FMRP-mediated regulation of *Syngap1* translation and its impact on NMDAR-mediated signalling in a model of Intellectual Disability, *Syngap1*$^{-/+}$

Thesis submitted for the degree of

DOCTOR OF PHILOSPOPHY

by

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Certificate

This is to certify that work described in this thesis entitled **'FMRP-mediated regulation of *Syngap1* translation and its impact on NMDAR-mediated signalling in a model of Intellectual Disability, *Syngap1*$^{+/-}$ '** is the result of investigations carried out by Mr Abhik Paul under my guidance and that the results presented here have previously not formed the basis for the award of any other diploma, degree, or fellowship.

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Author's Declaration

I declare that the work described in this thesis was carried out in accordance with the regulations of Jawaharlal Nehru Centre for Advanced Scientific Research. The work is original, except where indicated by references in the text. No part of this work has been submitted for any other academic award. Any views expressed in this thesis are those of the author.

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Abbreviations

SYNGAP1: Synaptic RAS-GTPase Activating Protein

FMRP: Fragile X Mental Retardation Protein

FXS: Fragile X Syndrome

NMDAR: N-methyl D-aspartate receptors

AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors

mGluR: Metabotropic glutamate receptors

NDD: Neurodevelopmental Disorders

ID: Intellectual Disability

ASD: Autism Spectrum Disorder

CAMKII: Calcium-calmodulin-dependent protein kinase II

PKC: Protein kinase C

LTP: Long-term potentiation

LTD: Long-term depression

PI3K: Phosphatidylinositol 3-kinases

AKT: Protein kinase B

ERK: Extracellular signal-regulated kinase

mTOR: Mechanistic/Mammalian target of Rapamycin

FOXO: Fork-head Box Protein O

BDNF: Brain-derived neurotrophic factor

CREB: cAMP response element-binding protein

CNS: Central nervous system

EPSC: Excitatory postsynaptic current

EPSP: Excitatory postsynaptic potential

IPSC: Inhibitory postsynaptic current

REST: Repressor Element-1 Silencing Transcription factor

eIF4E: Eukaryotic translation initiation factor 4E

4EBP: Eukaryotic translation initiation factor 4E binding protein

eEF2: Eukaryotic translation elongation factor 2

GPCR: G-Protein Coupled Receptors

PAM: Positive Allosteric Modulator

NAM: Negative Allosteric Modulator

PLC: Phospholipase-C

MAPK: Mitogen associated protein kinase

CDK: Cyclin-dependent kinases

S6K: p70 Ribosomal protein S 6 kinase

ARC: Activity Regulated Cytoskeleton Associated Protein

STEP: Striatal-enriched tyrosine phosphatase

GWAS: Genome-Wide Association Studies

UTR: Untranslated region

NLS: Nuclear Localization Signal

NES: Nuclear Export Signal

SoSLIP: Superoxide Dismutase 1 stem-loop

HFS: High frequency stimulus

TBS: Theta burst stimulus

E/I: Excitation to inhibition ratio

I/O: Input to output ratio

mPFC: medial Prefrontal cortex

PSD: Post Synaptic Density

FUNCAT: Fluorescent non-canonical amino acid tagging

AHA: Azidohomoalanine

KD; Knock-down

KO: Knock-out

HITS-CLIP: High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation

IP: Immunoprecipitation

RPLP0: Ribosomal large subunit protein P0

QGRS Mapper: Quadruplex forming G-Rich Sequences Mapper

Thesis Synopsis

FMRP-mediated regulation of *Syngap1* translation and its impact on NMDAR-mediated signalling in a model of intellectual Disability, *Syngap1*$^{-/+}$

Introduction

Synaptic RAS-GTPase Activating Protein (SYNGAP1) acts downstream of N-Methyl D-Aspartate Receptors (NMDAR), and negatively regulates the activity of RAS-GTPase and AMPAR insertion in the postsynaptic membrane (Kim, Liao et al. 1998, Komiyama, Watabe et al. 2002). When phosphorylated by Ca^{2+}/Calmodulin-dependent Kinase II (CAMKII), SYNGAP1 dispersed rapidly from dendritic spine to the dendritic shaft resulting in the activation of downstream signalling proteins in dendritic spines (Krapivinsky, Medina et al. 2004, Araki, Zeng et al. 2015). CAMKII-mediated phosphorylation of SYNGAP1 in-turn increases the activity of Extracellular Signal-Regulated Kinases (ERK) via RAS (Rumbaugh, Adams et al. 2006), which further allows α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Receptors (AMPAR) insertion on the post-synaptic membrane (Zhu, Qin et al. 2002). Heterozygous mutation in *SYNGAP1* gene (*SYNGAP1*$^{+/-}$) is known to cause Intellectual Disability (ID), Autism Spectrum Disorder (ASD), and Epilepsy such as Developmental and Epileptic Encephalopathy (DEE) in children (Hamdan, Gauthier et al. 2009, Hamdan, Daoud et al. 2011, Rauch, Wieczorek et al. 2012, Vlaskamp, Shaw et al. 2019).

Studies using *Syngap1*$^{+/-}$ mouse model have shown that haploinsufficiency of *Syngap1* causes early maturation of dendritic spines in the hippocampus during development (Clement, Aceti et al. 2012), and altered critical period of plasticity in thalamocortical synapses (Clement, Ozkan et al. 2013). These studies suggest that SYNGAP1 regulates synaptic connections and strength by allowing remapping of neuronal connections during the critical period of development (Clement, Ozkan et al. 2013, Aceti, Creson et al. 2015, Jeyabalan and Clement 2016).

A study by Wang et al. showed that knocking down of *Syngap1* in cortical neurons led to increased basal protein synthesis (Wang, Held et al. 2013). A similar observation was made in a study using acute hippocampal slice preparations in *Syngap1*$^{+/-}$ mice (Barnes, Wijetunge et al. 2015). In addition, these studies suggested that SYNGAP1 modulates synthesis and insertion of AMPARs at the post-synaptic membrane, thereby, regulating synaptic plasticity (Rumbaugh, Adams et al. 2006, Wang, Held et al. 2013). However, the molecular mechanisms for the regulation of protein synthesis mediated by SYNGAP1, particularly during development, are unclear.

Synaptic plasticity mechanisms were shown to be dependent on activity-mediated local protein synthesis in neurons (Klann, Antion et al. 2004, Pfeiffer and Huber 2006). Local protein synthesis is regulated by many protein synthesis regulators, such as Fragile X Mental Retardation Protein (FMRP), encoded by the *Fmr1* gene (Huber, Gallagher et al. 2002). Similar to *SYNGAP1*$^{+/-}$, a mutation in the *FMR1* gene causes ID and ASD (Garber, Visootsak et al. 2008, Hamdan, Gauthier et al. 2009). A recent report showed that protein synthesis-independent mGluR-LTD (Metabotropic glutamate receptor-dependent long-term depression) was increased in *Syngap1*$^{+/-}$ (Barnes, Wijetunge et al. 2015) which is another pathophysiological hallmark of FMRP associated synaptic deficits (Huber, Gallagher et al. 2002). Thus, to regulate synaptic protein synthesis, SYNGAP1 may crosstalk with FMRP.

Aims and Scopes of the study

Extensive studies have been done to understand ID and ASD related pathophysiology. Many studies showed progress in the preclinical mouse models of these diseases. However, until now, there is no effective cure available for ID and ASD. Advancement in the cellular, molecular, and biochemical techniques have opened up new potential strategies to target these diseases. A report by Auerbach et al. showed that genetic cross between two transgenic animals having mutations leading to opposing effect on synaptic function could ameliorate the pathophysiology manifested by both the mutants (Auerbach, Osterweil et al. 2011). Further, the authors showed that the application of Positive Allosteric Modulators of mGluRs (mGluR PAMs) could correct the pathophysiology of *Tsc2*$^{+/-}$ mice, and Negative Allosteric Modulators (NAMs) could rectify the pathology associated with *Fmr1* KO (Auerbach, Osterweil et al. 2011). This study proposes that, by targetting complementary pathways, behavioural and physiological deficits can be restored in certain forms of ID and ASD.

Similarly, Barnes *et al.* crossed *Fmr1*$^{-/Y}$ with *Syngap1*$^{+/-}$ but failed to rescue the neurophysiological deficits observed in *Syngap1*$^{+/-}$ (Barnes, Wijetunge et al. 2015). This study indicates that chronic depletion of these genes may not be a useful measure to rescue the pathophysiology observed in *Syngap1*$^{+/-}$, as both *Fmr1* and *Syngap1* genes are essential for brain development. Since SYNGAP1 is known to regulate synaptic maturation during a specific developmental window (Clement, Aceti et al. 2012, Clement, Ozkan et al. 2013, Aceti, Creson et al. 2015), thus, targeting it during that window is necessary.

The overall aim of this project is to understand the convergent biochemical pathway between SYNGAP1 and FMRP. Electrophysiology, biochemical, and molecular techniques were employed to study the complementary biochemical pathway between SYNGAP1 and FMRP.

1. To determine the FMRP expression level in *Syngap1*$^{+/-}$ during post-natal development
2. To evaluate FMRP-*Syngap1* mRNA interaction during development

3. To study the NMDAR-mediated protein synthesis response in *Syngap1*$^{+/-}$ during development

Chapter 1 is an introduction and background to the hypothesis mentioned above. In this chapter, the function of NMDAR, AMPAR, and mGluRs in synaptic plasticity, and their downstream signalling pathway is discussed in details. How protein synthesis is regulated downstream of NMDAR and mGluR was also highlighted in this section. Dysfunctioning of such receptors are associated with many neurological disorders, including Neurodevelopmental Disorders (NDD) such as ID and ASD. Aetiology, sign and symptoms, potential molecular pathways involved in ID and ASD are discussed in this chapter. Also, pathophysiological details of *Syngap1*$^{+/-}$ and *Fmr1* KO is discussed. Also, the results obtained *in vivo* and *in vitro* studies performed on *Syngap1*$^{-/+}$ and *Fmr1* KO are discussed in this chapter. The function of SYNGAP1and FMRP is known to be crucial during the critical period of brain development. Hence, the concept of the critical period of synaptic plasticity is discussed in detail and how these genes implicated in ID and ASD and affect the critical period of development is also mentioned.

Chapter 2 describes the methods and materials used in this study. A detailed description of acute brain slice preparation, brain lysate preparation, immunoblot, immunoprecipitation, polyribosome profiling assay, and synaptoneurosome preparation and stimulation of these preparations are mentioned in this chapter. Besides, all the buffers, salts, and other consumables used are summarised in tables. Similarly, all antibodies used in this project are summarised in the table.

Chapter 3 discusses the results obtained based on the objectives mentioned above. Results from a developmental profile for FMRP expression showed reduced FMRP level in the hippocampus of *Syngap1*$^{+/-}$ at Post-natal day (PND)21-23. Immunoprecipitation of FMRP showed *Syngap1* mRNA is enriched in the pellet fractions. This result demonstrated that FMRP interacts with and regulates the translation of *Syngap1* mRNA. As a proof of principle, knocking down of FMRP in Hela cells led to an increased SYNGAP1 level. Also, reduced *Fmr1* translation led to decreased FMRP level during development in *Syngap1*$^{+/-}$ that elevated *Syngap1* translation. Thus, FMRP downregulation may lead to upregulation of *Syngap1* mRNA translation.

Further, the altered response of eEF2 phosphorylation downstream of NMDA Receptor (NMDAR)-mediated signalling was observed during the given time window. In this study, a cross-talk between FMRP and SYNGAP1-mediated signalling was demonstrated and could explain the compensatory effect of impaired signalling observed in *Syngap1*$^{+/-}$ mice. Hence, modulating the level of FMRP can have a direct impact on the SYNGAP1 protein level in the brain.

Chapter 4 encompasses the overall discussion of the study. A previous study proposed that FMRP interacts and regulates the translation of *Syngap1* mRNA. FMRP was known to have a high affinity towards the G-quadruplex structures formed in the mRNA. Bioinformatics analysis using QGRS

mapper predicted the presence of a high G score containing G quadruplex motifs in the *Syngap1* mRNA. Immunoprecipitation experiments were performed to validate the interaction of FMRP with *Syngap1* mRNA and is the first to confirm FMRP-*Syngap1* mRNA interaction. Apart from that, the current study demonstrated a developmental regulation in the translation of *Syngap1* by FMRP, which in-turn differentially modulates NMDAR-mediated protein synthesis response. Also, the findings explain why genetic cross between *Fmr1* KO and *Syngap1*$^{+/-}$ failed to rescue the phenotype as both the proteins were developmentally regulated and crucial for early brain maturation. Thus, depleting both the genes could be deleterious.

Chapter 5 summarises the findings and proposes future experiments to further elucidate the mechanisms and implication of translation regulation of *Syngap1* (mRNA) by FMRP. One of the major questions is to rescue the pathophysiology observed in *Syngap1*$^{+/-}$ and how targeting FMRP would facilitate this question. This project has shown that downregulation of FMRP led to increased *Syngap1* mRNA translation, thereby, to compensate for the reduced level of SYNGAP1 in *Syngap1*$^{+/-}$. Chronic depletion of both the genes, *Fmr1* and *Syngap1*, failed to rescue the phenotype. Based on these studies, knockdown of FMRP in *Syngap1*$^{+/-}$ hippocampus at an earlier age group such as PND7-9 and study NMDAR-mediated signalling in adults would be the probable approach. Based on the results from this thesis, the proposed model suggests the temporal regulation of these proteins is essential and could be an attractive therapeutic target to ameliorate the disease pathology associated with it.

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List of Publications

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- Verma, V., **Paul, A**., Amrapali Vishwanath, A., Vaidya, B. and Clement, J.P., 2019. Understanding intellectual disability and autism spectrum disorders from common mouse models: synapses to behaviour. *Open biology*, *9*(6), p.180265.

CHAPTER - 1 Introduction

1.1. History of brain research

The ancient Egyptians were the first known people to use the word 'Brain' when they mentioned about some of the “head-related” illnesses in the papyrus scriptures. Strangely, they used to believe that the heart is the repository of memory and site of learning and not the brain (Győry 2008). Later, the famous Greek Physician, Hippocrates (460 B.C-370 B.C.), proposed that two halves of the brain can work independently (‘Mental duality’) and it was the seat of intelligence (WALSHE 1961) Since then, human beings were curious to know the function of the brain and started to explore and further understand the various functions of the brain.

In the early nineteenth century, Bell and Megandie proposed that nerves consisted of many wire-like structures; some send information from the periphery such as muscles to the brain, and some send information from the brain to the muscles (Jorgensen 2003) (http://faculty.washington.edu/chudler/hist.html#0). Based on research over many decades, neuroscience in recent years has emerged into an amalgamation of multidiscipline —from the molecules to cognition (higher level mental activity). Neuroscience can be classified in many subparts; such as Molecular Neuroscience, Cellular Neuroscience, Systems Neuroscience, Behavioural Neuroscience, and Cognitive Neuroscience (Bear, Connors et al. 2007, Kandel 2013). In spite of the studies on the brain for many years, it remains a puzzle, possibly because of its structural and functional complexity and heterogeneity, and due to lack of techniques to study neuronal function.

1.2. Advancement in the study of brain

The brain consists of many different types of cells, such as neurons, astrocytes, microglia, and oligodendrocytes (Berger 1998, Gasque, Jones et al. 1998, Spassky, Goujet-Zalc et al. 1998, NAVASCUÉS, CALVENTE et al. 2000, Zeisel, Munoz-Manchado et al. 2015). Until the last decade, neurons were considered to play a significant role in the brain functions, and the glia (non-neuronal cells present in the brain) were speculated to play a supportive role by providing nutrients to neurons. Due to lack of technical advancements, visualising these multiple cell types remains a challenge. German neurologist Franz Nissl, for the first time, stained brain tissue using Cresyl Violet that stained the nucleus (Kadar, Wittmann et al. 2009). He also observed clumps of materials surrounding the nucleus, named as Nissl bodies. These structures were the aggregated ribosomes associated with Endoplasmic Reticulum (Nievel and Cumings 1967). In 1873, Camillo Golgi invented Golgi stain (silver chromate) that stained the entire neurons (Wouterlood, Paniry et al. 1987). He proposed that

the neurites (extensions of neurons) are fused to form a continuous reticulum, later known as reticular theory (Cimino 1999). However, his contemporary, Santiago Ramon Y Cajal, opposed this theory and suggested that the neurites are not continuous; they must communicate through a contact, which we now know as synapses. This theory was known as the neurone doctrine. Later studies on brain highly favoured the theory of neuron doctrine (Jones 1999, Bock 2013).

1.3. Anatomy of the brain

Studies for centuries illustrated the anatomical structure of the mammalian brain. Broadly, the brain can be classified into three major parts, from posterior to anterior, brain stem, cerebellum and the cerebrum, respectively (Henery and Mayhew 1989, Rhoton 2007). These parts can be subdivided further depending on structure and function (Bottomley, Hart et al. 1984). Each of these parts plays their respective roles, which are necessary for normal brain functions. For example, brain stem helps in communicating with other parts of the brain from and to the peripheral nervous system via the spinal cord (Angeles Fernandez-Gil, Palacios-Bote et al. 2010). This structure consists of a very complex network of fibres and cells, which helps in the cross-communication. Apart from these, brain-stem is necessary for other vital functions such as breathing, consciousness, and maintenance of body temperature (Nicholls and Paton 2009). The cerebellum (which means ‘little brain’ in the Latin language) is the second largest part of the brain (D'Angelo 2018) and located behind the Cerebrum. The cerebellum is involved in movement and coordination, postures, eye and head movements, and maintaining body balance (Saab and Willis 2003, Glickstein and Doron 2008). Anatomically, the rostral and the substantial part of the brain is the cerebrum. The cerebrum can be split into two equal cerebral hemispheres morphologically. Right hemispheres get sensory input from the left side of the body and vice versa (Van Overwalle and Marien 2016). The cerebrum broadly controls sensory and motor functions and also intelligence. An outer layer of the cerebrum is known as Cerebral cortex, which mediates many essential features such as processing of sensory inputs, learning and memory, speech, and cognition (Sporns 2013). Some parts of the cortex have specialised functions, e.g. somatosensory cortex registers touch sensation from any part of the body (Borich, Brodie et al. 2015). Therefore, all brain parts play an essential role in helping us do our day to day activity.

1.4. The hippocampus

The hippocampus has a very well defined structure and appears as two sheets of neurons folded on top of each other, Cornu Ammonis (CA) and Dentate Gyrus (DG) respectively (Li, Mu et al. 2009). The anatomy and structure of the hippocampus have been studied for decades and has been an attractive model to study various functions of the brain, especially learning and memory, mainly due

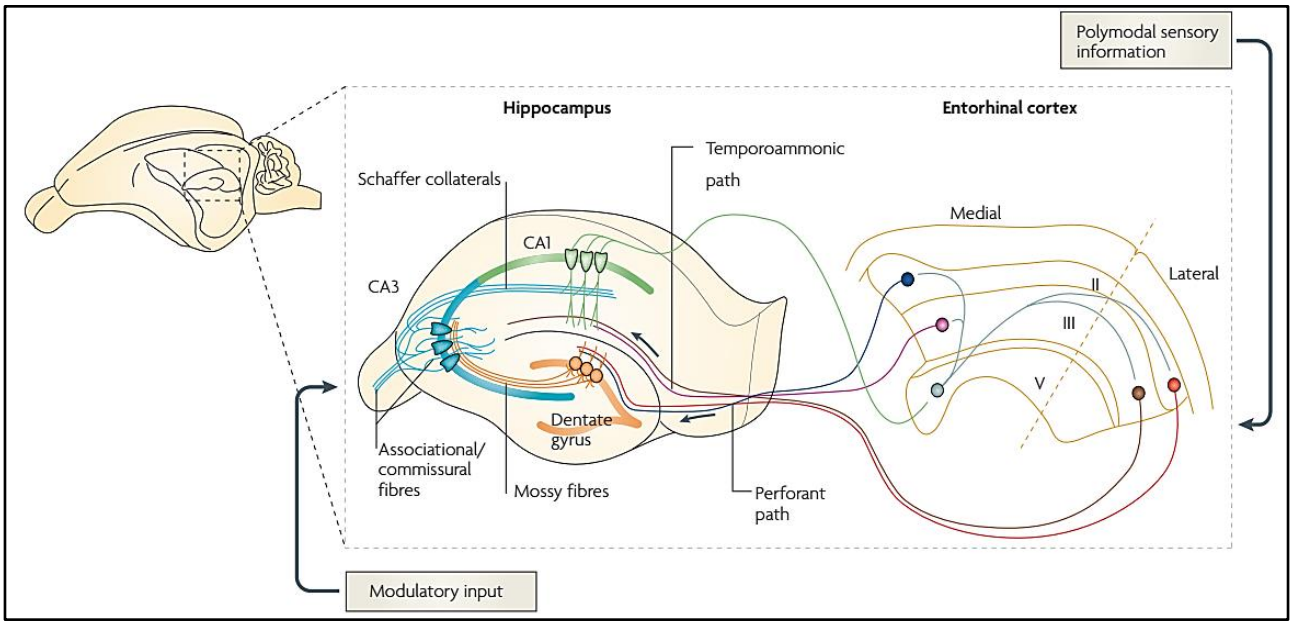

Figure 1-1: Trisynaptic pathway in the hippocampus

Location of the hippocampus is shown inside the rodent brain. The inlet shows different pathways present in the hippocampus. The major input comes from the layer II of the Entorhinal Cortex (EC) to the Dentate Gyrus (DG). That pathway is known as the Perforant Pathway (PP). Granule cells of the DG make projection on to the CA3 pyramidal neurons. This pathway is called the Mossy Fibre (MF) pathway. Further, the CA3 neurons project onto the dendrites of CA1 neurons via Schaffer Collateral (SC) pathway. The CA1 apical dendrites also receive direct input from EC. All the cells are organized and tightly packed in an interlocking C shaped orientation in the hippocampus. This figure is reproduced with permission from (Neves, Cooke et al. 2008).

to its well-defined circuitry and pathway. The primary input to the hippocamp DG comes from Entorhinal cortex, and the pathway is called the Perforant pathway (Vago, Bevan et al. 2007). DG neurons further form synapses on to the CA3 region and This pathway is known as Mossy fibre (Witton, Brown et al. 2010, Evstratova and Toth 2014). From the CA3 areas, axons bifurcated into two different directions; One branch leaves the hippocampus and projects onto the CA1 neurons of the contralateral hippocampus and another branch synapses on to the CA1 neurons. The first pathway

is known as the Associational Commissural (AC) pathway, and the latter pathway is called the Schaffer Collateral (Neves, Cooke et al. 2008). Together, there are three major pathways present in the hippocampus, i.e. Perforant, Mossy fibre, and Schaffer collateral is called the Trisynaptic pathway (Neves, Cooke et al. 2008)(Figure 1-1).

1.5. Importance of the hippocampus

The hippocampus is shown by many laboratories in the past decades to be essential for learning and memory, and, any damage or insult to the hippocampus has a profound effect on its function (Dede, Wixted et al. 2013). Most of the functions of the hippocampus were observed from a famous patient known as H.M (Shah, Pattanayak et al. 2014). He experienced seizures since his childhood, and later, it increased to severe generalised seizure, convulsion, and loss of consciousness (Squire 2009, Eichenbaum 2013). As medications did not work, he had undergone surgery where the medial temporal lobe was excised that contained part of the cortex, amygdala, and 2/3rd of the hippocampus (Milner 1972, Squire 2009). Although the surgery was successful, alleviating the seizures, he started to show profound amnesia, particularly anterograde amnesia (Milner 1972, Squire 2009, Eichenbaum 2013). He was able to recollect many incidences from his childhood, but he could not remember recent incidences, such as meeting someone 5 minutes ago (Bear, Connors et al. 2007). For example, he introduced himself every time he met his Doctor, Brenda Milner, who was a neuroscientist by profession, worked with H.M for almost 50 years. Each time Brenda met H.M, he had to introduce himself to her (Milner 1972, Bear, Connors et al. 2007). However, H.M could remember incidences of childhood, suggesting that he did not have any problem to recollect past incidences. Despite this, H.M was able to learn new tasks, indicating that procedural memory formation was normal. The only problem he faced was to form new declarative memory (Squire 2009, Eichenbaum 2013). Thus, the characteristics of H.M's amnesia suggests that there is a difference in neuroanatomy and mechanism for procedural and declarative memory formation as well as short-term and long-term memory. Therefore, for neuroscientists, the hippocampus is still a widely used model to study not only learning and memory but also other related questions related to brain function.

In this introductory chapter, Synaptic Plasticity Mechanisms, which are the cellular correlate of learning and memory, were discussed, including Hebb's postulate and BCM theory. Further, Long-term potentiation (LTP), and Long-term depression (LTD) and the role of different glutamate receptors in synaptic plasticity were discussed in details. Studies for decades have shown the role of protein synthesis in such synaptic plasticity mechanism is discussed in the following sections.

Moreover, an overview of how impairment in synaptic plasticity leading to defects in learning and memory affecting the critical period of plasticity is discussed. Further, how alteration in the critical period leads to neurodevelopmental disorders (NDD) such as Intellectual Disability (ID) and Autism Spectrum Disorder (ASD), particularly studies done using Fragile X syndrome and *SYNGAP1*$^{-/+}$ are discussed in detail.

1.6. Synaptic Plasticity in the hippocampus

Donald Hebb proposed that each synapse either becomes stronger when it successfully participates in the firing of the pre- and a post-synaptic neuron or weaker during a weak stimulation (Hebb 1949). This change in the strength of the synapse is termed as 'Synaptic plasticity'. The increase or decrease in the strength is referred to as Long-term potentiation (LTP) and Long-term depression (LTD), respectively. However, after more than two decades, Hebb's postulate was experimentally supported when, in 1973, Tim Bliss and colleagues showed that tetanic stimulation of perforant path elicited a persistent increase in the Dentate Gyrus (DG) granule cell responses in both anaesthetised and unanaesthetised rabbits (Bliss and Gardner-Medwin 1973, Bliss and Lomo 1973). Since then, synaptic plasticity was studied in many brain regions, including the hippocampus (Artola and Singer 1987, Alonso, de Curtis et al. 1990, Calabresi, Pisani et al. 1992). Hippocampus was particularly a well-established model to study plasticity due to its distinct cellular lining, and clear axonal pathway (Buzsaki 1980, Collingridge, Kehl et al. 1983, McNaughton and Miller 1986). Overall, synaptic plasticity is crucial and necessary for learning and memory. Such changes in the synaptic strength could lead to structural modification of synapses mediated by distinct molecular mechanisms (Ho, Lee et al. 2011).

Studies have shown that two major receptors in glutamatergic synapses mediate synaptic plasticity, LTP and LTD. These receptors are AMPAR (α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors) and NMDAR (N-methyl-D-aspartate receptors) and conduct Ca^{2+} (Wiltgen, Royle et al. 2010, Shepherd 2012, Maki and Popescu 2014, Carvajal, Mattison et al. 2016, Lalanne, Oyrer et al. 2018). NMDAR conducts Ca^{2+} only when glutamate binds to the receptor, and simultaneous unblocking of Mg^{2+} due to depolarisation of the membrane. The site of Mg^{2+} is within the cation binding site, and it is voltage dependent (Chahal, D'Souza et al. 1998). Hence, change in the

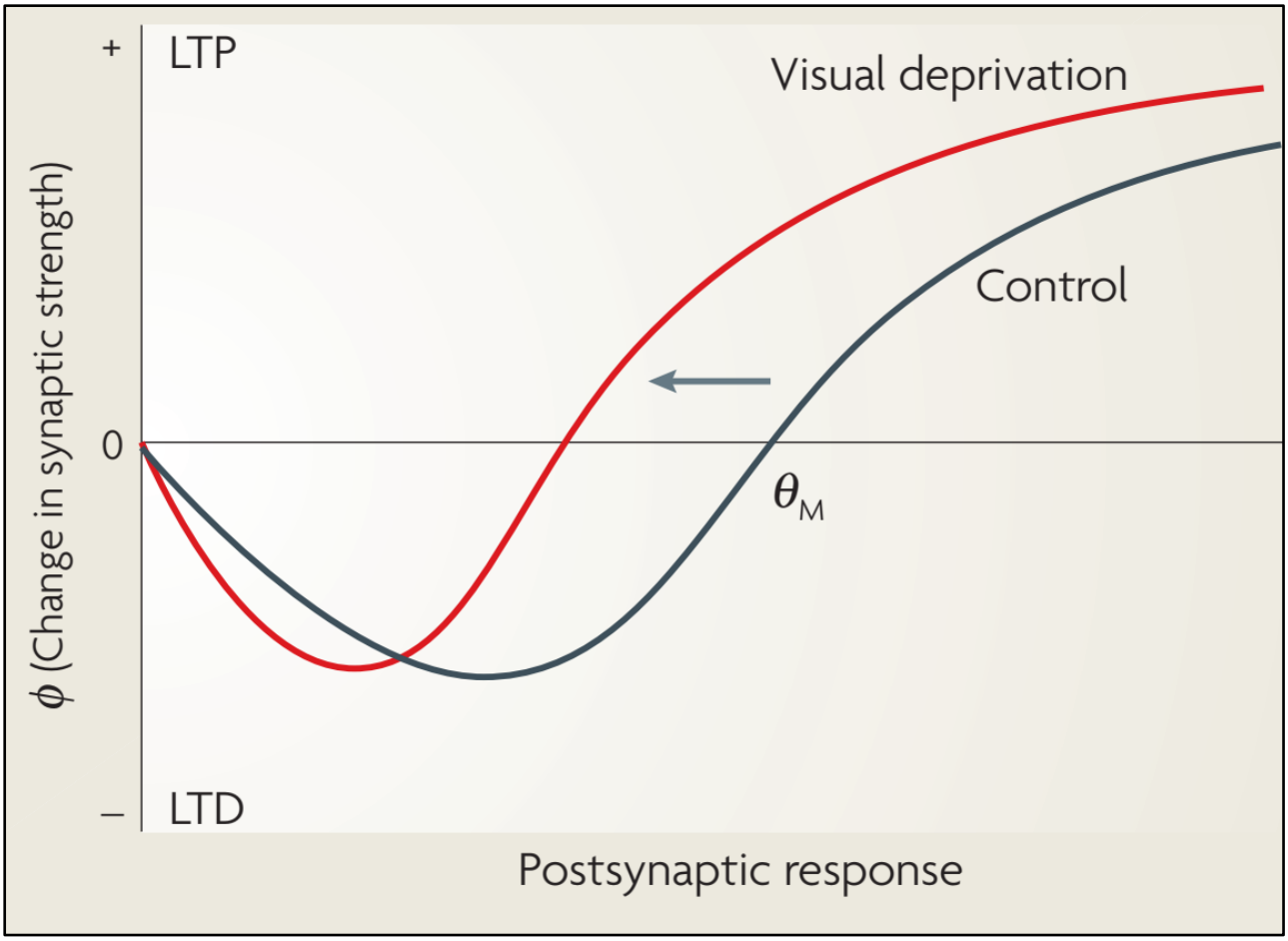

Figure 1-2: The Bienenstock, Cooper and Munro (BCM) computational model of synaptic plasticity

LTP and LTD are depicted as a function (φ) of post-synaptic strength. Modification threshold (θ_M) is the crossover point between LTD and LTP. The model describes that if the firing rate is high then the threshold shifts towards right which makes induction of LTP harder, and induction of LTD easier and vice versa. Monocular deprivation model shows that the threshold shifts towards left promoting LTP induction. The Figure was reproduced with permission (Abraham 2008) (see Appendix).

membrane potential can effectively remove the Mg^{2+} block. Evidence shows that the increase in post synaptic Ca^{2+} concentration link with the induction of LTP (Malenka 1991). Studies have shown that injecting calcium chelator in the post-synapse can inhibit the induction of the LTP (Yeckel, Kapur et al. 1999). Increase in Ca^{2+} concentration in the post-synapse activates two downstream proteins: Protein kinase C (PKC) and Calcium-calmodulin-dependent protein kinase II (CAMKII) (Huang 1989, Shifman, Choi et al. 2006, Bear, Connors et al. 2007). Studies suggest that these kinases activation leads to phosphorylation of AMPA receptors, which in turn increases the ionic conductance

of these receptors (Kristensen, Jenkins et al. 2011). Thus, it increases the efficacy of a postsynapse by inserting new AMPARs in the postsynaptic membrane. It is known that AMPA receptors are stored in vesicles in postsynapse, and upon CAMKII activity these vesicles fuse to the postsynaptic membrane inserting the AMPAR (Lu, Man et al. 2001, Bear, Connors et al. 2007, Hussain and Davanger 2015, Hangen, Cordelieres et al. 2018, Park 2018).

According to Elie **B**ienenstock, Leon **C**ooper, and Paul **M**unro (BCM) theory, synapses which are active when the postsynapse is weakly depolarised undergo depression, LTD, while stronger stimulation can lead to LTP (Bear, Connors et al. 2007). The BCM theory was developed to mathematically model the stimulus selectivity, orientation, and binocular interaction in cortical neurons. Although the BCM theory was initially designed to model modifications occurring in the cortex, it is now applicable to some experimental findings in the hippocampus synaptic plasticity (Bienenstock, Cooper et al. 1982). The model incorporates a modification threshold θ_M (activity-dependent modifications in the ability of synapses). Accordingly, under control conditions, in response to a low level of correlated presynaptic activity and postsynaptic cell firing LTD is induced, while LTP is induced by a high level of postsynaptic cell firing. In response to a high level of *previous* postsynaptic cell firing the LTD/LTP crossover point (θ_M) shifts to the right, inhibiting LTP, and facilitating LTD induction (Figure 1-2). In contrast, in response to a low level of previous postsynaptic cell firing θ_M shifts to the left, promoting LTP and inhibiting LTD induction (Abraham 2008).

Similar to LTP, LTD can be triggered by postsynaptic Ca^{2+} entry through the NMDA receptors (Luscher and Malenka 2012). Weak depolarisation of the membrane can allow the low concentration of Ca^{2+} to enter the postsynapse through NMDARs. This low concentration in the Ca^{2+} activates protein phosphatases instead of kinases. The induction of hippocampal LTD is associated with internalisation of AMPAR from postsynapse (Bear, Connors et al. 2007).

LTP has long been thought to be a key mechanism of storage of long-term memories; it has recently become clear that some mechanism is necessary for long-term depression (LTD) of synaptic transmission for several reasons. The bi-directional synaptic plasticity senses appropriate amounts of presynaptic input that is temporally coincident with postsynaptic firing maximises the strength of the synapses in a way that balances (i.e., avoid saturation of synaptic strength) the synaptic networks (Vose and Stanton 2017). Similar to LTP, two mechanistically distinct forms LTD coexist at synapses in the CA1 region of the hippocampus; induction of one form depends on activation of NMDARs, and of the other on activation of mGluRs (Dudek and Bear 1992, Oliet, Malenka et al. 1997).

