of Synapses[a]

The primary function of neurons, information transfer and signal conduction, is dependent on their unique physical structure. Neurons are polar in form and function and this polarity enables the signal to travel from one end of the cell to the other in the form of an action potential. This signal then travels from the presynaptic neuron through the synaptic cleft to the postsynaptic neuron. Major components of the pre-

[a] An evolutionary preserved gene, protein, function, structure or expression pattern emphasizes the importance of the preserved element for proper functioning of an organism. Particularly relevant to this study, it justifies use of an animal model for studying the effect of Lrrtm1 deletion and suggests that observed phenomena in the animal model maybe used to explain the effects seen in human patients.

and postsynaptic protein machinery evolved from prokaryotic and eukaryotic cells long before the advent of metazoans or the appearance of neurons. The common ancestral origin of synaptic machinery in unicellular and multicellular organisms, is called the proto-synapse. The proteins that contribute to the development of proto-synapse are referred to as proto-synaptic proteins, many of which are homologs of modern synaptic proteins[6-10]. Many of these molecular, cellular, and physiological features of the synapses are not specific to metazoan synapses or animal neurons. For instance, voltage-gated channels are abundant in bacteria and even viruses[11-13]. Unicellular protists also perform a function similar to rapid sodium-based action potentials[14]. Many plants use ionotropic glutamate receptors (iGluRs) in development, ion transportation, reproduction, and chemotaxis[15-17]. Figure 1.1 summarizes the estimated emergence time of different synaptic proteins during evolution.

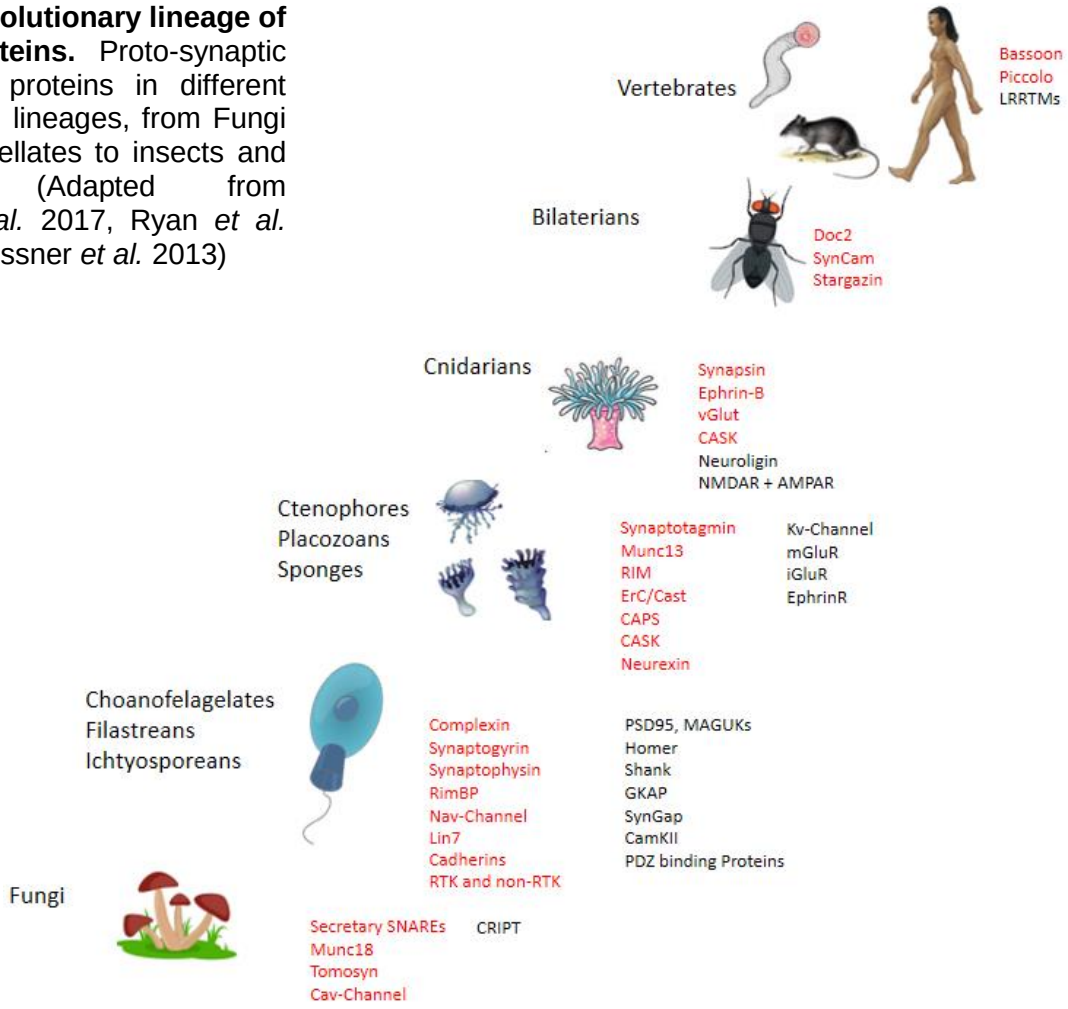

Figure 1.1. Evolutionary lineage of synaptic proteins. Proto-synaptic and synaptic proteins in different Kingdoms and lineages, from Fungi to Choanoflagellates to insects and vertebrates. (Adapted from Burkhardt *et al.* 2017, Ryan *et al.* 2009, and Reissner *et al.* 2013)

Ursynapse is introduced as the last common ancestor of all synapses that emerged later[18]. A comparison between synaptic proteins in vertebrates, bilaterians and non-bilaterians with the nervous system can lead to a better understanding of synapse evolution at its early emergence and later stages from reptiles to mammals[19-23]. The mammalian nervous system has two types of synapses, the electrical synapse and the chemical synapse. As this study focuses on structure and function of chemical synapses in the mammalian nervous system, electrical synapses we will not be discussed.

1.1.2 Chemical synapses

Chemical synapses are complex highly specialized cellular compartments that convey a signal from a neuron to another cell. The postsynaptic cell can be a skeletal muscle cell, a gland, or another neuron. Chemical synapses do not require a direct connection between the cytoplasm of presynaptic and postsynaptic cells. In a chemical synapse, presynaptic cells produce, and release messenger molecules named neurotransmitters following arrival of an action potential. Neurotransmitters diffuse through the synaptic cleft and are recognized by their receptors on the postsynaptic membrane. This is followed by the opening of the corresponding ion channels and influx of ions, causing the depolarization (activation) or hyperpolarization (silencing) of the postsynaptic cell[b]. For instance, glutamate, an excitatory neurotransmitter, is recognized by glutamate receptors on the postsynaptic membrane, triggering influx of Na^+, Ca^{2+}, or both; this can then depolarize the postsynaptic cell. Gamma amino butyric acid (GABA) on the other hand, is an inhibitory neurotransmitter. When GABA binds to $GABA_A$ receptors, which are permeable to Cl^-. Influx of Cl^- hyperpolarizes the postsynaptic cell.

The chemical synapse is the primary means of information transfer in the CNS. CNS neurons can form thousands of chemical synapses. It is estimated that the adult human brain contains 10^{14} to 5×10^{14} chemical synapses[24]. Most chemical synapses are formed by axons on dendrites (axodendritic). However, other less abundant forms of synapses have also been observed. Axoaxonic, dendrodendritic and axosomatic (axon on cell body) are three other forms of synapses[25].

[b] Whether a neuron becomes activated (fires an action potential) will depend on whether there is summation of enough number of inputs to a given neuron at a certain point in time.

1.1.2.1 Structure and function of chemical synapses

A chemical synapse is an intercellular junction with an asymmetric arrangement. Synapses vary in their neurotransmitter type, probability of neurotransmitter release, the composition of their postsynaptic receptors and mechanism of short- and long-term synaptic plasticity. Typically, a chemical synapse transfers information in a unidirectional manner, from a presynaptic to a postsynaptic cell[2].

A specific class of cell surface proteins called cell adhesion molecules (CAM) are essential for synapse formation, development, and maintenance. Multiple CAMs are particularly important in the initiation, formation, and alignment of presynaptic and postsynaptic compartments. These proteins are often also essential in developing short-term and long-term plasticity (Fig. 1.2)[2,3].

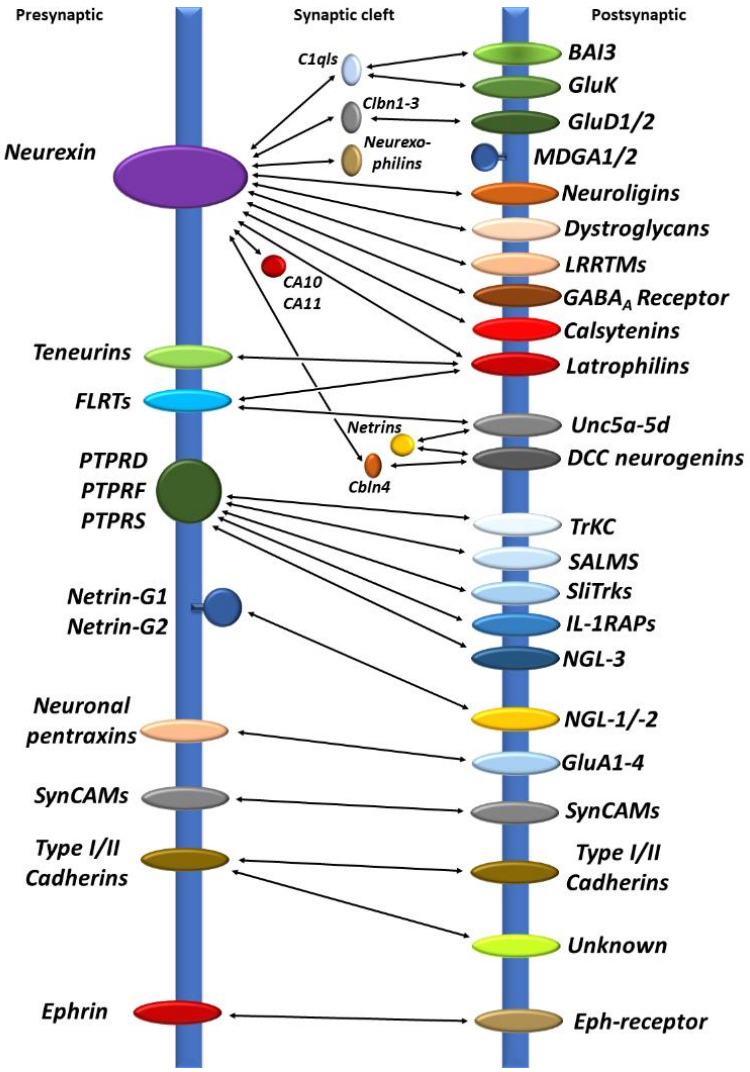

Figure 1.2. Schematic representation of CAMs interactions with their transsynaptic partners. Placement of CAMs on pre or postsynaptic side is based on published literature; however, it should be kept in mind that for many of CAMs a firm assignment cannot be yet made. A subset of these interaction partners was determined by rigorous biophysical evidence such as binding assay (for instance LAR-type binding between RPTPs and their ligands), and yet some have weaker evidence e.g. binding of neurexins to latrophilins of C1qls. (Adapted from Südhof *et al.* 2018 and Reissner *et al.* 2013)

Neuronal circuit formation is a result of a series of developmental events. For an embryonic cell to differentiate into a mature neuron with functional connections, the following steps must be taken: 1. Cell fate determination and proliferation, 2. Cell migration, 3. Axon guidance, 4. Synapse formation, and 5. Synapse maturation. Accurate synapse formation is fundamental for perception, learning and memory. The

accurate wiring of neurons leads to the formation of specialized neuronal circuits, which is only possible by the coordinated assembly of specialized synaptic proteins.

Most of the initial synaptic assembly in humans occurs during pre- and postnatal development. Nearly half of all synapses that form during these periods will be pruned in the next two decades of life, with pruning continuing well into the third decade. For instance, in the adult human prefrontal cortex (PFC), the density of dendritic spines is 2-3 times lower compared to a prepubescent person[26]. Although a fraction of synapses continues to be eliminated and formed throughout an individual's life, most synapses that survive the post pruning phase are stably maintained. The formation of synapses during the developmental period is primarily independent of neuronal activity. Activity-dependent synapse formation is more similar to artificial synapse formation during which various signals induce specialization of the synapses[2].

Structural and functional integrity of chemical synapses depend on proper expression, synaptic presence and function of synaptic proteins. These proteins ensure accurate development and maintenance of the synapses. Understanding the complex interplay amongst the various components of synaptic protein machinery is essential for elucidating the molecular mechanisms that regulate the formation, maturation and maintenance of synapses in the CNS. Disrupted function of these proteins is postulated as a mechanism that could lead to development of neuropsychiatric diseases[27]. Here presynaptic proteins will be briefly discussed, and two important presynaptic synapse organizers will be reviewed followed by

introduction of postsynaptic proteins with a more detailed introduction of neuroligins and Leucine Rich Repeat Transmembrane Neuronal Proteins (LRRTMs)[c].

1.1.3 Synaptic transmission and neuronal communication

Neurons primarily communicate through chemical synapses. First, a strong enough stimulation, through synaptic transmission or sensory receptors, is required. This stimulation depolarizes the neuronal membrane, opening voltage-gated Na^+-channels (VGSCs[d]) and pushing the membrane potential past the threshold (usually from -60 mV at rest to +55 mV). A depolarizing wave of action potential would then propagate through the neuron. Once the depolarizing wave reaches a presynaptic terminal, it opens voltage-gated Ca^{2+}-channels (VGCCs[e]). The influx of calcium triggers synaptic vesicle fusion and neurotransmitter release into the synaptic cleft. The released neurotransmitters activate their receptors on the postsynaptic membrane, which allows an influx of ions through the receptor channels (e.g, Na^+ and Ca^{2+} for excitatory neurotransmission, and Cl^- in case of inhibitory neurotransmission)[28,29].

Vesicle fusion for neurotransmitter release occurs in five steps: 1. The vesicles are brought to the vicinity of the presynaptic membrane with the help of vesicle fusion proteins, 2. The plasma membrane bilayers bend and subsequently destabilize and fuse with each other. 3. An intermediate stalk forms where the proximal bilayer leaflets

[c] Leucin Reach Repeat Transmembrane neuronal proteins. A family of four postsynaptic proteins involved in synapse formation and organization. To be discussed in detail in section 1.1.5.2.

[d] VGSCs are present in many cells including cardiac, striated muscle cells and neurons. Have three configurations, open, closed, and deactivated. VGSCs and are primary channels responsible for production of an action potential.

[e] VGCCs are fundamental channels in translating the of membrane potential changes into intracellular Ca^{2+} transients that set many physiological events in motion. They have ten family members in mammals.

have fused, 4. Distal leaflets merge and form a fusion pore. 5. The fusion pore expands and neurotransmitters are released[30].

The most essential proteins for synaptic membrane fusion are SNAREs. SNAREs are present in all cell types and are involved in membrane traffic. Neuronal SNAREs mediate vesicle fusion and neurotransmitter exocytosis and include synaptobrevin/VAMP (vesicle-associated membrane protein), the plasma membrane proteins SNAP-25 (synaptosomal-associated protein of 25 kDa) and Syntaxin-1. Synaptobrevin is called v-SNARE for “vesicular,” and SNAP-25 and syntaxin-1 are called t-SNAREs for “target”. SNARE proteins are characterized by an approximately 65-residue long sequence which is called a SNARE motif. The energy released upon assembly of these three SNAREs helps the membranes overcome the energy barrier for their fusion. After completion of the membrane fusion, N-ethylmaleimide-sensitive factor (NSF) and α-SNAP disassemble the complex with the help of SNAPs in a process that is powered by ATP hydrolysis and prepares SNAREs for another reaction[31,32].

1.1.4 Synapse-organizing proteins

1.1.4.1 Presynaptic synapse organizers proteins

Presynaptic proteins are essential for specificity and proper formation of excitatory and inhibitory synapses. Presynaptic proteins also play a role in neurotransmission by controlling presynaptic compartment-specific activities and regulate synaptic fusion and neurotransmitter release and synaptic plasticity[33,34]. Presynaptic proteins contribute to neurotransmission and control the cycle of synaptic vesicles through docking, priming, and vesicle fusion[35]. Neurexins and presynaptic

receptor protein tyrosine phosphatases (RPTPs) interact with variety of postsynaptic partners and are involved in formation and maintenance of synapses throughout the mammalian CNS.

1.1.4.1.1 Neurexins

Neurexins are a family of single-pass transmembrane, trans-synaptic, synapse organizers. In mammals, three different genes encode members of the neurexin family (*NRXN1,2* and *3*[f])[36-38]. Neurexin genes have two different promotor sites that give rise to α-neurexin (longer extracellular region, upstream promoter), and β-neurexin (shorter extracellular region, downstream promoter), comprising six different principal isoforms, Neurexin1-α to Neurexin3-β. Recently a unique, much shorter neurexin1 isoform, Neurexin1-γ, has been reported[39,40]. Each neurexin isoform can be alternatively spliced; alpha neurexins have five alternative splice sites, while beta neurexins have two. Alternative splicing generates over 3000 different variants, giving neurexins a wide range of synaptic partners, and suggesting an essential role for neurexins in synaptic specificity[41].

The neurexin C-terminal domain binds to synaptotagmin and PDZ domains of CASK (through PDZ binding domain) and then through CASK with Veli and Mint1. These connections facilitate the interaction between synaptic vesicles and fusion proteins such as Munc18-1, an essential component in synaptic vesicle exocytosis[3]. Neurexins can initiate and organize two fundamental synaptogenesis processes: 1. Nucleation of actins, and 2. Recruiting synaptic vesicles[42].

[f] Although a member of *Caspr*/*parandonin*/*CTNAP* family is named *NRXN4* for historical reasons, its domain structure makes it evolutionary and functionally more distant [36,37]

Alpha- and beta-neurexins have a signal peptide at the end of their N-terminal region. After the signal peptide, there are two Laminin-Neurexin and Sex hormone-binding globulin (LNS) domains flanking an Epidermal growth factor-like (EGF) domain. This motif is repeated three times in alpha-neurexins. α-neurexins have five splicing sites SS1-5; among these splicing sites, alternative splicing site 4 (S4) is critical in determination of specificity of neurexin binding to its postsynaptic partners. In addition, neurexins are *N*-glycosylated and *O*-glycosylated (Fig. 1.3a). β-neurexins lack EGF domains, and at the end of their N-terminal, they have a signal peptide followed by a 37-residue histidine-rich region. The only LNS domain of beta-neurexins has a single *N*-glycosylation site. There are two alternative splicing sites on β-neurexins (Fig. 1.3b). Neurexin1γ lacks LNS domains (Fig. 1.3c)[g].

[g] A soluble form of NRXN3 has also been reported[43]. These soluble isoforms are believed to act as extracellular molecules, antagonizing the activity of transmembrane neurexins[38].

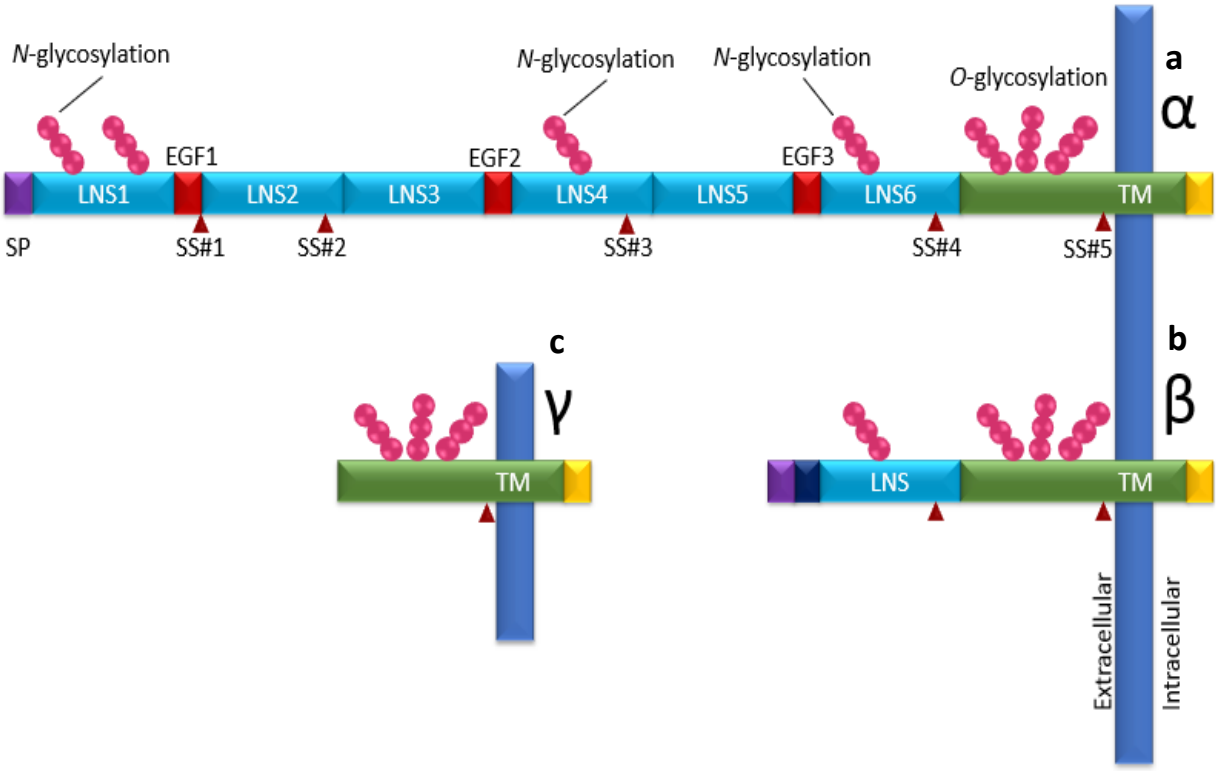

Figure 1.3. Structural domains of neurexins. Two LNS domains flanking an EGF domain is the distinctive feature of all alpha neurexins. This motif is repeated three times in alpha neurexins and is absent in beta isoforms. Both alpha and beta neurexins have a signal peptide (SP) domain at their N-terminal. In beta isoforms SP is followed by a unique 37 histamine-rich residues (dark blue). Neurexin-gamma lacks LNS domains altogether, and is only comprised of intracellular C-terminal, transmembrane region, and a small extracellular O-glycosylated N-terminal. SS# indicate alternative splicing sites and their designated numbers. TM: transmembrane region.

Apart from LNS domains, the Heparan sulfates (HS) modifications of neurexins play a key role in interaction of neurexins with some of their postsynaptic partners. Heparan sulfates are linear polysaccharides that occur as proteoglycans (Heparan Sulfate ProteoGlycan (HSPG)). HSPGs have two or three HS chains attached in close proximity to cell surface or extracellular matrix proteins. HSPGs play a role in controlling of CAM interactions. For instance, in development of neuronal processes and synapse formation[44-46]. HS modifications of neurexin are important in specificity of their interactions with their postsynaptic partners. Combined with splicing site variations, HS modifications create another layer of interaction specificity[40]. For instance, HS modifications of neurexins are essential for their interactions with

LRRTM3 and LRRTM4. Although LRRTM1 and LRRTM2 also interact with neurexin HS domains, this interaction is not necessary for their interactions with neurexins. Neurexin LNS domain is the necessary interaction site for LRRTM1 and LRRTM2. HS modification can also help neurexins recruit receptor-type protein tyrosine phosphatases (RPTP) in the process of transsynaptic interaction[40]. Figure 1.4 shows how splice variation of neurexins control their interactions with postsynaptic partners.

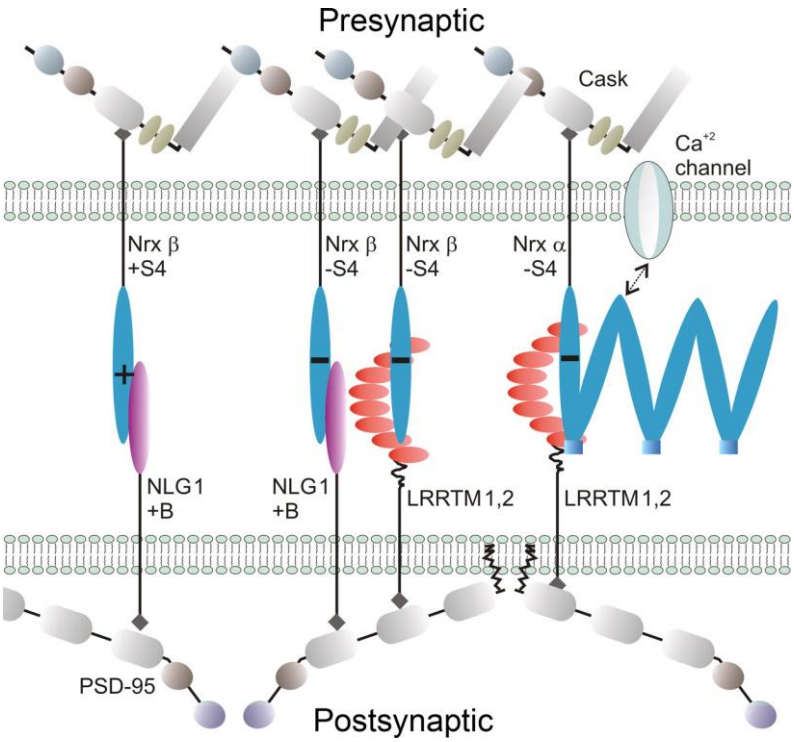

Figure 1.4. Splice variation of neurexins control their interactions with postsynaptic partners. In glutamatergic synapses -S4 neurexins interact with LRRTM1 and 2 (in presence of Ca^{2+}) as well as neuroligin1, whereas +S4 variants interact with neuroligin1 exclusively (Adapted from Siddiqui *et al.* 2010).

1.1.4.1.2 Receptor-type tyrosine-protein phosphatase family

The presynaptic receptor protein tyrosine phosphatases (RPTPs), regulate the development of synapses mainly through interaction with postsynaptic proteins with extracellular leucine-rich repeats (LRR) domains, including Slit- and Trk-like family of synaptic proteins (Slitrks), Netrin-G like-3 protein (NGL-3), and TrkC, among many

others[47]. Type IIa RPTPs have three members in vertebrates (Leukocyte antigen-related) LAR, PTPσ, and PTPδ. Three independent genes encode them, and each has three extracellular immunoglobulin-like (Ig) domain and four or eight Fibronectin III (FNIII) domains. RPTP pre-mRNAs can be alternatively spliced[48-50]. RPTPs also participate in parallel interactions with neurexins that interact with neuroligins, LRRTMs, or cerebellin–glutamate receptor-δ (GluRδ)[51]. For instance, RPTPs facilitate interaction of LRRTM3 and 4 with neurexins[40]. Since neurexins and RPTPs have many complementary roles, mutations in their encoding genes can often lead to development of similar disorders; for instance, mutations of RPTPs, neurexins and their postsynaptic partners are causative in the development of schizophrenia and autism spectrum disorders[51]. Regulated ectodomain shedding of RPTPs creates a soluble protein that negatively regulates the synaptic organizing activity of RPTPs[52]. RPTPs have two cytoplasmic domains; the membrane-proximal D1 domain has a strong catalytic activity, and the membrane-distal D2 domain, which shows minimal to no catalytic activity (Fig. 1.5 is a schematic representation of RPTPs' molecular structure).

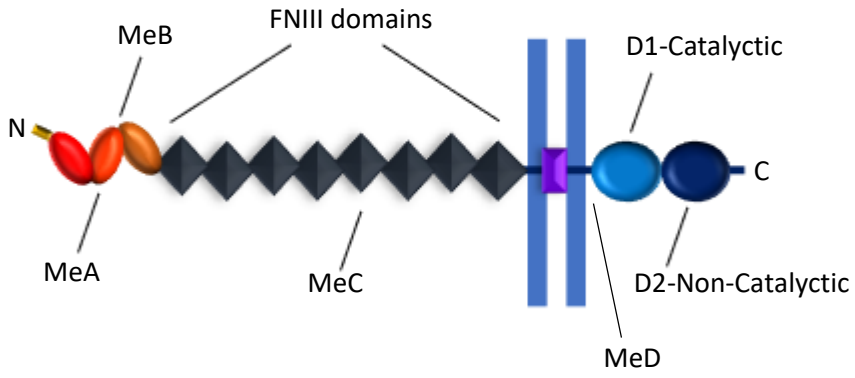

Figure 1.5. Protein structure and alternative splicing sites of RPTPs. Extracellular region of RPTPs has three Ig domains and four or eight FNIII domains depending on the alternative splicing. The intracellular region includes D1 (catalytically active) and D2 (catalytically inactive) domains. Isoforms of RPTPs are generated by alternative splicing of the four mini-exons (meA-D). (Adapted from Takahashi *et al.* 2013).

Alternative splicing of RPTPs at meA and meB sites is essential in determining the affinity of the interaction of RPTPs with all their postsynaptic partners except NGL-3. Partner-selective interactions make RPTPs a synaptic hub similar to the neurexins[51]. RPTPs play three prominent roles in trans-synaptic complexes: 1. to mediate cell-cell adhesion at synapses, 2. to mediate presynaptic differentiation, through recruiting the machinery for release and recycling of synaptic vesicles and triggering retrograde synaptogenic signalling through binding of the postsynaptic partners to axonal RPTPs, and 3. to trigger postsynaptic development, through local recruitment of neurotransmitter receptors and scaffolding and signalling proteins (anterograde synaptogenic signalling through binding of the presynaptic RPTP to dendritic binding partners)[49-51,53,54].

Figure 1.6 demonstrates the postsynaptic binding partners of RPTPs, and displays the interaction specificity that helps postsynaptic proteins to selectively interact with different RPTP family members[51].

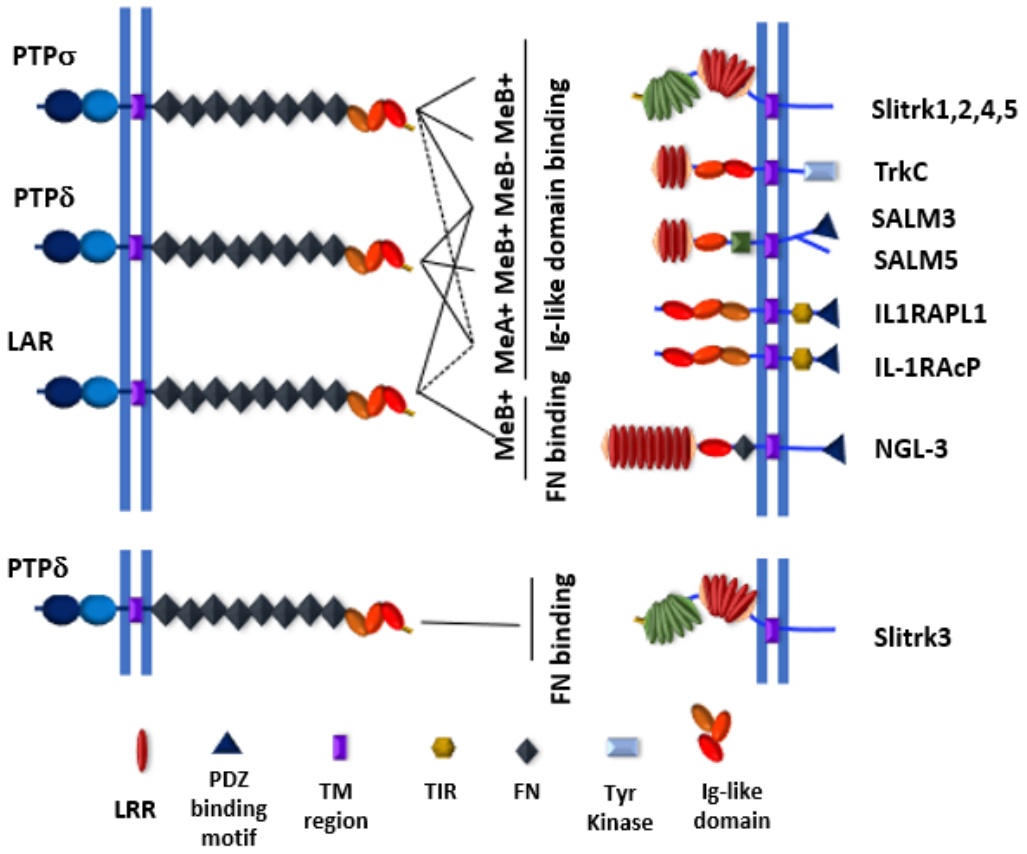

Figure 1.6. Postsynaptic partners of RPTPs. Individual RPTPs interact selectively with overlapping postsynaptic partners. The dashed lines show interactions that can take place in vivo but are not believed to have any physiological relevance. NGL-3 attaches to LAR, PTPσ, and PTPδ via their first two FNIII domains. TrkC selectively interacts with PTPσ, IL1RAPL1 to PTPδ and IL1RAcP to LAR, PTPσ, and PTPδ, and Slitrks selectively to PTPδ and PTPσ, through the Ig domains of the RPTPs. (Adapted from Won *et al.* 2018).

1.1.5 Postsynaptic synapse-organizing proteins

1.1.5.1 Neuroligins

Neuroligins are a family of postsynaptic type I (single-pass) transmembrane cell adhesion proteins. They are involved in the formation, maturation and maintenance of neuronal synapses and are among the binding partners of neurexins. Four neuroligins have been identified (NLGN1-4), with differential enrichment in the mammalian brain. Neuroligin 4 has two distinct isoform neuroligin 4Y and 4X (located on the Y and X chromosomes respectively)[55,56]. Neuroligin1 localizes to the excitatory postsynaptic

membranes, neuroligin2 to inhibitory and aminergic synapses, and neuroligins3 and 4 localize to both inhibitory and excitatory synapses[57]. Neuroligins like neurexins are potent synaptogenic proteins.

Mammalian neuroligins show a 70% sequence similarity in their extracellular N-terminal region. The N-terminal region is followed by a highly *O*-glycosylated region which is connected to the transmembrane area. The transmembrane region is followed by the intracellular C-terminal. The neuroligin extracellular region has 32-36% sequence identity and some similarity in shape to globular domains of acetylcholinesterase[h] (AChE)[58]. The intracellular region of neuroligins includes a PDZ binding domain at the end of the C-terminal region. Neuroligin PDZ domain interacts with PSD95, Shank and Gephryn scaffolding proteins[59,60]. Apart from the S4 splicing site on neurexins, an alternative splicing site on neuroligins (site B) also influences α- and β-neurexin binding to neuroligins[61,62]. Neuroligin splice site B acts as a "master switch" in neurexin-neuroligin interaction but has a small role in β-neurexin interactions[61]. On the other hand, affinity of neuroligin1 (+B) for neurexin-1β (-S4) is significantly higher than for the +S4 version[61,63,64]. Relatively less is known about splice site A. However, Oku *et al*.[65] discovered that presence of the positively charged A1 inserts in mouse neuroligin1 increases its binding affinity to heparan sulfate modifications of the neurexins. Presence of A1 insert in neuroligin1 leads to increased recruitment of neurexins, presynaptic differentiation, and neuroligin1-mediated synaptic transmission[65].

[h] **Acetylcholinesterase** breaks down acetylcholine neurotransmitter to acetic acid and choline at the synaptic cleft of cholinergic synapses.

1.1.5.2 Leucine-rich-repeat transmembrane neuronal proteins (LRRTMs)

LRRTMs are a family of four type-I transmembrane neuronal proteins (LRRTM1-4). Postsynaptic LRRTMs and their presynaptic binding partners are strongly associated with multiple psychiatric disorders such as schizophrenia[i] and autism. *LRRTM1* and *LRRTM4* genes are located on chromosome 2p12 (On the 12th band of short arm of chromosome number 2), 3 Mb apart, with *LRRTM4* being located closer to the telomere[66]. *LRRTM2* is located on chromosome 5q31.2 and *LRRTM3* on chromosome 10q21.3[67]. *LRRTM1*, *LRRTM2*, and *LRRTM3* genes are nested within the large, conserved intron of three α-catenin genes, *CTNNA2*, *CTNNA1* and *CTNNA3*, respectively[67,68].

LRR containing proteins are abundant in eukaryotic and prokaryotic organisms. In humans, LRR proteins make about 2% of the genome[69]. LRRs include 20-30 amino-acid repeats with the following arrangement LxxLxLxxN/CxL. L: Leucine, Valine or isoleucine, N: asparagine, cysteine, threonine, or serine, C: Serine or Cysteine x: any amino acid[70]. LRR proteins have a variable number of LRR tandem repeats ranging from 2 to several dozens. In LRR proteins with extracellular expression, tandem LRR repeats are capped at their C-terminal and N-terminal by Cys-rich domains, called LRRCT (LRR C-terminal cap) and LRRNT (LRR N-terminal cap)[63,71,72]. The LRR tandem repeats usually assume a curved or arched configuration with β strands aligned with the concave surface and the α helices with the convex surface[71,72]. This configuration makes the LRR proteins an ideal structure for mediating protein-protein interactions and diverse cellular functions.

[i] To be discussed in more detail in section 1.2.1

LRRTMs have 10 LRR repeats in their extracellular region flanked by LRRNT and LRRCT domains. Crystal structure analysis of the extracellular region of LRRTM2 indicates that it interacts with neurexin via concave surface of its LRR region[73].The LRRTMs C-terminal regions are comparatively short and can interact with various postsynaptic scaffolding partners and signalling molecules[63,74]. PSD95 is one of the most crucial scaffolding proteins with which LRRTMs interact. This interaction is mediated by the C-terminal end of LRRTMs which contain a PDZ binding E-C-E-V motif (similar to type I PDZ binding consensus sequence)[67,75]. Alternative splicing at the C-terminal of LRRTM3 and 4 can remove their PDZ binding motif[73].

Binding specificity of LRRTMs to presynaptic neurexin variants

LRRTM2 and Neuroligin1 both bind to an overlapping area on neurexins lacking an S4 insert. LRRTM2 and Neuroligin1, therefore, cannot bind to neurexins at the same time[64]. This competition leads to recruitment of different neurexin variants with differential intracellular binding partners and signalling molecules[64].

LRRTM1 and LRRTM2 bind to α and β variants of neurexins1-3 lacking the S4 insert. However, X-ray co-crystal structure of the LRRTM2-Nrx1β ectodomain revealed that LRRTM3 and LRRTM4 lack the neurexin LNS domain binding site of LRRTM2. LRRTM4 induces presynaptic differentiation through HS-dependent interactions(Fig. 1.7). LRRTM4 can induce presynaptic differentiation through interactions with neurexins including the neurexin-1γ variant [40,64]. Roppongi *et al.* showed that knock-in mice expressing a variant of LRRTM4 lacking the HS binding site have reduced levels of neurexin and PTPσ in hippocampal dentate gyrus (DG) synapses and show reduction in number of excitatory synapse and decreased synaptic transmission in the DG[40].

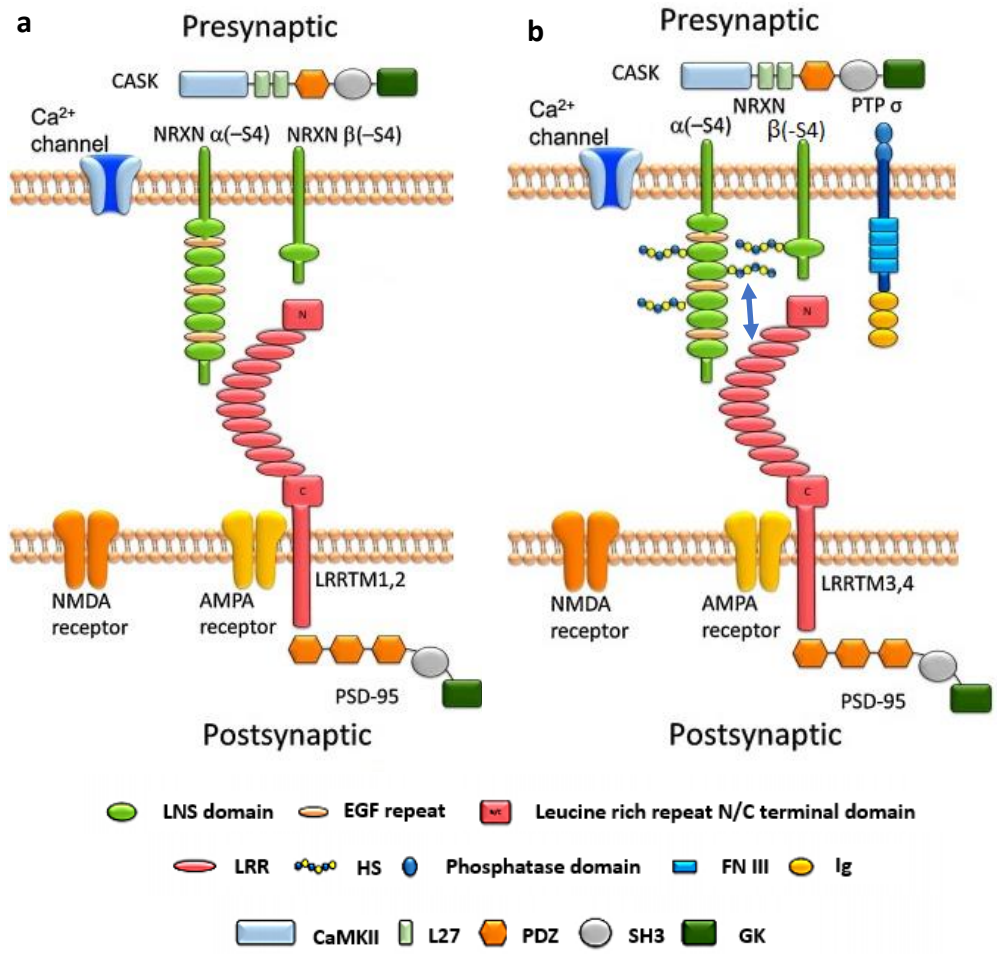

Figure 1.7. LRRTM1 and LRRTM2 bind to Neurexins (-S4) through the concave surface of their RR region in presence of Ca^{2+}. a. LRRTM1 and LRRTM2 interact with LNS domains of neurexins (-S4) in presence of Ca^{2+}. **b.** LRRTM3 and LRRTM4 on the other hand, interact with HS modifications on the neurexins in presence of presynaptic PTPs (Adapted from Roppongi *et al.* 2017).

1.1.5.2.1 Tissue expression of LRRTMs

Developing brain

In humans, *LRRTM1* is an imprinted gene that shows differential degrees of maternal downregulation. *Lrrtm1* expression in mice can be detected as early as nine days *post coitum* (dpc). Around this time, *Lrrtm1* is expressed in the dorsal otic vesicle and the overlying ectoderm of the limb buds. During development, *Lrrtm1* is detected in the midbrain and forebrain. *Lrrtm1* expression is also detected in granule cells of

the cerebellum around E14.5. Earliest expression of *Lrrtm2* is observed around ten dpc in the neural tube in motor horn cells. *Lrrtm3* mRNA is detected as early as 8.5 dpc in the anterior neural plate (in neural progenitor cells) and in the forebrain and a relatively narrow stripe in the hindbrain. *Lrrtm3* expression is reported to expand to a subset of motor horn cells around 11 dpc. Expression of *Lrrtm4* is detected as early as 9.5 dpc in the neural tube, limb buds and other regions of the head.