Hippocampal LTD was initially demonstrated to be heterosynaptic in nature; following the induction of LTP *in vitro* in the CA1 area by stimulation of one pathway a depression of responses at the quiescent input was observed (Lynch, Dunwiddie et al. 1977). Subsequent studies showed that by repetitive low frequency (1 Hz) synaptic stimulation in the CA1 region of the hippocampus, the synapses readily exhibited LTD. Additionally, this form of LTD was found to be synapse-specific and required activation of postsynaptic NMDA receptors (Dudek and Bear 1992, Mulkey and Malenka 1992). Similar to LTP, LTD required Ca^{2+}; loading CA1 cells with the Ca^{2+} chelator BAPTA prevented LTD (Mulkey and Malenka 1992). LTD can also be produced by low-level Ca^{2+} entry through L-type VDCCs or by uncaging of Ca^{2+} (Cummings, Mulkey et al. 1996, Yang, Tang et al. 1999). Although, a rise in intracellular Ca^{2+} initiates both LTP and NMDAR-dependent LTD. An important mechanism that distinguishes LTP from LTD is the induction of LTD leads to protein phosphatase activation (Mulkey, Herron et al. 1993, Mulkey, Endo et al. 1994). In general, Protein Phosphatase 2B is activated as they have a higher sensitivity for Ca^{+} than kinases, lower levels of intracellular Ca^{2+} elevation preferentially activates phosphatase cascades. In contrast, higher Ca^{2+} levels will both activate kinase phosphorylation cascades and inhibit phosphatase activity (Lisman 1989, Lisman 2001). LTP can also be converted to LTD by reducing the concentration of Ca^{2+} entry through NMDARs such as by application of a low dose of the NMDAR antagonist APV (Cummings, Mulkey et al. 1996).

1.7. NMDA receptor composition and stoichiometry

Structurally, NMDAR subunit can be classified into four distinct domains: Amino-terminal Domain, Agonist Binding Domain, Transmembrane Domain, and C-terminal Domain (Traynelis, Wollmuth et al. 2010, Mayer 2011, Paoletti 2011). Amino-terminal domain (NTD) of the subunit involved in subunit assembly and allosteric modulation. Agonist binding domain (ABD) is the site for the ligand binding such as Glycine (for GluN1 and GluN3), and Glutamate (for GluN2). The transmembrane domain (TMD), which consists of three transmembrane helices and one pore loop. And, the carboxy-terminal domain (CTD), plays a vital role in downstream signalling, receptor trafficking, and anchoring to the membrane.

Subfamilies	Subunits	Gene	Isoform	Region and Developmental period
GluN1	GluN1	*GRIN1*	GluN1 1a	Rostral region inc Cortex, Hippocampus
			GluN1 2a	Whole brain
			GluN1 3a	All principal cells in Hippocampus
			GluN1 4a	Mainly caudal regions
			GluN1 1b	Rostral region inc Cortex, CA3 in Hippocampus
			GluN1 2b	Whole brain, only CA3 in Hippocampus
			GluN1 3b	Only the CA3 layer
			GluN1 4b	Mainly caudal regions (all isoforms are expresssed from E14 to adult)
GluN2	GluN2A	*GRIN2A*	GluN2A	Whole brain (adult)
	GluN2B	*GRIN2B*	GluN2B	Forebrain (First-week post-natal)
	GluN2C	*GRIN2C*	GluN2C	Cerebellum, olfactory bulb (>P10)
	GluN2D	*GRIN2D*	GluN2D	Embryonic brain (caudal region)
GluN3	GluN3A	*GRIN3A*	GluN3A	Whole brain except cerebellum, olfactory bulb (P14)
	GluN3B	*GRIN3B*	GluN3B	Majorly motor neurons (adult)

Table 1-1: NMDA receptor subunit composition

The table summarises all receptor subunits of NMDAR. Different subunits show distinct spatiotemporal expression pattern in the brain (Watanabe, Inoue et al. 1992, Akazawa, Shigemoto et al. 1994, Monyer, Burnashev et al. 1994, Paoletti 2011)

Functionally, NMDAR is a tetrameric complex consisting of four subunits, out of which two GluN1 subunits are obligatory. Other two subunits could be GluN2 and/or GluN3 (Sheng,

Cummings et al. 1994). Combination of different subunits in the receptor complex brings the concept of Di-heteromeric/Tri-heteromeric receptors. For example, Di-heteromeric receptors consist of GluN1/GluN2A or GluN1/GluN2B, whereas tri-heteromeric consists of either of these combinations: GluN1/GluN2A/GluN2B or GluN1/GluN2/GluN3. Majorly, this diversity in the receptor subunit composition plays an essential role in the function of the NMDAR (Cull-Candy and Leszkiewicz 2004, Traynelis, Wollmuth et al. 2010). Different NMDAR subunits have a preferential expression in different types of neurons (Monyer, Burnashev et al. 1994, Cull-Candy and Leszkiewicz 2004). Not only cell type specificity, but NMDAR subunit composition varies depending on the subcellular localisation. For example, GluN2B containing receptors are concentrated in the peri- and extra-synaptic region in the post-synapse, whereas, GluN2A containing NMDARs are present in the synaptic region (Hardingham and Bading 2010, Gladding and Raymond 2011). The spatiotemporal expression of these receptor subunits is summarised in Table 1-1.

NMDAR-mediated signalling is known to induce both cell death as well as survival signal (Luo, Wu et al. 2011). The synaptic region of mature neurons contains NR2A containing NMDAR. Activation of such receptors triggers two distinct signalling pathways: PI3K, and CAMK, as described in the earlier section. PI3K activates AKT, which is a negative regulator of Forkhead Box Protein O (FOXO) (Brunet, Bonni et al. 1999, Al-Mubarak, Soriano et al. 2009). Thus, reduced FOXO activity leads to reduced expression of pro-death genes such as *Bim*, *Txnip* (Dick and Bading 2010). CAMKIV activity downstream to NR2A-NMDAR activation also induces the expression of pro-survival genes such as *Bdnf*. On the contrary, NR2B containing NMDARs populate the extra-synaptic regions predominantly. This subset of NMDAR activity leads to downregulation of CREB; in-turn inhibits the expression of pro-survival genes (Hardingham, Fukunaga et al. 2002). Also, activation of FOXO results in the expression of pro-death genes. Thus, increased activation of NR2B-containing NMDARs can lead to apoptosis and neuronal death (Hardingham, Fukunaga et al. 2002).

1.8. Developmental switch in the NMDAR subunit composition

During the early postnatal development, the NMDAR subunits undergo a switch in their expression. In the early stage of development, the majority of the NMDARs contain GluN2B subunit, which switches towards GluN2A during development (Williams, Russell et al. 1993, Xing, Wang et al. 2006). This developmental switch from GluN2B to GluN2A comprising NMDAR is observed in almost all part of the CNS. Additionally, this phenomenon is conserved amongst different species (Dumas 2005, Paoletti, Bellone et al. 2013). The switch in NMDAR subunit is not an absolute shift in the expression pattern of these two subunits, i.e. predominantly NR2A but has a small population

of NR2B subunits as well. This switchover suggests that the subunit expression level may not be correlated directly with the switching phenomenon.

One of the early electrophysiological studies by Williams et al., 1993 first noticed the presence of a subset of NMDARs which had 100 low fold affinity towards ifenprodil, a potent antagonist of NR1-NR2B containing NMDARs (Williams, Russell et al. 1993). Whereas insensitivity to ifenprodil suggests incorporation of a new subset of NMDARs that contains NR1-NR2A, which represents around 50% of NMDARs in adult rat brain (Williams, Russell et al. 1993). These observations were recapitulated in different brain regions later (Lopez de Armentia and Sah 2003). However, a study by Lopez de Armentia showed that Central Amygdala does not follow the same developmental switch pattern of NMDAR. NMDAR-mediated synaptic currents from both immature and matured neurons in the central amygdala manifested slow kinetics and were blocked effectively by ifenprodil indicating that there was no change in the subunit composition between immature and mature neurons in the central amygdala ((Lopez de Armentia and Sah 2003). However, in the immature synapses of the lateral amygdala, NMDAR EPSCs were slow and could be blocked by NR2B specific antagonist such as ifenprodil. Whereas in the mature synapses NMDAR EPSCs were fast and substantially less sensitive to NR2B selective antagonist (Lopez de Armentia and Sah 2003). Thus, the NMDARs in the lateral amygdala undergo the developmental switch of the receptor subunit, whereas in central amygdala they do not. Cellular and molecular mechanisms behind such developmental switch are discussed in the later section. Using cultured neurons, it has been possible to study the timescale of such switch. Not only developmentally NMDAR subunit composition can alter depending on the activity of the neurons but also in a much faster timeline. Under basal conditions, there is a turnover of these receptor subunits. However, NR2B and NR2A containing NMDAR turnover were shown to be comparable under the basal state (von Engelhardt, Doganci et al. 2009). Blockade of NMDAR and AMPAR activity for 8 hours led to the increased insertion of NR2A containing NMDAR on the post-synaptic surface. Whereas, there was no change in the surface expression level of NR2B containing NMDARs (von Engelhardt, Doganci et al. 2009). Electrophysiological recordings from cultured hippocampal neurons as well as from CA1 neurons in slice post 8-hour NMDAR block showed increased NMDAR EPSCs (von Engelhardt, Doganci et al. 2009). These data suggest incorporation of new NR2A containing NMDARs in the synapses but not NR2B containing receptors.

1.9. NMDAR subunit switch and critical period of plasticity

Earlier studies on somatosensory cortex gave indications about NMDAR's role in the critical period of plasticity. *Grin1* and *Grin2B*-null mice lacked whisker-related mapping of the thalamocortical region (Li, Erzurumlu et al. 1994, Iwasato, Erzurumlu et al. 1997). These studies provided the first

evidence to show direct involvement of NMDAR in the formation of sensory-related neuronal patterning in the mammalian brain. Further, pharmacological studies showed whisker-related thalamocortical map plasticity was reduced in the neonatal mice when NMDAR activity was blocked by APV (Fox, Schlaggar et al. 1996, Iwasato, Erzurumlu et al. 1997, Rema, Armstrong-James et al. 1998). These studies suggest that the development of the plasticity of rodent somatosensory cortex was mediated by NMDAR. In addition to these findings, NMDAR subunit composition was shown to be differentially regulated by neuronal activity (Hoffmann, Gremme et al. 2000) and sensory experience (Quinlan, Olstein et al. 1999, Quinlan, Philpot et al. 1999, Philpot, Sekhar et al. 2001) suggesting that the dynamic change in the subunit composition might play a crucial role in the development of plasticity. One of the apparent hypothesis about the molecular mechanism was expression level change in the *Grin2b* and *Grin2a* genes. However, the findings of Liu et al. 2004 was not consistent with the existing hypothesis. Authors found a developmental increase in NR2A mRNA and protein in the synapse, and a decrease in NR2B containing NMDARs even though there was no decrease in *Grin2b* expression (Liu, Murray et al. 2004). Thus, the change in the synaptic NMDAR composition in the somatosensory cortex is not correlated directly with the gene expression level. The detailed mechanisms for the developmental switch are discussed below.

Similar to the somatosensory cortex, NMDAR subunit switch was also shown to have an impact on the critical period of plasticity in Visual cortex. Erisir and Harris had quantified volumetric densities of NR1-, NR2B-, and NR2A-containing synapses in the layer 2/3 and layer 4 of the visual cortex of ferrets (Erisir and Harris 2003). They observed that NR2A-containing NMDARs in synapses increased significantly from eye-opening till PND 34, and these receptors became predominant in adulthood in both somatosensory and visual cortex. Whereas, NR2B was at peak during the critical period and reduced significantly at the closure of the critical period (Erisir and Harris 2003). These data suggest that increased level of NR2B-containing NMDARs is the permissive factor for the ocular dominance plasticity observed in developing visual cortex. The ratio of NR2A- and NR2B-containing NMDARs in the visual cortex is a key to the plasticity during the critical period. The study showed NR2A knockout mice exhibited precocious potentiation of non-deprived eye response and failed to manifest deprivation induced depression in monocular deprivation paradigm (Cho, Khibnik et al. 2009). Yoshimura et al. showed a reduction in the incidence of excitatory LTP in the visual cortex, which is corroborated with the decline in NR2B-containing NMDARs. Both of these changes were abolished by rearing the pups in the dark (Yoshimura, Ohmura et al. 2003). Therefore, based on these studies, critical period of plasticity in the visual cortex depends on age, experience, and change in NMDAR subunit expression.

1.10. Cellular/Molecular mechanisms behind the NMDAR subunit switch

During post-natal development, there is a concomitant increase in the GluN2A containing synapses, whereas synapses containing GluN2B still populate many parts of the CNS (Paoletti 2011). Initially, several hypothesise have suggested that there could be a decrease in the number of GluN2B containing synapses with a subsequent increase in GluN2A containing synapses (Paoletti 2011). It was believed that activity-mediated changes in the trafficking as well as the transcription of these subunits responsible for the switch (Hoffmann, Gremme et al. 2000, Barria and Malinow 2002). By overexpressing GFP tagged NMDAR subunit in the hippocampal slice neurons, Barria et al. showed NR1 containing NMDAR trafficking into the spines required NR2 expression (Barria and Malinow 2002). These results indicate an interplay between different subunit expression and its traffic to the spines. Many studies have consistently shown GluN2A mRNA, and protein expression in the brain increases with age (Zhong, Carrozza et al. 1995, Liu, Murray et al. 2004). One of the hallmark studies by Liu et al. demonstrated the cellular level of GluN2B was unchanged throughout development even though there was a reduction in GluN2B containing synapses. Using in situ hybridisation, immunohistochemistry, and immunoelectron microscopy authors have shown that the expression in the GluN2B containing synapses was not altered. Instead, it might occur because of the formation of new synapses lacking GluN2B (Liu, Murray et al. 2004).

For decades, understanding the mechanisms of the shift in NMDAR subunit composition during development was the major focus of many neuroscientists. The post-natal developmental switch of NMDAR subunit was regulated in an activity-dependent manner. In the hippocampal neurons, GluN2A insertion at the synapse requires neuronal activity, but not GluN2B trafficking to the post-synapses (Barria and Malinow 2002, Rebola, Srikumar et al. 2010). It has also been shown that activity mediated phosphorylation of the PDZ binding domain of GluN2B led to the removal of NMDAR containing GluN2B from the synapse (Tomita, Nicoll et al. 2001, Sanz-Clemente, Matta et al. 2010, Lu, Fang et al. 2015). At the level of gene regulation, epigenetic modifications play a crucial role. For example, Repressor Element-1 Silencing Transcription factor (REST), which is a transcriptional repressor involved in the post-natal switch of NMDAR subunit. REST participates in GluN2B downregulation by epigenetic remodelling of *Grin2b* gene. Hence, epigenetics plays a vital role in regulating the NMDAR subunit expression level in the brain (Rebola, Srikumar et al. 2010).

Further evidence showed that the shift in NMDAR subunit is determined by sensory experience *in vivo* and suggests to coincide with critical period of development. A study by Philpot et al. showed sensory experience was recorded in the visual cortex as a change in the subunit composition of NMDAR, eventually modulating its functional properties. Further, they demonstrated that visual

experience was associated with a reduction in GluN2B containing NMDARs, whereas visual-deprivation exhibited an opposing effect. However, when the visually deprived animals were exposed to visual stimuli for 2 hours, subunit switching was induced rapidly (Philpot, Sekhar et al. 2001). In addition, stressful and pathologic experience during the critical period of development affects GluN2B-GluN2A switch during development. Apart from that, maternal deprivation during early life delays the NMDAR subunit switch by impairing REST activation (Rodenas-Ruano, Chávez et al. 2012). Thus, experience shapes the NMDAR functional properties modulating subunit composition, achieved at gene level regulation.

1.11. Molecular mechanisms behind NMDAR-dependent plasticity

Long-term synaptic plasticity is thought to be dependent on gene expression downstream to NMDAR. The existence of NMDAR-mediated protein synthesis in the synapse was not known for a long time. Different mechanisms underlying protein synthesis downstream to NMDAR-mediated signalling is mentioned in the below section.

Protein synthesis or translation can be classified broadly into three main steps: Initiation, Elongation, and Termination. The initiation phase is the rate-limiting step, and most of the regulation occurs in this step. NMDAR-mediated translation is regulated in two main mechanisms; via calcium signalling and downstream signalling protein complexes such as PI3K (Husi, Ward et al. 2000, Hardingham, Arnold et al. 2001, Papadia and Hardingham 2007, Luo, Wu et al. 2011, Fan, Jin et al. 2014)

Many pathways act downstream to NMDAR-mediated signalling. Calcium signalling is one of the most potent pathways. Ca^{2+} entry through NMDAR activates cAMP, IP3, and DAG pathways (Sebatini BL, 2002; Vanhoutte P, 2003). NMDAR activation is coupled to ERK signalling via RAS-RAP. ERK regulates many translational regulators such as MNK1, MNK2, which can phosphorylate eIF4 (Waskiewicz AJ, 1997 and 1999). Also, it has been shown that NMDAR-mediated signalling leads to ERK-dependent phosphorylation of eIF4E. These results suggest NMDAR-mediated regulation of protein synthesis occurs via different pathways.

Though ERK is the primary signalling cascade downstream to NMDAR activation, the involvement of other ERK-independent pathways was also demonstrated concerning translation regulation (Krapivinsky, Krapivinsky et al. 2003, Pochwat, Rafalo-Ulinska et al. 2017). NMDAR-mediated signalling has been shown to activate RSK (Ribosomal S6 Kinase)-mediated translation (Kaphzan H, 2007). Apart from that, many upstream and downstream components of the mTOR pathway have also been implicated in NMDAR-mediated signalling (Burket, Benson et al. 2015, Tang, Xue et al. 2015). The study showed that dendritic protein synthesis occurs on NMDAR stimulation, and that

was sensitive to Rapamycin, which is a potent inhibitor of mTOR (Gong R, 2006). However, the role of mTOR downstream to NMDAR is still not clear.

NMDAR-mediated signalling has also been shown to regulate the elongation phase of the translation (Scheetz, Nairn et al. 2000). Ca^{2+} entry via NMDAR governs the activity of the eEF2 kinase (Cossenza, Cadilhe et al. 2006, Iizuka, Sengoku et al. 2007, Autry, Adachi et al. 2011). Further, studies have shown that NMDAR-mediated phosphorylation of eEF2 leads to inhibition of protein synthesis (Ryazanov AG, 1988; Nairn AC, 1987), results in a paradox where NMDAR activates translation initiation and represses translation elongation. Thus, NMDAR's role in the regulation of translation was inconclusive. More elaborate studies have indicated a robust translation regulation in the elongation phase, which followed a temporal dynamic of translation response. NMDAR stimulation on rat synaptoneurosome preparation showed decreased translation within 5 minutes, accompanied by an increase in protein synthesis at a later time point which could corroborate with the phosphorylation pattern of eEF2 (Scheetz AJ, 2000). These studies suggest that NMDAR activation, in general, downregulates protein synthesis but at the same time upregulate translation of a specific subset of mRNA.

1.12. AMPAR subunit composition and stoichiometry

AMPARs are present in the excitatory synapses, and also account for majority all of the excitatory postsynaptic potentials (EPSPs) elicited under basal conditions (Andreasen, Lambert et al. 1989). AMPARs are tetramers and composed of four subunits (GluR1-4). GluA1 and GluA2 subunit containing AMPARs are highly abundant; approximately 80% of synaptic AMPARs in the CA1 region of the hippocampus are GluA1-GluA2 heteromers (Lu, Shi et al. 2009). GluA3 containing receptors are present at a significantly lower level than GluA1 or GluA2 (Sans, Vissel et al. 2003). However, GluA4 containing AMPARs are sparsely expressed in the excitatory neurons (Zhu, Esteban et al. 2000). Each subunit of AMPAR consists of four membrane domains (M1-4), an extracellular N-terminal domain and a C-terminal intracellular domain (Mayer and Armstrong 2004). The extracellular domain comprises of an N-terminal domain (NTD) and an agonist binding domain (ABD). ABD is the site for glutamate binding (an excitatory neurotransmitter). They are permeable to monovalent cations such as sodium (Na^+) and potassium (K^+), and $Ca^{2+,}$ but the GluR2 subunit is impermeable to Ca^{2+} (Hollmann and Heinemann 1994).

These subunit expression profiles and the composition is differentially regulated during development. Early in development majority of the AMPARs are GluA2 lacking which are Ca^{2+}-permeable. These receptors are exchanged with GluA2-containing Ca^{2+}-impermeable ones after PND14 (Pellegrini-

Giampietro, Bennett et al. 1992). Soon after birth, GluA2 expression is low compared to GluA1 (Pickard, Noel et al. 2000). However, by PND14 most of the AMPAR positive synapses express GluA2 (Monyer, Seeburg et al. 1991). Such an expression pattern is particularly important for the development as Ca^{2+}-permeable AMPARs are necessary for neonatal synaptic functions (Pickard, Noel et al. 2000). GluA4 subunits were shown to be inserted in the silent synapses in an activity-dependent manner at PND5-7 and subsequently exchanged for GluA2 containing AMPARs (Zhu, Esteban et al. 2000). Along with these, GluA3 subunit expression also increases by PND21 (Suzuki, Kessler et al. 2008). Such developmental regulation of subunit composition and expression profile in-turn modulates the synaptic plasticity associated with the AMPAR.

1.13. AMPAR-mediated synaptic plasticity

Further studies have shown that GluR2 Knock-out (KO) mice had enhanced LTP (Jia, Lu et al. 2001), whereas GluR1 Knockout mice have attenuated LTP in the hippocampus (Morales and Goda 1999). This study suggests the expression of LTP involves an increment in the number of postsynaptic AMPARs via activity-dependent changes in AMPAR trafficking (Malenka and Nicoll 1999, Malinow and Malenka 2002, Song and Huganir 2002, Bredt and Nicoll 2003). Binding of glutamate to AMPARs allows rapid cation flow, predominantly inward flow of Na^+ and outward flow of K^+ to depolarise the postsynaptic cell (Jonas 1993). Usually, the excitatory postsynaptic current (EPSC) mediated by AMPAR is of relatively for a shorter duration, as AMPARs are rapidly deactivated following the clearance of glutamate, or desensitised to glutamate (Colquhoun, Jonas et al. 1992, Mosbacher, Schoepfer et al. 1994).

1.14. Role of GluA2 and RNA editing in AMPAR-mediated synaptic plasticity

AMPAR subunit composition is vital for trafficking and conductance. Mainly, the presence or absence of RNA edited form of GluA2 determines these properties of the AMPARs. In the brain, the majority of the *GluA2* mRNAs are edited, where glutamine is changed to arginine at 607 positions (Sommer, Kohler et al. 1991). Such a change of charge in the channel pore of the receptor makes it impermeable to Ca^{2+} (Verdoorn, Burnashev et al. 1991). A study by Donevan and Rogawski showed a majority of the cultured hippocampal neurons contain Ca^{2+} impermeable outwardly rectifying AMPAR. However, a small proportion of the neurons contain inwardly rectifying Ca^{2+} permeable AMPAR. Further, these Ca^{2+} permeable AMPARs were blocked by Spermine, whereas the impermeable subset was not blocked (Donevan and Rogawski 1995). These data suggest that the Ca^{2+} permeable GluA2 lacking subunits are inwardly rectifying (Henley and Wilkinson 2016).

GluA2 lacking Ca^{2+} permeable AMPARs are thought to play an important role in LTP induction. Washburn and Dingledine used Philanthotoxin-433 (PhTX-433) which selectively blocks the GluA2-lacking AMPARs, in their experiments. They showed the application of PhTX immediately after LTP induction prevents LTP expression. However, if PhTX is used once LTP is established, it does not have any effect (Washburn and Dingledine 1996). These results indicate that transient incorporation of GluA2-lacking Ca^{2+} permeable AMPARs is required for LTP. Later studies have shown that transiently incorporated Ca^{2+} permeable AMPARs are subsequently replaced with GluA2-containing Ca^{2+} impermeable AMPARs (Plant, Pelkey et al. 2006, Yang, Wang et al. 2010, Jaafari, Henley et al. 2012). During LTP induction Ca^{2+} permeable AMPARs are incorporated in the synapses which in-turn allows Ca^{2+} to enter and facilitate the further incorporation of Ca^{2+} impermeable AMPARs (He, Song et al. 2009, Jaafari, Henley et al. 2012). It is believed that until the incorporation of Ca^{2+} impermeable AMPARs, LTP is labile and vulnerable to reverse.

1.15. Cellular/Molecular mechanisms behind AMPAR-mediated synaptic plasticity

Similar to LTP, LTD is also believed to produce postsynaptic changes by internalisation of AMPARs and NMDARs from the synapse (Snyder, Philpot et al. 2001). NMDAR-LTD expression predominantly postsynaptic in expression; however, imaging using FM1-43 loading of vesicles showed that presynaptic neurotransmitter release was reduced (Stanton, Heinemann et al. 2001, Stanton, Winterer et al. 2003). Evidence of postsynaptic expression mechanisms for NMDAR-LTD includes alterations of the existing AMPARs through dephosphorylation and removal of AMPARs from the synapse. Interestingly the dephosphorylation of AMPARs occurs at ser-845, unlike in LTP, where it happens at ser-831. This alteration reduces the affinity of AMPAR towards glutamate and also reduces the probability of opening of the receptor (Banke, Bowie et al. 2000). Dephosphorylation of ser-831 occurs following depotentiation of LTP while phosphorylation of ser-845 occurs following de-depression. In summary, these results show that the phosphorylation state of AMPARs is not just bidirectionally modified. Additionally, LTD also results in lateral diffusion of AMPARs to extra-synaptic sites and internalisation of AMPARs through a dynamin- and clathrin-mediated process.

1.16. Metabotropic glutamate receptor (mGluR) composition and stoichiometry

mGluRs belong to the G-Protein Coupled Receptors (GPCR) superfamily, the most abundant receptor gene family in the human genome (Niswender and Conn 2010). In 2012, Nobel Prize in Chemistry was conferred to Brian Kobilka (Stanford University) and Robert Lefkowitz (Duke University) for discovering the structure GPCRs which led to a better understanding of the functions of GPCRs. Binding of ligand to the GPCR results in activation of G-protein, which is composed of α, β, and γ subunits (Kobilka 2007). At stable conditions, G-proteins are bound to GDP, whereas, upon

activation, GDP is replaced with GTP within α-subunit and leads to activation of proteins in the downstream signalling pathway (Niswender and Conn 2010). In 1985, one of the earliest studies showed Glutamate stimulation led to a 3 to 4-fold increase in inositol phosphate formation in the striatal neurons. Authors proposed that the increase in inositol phosphate formation might be a result of Quisqualate (QA) and NMDAR activity (Sladeczek, Pin et al. 1985). Later, Sugiyama et al. injected mRNA extracted from rat whole brain into *Xenopus* oocytes and characterised functional properties of two types of responses; one responding to QA/Glutamate, another to Kainate (KA). Authors reported the presence of a new group of receptors which preferred QA as an agonist and activated inositol phosphate metabolism via G-protein (Sugiyama, Ito et al. 1987). Thus, these receptors were proposed as metabotropic glutamate receptors. A follow up pharmacological study showed these new receptors did not share antagonists with the known cation channel coupled glutamate receptors known at that time (Sugiyama, Ito et al. 1989). Thus, these newly proposed metabotropic glutamate receptors were thought to be part of an entirely different receptor category. Pharmacokinetic studies on these mGluRs led to the discovery of many selective agonists/antagonists. Gereau and Conn showed DHPG activates mGluR5, and DCG-IV activates both mGluR2 and mGluR3 (Gereau and Conn 1995). The study suggested the specificity of DHPG and DCG-IV towards Group I and Group II mGluRs.

Further study by Desai et al. showed trans ACPD induced physiological effects in the hippocampus is mediated by mGluRs. The authors also reported that L-AP3, an antagonist of trans ACPD induced phosphoinositide hydrolysis, failed to inhibit physiological response conferred by trans ACPD in the hippocampus (Desai, Smith et al. 1992). These data suggest that the ACPD induced physiological changes are mediated by metabotropic glutamate receptor-mediated and distinct from AP3 sensitive phosphoinositide hydrolysis linked glutamate receptors (Desai, Smith et al. 1992). These findings have significant implications on drug development and therapeutics study (Conn and Pin 1997).

In 1991, a metabotropic glutamate receptor was cloned successfully, and these receptors were found to be abundant in the dentate gyrus, CA2-CA3 neurons, and in Purkinje cells of the cerebellum (Masu, Tanabe et al. 1991). Sequence analysis showed these receptors had unique hydrophilic sequences at both ends of the putative seven transmembrane domains (Masu, Tanabe et al. 1991). Depending on sequence homology, signalling pathway, and G-protein coupling, the receptors were classified as Group, I include mGluR1 and 5, Group II includes mGluR2 and 3, and Group III includes mGluR4,6,7,8 (Havlickova, Blahos et al. 2003, Trepanier, Lei et al. 2013, Ribeiro, Vieira et al. 2017).

mGluRs contain a large extracellular N-terminal domain, which is called Venus Flytrap Domain (VFD), consists of the glutamate binding site (Pin, Galvez et al. 2003). Crystal structure analysis showed VFD consists of two lobes and it brings about substantial conformational changes when bound by ligands (Kunishima, Shimada et al. 2000, Tsuchiya, Kunishima et al. 2002, Jingami, Nakanishi et al. 2003). Apart from glutamate, divalent cations such as magnesium or calcium can bind to VFD, which can activate the receptors (Kubo, Miyashita et al. 1998, Francesconi and Duvoisin 2004). Further, the conformational changes upon glutamate binding occur at the C-terminal via a Cysteine-Rich Domain (CRD). This domain contains nine cysteine residues out of which eight are linked by disulphide bonds (Muto, Tsuchiya et al. 2007). Mutations in Cys-234 residue in the mGluR suggested that these disulphide bonds in the CRD are connected to VFD and regulate the propagation of signals (Rondard, Liu et al. 2006). In addition, mGluRs consists of seven transmembrane domains, also called as Heptahelical Domain (HD). This domain participates in binding with Positive Allosteric Modulator (PAM) or Negative Allosteric Modulator (NAM) (Hampson, Rose et al. 2008). The C-termini are one of the most important domains of mGluRs. It takes part in G-protein coupling with the receptor. Also, this region is subject to regulatory protein-protein interaction such as mGluR-Homer, and modulation by post-translational modifications (Niswender and Conn 2010).

Group I mGluRs are predominantly present on the postsynapses and, thereby, excitatory (Shigemoto, Kinoshita et al. 1997, Endoh 2004). Downstream signalling involves phospholipase-C activity leading to the generation of IP3 and DAG (Bonsi, Cuomo et al. 2005). They are also associated with Sodium and Potassium channel, contributing to its excitatory nature (Chu and Hablitz 2000). Group II mGluRs are distributed in both pre and postsynapse and downregulate the formation of cAMP by inhibiting Adenylate Cyclase activity (Endoh 2004). Group III receptors are predominantly localised in the presynapse and inhibitory in function. These receptors also inhibit the Adenylate Cyclase and Calcium channels (Chu and Hablitz 2000). These summarised in Table 1-2. In the later sections, Group I mGluR-mediated signalling and plasticity is discussed.

Group	Receptor	Gene	Signalling
Group I mGluR	mGluR1	*GRM1*	Present in the postsynapse. Activates Phospholipase C, and ERK-MAPK pathway.
	mGluR5	*GRM5*	
Group II mGluR	mGluR2	*GRM2*	Present on both pre and postsynapse. Inhibits the activity of Adenylate Cyclase.
	mGluR3	*GRM3*	
Group III mGluR	mGluR4	*GRM4*	Predominantly present on the presynapse. Inhibits Adenylate Cyclase activity. They also impede Calcium channels.
	mGluR6	*GRM6*	
	mGluR7	*GRM7*	
	mGluR8	*GRM8*	

Table 1-2: Metabotropic glutamate receptor subunit composition

The table summarises all receptor subunits of mGluR. Different groups of mGluR show distinct signalling cascade downstream to their activation in the brain (Chu and Hablitz 2000, Havlickova, Blahos et al. 2003, Endoh 2004, Bonsi, Cuomo et al. 2005, Niswender and Conn 2010) (https://www.genenames.org/data/genegroup/#!/group/281).

1.17. Biochemical Pathways downstream to Group I mGluR

Group I mGluRs are G-protein coupled receptors involved in the modulation of synaptic transmission (Kim, Lee et al. 2008). Upon binding of neurotransmitter glutamate to its extracellular domain, mGluRs transmit signals through the receptor protein to the intracellular signalling molecules (Niswender and Conn 2010). mGluRs can regulate downstream gene expression at both translational, as well as the transcriptional level (Wang and Zhuo 2012). These regulations occur through a range of downstream signalling processes such as MAPKs, CAMKs, or PKA. In this section, how both the arms of Group I mGluR-mediated signalling and how it modulates synaptic plasticity are discussed.

Group I receptors are demonstrated to couple to the activation of $G\alpha_q$ via PLCβ (Floyd, Rzigalinski et al. 2004). It leads to intracellular accumulation of IP3 and DAG, which signals in an intracellular Ca^{2+}-dependent manner. Depending on the cell type and region, Group I mGluRs can activate an array of downstream signalling pathways such as Phospholipase D, Casein kinase, Protein kinases, Cyclin-dependent kinases (CDK), Jun kinases, Mitogen-Activated Protein kinases/ Extracellular Receptor-mediated kinases (MAPK/ERK), and the mechanistic Target of Rapamycin (mTOR)/ p70 S6 kinase (Hou and Klann 2004, Page, Khidir et al. 2006, Li, Li et al. 2007). All of these downstream

pathways are vital for cell survival and function. However, MAPK/ERK and mTOR/S6K pathways are specifically essential for synaptic plasticity and learning and memory (Graber, McCamphill et al. 2013).

1.18. Distribution of Group I mGluRs in the brain

The expression pattern of mGluR1 and mGluR5 are complimentary in the brain (Luscher and Huber 2010). The expression of mGluR1 is predominant in the Purkinje neurons of the cerebellum, and tufted cells of the olfactory bulbs, along with the pallidum, and in the thalamus. mGluR5 is expressed in the cerebral cortex, the subiculum, nucleus accumbens, and in the hippocampus. Stratum radiatum of the hippocampal dendritic field majorly expresses mGluR5, whereas the cell bodies have mGluR1 on the surface (Ferraguti and Shigemoto 2006). However, studies have shown that these receptors present in the peri-synapse surrounding the ionotropic receptors (Lujan, Nusser et al. 1996). Thus, localisation of Group I mGluR in the excitatory synapses is vital for the regulation of synaptic plasticity.

1.20. Group I mGluR-dependent plasticity

Due to the distribution of these receptors, it was believed that they might regulate the re-distribution of AMPAR and NMDAR on the synapse. Further studies have shown that the Group I mGluRs facilitates and induces Long-term depression (LTD) (Anwyl 1999, Bellone, Luscher et al. 2008). Studies have shown that the Group I mGluRs influences neuronal excitability (Wong, Chuang et al. 2004). mGluR-dependent LTD was first demonstrated at the parallel fibre (PF) synapses onto the Purkinje cells in the cerebellum (Neale, Garthwaite et al. 2001). Subsequent studies have found mGluR-LTD in diverse brain regions such as Hippocampus, neocortex, striatum, and spinal cord (Jorntell and Hansel 2006, Bellone, Luscher et al. 2008, Gladding, Fitzjohn et al. 2009). The occurrence of such plasticity in the brain region and cell-types shows the importance of mGluR-mediated synaptic plasticity. However, substantial evidence showed a potential role in goal-directed learning, Parkinson's disease, and drug addiction. A preclinical model of Parkinson's Disease (PD) manifested improved motor symptoms by administering Group I mGluR antagonists (Amalric 2015). Group I mGluR-mediated synaptic plasticity is thought to regulate excitation and inhibition balance in Basal Ganglia, which in-turn is a target in PD (Amalric 2015). A study showed that, *in vivo* exposure of Cocaine, impaired mGluR-LTD (Fourgeaud, Mato et al. 2004). The expression of mGluR5 was shown to be reduced due to drug intake, which in turn affects the mGluR-dependent plasticity (Fourgeaud, Mato et al. 2004). Thus, drug addiction has an impact on mGluR-mediated synaptic plasticity.

1.21. Molecular mechanisms for Group I mGluR-dependent plasticity

In hippocampal CA1 pyramidal neurons, mGluR-LTD is induced either by low-frequency electrical stimulation (1-3 Hz for 15 minutes) of Schaffer-Collateral axons or by bath application of R,S-Dihydroxyphenylglycine (DHPG) for 5-10 minutes (Bolshakov and Siegelbaum 1994, Kemp and Bashir 1999, Huber, Kayser et al. 2000, Clement, Randall et al. 2009). Not only in hippocampal slices the relevance of mGluR-LTD in learning and memory was shown but also demonstrated *in vivo* in free moving rodents (Naie and Manahan-Vaughan 2004, Naie and Manahan-Vaughan 2005). In their study, authors chronically implanted electrodes to evoke potentials at the DG synapses of the perforant pathway. Ventricular injection of DHPG or anisomycin were done to measure mGluR-LTD (Naie and Manahan-Vaughan 2005). mGluR-LTD induction requires the activation of phospholipase C (PLCβ), inositol triphosphate (IP3) generation, release of Ca^{2+} from intracellular stores, and protein kinase C (PKC) activation (Kano, Hashimoto et al. 2008). However, hippocampal mGluR-LTD can occur independently of intracellular Ca^{2+} increase shown by the application of calcium chelator BAPTA in the cultured neurons (Fitzjohn, Palmer et al. 2001). Most of these studies were done by bath application of DHPG in acute hippocampal slices. Recent advancement in techniques such as glutamate uncaging has further increased the understanding of these mechanisms at the synaptic level. A study by Holbro et al. showed activation of Group I mGluR induced an increase in the intracellular Ca^{2+} load in the individual spines of CA1 neurons showing that mGluR activity leads to an intracellular increase in Ca^{2+} ion concentration (Holbro, Grunditz et al. 2009).

mGluR-mediated signalling triggers the endocytosis of ionotropic receptor AMPAR subunits and long-term reduction in the number of postsynaptic AMPARs (Snyder, Philpot et al. 2001, Steinberg, Huganir et al. 2004, Moult, Gladding et al. 2006). The mGluR-mediated AMPAR endocytosis is best explained in the Purkinje cells (PC) of the cerebellum. In PCs, mGluR activity leads to an increase in intracellular Ca^{2+} and, hence, PKC activation results in phosphorylation of GluA2 at Ser880 (Chung, Steinberg et al. 2003, Steinberg, Takamiya et al. 2006). Thus, phosphorylated GluA2 loses its affinity towards AMPAR scaffold GRIP and facilitate its endocytosis contributing to the reduction of surface AMPAR (Chung, Steinberg et al. 2003). On the contrary, in the CA1 region, mGluR-LTD and AMPAR endocytosis do not require PKC activity (Schnabel, Kilpatrick et al. 1999, Fitzjohn, Palmer et al. 2001). Group I mGluR-mediated LTD requires a rapid translation of *Arc*, which is a component of AMPAR endocytosis machinery (Waung, Pfeiffer et al. 2008). The study showed that knocking down of *Arc* prevented AMPAR endocytosis and LTD induction downstream of mGluR (Waung, Pfeiffer et al. 2008). ARC specifically interacts with Endophilin, which is BAR domain-containing protein and recruits Dynamin to the complex to facilitate the endocytosis of AMPAR (Chowdhury, Shepherd et al. 2006, Hanley 2018). Further studies have shown that mGluR activity leads to GluA2

dephosphorylation by Striatal-enriched tyrosine phosphatase (STEP) (Moult, Schnabel et al. 2002, Moult, Gladding et al. 2006). Which in turn, helps in AMPAR endocytosis and LTD.

1.22. mGluR-LTD and Local translation control

mGluR-LTD requires local protein synthesis downstream to mGluR activity (Huber, Kayser et al. 2000, Karachot, Shirai et al. 2001, Waung and Huber 2009). Huber et al. was the first to demonstrate that the local protein synthesis occurs in the dendrites of CA1 pyramidal neurons upon activation of Group I mGluRs (Huber, Kayser et al. 2000, Waung and Huber 2009, El-Hassar, Hagenston et al. 2011). Besides, how disruption of this translational control leading to synaptic dysfunction and impairment in plasticity under diseased conditions was demonstrated by many researchers (Muddashetty, Kelic et al. 2007, Costa-Mattioli, Sossin et al. 2009, Waung and Huber 2009). However, mGluR-LTD can occur independently of protein synthesis under some circumstances such as in the diseased state (Huber, Kayser et al. 2000, Zang, Nosyreva et al. 2009, Abbas 2016). Studies showed that Fragile X Syndrome mouse model exhibited mGluR-LTD independent of protein synthesis in the CA1 hippocampal neurons (Oliet, Malenka et al. 1997, Hou, Antion et al. 2006, Nosyreva and Huber 2006, El-Hassar, Hagenston et al. 2011). These studies suggest that new protein synthesis was not required for mGluR-LTD in situations where the level of constitutively protein synthesis occurs at basal level and is sufficient to maintain reduced surface expression of AMPAR leading to increased LTD.

Extensive research has been done in the last few decades to understand the identity and nature of the LTD-proteins. Primarily, Group I mGluR activity synthesises a group of proteins, Activity-Regulated Cytoskeletal Associated Protein (ARC), that regulates AMPAR trafficking (Park, Park et al. 2008, Waung, Pfeiffer et al. 2008). Studies from multiple labs have shown that ARC increases AMPAR endocytosis by interacting with Dynamin2 and Endophilin, which are components of AMPAR endocytosis machinery (Huber, Kayser et al. 2000, Chowdhury, Shepherd et al. 2006, Rial Verde, Lee-Osbourne et al. 2006, Shepherd, Rumbaugh et al. 2006). *Arc* mRNA rapidly localises to the dendritic spine upon the activity, and *Arc* gene is a well established activity-mediated immediate early gene (Link, Konietzko et al. 1995, Steward and Worley 2001). Upon activation of Group I mGluR, *Arc* mRNA is rapidly translated in dendrites and is required to maintain low surface AMPAR expression leading to LTD (Chowdhury, Shepherd et al. 2006, Shepherd, Rumbaugh et al. 2006, Park, Park et al. 2008, Waung, Pfeiffer et al. 2008, DaSilva, Wall et al. 2016). Hence, *Arc* gene expression is a vital mechanism for mGluR-LTD.

1.23. Translational activation regulated by mGluR

Translation regulation is the primary mechanism by which Group I mGluR induces synaptic plasticity. Evidence suggests that the Group I mGluR can control translation at either initiation or elongation phases (Costa-Mattioli, Sossin et al. 2009, Waung and Huber 2009). Translation initiation is the rate-limiting step in the entire process. Activation of Group I mGluR leads to translation initiation through two significant pathways such as ERK-MAPK and PI3K-mTOR. Activation of both these pathways trigger phosphorylation of Eukaryotic Initiation Factor 4E (eIF4E), eIF4E Binding Protein (eIF4EBP, also known as 4EBP), as well as enhances the formation of initiation complex (eIF4F) (Banko, Hou et al. 2006, Monick, Powers et al. 2006, Ronesi and Huber 2008, Waung and Huber 2009, Roux and Topisirovic 2012, Pernice, Schieweck et al. 2016). Also, Group I mGluR signals via ERK, PI3K, mTOR that activates and phosphorylates Ribosomal S6 protein by p70 Ribosomal S6 Kinase (RSK). Phosphorylation of RSK leads to translation of a specific subset of RNAs containing 5' Terminal OligoPyrimidine tract (5'TOP, also known as TOP dependent translation) which encodes ribosomes and translation factors (Antion, Hou et al. 2008, Ronesi and Huber 2008). Thus, it increases the overall translation of the neurons. In the hippocampal CA1 neurons, mGluR-LTD activates both the PI3K-mTOR and ERK pathway to exhibit translational control (Gallagher, Daly et al. 2004, Hou and Klann 2004). In addition to these, mGluR-HOMER interaction-mediated regulation of the translation elongation was shown (Davidkova and Carroll 2007, Park, Park et al. 2008). Thus, it is clear that activation of Group I mGluR leads to an increase in protein synthesis in neurons, thereby, regulating synaptic function.

1.24. Critical period

One of the unique properties of the brain is the ability to undergo plasticity in response to external stimuli by a process called synaptic plasticity. The brain is highly plastic during the early stages of development as neurons are sensitive to environmental cues. The critical period of development is a time window during development of the brain when the brain is most sensitive to environmental stimuli (Stiles and Jernigan 2010). Critical period of development is essential to acquire a concerned skill at an early stage of development that will assist us to survive later in life. Takao Hensch, a renowned neuroscientist, said, "I was always wondering what is it that makes it so easy to learn languages when you are young and so hard once you begin to get older?" (Hensch 2004, Bardin 2012). Thus, critical period is an interval during development when the neural circuit responsible for a process can be sculpted and modified by experience (Hensch 2004, Bardin 2012, Meredith, Dawitz et al. 2012). In 1978, Nash, J. described three criteria to consider any time window as critical period: 1. An identifiable onset and terminus; 2. An intrinsic component (some in-built maturational event of

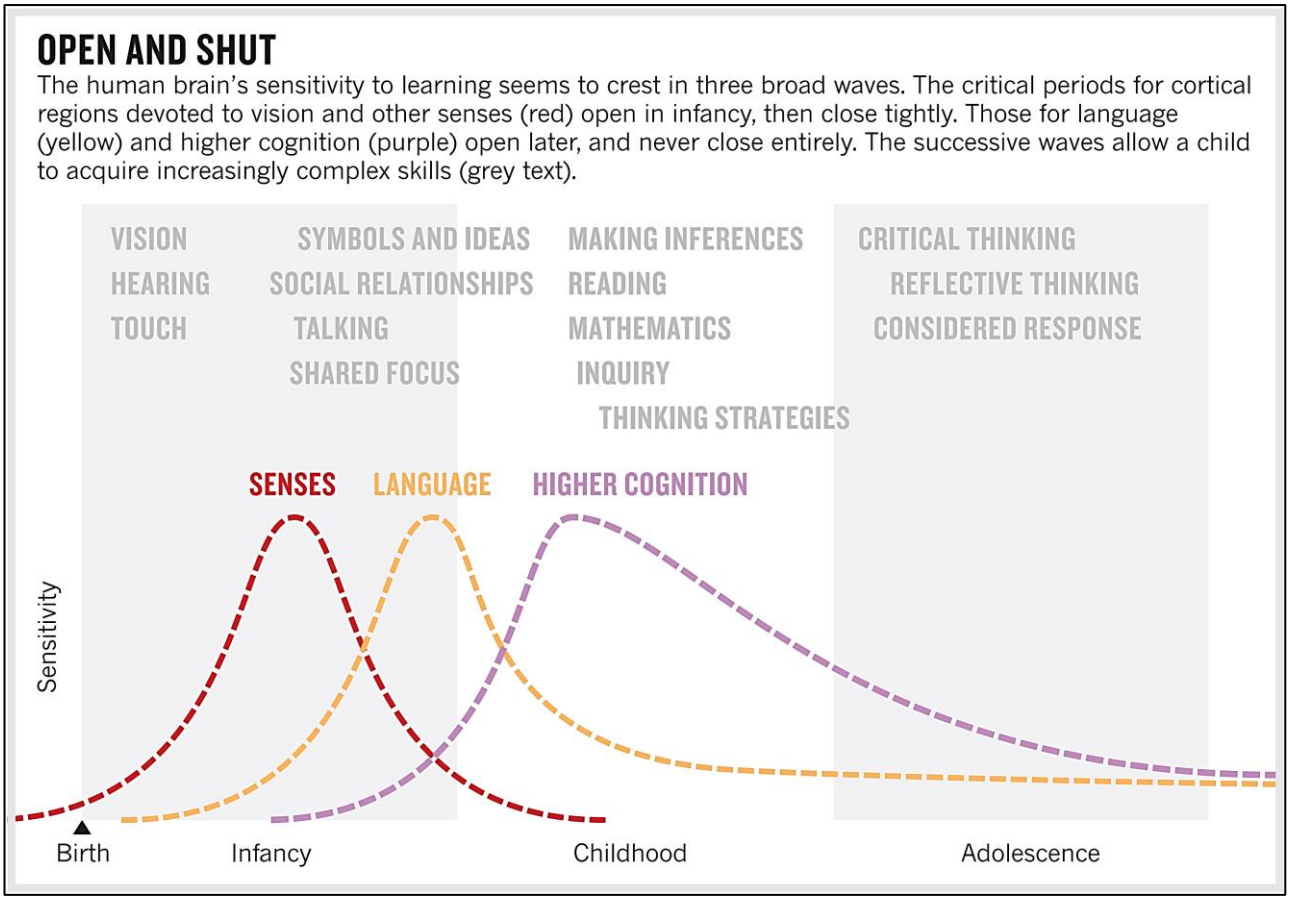

Figure 1-3: Open and closing of critical period of development

Three main modalities were shown in this figure. The first curve is for the sensory input dependent critical period in the cortex which starts early in the development and shuts completely. However, other two curve for language and higher cognition never closes. Shows that the ability to learn a language and other higher cognitive tasks persists in adulthood. The figure is taken from (Bardin 2012).

the organism); 3. An extrinsic component (such as environmental cues to which the system is sensitive) (Nash 1978, Spreen, Risser et al. 1995, Frampton and Warner-Rogers 2011).

However, the initial concept of the critical period was based on the experiments performed by Austrian biologist, Konrad Lorenz. In 1935, he investigated imprinting in animals that suggest animals start following any moving object whenever they see it for the first time (Moltz 1963, Lorenz 1971). Based on this, he divided a large clutch of goose eggs into two parts; some eggs were kept with the mother, and the remaining were kept in an incubator. The naturally hatched baby goslings followed their mother, and the remaining eggs hatched in an incubator followed Lorenz. This experiment suggests that the baby goslings recognised Lorenz as their mother. The possible reason could be the visual input from any moving object during very early life can be considered as a mother,

hence followed. He proposed that the phenomenon is irreversible (Moltz 1963, Hailman 1970, Lorenz 1971). He received Nobel prize in 1973 for his pioneering work on behaviour. In the later years (1946), McGraw suggested a similar critical period for motor learning skills in children (McGraw 1939, Spreen, Risser et al. 1995). Although behaviourally, the existence of the critical period of development was observed, the neural basis was yet to be understood.

The critical period of cortical development is a dedicated time window for learning multiple tasks in response to different senses such as visual, tactile, and auditory (Bardin 2012). During early life, the sensitivity of the brain and neural circuitry is high, then it decreases (Hensch 2004, Bardin 2012). This observation suggests an inability to learn to respond to various sensory inputs during the critical window of development can impair the capabilities to learn and adapt during a post-critical period of development. However, other modalities such as learning a language or higher cognition open in adolescent and does not close entirely in adult stages but reduce to a level that makes learning a new language difficult (Snow and Hoefnagel-Höhle 1978, Grimshaw, Adelstein et al. 1998, Hensch 2004, Bardin 2012). This ability to learn throughout adulthood, although at a slower pace, suggests we have a lifelong ability to learn a new language or a cognitive task. The various modalities of brain development from birth until adulthood are depicted in Figure 1-3.

David Hubel and Torsten Wiesel from Harvard Medical School started exploring the neural pathways underlying the critical period around the 1960s. They first demonstrated that many neurons in the visual cortex responded to only one specific eye. They showed that single cortical units displayed impulse activity in the absence of any alteration in the retinal illumination in freely moving light adapted cats. They found a restricted area in the retina from which firing could be influenced by lighting and these neurons responded to only to a specific angle of light. These areas were termed as the receptive field (Hubel and Wiesel 1959, Hubel and Wiesel 1962). Also, in the kittens, they sutured closed one eye to prevent light-based stimulation of neurons in that particular eye. They observed that the neurons which were supposed to fire in response to the closed eye started firing in response to the open eye and developed Amblyopia (Wiesel and Hubel 1963). This observation suggests that the cortical neurons respond to the external stimuli and further can remap the neuronal connections in response to the stimuli. Later, in 1981, Hubel and Weisel received Nobel prize for their work on the role of visual information processing in the visual cortex development.

During development, keeping one eye shut for a brief period leads to Amblyopia in the absence of any damage to the retina or visual cortex (Berardi, Pizzorusso et al. 2000). A minimum lack of stimuli is sufficient to induce amblyopia during the critical period of development, suggesting the importance

of sensory inputs to form neuronal connections. Further, studies by Antonini et al. had shown that monocular deprivation induced expansion of open eye columns to compensate for the loss of stimuli from deprived eye afferents. The neuronal morphology and the receptive field of the deprived eye afferents were reduced and less complex (Antonini and Stryker 1996, Antonini, Fagiolini et al. 1999).

For a long time, researchers presumed that the synaptic plasticity observed during the critical period of development is mediated majorly by excitatory neurons (Hensch 2003, Espinosa and Stryker 2012). Work from Stryker's group indicated that the activity of inhibitory neurons dampens the activity of neighbouring excitatory neurons (Fu, Tucciarone et al. 2014). Reiter et al. showed that application of muscimol inhibited the cortical neuron discharges in the visual cortex (Reiter and Stryker 1988). Muscimol is a potent agonist of $GABA_A$ receptors (Chandra, Halonen et al. 2010). Thus, increasing the activity of inhibitory neurons suppresses the activity of excitatory cells.

Further, Reiter and Stryker demonstrated that there was a shift in the cortical activity towards the less active closed eye (Reiter and Stryker 1988). There are three proposed underlying mechanisms behind such plasticity (Smith, Heynen et al. 2009). First, due to the lack of stimuli, deprived-eye responses weaken, leading to LTD. Second, as the deprivation progresses, the modification threshold (described in an earlier section) reduces which favours LTP. Third, LTP is facilitated in the open-eye response by the reduced modification threshold (Smith, Heynen et al. 2009). However, in normal conditions, during monocular dominance plasticity, the activity shift favours the more active open eye. It suggests the involvement of the inhibitory interneurons in the critical period of the plasticity of the visual cortex.

Similarly, a hallmark study by Hensch et al. showed that the mice having γ-aminobutyric acid (GABA) failed to develop Amblyopia, suggesting the absence of a critical period of development in these mice (Hensch, Fagiolini et al. 1998). Further, the authors rescued the synaptic plasticity during the critical period of development between postnatal day 25-27 by administering Benzodiazepine that increased the activity of GABA for next 4 days while one eye was sutured (Hensch, Fagiolini et al. 1998). These results suggest that inhibitory activity could be the potential driving force for the onset of critical period of plasticity (Fagiolini and Hensch 2000, Iwai, Fagiolini et al. 2003).

1.24.1. Mechanisms underlying the critical period

The change in the visual responsiveness was shown to be represented by synaptic plasticity mechanisms (LTP/LTD) in the visual circuit. It was proposed that NMDAR mediates the LTP/LTP and, in turn, regulates experience-dependent plasticity. NMDAR subunit switch is demonstrated to be one of the mechanisms regulating critical period of plasticity. The ratio of NR2A- and NR2B-

containing NMDARs is a key molecular switch during the critical period of development of the visual cortex. Erisir and Harris showed that NR2A-containing NMDARs in synapses increased significantly from eye-opening till postnatal day 4 in the visual cortex. Studies have shown that progressive insertion of NR2A subunit-containing NMDAR shortens the NMDAR-mediated current which marks the end of the critical period in the visual cortex (Quinlan, Philpot et al. 1999, Philpot, Sekhar et al. 2001). Philpot et al. showed that the NMDAR-EPSC kinetics could be modified by visual experience and deprivation. In their study, authors pharmacologically isolated NMDAR-mediated EPSC by voltage clamping the neuron at +40 mV from two groups of rodents, one was dark-reared, and another group was dark-reared exposed to light for 2 hours. They found that 2 hour light exposure significantly shortened the NMDAR-mediated current decay (Philpot, Sekhar et al. 2001). Targeted deletion of NR2A led to a prolonged NMDAR-mediated response in the mouse visual cortex (Fagiolini, Katagiri et al. 2003). In addition to that, ocular dominance plasticity was restricted in the visual cortex upon NR2A deletion (Fagiolini, Katagiri et al. 2003). These data further suggest the importance of NR2B to NR2A switch in the visual cortex during development.

In 1987, Arola and Singer showed NMDAR-dependent LTP could be induced in the slices rat visual cortex by high-frequency stimulus (Artola and Singer 1987). Further study by Daw *et al.* demonstrated that LTP induction is dependent on various types of glutamate receptors present on different layers of the visual cortex. At layer II and layer III is dependent on NMDAR, at layer V LTP is dependent on NMDAR and mGluR5, at layer VI LTP is dependent on mGluR1 and not on NMDARs (Daw, Rao et al. 2004). Experience plays a crucial role in the plasticity mechanisms observed in different layers of the visual cortex. A study by Jiang et al. showed that LTP and LTD were lost in the layer IV principle cells immediately after eye opening whereas it persisted in layer II/III beyond puberty (Jiang, Trevino et al. 2007).

However, similar to LTP, synaptic depression (LTD) plays a vital role in the plasticity of the visual field. Recent studies have indicated that LTD is critical for the ocular dominance plasticity (Heynen, Yoon et al. 2003, Yoon, Smith et al. 2009). A study from Mark Bear's group showed that, in the visual cortex of light deprived rats, LTP was enhanced and LTD was reduced, and these effects were reversed post light exposure for two days (Kirkwood, Rioult et al. 1996). Using Monocular deprivation (MD) as a model, Heynen et al. showed that prior synaptic depression because of MD occluded the induction of LTD (Heynen, Yoon et al. 2003). Further study by Yoon et al. dissected out the mechanisms behind the plasticity observed post MD. Authors blocked AMPAR internalisation and showed it lad to blockade of ocular dominance (OD) shift and depression of the deprived eye

responses (Yoon, Smith et al. 2009). These results suggest that LTP caused by deprivation from visual experience is mediated by the internalisation of AMPARs.

Synaptic depression was shown to be the underlying mechanism for the loss of cortical responsiveness from a deprived eye during critical period of development (Hubel and Wiesel 1970, Hensch and Stryker 1996, Frenkel and Bear 2004). In the year 1989, Dudek and Bear demonstrated that the effect of ibotenate treatment on phosphoinositide turnover was correlative with the critical period of the ocular dominance plasticity (Dudek and Bear 1989). They prepared synaptoneurosomes from the kitten striate cortex and treated with ibotenate and showed that during 2nd and 3rd postnatal month phosphoinositide turnover was stimulated, but NMDA or AMPA treatment did not show any effect (Dudek and Bear 1989). At that time, the receptors involved in the process of phosphoinositide turnover was not elaborated. Later, it was known mGluR1, and mGluR5 receptors were involved in that process. Considering these results, in 1996, Daw and Reid hypothesised that Group I mGluR level in the visual cortex might be modulated during the critical period for ocular dominance plasticity. Thus, they analysed the level of these mGluRs present in the visual cortex of the kittens grown normally or dark-reared (Daw and Reid 1996). mGluR1 and mGluR5 levels dropped as age increased from the time of birth. However, there was no correlation between these receptors level in the visual cortex with the critical period of ocular dominance plasticity (Daw and Reid 1996). Whereas, ACPD treatment led to the activation of cAMP, a secondary messenger during the critical period of ocular dominance plasticity (4-6 weeks of age). In addition to that, the basal level of cAMP was also increased during the critical window (4-6 weeks of age) (Daw and Reid 1996). These results suggest that the heightened plasticity in the visual cortex during the critical period might be because of the heightened level of secondary messenger activation, or because of increased coupling of mGluRs with the secondary messenger (Daw and Reid 1996).

It was believed that like LTP, NMDAR-mediated LTD is necessary for the visual cortex during the critical period of plasticity. Indeed, NMDAR activation led to LTD in the layer 2-4 of the visual cortex (Daw, Rao et al. 2004). However, LTD induction in layer 6 of the visual cortex required mGluR5 activation (Daw, Rao et al. 2004). These observations surfaced the importance of mGluR5 activity in the visual cortex. Further study using *Grm5*$^{+/-}$ mice showed that deprived-eye depression failed to occur in layer 4 of the visual cortex in these mutant mice (Dolen, Osterweil et al. 2007). The failure to induce depression in the deprived eye responses in *Grm5*$^{+/-}$ mice visual cortex was contradictory to the previous studies showing the existence of NMDAR-mediated LTP in layer 4. More recently, Sidorov et al. demonstrated that NMDAR-mediated LTD and depression in the deprived eye response needed mGluR5 activity during the postnatal development (Sidorov, Kaplan

et al. 2015). Therefore, Group I mGluR activity is indispensable in the visual cortex during postnatal brain development, more precisely during the critical period of ocular dominance plasticity.

Group II mGluRs, particularly mGluR2, play an essential role in visual cortex plasticity. Activation of mGluR2 receptors by Dicarboxycyclopropylglycine (DGC-IV) depresses the field potentials, LTD, at the layer 2/3 of the mouse visual cortex (Renger, Hartman et al. 2002). Gene targeting mutation or application of antagonists against mGluR2 prevented LTD induction in the visual cortex by LFS (Renger, Hartman et al. 2002). However, monocular dominance plasticity remained unaffected in the mouse model lacking mGluR2 (Renger, Hartman et al. 2002). In conclusion, mGluR2-mediated LTD is a crucial plasticity mechanism that occurs in the visual cortex. However, the role of mGluR2-mediated LTD is unclear in the ocular dominance plasticity observed in the visual cortex during the critical period of development.

Excitation to inhibition balance is required to process various information in the cortex (Desai, Cudmore et al. 2002, Turrigiano and Nelson 2004). Thus, the focus of the research was shifted towards the activity of GABA, which is a major inhibitory neurotransmitter in the brain. Deletion of GABA synthesising enzyme, GAD65, in mice showed no eye preference post monocular deprivation, and Benzodiazepine rescued the condition (Hensch, Fagiolini et al. 1998). Further studies have shown that Benzodiazepine infusion in the visual cortex of mice could accelerate the onset of critical period (Fagiolini and Hensch 2000, Fagiolini, Fritschy et al. 2004). Similarly, overexpression of BDNF led to the maturation of GABAergic and induced the early onset of critical period of plasticity in the visual cortex of mice (Hanover, Huang et al. 1999). These studies suggest an inter-relationship amongst GABA, BDNF, and experience-dependent synaptic activity, indeed, explain the lack of ocular dominance plasticity in the visual cortex due to the dark-rearing of the rodents. In addition to that, the GABAergic transmission was shown to be reduced in the visual cortex of the animals raised in the dark (Chen, Yang et al. 2001, Morales, Choi et al. 2002).

Apart from the synaptic plasticity and circuitry associated mechanisms, the extracellular matrix is considered as one of the essential determinants of the critical period of development (Berardi, Pizzorusso et al. 2004, Frischknecht and Gundelfinger 2012, Kelly, Russo et al. 2015, Hou, Yoshioka et al. 2017, Chao, Warren et al. 2018). Sensory experience is known to rewire the neuronal connections during the early stages of postnatal development. To rewire the neuronal connections, the Extracellular Matrix (ECM) should be dissolved, and, proteases are particularly required to form new neuronal connections. Many enzyme proteases play a crucial role to make way for a new neuronal connection in the brain. Tissue Plasminogen Activator (tPA) is one of the major serine

proteases, which can breakdown ECM, and expressed in the postnatal brain (Lee, Tsang et al. 2008). A study showed that tPA activity gradually upregulated by 2 days post-monocular deprivation during the critical period of development (Mataga, Nagai et al. 2002). Also, functional ocular dominance plasticity was impaired when tPA activity was blocked, and it was rescued by exogenous tPA, and not by Benzodiazepine (Muller and Griesinger 1998, Mataga, Nagai et al. 2002). These results suggest that tPA plays essential role in dissolving the ECM in the brain and in-turn make way for new connection formation leading to plasticity.

Such critical period of plasticity is not observed only in the visual cortex but also in other cortical regions such as somatosensory cortex. The same phenomenon of synaptic plasticity mediated by NMDAR is observed in somatosensory cortex (roughly between postnatal day 3 to postnatal day 9). Sensory experience is known to regulate the organisation of the brain circuit. Studies on rodents showed perturbations or damage in the whisker follicles led to a structural alteration in the patterning of whisker-related neuronal circuitry (Van der Loos and Woolsey 1973, Durham and Woolsey 1984). A study by Minlebaev et al. recorded the field potentials at P4 rat superfused barrel cortex in >10 Hz high pass AC mode. Authors showed that the thalamocortical inputs were associated with large amplitude NMDA receptor-dependent delta (ð) waves in the newborn rat barrel cortex (Minlebaev, Ben-Ari et al. 2009). A later study by infusing APV in the brain followed by cytochrome oxidase staining of the brain sections showed that even the structural (columnar) organisation of the barrel cortex is dependent on both NMDAR and non-NMDAR-mediated neuronal activity (Fox, Schlaggar et al. 1996).

Somatosensory barrel cortex is one of the remarkable sensory systems in rodents. Whiskers are represented by barrels in layer 4 of the somatosensory cortex (Woolsey and Van der Loos 1970). The sensory neurons from the hair follicle of whiskers make excitatory glutamatergic synapses in the brainstem trigeminal nuclei. The neurons present in the principal trigeminal nucleus are arranged somatotopically as barrellets, each receiving inputs from a single whisker (Veinante and Deschenes 1999). Further, the principal trigeminal neurons project on to the ventral posterior medial (VPM) nucleus of the thalamus, and the VPM neurons respond quickly and precisely to the whisker stimulation (Simons and Carvell 1989, Friedberg, Lee et al. 1999, Brecht and Sakmann 2002). The VPM neurons present in the barrelletes project to layer 4 of the primary somatosensory cortex and form the barrels. The barrel map is arranged quite identically to the whiskers present on the snout of the rodents (Woolsey and Van der Loos 1970, Petersen and Sakmann 2000). The detailed connections and the basic structure of the barrels are depicted in Figure 1-4.

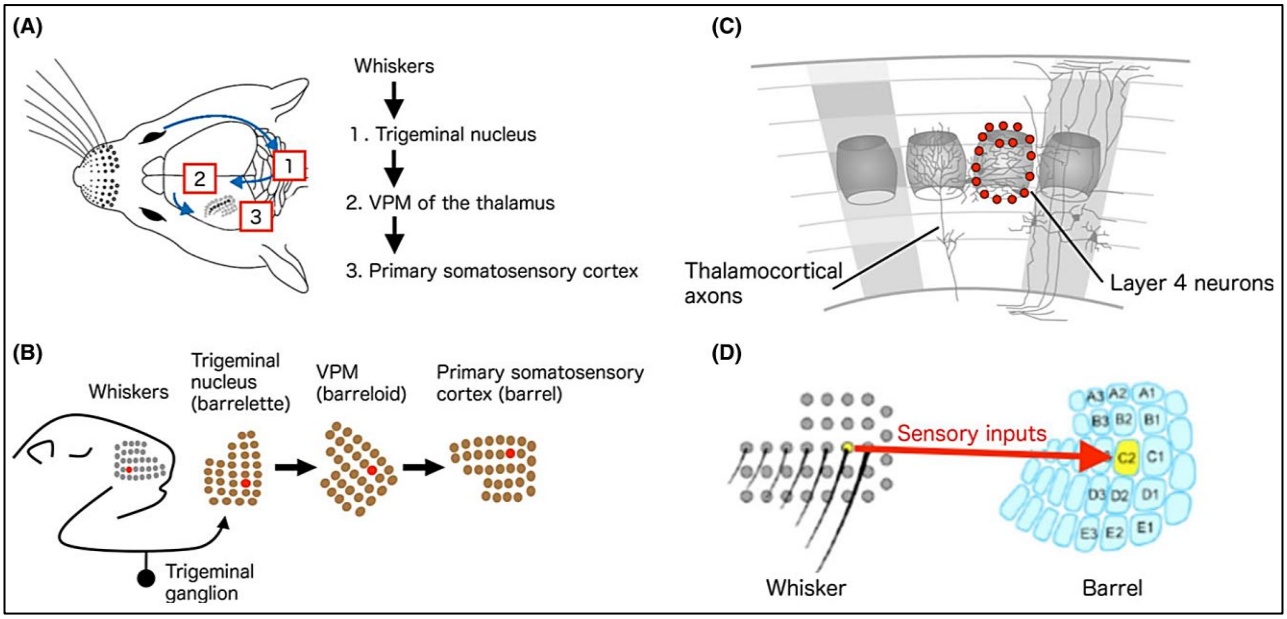

Figure 1-4: Development of Somatosensory system in rodents

A. Schematic route shows the tactile information path from whisker to somatosensory cortex. B. Somatosensory maps corresponding to the whiskers present in the somatosensory cortex and finally take shape of barrels. C. Schematic structure of barrels in the primary somatosensory cortex. D. Information from one whisker is specifically transferred to the corresponding barrel. Figure is adapted from (Kawasaki 2015) with permission.

The responses corresponding to the whiskers to the somatosensory cortex is dependent on the sensory inputs. Thus, cutting off or removal of facial vibrissae is an excellent model for sensory deprivation in the rodents. Local elimination of excitatory input to the cortical barrels led to the retraction of thalamocortical connection from the sensory deprived barrels (Wimmer, Broser et al. 2010). The reason could be an altered balance of excitation and inhibition. Another hallmark feature of such sensory deprivation is rewiring of the cortical connections (Hickmott and Steen 2005). Studies have shown that perturbation of sensory input led to the remodelling of axons and dendrites (De Paola, Holtmaat et al. 2006, Cheetham, Hammond et al. 2007).

Synchronous cortical neuronal discharge is crucial for the function of thalamocortical connections. A study from Ford Ebner's group showed that the synchronous discharge of the barrel cortex neurons failed to develop post sensory deprivation by bilateral whisker trimming from birth till postnatal day 60 in rats (Ghoshal, Pouget et al. 2009). The authors concluded that low level of synchrony could be the reason for the reduced plasticity observed in the barrel cortex of rodents due to the early sensory deprivation (Ghoshal, Pouget et al. 2009). Synaptic inhibition also plays a crucial role in the development of the thalamocortical connection of the somatosensory barrel cortex (White and Rock

1981). The synaptic inhibition was shown to be caused by the development of $GABA_A$-specific circuit in that region (Fritschy and Brunig 2003). Later, Li at al. performed whole-cell recordings to study the synaptic inhibition in the mouse barrel cortex (Li, Rudolph et al. 2009). They demonstrated that the inhibitory postsynaptic current (IPSC) was dependent on the expression of the α1 subunit. Authors observed that the amplitude of IPSC was increased and the decay rate was sped up in the Low-threshold spiking cells, a type of inhibitory neurons, on the removal of the whiskers (Li, Rudolph et al. 2009). Overall, these changes in the properties of IPSCs combining with further genetic manipulations suggest an alteration in the configuration of $GABA_A$-specific circuit post whisker trimming (sensory deprivation) (Li, Rudolph et al. 2009). Recently a study by Feldman's group has shown sensory deprivation in the barrel cortex weakened the inhibitory response in two distinct phases. Authors trimmed whiskers of rats at postnatal day 7 and measured the dynamics of synaptic inhibition (Gainey, Wolfe et al. 2016). In deprived columns of the barrel cortex, miniature inhibitory postsynaptic currents (mIPSCs) and evoked IPSCs normally developed till postnatal day 15. However, the IPSC amplitude, which was transiently reduced during early development, also recovered by postnatal day 16, despite of having deprivation (Gainey, Wolfe et al. 2016). Subsequently, after postnatal day 22, a second round of weakening of IPSC was observed. Therefore, they concluded that sensory deprivation drove two distinct round of IPSC weakening instead of the chronic arrest of synapse maturation (Gainey, Wolfe et al. 2016).