Role of LRRTM1 in brain development

The role of LRRTM1 in the brain development is relatively unexplored, however the studies so far suggest a role for LRRTM1 in organization of glutamatergic synapses and microanatomy of the brain. For instance, using fibroblast-neuron co-culture Linhoff *et al*[76]. identified LRRTM1 as a synaptogenic factor and report that *Lrrtm1* global deletion in mice causes a modest yet significant disorganization of the synapses and "anomalous ventriculomegaly". They report that *Lrrtm1*$^{-/-}$ mice show a selective increase in the VGLUT1 puncta size in the hippocampal CA1 stratum radiatum and stratum oriens. Takashima *et al.*[77] performed MRI scanning on *Lrrtm1*$^{-/-}$ adult mice and found out that hippocampus volume ($P=0.029$) and the hippocampus volume relative to the total brain volume ($P=0.046$) were significantly reduced. Their measurements also revealed a modest (6.6%) yet significant reduction ($P<0.001$) in the somatosensory cortex thickness. Using P0 injection of shRNA expressing lentiviral agents, Schroeder and colleagues knocked down LRRTM1 in mice hippocampal CA1 region. They reported reduced frequency of spontaneous post-synaptic currents (sEPSCs), reduced number of dendritic spines and reduced quantal[j] size after

[j] They "simultaneously recorded asynchronous EPSCs (aEPSCs) in infected and neighboring, non-infected CA1 neurons. aEPSC frequency provides information about quantal content—the number of vesicles released in response to a stimulus—whereas aEPSC amplitude provides information about quantal size—the synaptic response to release of a single vesicle."

LRRTM1 KD[78]. These results suggest a role for LRRTM1 in formation and organization of glutamatergic synapses during developmental stages.

Adult brain

LRRTMs have distinct expression patterns in the adult mouse brain, suggesting a region-specific function. Figure 1.12 shows mRNA expression in adult mouse brain for all LRRTMs. *Lrrtm1* (Fig. 1.8a) shows the highest expression in the thalamus, prefrontal cortex (except layer I), hippocampus *cornus ammonis* (CA) and dentate gyrus (DG) regions, and striatum. However, it is minimally expressed in the cerebellum and olfactory regions except for the mitral and glomerular layers[67]. *Lrrtm2* (Fig. 1.8b) shows a broader expression pattern with strong expression in hippocampus CA regions, and DG, and moderate expression in the striatum, thalamus, and granular cell layer of the cerebellum. Expression of *Lrrtm2* in the olfactory bulb seems to be confined to the plexiform layer. *Lrrtm3* (Fig. 1.8c) also shows a broad expression pattern, albeit at a lower level than *Lrrtm2* in hippocampus CA regions. *Lrrtm3* is also highly expressed in mitral and granule layers of the olfactory bulb[67]. *Lrrtm4* (Fig. 1.8d) is expressed in all regions with minimal expression in CA1, cerebellar cortex and thalamus, and its expression in the olfactory bulb is more prominent in the accessory olfactory bulb region and anterior olfactory nucleus[67]. *Lrrtm4* is also highly expressed in the hippocampal DG. Messenger RNA for all *Lrrtm* genes were detected in amygdala, caudate nucleus, corpus callosum, hippocampus, and thalamus[67], albeit in varying levels. For instance, *Lrrtm4* expression in the anterior olfactory nucleus and accessory olfactory bulb region is so high that the thalamus seems relatively void of *Lrrm4* in ISH radiographs. On the other hand, *Lrrtm1* is so highly expressed in the thalamus, cortex, and hippocampal CA regions that the olfactory bulb's granular layer appears to have no expression of *Lrrtm1* mRNA.

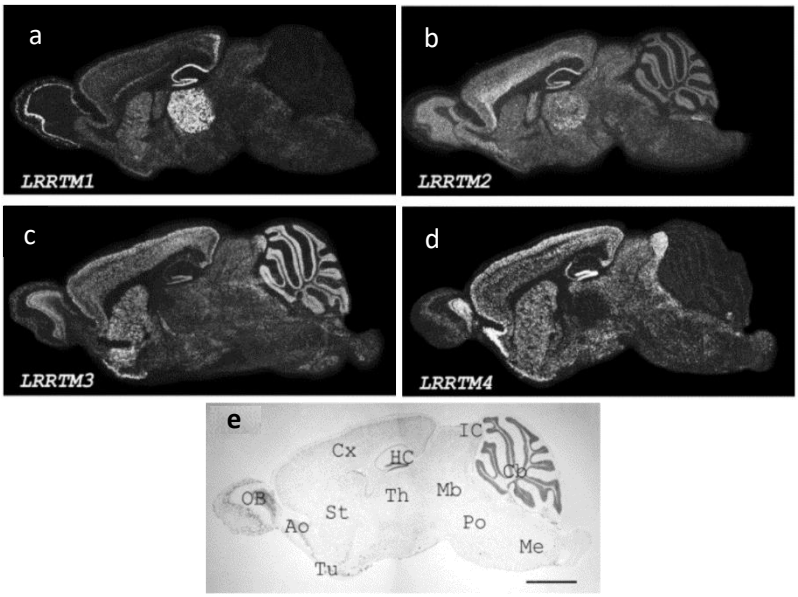

Figure 1.8. Messenger RNA Expression pattern of Lrrtm genes in adult mouse brain. Dark-field emulsion radiographs are shown. **a**. Lrrtm1, **b**. Lrrtm2, **c**. Lrrtm3, and **d**. Lrrtm4. **e**. Bright field image: OB: olfactory bulb, Cx: Cerebral cortex, IC: inferior colliculi, PO: pons, Me: Medulla, Th: thalamus, HC: hippocampus, Ao: anterior olfactory nucleus, Mb: midbrain, St: striatum. Scale bar 1mm, (Adapted from Lauren *et al.* 2003).

1.1.5.2.2 Synaptic Functions of LRRTMs

LRRTMs are almost exclusively localized to postsynaptic membranes of glutamatergic synapses[79,80]. When LRRTMs are overexpressed in neurons, they increase excitatory synapses on the transfected dendrites, but inhibitory synapse numbers remain unchanged[76,81]. Recently, however, a new study on the role of *Lrrtm4* in the mouse retina showed that *Lrrtm4* functions at inhibitory synapses as well[82]. Although LRRTMs are expressed in principal and interneurons, their roles in interneurons are relatively unknown. When expressed in non-neuronal cells in Fibroblast-neuron co-culture assays, LRRTMs induce presynaptic differentiation and development of excitatory hemi-synapses. LRRTM2 shows the strongest synaptogenic activity among all LRRTMs, followed by LRRTM1 and LRRTM4[63]. LRRTM2 recruits presynaptic scaffolding proteins bassoon and synaptophysin and

postsynaptic density protein PSD95 more strongly than neuroligin1 and is, therefore, a more potent presynaptic inducer[64].

LRRTMs contribute to regulation of synaptic strength through recruiting and maintaining of AMPARs at the synapses. The mechanism for this process is not fully understood. However, it is believed to be one of these three mechanisms: 1. Direct association with AMPAR subunits, 2. Indirect recruitment through scaffolding proteins such as PSD95, and 3. Through trans-synaptic interaction with presynaptic neurexins. The LRR domain of LRRTM2 is necessary for both its interaction with neurexins and recruitment of AMPARs. Blocking interaction of neurexins and LRRTMs blocks AMPAR recruitment by LRRTMs[79].

In cultured neurons a reduction in surface expression of endogenous AMPARs indicates that following induction of long-term potentiation (LTP)[k], LRRTMs keep the newly delivered AMPARs at synapses and are required for LTP induction in mature synapses on adult CA1 pyramidal neurons[83]. *In vitro* and *in vivo* Studies by De Wit *et al.*[79] and Schwenk *et al.*[84] suggest that LRRTMs may directly bind to subunits of AMPARs, thereby maintaining them at the synapses. Following LTP induction in cultured hippocampal neurons, LRRTMs seem essential for the stabilization of AMPARs at synapses, which is consistent with the findings that at early stages of LTP LRRTMs are required for retaining or trapping of AMPARs at synapses.

Cre-dependant deletion of LRRTM1 and LRRTM2 in cultured CA1 neurons significantly impaired LTP, which could be rescued by re-expression of LRRTM2[85]. A mutated form of LRRTM2 with deleted intracellular domain could also rescue LTP impairment. However, LRRTM2 mutations that impair its binding to presynaptic

[k] Discussed in detail in section 1.1.7.2.1

neurexins prevented rescue of LTP impairment. Consistently, deletion of LRRTM1 and LRRTM2 in mature synapses decreased in AMPA receptor-mediated, (but not NMDA receptor-mediated) synaptic transmission with no significant effect on presynaptic function. Figure 1.9 summarizes the methods for recruitment of AMPARs by LRRTMs[85].

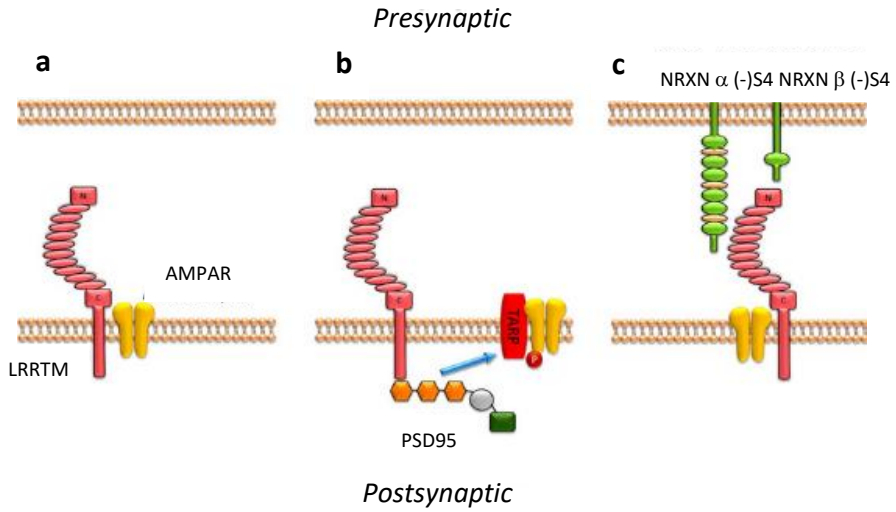

Figure 1.9. LRRTMs recruit AMPARs through three potential mechanisms. a. AMPARs are directly recruited to synaptic membrane by LRRTMs. **b.** AMPARs are indirectly recruited to synaptic membranes through interacting with PSD95 through their type I PDZ domain which in turn interacts with auxiliary AMPAR subunits and therefore recruits and maintain AMPARs at the synapses. **c.** Interaction of LRRTMs through their LRR domains with presynaptic neurexins is necessary for recruiting AMPARs (Adapted from Roppongi *et al.* 2017).

LRRTMs have recently emerged as essential synaptic organizers for maintaining AMPARs at the synaptic membrane in long-term potentiation (LTP). Chemically induced LTP in DG dissociated neurons after knocking out *Lrrtm4* and knocking down *Lrrtm3* failed to recruit GluA1 AMPAR subunit to the synaptic surface[83]. Moreover, AMPAR stability at the synapses was reduced in dissociated neurons after *Lrrtm1* or *Lrrtm2* knock down. knocking down *Lrrtm1* or *Lrrtm2* also blocked LTP induction in both young (P14-18) and mature (P35-39) CA1 pyramidal neurons[83,85,86].

Mutations of LRRTMs and their synaptic partners, such as neurexins and HSPGs, are associated with neuropsychiatric disorders and conditions[87,88]. Single Nucleotide Polymorphisms (SNP) of *LRRTM1* are associated with schizophrenia if inherited from the father[87]. In mice, deletion of *Lrrtm1* leads to behavioural abnormalities, altered response to environmental stimuli, and impaired cognitive abilities[77]. *Lrrtm1* deletion in mice leads to reduced hippocampal volume, which interestingly is one of anatomical manifestations of schizophrenia in humans[77]. Some of the impairments caused by *Lrrtm1* mutations are also mirrored in *Nrxn-1α* KO mice[89].

LRRTM2 is associated with bipolar disorder[90] and a 240 kb deletion in chromosome 5q31, containing *LRRTM2* and *CTNNA1*, is linked to delayed development and intellectual disability[91]. *LRRTM3* mutations contribute to development of late-onset Alzheimer's disease[92], whereas *LRRTM4* mutations are associated with ASD and an increased risk of suicide in females[93,94].

Mutations of LRRTMs and their synaptic partners, such as neurexins and HSPGs, are associated with neuropsychiatric disorders and conditions[87,88]. Single Nucleotide Polymorphisms (SNP) of *LRRTM1* are associated with schizophrenia if inherited from the father[87]. In mice, deletion of *Lrrtm1* leads to behavioural abnormalities, altered response to environmental stimuli, and impaired cognitive abilities[77]. *Lrrtm1* deletion in mice leads to reduced hippocampal volume, which interestingly is one of anatomical manifestations of schizophrenia in humans[77]. Some of the impairments caused by *Lrrtm1* mutations are also mirrored in *Nrxn-1α* KO mice[89].

1.1.6 Synaptic neurotransmitter receptors

1.1.6.1 Glutamate receptors

Glutamate receptors are a group of synaptic and non-synaptic receptors that are expressed in neuronal cells and glia throughout the mammalian brain. Glutamate receptors can be classified as ionotropic and metabotropic receptors. Ionotropic receptors respond to ligand binding by opening an ion channel, while metabotropic receptors trigger a slower and yet more lasting response by triggering a series of intracellular signaling pathways. Metabotropic receptors are member of G-protein coupled receptors (GPCRs)[95].

1.1.6.1.1 α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPARs)

AMPARs are transmembrane glutamate receptors that mediate fast synaptic transmission in the mammalian nervous system. AMPARs have four subunits, each encoded by a different gene, designated as *GRIA1*, *GRIA2*, *GRIA3*, and *GRIA4*. AMPAR subunits join together as tetramers to create a functional AMPAR. Most AMPARs are heterodimers, and the subunits usually assemble to create symmetric dimers of dimers[96]. The dimerization process begins in the rough endoplasmic reticulum (RER) and finishes when the dimerization domains zip up while the assembly is taking place in the membrane. AMPARs subunits have an extracellular N-terminal followed by four hydrophobic domains (M1-M4). M1, M2, and M3 form the channel with a part of the M2 re-entering loop. The C-terminal regions of AMPARs are intracellular[97].

AMPAR subunits may undergo RNA editing. GluA2 subunits undergo the most functionally significant RNA editing. GluA2 RNA editing changes the codon from CAG

to CGG at residue 607 near the M2 re-entering region. This modification means that in the translation process the arginine (R) at the 607 amino acid position is substituted by a glutamine(Q)[98]. Although all mammalian AMPAR genes contain the Q/R code region, only GluA2 mRNA is edited at this region. Adenosine deaminase (ADAR) enzyme is essential for conversion of the codon.

A functional AMPAR with a Q GluA2 subunit is permeable to Ca^{2+}, while R-containing GluA2 subunit renders the AMPARs impermeable to Ca^{2+}, in part because the more positively charged channel is not suitable for Ca^{2+} to pass through. Not being edited for Q/R in GluA2 makes mice susceptible to seizures, and they die by three weeks of age[99]. Majority of functional AMPARs in adult brain contain R-GluA2 subunits and are therefore impermeable to Ca^{2+} [100].

AMPARs may be assembled in homomeric or heteromeric forms. GluA1-4 can be assembled in variety of stoichiometric types and form various subtypes with distinct channel properties. In adult mammalian CNS, specially in hippocampus and cortex appear to be a combination of GluA2/GluA1 or GluA2/GluA3. As expression of GluA3 subunits are relatively lower, more than 70% of AMPARs are GluA2/GluA1[101,102].

Phosphorylation, Palmitoylation, and glycosylation are among other modifications which regulate and change activity, permeability, and interaction kinetics of AMPAR subunits. Palmitoylation is the addition of a 16-carbon fatty acid to a cysteine amino acid. Palmitoylation of AMPAR subunits can occur at two cysteine residues on the intracellular side; one is close to M2 and another closer to the M4. Palmitoylation occurs in the Golgi apparatus by Golgi-associated palmitoyl transferase called GODZ. Palmitoylation regulates the presence of AMPARs at different membrane compartments[103-105].

Several residues on C-terminal tail of AMPARs are targets for phosphorylation including serine (S), threonine (T), and tyrosine (Y) amino acids. The probability of channel opening, and surface expression increases by PKA phosphorylation of GluA1 at S845, but S831 phosphorylation increases single-channel conductance. GluA1 phosphorylation is necessary for synaptic plasticity and spatial memory consolidation[106,107].

Glycosylation of AMPARs occurs at 4-6 specific *N*-glycosylation regions of GluA subunits. AMPARs glycosylation progresses as they move through the protein secretory pathways (Golgi apparatus and Rough endoplasmic reticulum)[108]. These oligosaccharides can be sulfated, have an effect on ligand binding and are also likely to affect other characteristics. Figure 1.10 illustrates the common structural modifications of AMPAR subunits.

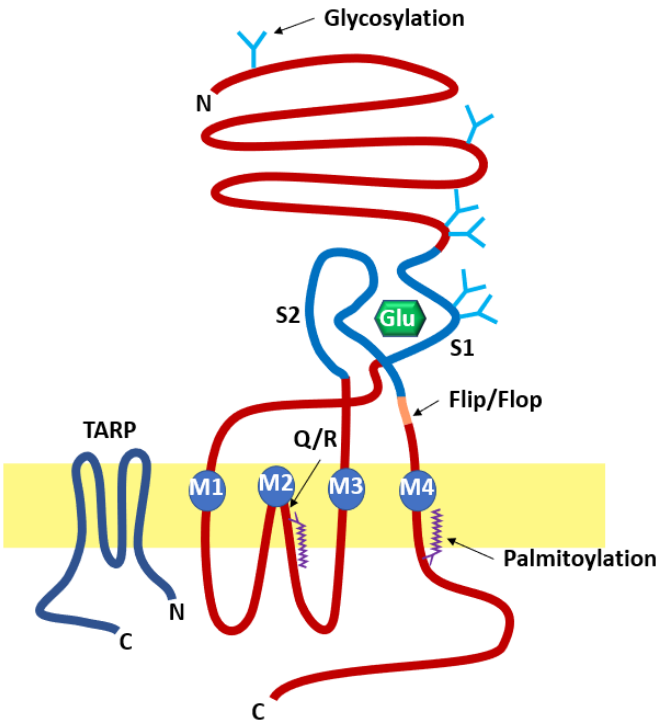

Figure 1.10. Structural modifications of AMPAR subunits. The diagram depicts common modifications of an AMPAR subunit and their corresponding locations on the protein. These modifications regulate the expression, binding ability, membrane compartmentalization, kinetic properties, and synaptic presence of AMPARs.

Transmembrane AMPA Receptor Regulatory Proteins (TARPs)

AMPARs are dependent on a large number of other proteins that regulate their activity, interaction with intracellular proteins, and trafficking. TARPs play a critical role in regulating both trafficking and functional properties of AMPARs in the vast majority of excitatory synapses of the CNS. The importance of regulatory role of TARP in AMPAR dependent synaptic transmission was revealed by lack of TARP2 or stargazin in cerebellar granule cells of the stargazer mice. Stargazer mice lack synaptic transmission in the mossy fiber synapses on the granule cells[109-111]. TARP2 is a four-transmembrane protein, TARPs, including stargazin, are all part of a larger family which were previously thought to be calcium channels. Other TARPs such as TARP3 and TARP4 are all important for AMPAR functions as well; their most critical role is

maintaining AMPARs at the surface. TARPs also help AMPARs to interact with PSD95 through their PDZ binding domain. This is particularly important for regulating LTP and LTD. TARPs also help regulate the pharmacology, gating, and channel-conductance of AMPARs[112-114] .

AMPAR trafficking and LRRTMs

AMPA receptors are the main glutamate receptors mediating fast excitatory synaptic transmission in the CNS and changes in their synaptic presence and subunit composition are major modulation mechanisms for plasticity of excitatory transmission in the brain[115]**.** AMPAR composition, channel properties, interaction partners and synaptic presence change, during development, synapse formation and maturation and induction of LTP and LTD[116,117]. One of essential indicators of LTP induction is the increase in number of AMPARs in the synapses following a repeated high-frequency stimulation. AMPARs are transferred from the reserve pools inside the dendrites and incorporated in the synapses through a specific signaling cascade[118,119]. Longer-term plasticity also involves secondary messengers that promote up regulation of genes expressing AMPAR subunits through cyclic AMP (cAMP) signaling. The mRNAs will be translated in the rough endoplasmic reticulum (RER)[120,121], and the composition of subunits will be determined. After further modifications and processing in the Golgi apparatus (GA), AMPARs will be released into a perisynaptic membrane where they will remain until they are recruited to the synapses[120,121].

Phosphorylation by Protein kinase A (PKA) and interaction with SAP97[122] promote trafficking of perisynaptic AMPAR subunits to the synaptic membrane. Phosphorylation by PKA is essential for targeting of AMPAR subunits to the synaptic membrane[123]. SAP97 interaction anchors and traps AMPAR subunits in the

postsynaptic density (PSD). SAP97 is also involved in lateral trafficking of AMPARs from extrasynaptic membrane to the synapses after induction of LTP[124]. Another important protein in AMPAR trafficking is the TARP2. TARP2 binds to AMPARs in perisynaptic and synaptic regions. TARP2 immobilizes AMPARs, entrapping them at the PSD through interaction with PSD95[125,126].

The C-terminal domains of AMPAR subunits contain a PDZ binding motif that interacts with a large number of proteins that play a role in their trafficking and targeting them for synapses or away from them[127]. The PDZ binding domain interacts with postsynaptic scaffolding proteins as well as AMPAR auxiliary proteins such as PICK1 and GRIP[128,129]. GRIP's function is to hold AMPAR subunits in place while PICK1 contributes to movement of AMPAR into the plasma membrane and out of it. An equilibrium must be stablished in interaction of these proteins with AMPAR subunit C-terminals for recruitment of AMPARs to the synapses or their internalization and removal[130,131].

GluA2 subunit has two other binding sites for NSF[l] and AP2[m]. NSF contributes to delivery of GluA2-containing AMPARs to the cell surface, and AP2 is associated with clathrin-mediated AMPAR internalization. AP2 is also responsible for NMDAR associated Long-term depression[n]. Thus, AP2 and NSF function in opposing roles to regulate AMPAR synaptic levels[131-133].

LRRTMs play a regulatory role in AMPAR trafficking. LRRTM2 binds directly to PSD95 and cooperates with TARPs in maintaining a pool of surface AMPARs[79] (Fig. 1.11). Knock down of both LRRTM1 and LRRTM2 in adult mice[83] impaired LTP and

[l] N-ethylmaleimide Sensitive Factor

[m] Clathrin adaptor protein 2

[n] Discussed in section 1.1.7.2.2

reduced AMPAR surface expression in hippocampal Schaffer collaterals[o]. Double Knock out of LRRTM1 and LRRTM2 by Bhouri *et al*.[85] also lead to reduced AMPAR basal transmission suggesting a role for LRRTMs in maintaining synaptic AMPAR levels.

Deletion of LRRTM4 in hippocampal dentate granule cells of mice, causes reduction in number of excitatory synapses and lower synaptic levels of PSD95, and impaired recruitment of new AMPARs to the surface following LTP induction[134]. Overlapping expression pattern of LRRTMs suggests that despite overlapping functional roles, there are member-specific functions that should be investigated[1].

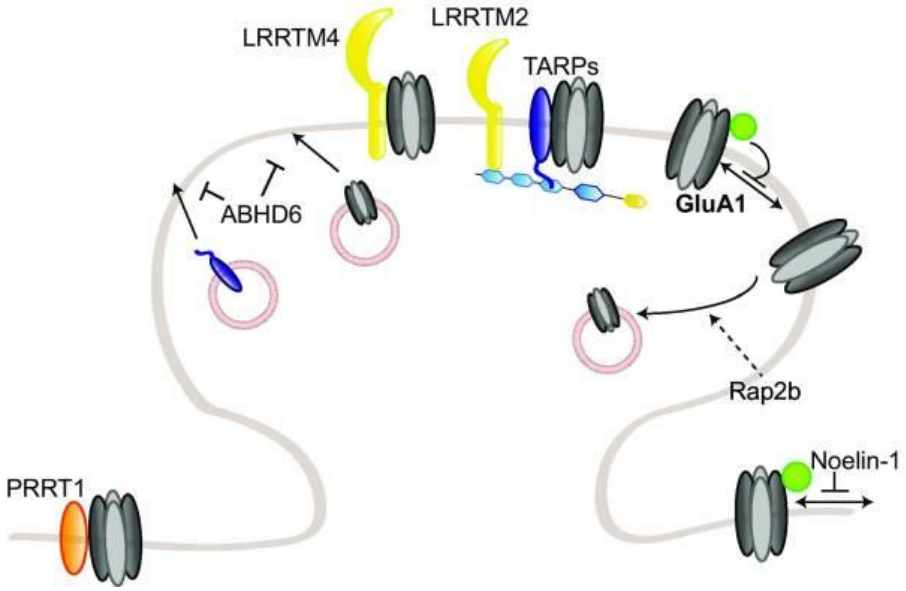

Figure 1.11. Role of LRRTMs in AMPAR trafficking and synaptic presence. LRRTMs may bind directly to PSD95 and cooperates with TARPs in maintaining a pool of surface AMPARs. ABHD6, negatively regulates surface AMPARs and stargazing (TARP2); Noelin-1, is involved in negative regulation of lateral AMPARs mobility. GluA1- and Rap2b, triggers AMPAR endocytosis through direct recruitment[1].

[o] Discussed in section 1.2.2.3

1.1.6.1.2 N-Methyl-D-Aspartate receptors (NMDAR)

NMDA receptors are a family of glutamate ionotropic receptors. NMDARs are expressed in neurons and glial cells[135,136]. N-Methyl-D-Aspartate (NMDA) is a specific NMDAR agonist and mimics the action of glutamate. However, unlike glutamate, NMDA does not interact with other glutamate receptors[137]. NMDAR subunits have seven members: GluN1, GluN2A-D and GluN3A-B. The genes for these subunits often have more than one splice variants: ***GluN1*:** GluN1-1a (Shows the highest expression), GluN1-1b, GluN1-2a, GluN1-2b, GluN1-3a, GluN1-3b, GluN1-4a, GluN1-4b; ***GluN2*:** GluN2A, GluN2B, GluN2C, GluN2D; and ***GluN3*:** GluN3A, GluN3B. NMDAR subunits share many structural and functional features with other ionotropic receptors such as AMPAR subunits[138,139]. On the extracellular side, NMDAR subunits have their N-terminal region, the S1 ligand-binding domain, and the S2 ligand-binding domain. There are two alternative splicing regions on NMDAR subunits, one in the N-terminal and one in the C-terminal region[139] (Fig. 1.12).

typical NMDARs form di-heteromeric tetramers that are composed of two GluN1 subunits and two GluN2 or GluN3 subunits, however, NMDARs are capable of forming other subunit compositions as well, for instance, GluN1/GluN2B/GluN3A or GluN1/GluN2B/GluN2D at early developmental stages or GluN1/GluN2A/GluN2B or GluN1/GluN2A/GluN2C in adulthood[140].

GluN1 is a single gene protein and is a mandatory subunit of all endogenous NMDA receptors. GluN1 is a glycine binding subunit. C-terminal region of the GluN1 contains several motifs that are associated with regulation of NMDAR trafficking and can bind to other proteins including calmodulin and CaMKII[141]. GluN2 subunits have four different isoforms in vertebrates, are responsible for glutamate binding, and

control the electrophysiological properties of NMDARs[6,142]. Each GluN2 isoform has a unique C-terminal region, that is capable of interacting with different sets of intracellular molecules[6]. GluN2 subunits show differential expression patterns across cell types and developmental stages. While GluN2B is highly expressed in the brain during early post-natal stages, GluN2A will gradually become the predominant subunit[143]. GluN2C expression becomes detectable after birth and is enriched in adult cerebellum. GluN2D shows highest expression in diencephalon, mesencephalon and spinal cord during adulthood[140]. GluN3 subunits are capable of biding to glycine, GluN3A peaks in early postnatal life and GluN3B increases throughout development[140].

NMDARs have three unique features. **First**, they require the simultaneous binding of two ligands to change their conformation to the open state. The first ligand is glutamate, and the other is glycine. Therefore, the presence of at least one GluN2 (glutamate binding subunit) and one GluN1 or GluN3 (glycine binding subunits) is essential for the proper function of NMDARs[144,145]. **Second**, due to the presence of a blockage created by a Mg^{2+} ion, the membrane has to be depolarized to repel the Mg^{2+} and allow influx of cations such as Ca^{2+} through the NMDARs. Therefore, NMDAR channel opening is ligand-dependent, while the current flow is voltage-dependent[146,147]. **Third**, they are the only glutamate receptors that are not only permeable to Na^{+} and K^{+} but are also permeable to Ca^{2+}.

Zinc and pH (proton concentrations) are allosteric modulators of NMDARs. For instance, NMDARs with GluN2A subunits can be inhibited by nanomolar scale changes in zinc ions concentration in low pH[148,149]. Using cryo-electron microscopy, Jalali-Yazdi *et al.*[150] showed that zinc binding to the amino-terminal domain of

NMDARs induces structural changes in the ligand-binding domain and result in constriction of the ion channel.

NMDA receptors can go through a series of posttranslational modifications, including phosphorylation, glycosylation, nitrosylation, ubiquitination, and calpain (calcium-dependent, non-lysosomal cysteine proteases) cleavage[151]. **Phosphorylation** of serine or tyrosine residues in the C-terminal region significantly impacts the activity of NMDAR activities[152]. The N-terminal domain of NMDARs is ***N*-glycosylated**, which occurs when the subunits travel through the Golgi apparatus and rough endoplasmic reticulum. GluN1 is the most extensively glycosylated subunit with 12 *N*-glycosylation sites[153]. This extensive glycosylation is essential for the oligomerization of GluN1 with GluN2 subunits. ***S*-nitrosylation** of cysteines on GluN2A via nitric oxide (NO) reduces channel activity. NO is usually synthesized after activation of NMDARs which is facilitated by coupling of NMDARs and NO synthase to PSD-95[154]. The calcium-dependent protease **calpain** may cleave GluN2 subunits; this is important in modulating the activity and function of NMDARs. Susceptibility of NMDARs to calpain, changes with neuronal maturity. Similar to ubiquitination, cleaving NMDAR receptors by calpain, destines the protein for degradation. However, interaction with PSD-95 ensures that NMDAR stays intact; this, nevertheless, can change if NMDARs are internalized[155].

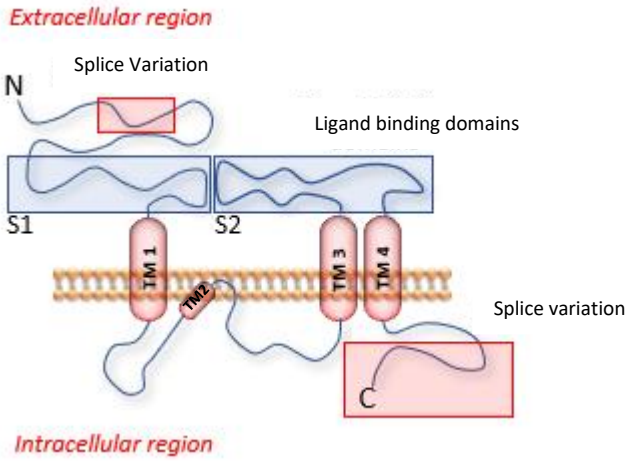

Figure 1.12. Schematic representation of structural features of NMDAR subunits. NMDAR subunits share many structural and functional features with other ionotropic receptors such as AMPARs. On the extracellular side, they have an N-terminal, the S1 ligand-binding domain, and the S2 ligand-binding domain. There are two alternative splicing regions, one in the N-terminal and one in the C-terminal region.

1.1.6.2 Gamma-(γ)-aminobutyric acid receptors (GABAR)

Gamma(γ)-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the mammalian brain. GABARs are a class of receptors activated by GABA. GABARs have two subclasses $GABA_A$ and $GABA_B$ receptors. While $GABA_A$ receptors are ionotropic, $GABA_B$ receptors are metabotropic G-protein coupled receptors[156,157]. $GABA_A$ receptors are large proteins consisting of five subunits. Binding GABA or GABA-like molecules opens the receptor channel allowing Cl^- ions into the cells[158,159]. This hyperpolarizes the cell, making its excitation harder, resulting in sedation and inhibition. $GABA_A$ receptor subunits have a large extracellular region, four transmembrane regions and several intracellular regions. The second transmembrane alpha-helices from each subunit form the channel[160,161]. The extracellular regions can be phosphorylated by kinases such as PKC, regulating the receptor function. There are three main classes of $GABA_A$ receptor subunits α, β, and γ[162-165].

There are two GABA binding sites on $GABA_A$ receptors located at the interface of α and β subunits[166,167]. Benzodiazepines (BDZ) are another $GABA_A$ receptor ligand; they are allosteric modulators of the $GABA_A$ receptor (bind directly to the receptor but do not compete for the binding site) and cause conformational changes; however, they cannot assert their effect in the absence of GABA. BDZs increase the Cl^- conduction rate by increasing the rate of channel openings without affecting the open state's duration[168].

$GABA_B$ receptors are G-protein coupled metabotropic GABA receptors. $GABA_B$ receptors are members of the same family of receptors as metabotropic glutamate receptors and have a similar structure. They have two subunits, $GABA_{B1}$ and $GABA_{B2}$. They assemble as heterodimers by linking their C-terminal domains in the neuronal membrane[169]. $GABA_B$ receptors are linked via G-proteins to potassium channels and are expressed in the central and the autonomic nervous systems[170]. $GABA_B$ receptors hyperpolarize the cell by changing potassium concentrations after an action potential. $GABA_B$ receptor opening decreases adenylyl cyclase activity and dampens that of Ca^{2+} channels using G-proteins with Gi/Go α subunits signalling. $GABA_B$ receptors contribute to the behavioural actions of ethanol and may be involved in pain physiology[171].

1.1.7 Synaptic plasticity

Plasticity is the process by which neuronal circuits adapt to the appropriate stimuli and accordingly modify the subsequent response. Synaptic plasticity is therefore activity-dependent and changes the strength and efficacy of synaptic transmission in the pre-existing synapses[172]. Synaptic plasticity is the underlying

mechanism for turning transient experiences into long-lasting memories[172]. There are two major forms of plasticity, Hebbian plasticity (first introduced by Donald Hebb in 1949) and homeostatic plasticity. Hebbian theory of synaptic plasticity describes a mechanism for induction of long-lasting activity-dependent changes in the synapse strength which includes long-term potentiation (LTP) and long-term depression (LTD) forms of plasticity[173]. Hebbian plasticity is input-dependent, can be induced rapidly, and requires coordinated activity between pre-and postsynaptic neurons[173]. Without proper safeguards, Hebbian plasticity can be damaging to a neuronal network. Neurons can sense their own excitability and trigger an inhibitory homeostatic response that counteracts the changes in synaptic activity and confines them within a dynamic but physiologically sustainable range[173].

1.1.7.1 Short-term synaptic plasticity (STP)

Postsynaptic responses undergo alternate increases and decreases as a response to changes in presynaptic activity. Short-term plasticity includes short-term synaptic changes that result from previous experiences and activities and last for a maximum of few minutes[172]. Some forms of short-term synaptic enhancements such as augmentation, facilitation and post-tetanic potentiation can be attributed to episodes of increased Ca^{2+} concentration. Increased Ca^{2+} concentration acts on distinct presynaptic proteins such as synaptotagmin to increase the speed and volume of neurotransmitter release from the presynaptic membrane. increased neurotransmitter release will elicit a stronger response from the postsynaptic cell following an action potential in the presynaptic neuron[174].

STP plays a crucial role in short-term adaptation to sensory inputs, short-lasting changes in behaviour, and short-term memory. Most STPs are induced following short bursts of activity that cause accumulation of Ca^{2+} at the presynaptic site and increase

the probability of neurotransmitter release[172]. Two of the most widely studied short-term plasticity forms are Paired-Pulse Facilitation (PPF) and Depression (PPD). The paired-pulse response is triggered when two stimuli stimulate a cell at a very short interval. This stimulation may create a more substantial response (Facilitation) or a weaker response (Depression) to the second stimulus as compared to the first one[175].

PPD can be elicited in almost all types of synapses, and usually the stimulations must be less than 20 ms apart[172]. The mechanism behind PPD can be the inactivation of voltage-dependent calcium channels or voltage-dependent sodium channels or a temporary decline in the number of release-ready vesicles. Prolonged stimulation intervals (20-500 ms) can lead to PPF in most synapses.

1.1.7.2 Long-term synaptic plasticity

Any type of experience can induce long-lasting changes in individual synaptic strength. Through collective action of long-lasting changes in large neuronal groups, the brain encodes and stores spatio-temporal events in the form of memories in specified neuronal networks. This notion was first put forward by Santiago Ramon Y Cajal in the late 19th century[176] and was further advanced in the 1940s by Donald Hebb. Hebb's theory states that associative memories form in a brain by modifying synaptic strength when the presynaptic neuron and postsynaptic neuron fire in a coordinated manner[177]. In the 1970s, Bliss and colleagues conducted a series of experiments that showed repetitive activation of hippocampal excitatory synapses led to induction of Long-Term potentiation (LTP)[178]. Other forms of long-term synaptic change also exist, for instance, the ability to weaken a specific synapse in response to reduced stimulation in a process that is termed long-term depression (LTD) (Figure 1.13).

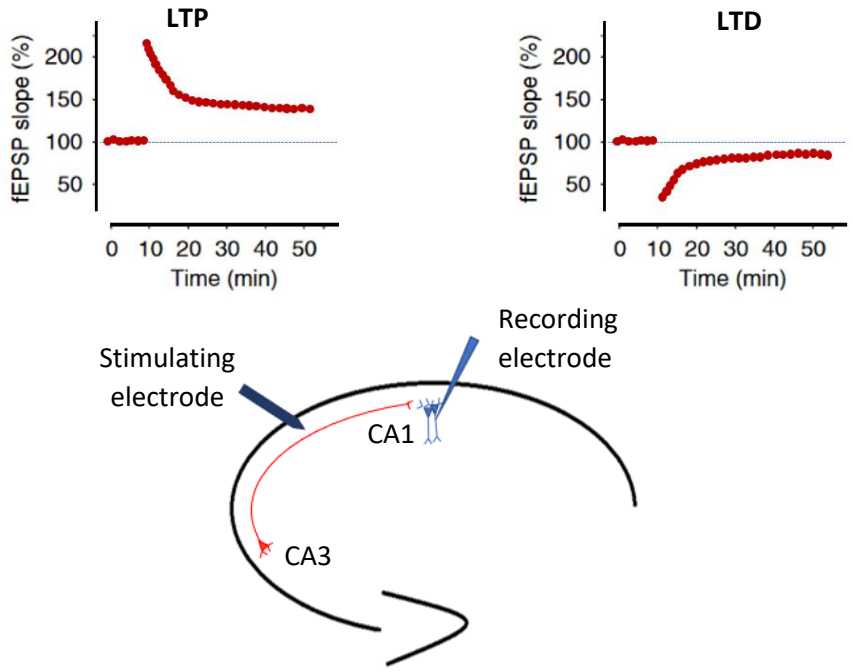

Figure 1.13. Long-term synaptic plasticity. Release of neurotransmitter 5-15 ms before the back-propagating AP induces LTP, whereas if the stimulus occurs 5-15 ms after the back-propagating AP it leads to induction of LTD.

Homeostatic plasticity and metaplasticity are two other forms of long-term synaptic plasticity. Synaptic scaling is the major form of homeostatic plasticity. During synaptic scaling, all synapses in a particular cell is adjusted to the firing properties of a given cell. Homeostatic plasticity may therefore scale a synapse up or down. Homeostatic plasticity works in a much longer time scale than LTP or LTD and is believed to be critically important during nervous system development. Metaplasticity is the plasticity of synaptic plasticity; in other words, it is the idea that the history of synaptic changes in a synapse determines its current state of plasticity. This is particularly important in the modulation of LTP and LTD[178-184].

1.1.7.2.1 Long-term potentiation (LTP)

There are three distinct phases in mammalian LTP: Induction, consolidation, and expression[185]. For induction of LTP specially in hippocampal CA3-CA1 synapses

and through high frequency stimulation a sequence of events should take place: simultaneous activation of synapses of single presynaptic cell on a postsynaptic neuron leads to depolarization of the postsynaptic cell, removal of Mg^{2+} blockage from NMDARs and influx of Ca^{2+}. Increased Ca^{2+} concentration leads to further Ca^{2+} release from the smooth endoplasmic reticulum (SER) and activation of protein kinases, such as Ca^{2+}-dependant calmodulin kinase II (CaM-KII), this in turn increases the synaptic presence of ionotropic (e.g., AMPAR) and metabotropic receptors (mGluRs), and modification of sensitivity and efficacy of AMPARs[185,186]. In later stages production and release of secondary messengers such as cAMP leads to changes in gene expression and synthesis of necessary proteins which strengthen the synaptic potentiation[185,187,188].

NMDAR-Dependent LTP

Among all forms of plasticity, the hippocampal NMDAR-dependent LTP is the most extensively studied. LTP in the hippocampus can be directly attributed to the mechanism through which the hippocampus acts as a memory hub. LTP in the hippocampus clearly demonstrates input specificity, cooperativity, and associativity. Input specificity implies that LTP is only induced in the synapse that is being stimulated and not in inactive synapse in that proximity. Cooperativity reflects the fact that there is a critical number of synapses that, if activated, can induce LTP. A commonly used protocol for induction of NMDAR-dependent LTP in hippocampus CA1, is a 100 Hz. stimulation for a duration of 1s[189,190]. Associativity denotes that a weaker signal from a lower number of synapses can be potentiated if associated with a larger number of synapses. For induction of LTP, two primary glutamate receptors are necessary. AMPARs and NMDARs. AMPARs and NMDARs are often found on the same synapses on dendritic spines.

The majority of cation-influx, such as influx of Na^+, occurs through AMPARs, which leads to increased positivity in the postsynaptic membrane. On the other hand, the membrane depolarization is necessary for removing Mg^{2+} ion blocking the NMDAR channel, to allow passage of Ca^{2+} and other cations. This Mg^{2+} blockage means that NMDARs cannot participate in basal synaptic activity; however, once the Mg^{2+} blockage is removed, the NMDAR will allow not only sodium and potassium to go through but Ca^{2+} ions as well. Ca^{2+} is necessary for induction of LTP. The increase in Ca^{2+} influx following NMDAR activation pushes the intracellular concentration of Ca^{2+} towards a specific threshold. Passing this threshold is essential for downstream biochemical signalling required for induction of long-lasting potentiation[189,191].

NMDAR-independent LTP

NMDAR-independent form of plasticity exist that dependent on activation of voltage-gated Ca^{2+} channels[192], or entry of Ca^{2+} through GluA2-lacking AMPARs. Unlike NMDARs, calcium-permeable AMPARs do not require membrane depolarization to be activated. Several studies have reported forms of LTP that are not only independent of NMDARs but are triggered by a mechanism independent of all ionotropic receptors all together[193,194]. These slow-developing LTPs probably involve activation of glutamate mGluRs and acquire the necessary Ca^{2+} from sources such as voltage-gated calcium channels. Synapses that may undergo NMDAR-independent LTP include among others, Synapses of mossy fibers on hippocampal CA3 principal neurons[195], Cortico-thalamic synapses[196], and mossy fiber synapses on interneurons in CA3[197].

1.1.7.2.2 Long-term depression (LTD)

Similar to LTP, LTD occurs in three distinct phases: induction, consolidation, and expression. Many of the steps that comprise these phases are shared with LTP, suggesting their mechanisms share many of the same features[198]. The initial steps are very similar to early phases of LTP. Synapses of single presynaptic cell on a postsynaptic neuron are simultaneously activated, leading to depolarization of the postsynaptic cell and removal of Mg^{2+} blockage from NMDAR and Ca^{2+} influx. LTD consolidation may also occur through metabotropic receptors such as (mGluRs), and activation of protein phosphatases such as phospholipase C (PLC) to produce triphosphoric inositol (IP3). Late phases of LTD also seem to require protein synthesis as well[185,199].

1.2 Pathophysiology, anatomy, and neuronal circuitry of schizophrenia

The main focus of this study is to investigate the role of LRRTM1 in the mammalian brain. *In situ* hybridization studies and mRNA expression analysis using qPCR have indicated the thalamus, hippocampus CA regions, hippocampal dentate gyrus, and prefrontal cortex (PFC) as brain regions with high expression levels of LRRTM1 across different species, including mice, pigs, and humans[66,200,201]. Point mutations and copy number variations of LRRTM1 have also been implicated in schizophrenia development and human handedness[p] [76,203,204]. A quantitative analysis of human handedness in dyslexic siblings, showed a significant association between haplotype upstream of the LRRTM1 gene with human handedness when it was paternally inherited (P=0.00002)[66]. In the same study, genomics analysis of 1002 affected families, showed that paternal overtransmission of the same haplotype was associated with schizophrenia/schizoaffective disorder (P=0.0014)[66]. Hypomethylation of LRRTM1 promoter is also linked with schizophrenia[205].

Schizophrenia is usually diagnosed in the late teen years to the early twenties and often manifests later in females. Schizophrenia is usually diagnosed after the first episode of psychosis. However, gradual changes in mood, thoughts, and social affiliations often manifest earlier, in mid-adolescence. Although schizophrenia has been diagnosed in children, its occurrence before adolescence is rare[206,207].

[p] Non-righthandedness has been shown to be an empirical effect that reflects a genetic link between schizophrenia and brain lateralization[202].

Common symptoms of schizophrenia can be classified into one of three groups: Positive symptoms, negative symptoms, and cognitive symptoms. Symptoms with psychotic nature are called positive, symptoms that are disturbing the normal functions fall under negative symptoms, and cognitive symptoms are those that are particularly disturbing to the cognitive faculties of an affected individual including Imagination, memory, and Intellectual capability[208-211].

Positive or psychotic symptoms manifest as altered perceptions in vision, hearing, smell, touch, and taste, accompanied by unusual thinking and strange and abnormal behaviour[206,212,213]. **Negative symptoms** include lack of motivation and interest, anhedonia, social withdrawal, difficulty in showing emotions and performing normal daily functions[208,213]. **Cognitive symptoms** often involve impaired attention, concentration and memory, and the degree of difficulties is variable from individual to individual[214-216].

Schizophrenia is accompanied or preceded by other neuropsychiatric diseases and comorbidities such as depression, obsessive-compulsive disorder (OCD), and anxiety, as well as seemingly somatic diseases such as diabetes[217] and increased inflammation[218,219]. These observations strengthen the theory that schizophrenia is a multisystem disease.

Risk factors in the development of schizophrenia

Genetics, environmental and physiological risk factors all contribute to the development of schizophrenia.

Genetics: Schizophrenia often occurs in individuals with a family history of the disease. A large list of genes has been identified as schizophrenia risk factors and therefore, schizophrenia cannot be considered a single-gene disease[220,221].

Environmental: Interaction between genetics and environment factors seems to be a more likely etiology in the development of schizophrenia. Having a family history of schizophrenia and being exposed to environmental stressors such as poverty, malnourishment, childhood trauma, and intrauterine infection during pregnancy have been reported in many schizophrenia patients. Nevertheless, schizophrenia remains a dominantly hereditary disease[222,223].

1.2.1 Pathophysiology of schizophrenia

Schizophrenia is a complicated chronic mental disorder specified by a range of symptoms, including hallucinations, delusions, disorganized speech and behaviour, and impaired cognitive function[210]. The fundamental pathophysiological basis of schizophrenia has been associated with abnormal neurotransmission in dopaminergic, serotoninergic, glutamatergic, GABAergic, and cholinergic systems. Aspartate and glycine have also been implicated, albeit to a lesser extent[224].

1.2.1.1 The dopamine hypothesis of schizophrenia

A wide range of schizophrenia symptoms have been associated with abnormal dopamine transmission, specifically through D2 receptors. Four major dopamine pathways have been identified in the human brain (Fig. 1.14). 1. Mesocortical pathway, 2. Nigrostriatal pathway, 3. The mesolimbic pathway, and 4. Tuberoinfundibular pathway. Initially, it was believed that positive symptoms resulted from hyperactivity in the mesolimbic dopamine (DA) system, whereas negative symptoms were associated with hypoactivity in the mesocortical DA pathway[225-227].

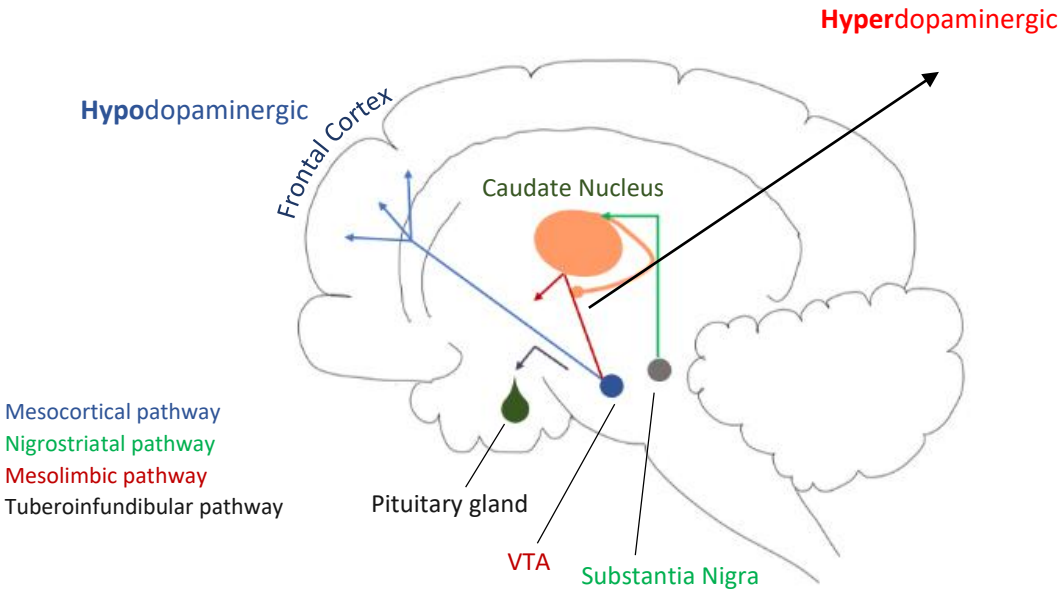

Figure 1.14. Major dopaminergic pathways of the brain. Four major dopamine pathways have been identified in the brain. 1. Mesocortical pathway, 2. Nigrostriatal pathway, 3. The mesolimbic pathway, and 4. Tuberoinfundibular pathway. Initially, it was believed that positive symptoms resulted from hyperactivity in the mesolimbic dopamine (DA) system, while negative symptoms were associated with hypoactivity in the mesocortical DA pathway. (Adapted from Schwartz *et al.* 2012)

The nigrostriatal dopaminergic pathway consists of substantia nigra dopaminergic neurons projecting to the caudate nucleus. Subnormal dopamine transmission in this pathway leads to extrapyramidal related motor system deficiencies similar to the symptoms observed in Parkinson's disease[228,229]. The mesolimbic pathway includes midbrain dopaminergic neurons originating from the Ventral tegmental area (VTA) and projecting to the limbic regions. The VTA dopaminergic neurons projecting to the frontal cortex comprise the Mesocortical pathway, and the Tuberoinfundibular pathway comprises the dopaminergic neurons of the hypothalamus projecting to the pituitary gland. Reduced inhibitory blockade of

tuberoinfundibular dopamine leads to hyperprolactinemia and consequently reduced libido, amenorrhea[q], and galactorrhea[r][230].

The dopamine hypothesis was proposed by Jean Delay and Pierre Deniker in 1952. This was motivated by their discovery that *chlorpromazine* manifest antipsychotic effects in schizophrenia patients. This was later reproduced by others, that showed that chlorpromazine and another antipsychotic *haloperidol* increase DA metabolite concentration in the mouse brain without changing DA concentration. Further studies found that *amphetamine*[s] administration to healthy individuals produces episodes of acute psychosis essentially indistinguishable from psychotic episodes observed in paranoid subtypes of schizophrenia patients. Together, these observations made the DA hypothesis the most popular hypothesis for the pathophysiology of schizophrenia[231,232].

Dopamine receptors in schizophrenia pathology

DA binds to the target cell through five DA receptors that are further classified into two subfamilies[233-235]: D1-like DA receptors (includes D1 and D5 receptors) and the D2-like receptors (includes D2, D3 and D4). The D1-type receptors are excitatory metabotropic receptors and pair primarily with the $G_{\alpha s}$ family of G proteins. On the other hand, the D2-type receptors are inhibitory and primarily pair with the $G_{\alpha i}$ family. Medications that block D2 receptors, such as antipsychotics, are used in treating positive and psychotic symptoms of schizophrenia and other psychoses. Medications that stimulate D1/D2-type receptors, on the other hand, are used to remedy some of

[q] Absence of menstruation during the reproductive years.

[r] A milky discharge from breasts unrelated to the normal milk production as a result of hyperprolactinemia.

[s] Amphetamine (alpha-methylphenethylamine) is a CNS stimulant. It increases monoamine and excitatory neurotransmission in the brain through inhibiting neurotransmitter reuptake and blocking the monoamine oxidase.

the symptoms of motor dysfunction in Parkinson's diseases that are a result of degeneration of dopaminergic neurons. Many of the conditions associated with schizophrenia pathology are believed to result from excessive stimulation of D2-type receptors in the striatum and subnormal activity of D1-type receptors in the prefrontal cortex[236-238].

1.2.1.2 The serotonin (5-hydroxytryptamine, 5-HT) hypothesis of schizophrenia

Excessive serotonergic neurotransmission from dorsal raphe nuclei (DRN), in response to elevated stress levels, disrupts the normal function of cortical neurons in schizophrenia. According to this hypothesis, elevated stress levels in schizophrenia leads to upregulation of serotonin release in the DRN in a potentially permanent manner. The anterior cingulate cortex (ACC) and dorsolateral PFC are particularly affected in this scenario[239].

Serotonin receptors (5-HTR) are a family of several G-protein coupled receptors and one ion channel receptor. They show differential expression patterns in the brain and display different degrees of involvement in the pathology of schizophrenia. Table 1.1 lists all serotonin receptors and their mechanisms of action.

Table 1.1. Serotonin receptors and their mechanisms of action[240].

G-protein component of 5-HTRs	Subtype	Mechanism of action and effect
Gi/Go	1a 1b 1d 1e 1f	Adenylate cyclase inhibition
Gs	5 4 6 7	Adenylate cyclase activation
Gq/G11	2a 2b 2c	Phospholipase C activation
No G-protein	3	Ligand-gated ion channel

German psychiatrist Kurt Beringer (1923) was the first to suggest that *mescaline* (a hallucinogen) can be used as an effective experimental model of psychosis. Later, mescaline was found to be an agonist of serotonin-2A (5-HT2A) receptor. In 1943, Albert Hofmann discovered the psychotomimetic effects of D-lysergic acid diethylamide (LSD); Hofmann's colleague Walter Stoll later found that the effects of LSD were similar to the symptoms of schizophrenia. LSD is a 5-HT2A receptor agonist and can stimulate D2 Dopamine receptors and Trace Amine Associated receptors[t] (TAARs) at higher doses as well[241].

Serotonin is also central in another hypothesis on schizophrenia development, the transmethylation hypothesis. According to this hypothesis, schizophrenia results from a biochemical malfunction in the stress system of the brain. This malfunction leads to aberrant endogenous biosynthesis of methylated indoleamine hallucinogens such as N, N, dimethyltryptamine (DMT). Stress elevates DMT levels in rodents, and

[t] A class of G protein-coupled receptors. They bind to endogenous amines found at trace concentrations in tissues. TAAR1 plays modulatory roles in neurotransmitters system specially in dopaminergic systems.

it is associated with the occurrence of positive symptoms in schizophrenia patients. DMT administration to healthy subjects can also lead to experiencing positive symptoms. DMT is structurally similar to serotonin and can easily dock and activate its receptors. DMT is highly enriched in the blood and urine of schizophrenia patients[242,243]. DMT was detected in the urine of 47% of those diagnosed with schizophrenic, 38% of patients with non-affective psychoses, 13% of patients with affective psychoses, 19% of patients with neurotic and personality disorders and 5% of normal individual[244]. Figure 1.15 demonstrates the brain's major serotonergic pathways, consisting of DRN serotonergic projections to the frontal brain regions, including ACC, PFC, as well as cerebellum, and diencephalon.

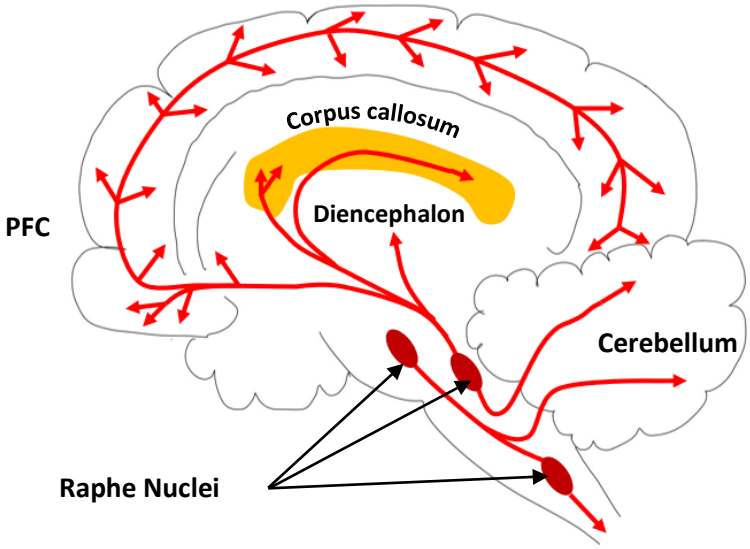

Figure 1.15. Major serotonergic pathways of the brain. Serotonergic pathways of the brain consist of Raphe nuclei serotonergic projections to the major brain regions including ACC, PFC, cerebellum, and diencephalon.

1.2.1.3 The Glutamate hypothesis of schizophrenia

The dopamine hypothesis of schizophrenia was the dominant narrative on the pathophysiology of schizophrenia since the 1970s. The positive symptoms were attributed to hyperactivity of the mesolimbic pathway, and negative and cognitive symptoms were thought to be associated with hypoactivity of the mesocortical pathway. However, administration of anti-psychotics (D2 DA receptor blockers) did not alleviate negative or cognitive symptoms. Blocking both dopamine and serotonin receptors, can be effective in alleviating the positive and some negative symptoms of schizophrenia without causing extrapyramidal[u] symptoms, however cognitive symptom remain mostly untreated[245].

More than 60% of neurons produce glutamate, and nearly all of them have glutamate receptors. NMDAR antagonists such as phencyclidine (PCP) and ketamine (a powerful anesthetic) could cause schizophrenia-type hallucinations, among other symptoms in otherwise healthy individuals. All brain areas, including those affected by schizophrenia, are directly or indirectly interconnected with glutamatergic projections. Glutamate is necessary for LTP induction and, therefore, plays a vital role in cognition and learning. NMDAR subunit GluN1 shows abnormal expression levels in the cortical areas of schizophrenia patients. Together these findings provide the basis for the glutamate hypothesis of schizophrenia[246,247].

The glutamate hypothesis of schizophrenia proposes that hypofunction and malfunction of NMDARs, AMPARs and Kainate receptors underlie the development of schizophrenia[248-250]. Research so far suggests that either hyperproduction or

[u] Extrapyramidal symptoms are result of dopamine blockade or depletion in the basal ganglia. The extrapyramidal symptoms include dyskinesias, tardive dyskinesia, Parkinsonism, akinesia, akathisia, and neuroleptic malignant syndrome.

insufficient glutamate neurotransmission may lead to schizophrenia symptoms. This is probably at least in part through the interaction of glutamate with other neurotransmitters like DA and GABA.

Building upon the knowledge from PCP and Ketamine, researchers tried targeting NMDAR for remediation of schizophrenia symptoms A meta-analysis on clinical trials suggested that administration of D-serine may help alleviating negative symptoms of schizophrenia[251], while another study major concluded that none of the studied NMDAR agonists (such as D-cyclo serine or glycine) were more successful than the placebo in improving any of schizophrenia symptoms[252]. The reason for this may be the relatively complicated nature of the glutamate pathway in the brain and the vast circuitry that depends on its transmission. More medications are going through different trial stages, and researchers are testing their effectiveness in alleviating negative and cognitive symptoms. Some like D-cycloserine have proven beneficial in combination with psychotherapy[253]. LY2140023 (also called *pomaglumetad*) is another medication that is being tested. LY2140023 is a highly selective agonist of metabotropic glutamate receptors $mGluR_2$ and $mGluR_3$. In a study on 196 schizophrenia patients, LY2140023 decreased the mean Positive and Negative Syndrome Scale (PANSS) by 13 points, and unlike antipsychotics such as Olanzapine did not show any side effects such as hyperprolactinemia (causes breast enlargement in men and amenorrhea in women) or weight gain. Figure 1.16 demonstrates the major glutamatergic pathways between brain regions affected in schizophrenia.

Ampakines are a relatively new group of medications that have produced promising results in animal trials and are currently going through different stages of clinical trials for treatment of schizophrenia symptoms. Ampakines enhance AMPAR

function and partially compensate for abnormal function of NMDARs by activating VGCCs[254-256]. Statistical power remains a major issue in clinical trials with medications that target glutamate neurotransmission.

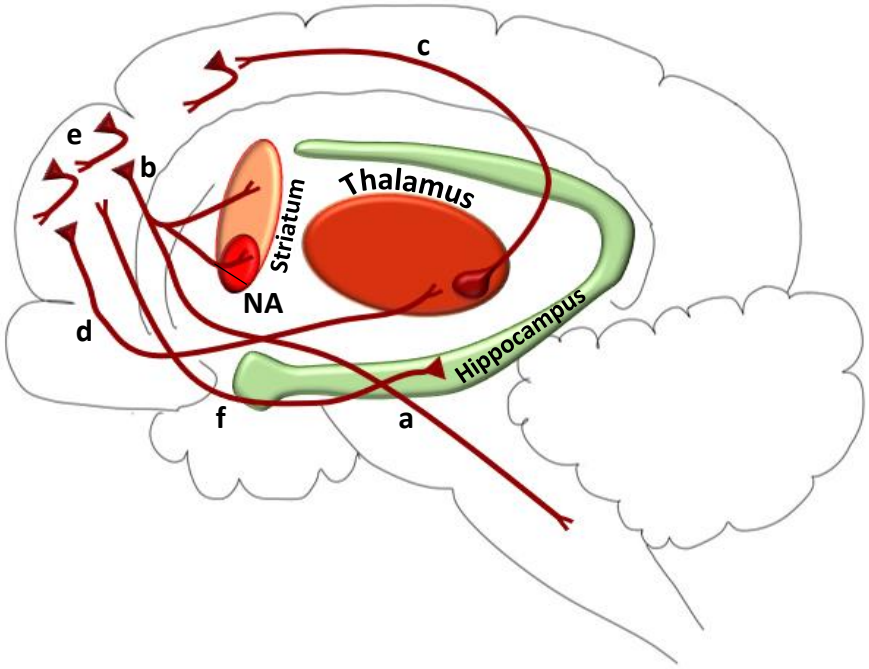

Figure 1.16. Major glutamatergic pathways in schizophrenia. a. The descending glutamatergic projections of cortical pyramidal neurons to the brainstem, targeting Raphe nucleus, locus coeruleus, VTA and substantia nigra). **b.** Descending glutamatergic pathway from the PFC to the nucleus accumbens (NA, Dorsal striatum). Constituting the corticostriatal portion of cortico-striatal-thalamo-cortical loops (section 1.2.2.1). **c.** Thalamocortical ascending glutamatergic projections from thalamic relay cells to the pyramidal neurons in the PFC. **d.** Descending corticothalamic glutamatergic pathway from the PFC to thalamic nuclei. **e.** Intracortical pyramidal neurons transferring signals among different areas of the cortex, known as corticocortical glutamatergic pathway. **f.** Descending cortical projections from the PFC to the hippocampal CA1. (Adapted from Schwartz *et al.* 2012)

Glutamate receptors in schizophrenia

Each subtype of glutamate receptor plays a specific and distinct role in the pathophysiology of schizophrenia. Although NMDARs show the highest degree of involvement in schizophrenia pathophysiology, disturbance in the normal function of any glutamate receptor can lead to the development of schizophrenia, especially since malfunction of other receptors can manifest as NMDAR abnormality. For instance,

changes in AMPAR function due to decreased expression, changes in subunit composition (e.g., GluA2 Q/R editing), or reduced membrane presence can, in turn, cause a reduction in firing of NMDARs since Mg^{2+} removal will be much harder. NMDARs are also expressed on presynaptic neurons; therefore, in addition to being responsible for slow glutamate response, NMDAR malfunction can also affect presynaptic glutamate release.

Kainate receptors are also potentially important in schizophrenia pathophysiology, albeit through a different mechanism since they act mostly as presynaptic regulators of glutamate release. Kainate receptors influence postsynaptic glutamate receptor function by modifying glutamate release through subunit composition changes or changes in the number of presynaptic kainate receptors. Changes in glutamate release not only directly affects NMDARs but can indirectly influence their function through the impact on AMPARs. Protein and mRNA level expression studies indicate that AMPARs are downregulated in the schizophrenic brain, especially in the hippocampus and medial temporal lobe. Abnormal functions of AMPAR regulatory proteins such as TARPs have also been reported in schizophrenia. Abnormal interaction between TARPs and AMPARs results in altered forward trafficking of AMPARs and leads to reduced synaptic presence and decreased glutamate transmission. Benesh *et al*.[257] report decreased Stargazin expression in ACC. Their results also indicate decreased GluA1 expression, increased GluA2:TARP2 ratio and reduced GluA1:TARP2 and GluA1:GluA2 ratios in synaptic fractions of ACC homogenates in postmortem brains samples of schizophrenia patients. In another study, Timpe *et al*.[258] showed that potentiating AMPARs in rats using glutamate could eliminate the effects of PCP administration by activating voltage-gated calcium channels, which would not be possible if voltage-gated calcium

channels were experimentally blocked. Together these studies suggest that AMPARs are affected in schizophrenia and their positive allosteric modulation or potentiation may help alleviate NMDAR malfunction in schizophrenia[259,260]. Kainate receptors seem to also be affected in schizophrenia, especially in the hippocampus, where they are reduced and in the frontal cortex, where they show an increased expression[261,262].

Glutamatergic metabotropic receptors are found on both presynaptic and postsynaptic neurons, albeit with different subunit compositions, and they interact with NMDARs[263-265]. Therefore, it seems highly probable that changes in subunit composition of metabotropic receptors on the presynaptic side can also influence glutamate release and causing NMDAR hypoactivity in postsynaptic neurons. These types of complex interactions between all subtypes of glutamate receptor on pre-and postsynaptic neurons and the difference in their expression between healthy individuals and schizophrenia patients strengthen the glutamate hypothesis of schizophrenia, and at the same time, explain the relatively poor outcomes of clinical drug trials in treating schizophrenia[264,265].

Interplay between glutamate and DA neurotransmitters in schizophrenia

Although dopamine hypothesis can explain the positive symptoms of schizophrenia, it is not as helpful in accounting for negative and cognitive symptoms. The same applies to glutamate hypothesis, as it cannot be directly attributed to increased presynaptic striatal dopamine function, or the clinical effectiveness of dopamine antagonists. Therefore, it is likely that both systems are involved in pathophysiology of schizophrenia and understanding their interplay is necessary in understanding the etiology of the disease[266].

Pharmacological studies in humans, such as administration of amphetamine has shown increase in cortical glutamate levels[267], however dopamine antagonists did not produce consistent effects on glutamate levels[268]. On the other hand, some PET[v] studies have reported that administration of ketamine would lead to increased dopaminergic disinhibition[269]. PET-MRI studies in healthy individuals found that increased dopamine synthesis in the ventral striatum is associated with reduced glutamatergic activity in cortical circuits while increasing it in the striatum[270]. Same relationship was observed between cortical glutamatergic projections and striatal dopamine system in high-risk and first-episode psychosis patients, but not in controls[271,272]. These results could potentially suggest that increased activity of glutamatergic projections to the striatum may lead to increased dopaminergic activity of the striatum.

Based on these findings, theories have been developed on the role of interactions between glutamate and dopamine neurotransmission systems in development of schizophrenia. These theories propose that defective glutamate neurotransmission, for instance, due to defective NMDARs on cortical GABA interneurons result in insufficient inhibition of cortical glutamatergic projections to the midbrain, increasing dopamine production in the striatum[273]. This theory also provides an explanation for dopamine deficiency in the cortical area. Excessive glutamatergic activity in the cortex may lead to overstimulated GABAergic activity in the VTA and overinhibition of mesocortical projections[273] (Fig. 1.17).

[v] Positron Emission Tomography

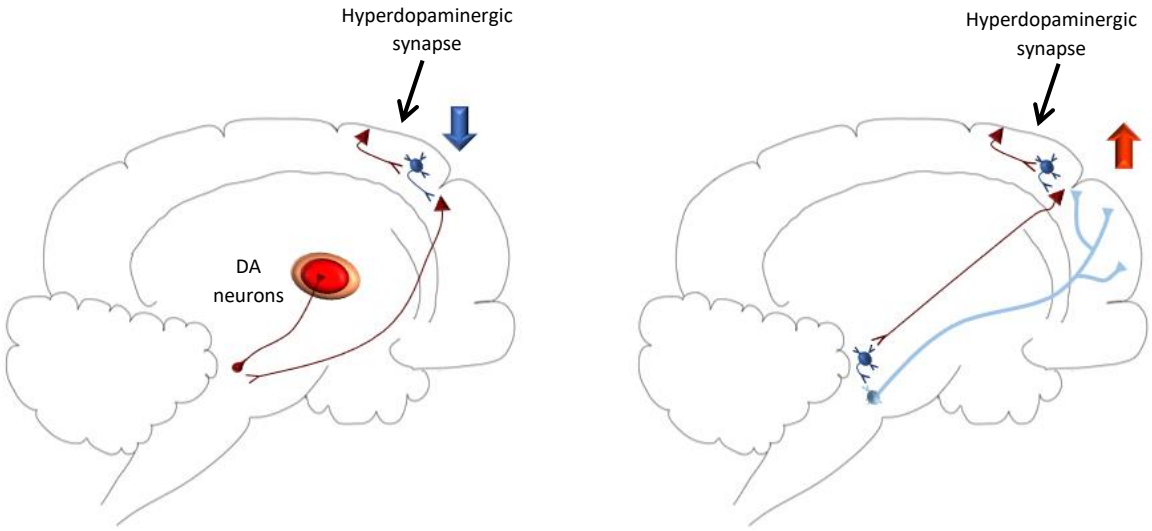

Figure 1.17. Glutamate receptor hypofunction leads to defective dopamine pathways in schizophrenia. a. hypofunctional NMDARs on PFC interneurons lead to decreased GABA release due to decreased excitation, which causes hyperfunctional glutamatergic activity in PFC pyramidal cells projecting to the brainstem dopaminergic cells, this then leads to hyperactivity in the mesolimbic pathway creating the positive symptoms of the schizophrenia. **b.** When the PFC projection neurons synapse on interneurons in the brainstem, this would lead to increased inhibition of dopaminergic projections to the PFC, causing hypoactivity of the mesocortical dopamine pathway leading to the development of negative and cognitive symptoms. (Adapted from Schwartz *et al.* 2012)

Role of the GABA system in the pathophysiology of schizophrenia

Deficiencies in GABA signalling have been linked with the pathophysiology of schizophrenia. For instance, blockade of the α5 subtype of the $GABA_A$ receptors (α5-$GABA_A$Rs) creates behavioural phenotypes usually linked with schizophrenia. Post-mortem analysis of brain samples from schizophrenia-affected individuals show reduced α5-$GABA_A$Rs in the hippocampus[274]. Post-mortem studies also showed reduced GABA signalling due to reduced $GABA_B$ receptors in lateral cerebella of individuals with schizophrenia, bipolar disorder and major depression compared to healthy controls[275]. GABA deficiency hypothesis of schizophrenia has also been confirmed by imaging studies, especially in the frontal lobe, occipital lobe, and basal ganglia[276]. However, this may be a mixed effect of relatively advanced age, exposure to antipsychotics, and GABA modulating medications such as benzodiazepine[276].

Role of the acetylcholine system in the pathophysiology of schizophrenia

Acetylcholine and its receptors are also associated with pathophysiology of schizophrenia. Deficits in P50 auditory gating have been associated with acetylcholine nicotinic receptor-related attentional impairments. P50 auditory response occurs 40-75 ms after an auditory stimulus. In a healthy individual, when two stimuli are presented 500 ms apart, the startle response to the second stimulus should be significantly reduced; this phenomenon is called auditory pre-pulse inhibition (PPI)[277]. PPI response is impaired in schizophrenia patients. Impaired auditory sensory gating has been linked to the α7 nicotinic receptor gene, located on chromosome 15q14. α7 nicotinic receptors mediate neuronal inhibition by enhancing GABA release from interneurons through a postsynaptic calcium-dependent mechanism. GABA release stimulates $GABA_B$ receptors and reduces glutamate release in return. NO release can help prolong this effect. Nicotinic inhibition is the underlying mechanism that prevents the hippocampal CA3 neurons from responding to the second stimulus and also plays a role in the efficiency and communication patterns of the cortex and the hippocampus[278-280]. The majority of schizophrenia patients are heavy smokers as nicotine can temporarily reverse the sensory gating impairment, but this effect will eventually be lost as the receptors will be desensitized[281,282]. Figure 1.18 illustrates the interaction between glutamate, GABA and Dopamine neurotransmission systems in rodent brain[283].

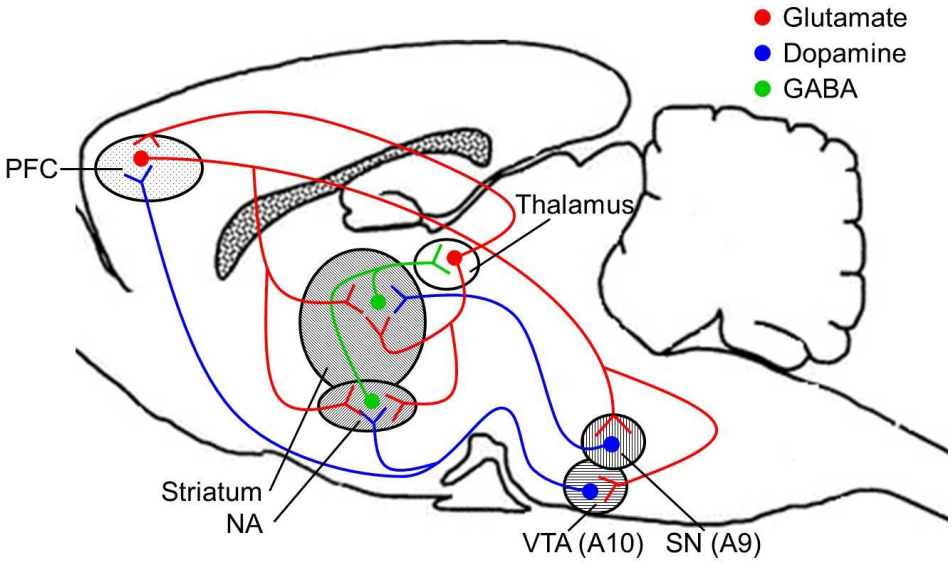

Figure 1.18. Interaction between glutamate, GABA and Dopamine neurotransmission systems in rodent brain. Dopaminergic (blue) projections exert their modulatory effect on the dorsal striatum via substantia niagra (SN), and on the ventral striatum and the PFC via the VTA. GABAergic (green) projections from the striatum then extend to different regions such as the thalamus. Thalamus has reciprocal glutamatergic (red) projections with the striatum and the PFC. In turn glutamatergic projections from the PFC are sent to the nucleus Accumbens, thalamus, substantia nigra and the VTA.

1.2.2 Anatomy and neuronal circuitry of schizophrenia

The complex nature of schizophrenia etiology, as manifested by the vast number of genes that are involved in its pathology, the interaction of environmental factors with those genes, and involvement of major neurotransmitter systems, means that many brain regions and the neuronal circuitry they use for communication are affected in schizophrenia patients. These areas include, among others, the prefrontal cortex (PFC), the anterior cingulate cortex, hippocampus, striatum, amygdala, and the mediodorsal nucleus of the thalamus (MD)[284-288].

1.2.2.1 The Prefrontal cortex

From evolutionary and developmental points of view, the cerebral cortex has a hierarchical physiological organization. At the lowest level, are the sensory and motor cortices, while the prefrontal cortex (PFC) constitutes the highest hierarchical level and

is one of the last areas to mature[289]. PFC is part of the neocortex (neo: relatively new in the evolutionary path) and is located in the most anterior part of the frontal lobe, in front of the motor and premotor areas[290]. PFC is associated with the expression of personality, decision making (by applying past knowledge and experience), complex behavioural planning, and suitability of social behaviours. From a neuronal circuitry angle, PFC is the projection zone of the of the mediodorsal nucleus of the thalamus (MD)[291,292]. Physiologically it is the frontal cortex area in which electrical stimulation does not cause observable movements[293]. The PFC also receives projections from other brain regions such as the VTA and the DRN and sends projections to other cortical and subcortical areas.

Prefrontal cortex in primates consists of three major subdivisions, orbital, medial and lateral areas. The orbitofrontal cortex (OFC) is involved in processing of primary reinforcing stimuli such as taste or touch. OFC is also associated with learning and reversal and is involved in controlling and modifying behaviour based on outcome of an action in a reward or punishment based manner, and therefore plays a strong role in processing emotions[294].

It is believed that medial prefrontal cortex (mPFC) is involved in decision making, memory consolidation and retrieval of long-term memory. However, it is suggested that mPFC's primary task is to learn association between contexts, locations, events and the corresponding adaptive responses[295]. Probably because proper fulfilment of these tasks requires the ability to decide on the best reaction or emotional response to a particular stimulus at a given time and place[295]. This level of complex decision making requires the mPFC to be able to correlate between different memory types over time and is the reason for the complex connectivity between mPFC, hippocampus and higher order thalamic nuclei such as the MD[295,296]. The

Lateral Prefrontal cortex consists of the dorsao-lateral prefrontal cortex (dlPFC) and ventro-lateral prefrontal cortex (vlPFC), which are present in primates only[297]. LPFC is associated with reasoning, planning and problem solving, and abstract representation. Damage to LPFC causes disorganization of thoughts, and context-inappropriate behaviour[298]. In primates these subregions can be further divided into regions with distinctive functional or anatomical properties.

In rodents PFC contains mPFC, OFC and agranular insular regions[299]. Dorsal part of the mPFC in rodents has functional links to motor cortex and is therefore, involved in motor and temporal processing[300,301] among other tasks. Rodent OFC is believed to be involved in associative learning and making predictions about the animal's environment based on previous experiences[302,303]. Agranular insular is mostly associated with processing of visceral sensory information such as gustation[304].

From a functional perspective the rodent mPFC is associated with working memory, attention, initiation of responses, emotions and autonomic control. Rodent mPFC corresponds with the same region in humans which is associated with neuropsychiatric diseases such as schizophrenia[305]. In rodents, the medial prefrontal cortex includes the ACC, prelimbic cortex (PL), and infralimbic cortex (IL) (Fig. 1.19). Functionally the primate ACC is associated with action monitoring and behaviour correction[306]. Multiple single-unit recording studies from rat PFC have reported this area to be correlated with motor planning, movement, and reward anticipation, similar to functional role of ACC and mPFC in primates[307,308].

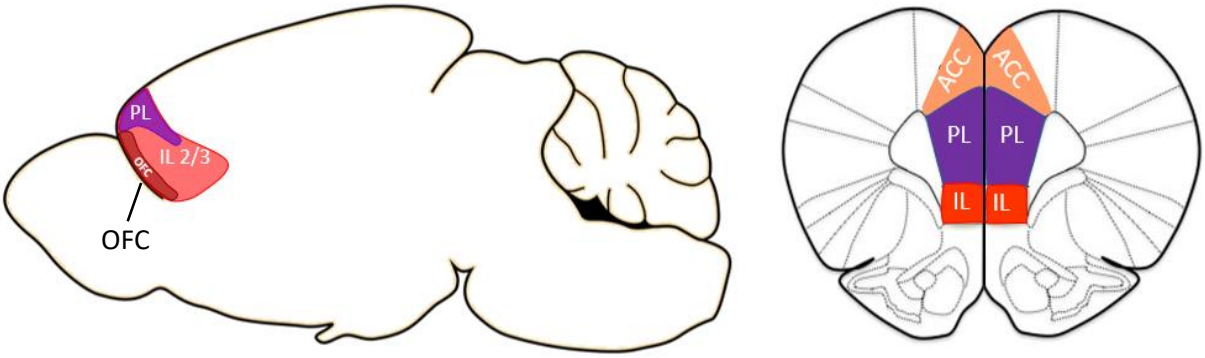

Figure 1.19. The prefrontal cortex of mouse brain. Mouse prefrontal cortex, midline sagittal view (left) and coronal right. The PFC can be divided into two distinct areas based on morphology, function, and evolutionary path. 1. The ventral-medial prefrontal cortex or (vmPFC), which itself consists of ventral PFC (vPFC) and medial PFC (mPFC) and is present in all mammalian brains. 2. Lateral prefrontal cortex (LPFC) which consists of dorsal-lateral prefrontal cortex (dlPFC) and ventral-lateral prefrontal cortex (vlPFC), which are present in primates only. Dorsal PFC is interconnected with brain regions that process attention, cognition, and action, while the ventral part is associated with brain areas processing emotions such as the limbic regions. Rodent PFC acts as a hub for cortical networks from motor, somatosensory, gustatory, auditory, and limbic areas. In rodents, the medial prefrontal cortex includes the ACC, prelimbic cortex (PL), and infralimbic cortex (IL). orbitofrontal cortex: OFC.

The general structure and connectivity patterns of the neocortex

Most of the cerebral hemispheres are covered by the neocortex. The neocortex has six layers; the cells in each layer are relatively distinct in morphology, function, and connectivity. There are, however, functional and structural similarities between neocortical circuits regardless of their task and anatomical position. This suggests a collective tactic in processing various types of information that the cortex deals with at any given time. The properties of the neurons that comprise the neocortex are also very similar between different cortical regions, including similarities in morphology, local and long-range connectivity, developmental origins, physiology, activity pattern, and gene expression. This similarity gives the cortical circuit a unique ability to take over the function of a damaged region or to expand a particular cortical area in case

of practice or repetitive action, for instance, by practicing playing a musical instrument[309].

Neocortical Layer I is almost entirely devoid of cells and contains the dendrites from cells in other layers and is thus called the molecular layer. Layer II and layer III Contain small pyramidal cells and receive mostly of the same inputs (primarily from layer IV, and project to layer V and horizontally to other cortical regions (corticocortical projections). Due to similarities in their morphology, function, and connectivity, layers II and III are often referred to as one functional unit, layer II/III. Layer IV and layer V contain large pyramidal cells and have a similar cytostructure but receive different inputs and project to different areas. Layer IV receives its inputs from thalamic regions (thalamocortical projections) and projects primarily to layer II/III, while layer V receives most of its projections from layer II/III, and projects to layer VI as well as the basal ganglia and other cortical regions. Layer VI receives its major projections from layer V and projects back to the same thalamic regions creating a feedback loop[310-314] (corticothalamic projections, Fig. 1.20).

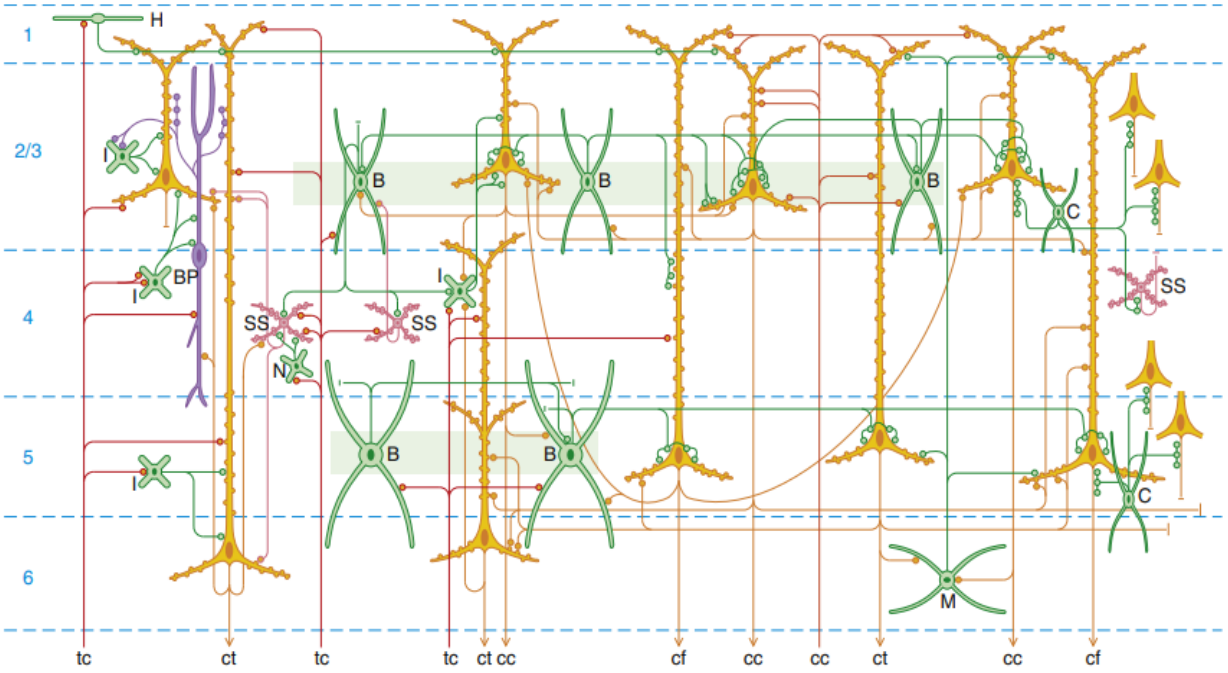

Figure 1.20. Common Connection patterns in the neocortex. Shared connection patterns of cortical neurons create repeated units of information processing across the neocortex. The pyramidal cells (orange), and spiny stellate cells (pink) are principle cortical neurons. The excitatory projections are depicted in red. Inhibitory interneurons and inhibitory projections are shown in green. Basket cells are connected by gap junctions (electrical synapses) creating an inhibitory network (green shading). This connectivity pattern can be seen in mice, rats, cats, non-human primates, and humans. B, basket cell; BP, bipolar cell; C, chandelier cell; cc, corticocortical (afferent or efferent); cf: corticofugal (to midbrain, hindbrain, spinal cord); ct: corticothalamic; H: horizontal inhibitory cell; I: interneuron; N: neurogliaform cell; SS: spiny stellate cell. (Adapted from Kirkcaldie, 2012).