Further study showed that postsynaptic NMDAR-mediated activity was required for the normal development of the receptive fields in the barrel neurons (Foeller and Feldman 2004). However, the LTP is often considered as a marker for the critical period of development in the somatosensory cortex. Crair and Malenka showed that LTP in thalamocortical synapses is dependent on NMDAR. They further showed that the NMDAR-dependent LTP in the barrel cortex was restricted to first postnatal week (Crair and Malenka 1995). In addition to that, they found a decrease in NMDAR-mediated current accompanied by loss of susceptibility to LTP with age (Crair and Malenka 1995). From these findings, they concluded that LTP is necessary for the development of cortical circuitry. A study by John Isaac's group demonstrated that not only LTP, NMDAR-dependent LTD is also important during the critical period of development of the thalamocortical synapses. Authors showed that the extent of LTD induction kept reducing as age increases. It was become difficult to induce LTD in the thalamocortical synapses by postnatal day 10-12 (Feldman, Nicoll et al. 1998). Therefore, NMDAR-mediated plasticity is crucial for the critical period of development in the somatosensory cortex.

These studies mentioned above on somatosensory cortex established the role of NMDARs in the critical period of plasticity. In addition to these findings, studies have shown that neuronal activity (Hoffmann, Gremme et al. 2000) and sensory experience (Quinlan, Olstein et al. 1999, Quinlan, Philpot et al. 1999, Philpot, Sekhar et al. 2001) differentially regulate NMDAR subunit compositions by inducing the incorporation of NR2A-containing NMDARs on the postsynapse. Thus, the dynamic change in the subunit composition might play a crucial role in the development of plasticity. One hypothesis about the cellular and molecular mechanism suggested a change in the expression pattern of NMDAR subunit genes such as *Grin2b* and *Grin2a* genes. However, the findings of Liu et al. 2004 contradicted the above-mentioned hypothesis of gene expression. Authors did not observe any alteration in the expression level of *Grin2b* gene even though there was a reduction in the NR2B-containing NMDARs in the synapses. However, they indeed observed an increment in the *Grin2a* mRNA level as well as in the NR2A protein expression level (Liu, Murray et al. 2004). Thus, the change in the synaptic NMDAR composition in the somatosensory cortex cannot be correlated directly with the gene expression level. Overall, such developmental switch is one of the underlying mechanisms for the critical period of plasticity observed in the somatosensory cortex.

During the course of development, synaptogenesis is one of the most crucial phenomena for brain maturation. Newly formed synapses are generally devoid of AMPA receptors (AMPAR). These synapses are called ‘Silent synapses’ (Isaac, Nicoll et al. 1995, Liao, Hessler et al. 1995, Durand, Kovalchuk et al. 1996, Feldman, Nicoll et al. 1999). Many of these synapses are eliminated during development, but few of them become functional by incorporating AMPAR. This process is called ‘AMPA Un-silencing’ (Hanse, Seth et al. 2013). The rate of conversion of silent to functional and vice versa is especially high during the early post-natal development (Feldman, Nicoll et al. 1999). Overall, these changes bring about plasticity in the brain.

Two broad models are postulated with respect to un-silencing of AMPAR.

AMPA un-silencing by correlated pre- and post-synaptic activity: Correlated pre- and post-synaptic activity is fundamental for NMDAR mediated LTP (Feldman, Nicoll et al. 1999). This activity leads to increase in intracellular Ca^{2+} concentration, which is necessary for AMPA un-silencing (Isaac, Nicoll et al. 1995, Liao, Hessler et al. 1995, Durand, Kovalchuk et al. 1996). Further studies have shown LTP leads to BDNF signalling, which also takes part in the AMPA un-silencing process (Itami, Kimura et al. 2003, Minichiello 2009). Physiologically, AMPA un-silencing is demonstrated as an increase in AMPAR mediated transmission. Studies have shown AMPA un-silencing is mediated by post-synaptic exocytosis of AMPAR (Montgomery, Pavlidis et al. 2001). This phenomenon is very

transient and unstable. During development, PKA activation mobilises GluA4 containing AMPAR to the post-synaptic membrane to un-silence (Zhu, Esteban et al. 2000, Esteban, Shi et al. 2003). Thus, stabilisation occurs in an activity-dependent fashion.

AMPA un-silencing process is often developmentally regulated. In the hippocampal CA3 to CA1 field, AMPA un-silencing is the primary way to generate LTP for the first two post-natal weeks; whereas this effect decreases after that (Abrahamsson, Gustafsson et al. 2008). In the thalamocortical synapses as AMPA un-silencing disappears, inducing LTP to become more and more difficult (Isaac, Crair et al. 1997). Thus, it can be marked as a correlate of critical period of plasticity. Hence, any perturbation in the AMPA un-silencing may perturb the critical period. A study by Clement et al. showed a reduced level of SYNGAP1 leads to early disappearance of LTP in thalamocortical slices which could be because of impairment in the AMPA un-silencing as SYNGAP1 is a good regulator of AMPA dynamic (Clement, Ozkan et al. 2013).

AMPA un-silencing via inactivity in the synapse: No presynaptic release for a limited duration (in minute scale) can also cause AMPA un-silencing (Strandberg, Wasling et al. 2009, Wasling, Strandberg et al. 2012). Inactivity-induced AMPA un-silenced synapses to revert to the silent state upon further activity.

The fate of these silent synapses depends on many factors such as developmental stage, brain region, synaptic activity status. Evidence suggests two distinct fates of these synapses; either they get eliminated, or they become functional by AMPA un-silencing and subsequent stabilisation process (Hanse, Seth et al. 2013).

Stabilisation completes the conversion of silent to functional synapse: Stabilization of unsilenced synapse is thought to be mediated by correlated synaptic activity mentioned above, whereas structural stability of the synapse is imparted by LTP induction (Katz and Shatz 1996, Hill and Zito 2013). Post-synaptic density scaffolding protein distribution and NMDAR subunit composition are thought to play an important role in the stabilisation of un-silenced synapses. During early development, hippocampal and cortical principle neurons majorly contain GluN2B containing synapses. As development proceeds, there is an activity-dependent switch towards GluN2A containing NMDAR incorporation in the synapses (Sanz-Clemente, Nicoll et al. 2013). GluN2A-NMDAR has reduced surface mobility resulting in a stable synapse, which coincides with the synapse maturation and further stabilisation (Groc, Heine et al. 2006, Groc, Bard et al. 2009).

Role of AMPA un-silencing in the critical developmental period is well elucidated. One such example of a thalamocortical critical period of development is mentioned above. Apart from somatosensory cortex, the role of silent synapses has been studied in visual cortex (Funahashi, Maruyama et al. 2013), and hippocampus (Smith and McMahon 2005). Any impairment in AMPA silencing and/or un-silencing process can lead to alteration in the critical period of development resulting in brain pathologies such as Neurodevelopmental disorders, Autism Spectrum Disorder, and Intellectual Disability (Hanse, Seth et al. 2013).

Further, studies have shown that different mutations may also affect the critical period in the somatosensory cortex leading to ASD/ID related pathophysiology (Bureau, Shepherd et al. 2008, Harlow, Till et al. 2010, Clement, Ozkan et al. 2013, He, Arroyo et al. 2018). Effect of mutations in *Fmr1* and *Syngap1* will be discussed in the next section in this context.

1.25. Neurodevelopmental Disorder

The human brain is one of the most plastic organs in our body. Begin plasticity allows neurons to reshape/remap in response to an external stimulus/cues (Johnson 2001). Neuronal activities play a significant role in learning and remembering different tasks/activities such as language, cycling, mathematics. During neuronal development, there is a time window when many new neuronal connections (synapses) are formed in response to an external stimulus (Tau and Peterson 2010). The concept of a critical period of development is discussed in the earlier section.

Neuronal connections are established through specialised junctions called synapses, which is the functional unit of the brain (Mayford, Siegelbaum et al. 2012). At the time of birth, there is less number of functional synapses and neuronal connections (Levitt 2003). During the early stages of development, the majority of synapses contain NMDARs, but, there is a gradual shift towards synapses containing both NMDARs as well as AMPARs (Durand, Kovalchuk et al. 1996). These AMPAR-containing synapses are denoted as functional synapses (Durand, Kovalchuk et al. 1996). As the brain develops, there is an increase in the number of functional synapses leading to a surge in the number of neuronal connections (Hensch 2004, Stiles and Jernigan 2010, Hensch and Bilimoria 2012). By the time the child reaches adolescence, the inactive/weak connections are removed, and active connections are strengthened, by then, learning anything new becomes difficult (Stiles and Jernigan 2010). However, the maturation process involving the formation of neuronal connections is executed at various time points in different parts of the brain during a child's development. This process of formation, strengthening, and elimination of synapses are regulated by many proteins present in both presynaptic as well as postsynaptic neurons (Brose 1999, Soltau, Berhorster et al.

2004, Yamaguchi and Pasquale 2004, Carlisle and Kennedy 2005, Gyorffy, Kun et al. 2018). Mutation(s) in any gene encoding a protein regulating synaptic function could lead to Neurodevelopmental Disorders (NDD) such as Intellectual Disability (ID) and Autism Spectrum Disorder (ASD) (Kishino, Lalande et al. 1997, Boeckers, Winter et al. 1999, Sahin and Sur 2015, Coe, Stessman et al. 2019). Of these genes, mutations in *SYNGAP1* (Hamdan, Gauthier et al. 2009) and *FMR1* (Pieretti, Zhang et al. 1991) are known to cause ID and ASD.

NDD is characterised by impairment in communication, cognition, motor learning, sociability and abnormal development of the brain (Reiss 2009). Categorisation and diagnosis of NDDs are complicated because of significant symptomatic overlaps among different diseases (Vahia 2013). For example, impaired social interaction is common in both ASD and Schizophrenia (Korkmaz 2011, Sugranyes, Kyriakopoulos et al. 2011). With the advent of technology such as Genome-Wide Association Studies (GWAS), Chromosomal microarray, whole-exome sequencing have led to the identification of many genes associated with NDD (Liu, Shimada et al. 2016, Sherr 2016, Hawi, Yates et al. 2018, Xu, Ji et al. 2018). Studies have shown that *De novo* autosomal dominant form of mutations accounts for 50% of patients (Sherr 2016, Wilfert, Sulovari et al. 2017).

Research over the past decade or so have identified or implicated mutations causing NDD. For example, trisomy at chromosome 21 causes Down's Syndrome (Down 1995, Hattori, Fujiyama et al. 2000), or single gene mutation in *MeCP2* caused Rett's Syndrome (Amir, Van den Veyver et al. 1999, Wan, Lee et al. 1999). Studies have shown that these mutations are hereditary which is approximately 90% for ASD (Freitag 2007), 80% for Schizophrenia (Cardno, Marshall et al. 1999), 60% for ADHD and 60% for Epilepsy (Kjeldsen, Kyvik et al. 2002, Faraone and Khan 2006). From the studies mentioned above, it is clear that gene mutations are one of the leading causes of these diseases. In addition to that many of the cases, these mutations are hereditary and carried to the next generation. Therefore, genetic mutations play a crucial role as a causative agent for these NDDs. Further, the creation of transgenic mouse models and studies on them have improved the knowledge of the biochemical, molecular, and cellular perturbations in various mutations implicated in NDD.

However, apart from common genetic mutations, epigenetic alterations are demonstrated in NDD such as Prader Willi Syndrome (PWS) and Angelman's Syndrome (AS) (Tran and Miyake 2017). The loss of function of the paternal genes located in the chromosome 15q11-q13 region is the cause for PWS (Bittel and Butler 2005, Angulo, Butler et al. 2015) and the symptoms include hypotonia during infancy, followed by hyperphagia and excessive appetite during childhood, behavioural problems such as temper tantrums, outburst, temperature instability, and endocrine abnormalities

(Butler 1990, Cassidy, Schwartz et al. 2012, Aycan and Bas 2014). Angelman's syndrome is caused due to the loss of expression of the maternally active region of chromosome 15q11-q13 (Lossie, Whitney et al. 2001). Majority of the cases the commonly affected identified gene was *UBE3A* (Albrecht, Sutcliffe et al. 1997, Kishino, Lalande et al. 1997, Matsuura, Sutcliffe et al. 1997). Dr Harry Angelman first reported Angelman's Syndrome in the year 1965 (Angelman 1965). He described the patients as 'puppet children' due to their characteristics posture and jerky movement (Angelman 1965). A later study showed patients suffer from severe developmental delay along with impairment in cognitive skills and language development (Andersen, Rasmussen et al. 2001, Gentile, Tan et al. 2010). Other symptoms manifested by patients include abnormal sleep-wake cycle, easily provoked laughter, and occurrence of seizures (Horsler and Oliver 2006, Pelc, Boyd et al. 2008, Pelc, Cheron et al. 2008, Thibert, Conant et al. 2009, Williams 2010) Both these diseases occur due to loss of function in either of the parental copy of the chromosome and are termed as imprinting disorders (Peters 2014). Genomic imprinting suggests that only one allele of a gene will be expressed depending on its parental origin (Bajrami and Spiroski 2016).

Apart from genetic and epigenetic alterations, the environment factors contribute to the occurrence of NDD. According to the United States Surgeon General's report, maternal smoking during pregnancy is detrimental for the health of the offspring and associated with adversities such as congenital anomalies or sudden infant death syndrome (2004). Tobacco smoke contains a variety of toxic substances, including Nicotine. Studies showed that prenatal exposure to nicotine is harmful, causing abnormal emotional and cognitive behaviour, as well as attention deficit (Schneider, Ilott et al. 2011, Alkam, Kim et al. 2013). The precise mechanisms behind the effect of nicotine on brain development are not known. However, a study in the rat model showed that nicotine treatment led to reduced expression of Cyclin-dependent Kinase 5 (*Cdk5*) (Shah and Lahiri 2014) that delayed neuronal migration (Zechel, Gamboa et al. 2005). Not only nicotine but the Tobacco Smoke Extract (TSE) was also shown to affect DNA synthesis (Slotkin, Skavicus et al. 2015).

Another contaminant, Bisphenol A or Phthalate Bisphenol A (BPA) was shown to cause impairment in behaviour, and learning and memory deficit in rodents by reducing synaptogenesis and altering synaptic structures (Xu, Zhang et al. 2010, Xu, Xie et al. 2013). Also, heavy metals such as methyl mercury (MeHg) could cause learning disabilities and behavioural abnormalities in rodent models (Cagiano, De Salvia et al. 1990, Sakamoto, Kakita et al. 2002).

Prenatal exposure to alcohol is another cause for the Neurodevelopmental disorders in children. Studies have shown that consumption of alcohol during pregnancy had a severe effect on the growth

and development of the foetus (Jones, Smith et al. 1973, Jones and Smith 1973). The whole spectrum of disabilities associated with prenatal alcohol exposure is known as Foetal Alcohol Spectrum Disorder (FASD) (Mattson, Roesch et al. 2010, Riley, Infante et al. 2011). Symptoms include growth delay, cognitive and social deficits, facial dysmorphology (Mattson, Roesch et al. 2010, Hoyme, Kalberg et al. 2016). Apart from these many studies have shown defects in motor skills, learning, attention, and language disabilities in children suffering from FASD (Gray, Mukherjee et al. 2009, Mattson, Crocker et al. 2011). Prenatal alcohol exposure led to increased oxidative stress, mitochondrial dysfunction, alteration in the gene expression (Goodlett and Horn 2001, Dikranian, Qin et al. 2005). A study in rodent showed that Ethanol exposure led to induction of neuroapoptosis in the cerebellum, and the brain stem, resulting in structural changes of these regions (Dikranian, Qin et al. 2005). Thus, alcohol exposure can have a life-long effect on the normal functioning of the brain by altering its physiology as well as structure. The prevalence of this disorder is highly variable depending on the geographical and socio-cultural status. A meta-analysis study showed partial fetal alcohol syndrome is higher in Croatia (~4.3%) compared to Australia (~1%), South Africa (~2.8%), and Italy (~3.6%) (Roozen, Peters et al. 2016). In the US and western Europian countries, the prevalence amongst young school going children ranges between 2-5% (May, Gossage et al. 2009, May, Baete et al. 2014). In conclusion, exposure to environmental contaminants also can exert long-lasting effects on the development leading to Neurodevelopmental Disorders.

1.26. Autism Spectrum Disorder (ASD) and Intellectual Disability (ID)

ASD was first identified in the 1940s by two scientists, Kanner in the US (Kanner 1968) and Asperger (Barahona-Correa and Filipe 2015) in Austria. However, this group of diseases were not known to people outside psychiatry for a long time until the 1980s. The prevalence of Autism before the 1990s was shown as 4-5 per 10000 individuals (Fombonne 2001). However, later study by Chakrabarty and Fombonne reported higher occurrences of ASD. They found that the prevalence of classical Autism was 22 out of 10000, and for all pervasive developmental disorders, it was 59 per 10000 children of below 6 years of age (Fombonne 2001). Two independent studies from the US in 2009 showed that 1 in 91 kids aged between 3- to 17 years old (Kogan, Blumberg et al. 2009), and 1 per 110 aged 8 years were diagnosed with Autism (Rice 2009). According to the recent WHO report (as on 2nd April 2018), 1 out of 160 children is affected with ASD, worldwide (https://www.who.int/news-room/fact-sheets/detail/autism-spectrum-disorders). These numbers kept increasing day by day, indicating a steady rise in the prevalence of ASD. Moreover, the advancement of diagnostic techniques has made identifying ASDs efficient.

Most of the cases of ASD develop before the age of 3 and often associated with avoidance of eye contact and delayed learning of language (Miles 2011). Repetitive and stereotyped behaviour is another core behavioural impairment associated with ASD (Miles 2011). The symptoms not only limited to the behavioural abnormalities. In many cases, motor skills were shown to be disrupted in kids (Miles 2011). Also, emotional impairment led to self-injurious behaviour in the patients (Miles 2011). Autism Spectrum Disorder is often co-diagnosed with other Neurodevelopmental Disorders such as Intellectual Disability (ID) (Rai, Heuvelman et al. 2018).

Intellectual Disability (ID) can be defined as a *significantly reduced ability to understand new or complex information, and to learn and apply new skills (impaired intelligence), starts before adolescent and continues into adulthood that results in reduced ability to cope independently (impaired social functioning)* (http://www.euro.who.int/en/health-topics/noncommunicable-diseases/mental-health/news/news/2010/15/childrens-right-to-family-life/definition-intellectual-disability). Clinically, ID can be characterised by the IQ values. The Individual having an IQ below 70 is considered as Intellectually disabled (2015, Girimaji and Pradeep 2018). The ID is classified into Syndromic ID (S-ID) and Non-Syndromic ID (NS-ID) (Kaufman, Ayub et al. 2010). S-ID patients show morphological and clinical features along with ID. NS-ID patients do not generally show any distinct morphological anomalies but cognitive and social disabilities (Kaufman, Ayub et al. 2010). *SYNGAP1* heterozygous mutation is implicated in NS-ID (Hamdan, Gauthier et al. 2009, Hamdan, Daoud et al. 2011), whereas Fragile X Syndrome (FXS) in S-ID (Kaufman, Ayub et al. 2010). However, a recent report suggested *SYNGAP1* heterozygous mutant individual manifest Syndromic form of ID (Parker, Fryer et al. 2015). Thus, further morphological studies are needed in human to establish the type of ID associated with *SYNGAP1* heterozygous patients.

1.27. Fragile X Syndrome

1.27.1. Mutations in the *FMR1* gene

Fragile X Syndrome is an X-linked condition described first by Martin and Bell as one of the leading cause of mental retardation (Martin and Bell 1943). Patients with a mutation in *FMR1* (Fragile X Mental Retardation) develop Fragile X Syndrome (FXS), a Syndromic ID due to lack of functional FMRP (Verkerk, Pieretti et al. 1991). The causative mutation in most of the cases is CGG repeat expansion at the 5' UTR (Untranslated region) of *FMR1* that leads to hypermethylation of UTR as well as the promoter region of the gene leading to transcriptional silencing (Fu, Kuhl et al. 1991, Coffee, Zhang et al. 2002). Majority of the FXS cases are due to trinucleotide repeat expansion, but the presence of rare point mutations (I304N and R138Q) in *FMR1* gene were also reported amongst

individuals suffering from the disease (De Boulle, Verkerk et al. 1993, Collins, Bray et al. 2010). R138Q mutation is located in the NLS motif suggesting FMRP's role is crucial in the nucleus. On the contrary, I304N mutation is located in the RNA binding domain, indicating FMRP plays a critical role in regulating translation via interacting with its mRNA targets. Different mutations found in FXS patients are summarised in Table 1-3.

Mutation	**Type of Mutation**	**Symptoms**	**References**
CGG repeat expansion	Trinucleotide repeat expansion; transcriptional silencing due to hypermethylation	Inherited Intellectual Disability, Fragile X Syndrome.	(Verkerk, Pieretti et al. 1991)
p.R138Q	Point mutation; Missense mutation	Developmental delay, Fragile X Syndrome, ID	(Collins, Bray et al. 2010)
lle367Asn	Point mutation (de novo)	FXS, FRAXA	(De Boulle, Verkerk et al. 1993)
CGG repeat expansion	Trinucleotide repeat expansion	FXS, Benign focal epilepsy of childhood (BFEC)	(Berry-Kravis 2002)
CGG repeat expansion	Trinucleotide repeat expansion	FXS	(Santa Maria, Aliaga et al. 2016)
CGG repeat expansion	Trinucleotide repeat expansion	FXS, Premature ovarian failure	(Tural, Tekcan et al. 2015)
c.80C>A	Point mutation, Nonsense mutation	FXS	(Gronskov, Brondum-Nielsen et al. 2011)

hg18, chr.X: 146801041–146801395 deletion	Deletion mutation	FXS	(Collins, Coffee et al. 2010)

Table 1-3: Major mutations found in *FMR1* gene

Trinucleotide repeat expansion, point mutation, nonsense mutation, and deletion mutation in the *FMR1* gene leads to Fragile X Syndrome. The associated symptoms often include Seizures, ataxia like symptoms, and ovarian failure.

Studies have shown that mutations in *FMR1* leads to pathologic conditions of FXS such as learning and memory deficit, increased susceptibility to seizures, macrocephaly, and macro-orchidism (Bernardet and Crusio 2006, Garber, Visootsak et al. 2008). However, the cognitive, behavioural, and morphological symptoms of Fragile X Syndrome are variable. The severity of ID ranges from moderate to severe, and further the IQ declines as age increases (Ashley, Wilkinson et al. 1993, Bernardet and Crusio 2006). Other morphological features include an elongated face, large and prominent ears, prominent jaw and forehead, high-arched palate, and loose connective tissue leading to hyperextensible joints, and flat feet (Hagerman and Hagerman 2002, Hagerman, Berry-Kravis et al. 2009). Additional medical problems, manifested by the patients include childhood seizures, sleep disorders, strabismus, a susceptibility to ear and sinus infections and gastrointestinal issues (Berry-Kravis and Potanos 2004, Hagerman, Berry-Kravis et al. 2009).

FXS was one of the first genetic causes of ID to be linked to ASD, which has highly variable behavioural manifestations that differ in severity. Fragile X Syndrome is the most common inherited cause of ASD. However, it only accounts for ~4 % of all ASD cases (Wang, Berry-Kravis et al. 2010). However, a vast majority of FXS males (~60-90%) displayed the core behavioural symptoms that are commonly observed in individuals with ASD including avoidance of eye contact, speech impairment, repetitive behaviour, hand flapping. Authors further showed 30% of FXS patients met the criteria of ASD, and 30% met the criteria of PDD-NOS (Harris, Hessl et al. 2008). Within the population suffering from FXS, there was a high incidence of epilepsy (10-20%), which was shown to be higher in male FXS patients than females (Berry-Kravis 2002). Authors also reported that FXS patients without a co-diagnosis of epilepsy manifested with abnormal EEG patterns indicating that they may also be at high risk of developing seizures (Berry-Kravis 2002).

1.27.2. Mouse models for *Fmr1* mutations

FMRP (Fragile X Mental Retardation Protein) is shown to be conserved throughout various species (Verkerk, Pieretti et al. 1991), which helped in developing the mouse model. However, an animal model consisting of expanded CGG repeats was made but failed to show the symptoms of FXS. In the available mouse model, CGG expansion did not undergo hypermethylation and transcriptional silencing (Brouwer, Mientjes et al. 2007). This lack of transcriptional silencing could be the reason behind the lack of manifestation of symptoms. The majorly used mouse model was made by deleting exon 5 of *Fmr1* by the Dutch-Belgian consortium (1994). These mice recapitulate many phenotypes shown by FXS patients such as cognitive and social deficits and increased susceptibility to seizures (Zang, Nosyreva et al. 2009). *Fmr1* KO mice also showed an increase in the number of immature dendritic spines and increased constitutive protein synthesis including the level of Calcium/Calmodulin-dependent Protein Kinase II (CAMKII), and Activity Regulated Cytoskeleton Associated Protein (ARC) (De Rubeis and Bagni 2011, Niere, Wilkerson et al. 2012).

1.27.3. Behavioural deficits in the mouse model of FXS

The generation of the mouse model of FXS was beneficial and has enabled the investigation of an extensive range of behavioural traits, which have revealed correlations between the human and mouse condition validating the *Fmr1* KO mouse as a model of Fragile X Syndrome. However, it was thought that many of the behavioural deficits observed in the *Fmr1* KO mouse are more moderate than those found in human patients and are not always consistently reported. These inconsistencies could be the result of strain variability from the effect of modifier genes. In addition to that, handling and housing, before testing, may have an adverse impact on the outcome of the test. The availability of the *Fmr1* KO rat may allow a more extensive set of behavioural tasks to be examined (Hamilton, Green et al. 2014). In terms of locomotive activity, *Fmr1* KO mice showed increased exploratory activity in the open field with a reduced tendency to remain close to peripheral zones (Mineur, Sluyter et al. 2002, Qin, Entezam et al. 2011). This increase in exploratory behaviour suggests that, in comparison to wild type (WT) controls, *Fmr1* KO mice were more hyperactive and have abnormal anxiety levels.

There was a subtle impairments in the Morris water maze when the platform was hidden, with *Fmr1* KO exhibiting increased escape latencies when the position of the platform was moved after learning from the initial position, as well as reduced rate of learning from trial to trial (1994, Kooy, D'Hooge et al. 1996). These observations suggest that *Fmr1* KO mice may have less flexibility in learning than their littermate WT control mice. In contrast, evidence from the radial maze has shown that the working memory is intact in the *Fmr1* KO mice (Mineur, Sluyter et al. 2002). However, Inhibitory avoidance (IA), a hippocampus-dependent memory, was similar between *Fmr1* KO and their WT

littermates. When IA extinction (IAE) was investigated, which was requiring new protein synthesis, the authors observed that IAE was exaggerated in the *Fmr1* KO mouse (Dolen, Osterweil et al. 2007). Majority of sensory stimuli responses in *Fmr1* KO have been found to be impaired. However, the response to nociception in response to heat was normal in *Fmr1* KO mice (Bernardet and Crusio 2006).

Further studies have shown *in vivo* that *Fmr1* KO mice have increased susceptibility to both partial and generalised seizures (Chen and Toth 2001, Osterweil, Krueger et al. 2010). In *Fmr1* KO mice, the rate of seizure progression was shown to be accelerated compared to WT littermates (Wang, Hessl et al. 2013). It was also consistently reported that *Fmr1* KO mice exhibit increased susceptibility to audiogenic seizures (AGS), upon exposure to a >130 dB alarm sound (Musumeci, Bosco et al. 2000, Yan, Asafo-Adjei et al. 2004, Osterweil, Krueger et al. 2010). There was also an increase in startle response in *Fmr1* KO mice on exposure to low-frequency auditory stimuli (Nielsen, Derber et al. 2002). Overall, these findings along with altered responses to IAE, point towards the similarities in hyper-sensory responses observed in *Fmr1* KO mice and FXS patients (Hagerman and Hagerman 2002, Dolen, Osterweil et al. 2007). *Fmr1* KO mice also exhibited higher pre-pulse inhibition suggesting an alteration in the sensorimotor processing (Frankland, Wang et al. 2004).

Furthermore the deficits in social interaction have been reported in the *Fmr1* KO mice exhibiting increased social anxiety in the mirror chamber test, reduced social dominance in the tube test to unfamiliar test mates (Spencer, Alekseyenko et al. 2011), and deficits in ultrasonic vocalizations in *Fmr1* KO pups that were isolated from their mothers (Roy, Watkins et al. 2012). In conclusion, based on these behavioural deficits observed in the *Fmr1* KO mice, it is clear that the mouse model of FXS is an effective and useful model to study FXS.

1.27.4. Dendritic spine morphology and synaptic function in *Fmr1* mutation

One of the hallmark phenotypes of the patients suffering from FXS is the excessive abundance of dendritic spines with a thin, filopodia-like immature morphology consistently reported in the autopsy samples (Rudelli, Brown et al. 1985, Hinton, Brown et al. 1991, Fiala, Feinberg et al. 1998). The abnormal spine phenotype was observed in various cortical regions, which were qualitatively analysed by rapid Golgi staining. Later, Irwin et al. (2001) observed an increase in the spine density in the visual and temporal cortices of the Fragile X Syndrome human brain, which was isolated to the most distal dendritic segments of Layer V pyramidal neurons (Irwin, Patel et al. 2001). This overabundance of immature spines and increased spine density suggests that there might be a failure in synapse maturation during the

development of the brain from Fragile X Syndrome patients. And, that impaired spine maturation persists throughout the entire lifespan. Also, synaptic pruning might be aberrant, leading to an increase in spine density in the brain of FXS patients.

Further, aberrant spine morphology was recapitulated in the mouse model of FXS. Abnormalities in the spine morphology was observed in the neocortex as well as in other brain regions such as hippocampus and the cerebellum of the *Fmr1* KO mice (Comery, Harris et al. 1997, Galvez and Greenough 2005, McKinney, Grossman et al. 2005, Grossman, Aldridge et al. 2006, Hayashi, Rao et al. 2007). Levanga et al. showed that mature hippocampal neurons from *Fmr1* KO mice contained an increased number of filopodia-like immature spines compared to the WT (Levenga, Hayashi et al. 2011). Therefore, this aberrant spine morphology and distribution suggest that FMRP may play a crucial role in regulating spinogenesis in the brain. Further, it was postulated that excessive protein synthesis due to the absence of FMRP could be the principle reason behind the abnormal spine morphology observed in the *Fmr1* KO mice as well as in the FXS patients.

Further examinations into the cellular mechanisms underlying learning and memory defects observed in *Fmr1* KO mice have revealed dysfunction in certain forms of synaptic plasticity. Group I mGluR-mediated Long-term depression (mGluR-dependent LTD) was shown to be enhanced in the hippocampus of *Fmr1* KO mice (Huber, Gallagher et al. 2002, Hou, Antion et al. 2006). However, investigations into long-term potentiation (LTP) have revealed that certain forms of NMDA receptor-dependent forms of LTP (NMDAR-dependent LTP) were impaired in FXS condition (Lauterborn, Rex et al. 2007, Hu, Qin et al. 2008, Shang, Wang et al. 2009). On the contrary, no differences were observed in NMDA receptor-dependent forms of LTD (NMDAR-dependent LTD) in the hippocampus of the *Fmr1* KO mice (Huber, Gallagher et al. 2002). Thus, FMRP plays an essential role in mGluR-mediated LTD but not in NMDAR-mediated LTD. Mechanistically, NMDAR- and mGluR-mediated LTD were distinct from one another indicating that FMRP may be specifically regulating the translation of proteins for the expression mGluR-dependent forms of synaptic plasticity but not for NMDAR-mediated plasticity.

Investigations on LTP mechanisms have shown contradictory results in the hippocampus of the *Fmr1* KO mice. Initial studies showed that late phase Long-term potentiation (LTP) induced by high-frequency stimulation (HFS) in *Fmr1* KO mice was similar to their WT littermate controls (Larson, Jessen et al. 2005, Li, Bassell et al. 2009, Auerbach and Bear 2010). However, other studies showed that NMDAR-dependent LTP was reduced in the CA1 of *Fmr1* KO mice (Lauterborn, Rex et al. 2007, Hu, Qin et al. 2008, Shang, Wang et al. 2009). The most probable reason for these discrepancies

could be the differences in the stimulation protocol, which can induce different forms of LTP (Abraham and Williams 2003). Further investigations on late phase LTP revealed that the lack of LTP in *Fmr1* KO mice was also accompanied by a failure in the trafficking of GluA1-containing AMPA receptors. Authors also showed that RAS-dependent activation of phosphoinositide 3-kinase (PI3K) was impaired which is a crucial pathway implicated in GluA1 insertion and LTP expression (Qin, Kang et al. 2005). Hence, PI3K-mediated GluA1 insertion is critical for the increase in synaptic strength post activation. However, uncoupling of this PI3K-mediated signalling to the GluA1 insertion led to impaired NMDAR-mediated LTP expression in the mouse model of FXS.

Investigations of basal synaptic properties in *Fmr1* KO hippocampal neurons revealed smaller excitatory postsynaptic currents (EPSCs) with no significant difference in mEPSC frequency compared to the WT controls (Braun and Segal 2000, Pfeiffer and Huber 2006). In addition to that, there was a reduction in AMPA/NMDA receptor ratio at postnatal day 14, which disappeared by 6-7 weeks of age in *Fmr1* KO mice (Pilpel, Kolleker et al. 2009). A study from Brian Christie's group reported that neurons with multiple primary dendrites that reside in the outer dentate granular cell layer (GCL) of *Fmr1* KO mice manifested significantly smaller NMDAR-mediated excitatory post-synaptic currents (EPSCs) and a higher AMPA to NMDA ratio in comparison to their wild-type littermate controls (Yau, Bettio et al. 2018). The authors further concluded that the loss of FMRP caused deficits in NMDAR-mediated signalling and reduced dendritic complexity in granule neurons (Yau, Bettio et al. 2018). Overall, all these studies suggest that during early development there may be delayed synapse maturation in *Fmr1* KO mice that are mediated by postsynaptic disruptions.

Not only on NMDAR-mediated plasticity, but the loss of FMRP also has a significant impact of Group I mGluR-mediated plasticity. Studies using *Fmr1*$^{-/Y}$ mouse model have shown that application of DHPG, a agonist of Group I mGluRs led to Group I Metabotropic Glutamate Receptors (Group I mGluR)-mediated Long-Term Depression (LTD) which was increased in the absence of FMRP due to internalisation of Amino 3-hydroxy 5-Methyl 4-isoxazole Propionic Acid (AMPA) type glutamate receptors (Snyder, Philpot et al. 2001, Huber, Gallagher et al. 2002). Earlier Study has shown that the number of the AMPAR present on the postsynaptic membrane was correlated with the Postsynaptic density (PSD) diameter (Takumi, Ramirez-Leon et al. 1999). As AMPAR number decreases, PSD size also decreases (Takumi, Ramirez-Leon et al. 1999). Another study has shown that the PSD size was corroborated with the spine head size (Harris and Stevens 1989). The authors also suggested the dimension of the spine head reflects synaptic efficacies (Harris and Stevens 1989).

Therefore, by modulating AMPAR on the postsynaptic membrane, the spine size and synaptic efficacy can be modulated.