Cortico-striatal-thalamo-cortical pathway (CSTC)

The CSTC is part of the brain salience network (SN). The SN is hypothesized as a large-scale brain network that primarily consists of the anterior insula[w] (AI), the dorsal ACC (dACC) and the dlPFC. In addition to the cortical nodes, SN also includes subcortical nodes, including the caudate nucleus, the MD and dopaminergic brain nuclei[315]; together, these create a distinct cortico-striatal-thalamo-cortical pathway[316-319] (Fig. 1.21).

[w] The Anterior insular cortex is involved in processing of emotions and feelings, including maternal and romantic feelings of love, anger, fear, unhappiness, happiness, sexual arousal, revulsion, reluctance, injustice, resentment, disbelief, social isolation, trust, empathy, aesthetics features, and hallucinogenic states.

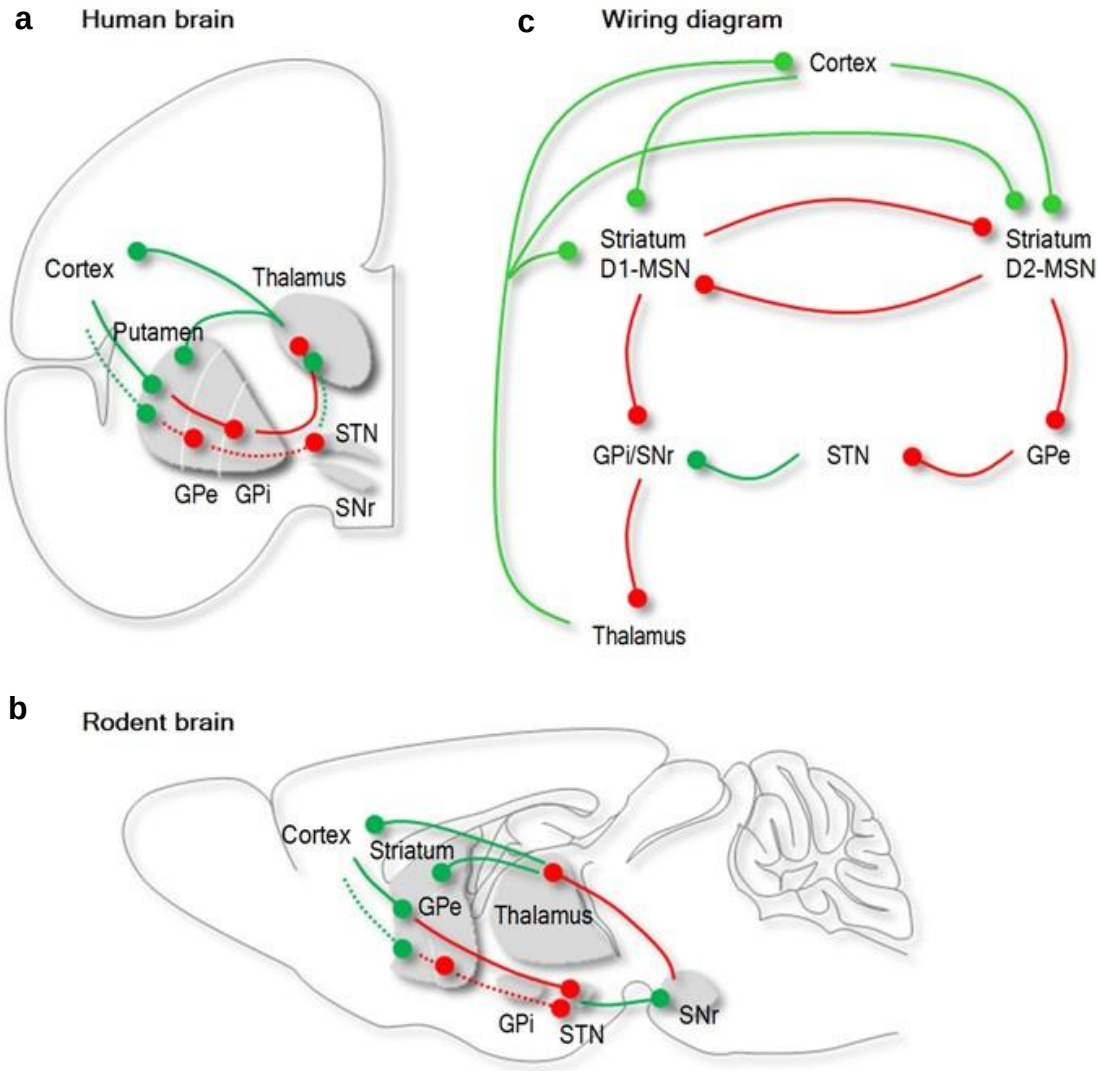

Figure 1.21. CSTC pathways and its synaptic systems in humans and rodents. a. Cortico-striatal-thalamo-cortical pathway in the human brain. Striatum sends GABAergic (red) projections to subthalamic nuclei (STN) and receives glutamatergic (green) projections from the thalamus and the cortex in return. **b.** The synaptic connections in the CSTC pathway in rodent brain. **c.** the general schematic representation of the synapses in the CSTC pathway in the mammalian brain. SNr (Substantia nigra pars reticulata), SNs (substantia nigra pars compacta), GPe (globus pallidus external part), GPi (globus pallidus internal part), STN (sub-thalamic nucleus). (Adapted from Rădulescu *et al.* 2017).

Salience network (SN) detects and filters salient stimuli[x] and engages the relevant networks such as the CSTC as required[320,321]. The salience network controls the execution of movement, formation of habits and risk-reward assessment.

[x] Salient stimulus refers to the features of an object in the environment that can attract an animal's attention. It can be bright colors, fast movement, a loud or distinctive sound or smell or something of personal relevance in case of humans.

Excessive or insufficient salience network activity has been attributed to the development of OCD, major depression, anxiety disorder, eating disorder, substance use disorder (SUD), bipolar disorder, and schizophrenia. The importance of salience network and CSTC in mental illness has been highlighted in a meta-analysis of studies involving 7000 patients affected with a variety of neuropsychiatric disorders. A pattern of decreased gray matter was reported in core areas of the salience network, including the CSTC resulting in impairment of the network's structural and functional integrity. The SN abnormalities in schizophrenia involve the insula, ACC, dlPFC, MD and the striatum[234,322].

The psychotic symptoms of schizophrenia are associated with salience network and has been proposed to involve the MD[323]. Auditory hallucinations that are a prominent psychotic feature of schizophrenia are associated with altered salience network dynamics which causes the internally generated stimuli to be abnormally perceived as salient stimulus[324,325]. Schizophrenia patients show reduced structural and functional integrity in the SN and the CTSC. This reduced integrity also results in interruption of information processing[322,326].

The sensory-motor gating deficiencies observed in schizophrenia patients are also attributed to the functional impairment of the CSTC. This is manifested by abnormal PPI responses in unmedicated schizophrenia patients. PPI deficits reflect the deficiencies in processing and integration of sensory and motor information and have been attributed to the development of OCD[319,327].

1.2.2.2 The thalamus

The thalamus is a paired structure with one pair in each hemisphere. It acts as a gating, preprocessing and relay center for the afferent information from other brain regions destined for the neocortex[328,329]. Thalamus consists of several nuclei, with distinct afferent regions and neocortical projection areas. The majority of thalamic cells are excitatory neurons that participate in the thalamic nuclei's gating and relay processes. The rest of thalamic cells are inhibitory interneurons that form inhibitory synapses on thalamic relay cells. The majority of thalamic interneurons are localized to the reticular thalamic nucleus (RTN), especially in the rodent thalamus, which except for the RTN, are devoid of inhibitory neurons[330-332].

The inputs to the thalamic nuclei can be divided into drivers and modulators. The drivers are the afferent fibres that transfer the information to the thalamus and form glutamatergic synapses on the relay neurons. Drivers form 10% of synapses, and the remaining synapses are modulator projections. Modulators form smaller synapses and regulate the transferring of information by the drivers. Modulators come from different brain regions, including the thalamic inhibitory interneurons, the RTN, the brainstem nuclei and cortical feedbacks[333,334].

Thalamic relay nuclei can be divided into two groups, the first-order thalamic nuclei and the higher-order thalamic nuclei. The drivers of the first-order thalamic nuclei arise from the brain stem or other subcortical brain regions and relay this information through the thalamus to the neocortex. Higher-order thalamic nuclei, on the other hand, receive their drivers from the neocortex itself and transfer this information to other neocortical regions. Therefore, the higher-order thalamic nuclei receive, process, and relay the information that has already been through primary cortical processing[335-338].

Subcortical regions send their projections to the first order relay cells, which in turn send their excitatory projections to layer IV of the neocortex. The first-order thalamic relay neurons also form excitatory synapses on RTN inhibitory interneurons on their way to the cortex; RTN inhibitory neurons then send inhibitory projections back to the thalamic relay cells. Neocortical cells from layer VI provide excitatory feedback to the thalamic relay neurons and the inhibitory interneurons of the RTN [333,339].

Thalamic relay neurons have two distinct firing modes, burst firing and tonic firing. Tonic firing is the standard firing mode shared by many other types of neurons. In tonic firing mode, the cell's excitation, beyond the threshold, elicits a train of action potentials. However, in bursting mode, thalamic relay neurons respond with short bursts of high-frequency spiking, followed by silent periods that contain very few spikes. Burst firing depends on the expression of a specific type of calcium channel called T-type calcium channels[340,341].

T-type calcium channels are double-gated channels; they have an activation gate and an inactivation gate. Both of these gates have to be open for calcium ions to pass through. The activation gate opens and closes by depolarization and hyperpolarization respectively, whereas the inactivation gate opens by hyperpolarization and closes by depolarization, albeit at a much slower rate. When the cell is hyperpolarized, the inactivation gate is open; therefore, when a depolarizing current arrives, it opens the activation gate, and for about 100 ms (before the inactivation gate closes), calcium ions can flow in causing burst firing, whereas when the cell is not hyperpolarized, the inactivation gate is closed, the calcium ions cannot flow in following the arrival of a depolarizing current, and the neuron fires in tonic mode[342-344] (Fig. 1.22). The RTN interneurons can control the firing mode of the relay cells. The inhibitory input of the RTN neurons to the thalamic relay cells hyperpolarizes

them and opens the inactivation gate, causing relay neurons to favour burst firing over tonic[345,346].

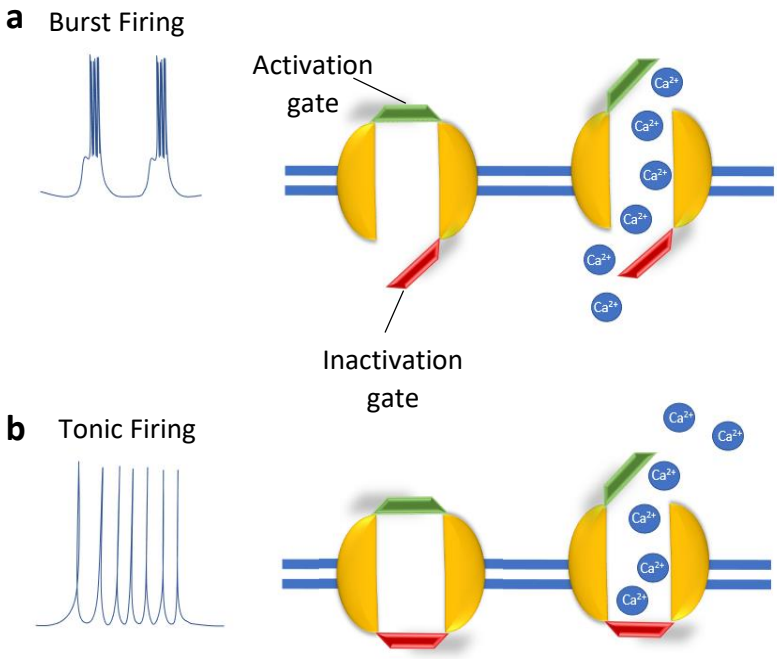

Figure 1.22. T-type calcium channels and their mechanism of function. a. When the cell is hyperpolarized the inactivation gate opens, therefore when a depolarizing current arrives, it opens the activation gate and for about 100 ms (before the inactivation gate closes) calcium ions can flow in causing burst firing, **b.** When the cell is not hyperpolarized, and the inactivation gate is closed, the calcium ions cannot flow in following arrival of a depolarizing current, and the neuron fires in tonic mode.

The mediodorsal nucleus of the thalamus (MD)

The mediodorsal nucleus of the thalamus is one of the largest of the thalamic nuclei, it shows the most extensive development in primates, but it is nevertheless an essential part of the thalamus in other mammals as well, with relatively well-defined boundaries. Its evolutionary size increase corresponds to the growth of the PFC, the association cortices and the cingulate regions[347,348]. In primates, MD consists of different cell groups that divide it into subregions of distinct morphology: magnocellular mediodorsal thalamus (MDmc), the parvocellular mediodorsal thalamus (MDpc), and the lateral group of the mediodorsal nucleus that includes the densocellular (MDdc)

and pars multiforms (MDmf) nuclei[349]. MDmc occupies most of the medial and rostral MD and is equivalent to the medial region of the MD in the rodents. MDpc is located in the central region of the MD and occupies most of the rostrocaudal extent[347,348]. MDmf is localized to the lateral region, and is part of the caudal MD[347,348].

MD in rodents has four subdivisions: Central, Medial, Lateral and Paralamellar[350,351]. The borders of each segment are rather well defined, especially for the central and lateral divisions. The dendrites of the cells in these two segments are limited to their own regions. Rodent subregions functionally and anatomically correspond to primate MD subregions. Medial MD to primate magnocellular MD, Central MD to the primate parvocellular MD, and the lateral MD to the primate lateral MD[349]. Figure 1.23 compare topography of the MD region in monkeys and rodents.

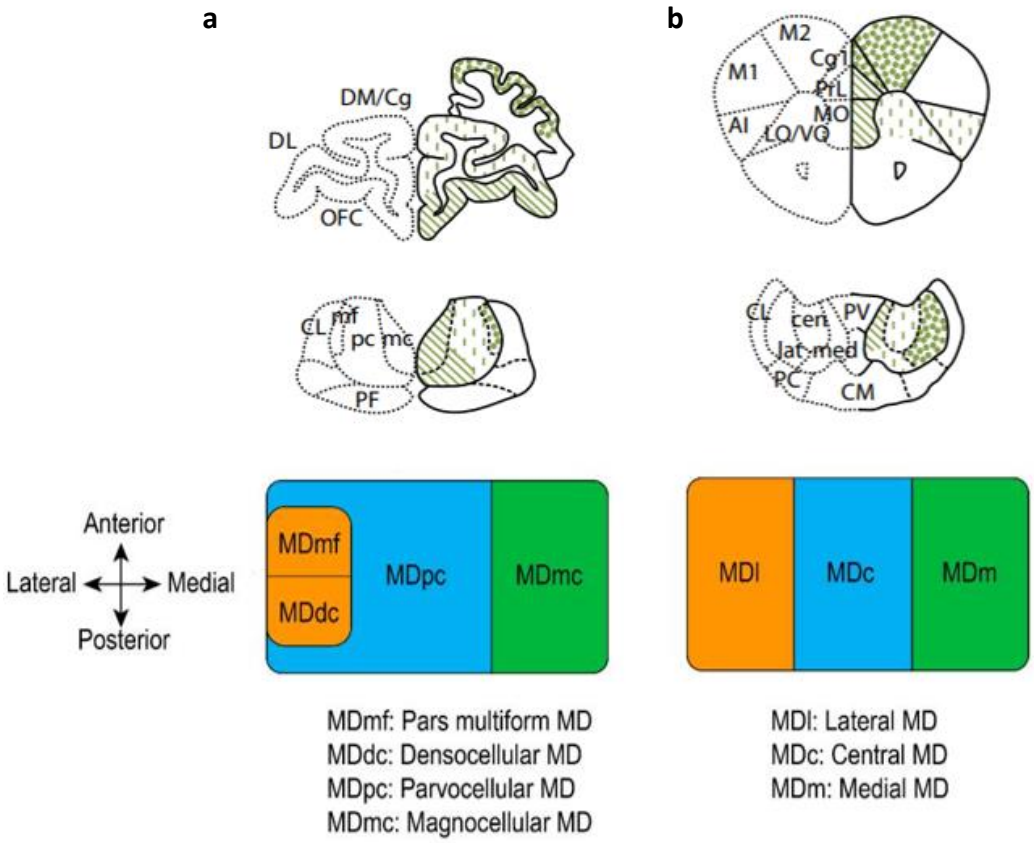

Figure 1.23. Schematic representation of the topographic organization of the MD subregions in non-human primates (a) and rodents (b). In non-human primates the MDmc is reciprocally connected with the medial OFC while the MDpc is interconnected with dorsolateral and multiform parts of the premotor cortex. In rodents on the other hand, medial segments conncets with vmPFC (PL and IL areas) and mPFC. The central part of the MD in rodents connects with lateral OFC and the lateral segment with dorsomedial PFC (ACC and the accessory motor cortices). Top panel adapted from Parnadeau *et al.* 2017, lower panel adapted from Georgescu *et al.* 2020.

MD-PFC reciprocal connections

Higher order thalamic nuclei, such as the MD are critical in providing a communication route between cortical regions. They also contribute to information relay and transmission from subcortical areas to the cortex[352]. Building Upon Brodmann's[353] cytoarchitectonic research on the prefrontal cortex in primates, recent studies suggest that prefrontal cortex in all mammalian species share many of the same functional characteristics[354]. Each MD subregion is bidirectionally linked with a specific cortical region. In primates and other mammals, the MD receives strong

projections from PFC layers V and VI, and projects back to layers I, III, IV, and V creating the cortico-thalamo-cortical circuits[355,356]. Deep layer PFC (V and VI) pyramidal cells form monosynaptic loops with the MD relay cells and are the driving projections of the MD. Both MD-PFC and PFC-MD projections form glutamatergic synapses (Fig. 1.24)[357]. MD and The PFC follow the same pattern as other areas of thalamus and cortex in coherence of their activity. Although MD regulates PFC activity, it is the PFC that exerts a top-down regulation on sensory, affective, and goal-directed processes through subcortical nuclei including the MD and thereby controls the behaviour and guides thoughts in a manner that is consistent with inner goals[358-361].

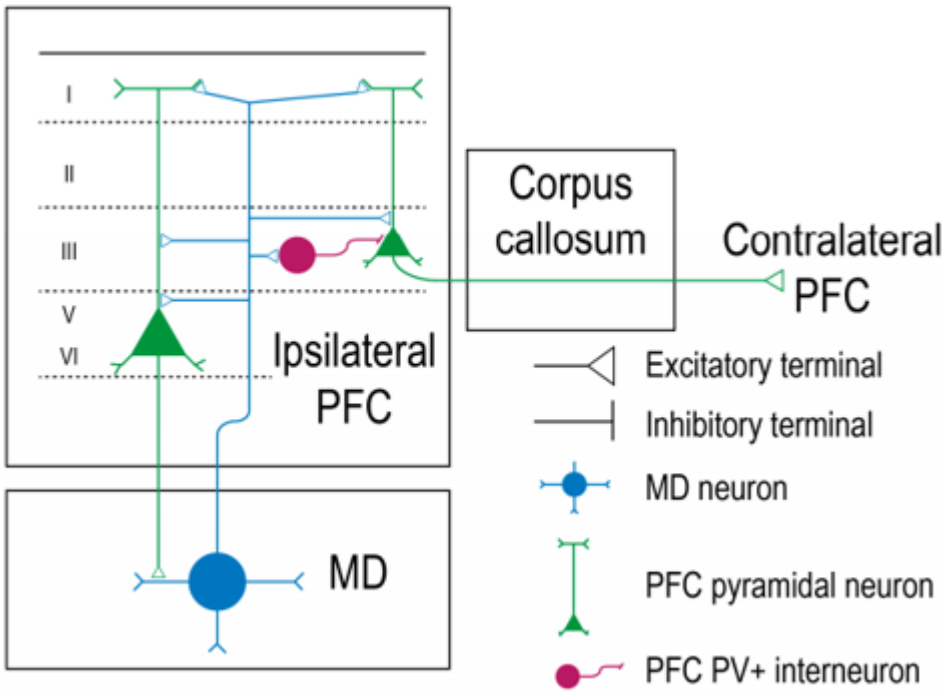

Figure 1.24. MD relays cells (blue) have reciprocal connections with the PFC pyramidal cells (green). PFC pyramidal cells located in layers V and VI project to the MD and form the driver synapses for this area. MD in return projects to layers II/III and V and form synapses with parvalbumine-expressing inhibitory interneurons (purple). Adapted from Georgescu *et al.* 2020.

MD associated behavioural phenotypes

In general, research over the past 18 years shows that MD has a distinct role in all cognitive behaviours controlled by the PFC, such as working memory, social interaction and reversal learning[296]. Thalamic lesions would lead to amnesia-related syndromes, similar to those observed in hippocampal lesions. This is probably due to damage to the mammillothalamic tract or the anterior thalamic nuclei, both essential parts of the Papez circuit, which is involved in processing emotion and memory[362]. However, when lesions are more confined to the MD region, the deficits are similar to phenotypes associated with lesions of the prefrontal cortex[363,364]. Controlled, more precise lesion studies on animals have indicated that MD lesions lead to deficits in working memory, behavioural flexibility, social interaction, and goal-directed behavior[365]. In rats, bilateral MD lesions show disrupted recognition memory, hypoactivity, anxiety-like behaviour, learning association deficits, reduced locomotor activity and reduced social interaction[366]. These lesions also reduced pyramidal cell density in the medial infralimbic cortex, the anterior dorsal cortex and the cingulate cortex, probably due to the failure in establishing a connection with their target cells in the MD[366,367].

Working memory is one of the main traits affected by schizophrenia and can also be impaired by lesions of MD, PFC, both or their reciprocal projections. Working memory is the ability to transiently hold, process or use information in a matter of seconds[368]. In animal models, it is defined as a delay-dependent short-term memory of an object, a location, or a stimulus presented to the animal in the experimental settings with a period of absence in between the trials. This is in contrast to reference memory tasks that require repeated training and usually last for days. Animal MD lesions indicated diminished working memory in rodents and primates[211]. The length

of delay period is an essential factor in the establishment of working memory, suggesting a role for MD in the maintenance of representations instead of general task learning[369-372]. Spatial working memory has been attributed to the mPFC; however, MD lesions cause very similar deficits, which has been attributed to the disrupted information flow between MD and the mPFC[373,374]. While encoding of spatial locations in the initial sampling phase is dependent on the direct projections of the ventral hippocampus to the mPFC, it is the reciprocal connection of MD and the mPFC that sustains the short-term memory maintenance during the delay in the working memory. Descending PFC projections to the MD are also involved in retrieval and choice selection (Fig. 1.25)[296,375,376].

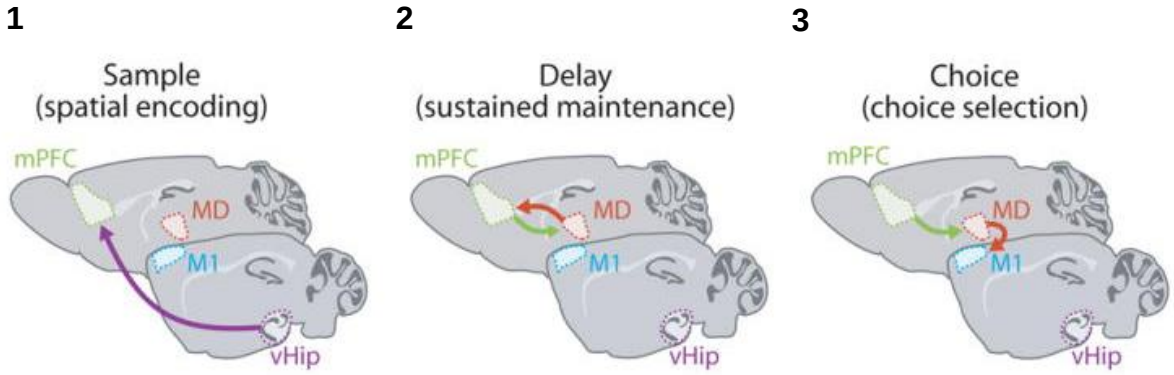

Figure 1.25. Thalamo-Prefrontal interactions in formation and retrieval of working memory. Schematic representation of MD-PFC interaction in encoding, maintenance, and selection phases of working memory. 1. In sampling phase ventral hippocampus inputs to the mPFC support encoding of spatial information. 2. MD is recruited by mPFC and then through its projections to the mPFC contributes to sustaining and amplification of cortical activity. This is critical for task performance. 3. In the selection phase, mPFC projections to the MD participate in retrieval of the encoded information may also relay the information to the motor areas. For instance, the primary motor area M1 (Adapted from Parnaudeau *et al.* 2018).

Reciprocal connections of the MD and the PFC are essential in the proper processing of social interactions. MD is believed to orchestrate PFC activity in response to social situations. In a study on rats by Jodo *et al.*[377], following

administration of PCP, 30% of centromedial MD neurons exhibited tonic firing, while only 7% were inhibited. The number of neurons activated following the systemic presence of PCP in PFC was more than double that of MD. Although this did not change the proportion of cells responsible for social interaction in either of the regions, as 90% of cells in PFC (vs 40% in MD) are activated in social settings, it nevertheless suggests that although PFC and MD are both required for proper social interaction, they process social information in distinct manners. Brumback and colleagues[378], Explored the role of MD and mPFC in social interaction using optogenetic techniques. They used a mouse autism model (through *in utero* valproic acid exposure, which leads to abnormal excitability of PFC layer V neurons). Their results indicated that optical excitement of either region alone leads to reduced social interaction. They also found that medial and lateral MD neurons show different electrical and anatomical properties, suggesting they would respond to stimuli of similar nature differently. The lateral MD neurons regain baseline electrical activity after activation relatively fast and have a more permeable membrane; this means these cells will be able to respond faster and more specifically to a stimulus. On the other hand, medial MD neurons need less current to activate, recover at a slower rate, and therefore are sensitive to different types of inputs. Taken together, this means that these cells send different information to the PFC.

1.2.2.3 The hippocampus

Hippocampus is one of the most complex and well-studied regions of the brain. It is a highly plastic structure, heavily involved in learning and memory and is affected in many neuropsychological diseases. Hippocampus is a paired structure, with one pair in each hemisphere. It is located in the allocortex and projects extensively to the neocortex. It has two distinct anatomical and functional regions, the hippocampus

proper, which contains the *cornua ammonis* (CA1-CA4) areas and the dentate gyrus (DG)[379-381]. The entorhinal cortex (EC) is the main input structure to the hippocampus and its leading output destination, and therefore, the primary interface between the hippocampus and the neocortex. EC is located in the medial temporal lobe and is an extensive network center for processing episodic memory, spatial navigation, and perception of time[251].

EC and hippocampus are major parts of the limbic system. The limbic system includes a wide range of cortical, subcortical, and diencephalic structures and is involved in the processing of emotions, feelings, motivation, memory, and learning. The cortical areas include the Limbic lobe, the OFC, piriform cortex, the EC, Hippocampus, and the fornix. Subcortical regions are the Septal nuclei, amygdala, and nucleus accumbens (NA, part of ventral striatum). Diencephalic regions are as follows: hypothalamus, mammillary bodies, and the anterior nucleus of the thalamus[382,383] (Fig. 1.26). The limbic regions are all interconnected and interact not only with other limbic regions but with other brain areas as well. The limbic system operates by manipulating the endocrine and autonomic nervous systems. For instance, NA is highly interconnected with all the limbic areas; it is involved in motivation, sexual arousal, and reward-risk assessment. NA is one of the major regions for euphoric sensation following the use of recreational drugs. The function of NA depends heavily on the dopaminergic system.

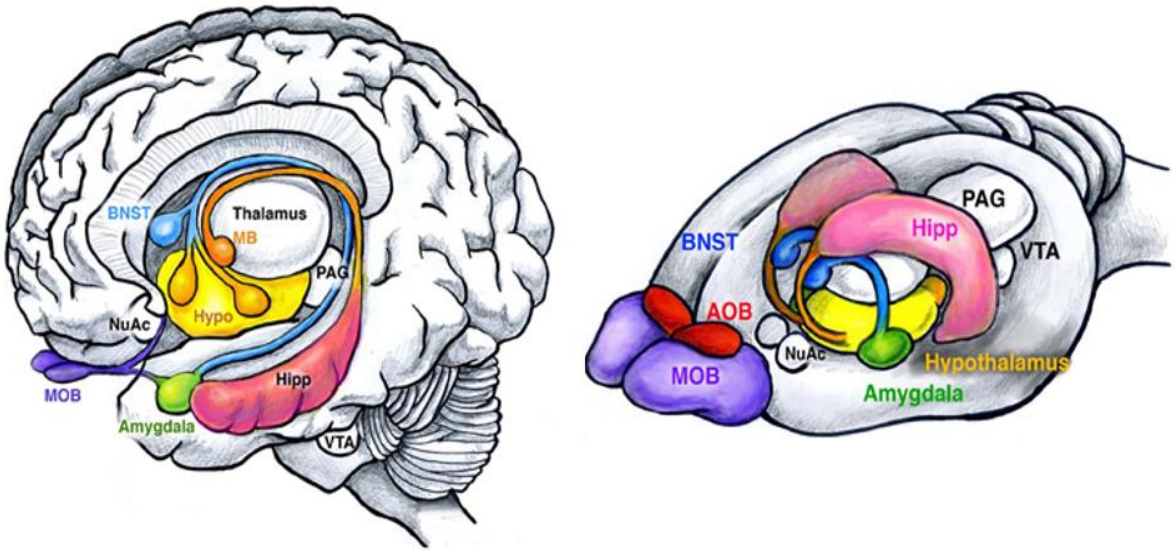

Figure 1.26. Main structures of the human and rodent limbic system. Left, Schema of human brain highlighting limbic structures; Right, schema of mouse limbic system. The amygdala is depicted in green, bed nucleus of stria terminalis (BNST) in blue), hypothalamus in yellow, and hippocampus in pink. Attached to the hippocampus through the fimbria-fornix are the mamillary bodies (in orange). Other structures are the nucleus accumbens (NuAc), The (VTA), the olfactory bulb (MOB), and the periaqueductal gray (PAG). (Adopted from Sokolowski *et al.* 2012)

Intrinsic circuitry of the hippocampus

In rodents, CA regions have four distinct layers. 1. **Stratum oriens** (SO), which contains the cell bodies of the basket cells, and the basal dendrites of the pyramidal cells. 2. **Stratum pyramidale** (SP), which contains the cell bodies of the pyramidal cells. 3. **Stratum Radiatum** (SR), Containing, among other fibres, the CA3 projection fibres to the CA1, the Schaffer Collaterals, and some interneurons. 4. **Stratum lacunosum moleculare** (SLM), which contains among other fibres the perforant pathway fibres, coming from EC layer III to the CA1[384].

The major inputs to the hippocampus come from layers II and III of the EC through the perforant pathway (PP), with minor contributions from layers IV and V. Layer III and IV projections synapse on the CA1 pyramidal cells and the subiculum through temporoammonic pathway. PP itself has two major components, the lateral

perforant pathway (LPP) originating from the lateral EC and the medial perforant pathway (MPP), originating from medial EC. The DG granular cells transfer the information they receive to the CA3 pyramidal cells through mossy fibres. Multiple granular neurons may synapse on a single pyramidal CA3 cell. CA3 pyramidal cells then transfer the information through Schaffer collaterals to the CA1 pyramidal cells, forming synapses on the SR layer. There are extensive ipsilateral and contralateral commissural projections from the CA3 regions to the CA1 areas in rodents. CA1 projects to the subiculum and to the entorhinal cortex layer V (Fig. 1.27). This network is extended to the perirhinal and post-rhinal cortices; the perirhinal cortex projects to and receives projections from the lateral EC while the post-rhinal cortex projects to the medial EC and receives its projections[385-387].

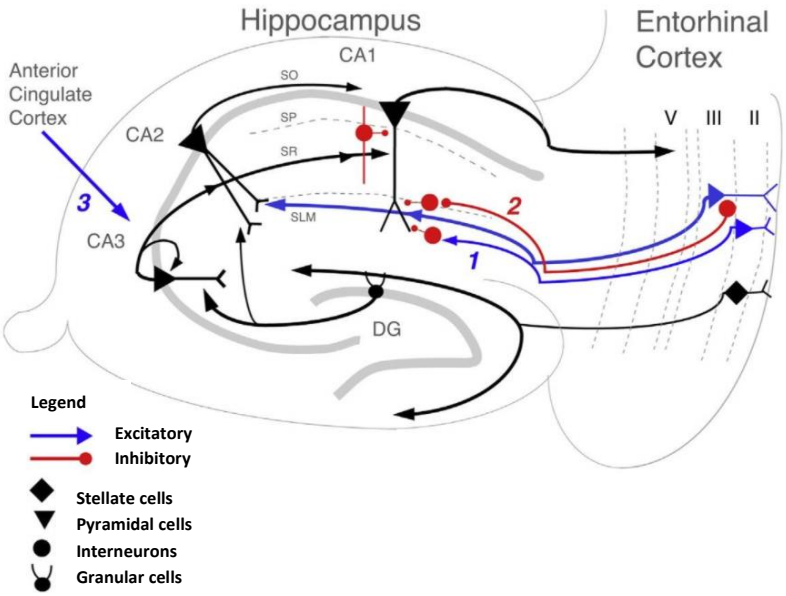

Figure 1.27. The information processing circuits in the hippocampus. Excitatory inputs from EC layer III project to the distal dendrites of the CA1 and CA2 pyramidal neurons in the SLM. EC layer II stellate cells directly project to the DG. DG granule cells then in turn project to the CA3 pyramidal neurons through the mossy fibers, with some weaker projections to the CA2 region. CA3 neurons are also recurrently connected and provide a major input to CA1 pyramidal neurons' proximal dendrites in the SR layer, through Schaffer collaterals. CA1 pyramidal neurons provide the major hippocampal output sent to the layer V of the EC, completing the hippocampus-EC loop. (Adopted from Zemla *et al.* 2017).

Differential role of dorsal and ventral hippocampus in regulating behaviour

Lesion, connectivity, and gene expression analyses suggest different roles for dorsal and ventral hippocampus regions. The dorsal hippocampus is involved in learning, memory, and spatial navigation, while the ventral hippocampus regulates motivated behaviour and emotions[388-390]. For example, the ventral hippocampus receives denser serotonergic projections and shows a higher expression for 5HT1A and 5HT2C serotonin receptors[389]. The ventral hippocampus has extensive connections with NA and participates in the modulation of reward circuitry and emotional behaviour[391]. The DG in both regions can produce new neurons in adulthood. However, the dual role of granule cells in learning and emotional behaviour suggests a possible dissociation between DG functions in dorsal and ventral regions[391]. Despite the separate roles and functions of these regions, they should not be thought of as fully separated areas, as they have extensive connections and operate in coordination with each other[389] (Fig. 1.28).

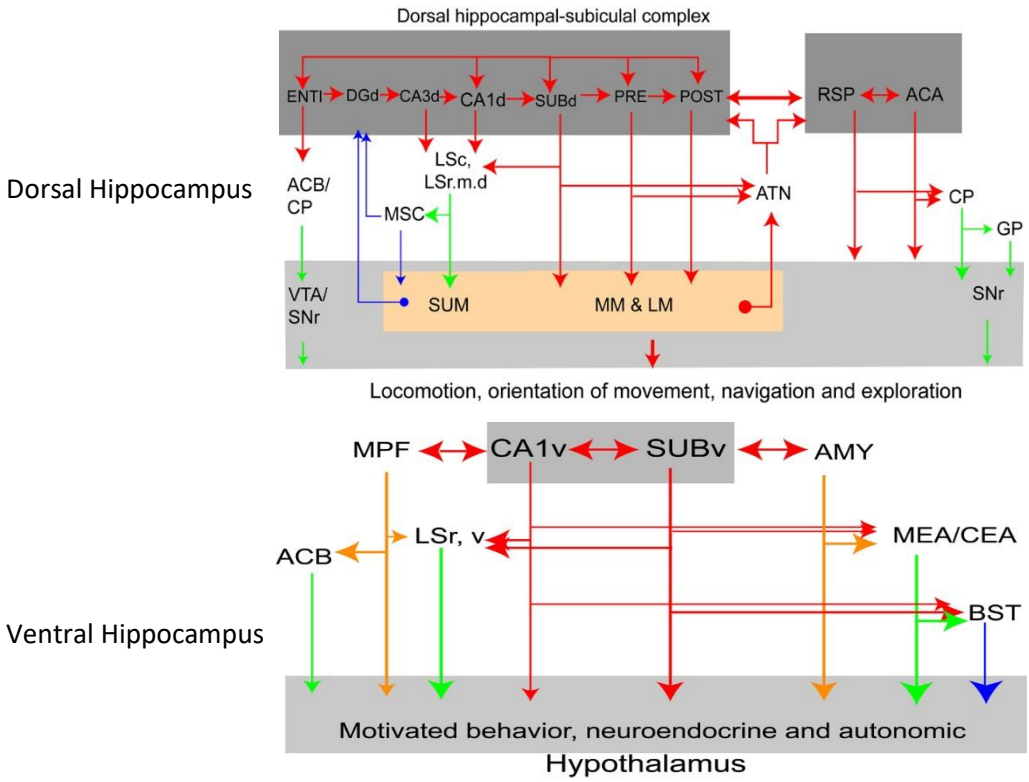

Figure 1.28. Organization of dorsal and ventral hippocampus neuronal circuits. Top panel. Efferent and afferent structures of dorsal hippocampus. "ACA anterior cingulated area; ACB, nucleus accumbens; ATN, anterior thalamic complex; CP, caudoputamen; DGd, dorsal domain of the dentate gyrus; ENTl, the caudolateral band of the entorhinal cortex; GP, globus pallidus; LM, lateral mammilary nucleus; LSc, the caudal part of the lateral septal nucleus; MM, medial mammilary nucleus; MSC, medial septal complex; PRE, presubiculum; POST, postsubiculum; RSP, retrosplenial cortex; SNr, reticular part of the substantial nigra; SUBd, dorsal subiculum; SUM, supramammillary nucleus; VTA, ventral tegmental area". **Lower panel.** Efferent and afferent structures of ventral hippocampus. "ACB, nucleus accumbens; AMY, cortical-like amygdalar areas (nuclei); BST, bed nuclei of the stria terminalis; CEA, central amygdalar nucleus; LSr, v, the rostral and ventral parts of the lateral septal nucleus; MEA, medial amygdalar nucleus; MPF, medial prefrontal cortex; SUBv, the ventral subiculum" (Adopted from Fanselow *et al.* 2010).

Hippocampus in the pathophysiology of schizophrenia

The plastic structure and function of the hippocampus make it a crucial component of the brain; this also means that the hippocampus is affected in a wide range of neuropsychological disorders, including temporal lobe epilepsy, amnesia, dementia, and schizophrenia. The most obvious and consistent change in the schizophrenia-affected hippocampus is the reduction in its volume[392]. Surprisingly, in contrast to many other neuropsychological disorders, the total number of neurons is

not significantly reduced in schizophrenia. However, there have been reports of abnormal function and reduced number of a selective population of hippocampal interneurons[393]. At the molecular level, investigations have indicated that $GABA_A$ receptors, AMPARs and kainate receptors and, to a lesser extent, NMDARs are abnormal in schizophrenia[394]. In addition, gene expression analysis has shown downregulation in genes related to the GABAergic system, neurodevelopment, and synaptic function[395].

Neuroimaging studies show increased hippocampal activity in schizophrenia. This is believed to be due to abnormal GABAergic activity. Post-mortem analyses of schizophrenia-affected brains have also confirmed the GABAergic abnormality of schizophrenia. Post-mortem analyses indicated decreased expression at the level of gene and protein in somatostatin-positive and parvalbumin-positive interneurons and reduced interneuron numbers. In animal models with decreased parvalbumin and reduced NMDAR function some of the schizophrenia-related cognitive deficits and hippocampal hyperactivity were observed[396-398].

1.3 Rationale and objectives

LRRTM1 is an important excitatory synapse organizer, is strongly associated with schizophrenia[88] and is highly expressed in the thalamus and hippocampus proper[200]. MD, a major thalamic nucleus, forms prominent reciprocal connections with the prefrontal cortex and is important for working memory and attentional tasks. *We hypothesize that LRRTM1 is critically important for excitatory synapse development and function in the MD and that its deletion in mice, similar to copy number deletions in human patients, may lead to disruption of information flow between the prefrontal*

cortex and MD and lead to cognitive deficits. To investigate this hypothesis, I have assessed cognitive function and behaviour in mice in which *Lrrtm1* had been selectively knocked out in the MD (Objective 1). I used Transmission Electron Microscopy, Immunocytochemistry and whole cell patch clamp recordings to study the potential morphological and functional changes in the MD following deletion of *Lrrtm1*. I also performed MRI assisted FDG-PET on the brain of mice after deletion of *Lrrtm1* in the MD to identify any changes that may have occurred in neuronal activity in the brain as a result of *Lrrtm1* deletion in the MD (Objective 2).

Building on the results from the MD study and based on LRRTM1 differential expression pattern in the CA1 Stratum Radiatum and Stratum Lacunosum Moleculare we further hypothesized that Lrrtm1 deletion in the dorsal hippocampal CA1 will disrupt dorsal-hippocampus-associated behaviour and can impair the responsiveness of CA1 pyramidal cells to stuimuli and Long-term potentiation. Therefore, I investigated the effect of *Lrrtm1* deletion in mouse dorsal hippocampal CA1 on their cognitive function and behaviour. In addition, I studied the impact of *Lrrtm1* deletion on input/output response and LTP using field excitatory postsynaptic potential (fEPSP) recordings in stratum radiatum and stratum lacunosum moleculare (Objective 3).

Objective 1. Assessment of cognition and behaviour in mice in which *Lrrtm1* is knocked out in the MD. LRRTM1 is a critical excitatory synapse organizer. Its loss leads to excitatory synapse number reduction in the mouse hippocampus. Moreover, these mice exhibit behavioral deficits such as avoiding small enclosures[399], altered behavioral responses to novel environments and novel social situations[400]. LRRTM1 is highly expressed in the thalamus including in the MD, which is enriched in glutamatergic synapses. The MD is required for working memory and working memory

related attentional tasks, social interaction and proper sensory motor gating. *We therefore hypothesized that mice lacking Lrrtm1 in the MD will show deficits in working memory, social interaction, and sensory motor gating.*

Objective 2. Analyses of morphology and function of MD excitatory synapses in MD-*Lrrtm1*-cKO mice. *Lrrtm1* global KO mice display wide-ranging deficits in excitatory synapse numbers and vesicular organization in the hippocampus[400]. However, role of LRRTM1 in synapse development and function in thalamic nuclei such as MD, where its expression is exceptionally high, was untested. *We hypothesized that deletion of Lrrtm1 from the MD will lead to reduced function of excitatory synapses in this region which may potentially lead to reduced PFC activity.*

Objective 3. Analysis of function of CA1 SR and SLM excitatory synapses in CA1-*Lrrtm1*-cKO mice, and its role in synaptic plasticity and behaviour. Double knock down of LRRTM1 and LRRTM2 blocks LTP at Schaffer colateral-CA1 synapses in neonatal hippocampus, and severely impairs it in young adult hippocampus[401]. LRRTMs are therefore required for LTP of mature synapses in adult CA1 pyramidal neurons indicating that blocking LTP in neonatal synapses by LRRTM1 KD is not due to impairment of synapse maturation[83]. Genetically deleting *Lrrtm1* and *2* in mature neurons dramatically impairs basal synaptic transmission and disrupts long-term potentiation[402]. Evidence suggest that tyrosine phosphorylation of internalization signal of GluR2 may be important in control of the strength of AMPA receptor-mediated synaptic transmission and may influence expression of certain forms of synaptic plasticity[403]. *We hypothesize that deletion of Lrrtm1 from the CA1 will reduce function of excitatory synapses in this region and compromises synaptic plasticity and leads to impairment of dorsal-CA1-associated behaviour. We also*

suggest that prevention of AMPAR internalization either through synthetic peptides or re-expression of LRRTM1 can rescue the LTP deficiency should it arise.