Also, a study by Harlow et al., demonstrated a temporal delay in the critical period of synaptic plasticity in the somatosensory cortex of *Fmr1*$^{-/Y}$, suggesting that mutations in *Fmr1* could lead to impaired neuronal connections during the critical period of development (Harlow, Till et al. 2010). Furthermore, Huber et al. had proposed that increased dendritic arborisation and spinogenesis, which are the hallmarks of FXS, can be caused due to a compensatory mechanism (Huber 2000, Huber, Gallagher et al. 2002). Decreased level of AMPAR on the post-synaptic membrane causes decreased electrical activity that is necessary for the survival of neurons. Generally, neurons restore this situation by inserting more glutamate receptors such as AMPAR on the post-synaptic membrane, which is not possible in FXS. Thus, neurons try to find presynaptic targets by increasing spinogenesis and dendritic arborisation as a compensatory mechanism (Kim and Cho 2014).

1.27.5. The critical period of plasticity

The critical period of plasticity is often found to be altered in many of the neurodevelopmental disorders and ASD. One of the first studies in the somatosensory cortex by Harlow et al. have shown that the *Fmr1* KO mouse model manifested impaired critical period of plasticity (Harlow, Till et al. 2010). *Fmr1* KO mice exhibited altered sensory processing, as discussed in the earlier section. Previous studies have also shown an increased abundance of long thin filopodia-like immature dendritic spines in the somatosensory cortex of *Fmr1* KO mice (Nimchinsky, Oberlander et al. 2001, Galvez and Greenough 2005). Whereas, these observations were highly correlative with respect to the mechanisms for various behavioural abnormalities observed in *Fmr1* KO mice. Harlow *et al.* measured the ratio of NMDAR- and AMPAR-mediated current (NMDA/AMPA ratio) from the spiny stellate cells of Somatosensory cortex using conventional voltage clamp recordings. NMDA/AMPA ratio decreased progressively from PND4 to PND7, marking the closure of the critical period in WT mice. On the contrary, in *Fmr1*$^{-/Y}$ mice, the NMDA/AMPA ratio increased between PND4 to P7 and returned to WT level at PND10-14 (Harlow, Till et al. 2010), indicating that the critical period of maturation of the somatosensory synapses is delayed.

In addition to the alteration in the NMDA/AMPA ratio, impairment in LTP induction was shown to be a hallmark of the closure of the critical period in the somatosensory cortex (Crair and Malenka 1995), which was delayed in *Fmr1*$^{-/Y}$ mice (Harlow, Till et al. 2010). These data suggest a delay in the maturation of the thalamocortical synapses in Fragile X Syndrome. This delay in the maturation

of the thalamocortical synapses might be the reason for the impaired sensory response, and defective learning and memory phenotype observed in *Fmr1*$^{-/Y}$ mice.

On the similar line, altered Ocular Dominance Plasticity was shown by measuring Visual Evoked Potentials (VEP) in the Visual Cortex of *Fmr1*$^{-/Y}$ mice (Frenkel and Bear 2004, Dolen, Osterweil et al. 2007). Later, maladaptive auditory response manifested by FXS patients and by *Fmr1* KO animals was shown to be a result of the impaired critical period of plasticity in the primary auditory cortex (Kim, Gibboni et al. 2013). Therefore, disruption in the critical period of synaptic plasticity is a significant contributor for the behavioural and synaptic pathophysiology observed in the Fragile X Syndrome.

1.27.6. Cellular/Molecular pathways involved in FMRP's function

Fragile X Mental Retardation protein (FMRP) is a polyribosome associated translational regulator of many essential plasticity-related genes in neurons (Stefani, Fraser et al. 2004) (Qin, Kang et al. 2005). FMRP is often considered as a renaissance protein with its diverse array of functions in the synapse. (Antar and Bassell 2003). Though it is known as a negative regulator of an essential subset of dendritically localised mRNA translation, it also takes part in mRNA shuttling (Dictenberg, Swanger et al. 2008), mRNP granule formation (Gareau, Houssin et al. 2013), and alteration in synaptic structure (Edbauer, Neilson et al. 2010). FMRP is ubiquitously expressed but enriched in brain and testis (Hinds, Ashley et al. 1993). In the brain, the presence of FMRP is predominantly observed in neurons as well as in glial cells. In neurons, it is seen throughout the cytoplasm, nucleus, and dendrites (Feng, Gutekunst et al. 1997). Structurally, FMRP contains 2K Homology domains (KH1 and KH2), and an Arginine-Glycine-Glycine (RGG) box. These two domains play a critical role in a sequence-specific RNA binding function of FMRP (Blackwell, Zhang et al. 2010). Also, FMRP consists of a Nuclear Localization Signal (NLS) and Nuclear Export Signal (NES), suggesting that it can shuttle mRNAs in and out of the nucleus (Eberhart, Malter et al. 1996). Previous studies have shown that FMRP can interact with its target mRNAs via adaptor molecules such as BC1, which is a noncoding RNA (Zalfa, Adinolfi et al. 2005). However, FMRP's direct interaction with its target mRNAs is also well established (Joachimi, Benz et al. 2009). Further, FMRP can recognise and bind to mRNAs containing a signature secondary structure such as G-quadruplex (Darnell, Jensen et al. 2001), SoSLIP (Superoxide Dismutase 1 stem-loop) (Bechara, Didiot et al. 2009), Kissing complex, and regulates their translation.

From the *Fmr1* KO *in vitro* and *in vivo* studies have revealed that there was an increase in protein synthesis rate in the hippocampus and other brain regions (Qin, Kang et al. 2005, Dolen, Osterweil

et al. 2007, Osterweil, Krueger et al. 2010). Furthermore, the activation of Group I mGluRs failed to increase elevated protein synthesis rates further in the *Fmr1* KO mice (Osterweil, Krueger et al. 2010). This failure to elevate protein synthesis rate after the activation of Group I mGluR suggests that in the *Fmr1* KO hippocampus, mRNA translation is already saturated downstream of constitutive mGluR activation due to the loss of FMRP which acts as translational repression.

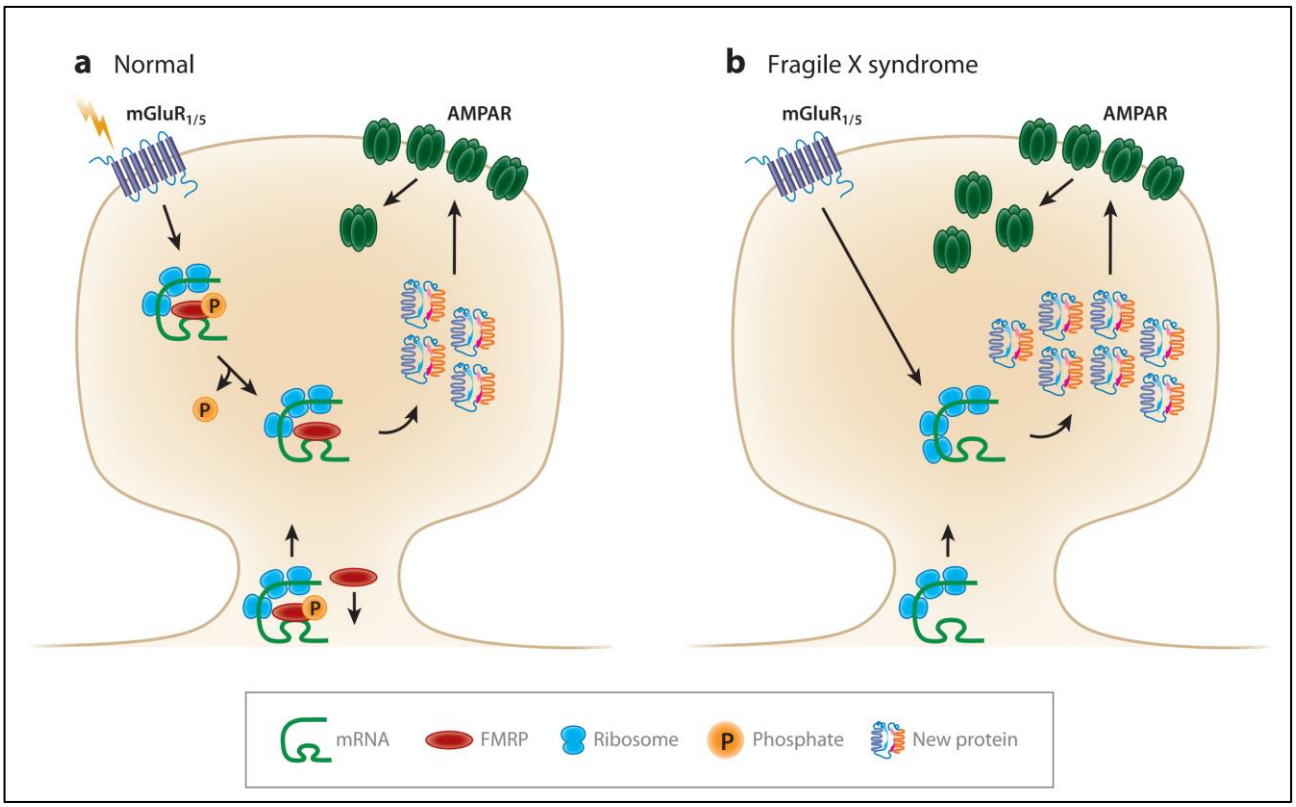

Figure 1-5: Molecular mechanism and mGluR theory of Fragile X Syndrome

FMRP interacts with its target mRNAs and shuttle them from nucleus to the dendritic spines. Following mGluR activation, FMRP gets dephosphorylated and the target mRNAs are translated. The protein synthesised help in the internalisation of the AMPARs from the postsynaptic membrane. In absence of FMRP (under Fragile X condition), the mRNAs are translated constitutively leading to increased overall protein synthesis. In turn, internalisation of AMPARs from postsynaptic membrane is increased. The image is produced with permission from (Santoro, Bray et al. 2012).

The translational efficiency of many of the FMRP target mRNAs has been studied in the hippocampus. mRNAs of *Psd-95*, *CamKII*, and *GluA1* were shown to be excessively translated in the absence of FMRP (Muddashetty, Kelic et al. 2007, Osterweil, Krueger et al. 2010). Therefore, abnormal translation of FMRP's target mRNAs in *Fmr1* KO brain indicates that FMRP represses translation of its target mRNAs under steady state condition.

So far, two critical intracellular signalling pathways were identified which are thought to couple to Group I mGluR activation to mRNA translation. The first pathway is the mammalian target of rapamycin (mTOR)-mediated pathway, and the second one is extracellular signal-regulated kinase 1/2 (ERK1/2)-mediated pathway. Both ERK1/2 and mTOR can stimulate cap-dependent protein synthesis (translation of mRNA targets having a 5' cap) by targeting the regulatory components of the initiation complex, primarily the eukaryotic translation initiation factor (EIF)-4E and the EIF4E binding protein (4E-BP) (Banko, Hou et al. 2006, Ronesi and Huber 2008, Ronesi and Huber 2008). The initiation step of mRNA translation starts with the recognition of 5' mRNA cap by EIF4E. This mRNA-4F interaction leads to the formation of the EIF4F complex, which consists of EIF4E, EIF4F and EIF4A. Once 4F complex binds to the mRNA, it further triggers the recruitment of the small ribosomal subunit and initiates the translation process (Richter and Sonenberg 2005). Alongside with that, both mTOR and ERK1/2 can activate 5'TOP-dependent mRNA translation (mRNAs containing a 5' **T**erminal **O**ligo-**P**yrimidine tract) through phosphorylation of S6K1/2 and RSK, respectively. Activation of TOP-dependent translation in-turn increases the translational capacity of the cell by synthesising ribosomal subunits and translation factors (Levy, Avni et al. 1991, Costa-Mattioli, Sossin et al. 2009). The model for the mGluR theory of Fragile X Syndrome is described in Figure 1-5.

1.28. *SYNGAP1*-related Intellectual Disability

1.28.1. *Syngap1* heterozygous mutation

Mutation in *SYNGAP1* gene was shown to cause Intellectual Disability (ID) in human (Hamdan, Gauthier et al. 2009). This initial study involved 94 patients with NSID who underwent *SYNGAP1* sequence analysis, for which three point mutations (K138X, R579X and L813RFSX22) were identified. Two patients were heterozygous for nonsense mutation, K138X and R579X. The study also predicted that the mutations result in a truncated protein formation as the frameshift mutation introduced a premutation stop codon. Interestingly, all mutations were absent from parental DNA samples suggesting that these mutations were *de novo* in nature. Only one of the three mutations were found in the RAS-GAP domain (R579X), while K138X and L813RFSX22 were located in the N-terminal and SH3 domains, respectively. Not only ID but Studies from human patients have also shown that loss-of-function mutations in *SYNGAP1* resulted in Intellectual Disability (ID), Autism Spectrum Disorder (ASD), as well as epilepsy (Hamdan, Gauthier et al. 2009, Hamdan, Daoud et al. 2011, Rauch, Wieczorek et al. 2012, Berryer, Hamdan et al. 2013,

Prchalova, Havlovicova et al. 2017, Vlaskamp, Shaw et al. 2019). Different mutations identified in patients are summarised in Table 1-4.

Clinical investigations on these patients have revealed a spectrum of symptoms that vary in severity. It is widely reported that all patients manifested a psychomotor delay early on in development and language is moderate to severely impaired. The recent identification of ID patients with mutations in the *SYNGAP1* gene has also shown that epilepsy is frequent comorbidity (Hamdan, Gauthier et al. 2009, Pinto, Pagnamenta et al. 2010, Klitten, Moller et al. 2011, Berryer, Hamdan et al. 2013, Writzl and Knegt 2013). Also, mutations in *SYNGAP1* gene have been linked to a form of epileptic encephalopathy, an extremely debilitating group of epilepsies characterised by refractory seizures and cognitive arrest (Carvill, Heavin et al. 2013). According to the study by Hamdan et al., three patients out of 94 were found to have a mutation in the *Syngap1* gene that leads to a truncated protein (Hamdan, Gauthier et al. 2009). Strikingly, the mutations are not present in the parents, and all the affected individuals are aged between 4 to 11 years. This pattern of incidence indicates the mutations found in *SYNGAP1* were *de novo* (Hamdan, Gauthier et al. 2009, Jeyabalan and Clement 2016). However, the existence of SYNGAP1 protein was first reported in 1998 in the rat brain (Chen, Rojas-Soto et al. 1998, Kim, Liao et al. 1998).

Mutation	**Type of mutation**	**Symptoms**	**References**
K138X	Pre-mature truncating mutation	Autosomal dominant Intellectual Disability	(Hamdan, Gauthier et al. 2009)
R579X	Pre-mature truncating mutation	Autosomal dominant Intellectual Disability	(Hamdan, Gauthier et al. 2009)
L813RfsX22	Frame shift premature truncating mutation	Autosomal dominant Intellectual Disability	(Hamdan, Gauthier et al. 2009)

c.2677delC/p.Q893RfsX184	Out of Frame deletion mutation	NSID, Generalised Epilepsy	(Hamdan, Daoud et al. 2011)
c.321_324delGAAG/p. K108VfsX25	Out of Frame deletion mutation	NSID, Generalised Epilepsy	(Hamdan, Daoud et al. 2011)
c.2294 + 1G>A	De novo splicing mutation	NSID, Autism	(Hamdan, Daoud et al. 2011)
c.1084T>C [p.W362R]	Missense mutation	NSID, Autism, Ataxia, generalised Epilepsy	(Berryer, Hamdan et al. 2013)
c.1685C>T [p.P562L]	Missense mutation	NSID, Autism, Ataxia, generalised Epilepsy	(Berryer, Hamdan et al. 2013)
c.283dupC [p.H95PfsX5]	Frame-shift truncating mutation	NSID, Autism, Ataxia, generalised Epilepsy	(Berryer, Hamdan et al. 2013)
c.2212_2213del [p.S738X]	Frame-shift truncating mutation	NSID, Autism, Ataxia, generalised Epilepsy	(Berryer, Hamdan et al. 2013)
c.2184del [p.N729TfsX31]	Frame-shift truncating mutation	NSID, Autism, Ataxia, generalised Epilepsy	(Berryer, Hamdan et al. 2013)

6p21.3 microdeletion	Deletion mutation	ID, seizures, speech impairment	(Writzl and Knegt 2013)

Table 1-4: Major mutaations found in *SYNGAP1* gene

Frame-shift, truncating, Missense, and deletion mutation in the *SYNGAP1* gene leads to Intellectual disability. Many cases, the symptoms include Epileptic seizures, ataxia, and autism.

1.28.2. Mouse models for *Syngap1*$^{+/-}$

Mouse models are particularly helpful in dissecting out the physiological and molecular functions of many genes. Being a gene affected in ID/ASD, it was essential to understand the role of *Syngap1* in the brain. Till date, many mouse models have been developed to study SYNGAP1 (Komiyama, Watabe et al. 2002, Kim, Lee et al. 2003, Vazquez, Chen et al. 2004). *Syngap1*$^{-/-}$ mice die by one week of age, which made it difficult to study the effect of complete loss of SYNGAP1 in postnatal development (Kim, Lee et al. 2003). These homozygous knockout mice have shown activation of apoptosis via increased activity of Caspase 3. Therefore, excessive apoptosis could be one of the reasons for the lethality of this mouse model (Knuesel, Elliott et al. 2005).

Syngap1$^{+/-}$ mice developed by replacing the exon 7 and 8 with a neomycin cassette (Komiyama, Watabe et al. 2002, Kim, Lee et al. 2003). *Syngap1*$^{+/-}$ mice were shown to be associated with the several behavioural abnormalities including cognitive and learning deficits, hyperactivity, reduced seizure threshold and enhanced locomotion (Guo, Hamilton et al. 2009, Ozkan, Creson et al. 2014, Parker, Fryer et al. 2015). Behavioural tests like Morris water maze, radial arm maze, Y-maze novel arm test shown impairments in learning and memory-related functions in the *Syngap1*$^{+/-}$ mice (Vazquez, Chen et al. 2004, Guo, Hamilton et al. 2009, Muhia, Willadt et al. 2012). Also, *Syngap1*$^{+/-}$ mice also showed a decline in the working memory (Guo, Hamilton et al. 2009, Muhia, Yee et al. 2010). These mice further manifested deficits in the remote memory when tested on the contextual fear learning procedure (Ozkan, Creson et al. 2014). Thus, *Syngap1*$^{+/-}$ mouse model is considered as an excellent model, suggesting that they effectively phenocopy the pathology/ symptoms observed in human patients (Guo, Hamilton et al. 2009).

1.28.3. Dendritic spine morphology and function in *Syngap1* mutation

Syngap1 mRNA expression levels increase during the first two weeks of postnatal life and peaks at postnatal day 14, which coincides with synaptogenesis (Clement, Aceti et al. 2012). Investigation of spine development in hippocampal cultures at DIV 10 showed that *Syngap1* KO pyramidal neurones had an increase in the number of protrusions,

which was absent in more mature cultures (DIV 21) (Vazquez, Chen et al. 2004). However, the WT neurones displayed thinner elongated spines, but in *Syngap1* KO neurones the spine head width and area of protrusions were markedly increased. This increase in spine head size was also observed when more mature *Syngap1* KO neurones were examined (DIV 21), suggesting that abnormalities in spine morphology persist into adulthood in the absence of SYNGAP1. On a similar line, Carlisle et al. observed that the adult hippocampal neurones from *Syngap1* heterozygous mice manifested more mushroom-shaped spines with a larger head volume and length relative to their WT counterparts (Carlisle, Manzerra et al. 2008). Overall, there was abnormality associated with spine morphology when SYNGAP1 is depleted.

Further, Clement et al. investigated dendritic spine size, density, and dynamics in Granule cells of the dentate gyrus. At postnatal day 9, *Syngap1* heterozygote granule cells had similar spine head widths to the WT (Clement, Aceti et al. 2012). However, by postnatal day 14, the head diameter was significantly larger, and this phenotype persisted to adulthood. Authors found that in *Syngap1*$^{+/-}$ condition by postnatal day 14 higher proportion of spines became mushroom-shaped spines with fewer stubby spines. Whereas, overall spine density was unaltered in *Syngap1*$^{+/-}$ *versus* WT mice (Clement, Aceti et al. 2012). Further examination and analysis of dendritic branches revealed that dendritic arborisations were unaltered in *Syngap1*$^{+/-}$ mice but overall spatial volume occupied by these arborisations were decreased relative to WT counterparts (Clement, Aceti et al. 2012). Therefore, the replication of spine phenotype in slice provides further support for SYNGAP1's role in the maturation rate of dendritic spines in multiple brain regions perturbing the enlargement of spines during brain development.

SYNGAP1 plays a crucial role in regulating glutamatergic signalling at the synapse during the early developmental window. A later study by Clement *et al.* showed that AMPAR-mediated responses increased in P14-16 compared to the wild-type littermates. However, the AMPA/NMDA ratio remained unaltered in young (P7-9) and the adult mice (Clement, Aceti et al. 2012). These changes in the AMPA/NMDA ratio suggested that the alteration in the insertion of AMPA receptors could lead to changes in excitatory and inhibitory balance. In addition, this increase in AMPA/NMDA could be because of increased incorporation of AMPA receptors on the postsynaptic membrane. However, by postnatal day 21 synaptic transmission in *Syngap1* heterozygous reached the same level observed the WT slices. Other reports also found that basal synaptic transmission in the hippocampus of adult *Syngap1* heterozygous mice was intact (Komiyama, Watabe et al. 2002, Kim, Lee et al. 2003).

Furthermore, electrophysiological recordings in hippocampal CA1 have shown that LTP, induced by a variety of stimulation paradigms, is consistently impaired in *Syngap1* heterozygous mice (Komiyama, Watabe et al. 2002, Kim, Lee et al. 2003).

There was significantly less potentiation observed in *Syngap1* heterozygous mutants compared to the WT slices, upon application of HFS (2 × trains of 100 Hz and 6 × trains of 100 Hz) or theta-burst stimulation to the Schaffer collateral pathway. Theta burst is more physiological stimulation protocol as compared to the HFS. One of the earlier studies from Kandel's group showed 3-second Theta burst stimulation was sufficient to induce hippocampal LTP. The brief theta burst protocol includes 15 trains of four pulses at 100 Hz with an inter-trial interval of 200 ms (Nguyen and Kandel 1997). This LTP deficit occurred in the absence of any alterations in basal synaptic transmission, paired-pulse ratio, or NMDA receptor-mediated synaptic currents (Komiyama, Watabe et al. 2002). Overall, these findings indicate that impairment in the synaptic functions and synaptic plasticity, in turn, led to learning and memory deficits in *Syngap1*$^{+/-}$ mice.

SYNGAP1 protein is known to regulate postsynaptic cytoskeletal changes and AMPA receptor trafficking on to the surface of the post-synaptic membrane (Kim, Lee et al. 2003). Authors showed a reduction in LTP in comparison to the wild-type littermates suggesting impaired learning and memory abilities (Kim, Lee et al. 2003). Later studies have shown that *Syngap1*$^{+/-}$ mice exhibited early spine maturation leading to an overall increase in the neuronal excitability (Vazquez, Chen et al. 2004, Clement, Aceti et al. 2012). Research had also pointed out at the involvement of SYNGAP1 in the actin-mediated steady-state regulation of spine morphology, which is necessary for spine maturation (Carlisle, Manzerra et al. 2008). A study by Clement et al. suggested that SYNGAP1 is particularly vital during the critical period of development in the somatosensory cortex (Clement, Ozkan et al. 2013). The similar observation made in the prefrontal cortex validated the role of SYNGAP1 during the critical period (Aceti, Creson et al. 2015).

1.28.4. The critical period of plasticity

The expression of *Syngap1* gene is developmentally regulated, and the mRNA level peaks at PND14 and stabilises subsequently (Clement, Aceti et al. 2012). SYNGAP1 expression in the brain of *Syngap1*$^{+/-}$ was shown to be almost 50% of that of WT (Clement, Aceti et al. 2012). As discussed in the above sections, SYNGAP1 is crucial for the NMDAR-mediated signalling as well as for the synaptic functions and plasticity. Electrophysiological recordings in the acute hippocampal brain slices have shown increased AMPA/NMDA ratio in the hippocampus of *Syngap1*$^{+/-}$ (Clement, Aceti et al. 2012). This increase in the ratio of AMPA/NMDA can further be corroborated with increased basal synaptic transmission, increased mEPSC amplitude as well as frequency, and finally with an increased number of mushroom-shaped spines observed at postnatal day 14 in *Syngap1*$^{+/-}$ as compared to WT littermates (Clement, Aceti et al. 2012).

Further, Somatosensory cortex was also used as a model region to study critical period of development in rodents. A study by Crair and Malenka showed that LTP was unable to be induced in the thalamocortical synapses by postnatal day 7 (Crair and Malenka 1995). Alongside, the AMPA/NMDA ratio gradually increased in the excitatory synapses from postnatal day 3 to postnatal day 7. There was an inverse relationship between the AMPA/NMDA ratio with the induction of LTP in the thalamocortical synapses (Crair and Malenka 1995). They also reported that the time window for the loss of LTP induction in the primary somatosensory cortex coincides with the critical period of development of the thalamocortical synapses (Crair and Malenka 1995). Later, Clement *et al.* extensively investigated the AMPA/NMDA ratio in the thalamocortical synapses during the early postnatal age in *Syngap1*$^{+/-}$ mice (Clement, Ozkan et al. 2013). Consistent with the previous studies, authors also showed that the AMPA/NMDA ratio increased during the first postnatal week in WT (Clement, Ozkan et al. 2013). However, the AMPA/NMDA ratio did not change from postnatal day 4 until postnatal day 9 in *Syngap1*$^{+/-}$ mice (Clement, Ozkan et al. 2013). One probable reason for unaltered AMPA/NMDA ratio in *Syngap1*$^{+/-}$ mice could be pre-existing increased AMPA/NMDA ratio at earlier postnatal days such as postnatal day 4-5, compared to WT. Further, authors demonstrated that AMPA/NMDA ratio was significantly high at the thalamocortical synapses of *Syngap1*$^{+/-}$ mice compared to their WT littermate controls at postnatal day 5 (Clement, Ozkan et al. 2013). Therefore, the thalamocortical synapses of the somatosensory cortex were matured early in *Syngap1*$^{+/-}$ mice compared to WT.

To validate the alteration of the critical period of synaptic plasticity in *Syngap1*$^{+/-}$ mice, Clement *et al.* investigated the LTP at the thalamocortical synapses. As established by Crair and Malenka, the authors showed that LTP was successfully induced at postnatal day 5, but it was lost by postnatal day 8 in WT. However, the thalamocortical synapses were unable to induce LTP from postnatal day 5 in *Syngap1*$^{+/-}$ mice indicating a precocious maturation of the synapses. Overall, the study by Clement *et al.* demonstrated that the critical period of development was altered in the thalamocortical connections in *Syngap1*$^{+/-}$ mice during development. Hence, the alteration of critical period of thalamocortical synapse maturation further validated the early excitatory synaptic maturation (Clement, Ozkan et al. 2013).

In conclusion, these two studies suggest that a mutation in the *Syngap1* gene led to an altered critical period of plasticity and early maturation of dendritic spine structures. This alteration in critical period and early maturation of synapses might prevent remapping of connections, particularly to any experience, during development. Thus, the effect of *Syngap1* mutation is long-lasting on the development of the brain. Hence, mutation in the *Syngap1* gene led to impaired synaptic plasticity, altered critical period of development, in-turn learning and memory deficits.

More recently, a study by Aceti *et al.* showed that the neurons of the somatosensory cortex manifested higher order branching, arborization, and adult-like dendritic length at postnatal day 21. The manifestation of such adult-like features further indicates early maturation of neurons in *Syngap1*$^{+/-}$ (Aceti, Creson et al. 2015). Filopodia to mushroom-shaped spine structure transition are often associated with functional remapping of sensory neural circuits in response to the experiences (Trachtenberg, Chen et al. 2002). Further study by whisker trimming indicated a 2.5-fold increase in filopodia-like immature spine density at postnatal day 21 in WT. However, such increase in filopodia-like spine formation was shown to be absent in *Syngap1*$^{+/-}$ that further suggests the limited capacity to organise cortical circuits or remapping cortical circuits in *Syngap1*$^{+/-}$. Aceti *et al.* further investigated the mPFC region in *Syngap1*$^{+/-}$ mice. The overall anatomical features of mPFC were unaltered in *Syngap1*$^{+/-}$ mice when compared to WT (Aceti, Creson et al. 2015). The authors also investigated the neuronal connection level. There was no change in the projections on to the mPFC from different brain regions in *Syngap1*$^{+/-}$ mice. They concluded that the gross anatomical connections were normal under *Syngap1*$^{+/-}$ condition (Aceti, Creson et al. 2015). However, the authors observed a trend of hyperconnectivity from the amygdala, secondary auditory cortex, and perirhinal cortical inputs into the mPFC indicating an alteration of the prefrontal cortical circuit in *Syngap1*$^{+/-}$ mice (Aceti, Creson et al. 2015). They further concluded that the disruption in the cortical circuit could be one of the routes for impaired cognition in *Syngap1*$^{+/-}$ mice (Aceti, Creson et al. 2015). Overall, these investigations indicate that there is indeed a heightened period of plasticity during development. Mutation in *Syngap1* gene alters such critical period. The effect of such alteration in the critical period of development is irreversible.

1.28.5. Cellular/Molecular pathways involved in SYNGAP1's function

SYNGAP1 encodes for Synaptic RAS-GTPase Activating Protein1 (SYNGAP1). SYNGAP1 is associated with Post Synaptic Density (PSD) complex downstream of N-Methyl-D-Aspartate Receptors (NMDAR) in neurons (Kim, Liao et al. 1998). SYNGAP1 negatively regulates RAS-GTPase activation (Kim, Liao et al. 1998), and AMPA Receptors (AMPAR) insertion on to the post-synaptic membrane via ERK (Rumbaugh, Adams et al. 2006). SYNGAP1 is a ~135 kDa protein and is exclusively expressed in the brain (Chen, Rojas-Soto et al. 1998, Kim, Liao et al. 1998). SYNGAP1 contains 1135 amino acids and has several regulatory domains (Kim, Liao et al. 1998). SYNGAP1 consists of a RAS-GAP domain, a Pleckstrin Homology domain and one C2 domain (Kim, Liao et al. 1998). The RAS-GAP domain contributes to the RAS-GTPase activity, C2 domain might help in Ca^{2+} or phospholipid binding. The PH domain of SYNGAP1 helps it to interact with PDZ domains of different scaffolding proteins present in the postsynapse such as

PSD-95, SAP 102. The C-terminal region contains a QTRV motif which also mediates the interaction with PSD-95 and SAP 102. SYNGAP1 is a cytosolic protein with no transmembrane domain (Kim, Liao et al. 1998). SYNGAP1 is expressed exclusively in the brain but not in any other organ (Kim, Liao et al. 1998).

Extensive studies using *Syngap1*$^{-/+}$ have shown that SYNGAP1 regulates NMDAR-mediated signalling in neurons (Oh, Manzerra et al. 2004). Neurons contain several types of ionotropic Glutamate receptors such as AMPA, NMDA, and Kainate receptors. When neurotransmitter glutamate is released from the presynapse, it binds to AMPAR, leading to depolarisation of postsynaptic membrane. As the depolarisation occurs, the Mg^{2+} block is released from NMDAR, and, thus, NMDARs are permeable to Na^{+} and Ca^{2+} that results in the activation of different Kinase cascades such as CAMKII (Krapivinsky, Medina et al. 2004). The influx of Ca^{2+} and activation CAMKII phosphorylates SYNGAP1, thereby, regulating synaptic function (Chen, Rojas-Soto et al. 1998).

Previous studies have shown that NMDAR-PSD complex plays a significant role in regulating dendritic spine structure and function (Pak, Yang et al. 2001, Tao and Johns 2001, Ultanir, Kim et al. 2007, Andres, Regev et al. 2013, Chen, Levy et al. 2015, Wu, Sun et al. 2017). In line with this, a recent study demonstrated that CAMKII mediated phosphorylation of SYNGAP1 leads to rapid dispersion of SYNGAP1 from dendritic spine upon stimulation resulting in the activation RAS-GTPase (Oh, Manzerra et al. 2004, Yang, Tao-Cheng et al. 2013, Araki, Zeng et al. 2015). This RAS-GTPase activity further phosphorylates ERK, imitates the process of AMPAR insertion on to the postsynaptic membrane (Rumbaugh, Adams et al. 2006). These studies suggest a significant role of SYNGAP1 in not only maintaining homeostasis of neuronal function but also in regulating synaptic plasticity.

Along with SYNGAP1's role in NMDAR-mediated signalling as well as in the postsynaptic scaffolding, recent studies have also indicated that SYNGAP1 might play a critical role in synaptic protein synthesis. However, the mechanisms behind the same are still unclear. In *Syngap1* KD cortical cultures, basal levels of protein synthesis were assessed using Fluorescent non-canonical amino acid tagging (FUNCAT) (Wang, Held et al. 2013). In their study, authors replaced the endogenous methionine with azidohomoalanine (AHA), which was incorporated into newly translated proteins in methionine's place. In *Syngap1* KD neurones authors observed an increased AHA signal in dendrites suggesting that during basal conditions SYNGAP1 might suppress mRNA translation at the synaptic sites. The introduction of WT *Syngap1* gene copy in KD neurones, rescued enhanced protein synthesis rates, however overexpression of *Syngap1* in WT neurones did not suppress protein

synthesis rates further (Wang, Held et al. 2013). More recently, A study by Barnes et al. showed that the overall protein synthesis was enhanced in the hippocampal slices obtained from the *Syngap1*$^{+/-}$ mice compared to their littermate WT controls (Barnes, Wijetunge et al. 2015). Similarly, enhanced protein synthesis rates in *Syngap1* KD neurones could be corrected by either mTOR or ERK1/2 inhibition, suggesting that increased translational rates lie downstream of ERK1/2 and mTOR-mediated signalling. Synaptic deficits due to *Syngap1* loss of function is described in Figure 1-6.

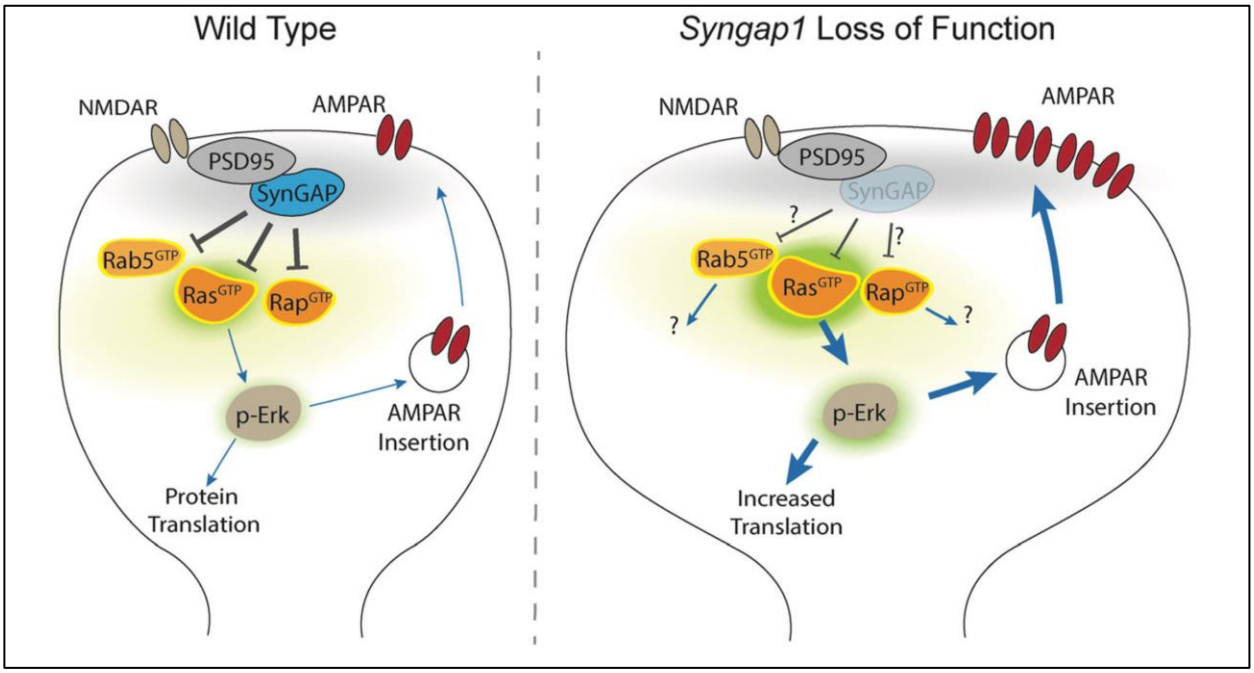

Figure 1-6: Molecular pathway affected in *Syngap1* heterozygous condition

SYNGAP1 regulates a variety of GTPases including RAS-GTPase. It regulates the AMPAR insertion on the postsynaptic membrane via RAS-ERK-mediated signalling pathway. In *Syngap1* heterozygous condition, RAS-ERK-mediated signalling pathway become highly active leading to increased protein synthesis and increased AMPAR insertion on the postsynaptic membrane. The image is produced with permission from (Weldon, Kilinc et al. 2018).