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Chapter 2[y]

Schizophrenia-associated LRRTM1 regulates cognitive behavior through controlling synaptic function in the mediodorsal thalamus

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2.1 Abstract

Reduced activity of the mediodorsal thalamus (MD) and abnormal functional connectivity of the MD with the prefrontal cortex (PFC) cause cognitive deficits in schizophrenia. However, the molecular basis of MD hypofunction in schizophrenia is not known. Here, we identified leucine-rich-repeat transmembrane neuronal protein 1 (LRRTM1), a postsynaptic cell-adhesion molecule, as a key regulator of excitatory synaptic function and excitation-inhibition balance in the MD. *LRRTM1* is strongly associated with schizophrenia and is highly expressed in the thalamus. Conditional deletion of *Lrrtm1* in the MD in adult mice reduced excitatory synaptic function and caused a parallel reduction in the afferent synaptic activity of the PFC, which was reversed by the reintroduction of LRRTM1 in the MD. Our results indicate that chronic reduction of synaptic strength in the MD by targeted deletion of *Lrrtm1* functionally disengages the MD from the PFC and may account for cognitive, social, and sensorimotor gating deficits, reminiscent of schizophrenia.

2.2 Introduction

The mediodorsal nucleus of the thalamus (MD) has profuse reciprocal connections with the prefrontal cortex (PFC)[1-3] and serves as a hub for information processing and transfer to the PFC. The MD also receives synaptic inputs from the basal ganglia, basolateral amygdala, and other cortical and thalamic regions, thus integrating, organizing, and relaying diverse signals to the PFC[2,4,5]. Morphological abnormalities in postmortem brains, and imaging and lesion studies indicate that MD is a key brain region impaired in schizophrenia[6-11]. Reduced MD activity and its impaired functional connectivity with the PFC contribute to cognitive impairment in

schizophrenia[8,12,13]. In accord, reduced MD activity by chemogenetic or optogenetic silencing of MD projection neurons in mice mimic some of the core cognitive deficits in schizophrenia[13-15]. However, the molecular pathways disrupted in the MD that lead to cognitive deficits in schizophrenia have not been identified.

To identify candidate molecular targets the disruption of which could account for cognitive impairment in schizophrenia, we applied three stringent criteria: one, high relative expression in the MD; two, strong genetic association with schizophrenia, and; three, evidence for function at glutamatergic synapses because PFC projections to the MD are largely glutamatergic[16]. We confirmed our top candidate, leucine-rich-repeat transmembrane neuronal protein 1 (LRRTM1), as a critical regulator of excitatory synaptic function in the MD and found that it not only regulates the excitability and synaptic properties of MD neurons but also influence how the MD, in turn, controls PFC activity[15,17].

LRRTMs are a family of four type 1 transmembrane proteins with discrete expression patterns in the brain[18]. LRRTM1 is enriched in the thalamus, including the MD, across species, from mouse to pig to human[18-20], indicating that its function in thalamic nuclei is evolutionarily conserved. LRRTMs are localized at excitatory postsynapses and contribute to synapse organization and long term forms of plasticity in the hippocampus[21-26]. We previously reported that in cell culture, retention of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) at synapses was reduced in the absence of LRRTMs[23]. However, the molecular mechanism by which LRRTM1 contributes to synaptic function at physiological synapses in the brain is not understood.

LRRTM1, on chromosome 2p12, was associated with schizophrenia/schizoaffective disorder in a meta-analysis study of 1002 affected

families (P=0.0014)[20] and supported in subsequent studies[27,28]. Likewise, genes encoding the neurexins, presynaptic partners of LRRTM1[29], are major risk factors in schizophrenia[30-32]. Constitutive deletion of *Lrrtm1* in mice altered hippocampal synapse morphology, and produced behavioral deficits in response to novel objects, social behavior, and spatial memory, and aversion to closed spaces[21,25,33]. However, these conclusions are confounding because *Lrrtm1* global deletion also leads to disrupted visual behavior due to impaired retinothalamic convergence[34].

To elucidate the molecular and circuit mechanisms through which *Lrrtm1* deletion impairs cognitive function, we generated *Lrrtm1* conditional knockout restricted to MD projection neurons. We found that the deletion of *Lrrtm1* in MD projection neurons of mice reduced excitatory synaptic transmission and altered firing pattern in the MD. Remarkably, *Lrrtm1* deletion in the MD caused a parallel impairment of afferent synaptic activity in the PFC. This deficit was fully rescued and reversed by the reintroduction of LRRTM1 in the MD. Mice with *Lrrtm1* deleted in the MD displayed cognitive and social deficits, and had impaired sensorimotor gating, a known endophenotype of schizophrenia. At the mechanistic level, we found that LRRTM1 strongly associates with AMPARs in native brain synaptic fractions and that MD synapses lacking *Lrrtm1* have significantly reduced levels of AMPARs, indicating that LRRTM1 maintains excitatory synaptic strength in the MD through maintaining the normal complement of AMPARs at MD synapses. Our study identifies reduced expression of LRRTM1, observed in human patients[20,27], as a plausible molecular basis of MD hypofunction in schizophrenia. We further propose that LRRTM1 and its interactome are potential targets for therapeutic intervention in a subset of schizophrenia patients.

2.3 Materials and methods

2.3.1 Mice and Stereotaxic Injection

All animal procedures conformed to the guidelines of the Canadian Council on Animal Care (http://www.ccac.ca) and were approved by the Animal Care Committee of the University of Manitoba. *Lrrtm1*$^{floxed/floxed}$ mice have been described before[23]. *Lrrtm1*$^{floxed/floxed}$ were maintained on the C57Bl/6N background. All experiments were conducted on cohorts of both male and female mice. All behavior studies were conducted and analyzed blind to genetic manipulation. 5-10 weeks old mice were bilaterally injected with adeno-associated viruses (AAVs) expressing Cre or control (Fig. 2.1c, AAV-CamKII-eGFP-Cre, AAV-CamKII-eYFP, rAAV pmSyn1-EBFP-Cre, rAAV pmSyn1-EGFP). Table S2.1 lists the details of the viruses used. Stereotaxic injections (AP:ML:DV (in mm from bregma): -1.30:±0.50:-3.20 (MD) and +2.1:±0.50: -1.5, -1.7, -2.0 (prelimbic cortex, mPFC)) were performed as described[23].

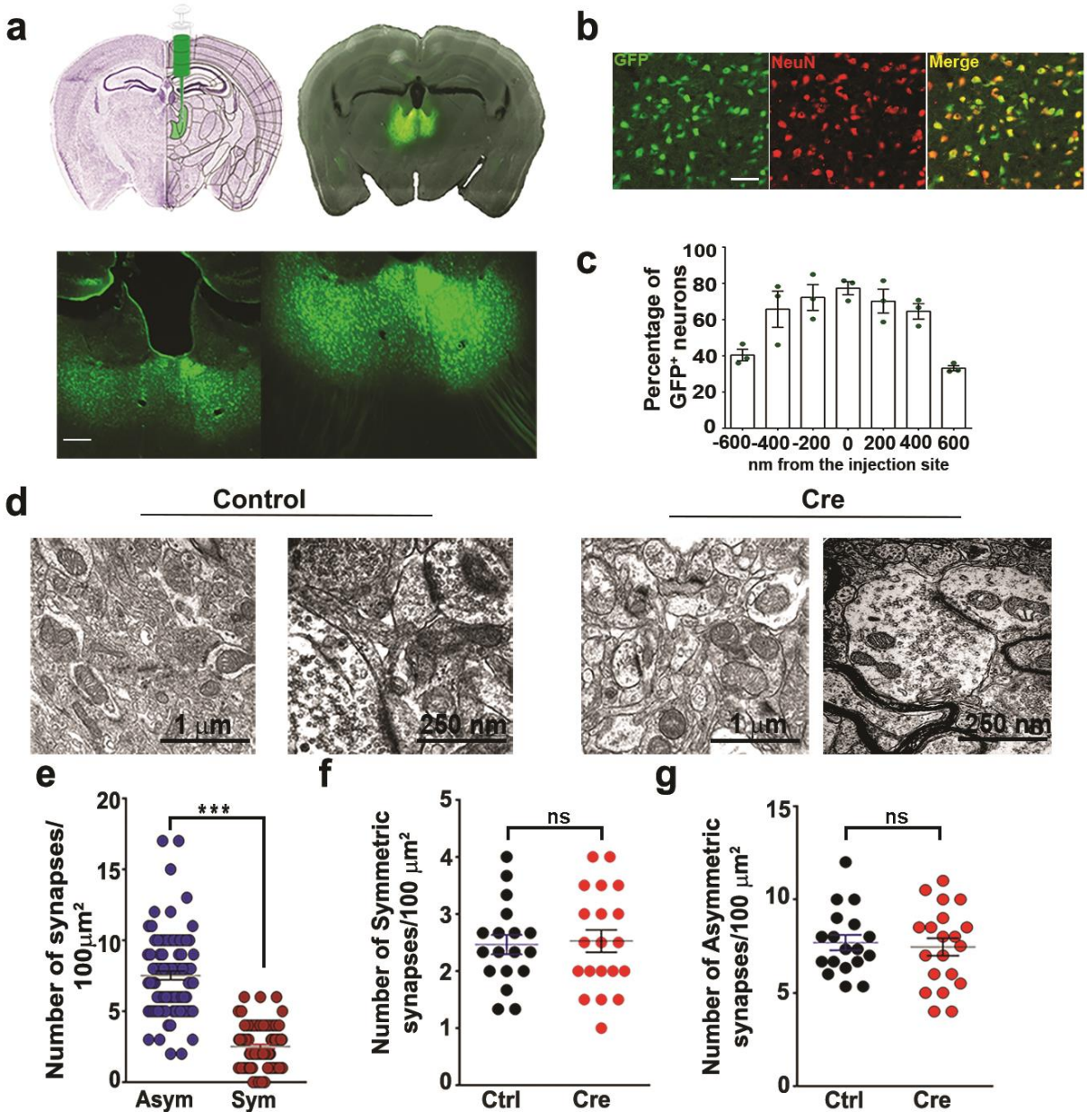

Figure 2.1. Conditional knockout of *Lrrtm1* in the MD. a. Injection of AAVs in the MD showing the site and spread of virus. b. Expression (GFP) was confined to neurons (NeuN). **c.** Infection efficiency of AAV in neurons across the anterior-posterior axis of MD, n=3 mice. **d.** Ultrastructural analysis by transmission electron microscopy revealed comparable number of synapses in control and MD-*Lrrtm1*-cKO (Cre) mice. **e.** Number of excitatory (asymmetric) synapses was significantly higher than inhibitory (symmetric) synapses in wildtype mice, ***p<0.0001, unpaired t-test. Number of asymmetric **(f)** and symmetric **(g)** synapses remained unchanged in the adult MD after acute deletion of *Lrrtm1*. Data represents mean ± SEM. n=3 mice per group, 15-20 fields per mouse. Scale bars in A: 200 µm, in B: 15µm.

2.3.2 Slice electrophysiology, Immunocytochemistry and Transmission electron microscopy (TEM)

Coronal vibratome brain slices were used for whole-cell patch-clamp recordings of miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs) in the presence of tetrodotoxin as described[23,35] (for more details, see supplementary methods). Immunocytochemistry of brain sections was performed and analyzed essentially as described[36]. Imaging was performed with Observer Z.1 equipped with Apotome 2.0 for optical sectioning (Zeiss) after staining with primary antibodies (GAD65; Developmental Hybridoma Bank, VGlut1; Synaptic Systems, anti-GluA1 and anti-GluA2; Frontier Institute, Japan and anti-NeuN; Millipore) and florescent secondary antibodies. TEM was performed on isolated MDs, and synapse numbers were measured per 100 μm^2 area at 10,500x and 46,000x magnification as described[37] using Philips CM10 electron microscope. Details on all the solutions used for voltage clamp recordings are listed in Table S2.2.

2.3.3 Behavioral tests

Accelerating rotarod, elevated plus maze, novel object recognition and Crawley's sociability and social novelty preference tests were conducted and analyzed as described [38-41] (for more details, see supplementary methods).

Acoustic startle reflex and pre-pulse inhibition (PPI) were assessed as described[37]. Background noise (70 dB), pre-pulse and pulse stimuli were provided by a speaker in the chamber. To assess the startle amplitude a 120 dB, stimulus was presented for 30-ms alone, without a pre-pulse. Mice were then presented with a 30-ms pre-pulse stimulus followed by PPI assessment at one of 73, 79 or 85 dB noise intensities.

2.3.4 FDG-PET

All positron emission tomography (PET) imaging acquisitions were performed on a 7T MR solutions flexiscan benchtop PET-MRI imaging system essentially as described[42]. Briefly, after 12-hour fasting, mice were anesthetized and intraperitoneally injected with 10 MBq of $^{[18F]}$fluoro-deoxyglucose (FDG). Fast spin echo T2 weighted MRI was acquired in axial slices with a TR of 5000 and a TE of 45, with a flip angle of 90^{o}. Images had a matrix size of 256 x 245 and a slice thickness of 0.5 mm. Static $^{[18]}$FDG-PET images had an acquisition time of 15 minutes and were reconstructed using 2D filtered back projection.

2.3.5 RT-qPCR, Immunoprecipitation, and Immunoblotting

mRNA quantifications were performed on total RNA isolated from freshly-dissected thalamus, as described[43]. TaqMan-based qPCR (Cells-to-C_T/TaqMan assay kits, Thermo-Fisher Scientific) was performed on neurons isolated from micro-dissected MD by fluorescent-associated cell-sorting (FACS). Immunoprecipitation and immunoblotting experiments were performed as described[36]. Co-immunoprecipitations were performed with a custom anti-LRRTM1 antibody and immunoblotted with anti-GluA1, anti-GluA2 (gifts from Yu Tian Wang, University of British Columbia), and anti-PSD95 (Neuromab) followed by a conformation specific secondary antibody. Table S2.3 lists all the primer sequences used in SYBR™ Green qPCR assays.

2.3.6 Statistical analyses

Statistical analyses were conducted using unpaired t-test or two-way ANOVA followed by Tukey's multiple comparison post-hoc test, or multiple t-tests using Sidak-Bonferroni method, with alpha=5%. Statistical significance was set to $p<0.05$. For

voxel-based FDG PET image analysis, $p<0.01$ with extent threshold of >20 was considered significant. Data shown are mean ± SEM. Microsoft Office Excel 365 and GraphPad Prism 6 were used to perform the analyses. To ensure that standard deviations were comparable in unpaired t-tests, an F test was performed. If variances were significantly different, unpaired t-test was done with Welch's correction.

See SI Appendix, for details on all procedures in this study.

2.4 Results

2.4.1 Conditional KO of *Lrrtm1* in the MD impairs excitatory synaptic transmission

To determine the expression levels of synaptic genes in the mouse MD, we extracted total RNA from micro-dissected MD and measured mRNA expression levels using custom TaqMan array© plates. We assessed the expression levels of 83 synaptic genes (Figure S1.1). Of the 42 postsynaptic synapse organizers, *Lrrtm1* had the highest expression. Only *Lrrc4b* and *Nlgn2* had comparable expression levels. Previous *in situ* hybridization studies confirmed enrichment of LRRTM1 in the thalamus (Fig. S2.2a)[18]. To determine the relative enrichment of LRRTM1 in the thalamus, we micro-dissected the thalamus and hypothalamus, a control region, from young adult mice and quantitated the levels of *Lrrtm1*, *Lrrtm4,* and control *Gapdh* transcripts. *Lrrtm1* was significantly enriched in the thalamus when compared to that in the hypothalamus (Fig. S2.2b). In contrast, *Lrrtm4* was expressed at a lower level in the thalamus.

The rodent MD is mostly devoid of interneurons and is comprised exclusively of excitatory projection neurons[44]. To delete *Lrrtm1* selectively in the MD, we stereotactically delivered AAV-CamKII-eGFP-Cre (MD-*Lrrtm1*-cKO) or AAV-CamKII-eYFP (control) bilaterally to the MD of *Lrrtm1*$^{floxed/floxed}$ mice[23] (Fig. 2.1a, S2.2c). Staining with neuron-staining anti-NeuN antibody revealed that AAV targeting, assessed by GFP expression, was exclusively neuronal (Fig. 2.1b). An average of 62% ± 4% of all NeuN-positive MD neurons expressed GFP, maximally 77% ± 7% at the targeted injection site (Fig. 2.1c). The virus spread along the entire expanse of the anteroposterior axis within the MD but remained largely confined within its dorsoventral and mediolateral axes, with only occasional spread beyond the MD.

To assess whether *Lrrtm1* was successfully deleted in Cre^{+ve} neurons, we performed fluorescence-assisted cell sorting (FACS) on micro-dissected MD from MD-*Lrrtm1*-cKO and control mice to collect GFP^{+ve} cells. We extracted total mRNA from the sorted cells and quantitated the levels of *Lrrtm1* and *Gapdh* transcripts using TaqMan-based RT-qPCR. The level of control *Gapdh* was comparable between the Cre^{+ve} and control cells. Whereas *Lrrtm1* was highly expressed in control cells, it was undetectable in Cre^{+ve} cells confirming effective deletion of *Lrrtm1* in MD projection neurons (Fig. S2.2d, e).

To investigate whether LRRTM1 contributes to synaptic maintenance and density in the mature MD, we performed immunocytochemical analysis of VGlut1 and GAD65, representing glutamatergic and GABAergic presynaptic terminals respectively, in the MD (Fig. S2.3a, c). Quantitative imaging revealed that both VGlut1 and GAD65 synaptic immunofluorescence puncta were comparable in the MD of MD-*Lrrtm1*-cKO and control mice (Fig. S3b, d), indicating that both excitatory and inhibitory presynaptic

inputs to the MD were unaltered. To examine synapse numbers with more stringent criteria at the ultrastructural level, we assessed asymmetric and symmetric synapse density, representing excitatory and inhibitory synapses, respectively, by transmission electron microscopy (TEM) (Fig. 2.1d). Consistent with previous reports, the number of asymmetric synapses outnumbered symmetric synapses in the MD by nearly four times (Fig. 2.1e). However, there was no difference in the number of asymmetric or symmetric synapses between the MD of MD-*Lrrtm1*-cKO and control mice (Fig. 2.1f, g). Thus, the deletion of *Lrrtm1* in mature MD neurons did not alter either excitatory or inhibitory synapse numbers.

To determine whether *Lrrtm1* deletion in the MD alters ultrastructural features of synapses, we assessed postsynaptic density thickness, active zone length, synaptic cleft width, total number of synaptic vesicles and number of docked vesicles/active zone length (Fig. S2.4). All of these parameters remained unchanged in the MD-*Lrrtm1*-cKO compared to control mice.

To determine the contribution of LRRTM1 to synaptic function, we performed whole-cell voltage-clamp recordings from GFP^{+ve} MD neurons in acute brain slices from MD-*Lrrtm1*-cKO and control mice (Figs. S2.5a, b). Miniature excitatory postsynaptic current (mEPSC) amplitude was significantly reduced (~18%) in projection neurons in MD-*Lrrtm1*-cKO mice relative to that in control mice (Fig. 2.2a-c). mEPSC frequency was not significantly different between the two groups, confirming our imaging and electron microscopy results. Miniature inhibitory postsynaptic current (mIPSC) amplitude and frequency were comparable between the MD-*Lrrtm1*-cKO and control mice (Fig. 2.2d-f). Thus, in the MD of adult mice, LRRTM1 controls excitatory synaptic transmission but not synapse numbers. The selective

reduction in excitatory synaptic function in the MD of MD-*Lrrtm1*-cKO mice indicates that LRRTM1 is a regulator of excitation-inhibition balance in the MD.

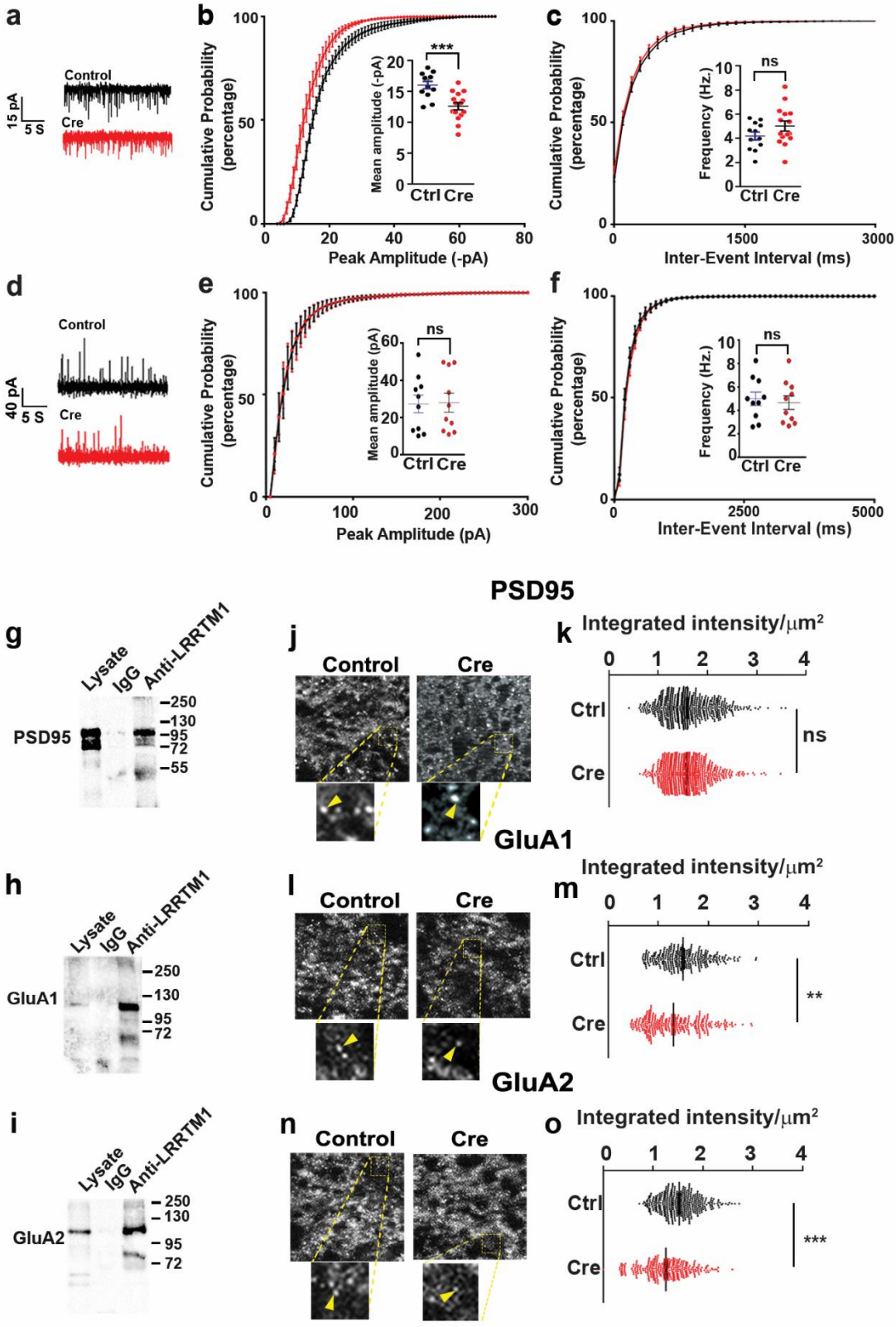
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Figure 2.2. *Lrrtm1* deletion in mature MD neurons reduces excitatory but not inhibitory synaptic strength and reduces synaptic levels of AMPAR subunits. a-c. mEPSC recording from MD neurons directly injected with AAVs, 12-15 neurons from 3-4 mice per group. **a.** Representative traces **b.** Cumulative frequency of event amplitudes recorded from GFP^{+ve} control and MD-*Lrrtm1*-cKO neurons. Inset bar graph represents mean amplitude, p=0.009 by the Kolmogrov-Smirnov test; bar graphs were analyzed by unpaired t-test, ***p=0.0005. **c.** Cumulative frequency of interevent intervals of GFP^{+ve} control and MD-*Lrrtm1*-cKO (Cre) neurons. Inset bar graph represents frequency of events. **d-f.** mIPSC recording from MD projection neurons injected with AAVs **d.** Representative traces **e.** Cumulative frequency of event amplitudes recorded from GFP^{+ve} control and MD-*Lrrtm1*-cKO neurons. Inset bar graph represents mean amplitude, assessed by the Kolmogrov-Smirnov test, bar graphs were analyzed by the unpaired t-test. **f.** Cumulative frequency of interevent intervals of GFP^{+ve} control and MD-*Lrrtm1*-cKO (Cre) neurons. Inset bar graph represents frequency of events, n=10 neurons for either group from 3 mice. **g-i.** An anti-LRRTM1 antibody co-immunoprecipitates PSD-95, GluA1 and GluA2 from synaptic fractions (1% input). **j-o.** Immunocytochemistry of coronal brain sections from MD-*Lrrtm1*-cKO (Cre) and control mice revealed comparable levels of PSD-95 but reduced AMPARs containing GluA1 and GluA2 subunits co-localized with PSD-95, **p=0.005 **(m)** and ***p<0.0001**(o)** by unpaired t-test, 3 mice per group, 8 sections per mouse, 8 images per section. Data represents mean ± SEM, Scale bar is 5µm.

2.4.2 Alteration of excitatory synaptic transmission in the absence of LRRTM1 is caused by a postsynaptic mechanism

Reduced mEPSC but not mIPSC amplitude in our experiments suggest that the deletion of *Lrrtm1* selectively leads to impaired function of glutamatergic postsynapses in the MD. LRRTM2 and LRRTM4 bind to PSD-95, a critical scaffolding and signaling protein at excitatory postsynapses, through their C-terminal PDZ-binding motif[21,36]. The PDZ-binding motif is conserved in LRRTM1, indicating that like LRRTM2 and LRRTM4, LRRTM1 may bind to PSD-95[18]. We first determined whether LRRTM1 associates with PSD-95 in the mouse brain. An anti-LRRTM1 but not a control antibody co-immunoprecipitated PSD-95 from synaptosomes (Fig. 2.2g). Loss of LRRTM1 and LRRTM2 in cultured neurons led to unstable AMPARs at synapses, but the mechanism underlying this phenomenon is unclear[23]. We, therefore, determined whether LRRTM1 might also associate with AMPARs. Indeed, anti-LRRTM1 but not a control antibody robustly co-immunoprecipitated both AMPAR subunits tested, GluA1 and GluA2 (Fig. 2.2h, i). To explore the mechanism underlying the impairment of

postsynaptic function in MD projection neurons lacking *Lrrtm1*, we performed quantitative imaging of PSD-95 and co-labeled AMPAR subunits GluA1 and GluA2 in the MD (Fig. 2.2j-o). Quantitative analysis revealed that the puncta immunofluorescence of PSD-95 was comparable between MD-*Lrrtm1*-cKO and control mice, and further confirming our assessment that *Lrrtm1* deletion did not alter synapse density in the MD. However, the synaptic levels of GluA1 and GluA2 in the PSD-95 co-labeled regions were significantly reduced in MD-*Lrrtm1*-cKO mice relative to that in control mice. Thus, loss of LRRTM1 leads to reduced levels of AMPARs containing GluA1 and GluA2 at MD synapses, likely because LRRTM1 captures and retains AMPARs at synapses. Reduced AMPARs at synapses lead to a reduction of synaptic efficacy and strength and account for impaired excitatory transmission in the MD projection neurons in MD-*Lrrtm1*-cKO mice.

2.4.3 Conditional deletion of *Lrrtm1* in the MD impairs cognitive function

To assess the impact of impaired synaptic function in the MD of MD-*Lrrtm1*-cKO mice on behavior, we performed a series of assays to examine motor, sensory and cognitive function. First, we subjected the mice to the rotarod motor learning task. Both the MD-*Lrrtm1*-cKO and control mice had normal motor learning ability (Fig. S2.6a, b), indicating that reduced excitatory synaptic transmission in the MD does not alter gross motor function.

Early postnatal lesions of the rat MD led to anxiety-related behavior in the elevated plus-maze (EPM) task[45]. To assess whether reduced synaptic function in the MD in mice phenocopied this behavior, we tested for anxiety-related avoidance behavior using the EPM (Fig. S2.6c-f). MD-*Lrrtm1*-cKO mice spent significantly less time than control mice in the open arms of the EPM even though the number of entries to the

open arms was comparable between the MD-*Lrrtm1*-cKO and control mice. The total distance traveled, and the average speed of both groups of mice was comparable. Thus, the conditional deletion of *Lrrtm1* in the MD led to anxiety-related avoidance behavior.

MD hypofunction or lesion leads to deficits in working memory, object recognition memory, and behavioral flexibility in rodents[13,14,45,46]. The novel object recognition (NOR) task (Fig. 2.3a) tests for allocentric memory without food deprivation or prolonged training, thus avoiding undue stress to mice. The NOR task also tests for behavioral flexibility because a mouse has to decide to choose between a novel object and a familiar one, thus making it a popular test used in animal models of schizophrenia[47]. We, therefore, tested memory and behavioral flexibility in MD-*Lrrtm1*-cKO and control mice using NOR. Whereas control mice spent considerably more time with the novel object as compared to the familiar object, there was no measurable difference in the time spent by MD-*Lrrtm1*-cKO between the familiar and novel objects (Fig. 2.3b, c). The total distance traveled, and the average speed of both groups of mice was comparable (Fig. S2.6g, h). Thus, the conditional deletion of *Lrrtm1* in the MD led to impaired object recognition memory.

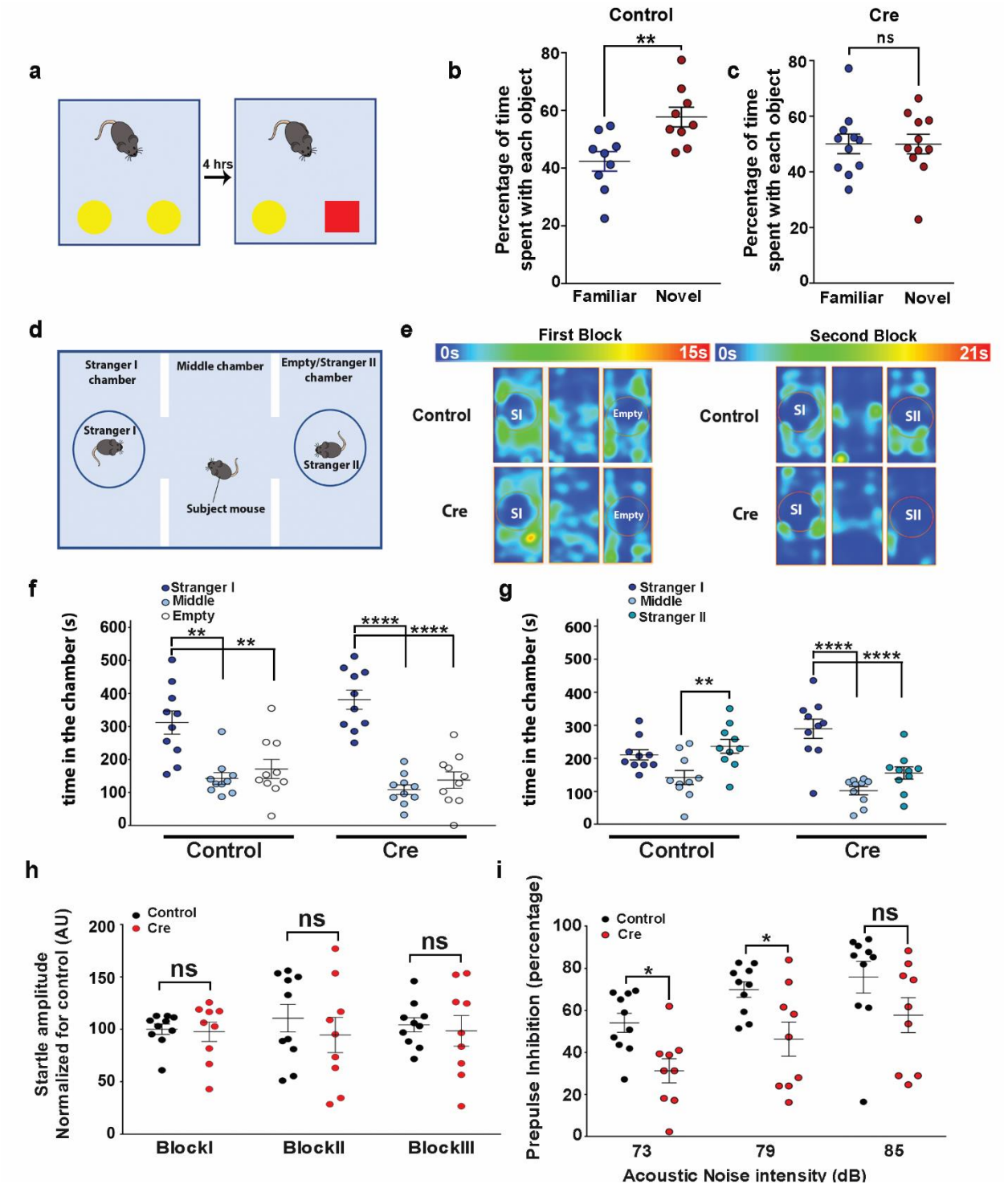

Figure 2.3. *Lrrtm1* deletion in mature MD neurons impairs cognitive and social behavior and disrupts sensorimotor gating. a-c. NOR test, n=10 mice per group. Control mice spent significantly more time with the novel object (**p=0.005 by unpaired t-test) whereas MD-Lrrtm1-cKO (Cre) mice displayed no preference for the novel object. **d-g.** Crawley's three-chamber social interaction and social novelty test, n=10 mice per group. **e.** Average heat map of time spent in each block for control and MD-*Lrrtm1*-cKO (Cre) mice. **f.** Both control and MD-*Lrrtm1*-cKO mice preferred Stranger I over the middle and empty chambers, (**p=0.001 and 0.009, ****p<0.0001, two-way ANOVA and post hoc Tukey's multiple comparison test). **g.** Control mice preferred Stranger II over the middle chamber (**p<0.005, two-way ANOVA and

post-hoc Tukey's test) whereas MD-*Lrrtm1*-cKO had minimal contact with Stranger II (****p<0.0001, two-way ANOVA and post-hoc Tukey's test). **h and i.** Sensorimotor gating is disrupted in MD-*Lrrtm1*-cKO mice, n=9-10 mice per group. **h.** The amplitude of startle response was comparable between the control and MD-*Lrrtm1*-cKO mice. **(i)**, MD-*Lrrtm1*-cKO mice showed reduced pre-pulse inhibition to noise intensities at 73 and 79 dB (*p=0.005 (73 dB), *p=0.014 (79 dB) by multiple t-test, significance determined by Holm-Sidak method. Data represents mean ± SEM.

2.4.4 Conditional deletion of *Lrrtm1* in the MD impairs social novelty but not social affiliation

Deficits in social interaction and social withdrawal are core negative symptoms of schizophrenia and are particularly resistant to treatment[47]. Lesion of MD in rats produced profound deficits in social behavior[45]. We assessed social behavior in MD-*Lrrtm1*-cKO and control mice using Crawley's sociability and social novelty preference three-chambered social approach test[41] (Fig. 2.3d-g). In the first block, both MD-*Lrrtm1*-cKO and control mice spent more time interacting with another mouse (Stranger I) placed in a cage in one of the chambers versus the middle and empty chambers. The number of entries to the chamber housing the Stranger I compared to the middle and empty chambers was higher for both groups of mice. In the second block, a second mouse (Stranger II) was placed in the cage in the third chamber. Control mice spent more time with Stranger II compared to the middle chamber. MD-*Lrrtm1*-cKO mice spent significantly less time with Stranger II when compared to the middle chamber or Stranger I. The total distance travelled and average speed of both groups of mice were comparable (Fig. S2.6i, j). Thus, conditional deletion of *Lrrtm1* in the MD did not alter sociability but reduced social novelty preference.

2.4.5 Absence of *Lrrtm1* in the MD impairs sensorimotor gating

The synchrony between the MD and mPFC is in the beta-frequency range, which has been linked to sensorimotor operations[13,48,49]. The MD, as an integral component of the cortico-striatal-thalamo-cortical circuit, is a major conduit for

processing and transmitting diverse signals to the frontal cortex, and therefore serves as a sensorimotor relay station in the brain[50,51]. Measuring sensorimotor gating models attention processing, which is disrupted in schizophrenic patients[52]. Prepulse inhibition (PPI), commonly used to measure sensorimotor gating deficits, is decreased in both schizophrenia patients and animal models of schizophrenia[52,53]. We used acoustic startle reflex to measure PPI in MD-*Lrrtm1*-cKO and control mice. Both groups of mice displayed comparable amplitudes of the acoustic startle reflex (Fig. 2.3h). However, only MD-*Lrrtm1*-cKO mice showed a significant reduction in the PPI of the acoustic response at specific decibels (at 73 and 70 dB, Fig. 2.3i). Thus, the conditional deletion of *Lrrtm1* in the MD impairs sensorimotor gating.

Our results showing deficits in cognitive function, social behavior, and sensorimotor gating in mice lacking *Lrrtm1* selectively in the MD indicates that these deficits likely stem from abnormal functional connectivity with the PFC. Therefore, we next tested the hypothesis that LRRTM1 regulates functional connectivity of the MD with the PFC.

2.4.6 LRRTM1 regulates excitatory synaptic transmission and firing pattern in MD neurons that project to the PFC

To assess whether LRRTM1 functions at glutamate synapses in the MD that send axonal inputs to the PFC, we injected retrograde (r)-AAV-pmSyn1-EBFP-Cre in the medial (m) PFC of *Lrrtm1*$^{floxed/floxed}$ mice (Fig. 2.4a). rAAV injected in the mPFC was taken up by synaptic terminals within the mPFC via synaptic projections from a sub-population of MD neurons, which expressed EBFP-Cre (Fig. S2.5c). We repeated whole-cell voltage-clamp recordings from EBFP-Cre^{+ve} or control MD neurons in acute brain slices derived from *Lrrtm1*$^{floxed/floxed}$ mice. mEPSC amplitude was significantly

reduced (~17%), but frequency was unchanged in EBFP-Cre^{+ve} relative to control MD neurons (Fig. 2.4b-d).

As intrinsic neuronal electrophysiological properties play a critical role in producing circuit activity[54], we sought to determine if these properties differed between the MD neurons of MD-*Lrrtm1*-cKO and control mice. We injected retrograde AAV pmSyn1-EBFP-Cre or rAAV pmSyn1-EGFP in medial prefrontal cortex of *Lrrtm1*$^{floxed/floxed}$ mice and subsequently performed whole-cell patch-clamp recordings in adult mice from control MD^{GFP+ve} neurons (n=15 from 6 mice) and from MD-*Lrrtm1*-cKO neurons (n=10 from 5 mice). All neurons were held at -60 mV to have a uniform baseline when measuring their responses to current injection. We characterized the spike patterns of MD neurons in response to sustained suprathreshold current steps. Three main patterns emerged in both groups of neurons with differences in the number of neurons for each pattern of spiking. In the control group, 3 of 15 neurons failed to elicit an action potential in response to current injection, six of fifteen neurons generated a single action potential (Fig. 2.4e) and six of fifteen neurons generated multiple action potentials in response to increasing steps of depolarizing current injection (Fig. 2.4f). MD-*Lrrtm1*-cKO neurons had a similar absolute number of neurons that did not respond (n=3, Fig. 2.4h) or generated a single action potential (n =7, Fig. 2.4i) in response to current injection. However, none of the 10 recorded neurons was capable of generating multiple action potentials in response to current injection. In response to hyperpolarizing current, neurons from both groups responded similarly with minimal to zero sag potential and post-inhibitory rebound responses seen in 7 neurons in the control group and 5 in the MD-*Lrrtm1*-cKO group (Figure 2.4g and 2.4j). These results indicate that unlike control MD neurons in which sustained firing was seen in ~1/3 of the recorded neurons, MD-*Lrrtm1*-cKO did not demonstrate the ability to generate

sustained firing in response to current injection. These results may explain why even modest reduction in synaptic strength in the MD neurons, which have reverberant resultant effects on PFC neurons, can lead to a clear decrease in PFC activity. The resting membrane potentials were similar between groups with resting membrane potentials of 51.3 ± 3.4 mV and 49.2 ± 4.5 mV from control and MD-*Lrrtm1*-cKO neurons respectively. Similarly, no difference between groups was seen in spike threshold (-39.9 ± 6.6 mV and-37.8 ± 7.3 mV, p= 0.63), input resistance (174 ± 108 MΩ and 105 ± 34 MΩ, p=0.15) or rheobase (176.8 ± 117 pA and 222 ± 63 pA, p=0.44) from control and MD-*Lrrtm1*-cKO neurons, respectively. Therefore, MD neurons in MD-*Lrrtm1*-cKO neurons had similar cellular properties compared to control MD neurons. We also recorded spontaneous (s) EPSCs from control and MD-*Lrrtm1*-cKO neurons and found that the sEPSC amplitude was significantly reduced (~22%) but sEPSC frequency remained unchanged (Fig. S2.7). Thus, LRRTM1 regulates excitatory synaptic transmission and influences firing pattern in MD neurons that project to the PFC. We then tested whether the gating of information transfer and afferent synaptic activity in the PFC is regulated by LRRTM1 in the MD.

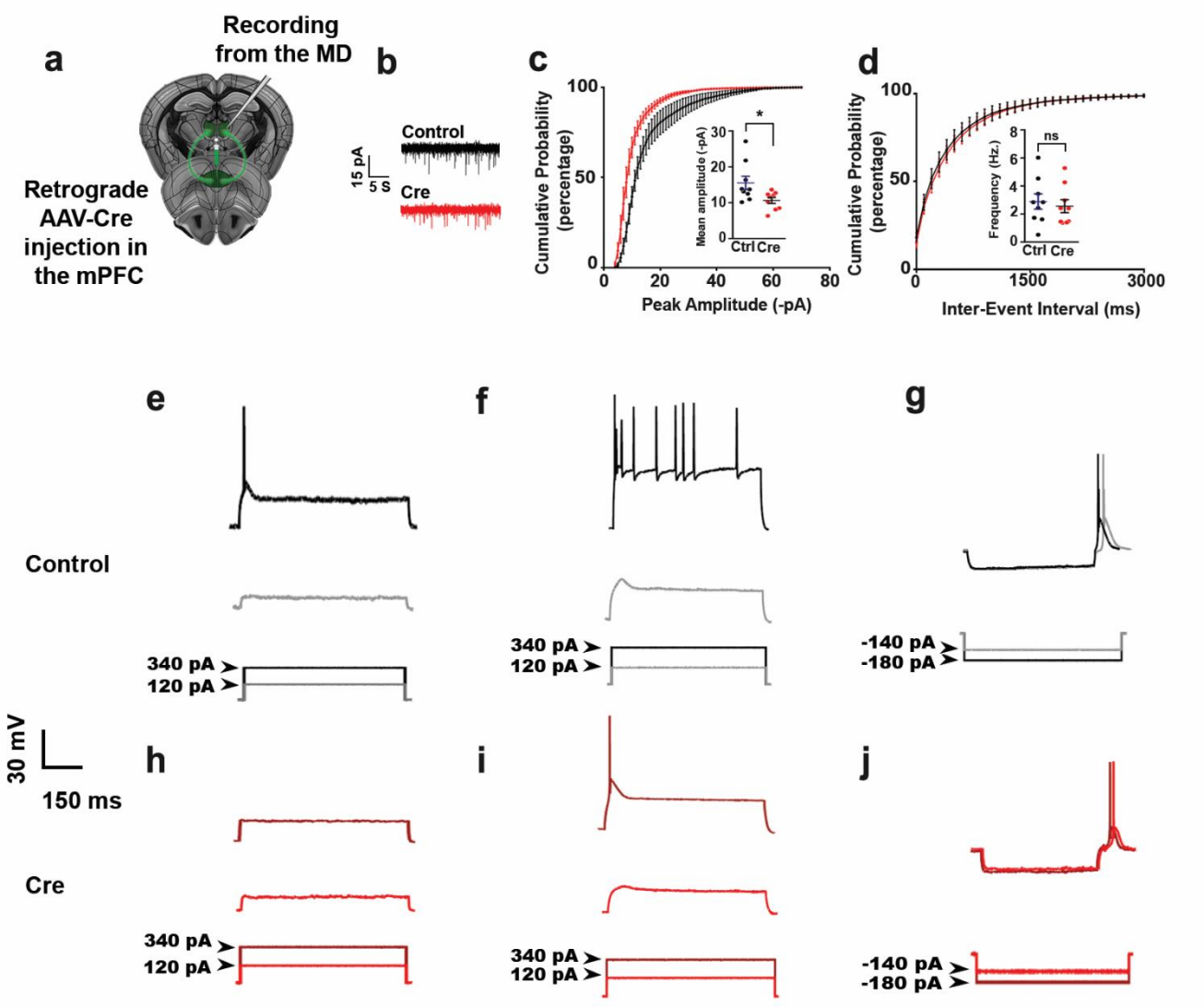

Figure 4. LRRTM1 controls synaptic function in MD relay neurons projecting to PFC. a-d. mEPSC recording from MD neurons from mice injected with retrograde AAV-Cre in the mPFC, 9 uninfected and 9 infected neurons from 3 mice. **b.** Representative traces **c.** Cumulative frequency of event amplitudes recorded from EBFP-Cre^{+ve} and uninfected neurons. Inset bar graph represents mean amplitude, p=0.0091 by the Kolmogrov-Smirnov test; bar graphs were analyzed by unpaired t-test, *p=0.0292. **d.** Cumulative frequency of inter-event intervals of EBFP-Cre^{+ve} and uninfected neurons. Inset bar graph represents frequency of events. **e-j**. Control neurons responded to current injection by generating one spike (**e**, n=6), multiple spikes (**f**, n=6) or failed to elicit a response (n=3). MD-*Lrrtm1*-cKO neurons responded to current injection with no spikes (**h**, n=3) or one spike (**g**, n=7). No MD-*Lrrtm1*-cKO neuron was able to generate multiple spikes. **g** and **j**. Both groups responded similarly to injection of hyperpolarizing currents with minimal to zero sag potential and post-inhibitory rebound response seen in 7 neurons in control group and 5 neurons in MD-*Lrrtm1*-cKO group. Also see Figure S7.

2.4.7 LRRTM1 expressed in MD regulates PFC synaptic activation

Since MD is a gating hub of information processing and transfer in the brain, we adopted an unbiased approach to assess how LRRTM1 expression in the MD affects afferent synaptic activity across the entire mouse brain. To this end, we performed MRI assisted FDG-PET on anesthetized MD-*Lrrtm1*-cKO and control mice. FDG-PET measures local metabolic activity and is a probe for presynaptic activity[55]. We conducted a voxel wise t-test for regions showing significant differences between the two groups of mice at a significance threshold of $p<0.01$ and for clusters greater than 20 voxels. We identified an 84-voxel cluster in the left prefrontal cortex showing significantly reduced FDG-uptake in the MD-*Lrrtm1*-cKO group compared to the control group (Fig. 2.5a-g). There was no region where increased FDG-uptake was observed in the MD-*Lrrtm1*-cKO group. In a separate series of experiments, we reintroduced LRRTM1 in MD-*Lrrtm1*-cKO mice via AAV (pAAV-Ef1a-mCherry-p2A-HA-LRRTM1-DIO) such that LRRTM1 would express exclusively in MD projection neurons in which *Lrrtm1* was deleted by Cre recombinase. Re-expressed LRRTM1 in MD rescued prefrontal hypofunction and increased synaptic afferent activity in the PFC (Fig. 2.5h-k). Thus, LRRTM1 expression in the MD directly influences prefrontal synaptic activity and may account for the behavioral deficits observed in MD-*Lrrtm1*-cKO mice. These results provide a molecular mechanism for how MD controls synaptic activity in the PFC[8,12,56].

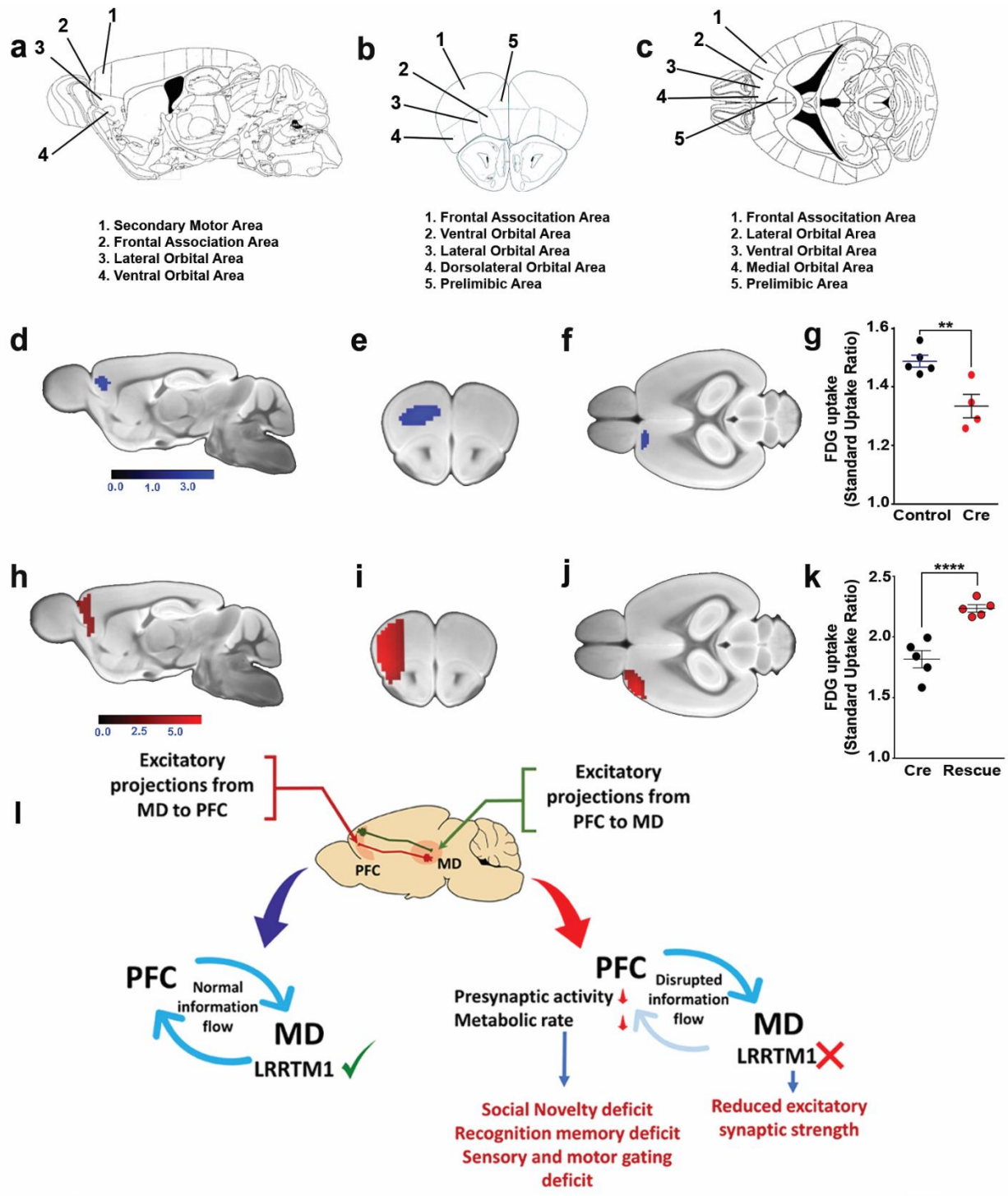

Figure 2.5. *Lrrtm1* deletion in the MD reduces afferent synaptic activity in the PFC. Thresholded t-map of FDG-PET scans showing hypometabolism in the PFC in the MD-*Lrrtm1*-cKO group compared to control mice and hypermetabolism in the PFC in MD-*Lrrtm1*-cKO mice rescued with LRRTM1. **a-c.** Annotated brain section diagrams corresponding to PET images. **d-f.** Average sagittal **(d)**, coronal **(e)** and horizontal **(f)** MRI fitted PET scan images, $p<0.01$ and $k_e>20$, unpaired t-test. Regions highlighted in blue are clusters with significant reduction in FDG uptake in MD-*Lrrtm1*-cKO (Cre, n=4) versus control mice (n=5). **g.** For visualization purposes, the average FDG uptake is extracted from the significant cluster and proportionally scaled to the whole-brain mean value, **p= 0.0082, unpaired t-test. **h-j**. T-map contrasting rescue of MD-*Lrrtm1*-cKO with LRRTM1 (rescue) vs. MD-*Lrrtm1*-cKO, p<0.01 and

k_e>20, unpaired t-test. Regions highlighted in red are clusters with significant increase in FDG uptake in Rescue (n=5) versus MD-*Lrrtm1*-cKO (Cre, n=5). **k**. For visualization purposes, the average FDG uptake is extracted from the significant cluster and proportionally scaled to the whole-brain mean value, ***p<0.0006, unpaired t-test. **l**. Schematic description of the role of LRRTM1 in controlling the MD-PFC circuit and associated behaviors.

2.5 Discussion

The MD is a central processing center and information gateway to the PFC, and impaired functional connectivity of the MD-PFC circuit profoundly alters cognitive function. Our study integrates molecular genetics with comprehensive morphological and functional approaches to provide novel insights into the mechanisms underlying these deficits (Fig. 2.5l). Our study has led us to reach the following conclusions. First, LRRTM1 mediates excitatory but not inhibitory function on MD projection neurons and is, therefore, a key regulator of excitation-inhibition balance in the MD. Second, LRRTM1 directly associates with AMPARs in the brain, and its loss leads to significantly reduced levels of AMPARs at synapses. Thus, our study shows that reduced excitatory synaptic strength at MD synapses is through a postsynaptic mechanism that could be potentially targeted in the development of therapeutics in schizophrenia. Third, reduced synaptic strength in the MD leads to reduced afferent synaptic activity in the PFC, which was fully reversed by the reintroduction of LRRTM1 into the MD of MD-*Lrrtm1*-cKO mice. Remarkably, our study demonstrates that synaptic circuit disruption by a single-gene deletion in the MD leads to attenuated afferent synaptic activity in a broad region of the PFC. Finally, our study demonstrates that local synaptic perturbation in the MD through targeted deletion of *Lrrtm1* produces cognitive, social, and sensorimotor gating deficits, reminiscent of schizophrenia. Thus, our findings provide a molecular logic for MD hypofunction in schizophrenia. These results may be interpreted to suggest that *Lrrtm1* deletion in the MD may lead to MD-

PFC circuit disruption, although further experimental validation may be required in future studies.

2.5.1 Uncovering the molecular basis of MD hypofunction in schizophrenia

Decreased function of MD has been demonstrated to be a critical causative feature of cognitive deficits in schizophrenia in humans, which is mimicked in non-human primates and rodents. While imaging studies in patients and lesion studies in rodents have underlined the contribution of MD to cognitive function, they do not provide insights into the causal relationship between MD and cognitive impairment in schizophrenia[9-11,57,58]. Chemogenetic and optogenetic silencing of the MD are more useful tools to demonstrate causality at the neural circuit level but do not address the molecular basis of altered cognition. Deconstructing the molecular underpinnings of disruption in psychiatric disorders would require investigating the role of specific disease-associated genes at the synaptic level, as we have demonstrated here for *Lrrtm1*. Although deficits in the mouse model may not be directly attributed to schizophrenia, the MD to PFC circuit in rodents and humans has a highly conserved topographic pattern of reciprocal interconnections enabling interrogation of the underlying neurobiology. Studies in humans have found LRRTM1 to have a paternal transmission pattern with effects on handedness. However, during the generation and maintenance of the *Lrrtm1*$^{floxed/floxed}$ or *Lrrtm1*$^{-/-}$ [23] mice lines, we did not observe such mode of sex-specific transmission in mice. Therefore, we focused our effort on altering the levels of LRRTM1 to reflect gene dosage changes in humans, as reported in schizophrenia.

2.5.2 Single gene deletion disrupts MD-PFC communication and alters cognitive function

A previous study by Takashima et al.[25] examining the role of LRRTM1 in cognitive function relied on global germline deletion of *Lrrtm1.* In the current study, we adopted a conditional knockout approach in a targeted neuronal population in mature animals. Takashima et al.[25] reported that constitutive knockout of *Lrrtm1* (*Lrrtm1*$^{-/-}$ mice) had reduced locomotor activity, whereas such deficit was not observed in MD-*Lrrtm1*-cKO mice. Interestingly, whereas MD-*Lrrtm1*-cKO mice had anxiety-related avoidance in the EPM test, Takashima et al. found the opposite to be the case in the *Lrrtm1*$^{-/-}$ mice. In the NOR test, *Lrrtm1*$^{-/-}$ mice showed a similar preference for a novel object compared to controls, whereas MD-*Lrrtm1*-cKO mice failed to distinguish between the familiar and novel objects. However, *Lrrtm1*$^{-/-}$ and MD-*Lrrtm1*-cKO mice had similar social novelty preference deficits, albeit in different experimental set-ups. LRRTM1 is co-expressed with other LRRTM family members in various brain regions such as the hippocampus. As such, functional compensation by other LRRTMs is a distinct and confounding possibility in constitutive *Lrrtm1* knockout mice. Moreover, the neural circuit origins of behavioral deficits cannot be determined in constitutive knockouts[59]. It is noteworthy that synapse organizers show input-, context- and tissue-specific expression and function. In the dentate gyrus, LRRTM4 functions at excitatory synapses whereas in the retina, it organizes inhibitory synapses between AII amacrine and rod bipolar cells[26,36,60,61]. Moreover, the best characterized synapses organizers, neurexins and neuroligins, have context-dependent functions in various brain regions and cell-types in the mammalian brain[62-64]. These observations underscore the importance of investigating the functions of individual synapse organizers in the context in which they are expressed.

Our study demonstrates that cognitive deficits due to disrupted MD-PFC communication may arise from the disruption of a single gene in the MD. The deletion of *Lrrtm1* in the MD leads to reduced synaptic strength, likely resulting in disruption of information flow to the PFC. Our FDG-PET imaging results demonstrate that conditional deletion of *Lrrtm1* in the MD leads to reduced synaptic activity in the PFC, findings which are consistent with human FDG-PET/functional MRI studies showing thalamofrontal hypofunction in schizophrenia[51,56,65]. Our study is consistent with previous reports that decreasing information flow from the MD to the PFC produces cognitive symptoms of schizophrenia[13,66,67]. MD-*Lrrtm1*-cKO mice show higher anxiety-like avoidance behavior in the EPM test, which can also arise from disrupted excitation-inhibition balance in the PFC[68]. The PFC is required for cognitive flexibility[69,70]. MD-*Lrrtm1*-cKO mice have increased perseveration for the familiar object in the NOR task, which may be interpreted as either impaired memory or reduced behavioral flexibility[71]. Further, MD-*Lrrtm1*-cKO mice have impaired social behavior and sensorimotor gating, with no difference in motor activity and coordination, implicating the MD-PFC circuit in contributing to these functions. Thus, deficits in MD-*Lrrtm1*-cKO mice likely recapitulate a subset of cognitive deficits of schizophrenia. Our results showing that reduced synaptic strength in the MD led to reduced afferent synaptic activity in the PFC may not be solely due to direct reciprocal connections between the MD and PFC but could additionally be a cumulative effect of the all other inputs to the PFC that the MD connects with.

2.5.3 Placing LRRTM1 within the glutamate hypothesis of schizophrenia

Genetic and proteomics studies implicate genes encoding components of glutamatergic signaling in schizophrenia[72]. Within the thalamus, the molecular composition of glutamate synapses was found to be abnormal in schizophrenia[73].

Recent studies implicate the disruption of AMPAR trafficking and localization at synapses in schizophrenia. Forward trafficking of AMPARs containing the GluA1 subunit was impaired in frontal cortex of schizophrenia patients[74], and GluA1 and GluA3 transcripts were found to be reduced in the thalamus of schizophrenia subjects[75,76]. Our results showing that the conditional deletion of *Lrrtm1* in the MD reduces synaptic strength due to reduced synaptic surface AMPAR subunits are consistent with these findings. We propose that modulating LRRTM1 or LRRTM1 interactome, including the AMPARs at MD synapses is a potential therapeutic strategy for cognitive impairment in schizophrenia.

Acknowledgments

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Author contributions: T.J.S conceived research; B.K. and T.J.S. designed research; B.K performed the bulk of the experiments and analysis (85%), P.S., S.B., N.P., S.D., D.Z. and N.Z. contributed to experiments and analysis; M.F.J., and J.W.C. advised on electrophysiology experiments; and G.K. advised on behavior experiments; J.H.K supervised FDG-PET experiments; B.K. and T.J.S. wrote the paper. All authors read and approved the manuscript.

2.6 Supplemental information

Detailed experimental procedures

Mice

All animal experiments complied with government and institutional requirements of the University of Manitoba and conformed to ethical and procedural guidelines of the Canadian Council on Animal Care (CCAC, http://www.ccac.ca). Transgenic C57BL/6 mice genetically engineered with *loxP* sequences of same orientation flanking the second exon of their *Lrrtm1* (Fig.S2.2c) were used in this experiment. Mice were housed with a 12-h light/dark cycle and *ad libitum* food and water access.

Animal injections, cDNA and viruses

Mice were weighed and then deeply anesthetized using 4% isoflurane supplied with oxygen to a holding chamber. Local (Marcaine) and general (Metcam) analgesics were administered according to the recommended doses. Mice were then placed in a stereotaxic frame with a nose cone supplying oxygen and 2% isoflurane. Stereotaxic Injections were performed using pulled 20 µl glass pipettes (puller; 700 D. Kopf, USA, pipette; Drummond scientific company, USA) with a narrow taper and a sharp tip with an opening adjusted to 30-40 µm using a light microscope. A picospritzer (Parker, USA) supplied with nitrogen gas through a cylinder was used to push the liquid through the pipette. Mice were housed individually post-surgery. pAAV-Ef1a-mCherry-p2A-HA-LRRTM1-DIO rescue construct was generated by cloning HA-LRRTM1 into the pAAV-Ef1a-mCherry-p2A-MCS-DIO construct. Viruses used are listed in Table S2.1.

Table S2.1. List of viruses used in stereotaxic injection

Virus	Injection volume	Supplier
AAV-CamKII-eGFP-Cre	75-100 *nl*	UPenn vector core
AAV-CamKII-eGFP	75-100 *nl*	UPenn vector core
AAV-Ef1a-mCherry-p2A-HA-LRRTM1-DIO	75-100 *nl*	Neurophotonics
Retrograde AAV pmSyn1-EBFP Cre	75-100 *nl*	Addgene
Retrograde AAV pmSyn1-EGFP	75-100 *nl*	Addgene

Behavior

Behavioral testing was done in a relatively soundproof room with constant white noise. The testing area was separated by thick curtains from the rest of the room and illuminated with a separate light source. The performance of the mice was recorded using a video camera mounted above the apparatus. Before the beginning of each test, the experimenter would leave the area, draw the curtains and remain outside until the experiment or the session was complete. Processing and analysis of behavior performance were done using AnyMaze® software (Stoelting, UK).

Accelerating Rotarod

An accelerated rotarod test was performed as a measure of gross motor skill. Since all other behavioral tasks required the mice to be able to move and interact with objects or other animals, it was crucial to ensure that their motor function remained intact following stereotaxic injections and *Lrrtm1* deletion in the MD. An accelerated rotarod machine (Harvard Apparatus, USA) was used to conduct this experiment. Each mouse was placed on the axis of the rod facing away from the direction of rotation. The rod was rotating at the speed of 4 rpm and acceleration began at 20 rpm/min after 10 s at 4 rpm. The speed of the rotarod at which the mouse fell and the latency of the fall were recorded. Ninety minutes after the first trial, all animals underwent a second trial. Mean values were used for analyses.

Elevated plus maze (EPM)

Mice were individually placed in the center of the plus-maze with the head facing a closed arm. Mice were allowed to move freely in the maze for 10 mins. The distance

traveled, average speed and the number of entries into each arm, as well as the time spent in each arm, was recorded and measured using AnyMaze software. Each mouse received one trial. The apparatus was cleaned with 10% ethanol after each trial.

Novel object recognition test (NOR)

The experiment was conducted in a 50 cm x 50 cm x 40 cm open field box made from black plexiglass. Three T25 flasks filled with yellow colored water and three scot bottle caps of the same color were used as objects. Two of each object type were used randomly for familiarization and the third was used for testing. Spatial cues (four different geometrical shapes each in different color) were placed on the walls of the testing area. To acclimate the mice, each mouse was placed in the empty open filed box facing the wall closest to the experimenter and allowed to explore the open field for 5 mins, then returned to its home cage. Habituation was repeated after 90 mins. 24 hours later, two randomly selected identical objects were placed in the box, 5 cm away from the same wall. The object pair were randomly assigned to each mouse and each group tested. Mice were allowed to behave freely until objects were explored for a total of 20 s. 4 hours after the familiarization session, mice were returned to the box, and one of the familiar objects was replaced with a novel object. Again, mice were allowed to move freely until 20 s of total object exploration time had been reached. The apparatus and objects were cleaned with 10% ethanol after every session.

Three-Chambered social interaction test

The apparatus for Crawley's sociability and social novelty preference test is comprised of a rectangular box with three chambers of equal size (20 x 40 x 22 cm) made from clear plexiglass. The middle section is open and is connected to the other chambers with gates with sliding doors that allow the test mouse free access to each chamber.

To perform the test, a subject mouse was placed in the middle chamber and was allowed to move about for 5 mins before the first stranger mouse (Stranger I) was introduced into the cage located in one of the side chambers. The cages allowed the mice to interact and sniff each other without allowing the strangers to leave the cage (the placement of Stranger I in the left or right side of the chamber was randomly altered between trials. Stranger mice would alternate between being Stranger I or II as well). After placement of the first stranger, the gates to the compartments were removed, allowing the subject mouse free access to all the chambers. After 10 minutes, a second stranger mouse ("Stranger II") was placed inside an identical cage on the opposite side chamber. The subject mouse was allowed to explore freely for another 10 minutes. The time spent by the subject mouse in each of the three chambers as well as total distance travelled and average speed of the mice was measured. The apparatus was cleaned with 10% ethanol after every session. Note: All Stranger mice were of the same gender as the subject mice and had no prior interaction with them.

Acoustic startle reflex and Pre-pulse inhibition test

Mice were harnessed in a Plexiglass cylindrical chamber, 8 cm in diameter and 16 cm long. The chamber was placed in a ventilated and sound-attenuating chamber. Background noise (70 dB), pre-pulse and pulse stimuli were provided by a speaker in the chamber. Before the startle stimulus, a 30-ms pre-pulse stimulus was presented, at one of 73, 79 or 85 dB. There was a 5 mins acclimation at the beginning of every trial (Blocks I to III). The first and third blocks each had 6 startle trials without a pre-pulse, while Block II had 28 trials, 8 of which were startle trials, 5 were PPI trials for each of the 3 pre-pulse intensities and 5 were trials with no stimuli. The trials were separated by variable, 5s to 30s, inter-trial intervals in a random order. The startle

response was measured by averaging the 8 startle trials in Block II. We also averaged 5 PPI trials at each of the pre-pulse intensities, which we expressed as percentage of the average response of the 8 startle trials.

Slice electrophysiology

Solutions

Three different solutions were used for cutting, holding and electrophysiological recordings in this study, a standard artificial cerebrospinal fluid (aCSF), a HEPES-buffered solution for recovery and holding of slices and an (N-Methyl-D-glucamine) NMDG-HEPES solution for cutting slices. Salts and other compounds comprising each of these solutions as well as intracellular recording solution (iCS) for voltage clamp recordings are listed in Table S2.2.

Table S2.2. List of salts and chemical compounds and their concentration in each of electrophysiological solutions used in this study.

Compound/salt	aCSF*¥ (mM)	HEPES holding buffer*¥	NMDG cutting Buffer*¥	iCS∓
NaCl (Fisher scientific)	124	95	-	6
NMDG	-	-	94	-
KCl (Fisher scientific)	3	3	3	-
NaH_2PO_4 (Fisher scientific)	1.25	1.25	1.25	-
$NaHCO_3$ (Fisher scientific)	26	30	30	-
$MgCl_2.H_2O$ (Fisher scientific)	1.3	1.3	5	2
$CaCl_2.H_2O$ (Fisher scientific)	2.6	2.6	0.5	-
HEPES (Fisher scientific)	-	20	20	10
Sodium Ascorbate (Sigma)	-	5	5	-
Sodium Pyruvate (Sigma)	-	3	3	-
Na-GTP (Sigma)	-	-	-	0.3
Mg-ATP (Sigma)	-	-	-	2
Glucose (Sigma)	10	25	25	-
K-gluconate/Cs-gluconate (Sigma)	-	-	-	135/125
EGTA (Sigma)	-	-	-	0.1
*Osmolality 300-310 osmol/kg ¥ pH: 7.4 ∓ Osmolality adjusted to 10 units less than aCSF, pH: 7.2				

Slice preparation

Mice were deeply anesthetized using isoflurane and then decapitated using a sharp guillotine. Brains were quickly removed and submerged in chilled oxygenated (95% O_2, 5% CO_2) NMDG cutting solution. After 2 minutes, brains were trimmed by removing frontal cortex and cerebellum, and mounted on the vibratome chuck (HM650V, Thermo-Fisher Scientific). Chuk and brain were then submerged in chilled, oxygenated NMDG cutting solution inside the vibratome cutting chamber. Brains were sliced coronally into 350 µm thick slices. Slices were incubated in oxygenated NMDG cutting buffer for 5 minutes at 32ºC, prior to being transferred to a chamber containing HEPES holding buffer, constantly perfused with 95% O_2 and 5% CO_2. Slices were allowed to recover in this solution at room temperature for 1 hour and were kept in this condition before being transferred to the recording chamber on the electrophysiology

workstation. The recording chamber contained constantly oxygenated aCSF circulating at 3ml/min. Recordings were performed at 31ºC.

Whole cell recordings

Slices were observed using Differential interference contrast (DIC) visualization (Olympus microscope BX51) for guiding the recording electrode and patching cells. Injections site was observed under fluorescence light (Colibri 7, ZEISS, Germany) to distinguish infected cells from uninfected ones; infected cells would express GFP. Infected cells were selected for recording by marking their place as well as their depth. Later under DIC, recording electrode was advanced to the fluorescent cell's marked location, cell was visualized at the specified depth and patched. Recording electrodes would be pulled with vertical puller (Narishige, Japan), filled with iCS, resistance 3-4 MΩ. Prior to recordings, slices were treated with tetrodotoxin (1 µM), applied into the bath, to record mEPSCs. Membrane potential was clamped at -70mV. For mIPSC recordings, in addition to tetrodotoxin (1 µM), CNQX (10 µM), and AP5 (50 µM) were applied to the bath, membrane potential was clamped at 0 mV, iCSF contained Cs-gluconate (125 mM) instead of K-gluconate (135 mM). Recordings were conducted using Clampex 9.2 and analyzed by Clampfit 10.7 (Molecular Devices), 500 events were analyzed for each recording. Events less than 3 RMS were discarded as noise. In case of retrograde injections, infected BFP expressing cells were patched along with their uninfected counterparts.

In a subset of experiments current clamp recordings were performed to examine the biophysical properties of MD projections neurons in control (injected in the mPFC with retrograde AAV pmSyn1-EGFP) and MD-*Lrrtm1*-cKO mice (injected in the mPFC with retrograde AAV pmSyn1-EBFP-Cre). Slice preparation, aCSF and pipette recording solutions described above were used. Whole cell patch clamp recordings

were made under current-clamp configurations using a Multiclamp 700B amplifier (Molecular Devices, California, USA). Recordings were low pass filtered at 10 kHz and acquired at 25 kHz with CED Power 1401 AD board and Signal software (Cambridge Electronic Design, UK). All properties were collected while holding the cell at -60mv. Input resistance was collected as the average response of the cell to repetitive (minimum 20 sweeps), small hyperpolarizing pulses (-10 pA, 100 ms). Rheobase, defined as the minimum current to elicit an action potential 50% of the time was collected from incremental 1 pA depolarizing current steps. Voltage threshold defined as the membrane potential at which depolarization increased at ≥10 V/s was determined from the first spiking response during rheobase. Repetitive firing was determined by applying 1 second depolarizing current pulses with incremental increase of 20 pA. Sag potential and post inhibitory rebound firing was collected during 1 second hyperpolarizing current pulses with increases of -20 pA. Sag potential was collected as the difference in the membrane potential at maximum minus steady state change during the hyperpolarizing pulse at -100 mV. Spontaneous EPSCs were recorded by holding the cells at -60 mV.

Transmission electron microscopy (TEM)

Mice were deeply anesthetized by intraperitoneal injections (IP) of chloral hydrate (600 mg/Kg). Later, cardiac perfusion was performed with chilled phosphate buffer saline (PBS), followed by chilled fixative (4% paraformaldehyde, 1.5% glutaraldehyde in PBS). Brains were removed and incubated at 4ºC in Sorensen's buffer overnight. Brains were cut into 1mm thick slices using a coronal brain matrix, MD was then isolated using a tissue puncher, and processed for downstream TEM imaging. Total number of synapses, number of excitatory and inhibitory synapses as well as postsynaptic length and width were measured.

Immunocytochemistry and Tissue Immunofluorescence

For VGlut1 and GAD65 staining, mice were deeply anesthetized using isoflurane and perfused with ice cold PBS, pH 7.4 followed by ice cold fixative solution (4% paraformaldehyde, 4% sucrose in PBS, pH 7.4). Brains were removed and stored in fixative at 4°C for 24 hours, and subsequently immersed in 20% sucrose in PBS overnight, and finally 30% sucrose in PBS for another 24 hours. Brains were then embedded in OCT and immediately frozen using dry ice. 20µm thick brain sections were collected on SuperFrost slides and processed for immunostaining. The slides were dried completely and incubated in blocking solution (5% bovine serum albumin (BSA) + 5% normal goat serum (NGS) + 0.25% Triton X100 in PBS) for an hour. The primary antibodies anti-VGlut1 (rabbit, 1:1000; Synaptic Systems; 135 302) and anti-GAD65 (mouse IgG2a, 1:100, GAD-6-c, Developmental Hybridoma) diluted in the blocking solution were added to the slides and incubated overnight at 4˚C. The slides were washed with PBS, three washes of fifteen minutes each. The appropriate secondary antibodies conjugated to Alexa 488 or 568 were diluted in the blocking solution and the slides were incubated with it for an hour at room temperature. The slides were washed thrice with PBS (fifteen minutes each) and mounted with Fluoromount-G with DAPI (4',6 diamidino-2-phenylindole) (Southern Biotech 0100-20). The slides were used for imaging after drying. 4 section per mouse, 8 images per section, 4-5 mice per group. For PSD95, GluA1 and GluA2 staining, mice were deeply anesthetized using isoflurane and perfused with ice cold pre-fixative solution (50mM phosphate buffer, 0.1% sodium nitrite and 1unit/ml heparin) followed by cold fixative solution (0.16M sodium phosphate buffer, pH 7.1, 0.2% picric acid and 1% paraformaldehyde) and finally with 10% sucrose (made in 25mM phosphate buffer, pH 7.4). The brains were carefully removed and immersed in cryoprotectant (25mM

phosphate buffer, pH 7.4, 10% sucrose, 0.04% sodium azide) and stored at 4°C for 48 to 72 hours. Before cryo-sectioning, brains were embedded in OCT and dry ice was used for immediate freezing. 20µm thick brain sections were collected on SuperFrost slides. Staining procedure for PSD95 (Thermo Scientific, 6G6-1C9, 1:2000), GluA1 (Frontiers Institute, Rb-Af690, 1:200), GluA2 (Frontiers Institute, Rb- Af1050, 1:200) was same as VGlut1 and GAD65.

Positron emission tomography (PET) imaging

All PET imaging acquisitions were performed with a 7T MR solutions flexiscan benchtop PET-MRI imaging system (MR Solutions Ltd.). Food was withdrawn from all animals 12 hours prior to scanning. Animals were anesthetized and injected [IP] with approximately 10 MBq of FDG and returned to their home cage. After a 45-minute uptake period, animals were then anaesthetized with isoflurane at 5% induction and 1.5-2% for maintenance. Animals were placed in the scanner in the axial position with the head in the center of the field of view and sequentially scanned with T2 structural MRI and static FDG-PET. Fast spin echo T2 weighted MRI was acquired in axial slices with a TR of 5000 and a TE of 45, with a flip angle of 90. Images had a matrix size of 256 x 245 and a slice thickness of 0.5 mm. Static FDG-PET images had an acquisition time of 15 minutes and were reconstructed using 2D Filtered back projection. Final image volumes were 81 x 81 x 245 with a voxel size of 0.84 x 0.84 x 0.42 millimeters.

Preprocessing

All preprocessing and imaging analysis were done using SPM12 (https://www.fil.ion.ucl.ac.uk/spm/software/spm12/) using the spmmouse small animal imaging toolbox (spmmouse), running on MATLAB 2018b (www.mathworks.com/). Each FDG-PET image was coregistered to that subjects T2 MRI. Then each T2 image

was segmented and spatially normalized to the template using the tissue probability maps provided in spmmouse. The resulting deformation fields from each subject were also applied to the coregistered FDG-PET image, thus each FDG-PET image was in standard space. FDG-PET images were then smoothed with a 0.5 x 0.5 x 0.5 mm Gaussian kernel. FDG uptake was normalized by proportional scaling to the mean of whole brain value. Thus, all subsequent image analysis was done with standard uptake ratio (SUR; referenced to the whole-brain mean value). One animal in the knockout group was excluded due to technical issues with the image.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed to measure expression and confirm expression levels of *Lrrtm1* in MD compared to hypothalamus as an area with potentially lower expression of *Lrrtm1*. RT-qPCR was also performed to confirm that GFP^{+ve} neurons isolated from injected mice were not expressing *Lrrtm1*.

Isolation of MD

Mice were deeply anesthetized using isoflurane and then decapitated. Brain was removed and submerged in chilled PBS for 2 minutes, then cut into 1mm thick slices using a coronal brain matrix. The MD was extracted using a tissue puncher. If the mice were injected with AAV extracted tissue was digested using trypsin solution in HBSS buffer and subjected to Fluorescence-associated cell sorting.

Lrrtm1 expression vs Lrrtm4 in MD and hypothalamus

After isolation of MD, total RNA was immediately extracted using PureLink™ RNA Mini Kit (Applied Biosystems, USA) followed by cDNA synthesis using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Invitrogen, USA). Quantitative PCR was performed using Powerup SYBR™ kit (Applied Biosystems, USA) in a QuantStudio 5

Real-Time PCR System (Thermo Fisher Scientific, USA). The table below contains details of qPCR primers (Table S2.3).

Table S2.3. Sequences of qPCR primers

Primer	**Sequence 5' to 3'**
Lrrtm1 forward	GTCACTCGCTGGAAAAGGAT
Lrrtm1 reverse	TGTAGACAGAGGCCGAGTAG
Lrrtm4 forward	AAACATTTCACCCAGTCCCC
Lrrtm4 reverse	TTGAAAAACCCGTATGGGCA
Gapdh forward	TCAGGAGAGTGTTTCCTCGT
Gapdh reverse	TTGAATTTGCCGTGAGTGGA

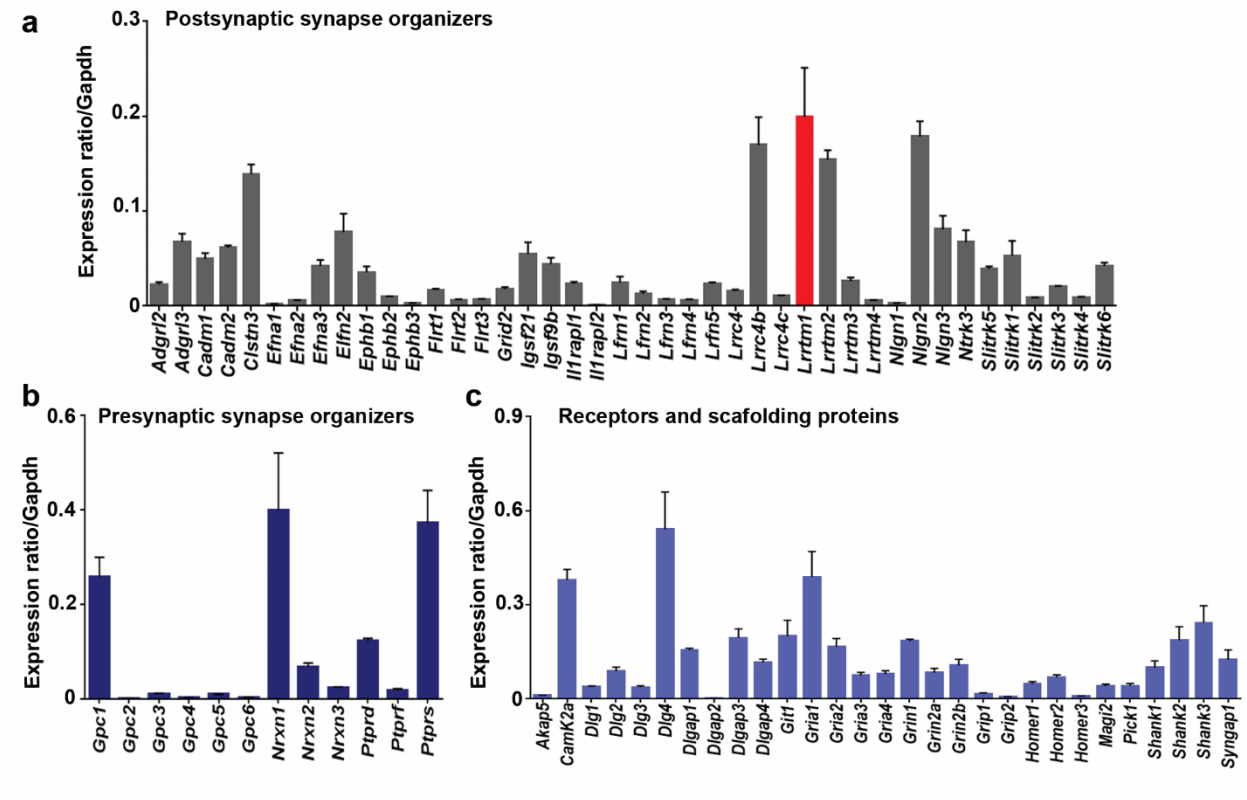
Processing GFP positive cells for gene expression

Cells-to-C_T kit (Thermo-Fisher Scientific) was used to process FACS sorted cells according to the manufacturer's protocol. The resulted lysate were them processed by DNase for removal of genomic DNA and cDNA synthesis using the same kit. The cDNA was later used for TaqMan qPCR analysis using *Lrrtm1* and *Gapdh* TaqMan™ assays (Thermo-Fisher Scientific). Results were normalized for *Gapdh* and compared between mice injected with Cre expressing AAV in the MD and those injected with virus not expressing Cre (three each).

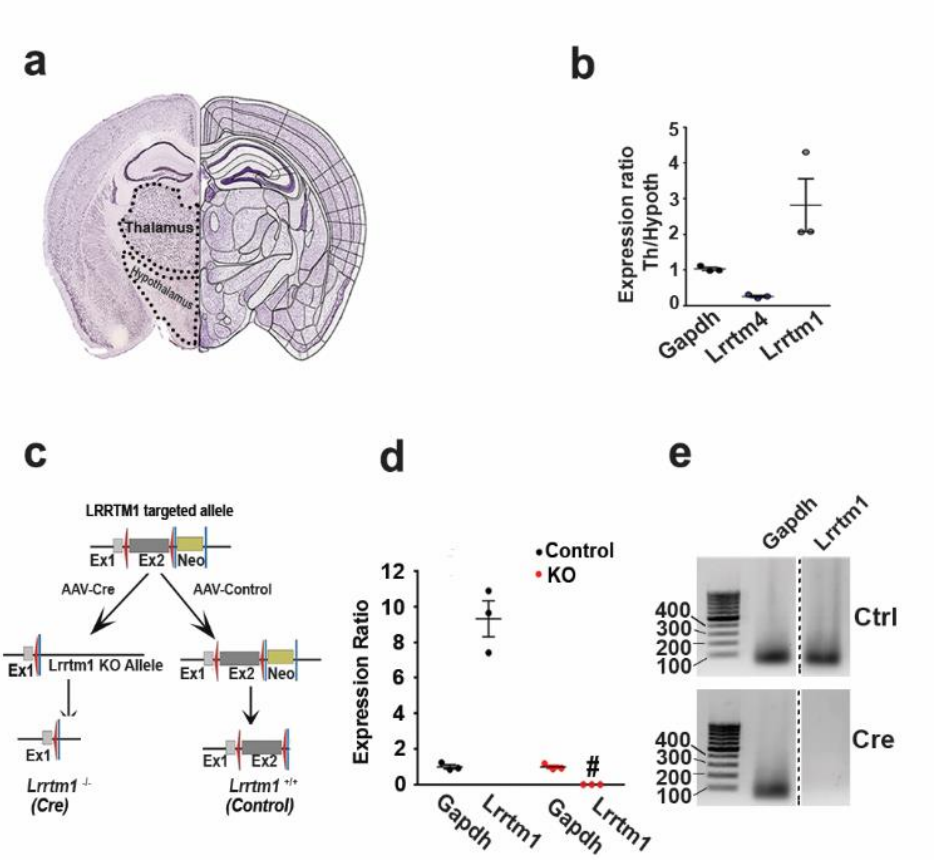
MD synaptic gene analysis

9 male mice (P60-P70) were deeply anesthetized with isoflurane and decapitated with a sharp guillotine. Brains were removed and MD was micro-dissected using a tissue puncher. Isolated MD tissues from 3 mice each were pooled to serve as biological replicates. RNA extraction and cDNA synthesis were performed as described above. Custom designed TaqMan™ array© plates (Thermofisher, USA) were used to measure gene expression of candidate synaptic genes.