1.29. The potential link between Fragile X Syndrome and *SYNGAP1*$^{+/-}$ mutation

The genetic aetiology of intellectual disability (ID) and autism spectrum disorder (ASD) is frequently associated with mutations in genes that encode synaptic proteins. A study on human patients has revealed that de novo mutations in the *SYNGAP1* gene are a potential cause of ID (Hamdan, Gauthier et al. 2009). *SYNGAP1* gene encodes the synaptic GTPase activating protein (SYNGAP1) a known regulator of RAS-mediated signalling (Kim, Liao et al. 1998, Jeyabalan and Clement 2016). Investigations on *Syngap1* haploinsufficiency (*Syngap1*$^{+/-}$) in mice have shown

abnormalities in behaviour, synaptic plasticity, and dendritic spine development (Komiyama, Watabe et al. 2002, Vazquez, Chen et al. 2004, Guo, Hamilton et al. 2009, Clement, Aceti et al. 2012, Jeyabalan and Clement 2016). These observations are similar to findings from the Fragile X Syndrome mouse model (FXS; *Fmr1*$^{-/y}$), the most common inherited form of ID (Santoro, Bray et al. 2012). One of the prominent phenotypes observed in the mouse model of FXS was that a form of hippocampal Group I metabotropic glutamate (mGluR) receptor-mediated long-term depression (LTD) was enhanced and independent of new protein synthesis (Huber, Gallagher et al. 2002, Nosyreva and Huber 2006). The reason behind these deficits in synaptic plasticity together with other cognitive abnormalities manifested in FXS were thought to arise, in part, from excessive protein synthesis, the consequence of altered Group I mGluR-mediated signalling via the RAS-/ERK1/2-mediated signalling pathway (Bear, Huber et al. 2004, Bear 2005, Wang, Snape et al. 2012). Either inhibiting mGluR5 receptors or reducing RAS-mediated and subsequent ERK1/2-mediated activity can correct increased rates of protein synthesis in *Fmr1*$^{-/y}$ mice (Osterweil, Chuang et al. 2013).

The cognitive, behavioural and morphological symptoms of FXS are highly variable, though they share a defining feature of ID. The severity of ID ranges from moderate to severe and the Intelligent Quotient (IQ) level was found to decline further as age increases (Eliez, Blasey et al. 2001). Individual affected with FXS usually show language delay, and additional behavioural symptoms, including hyperactivity, social anxiety, impulsivity, attention deficit hyperactivity disorder and autistic-like behaviours, such as poor eye contact and hypersensitivity to sensory stimuli, and shyness (Tranfaglia 2011, Smith, Barker et al. 2012). Apart from the morphological features, other medical problems associated with FXS include seizures and sleep disorders (Hagerman and Hagerman 2002, Kidd, Lachiewicz et al. 2014, Lozano, Azarang et al. 2016, Ciaccio, Fontana et al. 2017).

Hamdan *et al.* was the first to identify rare *de novo* mutations in *SYNGAP1 gene* in patients with NSID and proposed that they were likely to be pathogenic because of the production of the truncated versions of SYNGAP1 protein (Hamdan, Gauthier et al. 2009). Since the first report of *SYNGAP1*'s association with ID and ASD, many more mutations were found in the *SYNGAP1* gene resulting in moderate to severe form of ID (Krepischi, Rosenberg et al. 2010, Berryer, Hamdan et al. 2013, Writzl and Knegt 2013). Further clinical examinations of the patients affected with *SYNGAP1* mutation showed a varied level of severity. Similar to the FXS, patients with *SYNGAP1* mutation manifested impairment in language development as well as a severe psychomotor delay (Krepischi, Rosenberg et al. 2010, Prchalova, Havlovicova et al. 2017). Authors also reported severe mental

retardation, absent speech, and seizures in the patients with *SYNGAP1* mutation (Krepischi, Rosenberg et al. 2010, Prchalova, Havlovicova et al. 2017). In conclusion, patients suffering from FXS or *SYNGAP1* heterozygous mutation show significant overlap in the cognitive and behavioural symptoms associated with the ID.

Studies using *Fmr1* KO mice showed that they have increased exploratory activity in the open field arena and preferred to stay towards the periphery (Qin, Entezam et al. 2011, Spencer, Alekseyenko et al. 2011). These results suggest that the *Fmr1* KO mice are hyperactive compared to their littermate controls. Further, these *Fmr1* KO mice showed impairment in learning (Kooy, D'Hooge et al. 1996). Another hallmark feature of the *Fmr1* KO mice was shown to be the impairment in the sensory stimuli (Bernardet and Crusio 2006). In addition to that, *Fmr1* KO mice manifested increases susceptibility to both partial and generalised seizures (Qiu, Lu et al. 2009, Osterweil, Krueger et al. 2010, Osterweil, Chuang et al. 2013). Along with these symptoms, *Fmr1* KO mice also exhibited more significant pre-pulse inhibition suggesting an alteration in the sensory-motor processing (Frankland, Bontempi et al. 2004). Further, the social deficit was observed in the mouse model of FXS (Spencer, Alekseyenko et al. 2011).

Syngap1$^{+/-}$ mice show a range of behavioural deficits associated with the ID. *Syngap1*$^{+/-}$ mice manifested increased locomotor activity at both juvenile as well as in adult age (Guo, Hamilton et al. 2009, Muhia, Yee et al. 2010, Jeyabalan and Clement 2016). Similar to the *Fmr1* KO mice, *Syngap1*$^{+/-}$ mice showed hyperactivity in the open field arena (Guo, Hamilton et al. 2009, Jeyabalan and Clement 2016). *Syngap1*$^{+/-}$ mice also manifested impaired learning in Morris water maze and deficits in spatial memory which is analogous to *Fmr1* KO (Komiyama, Watabe et al. 2002, Muhia, Yee et al. 2010). *Syngap1*$^{+/-}$ mice showed an increased response to sensory stimuli. These mice also manifested reduced threshold to seizure, a feature also observed in *Fmr1* KO mice (Qiu, Lu et al. 2009, Osterweil, Krueger et al. 2010, Clement, Aceti et al. 2012). Further, Guo et al. also showed that *Syngap1*$^{+/-}$ mice had deficits in social interaction, also observed in *Fmr1* KO mice (Guo, Hamilton et al. 2009, Spencer, Alekseyenko et al. 2011).

One of the hallmark features of the patients with FXS is the excessive presence (increased density) of long, thin, immature filopodia shaped dendritic spines (Hinton, Brown et al. 1991, Fiala, Feinberg et al. 1998). These observations indicate that there might be a failure in synapse maturation in the patients suffering from Fragile X Syndrome. It was proposed that excessive protein synthesis due to the absence of FMRP may lead to abnormal spine morphology and maturation in the brain. A study

by Levanga *et al*. showed hippocampal CA1 neurons from *Fmr1* KO mice contain an increased proportion of filopodia-like immature spines, compared to WT (Levenga, Hayashi et al. 2011).

A study by Vazquez *et al*. showed that *Syngap1* KO pyramidal neurones had a subtle increase in the number of protrusions at DIV 10. However, this increase in dendritic protrusions was absent in more mature cultures at DIV 21 (Vazquez, Chen et al. 2004). The authors also demonstrated the WT neurons manifested long, thinner protrusions during the early developmental time window. In contrast, *Syngap1* KO pyramidal neurones contained dendritic protrusions (spines) having more wider head width (Vazquez, Chen et al. 2004). These observations suggested an alteration in the maturation rate of dendritic spines in the absence of SYNGAP1. Later, Carlisle et al. showed that adult hippocampal neurons of *Syngap1*$^{+/-}$ mice had an increased proportion of mature mushroom-shaped dendritic spines compared to their littermate controls (Carlisle, Manzerra et al. 2008).

Further, Clement *et al.* studied dendritic spine size, density and dynamics in the dentate gyrus. At postnatal day 7-9, *Syngap1* heterozygous neurons had similar spine head sizes, however by postnatal day 14-16, spine head diameter was significantly larger compared to WT, and this phenotype persisted into adulthood (Clement, Aceti et al. 2012). Therefore, the morphological classification was shifted towards more mushroom-like spines with fewer stubby-shaped spines by postnatal day 14-16 (Clement, Aceti et al. 2012). Whereas, overall spine density was unchanged in *Syngap1* heterozygous condition compared to WT mice. Further analysis of dendritic branches revealed that dendritic arborisations were unaltered in *Syngap1*$^{+/-}$ mice (Clement, Aceti et al. 2012).

Investigations into the cellular mechanisms underlying learning and memory deficits in *Fmr1* KO mice have shown that certain forms of synaptic plasticity were dysfunctional. Group I mGluR-mediated Long-term depression (LTD) was enhanced in the hippocampus of *Fmr1* KO mice (Huber, Gallagher et al. 2002, Hou, Antion et al. 2006). Whereas, investigations into long-term plasticity (LTP) have revealed that certain forms of NMDA receptor-dependent forms of LTP (NMDAR-dependent LTP) were impaired in *Fmr1* KO mice compared to the WT littermate controls (Hu, Qin et al. 2008, Shang, Wang et al. 2009). However, NMDA receptor-dependent forms of LTD (NMDAR-dependent LTD) was unaffected in the *Fmr1* KO mice (Huber, Gallagher et al. 2002). Thus, the absence of FMRP affects synaptic plasticity; in-turn affects learning and memory.

Electrophysiological recordings from hippocampal CA1 neurons have shown that LTP, was impaired in *Syngap1*$^{+/-}$ mice (Komiyama, Watabe et al. 2002, Kim, Lee et al. 2003). Application of high-frequency stimulation (HFS) or theta-burst stimulation to the Schaffer collateral pathway in the hippocampus of *Syngap1*$^{+/-}$ mice showed significantly less potentiation relative to WT slices

(Komiyama, Watabe et al. 2002). These results indicate impairment in the NMDAR-mediated synaptic plasticity in *Syngap1*$^{+/-}$ mice. Therefore, impairment in synaptic plasticity led to an alteration in the learning and memory observed in both *Fmr1* KO and *Syngap1*$^{+/-}$ mice.

Fmr1 KO mice also manifested increased AMPA/NMDA ratio similar to the *Syngap1*$^{+/-}$ mice (Clement, Aceti et al. 2012, Yau, Bettio et al. 2018). Increased AMPA/NMDA ratio observed in *Fmr1* KO mice could be because of decreased NMDAR-mediated activity (Yau, Bettio et al. 2018). However, in *Syngap1*$^{+/-}$ mice, an increase in the AMPA/NMDA ratio was also observed (Clement, Aceti et al. 2012). Further, it was shown that SYNGAP1 regulates the AMPAR trafficking to the postsynaptic membrane via ERK-mediated signalling pathway (Rumbaugh, Adams et al. 2006). Hence, in *Syngap1*$^{+/-}$ mice, increased AMPA/NMDA could be a reason for increased AMPAR trafficking and insertion to the postsynaptic membrane.

Alteration in the critical period of developmental plasticity is one of the essential features associated with many of the ID-related mutations. A study by Harlow *et al.* have shown that *Fmr1* KO mice manifested delayed maturation of the thalamocortical synapses in the primary somatosensory cortex compared to their WT littermates (Harlow, Till et al. 2010). However, in the same region, early maturation of the thalamocortical synapses was observed in *Syngap1*$^{+/-}$ mice leading to an alteration in the critical period of development (Clement, Ozkan et al. 2013). In conclusion, *Fmr1* KO showed a delay in synaptic maturation, and *Syngap1*$^{+/-}$ mice manifested early maturation. These results can be correlated with the developmental spine maturation, discussed in this section.

Overall, a mutation in *Fmr1* and *Syngap1*, both lead to deficits in synaptic function, cognition, and behaviour. In both cases, synaptic plasticity was shown to be altered. Patients affecting with either of these genes mutation have shown a significant level of behavioural overlaps. Even the mouse models manifested commonality in behavioural phenotypes in *Fmr1* KO and *Syngap1*$^{+/-}$ mice. However, there was a different effect observed with respect to spine maturity and synaptic maturation. In *Fmr1* KO mice, synapse maturity is delayed leading to the alteration in the critical period of plasticity. In contrast, an early maturation of dendritic spines and thalamocortical synapses was observed in *Syngap1*$^{+/-}$ mice leading to change in critical period of plasticity.

Earlier studies had shown when the pathophysiological effects of mutations in two different genes were opposing, then crossing the respective mutant might ameliorate the pathophysiology observed in any one of the mutations (Dolen and Bear 2008, Auerbach, Osterweil et al. 2011). These studies mentioned above led to the attractive hypothesis that a genetic cross between two mutations can indeed bring back the WT phenotype in the mouse models.

The mGluR theory of Fragile X Syndrome states that loss of FMRP led to exaggerated Group I mGluR-mediated signalling. Thus, it could be a plausible hypothesis to reduce the activity of Group I mGluR in Fragile X condition. Dolen *et al.* created a double heterozygous mouse model by genetic crossing *Fmr1*$^{-/Y}$ and *Grm5*$^{+/-}$ (Dolen and Bear 2008). Further, authors different aspects of the pathophysiology observed in the *Fmr1*$^{-/Y}$ /*Grm5*$^{+/-}$ (double heterozygous) mice (Dolen and Bear 2008). They categorised the phenotypes as either cognitive or syndromic features. Cognitive features include ocular dominance plasticity, dendritic spine density, and inhibitory avoidance extinction. Syndromic features include audiogenic seizures, body weight, and macroorchidism. Their results showed that the double heterozygous mice had ameliorated phenotypes, which are closer to the littermate WT controls (Dolen and Bear 2008). This study on double heterozygous mice opened up the possibility of rescuing disease phenotypes by a genetic cross.

In the later years, Auerbach *et al.* created another genetic cross between *Fmr1*$^{-/Y}$ and *Tsc2*$^{+/-}$ mice (Auerbach, Osterweil et al. 2011). The authors showed that *Tsc2*$^{+/-}$ mice fall in the opposite spectra of the pathophysiology observed in the *Fmr1*$^{-/Y}$ mice (Auerbach, Osterweil et al. 2011). In contrast with *Fmr1*$^{-/Y}$ mice, *Tsc2*$^{+/-}$ mice manifested reduced protein synthesis, and reduced Group I mGluR-mediated LTP. Surprisingly, the investigation on the double heterozygous mice showed that the Group I mGluR-mediated LTD was similar to the WT level, and it was significantly higher than the level observed in *Tsc2*$^{+/-}$ and considerably lower than the level found in *Fmr1*$^{-/Y}$ (Auerbach, Osterweil et al. 2011). Further analysis showed that the double heterozygous mutant mice performed better in context discrimination task compared to the mice carrying a single mutation of *Fmr1*$^{-/Y}$ and *Tsc2*$^{+/-}$ (Auerbach, Osterweil et al. 2011). Therefore, this cognitive improvement in the double heterozygous mice further strengthens the possibility of using the genetic cross as a potential tool to study ID/ASD to derive future treatment.

In conclusion, it is tempting to speculate that there might be a convergence between the signalling pathways associated with FMRP and SYNGAP1, respectively. Also, understanding the association and convergence between these two proteins might open new frontiers to target both FXS and *SYNGAP1*$^{+/-}$-related ID.

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Aims and Objectives

- **Elucidating FMRP expression profile during development in *Syngap1*$^{+/-}$ mice**

  Approach: Hippocampal lysates were prepared from *Syngap1*$^{+/-}$ and WT littermate mice of different age groups, starting from postnatal day 7-9 to adult (2 to 6 months). Further, quantitative immunoblotting was done to check the FMRP level.

- **Investigating the role of FMRP in *Syngap1* mRNA translation in *Syngap1*$^{+/-}$ mice**

  Approaches:

  1. FMRP was immunoprecipitated from the hippocampal lysates. Further, the associated mRNA was extracted, and qPCR for *Syngap1* was done.
  2. SYNGAP1 was transiently expressed in the HeLa cells. *FMR1* was knocked down using siRNAs. Further, the level of SYNGAP1 was checked post *FMR1* knockdown.
  3. Polyribosome profiling assay was done to investigate the translation of *Syngap1* and *Fmr1* mRNAs during development.

- **Investigating NMDAR-mediated protein synthesis in *Syngap1*$^{+/-}$ mice**

  Approach: Synaptoneurosomes were prepared from the hippocampus of both *Syngap1*$^{+/-}$ and WT littermate mice during development. The synaptoneurosomes were stimulated with NMDA for 1’ and 2’. Post-stimulation immunoblotting was done to check the phosphorylation of eEF2.

CHAPTER - 2 Materials and Methods

2.1. Animals

C57/BL6 Wild-type (WT) and *Syngap1*$^{+/-}$ mice were obtained from The Jacksons Laboratory (https://www.jax.org/strain/008890) (Kim, Lee et al. 2003), and bred and maintained in the Animal Facility, JNCASR, under 12-hour dark and light cycle. The *Syngap1*$^{+/-}$ mouse model was generated by targeting exon 7, and exon 8 of the *Syngap1* gene. SacII and EcoRI restriction endonuclease digested fragment of the *Syngap1* gene was replaced by a Neomycin resistant (neo^{R}) cassette. After the ES clones were obtained, they were injected into the blastocysts of C57/BL6 to get the transgenic mice. For further details about the transgenic mouse generation, please refer to Kim *et al.* 2003 (Kim, Lee et al. 2003). Study for this thesis was carried out according to the recommendations of the Institutional Animal Ethics Committee (IAEC). The protocol was approved by the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

The animals were tagged in one of the ears using Monel ear tags procured from Kent Scientific, USA (INS10005-1Z). A small piece of tail (approximately 2 mm) was clipped at the time of tagging. For Genomic DNA isolation, tails were chopped into small pieces and mixed with 180 µl of 50 mM NaOH (GRM467, HIMEDIA) solution, vortexed and kept in the dry bath at 95 ºC for 10 minutes. NaOH is a strong alkali which helps in the alkaline lysis method of the tail samples leading to rupture of the cell membrane, and DNA gets exposed. After that, 20 µl of 1 M Tris-HCl (Tris: 15965, Thermo Fisher Scientific; HCl: HC301585, Merck) having a pH of 8.0 was added to each tube, and the samples were centrifuged at 12000 RPM for 10 minutes. Addition of Tris is important as it dissolves DNA and maintains it in the soluble phase. Pellets were discarded, and supernatants were aliquoted into tubes and used as DNA template for genotyping. The combination of speed and time for centrifugation mentioned above is optimum for efficient DNA extraction. Further, Polymerase Chain Reactions (PCR) in Thermocycler (Mastercycler Nexus GX2, Eppendorf) were done using these DNA samples. To genotype WT or *Syngap1*$^{+/-}$, three separate primers were used. These primers were obtained from Sigma Aldrich.

Sequence1:ACCTCAAATCCACACTCCTCTCCAG;

Sequence2:AGGGAACATAAGTCTTGGCTCTGTC;

Sequence3:ATGCTCCAGACTGCCTTGGGAAAAG.

The PCR protocol is shown in Table 2-1.

Step	Temperature (°C)	Duration	Cycles
Initial denaturation	95	3 minutes	1
Denaturation	95	30 seconds	35
Annealing	61.6	45 seconds	
Extension	72	35 seconds	
Final extension	72	2 minutes	1
Hold	4	∞	1

Table 2-1: PCR protocol for *Syngap1* genotyping

2.2. Preparation of hippocampal slices

Acute brain slices were prepared from PND>90 male and female WT and *Syngap1*$^{+/-}$ mice. Mice were brought from the animal house and sacrificed by cervical dislocation, and the brain was dissected out. The brain was kept in ice-cold sucrose based artificial cerebrospinal fluid (aCSF; cutting solution) comprising of: 189 mM Sucrose (S9378, Sigma Aldrich), 10 mM D-Glucose (G8270, Sigma Aldrich), 26 mM $NaHCO_3$ (5761, Sigma Aldrich), 3 mM KCl (P5405, Sigma Aldrich), 10 mM $MgSO_4.7H_2O$ (M2773, Sigma Aldrich), 1.25 mM NaH_2PO_4 (8282, Sigma Aldrich) and 0.1 mM $CaCl_2$ (21115, Sigma Aldrich). The composition of sucrose aCSF is also summarised in Table 2-2. The brain was taken out of cutting solution and glued to the brain holder of the vibratome (Leica #VT1200), and 350 µm thick horizontal slices were prepared. Cortex was dissected out from each slice to obtain only the hippocampus. All the slices were kept in slice chamber containing aCSF comprising: 124 mM NaCl (6191, Sigma Aldrich), 3 mM KCl (P5405, Sigma Aldrich), 1 mM $MgSO_4.7H_2O$ (M2773, Sigma Aldrich), 1.25 mM NaH_2PO_4 (8282, Sigma Aldrich), 10 mM D-Glucose (G8270, Sigma Aldrich), 24 mM $NaHCO_3$ (5761, Sigma Aldrich), and 2 mM $CaCl_2$ (21115, Sigma Aldrich) in water bath (2842, Thermo Fisher Scientific) at 37 ºC for 45 minutes. Following recovery, slices were kept at room temperature (RT) in 25 °C till the experiment completed. Post dissection, every step was carried out in the presence of constant

bubbling with carbogen (5 % CO_2 and 95 % O_2; Chemix, India). All measurements were performed by an experimenter blind to the experimental conditions.

Component	Final concentration	Manufacturer	Catalogue number
Sucrose	189 mM	Sigma Aldrich	S9378
D-Glucose	10 mM	Sigma Aldrich	G8270
$NaHCO_3$	26 mM	Sigma Aldrich	5761
KCl	3 mM	Sigma Aldrich	P5405
$MgSO_4.7H_20$	10 mM	Sigma Aldrich	M2773
NaH_2P0_4	1.25 mM	Sigma Aldrich	8282
$CaCl_2$	0.1 mM	Sigma Aldrich	21115

Table 2-2: Composition of Sucrose-based aCSF solution

2.3. Extracellular field recordings

Field excitatory post-synaptic potential (fEPSP) were elicited from pyramidal cells of CA1 regions of stratum radiatum by placing concentric bipolar stimulating electrode (CBARC75, FHC, USA) connected to a constant current isolator stimulator unit (Digitimer, UK) at Schaffer-Collateral commissural pathway, and recorded from stratum radiatum of CA1 area of the hippocampus, with 3-5 MΩ resistance glass pipette (ID: 0.69 mm, OD: 1.2 mm, Harvard Apparatus) filled with aCSF. Signals were amplified using Axon Multiclamp 700B amplifier (Molecular Devices), digitised using an Axon Digidata 1440A (Molecular Devices), and stored on a personal computer. Online recordings and analysis were performed using pClamp10.7 software (Molecular Devices). Stimulation frequency was set at 0.05 Hz. mGluR-LTD was induced by 5 minutes bath application of the Group I mGluR agonist (*S*)-3,5-Dihydroxyphenylglycine (DHPG; Cat# 0805, Tocris, UK).

2.4. Lysate preparation

Brain lysates were prepared from Post-Natal Day (PND) 4-5, 7-9,14-16, 21-23, and adults (2-5 months). WT and *Syngap1*$^{+/-}$ mice were sacrificed by cervical dislocation, brain was dissected out, and hippocampus was separated in cold Phosphate Buffered Saline (PBS, Table 2-3) of pH 7.4 containing NaCl (137 mM, S6191, Sigma Aldrich), KCl (2.7 mM, P5405, Sigma Aldrich), Na_2HPO_4 (10 mM, 10028-24-7, Thermo Fisher Scientific), KH_2PO_4 (1.8 mM, GRM1188, HIMEDIA). The tissue was homogenised using RIPA buffer (Table 2-4) containing NaCl (150 mM, S6191, Sigma Aldrich,), Tris-HCl (50 mM, Tris: 15965,

Thermo Fisher Scientific; HCl: HC301585, Merck) pH 7.4, EDTA (5 mM, 6381-92-6, Thermo Fisher Scientific), Na-Deoxycholate (0.25 %, RM-131, HIMEDIA), Triton X (1 %, RM 845, HIMEDIA). RIPA buffer will help in the lysis process of the tissues and the cells. Thus, the intracellular components will be accessible. Additionally, 1 X Protease Inhibitor (P5726, Sigma Aldrich,), and 1 X Phosphatase Inhibitor Cocktail 2 and 3 (P0044, Sigma Aldrich) was added to the buffer to increase the stability of the lysate. Then, the homogenates were centrifuged at 16000 RCF for 30 minutes at 4 ºC. This step leads to pellet down of all cellular or extracellular debris, and the soluble proteins retained at the supernatant. The supernatants were collected, and the protein was estimated using Bradford (5000006, Bio-Rad) assay. The samples were aliquoted and stored at -80 ºC. Each aliquot was used for 3-4 times.

Component	**Final concentration**	**Manufacturer**	**Catalogue number**
NaCl	137 mM	Sigma Aldrich	S6191
KCl	2.7 mM	Sigma Aldrich	P5405
Na_2HPO_4	10 mM	Thermo Fisher Scientific	10028-24-7
KH_2PO_4	1.8 mM	HIMEDIA	GRM1188

Table 2-3: Composition of PBS

Component	**Final concentration**	**Manufacturer**	**Catalogue number**
NaCl	150 mM	Sigma Aldrich	S6191
Tris-HCl (pH 7.4)	50 mM	Thermo Fisher Scientific	15965
EDTA	5 mM	Thermo Fisher Scientific	6381-92-6
Na-Deoxycholate	0.25 %	HIMEDIA	RM-131
Triton X	1 %	HIMEDIA	RM-845
Protease Inhibit cocktail	1 X	Sigma Aldrich	P5726
Phosphatase Inhibit cocktail	1 X	Sigma Aldrich	P0044

Table 2-4: Composition of RIPA buffer

2.5. SDS-PAGE and Western blotting

The protein samples (50 µg in each lane) were electrophoresed onto SDS (161-0302, Bio-Rad) Polyacrylamide (161-0156, Bio-Rad) 5 % stacking gel for 30 minutes and 8 % resolving gel (for FMRP, SYNGAP1, MOV10, and PSD95) for around 2 hours or 10 % resolving gel (Phospho-eEF2, Total-eEF2, Phospho-ERK1/2, Total-ERK1/2, and RPLP0) for approximately 3 hours. The proteins were transferred for 3 hours at 80 V at 4 ºC onto Polyvinylidene Fluoride (PVDF) membrane (1620177, Bio-Rad) and blocked using 5 % skim milk (GRM 1254, HIMEDIA) or 5 % Bovine Serum Albumin (BSA, GRM105, HIMEDIA) in PBS for 1-hour at Room Temperature (RT) *i.e.* 25 ºC. 5 % BSA in PBS was used for blocking of all phospho-proteins. For MOV10, blocking was done for ~3-4 hours at RT. The blots were washed with 1 % PBST (PBS+ Tween 20; GRM156 HIMEDIA) three times for 10 minutes, and incubated with Primary Antibodies for FMRP (FMR1 C-terminal, F4055, Sigma Aldrich, 1:1000 dilution, raised in rabbit), β-ACTIN (PA116889, Thermo Fisher Scientific, 1:15000 dilution, raised in rabbit), SYNGAP1 (PA1-046, Thermo Fisher Scientific, 1:1000 dilution, raised in rabbit), and MOV10 (ab80613, Abcam, 1:1000 dilution, raised in rabbit), PSD95 (MA1-046, Thermo Fisher Scientific, 1:1000 dilution, raised in mouse), Phospho-eEF2 (Thr56, 2331S ,Cell Signalling Technology, 1:1000 dilution, raised in rabbit), Total-eEF2 (2332S, Cell Signalling Technology, 1:1000 dilution, raised in rabbit), and RPLP0 (ab101279, Abcam, 1:1000 dilution, raised in rabbit) overnight. Details of the antibodies used are summarised in Table 2-5. Overnight transfer at 20 V was done for the detection of Phospho-ERK1/2 (#9101, Cell Signalling Technology, 1:1000, raised in rabbit) and Total-ERK1/2 (#9102, Cell Signalling Technology, 1:750, raised in rabbit). Post-transfer Ponceau staining was done, and Methanol was used as a fixative, and further washed with PBS. Blots were incubated with primary antibody (Phospho-ERK1/2, and Total-ERK1/2) for 4 hours at RT under shaking condition. Phospho-ERK1/2 and Total-ERK1/2 antibodies were reused maximum twice, whereas all other antibody aliquots were reused up to 8 times. After primary incubation, all blots were given PBST wash for three times for 10 minutes each, then incubated with anti-Rabbit (1706515, Bio-Rad) or anti-Mouse (1706516, Bio-Rad) HRP conjugated Secondary antibody (1:10000 dilution). After subsequent washes with PBST, the blots were developed by a chemiluminescent method using ECL western clarity solution (1705060, Bio-Rad). Images were taken in Versa Doc (4000 MP, Bio-Rad), or ImageQuant (LAS 4000 from GE) further merged using ImageLab version 5.2.1 and bands were quantified using ImageJ software. Chemiluminescence involves a chemical reaction where energy is released in the form of light. The reaction happens when the Horse Radish Peroxidase (HRP) conjugated with the secondary antibody, oxidises luminol in the presence of

Hydrogen Peroxide (H_2O_2). The emitted light then detected by the charge-coupled device (CCD) camera present in the Versa Doc Molecular Imager. CCD is an integrated circuit which is photosensitive (http://www.specinst.com/What_Is_A_CCD.html). When photon falls on that surface, it is converted into charge. That charge is read by electronics and turned into a digital signal (https://electronics.howstuffworks.com/cameras-photography/digital/digital-camera2.htm). Thus, more protein in the blot will lead to binding of more primary and subsequently, the secondary antibody conjugated with HRP. That will lead to an increased chemiluminescence reaction and more production of photons. These photons will be detected by the CCD camera and displayed as intense bands in the western blot. For further details, please refer to http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5809.pdf.

Antibody	**Dilution**	**Manufacturer**	**Catalogue number**
FMR1 C-terminal	1:1000	Sigma Aldrich	F4055
SYNGAP1	1:1000	Thermo Fisher Scientific	PA1-046
PSD-95	1:1000	Thermo Fisher Scientific	MA1-046
MOV10	1:1000	Abcam	AB80613
RPLP0	1:1000	Abcam	AB101279
Phospho-eEF2 (Thr 56)	1:1000	Cell Signalling Technology	2331S
Total-eEF2	1:1000	Cell Signalling Technology	2332S
Phospho-ERK1/2	1:1000	Cell Signalling Technology	9101
Total-ERK1/2	1:750	Cell Signalling Technology	9102
Anti-rabbit HRP secondary	1:10000	Bio-Rad	1706515
Anti-mouse HRP secondary	1:10000	Bio-Rad	1706516

Table 2-5: Antibodies used for immunoblotting

2.6. Immunoprecipitation

Hippocampus was dissected out from PND14-16 and 21-23 WT (littermates) and *Syngap1*$^{+/-}$ (HET) as described earlier. Tissue was homogenised using Lysis buffer (Table 2-6) containing Tris-HCl (50 mM, Tris: 15965, Thermo Fisher Scientific; HCl: HC301585, Merck), NaCl (150

mM, S6191, Sigma Aldrich), $MgCl_2$ (5 mM, M8266, Sigma Aldrich), Dithiothreitol (DTT, 1 mM, 3483-12-3, Sigma Aldrich), NP40 (1 %, 127087-87-0, Sigma Aldrich), RNaseOUT (1 U/µl; 10777-019, Invitrogen), and 1 X Protease Inhibitor cocktail (P5726, Sigma Aldrich). All reagents were dissolved in Diethylpyrocarbonate (DEPC, D5758, Sigma Aldrich) treated autoclaved water. Immunoprecipitation was done using anti-FMR1 c-terminal antibody (F4055, Sigma Aldrich), Rabbit IgG (40159050MG, Millipore) and protein G Dyna-beads (10003D, Thermo Fisher Scientific). 30 µl of Dyna-beads were equilibrated with lysis buffer, and further 200 µl of lysis buffer containing 5 µg of antibody was added to Dyna-beads and incubated at room temperature for one hour with rotation. Afterwards, the antibody solution was removed from the beads by placing the tube in the magnetic stand. Tissue lysate was added to the antibody bound beads and was incubated for 1-hour at room temperature. The lysate was given five washes with lysis buffer. After the last wash, IP buffer was removed completely, and the sample was eluted in either 1 X Laemmli buffer (for protein detection) or Trizol (for RNA isolation). For the mRNA enrichment, mRNA copy number in the pellet was divided by mRNA copy number in the supernatant.

Component	**Final Concentration**	**Manufacturer**	**Catalogue number**
NaCl	150 mM	Sigma Aldrich	S6191
Tris-HCl (pH 7.4)	50 mM	Thermo Fisher Scientific	15965
$MgCl_2$	5 mM	Sigma Aldrich	M8266
Dithiothreitol	1 mM	Sigma Aldrich	3483-12-3
RNaseOUT	1 U/µl	Thermo Fisher Scientific	10777-019
Protease Inhibit cocktail	1 X	Sigma Aldrich	P5726

Table 2-6: Composition of IP-Lysis buffer

2.7. RNA extraction and qPCR

Total RNA was extracted from the polysome fractions by Trizol (15596026, Thermo Fisher Scientific) method (For each sample three times the volume of Trizol was added) and the mRNAs were converted to cDNA using iScript cDNA synthesis kit (1708891, Bio-Rad). The reaction system and the reaction protocol is described in Table 2-7 and 2-8. qPCR was performed for *Syngap1*, *Fmr1*, and *β-actin* (Primers were designed, and obtained from Sigma Aldrich) using

CFX384 Real-Time System from Bio-Rad. SYBR green was purchased from Bio-Rad (1725122). C_t values obtained from the reactions were converted to the copy number of the mRNA (Muddashetty, Kelic et al. 2007, Muddashetty, Nalavadi et al. 2011), and then the percentage of these copy numbers in each fraction was plotted. mRNA copy number was derived from the C_t values using the standard curve obtained. The equation for the standard curve is y=-1.44x+31.699; Here, y=average C_t value and EXP(x) is the mRNA copy number. List of primers used is mentioned in Table 2-9.