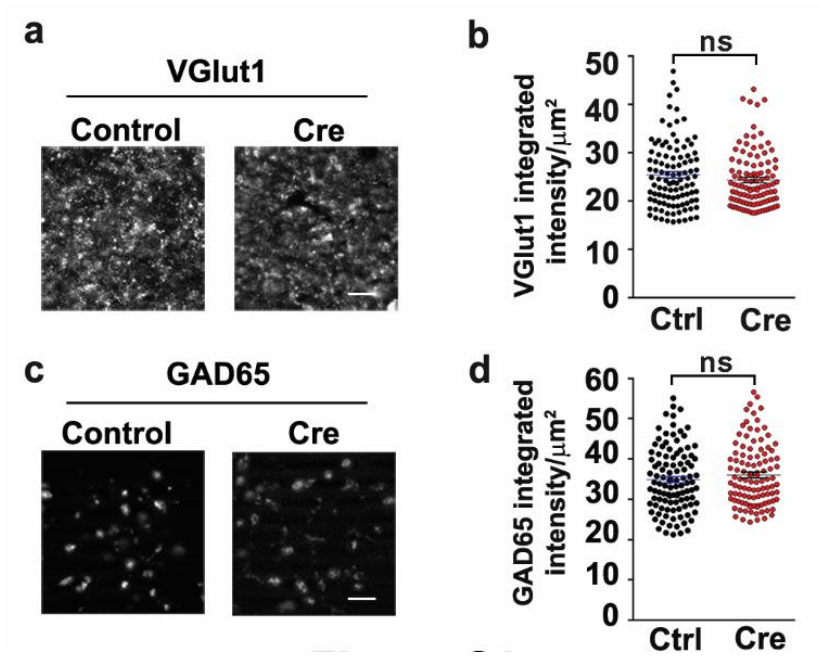
Supplementary figures and figure legends



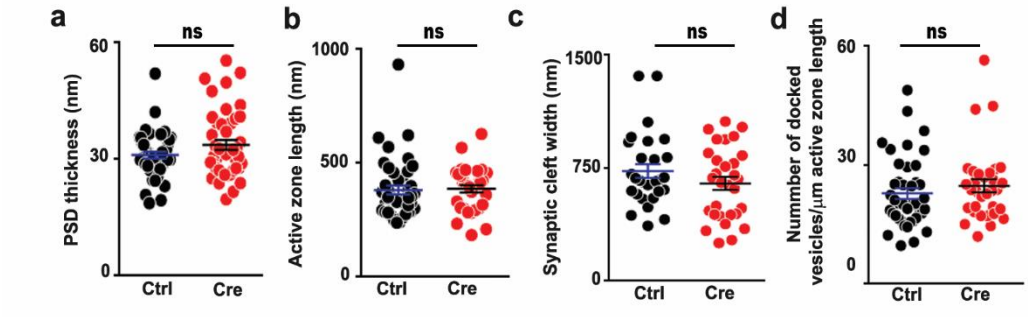
Supplemental Figure S2.1. Transcript expression analysis revealed that *Lrrtm1* is the most highly expressed postsynaptic synapse organizer in the mouse MD. Only *Lrrc4b* and *Nlgn2* had comparable expression levels**. a.** Expression profile of postsynaptic synapse organizers in mouse MD. **b** and **c.** Gene expression levels of presynaptic synapse organizers and glypicans (**b**) and receptors and scaffolding proteins (**c**). RNA was extracted from MD of 9 male mice and pooled in groups of three to serve as biological replicates (see Methods for details).



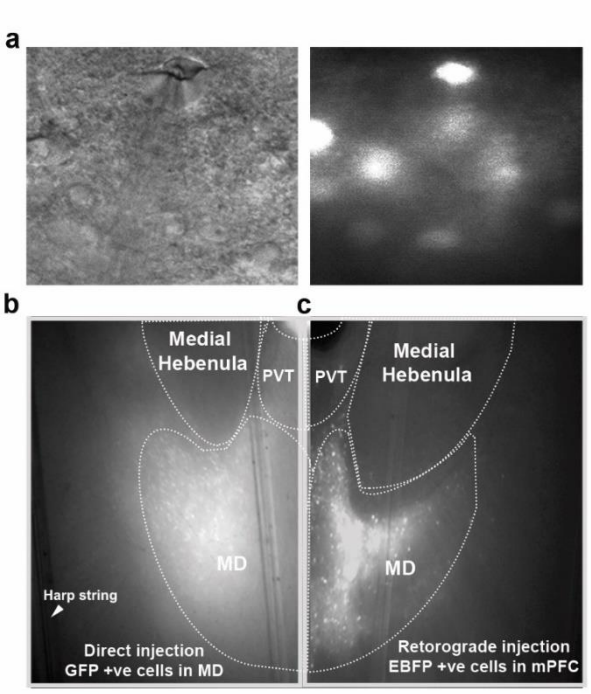
Supplemental Figure S2.2. LRRTM1 is strongly expressed in the mouse MD and selective deletion of *Lrrtm1* in the MD. a. *In situ* hybridization of *Lrrtm1* on mouse coronal brain section, reproduced from Allen Brain Atlas. **b.** *Lrrtm1* expression is ~3 times higher in thalamus than in the hypothalamus, whereas *Lrrtm4* expression is lower in thalamus. **c.** Schematic of conditional deletion of *Lrrtm1*. **d and e.** AAV-CamKII-Cre-EGFP effectively deleted *Lrrtm1* in MD projection neurons, as assessed by TaqMan-based RT-qPCR of fluorescence-sorted AAV-injected cells, and subsequently visualized on agarose gel.



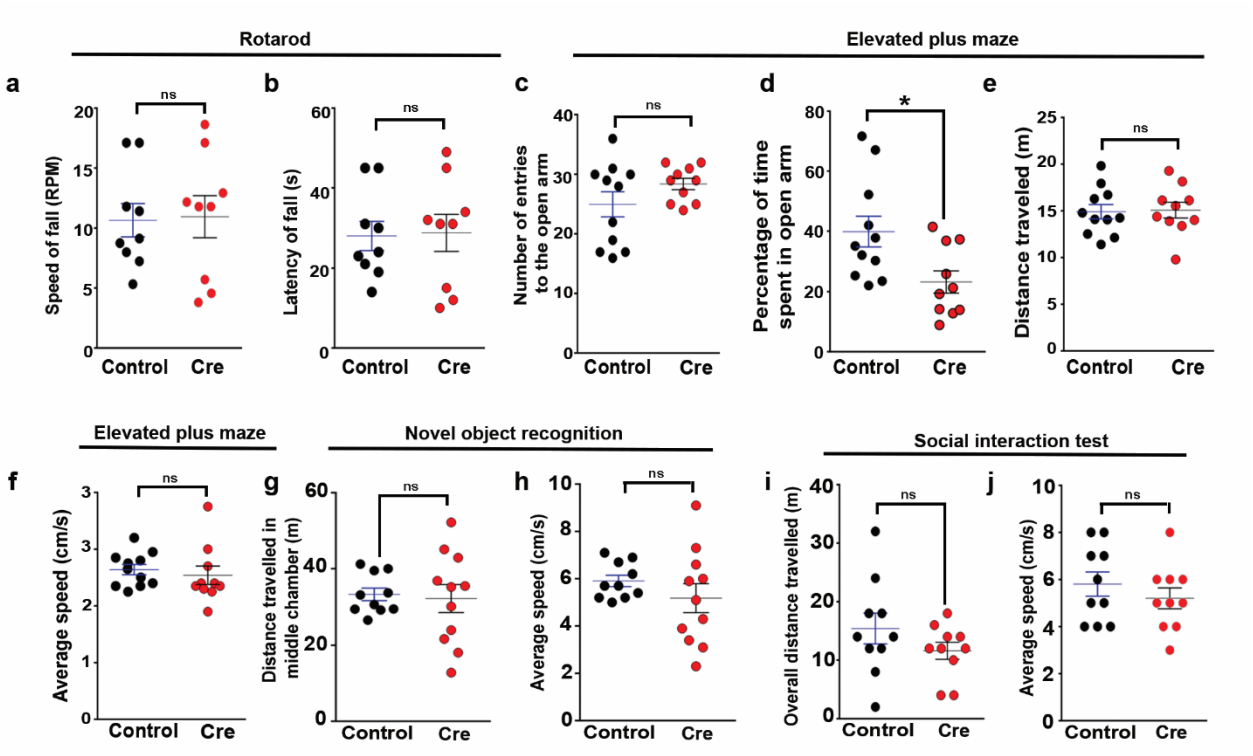
Supplemental Figure S2.3. Excitatory and inhibitory presynaptic inputs to MD in control and MD-*Lrrtm1*-cKO (Cre) mice are comparable. a and **c.** Representative images of VGlut1 (A) and GAD65 (C) staining of MD in control and MD-*Lrrtm1*-cKO mice. **b** and **d**. Quantitative analysis of integrated intensity for a and c, n=3 mice per experimental group, 6-8 sections per mouse, 4 images per section. Scale bar represents 5µm. Data represents mean ± SEM.



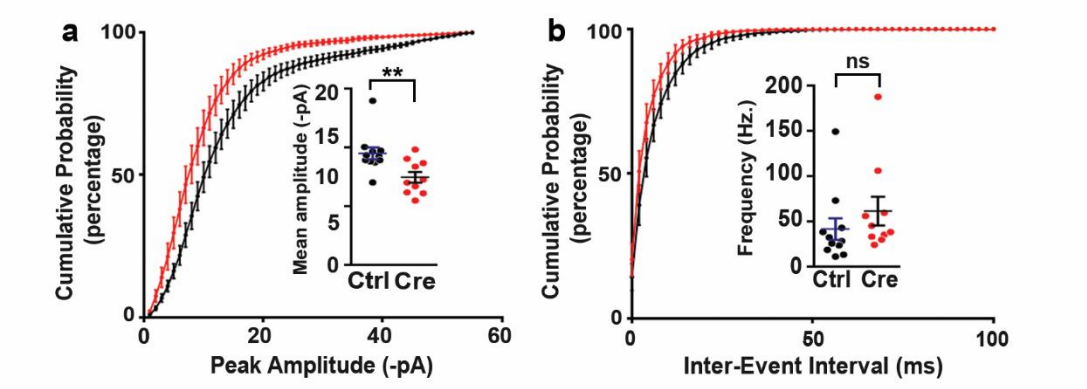
Supplemental Figure S2.4. Ultra-structural analysis of synapses using transmission electron microscopy. Post synaptic density thickness (**a**), active zone length (**b**), synaptic cleft width (**c**), and number of vesicles per µm length of active zone (**d**) remained unchanged (unpaired t-test) in the in MD-*Lrrtm1*-cKO mice compared to controls. 72-106 synapses from control and MD-*Lrrtm1*-cKO mice were analyzed.



Supplemental Figure 2.5. Whole-cell voltage clamp on MD neurons expressing GFP. (**a**) and restriction of directly injected (**b**) and indirectly injected (**c,** retrograde AAV in mPFC) to the MD, as visualized on coronal mouse brain slices.



Supplemental Figure 2.6. *Lrrtm1* deletion in mature MD neurons does not affect gross motor function and increases anxiety-related avoidance behaviour. a-b. Control and MD-*Lrrtm1*-cKO (Cre) mice have similar speed (a) and latency of fall (b) in the accelerating rotarod test. **c-f**. In the elevated plus maze (EPM) test, MD-*Lrrtm1*-cKO mice had similar number of entries to (c) but spent less time in the open arms (d) relative to control mice (*p=0.017, unpaired t-test), No significant difference was observed in distance travelled (**e**) or average speed (**f**). In the novel object recognition test (see Figure 4a-c) distance travelled (**g**) or average speed (**h**) were comparable between the MD-*Lrrtm1*-cKO and control mice. In three-chamber social interaction test (see Figure 4d-g), distance travelled (**i**) or average speed (**j**) were comparable between the MD-*Lrrtm1*-cKO and control mice.



Supplemental Figure S2.7. Spontaneous EPSC recordings from MD projection neurons in mice in which retrograde AAV-Cre or control retrograde AAV were injected into the mPFC. 10-11 neurons from 5-6 mice per group. **a.** Cumulative frequency of event amplitudes recorded from EGFP^{+ve} control and EBFP^{+ve} MD-*Lrrtm1*-cKO MD projection neurons. Inset bar graph represents mean amplitude, **p=0.0055 by the Kolmogrov-Smirnov test; bar graphs were analyzed by unpaired t-test, **p=0.0082. **b.** Cumulative frequency of interevent intervals of EGFP^{+ve} control and EBFP^{+ve} MD-*Lrrtm1*-cKO neurons. Inset bar graph represents frequency of events.

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Chapter 3

Stratum-specific role of LRRTM1 in regulation of long-term potentiation and dorsal CA1-associated behaviour

The results of this collaborative work are to be published as part of an original article, in 2022.

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3.1 Abstract

The hippocampus has a laminar organization with defined axonal inputs onto specific dendritic compartments of pyramidal neurons and granule cells. The characteristic laminar organization of the hippocampus is thought to be orchestrated in part by cell-surface synapse organizing proteins, some of which such as the leucine-rich-repeat transmembrane neuronal proteins (LRRTMs) are essential for mediating

enduring changes in synaptic efficacy such as long-term potentiation (LTP). LTP is differentially expressed in the proximal and distal dendritic compartments of the CA1 pyramidal neurons constituting the stratum radiatum and stratum lacunosum moleculare respectively However, the molecular mechanisms underlying differential expression of LTP in the hippocampal laminae is poorly understood. We show here that LRRTM1 expression is largely restricted to the stratum radiatum. Loss of LRRTM1 in CA1 pyramidal neurons impaired LTP in the stratum radiatum but not in the stratum lacunosum moleculare. These deficits were corrected by the reintroduction of LRRTM1 or perfusion with a peptide that interferes with the endocytosis of GluA2-containing AMPA receptors. Our results further indicate that chronic reduction of synaptic strength in the dorsal CA1 by targeted deletion of *Lrrtm1* in adult mice may account for memory deficits attributed to dorsal CA1.

3.2 Introduction

The mammalian brain is organized into discrete laminae that constitute dendritic compartments of neuronal populations[1,2]. Dendritic laminae are innervated by stereotyped axonal projections thus allowing neurons to receive differential inputs to produce a characteristic output[3]. Among the best studied laminae-rich structures are the retina and hippocampus. The hippocampal CA1 region has a compact pyramidal cell layer with shorter basal dendrites constituting the *stratum oriens*, and longer apical dendrites that form the proximal *stratum radiatum* and the distal *stratum lacunosum moleculare*[3-6] respectively. The *stratum radiatum (SR)* receives commissural excitatory inputs from Schaffer collaterals originating in the CA3 subfield. The *stratum lacunosum moleculare (SLM),* on the other hand, receives direct temporoammonic axonal excitatory projections from layer III of the entorhinal cortex[3-6]. The CA1 dendritic layers compute disparate synaptic inputs to orchestrate co-

ordinated neuronal activity underlying cognitive functions such as learning and memory[7].

There has been considerable progress in understanding the complement of molecular factors that dictate CA1 lamination. Netrin-G1 and -G2 binding to netrin-G-ligand-1 or -2 are selective for the laminar organization of the SLM and SR respectively[7,8]. Another well-known molecule that contributes to hippocampal lamination is reelin[9]. It is likely that lamination is orchestrated by not one but several molecular players that act in a co-ordinated manner. Prime candidates for conferring functionally unique properties on CA1 laminae are synapse organizing proteins such as the leucine-rich-repeat transmembrane neuronal proteins (LRRTMs). Whereas LRRTM2 is enriched in the SLM, LRRTM1 is concentrated in the SR[7,8,10]. The differential distribution of the LRRTMs in the hippocampal laminae indicates that they may functionally contribute differentially to CA1 laminae function.

The LRRTMs are localized at excitatory postsynapses and contribute to synapse organization and long-term forms of plasticity such as long-term potentiation (LTP)[11-16]. We previously reported that in cultured primary neurons, retention of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) at synapses was reduced in the absence of LRRTMs[13]. Other studies from our lab also showed that deletion of *Lrrtm1* in the mediodorsal thalamus or joint deletion of *Lrrtm1* and *Lrrtm2* in the dorsal CA1 leads to reduced synaptic strength accompanied by a wide range of behavioural abnormalities[13] (Siddiqui lab, unpublished). However, it is not known whether LRRTMs regulate LTP in a lamina-selective manner.

To determine whether LRRTM1 contributes to LTP in a lamina-selective manner, we generated *Lrrtm1* conditional knockout restricted to dorsal CA1 pyramidal neurons (CA1-*Lrrtm1*-cKO). We found that the deletion of *Lrrtm1* in dorsal CA1

pyramidal neurons of mice impaired synaptic transmission and impaired LTP in the SR but not in the SLM. LTP deficits in CA1-*Lrrtm1*-cKO mice was rescued and reversed by the reintroduction of LRRTM1 or by blocking endocytosis of GluA2-containing AMPARs. Our results identified for the first time a synapse organizer that has a lamina-selective role in synaptic plasticity. Moreover, mice lacking *Lrrtm1* in the dorsal CA1 had poor sociability and severely impaired memory. These results underscore the indispensable contribution of synaptic function in a single lamina of a brain region to its essential functions.

3.3 Materials and methods

3.3.1 Mice and Stereotaxic Injection

All animal procedures conformed to the guidelines of the Canadian Council on Animal Care (http://www.ccac.ca) and were approved by the University of Manitoba Animal Care Committee. C57Bl/6N *Lrrtm1*$^{floxed/floxed}$ mice line was described before[13]. All experiments were conducted on cohorts of male and female mice. All behaviour studies were conducted and analyzed blind to the genetic manipulation. 5-10 weeks old mice were bilaterally injected with adeno-associated viruses (AAVs) expressing Cre or control or rescue construct in the dorsal CA1 (Fig. 3.1a and 1b, AAV-CamKII-eGFP-Cre, AAV-CamKII-eYFP, AAV-Ef1a-mCherry-p2A-HA-LRRTM1-DIO, respectively). Table S3.1 lists the details of the viruses used. Stereotaxic injections (dorsal CA1 coordinates, AP:ML:DV: -2.1:±1.4:-2.0 mm from bregma) were performed as described[13].

3.3.2 Slice electrophysiology

Coronal vibratome brain slices were used to record field excitatory postsynaptic potentials (fEPSPs) and induction of long-term potentiation (LTP); for more details, see supplementary methods.

3.3.3 Behavioural tests

Accelerating rotarod, elevated plus maze, Crawley's three-chambered sociability and social novelty preference, and contextual fear conditioning tests were conducted and analyzed as described[17-20], for more details, see supplementary methods.

3.3.4 Statistical analyses

Statistical analyses were conducted using unpaired t-test, one-way ANOVA with Bonferroni's multiple comparisons test post-hoc test, two-way ANOVA followed by Tukey's multiple comparison post-hoc test, or multiple t-tests using Sidak-Bonferroni method, with alpha=5%. Statistical significance was set to $p<0.05$. Data shown are mean ± SEM. Microsoft Office Excel 365 and GraphPad Prism 6 were used to perform the analyses. To ensure that standard deviations were comparable in unpaired t-tests, an F test was performed. If variances were significantly different, unpaired t-test was done with Welch's correction.

See SI Appendix for details on all procedures in this study.

3.4 Results

3.4.1 Conditional KO of *Lrrtm1* in the dorsal CA1 impairs excitability and LTP in the SR but not the SLM layer.

To delete *Lrrtm1* selectively in the dorsal CA1, we stereotactically delivered AAV-CamKII-eGFP-Cre (CA1-*Lrrtm1*-cKO) or AAV-CamKII-eYFP (control) bilaterally

to the dorsal CA1 of *Lrrtm1*$^{floxed/floxed}$ mice. The virus spread along the entire expanse of the dorsal CA1 with minimal overflow to other areas such as CA2 and DG (Fig. 3.1a). To determine the stratum-specific role of LRRTM1 in dorsal CA1, we performed field recordings from acute slices containing dorsal CA1 by stimulating Schaffer collaterals and recording from the stratum radiatum or stimulating the temporoammonic pathway and recording from the stratum lacunosum moleculare. We recorded input/output responses and measured long-term potentiation (LTP). Recordings of field excitatory postsynaptic potentials (fEPSPs) from CA1-*Lrrtm1*-cKO showed reduced output responses to the increasing intensity of input stimuli in the SR (Fig. 3.1c) but not in the SLM (Fig. 3.1d). These results indicate that synaptic transmission in the dorsal CA1 lacking LRRTM1 is reduced in a lamina-specific manner.

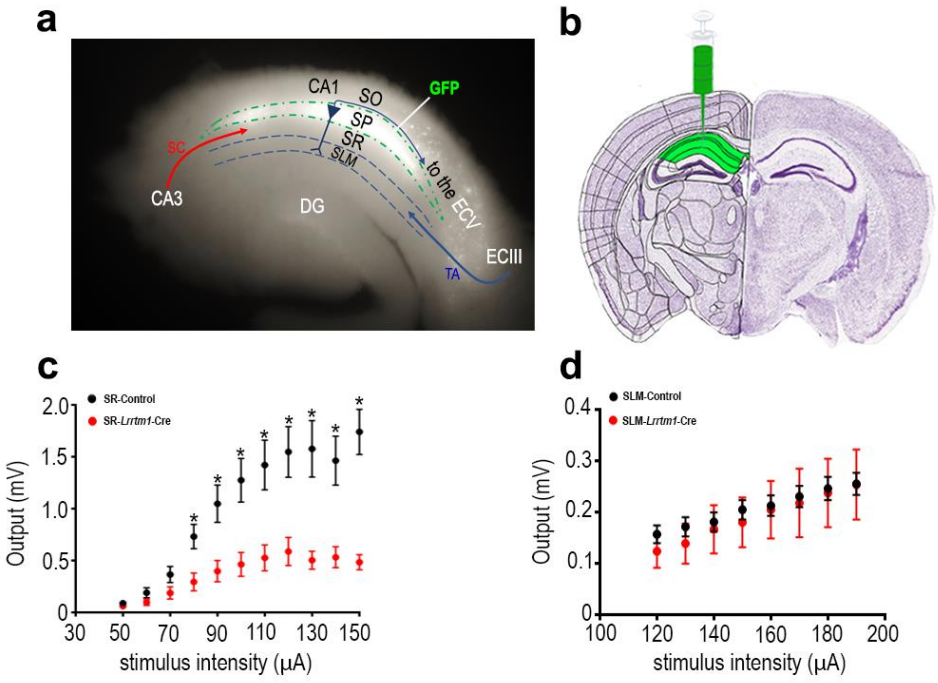

Figure 3.1. *Lrrtm1* deletion reduces excitability in SR but not SLM layer. a. A representative image of a dorsal hippocampal slice. Injections were confined to dorsal CA1 with not overflow to CA2, or DG areas. Picture also illustrates CA1 major input and outputs. CA1 Stratum radiatum (SR) receives Schaffer collateral (SC) inputs from CA3, and CA1 Stratum Lacunosum Moleculare (SLM) receives Temporoammonic (TA) projections from the entorhinal cortex layer III (ECIII). CA1 pyramidal cells (SP) send their projections to the EC layer V through the Stratum Oriens (SO). **b.** Schematic representation of dorsal CA1. Mice

were injected with CaMKII-driven Cre expressing AAV or control AAV in the dorsal CA1. **c.** Deletion of *Lrrtm1* significantly reduced the excitability of CA3-CA1 synapses (multiple t-test, statistical significance determined using the Holm-Sidak method. *p=0.007 for 80 µA, 0.004 for 90 µA, and 0.003 for 100 and 110 µA, 0.002 for 120 and 130, 0.004 for 140 µA and 0.001 for 150), whereas the excitability of ECIII projections on the SLM layer remained intact (**d**)**.** 2-3 slices per mice, 3-4 mice per genotype.

3.4.2 *Lrrtm1* deletion impairs long-term potentiation in the dorsal CA1 SR but not SLM

We investigated the effect of *Lrrtm1* deletion on LTP in the CA3-CA1 and ECIII-CA1 pathways. Following induction of LTP in the SR, both control and CA1-*Lrrtm1*-cKO mice showed an initial potentiation of the average fEPSP. However, whereas LTP after 60 min post-stimulation in the control SR was 140%±6% of the baseline, it was 116%±2% of the baseline in the SR of CA1-*Lrrtm1*-cKO. Thus, LTP was significantly impaired in the SR of CA1-*Lrrtm1*-cKO relative to controls (Fig. 3.2a and 3.2b). In contrast, LTP in the SLM was comparable between controls and CA1-*Lrrtm1*-cKO (Fig 3.2c and 3.2d). We then explored the possibility of rescuing this deficit by re-expression of LRRTM1. Re-expression of LRRTM1 in a Cre-dependent manner partially rescued the input/output responses (Fig. 3.3a), and LTP (Fig. 3.3b and 3.3c) in the SR of CA1-*Lrrtm1*-cKO mice (118%±4% vs 134%±4% of the baseline).

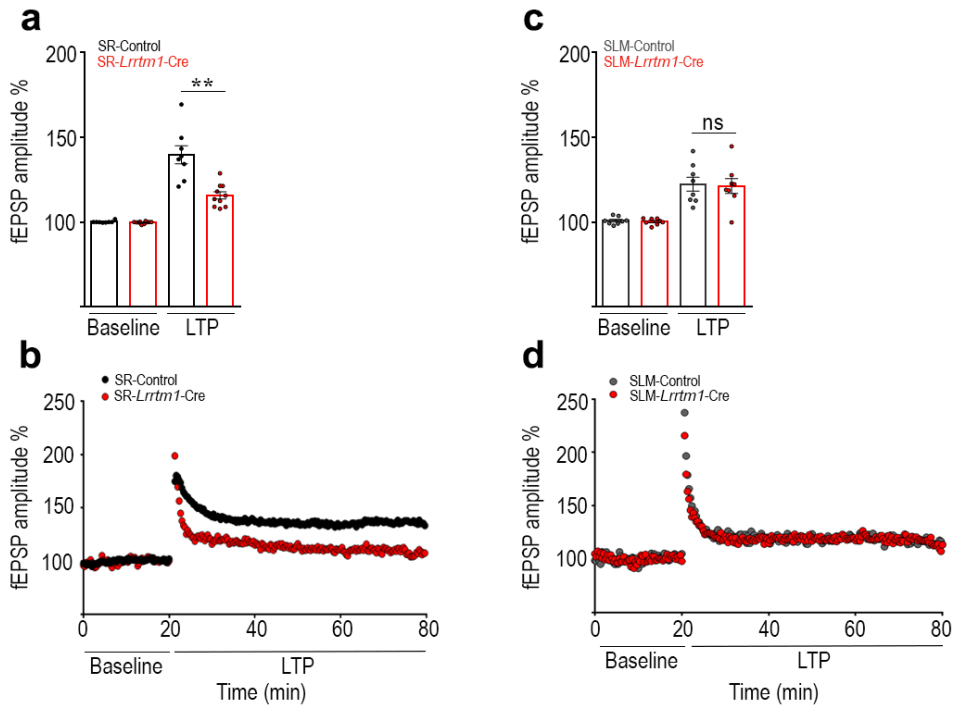

Figure 3.2. Conditional deletion of *Lrrtm1* impairs LTP in dorsal CA1 SR but not SLM layer. a and **b.** Deletion of *Lrrtm1* significantly reduced long-term potentiation in SR region following stimulation of CA3 Schaffer collaterals. 3-4 animals 2-3 slices each (unpaired t-test, **p=0.0045). **c** and **d.** After deletion of *Lrrtm1* LTP remain unchanged in SLM layer following stimulation of TA projection fibres. 3-4 animals per condition 2-3 slices each.

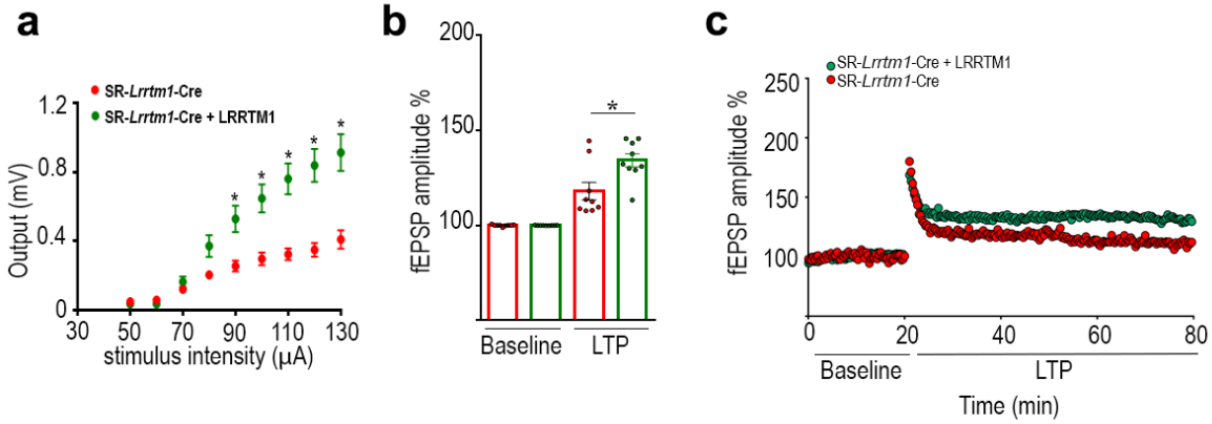

Figure 3.3. Re-expression of LRRTM1 in dorsal CA1 of CA1-*Lrrtm1*-cKO mice rescues excitability and LTP deficits. a. Re-expression of *Lrrtm1* through Injection of DIO-Lrrtm1 expressing AAV along with CaMKII-driven Cre expressing AAV in the dorsal CA1 partially rescued the input/output ratio (multiple t-test, statistical significance determined using the Holm-Sidak method), for stimulus intensities of 90-130 μA *P=0.006, 0.002, 0.0006, 0.0004, and 0.003 respectively). **b** and **c.** Re-expression of *Lrrtm1* in the dorsal CA1 of CA1-*Lrrtm1*-cKO partially rescued LTP and input/output responses (unpaired t-test, *P=0.012). 3-4 animal per condition, 2-3 slices each.

3.4.3 LRRTM1 contributes to LTP by stabilizing AMPARs at synapses.

We have previously shown that AMPARs are rapidly endocytosed in in primary cultured hippocampal neurons lacking LRRTM1 and LRRTM2[13]. However, this mechanism has not been tested in intact hippocampal slices. To test whether impaired LTP in the SR of *CA1-Lrrtm1-cKO* mice was a consequence of reduced levels of AMPARs at synapses, we incubated hippocamps slices in aCSF containing GluR2^{3Y} or a control scrambled peptide to investigate whether preventing the internalization of AMPARs can rescue the LTP deficits in *CA1-Lrrtm1-cKO* mice. GluR2^{3Y} interferes with endocytosis of GluA2-containing AMPARs[21]. GluR2^{3Y} did not rescue input/output responses in the SR of CA1-*Lrrtm1*-cKO mice (Fig. 3.4a). However, whereas GluR2^{3Y} restored LTP in the SR of CA1-*Lrrtm1*-cKO mice comparable to controls, the control scrambled peptide had no effect on LTP (Fig. 3.4b and 3.4c). These results indicate that LRRTM1 may contribute to synaptic transmission and long-term changes in synaptic efficacy via distinct mechanisms.

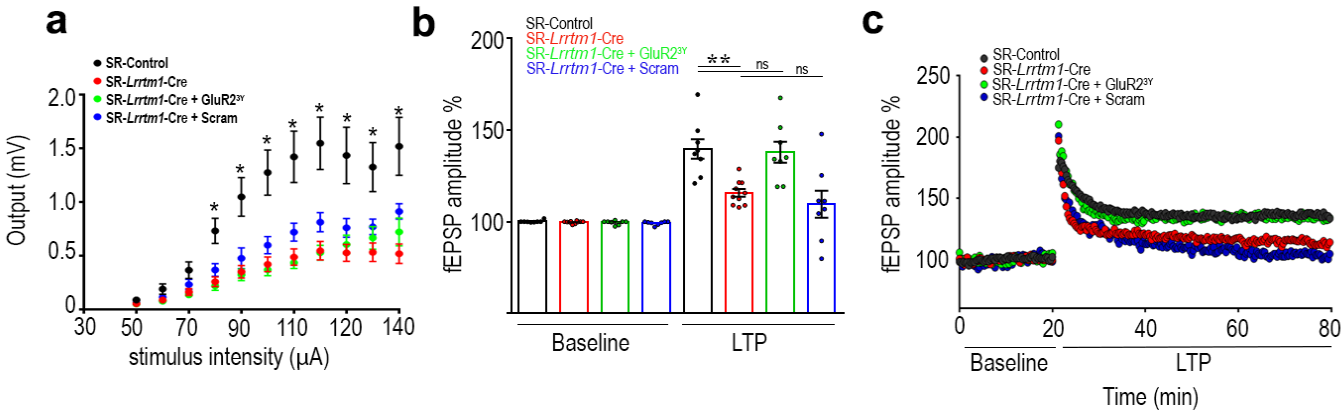

Figure 3.4. Preventing AMPARs internalization recues LTP but not the input/output responses in SR layer of CA1-*Lrrtm1*-cKO mice. a. GluR2^{3Y} incubation could not rescue the deficit in fEPSP (Two-way ANOVA, Tukey's multiple comparisons test. For 80 µA stimulus intensity, *p=0.0009 for Control vs. Cre, 0.001 for control vs. Cre+GluR2^{3Y} and ns for control vs. Cre+Scrambled; for 90 µA p<0.0001 for control vs Cre and Cre+GluR2^{3Y}, for 100-120 µA stimulus intensity *p<0.0001 for control vs. Cre, control vs. Cre+GluR2^{3Y}, and control vs. Cre+Scrambled. For 130 µA *p<0.0001 for Control vs. Cre, and control vs. Cre+GluR2^{3Y} and p=0.012 for control vs. Cre+Scrambled, for 140 and 150 µA *p<0.0001 for Control vs. Cre, and control vs. Cre+GluR2^{3Y} and p=0.03 for control vs. Cre+Scrambled). **b** and **c.** Incubation of slices in GluR2^{3Y} containing aCSF solution was able to rescue the LTP in SR layer (unpaired t-test, **p=0.0024).

3.4.4 Conditional deletion of *Lrrtm1* in the dorsal CA1 impairs contextual fear memory

To assess the impact of impaired LTP in the SR of dorsal CA1 of CA1-*Lrrtm1*-cKO mice on their behaviour, we performed a series of assays to examine hippocampus-associated behaviours. First, we subjected the mice to the rotarod motor learning task. The CA1-*Lrrtm1*-cKO and control mice had normal motor learning ability (Fig. 3.5a and 3.5b), indicating that reduced synaptic transmission in the CA1 does not alter gross motor function. Next, to test whether CA1-Lrrtm1-cKO mice displayed anxiety, we assessed their performance in the elevated plus maze task (EPM). Our results indicated no changes in EPM performance between CA1-*Lrrtm1*-cKO and control mice (Fig.3.5c-f).

The dorsal CA1 is best known to contribute to episodic memory, which is frequently tested using the contextual fear memory test[22]. Lesions or pharmaceutical interference with dorsal CA1, such as injection of $GABA_A$ receptor agonists or NMDA receptor antagonists, leads to impairment of performance in contextual fear conditioning, while lesions in the ventral hippocampus do not affect contextual fear memory performance[23,24]. To assess whether *Lrrtm1* deletion in dorsal CA1 and subsequent impairment of LTP would lead to impairment of contextual fear memory in mice, we compared performance of control and CA1-*Lrrtm1*-cKO mice in the contextual fear conditioning test. CA1-*Lrrtm1*-cKO froze for a significantly shorter duration (Fig 3.5g). They were also significantly less immobile (Fig. 3.5h) than the control, traveled longer distances (Fig. 3.5i) and moved faster (Fig. 3.5j).

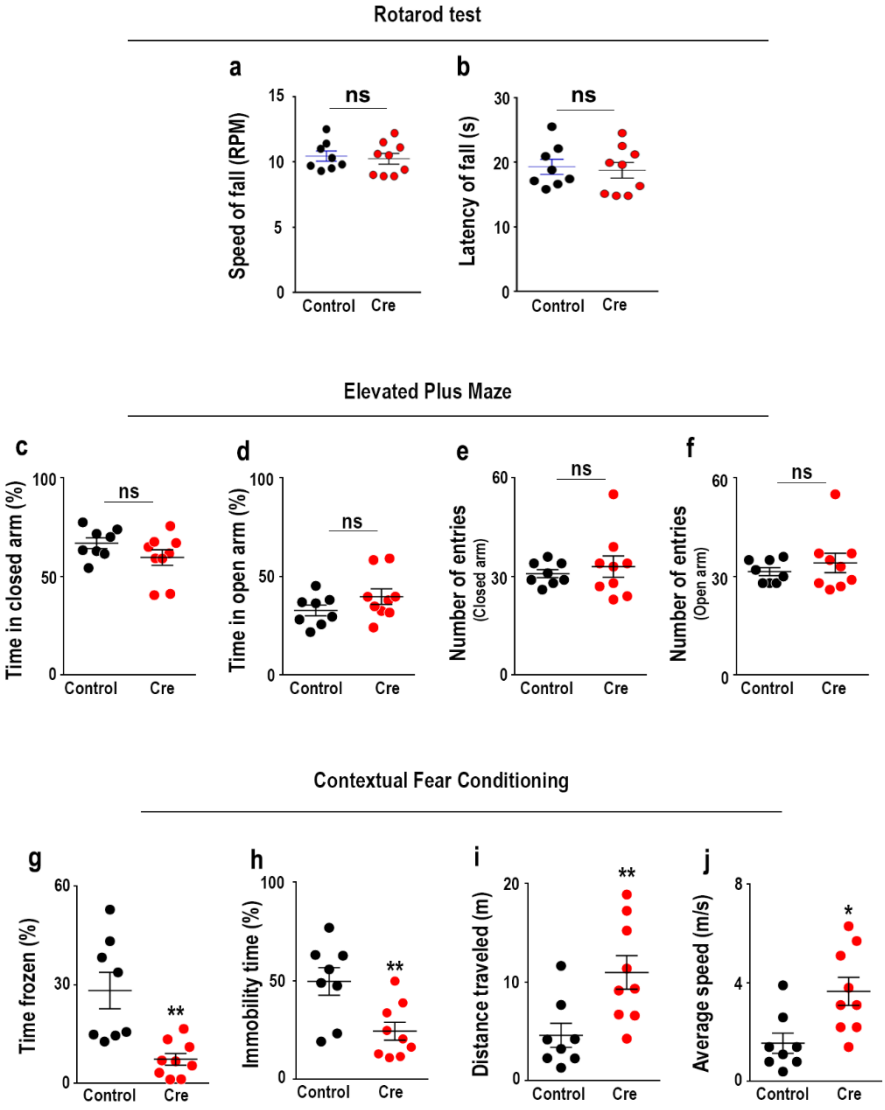

Figure 3.5. Deletion of *Lrrtm1* impairs contextual fear memory. a and **b.** Deletion of *Lrrtm1* in the dorsal CA1 does not affect gross motor skills. Speed of fall (**e**) or latency of fall (**f**) was not significantly different between CA1-Lrrtm1-cKO mice and the controls. **c-f.** No significant difference was observed in performance of CA1-*Lrrtm1*-cKO mice in elevated plus maze when compared to controls in terms of time spent in closed arm (**c**), time spent in open arm (**d**), or number of entries to closed (**e**) or open arm (**f**). *Lrrtm1* deletion impairs contextual memory in CA1-*Lrrtm1*-cKO mice. When placed in the same context, twenty-four hours after receiving foot shock in the chamber, CA1-*Lrrtm1*-cKO mice spent less time frozen (**g**, unpaired t-test, **p=0.001) and immobile (**h**, unpaired t-test, **p=0.007). Also, CA1-*Lrrtm1*-cKO mice traveled significantly more distance (**i**, unpaired t-test, **p=0.009) at faster speeds (**j**, unpaired t-test, *p=0.01), compared to controls; overall, indicating an impaired contextual memory following an unpleasant experience.

3.4.5 Conditional deletion of *Lrrtm1* in the CA1 impairs social interaction but not social novelty preference

Deficits in social interaction and social withdrawal are among the core negative symptoms of many neuropsychiatric disorders[25-27]. We performed Crowley's three-chamber sociability and social novelty test to investigate the effect of *Lrrtm1* deletion in the dorsal CA1 on social behaviour in mice. The test apparatus consisted of a rectangular box with three chambers of equal size. The middle chamber was connected to the side chambers with gates devised in the separating walls. In the first block, a stranger mouse of the same sex was placed in a cage in one of the side chambers, the subject mouse would then be allowed to explore freely for 10 min, before introduction of a second stranger and start of the second block. After the second stranger mouse was placed in the cage in the other side chamber, subject mouse would then be allowed another 10 min to explore the chambers. Our results indicated that deletion of *Lrrtm1* impairs the social interaction ability of CA1-*Lrrtm1*-cKO mice (Fig. 3.6a and 3.6b), while social novelty preference remains comparable between CA1-*Lrrtm1*-cKO mice and controls (Fig 3.6c and 3.6d). No significant difference was observed in speed of movement or distance traveled between CA1-*Lrrtm1*-cKO and control mice in the middle chamber (Fig. 3.6e and 3.6f).

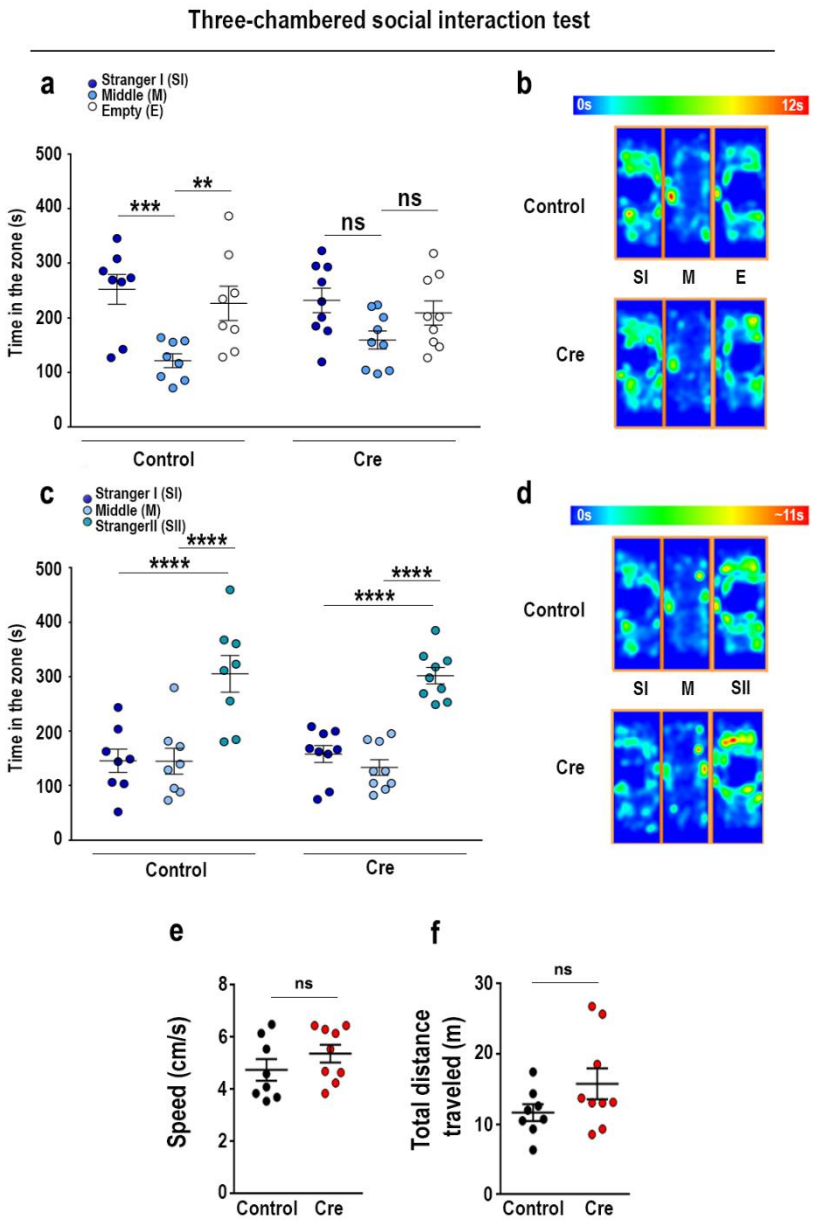

Figure 3.6. Deletion of *Lrrtm1* impairs social interaction in CA1-*Lrrtm1*-cKO mice. a. In block I, control mice preferred Stranger I over the middle chamber and empty chambers, (***p=0.0008, **p=0.007, two-way ANOVA and post-hoc Tukey's test), whereas CA1-*Lrrtm1*-cKO mice spent relatively more time in the middle chamber making the difference in time they spent in either one of the other chambers insignificant, this was also reflected in mean heat map for block I. **b.** Mean heat map of block I for Cre and control mice. **c.** Control and CA1-*Lrrtm1*-cKO mice preferred Stranger II over middle or Stranger I chamber, (****p<0.0001). **d.** Mean heat map of second block for Cre and control animals shows comparable pattern of interaction. **e** and **f.** No significant difference was observed in speed (**e**), and travelled distance (**f**) between control and CA1-*Lrrtm1*-cKO mice.

3.5 Discussion

The dorsal CA1 is an important processing center involved in mediating episodic memory for spatial information and encoding and retention of contextual memory. Our study integrates molecular genetics with behavioural and functional approaches to

provide novel insights into the role of LRRTM1 in dorsal-CA1-associated tasks. Our study has led us to reach the following conclusions. First, LRRTM1 contributes to excitatory transmission in synapses of CA3 Schaffer collateral projections made on apical dendrites of dorsal CA1 pyramidal neurons, but it does not seem to play a similar role in TA projections from the ECIII to the CA1. This is manifested by reduced input/output responses in the SR layer, but not the SLM. Second, LRRTM1 is pivotal to synaptic plasticity in the SR but not the SLM, indicating the stratum- and input-specific role of LRRTM1 in the dorsal CA1. Deficits in contextual fear memory and social interaction following the deletion of *Lrrtm1* in the dorsal CA1 further confirm the importance of LRRTM1 in the integrity of CA3-CA1 circuitry. Third, we have previously shown that LRRTM1 directly associates with AMPARs in the brain, and its loss leads to significantly reduced levels of AMPARs at synapses[28]. Here, we demonstrate that LRRTM1 contributes to maintaining synaptic strength and synaptic plasticity in the dorsal CA1 by retaining AMPARs at CA3-CA1 synapses. The role of LRRTM1 in stabilization of AMPARs was demonstrated using GluR2^{3Y}, a peptide that prevents endocytosis of GluA2-containing AMPARs. Overall, our study shows that LRRTM1 is lamina-specific regulator or synaptic strength and plasticity, and that it executes these functions by maintaining the normal complement of AMPARs at synapses.

3.5.1 Uncovering the molecular underpinnings of lamina-specific role of LRRTM1 in the mouse dorsal CA1

Decreased function of dorsal CA1 has been demonstrated to be associated with cognitive deficits in humans[29-32]. These deficits can be reproduced through pharmaceutical, genetic or lesion manipulations in non-human primates and rodents to variable degrees[6,33-37]. Using region-specific deletion of *Lrrtm1* in the mouse dorsal

CA1, we have demonstrated the role of LRRTM1 in maintaining synaptic strength and plasticity in the dorsal CA1 and the behavioural tasks associated with this region. While imaging and post-mortem studies in patients and lesion studies in rodents have underlined the contribution of dorsal CA1 to cognitive functions, analysing the molecular foundations of impairments in psychiatric disorders would need exploring the role of specific disease-associated genes in a region and circuit-specific manner, this has been our approach here for *Lrrtm1*. Although deficits in mouse models may not be directly attributed to neuropsychiatric disorders, the CA3-CA1 circuit in rodents and humans has a highly preserved topographic arrangement of reciprocal interconnections allowing for examination of the neurobiological foundations.

3.5.2 Single gene deletion disrupts dorsal CA1 synaptic transmission and plasticity and reiterates the region-specific role of *Lrrtm1*

A previous study by Takashima *et al.*[15] on the role of LRRTM1 in cognitive functions used global germline deletion of *Lrrtm1* as their experimental model. We adopted a conditional knockout approach in a targeted neuronal population in mature animals in the current study. Takashima *et al.* reported that the constitutive knockout of *Lrrtm1* (*Lrrtm1*$^{-/-}$ mice) had reduced locomotor activity, whereas such deficit was not observed in CA1-*Lrrtm1*-cKO mice. Interestingly, whereas CA1-*Lrrtm1*-cKO mice showed no anxiety-related avoidance in the EPM test, Takashima *et al.* found that *Lrrtm1*$^{-/-}$ mice were less anxious compared to the controls. Both *Lrrtm1*$^{-/-}$ and CA1-*Lrrtm1*-cKO mice showed deficits in sociability tests. However, social interaction was normal in *Lrrtm1*$^{-/-}$ mice but impaired in CA1-*Lrrtm1*-cKO mice. In contrast, social novelty preference was impaired in *Lrrtm1*$^{-/-}$ mice but unaffected in CA1-*Lrrtm1*-cKO

mice. These results indicate that region-specific acute deletion of synapse organizers is important to dissect their role in neural circuit function, without the confounding possibility of functional compensation in the developing brain.

Our study demonstrates that behavioural deficits may arise from reduced synaptic transmission and plasticity in the dorsal CA1 and arises from disrupting a single gene in CA1 pyramidal neurons. The deletion of *Lrrtm1* in the CA1 leads to reduced synaptic strength, likely resulting in disruption of memory encoding function. Our study is consistent with previous reports that disrupted CA1 activity results in disrupted cognitive abilities[38-40].

Lrrtm1 deletion in the hippocampal CA1 caused several behavioural abnormalities. CA1-*Lrrtm1*-cKO show reduced social interaction. This is in contrast to the phenotype observed after deletion of *Lrrtm1* in another brain region, the mediodorsal nucleus of the thalamus (MD). CA1-*Lrrtm1*-cKO mice show no signs of reduced interest in social novelty which was the phenotype observed when *Lrrtm1* was deleted from the MD. CA1-*Lrrtm1*-cKO also displayed normal exploratory drive and no signs of anxiety in the elevated plus-maze test. CA1-*Lrrtm1*-cKO mice, however, showed an abnormal performance in dorsal CA1-specific behaviour, the contextual fear conditioning. 24 hrs after receiving foot shock, CA1-*Lrrtm1*-cKO spent 25% less time in freezing mode, moved faster, and covered more distance in the same CFC chamber, compared to controls.

The hippocampus is a polar structure. The dorsal and ventral poles play distinct roles in information processing, have distinct gene expression profiles and connectivity[41]. The dorsal hippocampus is primarily involved in cognitive functions, whereas the ventral hippocampus is mainly associated with stress and emotion[41].

Acute dorsal CA1 inactivation disrupts fear memory acquisition and retrieval[42]. We observed the same in CA1-*Lrrtm1*-cKO mice which attests to the significance of LRRTM1 in dorsal-CA1-associated behaviour. The dorsal CA1 is involved in spatial memory[43] and unlike ventral CA1 is not known to be associated with social behaviour[44,45]. Our observation that CA1-*Lrrtm1*-cKO mice had impaired social behaviour may stem from impaired spatial memory. CA1-*Lrrtm1*-cKO mice may fail to utilize visual cues in the surrounding environment to return to the first stranger in the first block of three-chambered social approach test. However, their ability to rely on olfactory cues in the second block may have helped them with having a comparable performance in social novelty preference.

Therefore, our results suggest that LRRTM1 actively contributes to synaptic transmission and long-term potentiation in a lamina-specific manner in the dorsal CA1 and is associated with behavioural tasks performed by dorsal CA1. This is manifested by deficits in input/output response, impairment of LTP and diminished contextual memory following deletion of *Lrrtm1* in the dorsal CA1. We suggest that LRRTM1 is necessary for recruitment and maintaining of AMPARs at synapses and is therefore essential for induction of LTP and in turn consolidation of context dependent memory.

Acknowledgments

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Author contributions: T.J.S conceived research; B.K. and T.J.S. designed research; B.K performed the experiments and analysis. All authors read and approved the manuscript.

Competing interests: The authors declare that there are no competing interests.

Supplementary files contain one figure and detailed experimental procedures.

3.6 Supplemental information

Detailed experimental procedures

All animal experiments were conducted in accordance with government and institutional requirements of the University of Manitoba and were in line with the ethical and procedural guidelines of the Canadian Council on Animal Care (CCAC, http://www.ccac.ca). Transgenic mice were generated on C57BL/6N mice line as previously described. Same orientation *loxP* sequences were engineered to flank the second exon of the *Lrrtm1*. Mice had *ad libitum* access to food and water and were housed with a 12-h light/dark.

Animal injections and viruses

Mice were weighed and then deeply anesthetized in an induction chamber supplied with 4% isoflurane and oxygen. Mice were given local (Marcaine) and general (Metcam) analgesics according to the recommended doses. The hair was shaved, and area sterilized. Mice were then placed in a stereotaxic frame with a nose cone supplying oxygen and 2% isoflurane. Stereotaxic Injections were done using pulled 20µl glass pipettes (puller; 700 D. Kopf, USA, pipette; Drummond scientific company, USA) with a very narrow taper and a sharp tip, opening adjusted to 30-40 µm. A picospritzer pump (Parker, USA) equipped with nitrogen gas was used to push the liquid through the pipette. Post-surgery mice were housed individually. pAAV-Ef1a-mCherry-p2A-HA-LRRTM1-DIO rescue construct was generated by cloning HA-

LRRTM1 into the pAAV-Ef1a-mCherry-p2A-MCS-DIO construct. Viruses used are listed in Table S3.1.

Table S3.1. List of viruses used in stereotaxic injection

Virus	Injection volume	Supplier
AAV-CamKII-eGFP-Cre	75-100 *nl*	UPenn vector core
AAV-CamKII-eGFP	75-100 *nl*	UPenn vector core
AAV-Ef1a-mCherry-p2A-HA-LRRTM1-DIO	75-100 *nl*	Neurophotonics

Behavior

All behavioral tests were done in a relatively soundproof room with constant white noise. Thick curtains separated the testing area from the rest of the room. Testing area was equipped with a separate light source. For most experiments performance of the mice was recorded using a video camera for later processing and analysis using AnyMaze® software (Stoelting, UK). The experimenter would leave the area before the beginning of each test, draw the curtains, and remain outside until the experiment or the session was complete.

Accelerating Rotarod

All behavioral tasks required the mice to be able to move and interact with objects or other animals; therefore, it was critical to ensure that animals motor function was not affected after stereotaxic injections and *Lrrtm1* deletion in the dorsal hippocampus CA1. An accelerated rotarod test (Harvard Apparatus, USA) was performed for measuring gross motor skill. Mice were placed on the axis of the rod facing away from the direction of rotation. The rod would rotate at 4 rpm, after 10 s at 4 rpm acceleration would begin at 20 rpm/min. Two trials were conducted for each animal, ninety minutes after the first trial. Mean values were used for analyses. The speed of the rotarod at which the mouse fell and the latency of the fall were recorded.

Elevated plus maze (EPM)

Individual mice were placed in the center of the plus-maze facing one of the closed arms. Mice were given 10 mins to explore the maze. Each mouse received one trial. Number of entries into each arm and the time they spent in each arm, as well as the distance traveled, and average speed was measured using AnyMaze software. The apparatus was cleaned with 10% ethanol after each trial.

Three-Chambered social interaction test

The apparatus consisted of a clear plexiglass rectangular box with three chambers of equal size (20 x 40 x 22 cm). The middle chamber is accessible from other chambers, through gates with sliding doors. A subject mouse would be placed in the middle chamber and allowed to move about for 5 mins for acclimation. Later the first stranger mouse (Stranger I) would be placed in the cage located in one of the side chambers. The gates to the compartments were removed, allowing the subject mouse free access to all the chambers for 10 minutes. Another stranger mouse ("Stranger II") would be placed inside an identical cage on the opposite chamber. The subject mouse was allowed to explore freely for another 10 minutes. The time spent by the subject mouse in each of the three chambers as well as total distance they travelled and average speed of their movement was measured. The apparatus was cleaned with 10% ethanol after every session. Stranger mice were of the same gender as the subject mice with no prior history of interaction.

Contextual fear conditioning (CFC)

Contextual fear conditioning was performed in a black Plexiglas chamber. Each session was recorded by a camera mounted above for offline behavioral analysis. The mice were habituated to the context by being placed in the chamber for 2 min before administration of three electrical foot shocks with 30s intervals (0.7 mA foot shock, 2

s duration) administered through a stainless-steel grid on the chamber floor. Mice remained in the chamber for an additional 3 min to ensure association of context and shock is encoded. Mice were then returned to their home cage. Twenty-four hours later CFC was assessed by returning the mice to the conditioning chamber. Mice were remained in the chamber for 5 min. Freezing (Absence of movement except respiration) time was recorded and reported as freezing percentage (duration of freezing/5 min) to assess contextual memory for each mouse. Duration of immobility (in percentage), speed of movement and distance were also measured and reported. Analysis was done using AnyMaze software.

Slice electrophysiology

Solutions

Cutting, holding and electrophysiological recordings were performed in a standard artificial cerebrospinal fluid (aCSF), salts and other compounds comprising each of these solutions are listed in Table S3.2.

Table S3.2. List of salts and chemical compounds and their concentration in electrophysiological solutions used in this study.

Compound/salt	aCSF*¥ (mM)
NaCl (Fisher scientific)	124
KCl (Fisher scientific)	3
NaH_2PO_4 (Fisher scientific)	1.25
$NaHCO_3$ (Fisher scientific)	26
$MgCl_2.H_2O$ (Fisher scientific)	1.3
$CaCl_2.H_2O$ (Fisher scientific)	2.6
Glucose (Sigma)	10
*Osmolality 300-310 osmol/kg ¥ pH: 7.4	

Slice preparation

Mice were deeply anesthetized using isoflurane and then decapitated using a sharp guillotine. Brains were removed and submerged in oxygenated (95% O_2, 5% CO_2) chilled aCSF. Hippocampi were then removed and sandwiched in an agar block with dorsal side facing up on a vibratome chuck (HM650V, Thermo-Fisher Scientific). Chuk and hippocampi were then submerged in chilled, oxygenated aCSF inside the vibratome cutting chamber. Hippocampi were sliced into 350 µm thick slices. Slices were incubated in oxygenated aCSF, constantly perfused with 95% O_2 and 5% CO_2. Slices were allowed to recover in this solution at room temperature for 1 hour, before being transferred to the recording chamber on the electrophysiology workstation. The recording chamber contained constantly oxygenated aCSF circulating at 3ml/min. Recordings were performed at 31ºC. Extracellular field EPSPs (fEPSPs) were elicited by using concentric bipolar electrodes placed on stratum radiatum or stratum lacunosum moleculare of dorsal CA1 and recorded with a glass microelectrode filled with aCSF (resistances, 2–3 MΩ). Baseline responses were taken for 20 min prior to inducing LTP. High-frequency stimulation consisting of 1 x 100 Hz, 1 s duration was used to induce LTP. Figure S1, depicts the placement of electrodes for field recordings from SR and SLM layers. To assess if GluR2^{3Y} can rescue LTP deficits, slices were incubated for 10 min in oxygenated aCSF containing GluR2^{3Y} or scrambled peptides (tat-GluR2^{3Y} or tat-Scrambled peptide) at a concentration of 2 µM.

Supplementary figure and figure legend

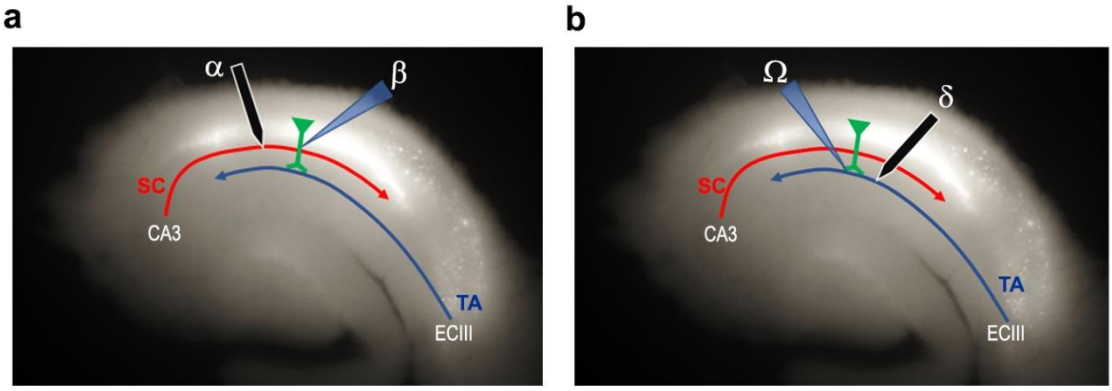

Figure S3.2. Electrode placements for SR and SLM field recordings. a. Electrode placement for SR field recordings, stimulating electrode was placed at point α and the responses were recorded through the recording electrode at β. **b.** Electrode placement for SR field recordings, stimulating electrode was placed at point δ and the responses were recorded through the recording electrode at Ω. Correct recording was ensured by switching the recording electrode between α and β. SC: Schaffer collaterals, TA: Temporoammonic pathway.

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Discussion

4.1 Synaptic proteins, synaptic specificity, and context-dependent function

To form accurate and specific connections among myriads of neurons is a unique characteristic of the nervous system. Through differentiation and migration, growing neurons find their ultimate projection destination in the nervous system. The neuronal processes travel significant distances from their cell body to find their targets in the brain. Neuronal connections are not only cell specific but are often restricted to specific subcellular compartments (e.g., dendritic spines or the cell body)[1,2].

The mammalian nervous system depends on various mechanisms to establish cell- and region-specific connections[3,4]. One of the main mechanisms is organizing the brain into layers (laminae) of distinct anatomical features. One strategy to limit the number of available postsynaptic targets is to direct the neuronal processes to specific lamina for synapse formation[5]. Lamina-specific targeting not only limits the cell type (e.g., principal neurons or interneurons) but also facilitates subcellular target selection. Once pre- and postsynaptic compartments are matched, synapses differentiate into functionally and structurally distinct synapse types[5].

Roger Sperry, American neurophysiologist, proposed the “chemoaffinity theory”. According to this theory adhesion molecules use a “lock and key” model to create specificity of neuronal wiring[6,7]. Sperry[7] and Langley’s[8] classical work showed that neurons can rewire following damage with remarkable specificity. They suggested that cells and fibres have specific identification tags that can aid them in not only forming synapses but re-stablishing them following an injury[7].

A large group of synaptic adhesion/organizing molecules (SAMs) are involved in synapse development and maintenance in the mammalian brain. SAMs bind their partners, to form cis- or trans-synaptic complexes. SAMs are essential components of cell adhesion and organizing networks[9]. They also contribute to recognition and generation of scaffolding proteins that take part in signaling processes. SAMs are regulated by different mechanisms that modify their protein levels, localization, stability, and synaptic partner availability. Interaction of SAMs with their partners can further be reinforced or weakened through alternative splicing, ectodomain shedding, competition with other synaptic partners, or astrocyte-secreted factors[10].

Through genomics and proteomics studies, a large number of cell-surface protein families including SAMs have been systematically catalogued. Many of these cell surface proteins can potentially act as surface tags envisioned by Langley and Sperry[11,12]. However, identification of the mechanisms by which this molecular diversity leads to encoding of wiring and synaptic diversity has been challenging[13,14]. Cell surface proteins that can act as regulators of diversity and specificity of synapses should be able to form trans-synaptic connections with their partners on the presynaptic or postsynaptic membrane[15,16]. These molecular interactions should also produce enough diversity to create cell- and synapse-type specificity. Moreover, cell surface proteins should have distinct expression patterns in different neurons and cell types. Several protein families including neurexins, cadherins, LRR and Ig proteins have what is required to act as synapse-specificity and -diversity regulators[16]. The molecular diversity of these proteins is a result of the large size of their gene family or alternative splicing[5].

In addition to creating synaptic diversity and specificity, synaptic adhesion proteins show a region-specific and variant-specific role. Different mutations of these

genes are believed to be associated with different diseases. Cadherins[17], neuroligins[18], Slitrks[9,19], RPTPs[20], neurexins[21] and LRRTMs[22,23] also show region- and variant -specific roles. For instance, whereas LRRTM4 functions at excitatory synapses in the dentate gyrus granule cells[22,24], it is associated with inhibitory synapses in the retinal bipolar cells[25]. Aoto *et al.*[26] found that neurexin-3 functions differentially in different brain regions. Specifically, in the hippocampus, presynaptic neurexin-3 was found to mediate trans-synaptic regulation of postsynaptic AMPA receptors whereas in the olfactory bulb, it was selectively required for GABA release. In another study, using pan-neurexin deletion of neurexins in different brain regions, Chen *et al.*[27] found that neurexins primarily function in a region-dependent manner. Neuroligins were also found to differentially contribute to synapses of the cerebellum Purkinje cells[27]. Therefore, with the body of evidence from multiple studies, it is clear that synaptic proteins have a context-dependent function. At the molecular level, the various modulators, regulators and trans-synaptic partners of synapse organizers, may also have cell-type expression patterns.

LRRTM1 as an excitatory synapse organizer protein, has a very distinct region-dependent function. Takashima *et al.*[28] performed behavior studies in global germline deletion of *Lrrtm1.* They showed that global deletion of *Lrrtm1* leads to reduced locomotor activity in the early dark phases of the light-dark box, altered behavioral responses to novel environments (as observed in open-field box, light-dark box, elevated plus maze, and hole board), reluctance to approach large inanimate objects, deficits in social discrimination and spatial memory. In another study Voikar *et al.*[29] reported that *Lrrtm1*-knockout mice avoid entering small enclosures. In the light-dark box, the *Lrrtm1*-knockout mice did not show any difference compared to controls, however when the access door was replaced with a smaller alternative, *Lrrtm1*-

knockout mice were less likely to pass through the small doorway. Moreover, *Lrrtm1*-knockout mice showed increase in social interaction, reduction in nest building behaviour and MK801-induced locomotion. They also swam slower but showed normal water maze learning. Monavarfeshani *et al.*[30] found that LRRTM1 contributes to retinal synaptic convergence on the lateral geniculate nucleus (visual thalamus). Therefore, although the pathology associated with a gene mutation or variation is the collective result of the changes in different brain regions, the role that product of such gene plays in any particular disorder can only be properly understood with region- and circuit-specific studies as we have done in this discussion.

4.2 *Lrrtm1* has region-specific function

4.2.1 *Lrrtm1* deletion in the mediodorsal nucleus of the thalamus

Lrrtm1 is consistently expressed at high levels in the brains of mice, pigs, monkeys, and humans, in the prefrontal cortex, hippocampus and the MD[31,32]. Considering the essential role of LRRTM1 in the organization of excitatory synapses and its high expression levels in the MD, Hippocampus and PFC, three key brain regions in schizophrenia pathology, and association of LRRTM1 mutations (copy number variations (CNV))[33-35] with development of schizophrenia, our goal was to explore the effect of its deletion in the MD and dorsal CA1 on synaptic integrity and behavioural profile in mice.

By stereotaxic injection of Cre expressing adeno-associated virus (AAV) in the MD of *Lrrtm1* floxed mice, we effectively deleted the *Lrrtm1* gene in the MD. We explored the effect of *Lrrtm1* deletion in the MD on social behaviour, working memory, anxiety-related avoidance, exploratory drive, and prepulse inhibition. We also used

voltage clamp and current clamp electrophysiological recordings in acute brain slices to investigate the effects of this deletion on basal synaptic transmission in the MD neurons and their intrinsic neuronal properties. In addition, we investigated the effect of *Lrrtm1* deletion on ultrastructural properties of MD synapses and expression and synaptic localization of AMPAR subunits (GluA1 and GluA2) and PSD95. Further, using brain-wide FDG-PET imaging we detected reduction in activity of prefrontal cortex after *Lrrtm1* deletion in the MD.

Using Stereotaxic injection of retrograde Cre expressing AAVs in the PFC we proved that the MD relay neurons projecting to the mPFC are heavily dependent on LRRTM1 expression for receiving information from PFC. Our results indicated that *Lrrtm1* deletion reduces mEPSC amplitude in MD relay neurons projecting to the mPFC. Reduction of mEPSC amplitude in turn, affected the regulatory role of MD relay neurons projecting to the PFC as *Lrrtm1* deletion in MD-*Lrrtm1*-cKO reduced the presynaptic activity in the PFC neurons, a condition that was rescued by re-expression of *Lrrtm1* using DIO-*Lrrtm1* expressing AAV in presence of Cre enzyme.

It is postulated that Decreased activity of MD maybe a causative characteristic of cognitive deficits in schizophrenia in humans which seems to be recapitulated in non-human primates and rodents[36,37]. Although imaging studies in patients and lesion studies in rodents have underlined the importance of MD to cognitive performance, they do not offer insights into the causal association between MD and cognitive dysfunction in schizophrenia[38,39]. Chemogenetic and optogenetic silencing of the MD are more effective methods to explain causality at the neuronal circuit level but may not resolve the biochemical foundation of altered cognition. Dissecting the molecular foundations of disruption in synaptic disorders entails exploring the function of

particular disease-associated genes at the synaptic stage, as we have shown here for *Lrrtm1*.

Our study demonstrates that cognitive and behavioural impairments due to impaired MD-PFC connectivity can emerge from the dysfunction of a single gene, *Lrrtm1*, in the MD. The deletion of *Lrrtm1* in the MD leads to decreased synaptic strength, potentially disturbing information flow to the PFC. Our FDG-PET imaging results indicate that conditional deletion of *Lrrtm1* in the MD leads to decreased synaptic activation in the PFC, findings which are compatible with human FDG-PET/functional MRI studies that show thalamofrontal hypofunction in schizophrenia. Our results are consistent with other studies showing that reduced information flow from the MD to the PFC causes cognitive symptoms of schizophrenia[40-42]. MD-*Lrrtm1*-cKO mice exhibit higher anxiety-like avoidance activity in the EPM test, which may also result from possible impairment of the excitation-inhibition balance in the PFC after *Lrrtm1* deletion. The PFC is essential for decision making and cognitive flexibility and MD projections to PFC relay the necessary information for regulation of inputs. MD-*Lrrtm1*-cKO mice fail to distinguish novel objects from the familiar target in the NOR task, which can be perceived as either diminished memory or decreased behavioural flexibility. Further, MD-*Lrrtm1*-cKO mice have disrupted social behaviour and sensorimotor gating, implicating the MD-PFC circuit in contributing to these functions. Thus, deficits in MD-*Lrrtm1*-cKO mice models a subset of cognitive deficits of schizophrenia.

Genetic and proteomics experiments implicate genes encoding components of glutamatergic signaling in schizophrenia. Within the thalamus, the molecular structure of glutamate synapses was shown to be abnormal in schizophrenia[43,44]. Recent

findings implicate the disruption of AMPAR trafficking and localization at synapses in schizophrenia. Forward trafficking of AMPARs containing the GluA1 subunit was disrupted in the frontal cortex of schizophrenia patients, and GluA1 and GluA3 transcripts were found to be decreased in the thalamus of schizophrenia subjects[45-47]. MD-*Lrrtm1*-cKO mice provide a model of schizophrenia with face, build and predictive validity. The endophenotype of MD-*Lrrtm1*-cKO mice mimic certain symptoms of schizophrenia such as anxiety, deficiency in social novelty, working memory, and sensorimotor gating. MD-*Lrrtm1*-cKO also show build and predictive validity. For instance, FDG-PET results indicated that *Lrrtm1* deletion in the MD results in reduced presynaptic activity in the mPFC which can be rescued by reintroduction of *Lrrtm1*.

Our results demonstrate the crucial role of LRRTM1 in integrity of PFC-MD-PFC circuitry. Absence of LRRTM1 in the MD leads to functional disconnection between MD and PFC. In particular, reduction of mEPSC amplitude, means that excitatory inputs from the prefrontal cortex layers to MD relay cells are weakened, which reduces the activity of MD neurons and leads to dysregulation of prefrontal pyramidal neurons as manifested by reduced PFC activity in the FDG-PET scans after deletion of *Lrrtm1* in the MD.

4.2.2 *Lrrtm1* deletion in dorsal hippocampus CA1 generates a different behavioural profile than its deletion in the MD

We investigated the effect of *Lrrtm1* deletion on social behaviour, contextual fear conditioning, and exploratory drive and anxiety in mice in which *Lrrtm1* was deleted in their dorsal hippocampus CA1. As hippocampus CA1 is one of the most well-characterized models for studying LTP, and its impairment in CA1 can have widespread cognitive effects, we explored the effect of *Lrrtm1* deletion on LTP in the

CA1 region in a layer-specific manner. CA1 stratum radiatum (SR) layer is the part of CA1 with the highest *Lrrtm1* expression, while the Stratum lacunosome moleculare (SLM) layer is almost void of LRRTM1. Deletion of *Lrrtm1* was achieved by stereotaxic injection of Cre expressing AAV in the dorsal CA1. SR is a functionally distinct part of CA1. SR receives excitatory afferents mainly from the CA3 via Shaffer collaterals. In contrast, SLM receives excitatory projections from entorhinal cortex (through the perforant pathway), lateral amygdala and the thalamus[48].

Deletion of *Lrrtm1* in CA1 region of C57Bl/6 *Lrrtm1* floxed mice (CA1-*Lrrtm1*-cKO) caused several behavioural abnormalities CA1-*Lrrtm1*-cKO show reduced social interaction as indicated by spending less time with the first stranger as opposed to the empty cage (an inanimate object). However, unlike MD-*Lrrtm1*-cKO, CA1-*Lrrtm1*-cKO mice show no signs of reduced interest in social novelty. CA1-*Lrrtm1*-cKO also displayed normal exploratory drive and no signs of anxiety in the elevated plus-maze test. CA1-*Lrrtm1*-cKO, however, show an abnormal performance in dorsal CA1-specific behaviour, the contextual fear conditioning test. 24 hrs after receiving a foot shock, CA1-*Lrrtm1*-cKO spent 25% less time in freezing mode, moved faster, and covered more distance in the same CFC chamber, compared to the controls. From a functional perspective, LTP and field excitatory post synaptic potential (fEPSP) amplitude were comparable in the SLM layer of CA1-*Lrrtm1*-cKO and control mice, whereas the SR layer in the CA1-*Lrrtm1*-cKO, showed impaired LTP maintenance and reduced amplitude of fEPSPs compared to the controls. Previously other studies have reported that LRRTMs contribute to synaptogenesis, help maintain AMPAR-mediated transmission in developing synapses, and are associated with NMDAR-triggered LTP[49-51]. More recently another study[52] from our group showed that deletion of *Lrrtm1 and Lrrtm2* in mice blocks LTP independent of NMDARs or Ca^{2+}. We found that *Lrrtm1*

and Lrrtm2 deletion decreases AMPAR-mediated transmission and impairs maintenance of AMPARS in dendritic spines in the cultured neurons.

We hypothesized based on our previous results and the results of our AMPAR subunit immunocytochemistry in MD-*Lrrtm1*-cKO mice that the reduced fEPSP amplitude and impaired LTP could be due to the reduced presence of AMPARs at the synapse as result of their internalization following *Lrrtm1* deletion. It was previously shown[53] that GluR2^{3Y} peptide can help maintain AMPARs at the synapses thereby enhancing LTP maintenance and preventing LTD. GluR2^{3Y}, is derived from GluA2 carboxyl tail (869YKEGYNVYG877), and blocks the expression of LTD in many brain areas by preventing phosphorylation of AMPARs and subsequently their internalization[54,55]. We incubated hippocampal brain slices from CA1-*Lrrtm1*-cKO mice with GluR2^{3Y} and scrambled peptide containing aCSF to rescue LTP induction and maintenance. Our results showed that after incubation with GluR2^{3Y}, LTP was rescued in SR layer of CA1-*Lrrtm1*-cKO mice, however, amplitude was not recovered. Similar results were not observed after incubation of slices with scrambled peptide. LTP and fEPSP amplitude could also be rescued by re-expression of LRRTM1 in the dorsal CA1 in a Cre-dependent manner. The inability to restore fEPSP amplitude could be attributed to the mechanism of GluR2^{3Y} peptide function. GluR2^{3Y} prevents phosphorylation of AMPARs that are present at the synapse, although this can help rescuing the maintenance of the LTP, it probably cannot recruit more AMPARs to the synapse which is probably what LRRTM1 does.

The hippocampus plays an essential role in memory formation and memory retrieval through the PFC[56-58]. The ventral hippocampus sends information related to working memory, anxiety and learned fear (e.g., CFC) to the prefrontal cortex, and in

turn, the dorsal hippocampus receives inputs from the PFC, assisting and regulating the retrieval process[59,60].

Abnormal interaction of PFC and temporal lobe structures has been reported to correlate with positive schizophrenia symptoms such as hallucinations. However, the magnitude and nature of disruption in the prefrontal-hippocampal interactions are somewhat inconsistent in schizophrenia patients[61]. Disrupted prefrontal-hippocampal communication is observed in various animal models of schizophrenia[61]. In animal models, disrupted synchrony of communications between PFC and hippocampus has been observed across a wide range of conditions such as during cognitive tasks performance, sleep, anesthesia, and wakefulness[62-64].

The ventral hippocampus projects to the mPFC, olfactory bulb and amygdala, and is traditionally believed to be responsible for social aspects of memory[65]. However, the dorsal hippocampus sends projections to septal nucleus and retrospenial area of the anterior cingulate cortex[66,67]. The cholinergic projections of septal nuclei to the CA1 region enable CA1 pyramidal cells to switch between memory encoding (Short-term storage) and consolidation (Long-term storage)[68,69]. ACh suppresses the EC to the CA1 pathway and therefore inhibits memory consolidation through interneurons in the SLM in favour of encoding. Projections of the dorsal hippocampus to septal areas are critical for the regulation of social behaviour[70,71].

Acute dorsal CA1 inactivation disrupts fear memory acquisition and retrieval[48]. The same phenotype is observed when *Lrrtm1* is deleted in the dorsal CA1 which attests to the significance of LRRTM1 in dorsal-CA1-associated behaviour. The dorsal CA1 is involved in spatial memory[72] and unlike ventral CA1 is not associated with social interaction ability[73,74]. Therefore, the impairments in social interaction, the

endophenotype displayed by CA1-*Lrrtm1*-cKO mice, may be more probably associated with impaired spatial memory than it is with social ability. CA1-*Lrrtm1*-cKO mice may fail to utilize visual cues in the surrounding environment to return to the first stranger in the first block of three-chambered social approach test. However, their ability to rely on olfactory cues in the second block may have helped them with having a comparable performance in social novelty preference.

4.3 The animal models of schizophrenia

To enhance our understanding of the neurobiological basis of complex psychiatric disorders like schizophrenia and to develop new drugs with enhanced therapeutic efficacy, we need to develop accurate, predictive animal models. Most available animal models of schizophrenia fall into one of four groups: developmental, drug-induced, lesion-induced, or hereditary. Most rodent studies exhibit behavioural phenotype modifications that are similar to positive symptoms of schizophrenia, probably due to impaired mesolimbic dopamine regulation; fewer models also show altered social interaction and learning and memory dysfunction, which are similar to negative and cognitive symptoms of schizophrenia, respectively[75].

A useful animal model for a neuropsychiatric disorder should have face, build and predictive validity. **Face validity** can be defined as existence of symptom homology. **Build validity** is the power of the model in replicating the theoretical neurobiological rationale and pathology[75,76], and **predictive validity,** which is the ability of an animal model to mimic the effect of a certain manipulation from one species to another (mouse to human) or from one condition to another condition (laboratory to the “real world”)[77]. Behavioural and neurochemical features of

schizophrenia include emergence after puberty in most cases, lack of hippocampal and cortical integration and control, dysregulation of limbic dopamine system, cortical glutamatergic hypofunction, hallucination, delusions, susceptibility to stress, impaired incentive response, abnormal sociability, and cognitive impairment[75,76].

4.4 Placing LRRTM1 in glutamate hypothesis of schizophrenia

Dopamine hypothesis of schizophrenia is the leading and most widely accepted hypothesis on pathophysiology of schizophrenia. However, the dopamine hypothesis can be reconciled with the glutamate hypothesis, and it is becoming increasingly evident that deficiency in glutamate may at least in part be responsible for dopamine pathology of schizophrenia[78,79]. The glutamate hypothesis of schizophrenia is based on the observations that deficiency in activity of glutamate at the glutamatergic synapses, especially in the PFC leads to development of schizophrenia-associated symptoms. In many brain regions, dopamine inhibits glutamate release, or glutamate leads to release of dopamine from dopaminergic neurons. Therefore, elevated dopamine levels lead to decreased levels of glutamate, while drugs that block glutamate receptors can in turn increase dopamine release[80-83].

Our results indicate that LRRTM1 plays an essential role in maintaining the integrity of glutamatergic synapses in two important information hubs in the mammalian brain, the mediodorsal nucleus of the thalamus (MD) and the dorsal hippocampal CA1. The MD and CA1 are also innervated by dopaminergic projections. MD receives heavy dopaminergic innervations and has a relatively high density of D_2–type dopamine receptors[84-86]. Dopamine is also required for long-term potentiation and memory formation in the hippocampus CA3-CA1 Schaffer collateral synapses[87].

Presence of dopaminergic innervations in the MD and hippocampal CA1 region suggests that changes in the action of dopamine in the MD and CA1 can affect PFC function through glutamatergic projections of the these areas to the PFC and *vice versa*[88]. CA1 projections to the PFC are necessary for the long-term synaptic plasticity in the neurons of the prefrontal cortex, and MD-PFC projections regulate the activity of PFC and contribute to processing of incoming information in the prefrontal cortex[59,89-91].

Enrichment of LRRTM1 in brain regions such as CA1 and MD appears to be in direct correlation with the specific functions of these regions. In the MD, the driver projections from the prefrontal cortex layers V and VI depend on the LRRTM1 as the major glutamatergic postsynaptic proteins for integrity of the synapse they make on MD relay cells. The importance of MD in PFC-MD synapses is attested by the reduced mEPSC amplitude and reduced synaptic presence of AMPAR subunits in MD-*Lrrtm1*-cKO neurons. Reduced strength of these synapses then in turn leads to reduction of the activity of neurons in mPFC, the major projection area of the MD. The essential role of LRRTM1 in integrity of MD-PFC and PFC-MD circuitry is stressed further when re-expression of LRRTM1 rescues PFC activity.

In the dorsal CA1, LRRTM1 is necessary for proper functioning of the synapses as shown by reduction of mEPSCs[52] and fEPSP amplitude and impaired induction and maintenance of LTP. In CA1, similar to the MD, re-expression of LRRTM1 rescues LTP and partially restores amplitude of fEPSPs.

These observations highlight the importance of LRRTM1 in PFC-MD-PFC and CA3-CA1 circuitry. Our results indicate that regardless of similar functional role of LRRTM1 in both circuits (organization of glutamatergic synapses), it fulfills region-

specific roles that conforms with the specific function of these circuits. LRRTM1 is essential for proper functioning of the MD which is vital for the regulatory role MD plays in regulation of PFC activity and PFC-related behaviours such as social behaviour (social novelty preference), anxiety, working memory, and prepulse inhibition[92].

In dorsal CA1, deletion of *Lrrtm1*, produces the same functional impairments, albeit with different behavioural indications. CA1-*Lrrtm1*-cKO mice show impaired contextual fear memory, a dorsal-CA1-associated behaviour. However, absence of LRRTM1 in dorsal CA1, unlike its absence in MD does not lead to development of anxiety, and although CA1-*Lrrtm1*-cKO mice show impaired social interaction, the manifestation is different from MD-*Lrrtm1*-cKO mice. CA1-*Lrrtm1*-cKO mice show impaired social interaction in the first block of three-chambered social approach test, and they have comparable performance in social novelty preference block of the test.

Concluding remarks

The region-specific deletion of *Lrrtm1* in the MD has all the proper attributes of a valid animal model of schizophrenia. MD-*Lrrtm1*-cKO mice provide a very strong face validity by manifesting many of schizophrenia symptoms such as deficiency in social affiliation and working memory, anxiety and reduced PPI. MD-*Lrrtm1*-cKO also show build validity and predictive validity. The build validity of MD-*Lrrtm1*-cKO mice is associated with ability of this model in providing a molecular explanation for central role of MD-PFC dissociation in pathology of schizophrenia as deletion of *Lrrtm1* in the MD was sufficient for manifestation of PFC-related symptoms such as social affiliation and working memory deficiency. The dissociation of MD-PFC circuitry as a result of *Lrrtm1* deletion which is a phenomenon seen in schizophrenia patients, lesion studies and MD-*Lrrtm1*-cKO mice provides predictive validity as well. The effect of *Lrrtm1*

deletion seems to be relatively consistent in the functional and physiological consequence it creates. For instance, MD and PFC hypoactivity are consistently observed in imaging studies on schizophrenia patient, duction of synaptic strength in MD can in turn reduce prefrontal activity. FDG-PET results in fact, indicate significant reduction in activity of PFC following deletion of *Lrrtm1* in the MD.

Although CA1-*Lrrtm1*-cKO mice do not seem to have all the validity criteria of an schizophrenia model, they support three important ideas, 1. LRRTM1 has a region-specific role, 2. CA1-*Lrrtm1*-cKO provide a molecular mechanism for associative[93] memory and social interaction deficits[94] in schizophrenia, and 3. Together with MD-*Lrrtm1*-cKO mice, they support the important role that LRRTM1 mutations play in development of symptoms in at least a subset of schizophrenia patients.

Finally, it should be taken into consideration that, as is the case with all studies, this study has its limitations and caveats. For instance, although *Lrrtm1* deletion in the dorsal CA1 and mediodorsal thalamus generate animal models with a reasonable degree of validity, to the best of our knowledge, mice, however similar to humans in their physiology, do not manifest complex multi-system neuropsychiatric diseases such as schizophrenia. Another limitation of this study was that our models only explored the function of LRRTM1 in mature animals and did not investigate the developmental role of LRRTM1 in the organization of synapses in the mammalian brain. Nevertheless, and at least in the case of schizophrenia, despite the presence of mutations since early stages of development, the pathological effects usually do not manifest until later stages of life, mostly during prepubescence and adolescence. That is why I believe that despite these limitations, our models help provide a possible molecular explanation for the involvement of LRRTM1 mutations in the pathology of schizophrenia.

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Appendix

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