Components	**Volume per reaction in µl**
5 X iScript reaction mix	4
iScript Reverse Transcriptase	1
Nuclease-free water	4
RNA template	11
Total volume	20

Table 2-7: Reaction system for cDNA synthesis

Priming	5 minutes at 25 °C
Reverse Transcription	20 minutes at 46 °C
RT inactivation	1 minute at 95 °C
Optional Step	5 minutes at 4 °C

Table 2-8: Reaction protocol for cDNA synthesis

Transcript	Forward sequence (5'→3')	Reverse Sequence (5'→3')
Psd-95	ATGGCAGGTTGCAGATTGGA	GGTTGTGATGTCTGGGGGAG
β-actin	GGCTCCTAGCACCATGAAGAT	AAACGCAGCTCAGTAACAGTC
Syngap1	CAACCGGAAGCTGGAAGAG	CATCAGCCTGCCAATGATGC
Fmr1	GCAGTTGGTGCCTTCTCTGT	GCTGCCTTGAACTCTCCAGT

Table 2-9: List of qPCR primers

2.8. Cell culture and transfection

HeLa cells were maintained in DMEM containing 10 % FBS at 37 °C in a 5 % CO_2 environment (3110, Thermo Fisher Scientific) passaged using 0.05 % trypsin-EDTA solution. Transfections were performed using lipofectamine 2000 (11668027, Thermo Fisher Scientific) as per the manufacturer's protocol. Lipofectamine is a specially designed cationic lipid consisting of one positively charged head group along with one or two fatty acid chains. These head groups interact with the phosphate backbone of the nucleic acid (DNA, siRNA, etc.) and in aqueous environment form liposome-like structure with a positive surface charge. Further, these positive surface charge containing liposomes interact with the cell membrane and fuse to deliver the nucleic acid. For further details, please refer to https://www.thermofisher.com/in/en/home/references/gibco-cell-culture-basics/transfection-basics/gene-delivery-technologies/cationic-lipid-mediated-delivery/how-cationic-lipid-mediated-transfection-works.html. For the knockdown experiments, commercially available siRNAs were used. *Fmr1* siRNA (4392421 S-5317, ThermoFisher Scientific), and Scrambled siRNA (AM 4635, ThermoFisher Scientific) were used for the experiments.

2.9. Plasmid construct and bacterial transformation

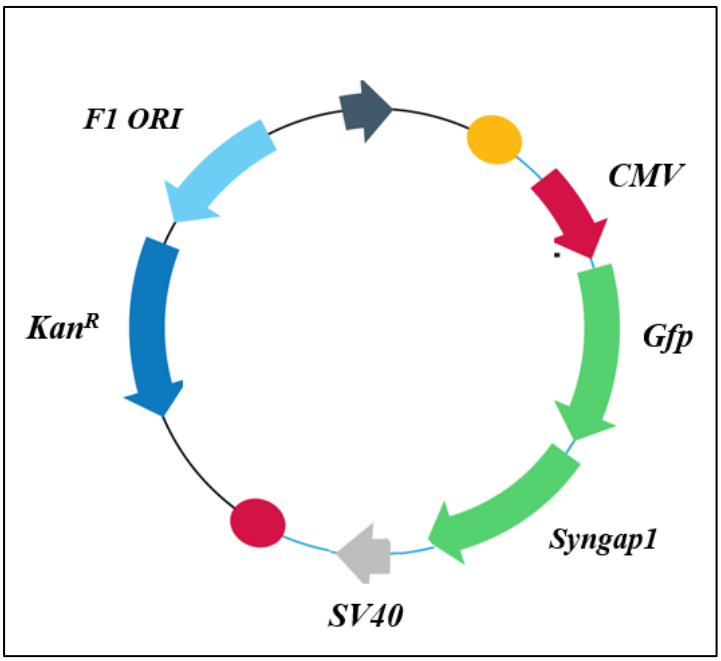

Figure 2-1: *Gfp-Syngap1* plasmid construct

Gfp-Syngap1 fusion protein was cloned in the plasmid under CMV promoter. The vector has a F1 ORI sequence for replication. In addition, the construct contains a Kanamycin resistance gene marker for positive selection.

The *Gfp-Syngap1* plasmid construct was kindly provided by Dr Gavin Rumbaugh (Scripps Research Institute, U.S.A.). The DNA was extracted from the filter paper using 1X TE buffer at room temperature for 1 hour. Once the DNA was extracted it was transformed into *E.coli* DH5α strains obtained from Molecular Mycology Lab, JNCASR. The vector plasmid construct carries a Kanamycin resistance marker. For the selection of the positive colonies, the bacteria were grown in LB agar plate containing Kanamycin. Once the positive colony was obtained, it was further scaled up for maxi-prep DNA extraction. Post-overnight incubation at 37 °C under shaking condition, when OD reached 0.6, maxi-prep plasmid DNA extraction procedure was done.

Further, the plasmid DNA extracted from the transformed strain was used for transfection into the HeLa cells. The plasmid construct map was obtained from Dr Deepak Nair's Lab at the CNS, IISc., India. The schematic plasmid map is depicted in Figure 2-1.

2.10. Polysome profiling assay

Hippocampus was dissected out from PND21-23 and PND14-16 *Syngap1*$^{+/-}$ (HET) and WT (littermates) as described earlier. Tissue was homogenised using Lysis buffer (Table 2-10) containing Tris-HCl (200 mM, Tris: 15965, Thermo Fisher Scientific; HCl: HC301585, Merck), KCl (100 mM, P5405, Sigma Aldrich), $MgCl_2$ (5 mM, M8266, Sigma Aldrich), Dithiothreitol (DTT, 1 mM, 3483-12-3, Sigma Aldrich), NP40 (1 %, 127087-87-0, Sigma Aldrich), and 1 X Protease Inhibitor cocktail (P5726, Sigma Aldrich). All reagents were dissolved in Diethylpyrocarbonate (DEPC, D5758, Sigma Aldrich) treated autoclaved water. Samples were aliquoted into two equal parts and treated with either of the protein translation inhibitors:

Cycloheximide (10 µg/ml, C7698, Sigma Aldrich) or Puromycin (1 mM, P9620, Sigma Aldrich). The lysates were then kept at 37 ºC for 30 minutes and centrifuged at 4 ºC for 30 minutes at 18213 RCF. The treatment mentioned above disrupts cell membrane and pellet down the cellular/extracellular debris. The supernatant was further loaded carefully on to the sucrose gradient prepared in polysome tubes. Sucrose (84097, Sigma Aldrich) gradient tubes were prepared 1-day before the day of the experiment. 15 % to 45 % gradients were made, and stored at -80 ºC. The supernatant was gently added to each polysome tubes (331372, BECKMAN COULTER), and ultra-centrifuged (Beckman, OptimaXL 100K) at 4 ºC at 39000 RPM for 1 hour and 40 minutes. That density gradient centrifugation step leads to separation of RNAs depending on their relative mass. The schematic is depicted in the Figure 2-2. Thus, high mass containing RNAs (polysomes) come at more upper sucrose density portion, and mRNPs come in the low sucrose density portion. The tubes were then transferred to UV Visible spectrophotometer (Model: Type 11 Optical unit with reference Flowcell/No bracket, Serial No: 213K20162 at National Centre for Biological Sciences, NCBS), and fractions were collected at A_{254} spectra using Fraction collector instrument (from TELEDYNE ISCO at NCBS). The bottom of the tube was pierced using a syringe attached to a pipe containing 60 % sucrose, and the fractions were collected in 1.5 ml tubes. Total of 11 fractions was obtained from each polysome tube, and these fractions were treated with SDS loading dye containing β-Mercaptoethanol (MB041, HIMEDIA) for immunoblot assays, or total RNA was extracted from each fraction by Trizol method for qPCR experiments.

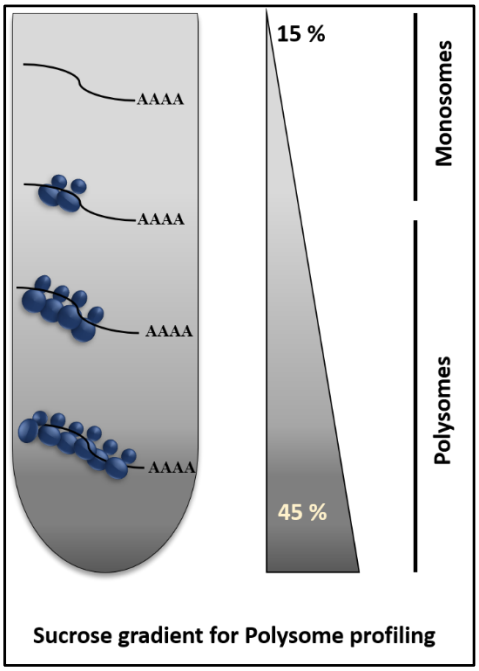

Figure 2-2: Schematic model for Polyribosome profiling

Component	Final Concentration	Manufacturer	Catalogue number
Tris-HCl	200 mM	Thermo Fisher Scientific	15965
KCl	100 mM	Sigma Aldrich	P5405
$MgCl_2$	5 mM	Sigma Aldrich	M8266

Dithiothreitol	1 mM	Sigma Aldrich	3483-12-3
NP40	1 %	Sigma Aldrich	127087-87-0
Protease Inhibitor	1 X	Sigma Aldrich	P5726
Cycloheximide	10 µg/ml	Sigma Aldrich	C7698
Puromycin	1 mM	Sigma Aldrich	P9620

Table 2-10: Composition of polysome lysis buffer

2.11. Synaptoneurosome preparation and NMDA stimulation

Hippocampus was dissected out as described earlier and homogenised in 1000 µl of Synaptoneurosome buffer containing NaCl (116.5 mM, S6191, Sigma Aldrich), KCl (5 mM, P5405, Sigma Aldrich), $MgSO_4$ (1.2 mM, M7506, Sigma Aldrich), $CaCl_2$ (2.5 mM, C5670, Sigma Aldrich), KH_2PO_4 (1.53 mM, GRM1188, HIMEDIA), Glucose (3.83 %, G8270, Sigma Aldrich), 1 X Protease Inhibitor Cocktail (P5726, Sigma Aldrich). Homogenate was filtered through three 100 µm filter (NY1H02500, Merck Millipore), and 11 µm filter once (NY1102500, Merck Millipore). These filtration steps are essential to remove high-density cellular debris. The filtrate obtained was centrifuged at 1500 RCF for 15 minutes at 4 ºC. That low-speed centrifugation pellet down the synaptoneurosomes. Pellet was resuspended in 1 ml Synaptoneurosome buffer (Table 2-11). NMDA receptor stimulation was done by applying NMDA (Final concentration 40 µM, M3262, Sigma Aldrich) for 1, 2, and 5-minutes respectively at 37 ºC in 350 RPM. In one set of experiments, AP-5 was applied. The synaptoneurosomes prepared from the hippocampus of PND21-23 mice were aliquoted into four tubes. Three aliquots were treated with NMDA (40 µM), NMDA+ AP-5 (100 µM, Cat#0105, TOCRIS), and only AP-5 respectively. One tube was left untreated and considered as Basal. Stimulation was done for 1-minute at 37 ºC in 350 RPM. After stimulation, the synaptoneurosomes were centrifuged at 11000 RPM for a short spin (approximately 21 seconds). The short spin pellet down the crude synaptoneurosome. The pellet was resuspended in Lysis buffer followed by centrifugation at 18213 RCF at 4 ºC for 30 minutes for further lysis. The supernatant was taken and denatured in loading dye containing SDS and β-Mercaptoethanol (MB041, HIMEDIA), and immunoblot assays were done.

Component	Final Concentration	Manufacturer	Catalogue number
NaCl	116.5 mM	Sigma Aldrich	S6191
KCl	5 mM	Sigma Aldrich	P5405
$MgSO_4$	1.2 mM	Sigma Aldrich	M7506
$CaCl_2$	2.5 mM	Sigma Aldrich	C5670
KH_2PO_4	1.53 mM	HIMEDIA	GRM1188
Glucose	3.83 %	Sigma Aldrich	G8270
Protease Inhibitor	1 X	Sigma Aldrich	P5726

Table 2-11: Composition of Synaptoneurosome buffer

2.12. Statistics

All graphs were plotted in Graph Pad Prism 7, and Microsoft Excel 2016. Extracellular field recordings were performed and analysed using Clampfit10.7, and Excel 2016. Time course data were plotted by averaging every 2 minutes. Example traces were those recorded for 1-2 min around the time point indicated. Error bars correspond to ±SEM (Standard Error of Mean). Unpaired Student's *t*-test and 2-way ANOVA were performed to test for the difference between groups and different age unless otherwise stated.

References

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Muddashetty, R. S., V. C. Nalavadi, C. Gross, X. Yao, L. Xing, O. Laur, S. T. Warren and G. J. Bassell (2011). "Reversible inhibition of PSD-95 mRNA translation by miR-125a, FMRP phosphorylation, and mGluR signaling." Mol Cell **42**(5): 673-688.

CHAPTER - 3 Results

3.1. Increased Group I mGluR-mediated LTD in *Syngap1*$^{+/-}$

Studies have shown that Group I mGluR-mediated LTD (mGluR-LTD) requires protein synthesis downstream to its activation (Huber 2000). Studies on *Fmr1*$^{-/y}$ mouse models showed that Group I mGluR-mediated LTD was increased due to the dysregulated protein synthesis (Huber, Gallagher et al. 2002). Previous studies also have shown increased protein synthesis in *Syngap1*$^{+/-}$ mice (Wang, Held et al. 2013, Barnes, Wijetunge et al. 2015). Thus, Group I mGluR-mediated LTD was investigated in the hippocampus of *Syngap1*$^{+/-}$ mice. mGluR-LTD was induced in the Schaffer-Collateral pathway of the hippocampus in adult mice by bath applying 50 µm (*S*)-DHPG, a Group I mGluR agonist, for 5 minutes. mGluR-mediated LTD was significantly enhanced in *Syngap1*$^{+/-}$ mice (*Syngap1*$^{+/-}$ referred as HET in Figures; 47±4% LTD) compared to their WT littermate controls (61±3% LTD; $p<0.05$ **Fig 3-1A**). The increased LTD indicates that mGluR-LTD in *Syngap1*$^{+/-}$ is similar to *Fmr1*$^{-/y,}$ as shown earlier by Barnes et al. (Barnes, Wijetunge et al. 2015). In conclusion, SYNGAP1 and FMRP might converge into a common biochemical pathway.

3.2. Reduced FMRP expression level in *Syngap1*$^{+/-}$ during development

As FMRP is known to regulate translation downstream to the Group I mGluR-mediated LTD, the expression level of FMRP was investigated in *Syngap1*$^{+/-}$ mice. Surprisingly, FMRP level was significantly reduced at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice compared to their WT littermate controls (HET=0.775±0.06; WT=1.00±0.07; $p<0.05$ **Fig 3-1B**). Further analysis of FMRP expression profile showed that FMRP level, normalising to β-ACTIN, and GAPDH, decreased with age in the WT **Fig 3-2A**). This reduction in the FMRP level at the later age group suggests that FMRP might play a crucial role during the early developmental time window.

3.3. Increased SYNGAP1 expression level in *Syngap1*$^{+/-}$ during development

Downregulation of FMRP expression at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice may be due to compensatory effect on the expression level of SYNGAP1. Therefore, SYNGAP1 protein level was investigated in the hippocampus of *Syngap1*$^{+/-}$ and WT mice during development. Using immunoblotting assays found that the SYNGAP1 level was increased during postnatal day 21-23 (1.12±0.09) compared to postnatal day 14-16 in *Syngap1*$^{+/-}$ (0.83±0.05; $p<0.05$; **Fig 3-1C**). However, no significant alteration in the SYNGAP1 protein level between postnatal day 14-16 (1.33±0.08) and postnatal day 21-23 (1.82±0.06) in littermate WT controls ($p>0.05$; **Fig 3-1C**) was observed. β-ACTIN was used as an internal control. However, FMRP and SYNGAP1 are known to regulate the

polymerisation-depolymerisation dynamics of ACTIN. Hence, to validate the results, the same experiments were performed using GAPDH as control. Immunoblotting analysis showed that the expression pattern of FMRP and SYNGAP1 were similar when either normalised to GAPDH or β-ACTIN (**Fig 3-2A, 3-2B**).

3.4. *Syngap1* mRNA forms putative G-quadruplex structures

The results so far showed that reduction in FMRP level and upregulation of SYNGAP1 protein level occurred at the same time window (at postnatal day 21-23) in *Syngap1*$^{+/-}$ mice. The contrasting expression level of both these proteins, FMRP and SYNGAP1, raised the question of whether FMRP regulates the expression of *Syngap1*. FMRP is a well-known regulator of synaptic protein synthesis (Osterweil, Krueger et al. 2010). To regulate translation, FMRP interacts with its target (cognate) mRNAs either directly or in a complex. Therefore, it is essential to understand whether FMRP can interact with *Syngap1* mRNA or not. A previous study using High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) has reported that FMRP might interact with *Syngap1* mRNA (Darnell, Van Driesche et al. 2011, Darnell and Klann 2013). However, this putative FMRP-*Syngap1* mRNA interaction was not validated further by the author. An earlier study has shown that G-quadruplexes are one of the secondary structures present in RNA, which could be recognised by FMRP to interact with the G-quadruplex forming RNA (Darnell, Jensen et al. 2001). Further, bioinformatic analysis was performed to find the possibility of G-quadruplex structure formation in *Syngap1* mRNA. Bioinformatic analysis using Quadruplex forming G-Rich Sequences (QGRS) Mapper predicted the presence of multiple putative G-quadruplex forming structures with high G-Score in *Syngap1* mRNA (**Fig 3-2C**). Further analysis showed that such G-quadruplex forming sequence was conserved in rat and human, indicating the possibility for a highly conserved interaction between FMRP and *Syngap1* mRNA (**Fig 3-2C).**

3.5. FMRP interacts with *Syngap1* mRNA in the hippocampus

We wanted to confirm our bioinformatic prediction by biochemical immunoprecipitation assay. We performed immunoprecipitation of FMRP (FMRP-IP) from mouse hippocampal lysates to investigate the enrichment of *Syngap1* mRNA by qPCR. Results showed around 5-fold enrichment of *Syngap1* mRNA in the FMRP-IP pellet over supernatant in comparison to *β-actin* mRNA (5.15±0.43, p=0.0009; **Fig 3-3A and Fig 3-4A)**. *Psd-95* mRNA was used as a positive control, which was shown to be a target mRNA of FMRP (Muddashetty, Nalavadi et al. 2011). We observed ~4.5-fold enrichment of *Psd-95* mRNA compared to *β-actin* mRNA in FMRP-IP pellet over supernatant (4.77±0.09; p=0.0001; **Fig 3-3A and Fig 3-4A**). Overall, these immunoprecipitation results indicate that FMRP interacts with *Syngap1* mRNA.

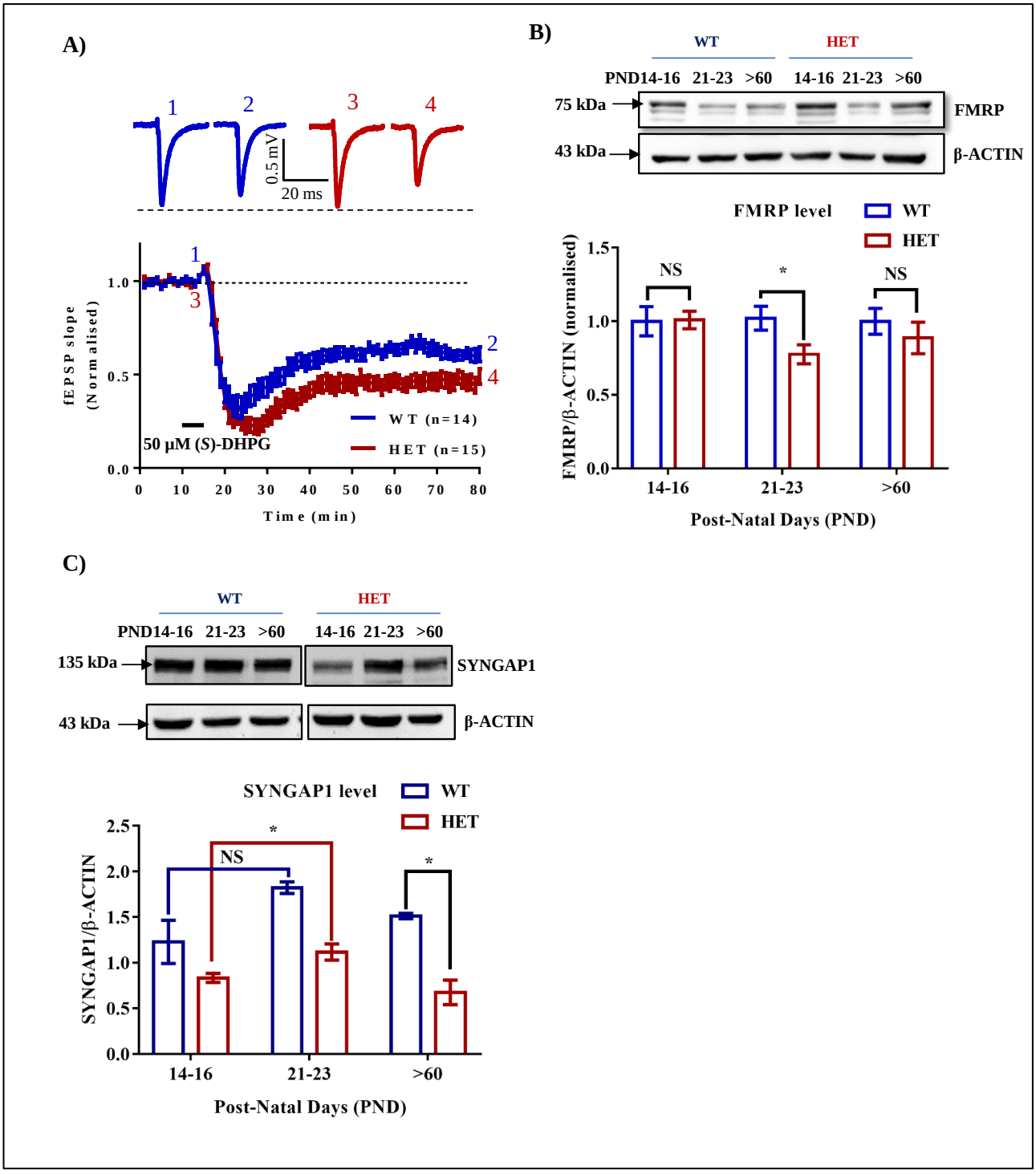
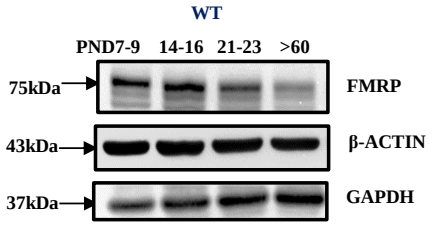
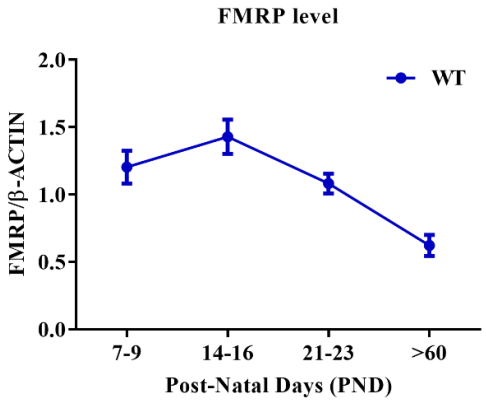

A)
1
2
3
4
0.5 mV
20 ms
fEPSP slope (Normalised)
1.0
0.5
0.0
1
3
2
4
50 µM (S)-DHPG
WT (n=14)
HET (n=15)
0 10 20 30 40 50 60 70 80
Time (min)
B)
WT
HET
PND14-16 21-23 >60 14-16 21-23 >60
75 kDa
FMRP
43 kDa
β-ACTIN
FMRP level
WT
HET
FMRP/β-ACTIN (normalised)
1.5
1.0
0.5
0.0
NS
*
NS
14-16
21-23
>60
Post-Natal Days (PND)
C)
WT
HET
PND14-16 21-23 >60 14-16 21-23 >60
135 kDa
SYNGAP1
43 kDa
β-ACTIN
SYNGAP1 level
WT
HET
SYNGAP1/β-ACTIN
2.5
2.0
1.5
1.0
0.5
0.0
*
NS
*
14-16
21-23
>60
Post-Natal Days (PND)

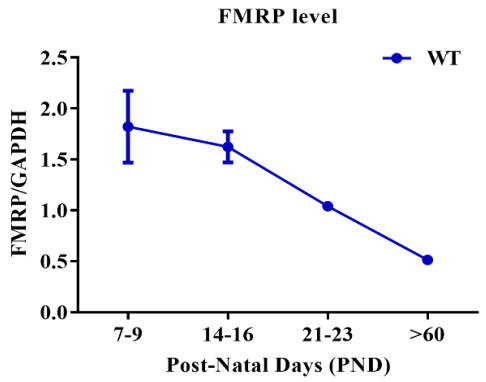
Figure 3-1: Altered expression of FMRP in the hippocampus of *Syngap1*$^{+/-}$ during development.

A. Application of 50 μm (*S*)-DHPG induced enhanced Group I mGluR-mediated LTD in the Schaffer-Collateral pathway of adult (PND90) *Syngap1*$^{+/-}$ (HET) compared to WT (WT) littermates. Sample traces obtained before and after the induction of LTD as indicated by time points (*top*). WT=61±3% LTD, n=14; HET=47±4% LTD, n=15; Unpaired Student's *t*-test; * $p<0.05$. **B**. Representative immunoblot for FMRP level in the hippocampus during development (*top*). Pooled data of FMRP level normalised to β-ACTIN in the hippocampus during development, normalised to the level of WT (*below*). PND14-16 (WT: N=10; HET: N=8), PND21-23 (WT: N=10; HET: N=8), PND>60 (WT: N=8; HET: N=10). *$p<0.05$; Unpaired Student's *t*-test. **C**. Representative Immunoblots for SYNGAP1 during development (*top*). Histogram depicts SYNGAP1 level normalised to β-ACTIN in WT and HET at PND14-16, PND21-23, and PND>60 (WT: N=4; HET: N=3). Bar graph shows increased SYNGAP1 level in HET during PND21-23 (WT: N=4; HET: N=5) when compared to PND14-16 (WT: N=5; HET: N=5) while no significant change was observed in WT. All WT and HET samples for individual age groups were run on the same gel. Two-way ANOVA with Bonferroni's comparison test; Unpaired Student's *t*-test; *$p<0.05$, NS= not significant.

A)







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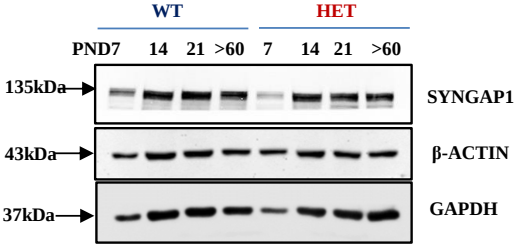

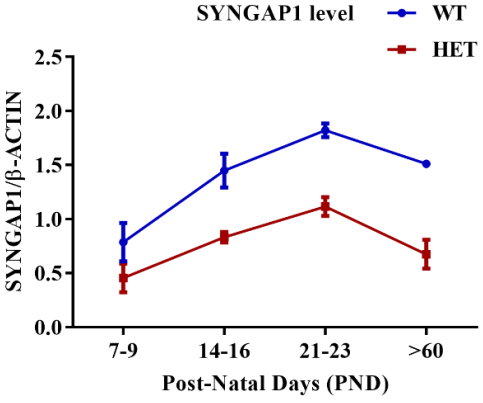

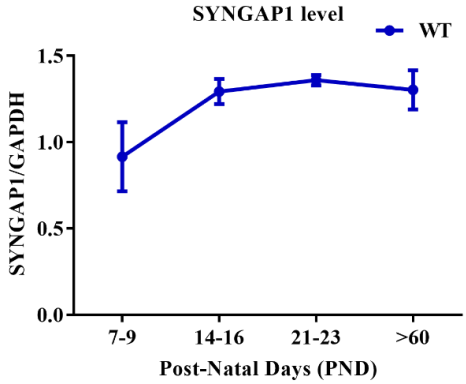

C)

QGRS sequences found (overlaps not included)

Position	Length	QGRS	G-Score
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270	10	GGAGGGGCGG	20
307	29	GGTCGCTCGAGGAGGAAGAGTGTCCCAGG	10
336	23	GGGGAAACAGTACAGCATGGAGG	7
380	29	GGCCCTCGCAAGGCTTCCTGAGCCGGAGG	11
900	27	GGCTTCAGGAGACACCGTCTTTTGGGG	7
1004	28	GGAAGAAGGACAAGGCTGGCTACGTTGG	18
1115	27	GGGGCTCTGGGGGCATGGGCTCGGGGG	42
1150	30	GGGTCAGGGGGTGGCTCAGGGGGCAAAGGG	40
1327	16	GGCAAGGAAGAGGTGG	18
1698	22	GGAGGTGTTTGCATCTTGGCGG	10
1736	26	GGGGCCGAGAGGACATTGCTGACAGG	9
1959	15	GGAGCTGGAGTGGGG	17
2532	27	GGACCTTCAGGGCGACGGGCCTGGAGG	20
2666	22	GGCGCCTCTCCCAGGGGAGTGG	10
2830	26	GGCCATGGAGGGAGCAGTGGTCATGG	18
2902	19	GGGGGAGAACCCCCAGGGG	11
3142	24	GGGCCGGGAGGGGCAGCGGCGGG	37
3166	14	GGCAGCGGTGGGGG	18
3346	30	GGGGGCAGCGGGGGCGGAGGGGGGTGGGGGG	82
3563	18	GGCTGGACAGGGTGAAGG	19
3588	28	GGAGGAGATACATTCGCTGAAGGAGAGG	6
3632	26	GGAAGCTGGAAGAGTATGAGCGGAGG	10
3795	11	GGTGGAGGAGG	21
3924	23	GGCCCCACCTTGGAACGGCCTGG	15

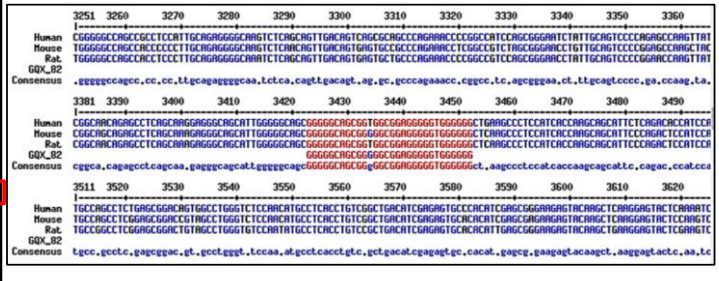


Figure 3-2: FMRP and SYNGAP1 expression during development.

A. Representative Immunoblots for FMRP in WT at PND7-9, PND14-16, PND21-23, and PND>60, normalised to β-ACTIN, and GAPDH (*top*). Line graph shows the expression profile of FMRP normalised to β-ACTIN (*middle*), and normalised to GAPDH (*below*) at PND7-9, PND14-16, PND21-23, and PND>60 (N=4 for all age groups, samples were run on the same gel); FMRP/ β-ACTIN: One-way ANOVA followed by Tukey's multiple comparison test; PND7-9 vs PND>60: **p<0.01; PND14-16 vs PND>60: ***p<0.001; PND21-23 vs PND>60: *p<0.05. FMRP/GAPDH: One-way ANOVA followed by Tukey's multiple comparison tests; PND7-9 vs PND>60: **p<0.01; PND14-16 vs PND>60: **p<0.01. **B.** Representative Immunoblots for SYNGAP1 in WT and HET at PND7-9, PND14-16, PND21-23, and PND>60, normalised to β-ACTIN, and GAPDH (*top*). The line graph shows the expression profile of SYNGAP1 normalised to β-ACTIN (*middle*) and normalised to GAPDH (*below*; only WT) at PND7-9, PND14-16, PND21-23, and PND>60 (N=4 for all age groups, samples were run on the same gel). SYNGAP1/ β-ACTIN: Two-way ANOVA followed by Bonferroni's multiple comparison test; WT vs HET at PND7-9: p=0.23; PND14-16: **p<0.01; PND21-23: ***p<0.001; PND>60: ***p<0.001. SYNGAP1/GAPDH: One-way ANOVA followed by Tukey's multiple comparison tests; NS= not significant across age. **C.** Multiple putative G-quadruplex was detected using QGRS Mapper in the validated sequence available for mouse *Syngap1* from NCBI (Gene ID: 240057). Three G- quadruplex sequences having high G-score were highlighted in the red box. All these sequences have been mapped in the Coding Sequence (CDS) (*left panel*). Multiple sequence alignment of the highest score G-quadruplexes of mouse *Syngap1* compared with Human and Rat. G score: 82 showing putative G-quadruplexes conserved among Human, Mouse, and Rat respectively (*right panel*)

However, it was not known if FMRP-*Syngap1* mRNA interaction level is consistent during development. To understand the extent of FMRP-*Syngap1* mRNA interaction during development, we performed the FMRP immunoprecipitation at postnatal day 14-16, as well as at postnatal day 21-23 in both *Syngap1*$^{+/-}$ mice and their WT littermate controls. The interaction between FMRP and *Syngap1* mRNA was significantly reduced in *Syngap1*$^{+/-}$ at postnatal day 21-23 ($p<0.05$; 0.63±0.04; **Fig 3-3B**) compared to their WT littermates (1.0±0.13). However, the level of FMRP-*Syngap1* mRNA interaction was unaltered at postnatal day 14-16, compared between *Syngap1*$^{+/-}$ and WT ($p>0.05$; *Syngap1*$^{+/-}$=2.3±0.66; WT=1.0±0.1; **Fig 3-3B).**

In addition to that, the interaction of FMRP with *Psd-95* mRNA was investigated at postnatal day 14-16 and 21-23. No alteration in the interaction of *Psd-95* mRNA with FMRP was found at any of these age groups (postnatal day 14-16: $p>0.05$; *Syngap1*$^{+/-}$=1.297±0.34; WT=1.0±0.2; postnatal day 21-23: $p>0.05$; *Syngap1*$^{+/-}$=0.8347±0.12; WT=1.0±0.07; **Fig 3-4B**). Therefore, the differential interaction between FMRP and associated *Syngap1* mRNA at postnatal day 21-23 may be a possible reason for the differential expression level of SYNGAP1 at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice.

3.6. FMRP regulates the translation of *Syngap1* mRNA

Further, the investigation was done to check if FMRP can regulate *Syngap1* translation using HeLa cells system. *Gfp-Syngap1* was expressed in HeLa cells followed by knocking down of *FMR1* (**Fig 3-4C, 3-4D, 3-4E**). Reduction in the level of FMRP resulted in an increase in the level of GFP-SYNGAP1 ($p<0.05$; *Scr* siRNA 0.58±0.05; *FMR1* siRNA 0.82±0.067; **Fig 3-3C**). In conclusion, FMRP indeed regulates the translation of *Syngap1* mRNA by interacting with it. Overall, the increased SYNGAP1 protein level at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice may be a result of reduced FMRP-*Syngap1* mRNA interaction, which, in turn, might upregulate *Syngap1* mRNA translation.

3.7. Steady-state translation might be unaltered in *Syngap1*$^{+/-}$ mice

Translation was assessed by Polysome profiling assay (**Fig 3-5A**) from hippocampal lysates of WT and *Syngap1*$^{+/-}$ mice at postnatal day 14-16 and postnatal day 21-23 (Muddashetty, Kelic et al. 2007). Based on the A_{254} traces from cycloheximide-treated samples, **Fig 3-5B** showed the distinct peaks corresponding to mRNP, monosome, and polysomes, respectively. A_{254} traces between WT and *Syngap1*$^{+/-}$ mice did not show any significant difference, suggesting that the global translation in the hippocampus might be unaffected in *Syngap1*$^{+/-}$ mice at postnatal day 14-16 and 21-23. As a proof of principle, polysome profiling assay was also performed with puromycin treated hippocampal lysates. Immunoblotting experiment for Ribosomal large subunit protein, RPLP0, has shown a shift in

puromycin treated samples as puromycin disassemble the ribosome from translating mRNA (**Fig 3-5B**). The similar shift was observed in *β-actin* mRNA distribution in polysome upon puromycin treatment (**Fig 3-6A**).

Further, the distribution of 18s rRNA in the polysomal fractions was investigated (both cycloheximide and puromycin treated) as a quality check for RNA integrity. A shift of rRNA towards non-polysomal fractions was observed upon puromycin-treatment as compared to cycloheximide-treatment (**Fig 3-6B, 3-6C**). By comparing the overall polysomal distribution of 18s rRNA, *β-actin* mRNA, and RPLP0 in the samples treated with cycloheximide and puromycin, fractions 1 to 6 are considered as a non-translating pool, and fractions 7 to 11 are translating pool (**Fig 3-6A, 3-6B, 3-6C**).

As a control, *β-actin* mRNA distribution was quantified in translating pool, and no significant difference was found between WT and *Syngap1*$^{+/-}$ at postnatal day 14-16 (WT=89.9±3%; *Syngap1*$^{+/-}$ =83.6±2.9%; $p>0.05$), as well as at postnatal day 21-23 (WT=97.9±0.6%; *Syngap1*$^{+/-}$=89.8±3.8%; $p>0.05$; **Fig 3-6D**). Next, the RPLP0 protein distribution was estimated in translating and non-translating pool of cycloheximide-treated polysomal fractions obtained from WT and *Syngap1*$^{+/-}$ mice hippocampus during postnatal day 14-16 (WT=1.06±0.18, *Syngap1*$^{+/-}$=0.71±0.15, $p>0.05$) and postnatal day 21-23 (WT=1.27±0.21, *Syngap1*$^{+/-}$=0.83±0.17, $p=0.14$), indicating no significant change in the distribution of RPLP0 (**Fig 3-5C, Fig 3-6E**). Hence, there may be no alteration in the steady state protein synthesis level in *Syngap1*$^{+/-}$ mice.

3.8. *Syngap1* mRNA translation is altered in *Syngap1*$^{+/-}$ mice

To further understand the mechanisms behind the compensatory increase in SYNGAP1 levels at postnatal day 21-23 in *Syngap1*$^{+/-}$, analysis of *Syngap1* mRNA translation status was done using polysome profiling assay. *Syngap1* mRNA present in translating pool was quantified by performing quantitative PCR (qPCR) using the total RNA isolated from both non-translating (Fractions 1-6) and translating fractions (Fractions 7-11) of the fractions obtained from the polysome profiling. The polysome analysis found a significantly reduced level of *Syngap1* mRNA was associated in the translating pool derived from the polysomal fractions of the postnatal day 14-16 *Syngap1*$^{+/-}$ mice compared to their WT littermate controls (WT=84.32±4%; *Syngap1*$^{+/-}$=65.77±2%; $p<0.01$; **Fig 3-5D**). On the contrary, no difference was observed concerning the *Syngap1* mRNA distribution in the polysome at postnatal day 21-23 between WT and *Syngap1*$^{+/-}$ mice (WT=92.9±3.5%; *Syngap1*$^{+/-}$ =87.9±2.5%; $p=0.3033$). Overall, the increase in the *Syngap1* mRNA distribution in the translating

pool at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice may lead to the increased SYNGAP1 protein level at this particular time window.

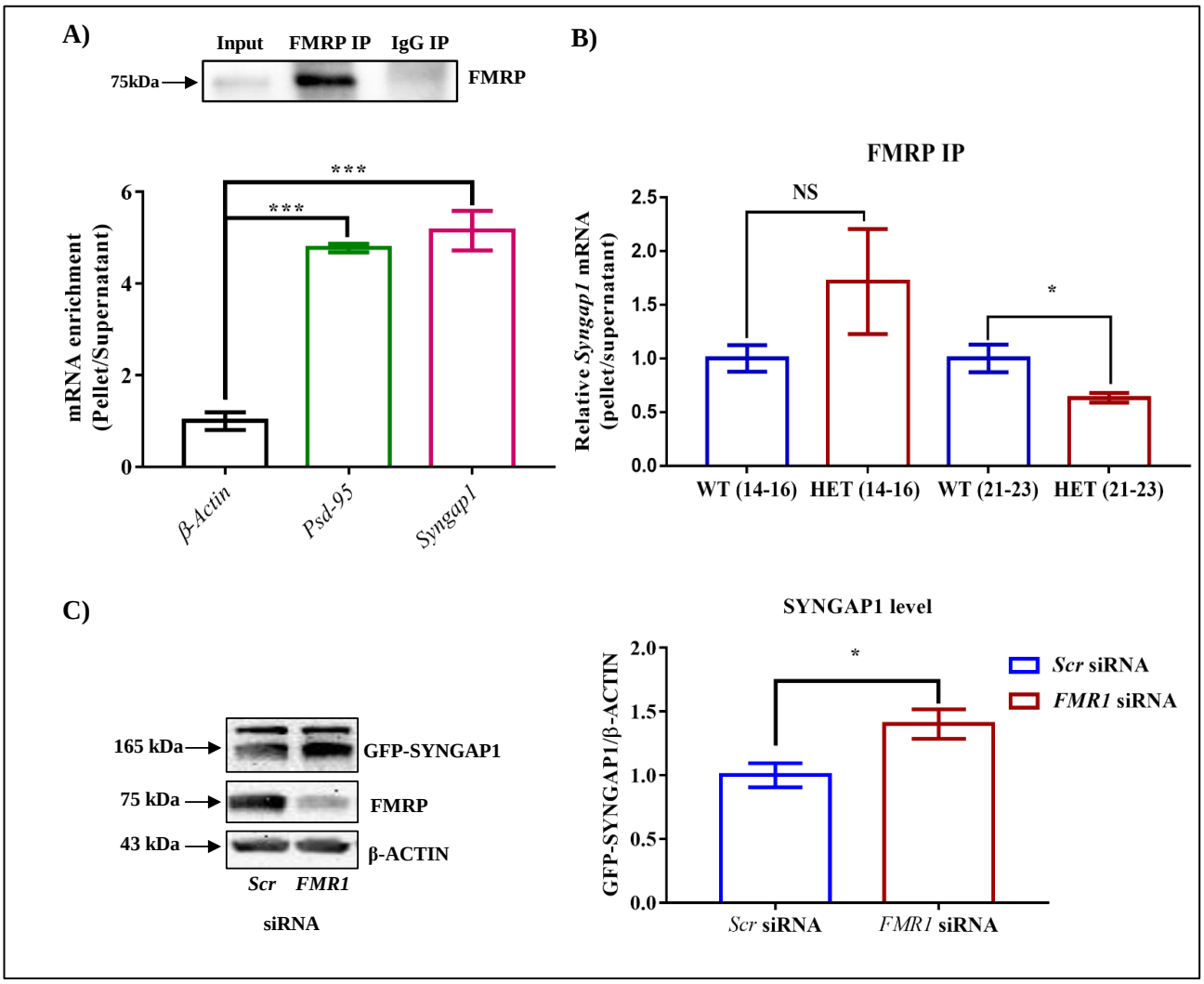
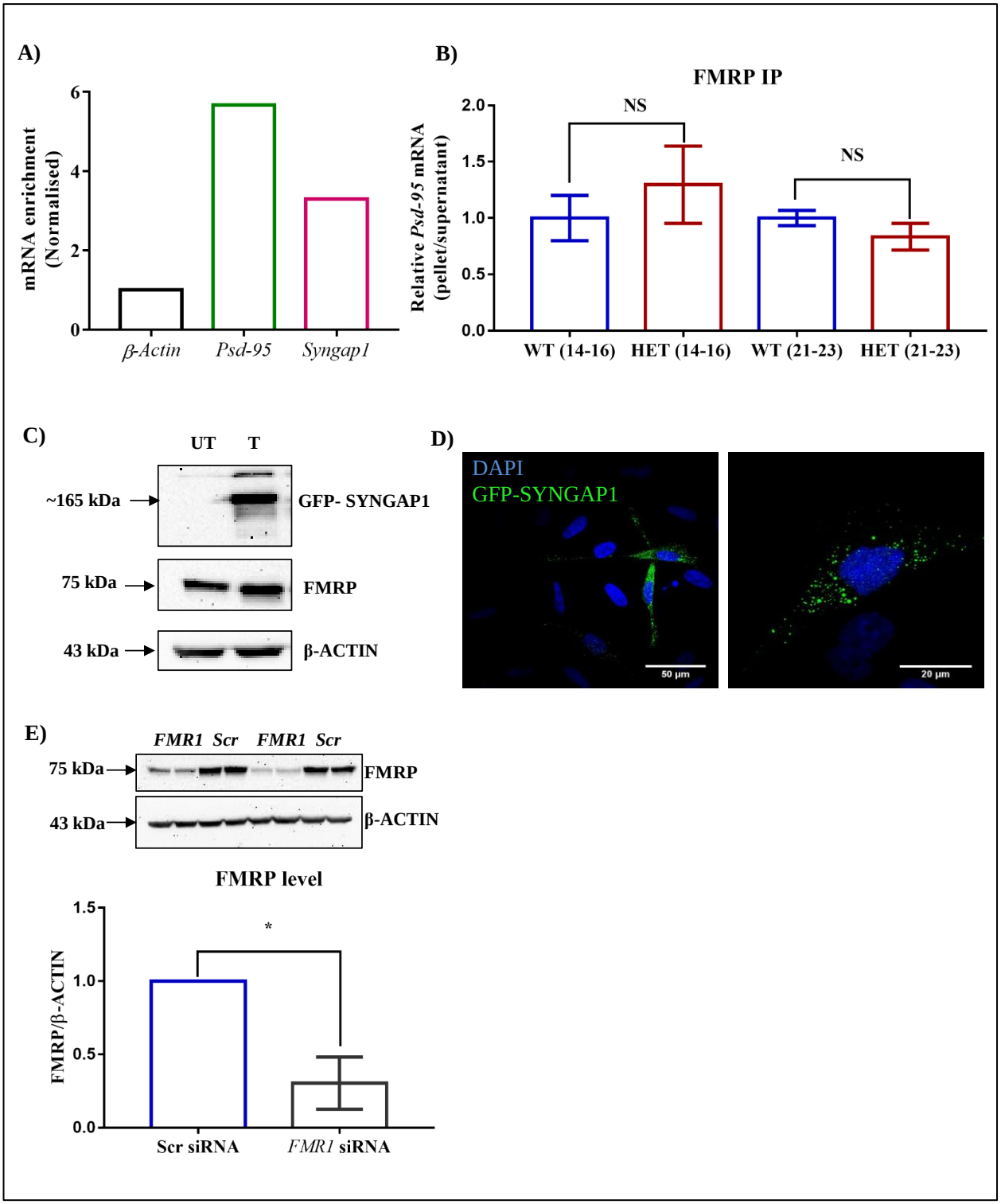


Figure 3-3: FMRP regulates *Syngap1* mRNA translation

A. Immunoblot for FMRP following FMRP-IP and IgG-IP (*top)*. Bar graph showing relative *Syngap1, Psd95* mRNA enrichment in FMRP IP pellet compared to Supernatant after normalising to *β-Actin* (WT: N=3; Below). Enrichment was calculated by the given formula: $2^{-(dCt\ FMRP\ IP)}$; dCt=Ct (pellet) – Ct (Supernatant); One-way ANOVA followed by Dunnett's multiple comparisons test was done to compare the enrichment of *Psd-95* and *Syngap1* mRNA association with FMRP, compared to FMRP- *β-Actin* mRNA association; ***p<0.0001. **B.** The bar graph shows relative *Syngap1* mRNA enrichment in FMRP IP pellet compared to supernatant from hippocampus at PND21-23 (WT: N=5; HET: N=4), and PND14-16 (WT: N=4; HET: N=3) normalised to WT. Unpaired Student's *t*-test; *p<0.05; NS= not significant. **C.** Representative immunoblot for SYNGAP1, FMRP, and β-ACTIN showing knock-down of FMRP leads to an increase in SYNGAP1 expression in Hela (*left*). The quantified bar graph shows an increase in the level of GFP-SYNGAP1 expression in the cells treated with *FMR1* siRNA compared to *Scr* siRNA treatment (*right*). Unpaired Student's *t*-test; *p<0.05.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Syngap1
B)
FMRP IP
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1.0
0.5
0.0
NS
NS
WT (14-16)
HET (14-16)
WT (21-23)
HET (21-23)
C)
UT
T
~165 kDa
GFP- SYNGAP1
75 kDa
FMRP
43 kDa
β-ACTIN
D)
DAPI
GFP-SYNGAP1
50 µm
20 µm
E)
FMR1 Scr FMR1 Scr
75 kDa
FMRP
43 kDa
β-ACTIN
FMRP level
FMRP/β-ACTIN
1.5
1.0
0.5
0.0
*
Scr siRNA
FMR1 siRNA

Figure 3-4: FMRP-*Syngap1* and FMRP-*Psd-95* interaction in the hippocampus.

A. Bar graph showing relative mRNA enrichment in FMRP IP pellet compared to supernatant from the hippocampus of WT at PND14-16 normalised to IgG IP. Enrichment was calculated by the given formula: $2^{-(dCt\ FMRP\ IP)}/2^{-(dCt\ IgG\ IP)}$; dCt=Ct (pellet) – Ct (Supernatant); N=1. **B.** Bar graph showing relative *Psd-95* mRNA enrichment in FMRP IP pellet compared to supernatant from hippocampus at PND14-16 (WT: N=7; HET: N=3) and PND21-23 (WT: N=5; HET: N=4) normalised to WT. Unpaired Student's *t*-test. NS=not significant. **C.** Representative immunoblot for SYNGAP1 and FMRP showing the expression of SYNGAP1 in transfected (T) compared to Un-transfected (UT) control. **D.** Representative images of Hela cells showing the expression of GFP-SYNGAP1 (Green). Cell nuclei are stained with DAPI (Blue). The right panel shows a higher magnification image where GFP-SYNGAP1 shows punctate structure. **E.** Representative immunoblot for FMRP normalised to β-ACTIN (*top*). The bar graph (*below*) shows a reduced level of FMRP in the *FMR1* siRNA treated cells compared to *scr* siRNA treated control (WT: N=4; HET: N=4). Unpaired Student's *t*-test; * $p<0.05$.

3.9. *Fmr1* mRNA translation is altered in *Syngap1*$^{+/-}$ mice

The initial results described in the earlier section showed that the FMRP level was reduced significantly at postnatal day 21-23 in *Syngap1*$^{+/-}$ as compared to WT (**Fig 3-1**). Further investigation was done to identify whether the reduced level of FMRP is due to altered *Fmr1* mRNA levels (transcription) or translation. *Fmr1* mRNA levels obtained from the hippocampal lysates was evaluated of WT and *Syngap1*$^{+/-}$ mice at both postnatal day 14-16 and 21-23. No significant difference in *Fmr1* mRNA levels was observed between WT and *Syngap1*$^{+/-}$ mice at postnatal day 14-16 (WT=0.019±0.008; *Syngap1*$^{+/-}$=0.029±0.008; $p>0.05$) and postnatal day 21-23 (WT=0.009±0.001; *Syngap1*$^{+/-}$=0.020±0.009; $p>0.05$; **Fig 3-8B**).

Next, investigation of the translation status of *Fmr1* mRNA was done during postnatal day 14-16 and postnatal day 21-23 by estimating the *Fmr1* mRNA present in translating fractions of the polysome profile. The polysome profiling analysis showed that *Fmr1* mRNA distribution in translating pool was unaltered at postnatal day 14-16 (WT=66.66±2.9%; *Syngap1*$^{+/-}$=66.03±4.1%; $p>0.05$). However, *Fmr1* mRNA distribution was significantly reduced in the translating pool obtained from the *Syngap1*$^{+/-}$ mice compared to WT at postnatal day 21-23 (WT=89.34±1.03%; *Syngap1*$^{+/-}$=73.38±4%; $p<0.05$; **Fig 3-7C**), suggesting that a reduced FMRP level was a consequence of decreased *Fmr1* mRNA translation at postnatal day 21-23 in *Syngap1*$^{+/-}$.

3.10. FMRP distribution in polysome is altered in *Syngap1*$^{+/-}$ mice

Fmr1 mRNA translation was altered at postnatal day 21-23, leading to decreased FMRP protein level and increased *Syngap1* mRNA translation at this age group of *Syngap1*$^{+/-}$ mice. Therefore, to further assess whether the changes in the levels of translating *Syngap1* mRNA is a result of the altered association of FMRP with polysomes, the distribution of FMRP was estimated in translating/non-translating pools from polysomal fractions. Polysome profiling assay showed that the FMRP protein distribution increased significantly in the translating pool at postnatal day 14-16 in *Syngap1*$^{+/-}$ mice (0.41±0.03) as compared to the age-matched WT controls (0.17±0.02; $p<0.01$; **Fig 3-7A, 3-7B**). However, FMRP distribution was reduced in the polysomal fraction of *Syngap1*$^{+/-}$ (0.23±0.03) at postnatal day 21-23 compared to WT (0.55±0.15; $p<0.05$; **Fig 3-7A, 3-7B**). This reduction in the polysomal distribution of FMRP might have a compounding effect on the translation of FMRP target mRNAs during postnatal day 21-23 in *Syngap1*$^{+/-}$ as the overall FMRP level was also reduced. As a control, PSD-95 levels were also analysed at postnatal day 14-16 (WT=2.03±0.35; *Syngap1*$^{+/-}$ =1.39±0.15; $p>0.05$) and postnatal day 21-23 (WT=0.98±0.05; *Syngap1*$^{+/-}$=0.98±0.12; $p>0.05$). However, PSD-95 level was similar between WT and *Syngap1*$^{+/-}$ (**Fig 3-8A**).

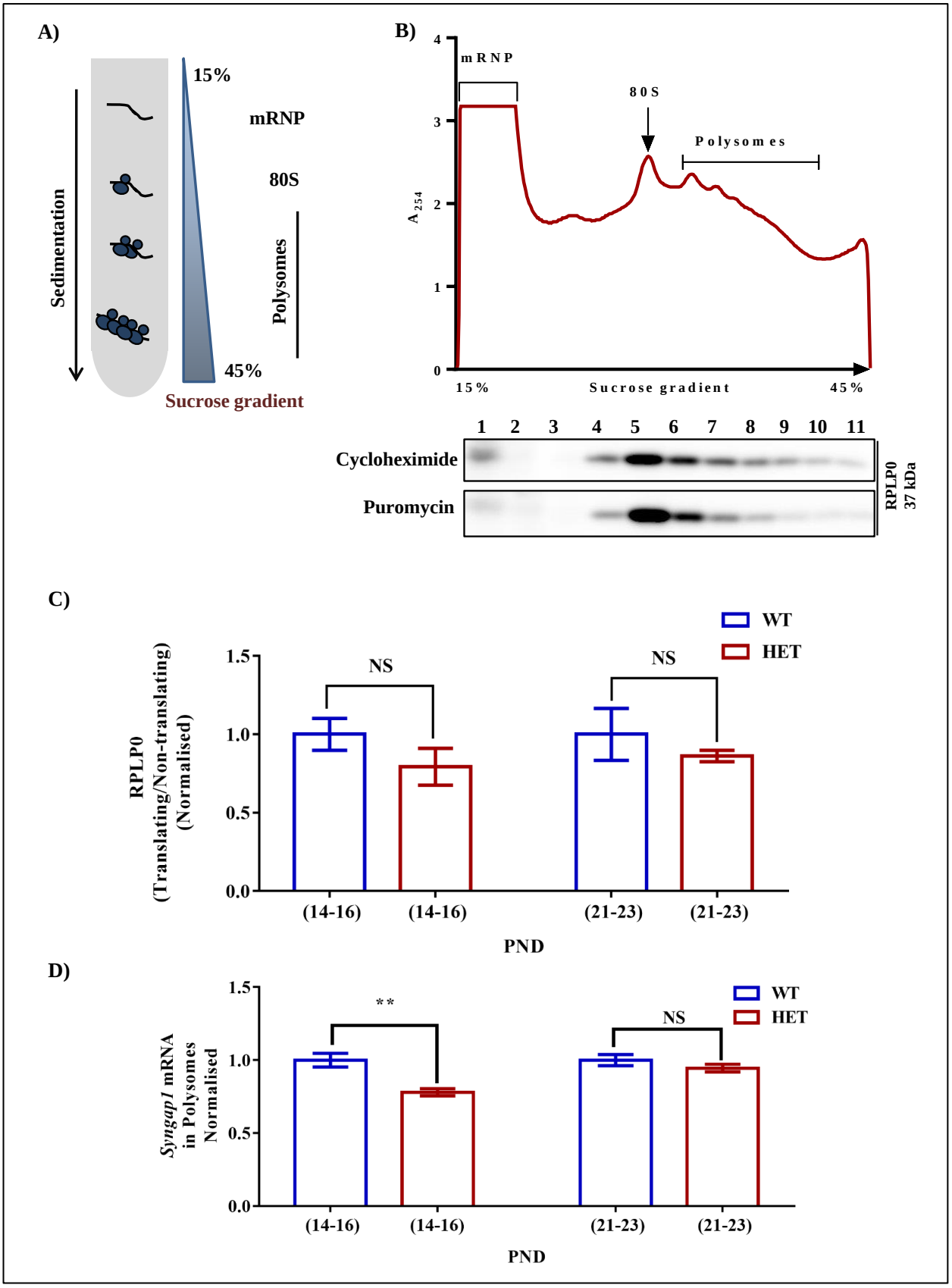
A)
15%
mRNP
80S
Polysomes
45%
Sedimentation
Sucrose gradient
B)
mRNP
80S
Polysomes
A254
15%
Sucrose gradient
45%
1 2 3 4 5 6 7 8 9 10 11
Cycloheximide
Puromycin
RPLP0
37 kDa
C)
WT
HET
RPLP0
(Translating/Non-translating)
(Normalised)
NS
NS
(14-16)
(14-16)
(21-23)
(21-23)
PND
D)
WT
HET
Syngap1 mRNA
in Polysomes
Normalised
**
NS
(14-16)
(14-16)
(21-23)
(21-23)
PND

Figure 3-5: Altered *Syngap1* mRNA translation in HET

A. Schematic depicting the sucrose gradient method used for polyribosome profiling (translation assay). **B.** Polyribosome profile obtained from Cycloheximide treated hippocampal lysate during PND14-16 in HET (*top*). Representative immunoblots for RPLP0 distribution in Cycloheximide and Puromycin treated polysome during PND14-16 (*below*). **C.** Bar graph shows RPLP0 distribution in Translating/Non-translating fractions during PND14-16 (WT: N=5; HET: N=3; $p>0.05$) and PND21-23 (WT: N=4; HET: N=4; $p>0.05$). Unpaired Student's *t*-test was done for both age groups. NS = not significant. **D.** *Syngap1* mRNA distribution in polysome in HET normalised to WT during PND14-16 (WT: N=4; HET: N=6; $p<0.01$) and PND21-23 (WT: N=3; HET: N=3; $p>0.05$); Unpaired Student's *t*-test.

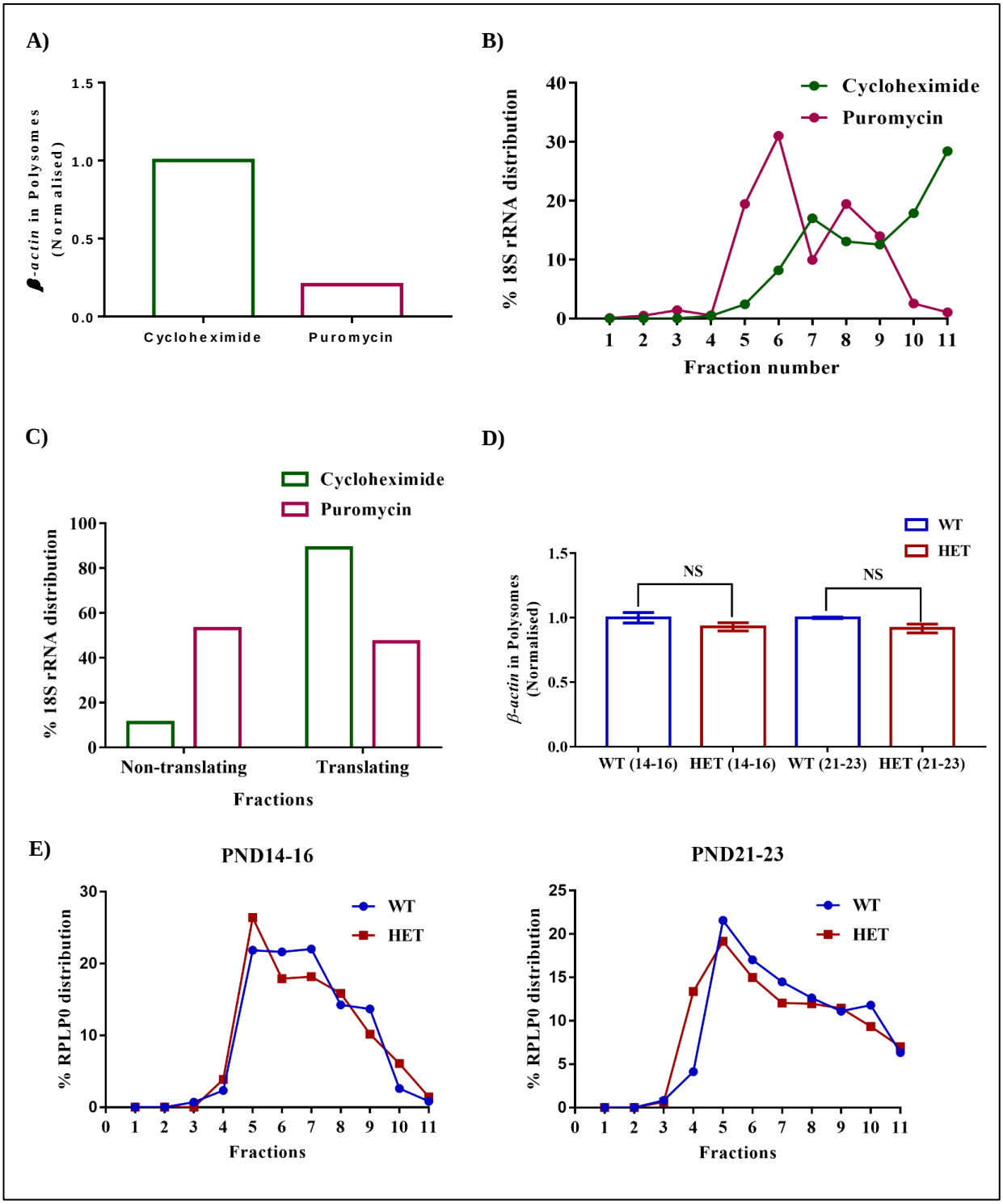
A)
β-actin in Polysomes (Normalised)
1.5
1.0
0.5
0.0
Cycloheximide
Puromycin
B)
% 18S rRNA distribution
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11
Fraction number
Cycloheximide
Puromycin
C)
Cycloheximide
Puromycin
% 18S rRNA distribution
100
80
60
40
20
0
Non-translating
Translating
Fractions
D)
WT
HET
β-actin in Polysomes (Normalised)
1.5
1.0
0.5
0.0
NS
NS
WT (14-16)
HET (14-16)
WT (21-23)
HET (21-23)
E)
PND14-16
% RPLP0 distribution
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
Fractions
WT
HET
PND21-23
% RPLP0 distribution
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11
Fractions
WT
HET

Figure 3-6: Steady-state protein synthesis is unaltered in HET.

A. *β-actin* mRNA distribution in polysomes treated with cycloheximide (CHX) and puromycin (PURO). **B.** Representative percentage distribution of 18S rRNA in the polysome fractions of CHX and PURO treated WT samples in PND14-16. **C.** Percentage distribution of 18S rRNA in the translating and non-translating pool of CHX and PURO treated polysome. **D.** *β-actin* mRNA distribution in CHX treated polysome HET normalised to WT in PND14-16 (WT: N=6; HET: N=6) and PND21-23 (WT: N=4; HET: N=5). NS = not significant. Unpaired Student's *t*-test. **E.** Representative percentage RPLP0 distribution line graph in PND14-16 (*left*) and PND21-23 (*right*).

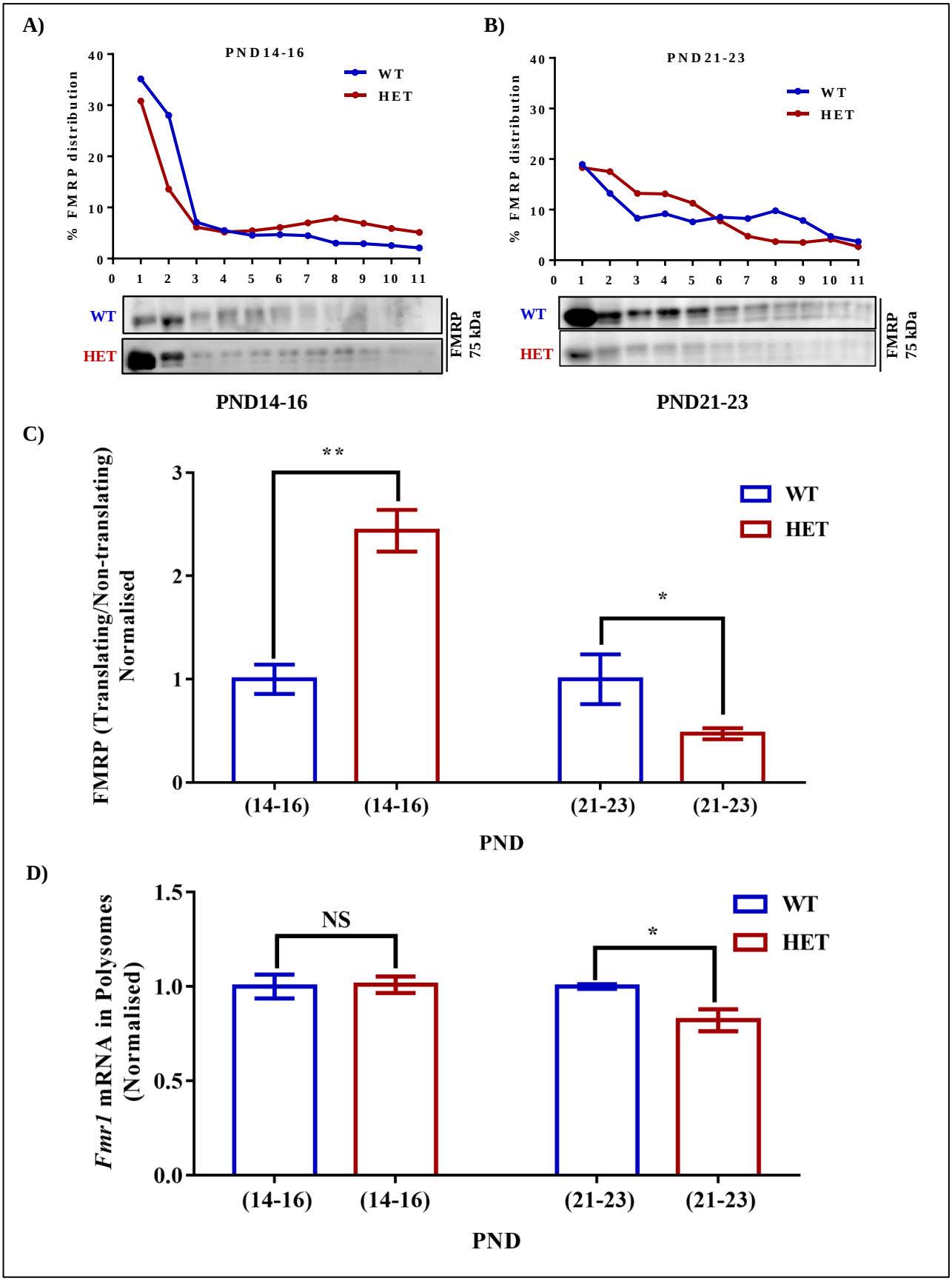
A)
PND14-16
WT
HET
% FMRP distribution
WT
HET
FMRP
75 kDa
PND14-16
B)
PND21-23
WT
HET
% FMRP distribution
WT
HET
FMRP
75 kDa
PND21-23
C)
FMRP (Translating/Non-translating)
Normalised
**
*
WT
HET
(14-16)
(14-16)
(21-23)
(21-23)
PND
D)
Fmr1 mRNA in Polysomes
(Normalised)
NS
*
WT
HET
(14-16)
(14-16)
(21-23)
(21-23)
PND

Figure 3-7: Altered *Fmr1* mRNA translation.

A. Representative line graphs showing percentage FMRP distribution in polysomes during PND14-16 (*top*) along with representative immunoblot for FMRP distribution (*below*). **B.** Line graph showing representative percentage FMRP distribution in polysomes during PND21-23 (*top*) and the corresponding representative immunoblot for FMRP distribution (*below*). **C.** Bar graph showing FMRP distribution in translating/non-translating fractions in HET normalised to WT during PND14-16 (WT: N=4; HET: N=4) and PND21-23 (WT: N=4; HET: N=5). *$p<0.05$, **$p<0.01$; Unpaired Student's *t*-test. **D.** Bar graph depicting relative *Fmr1* mRNA in translating fractions of HET normalised to WT during PND14-16 (WT: N=3; HET: N=3) and PND21-23 (WT: N=3; HET: N=5). *$p<0.05$, NS= not significant; Unpaired Student's *t*-test.

3.11. NMDAR activation leads to increased phosphorylation of eEF2

Previous studies have shown that basal protein synthesis level was increased in *Syngap1*$^{+/-}$ (Wang, Held et al. 2013, Barnes, Wijetunge et al. 2015). Also, SYNGAP1 is known to regulate synaptic maturation during a critical time window (Clement, Aceti et al. 2012, Clement, Ozkan et al. 2013), and the results described in the earlier sections further demonstrated altered expression of FMRP during a specific developmental stage in *Syngap1*$^{+/-}$. Therefore, it is crucial to investigate protein synthesis during development in *Syngap1*$^{+/-}$ mice. Polysome profile analysis indicated that there might not be any alteration in the steady state protein synthesis. Thus, further investigation was done on activity-mediated protein synthesis response on NMDAR activation. The phosphorylation status of eukaryotic Elongation Factor 2 (eEF2) was used as a read-out of translation response on NMDAR activation. Phosphorylation of eEF2 was shown to repress global protein synthesis (Scheetz, Nairn, & Constantine-paton, 2000). Analysis of phospho/total-eEF2 level in response to NMDAR stimulation in hippocampal synaptoneurosomes was done using immunoblotting. As a control, PSD-95 enrichment was estimated in the hippocampal synaptoneurosomes as compared to the total lysate (**Fig 3-10A**).

Further, NMDAR stimulation in synaptoneurosomes from WT mice manifested around a 1.5-fold increase in phospho/total-eEF2 on 1-minute stimulation with NMDA (Basal=0.84±0.11; Stimulated=1.3±0.12; $p<0.05$; **Fig 3-9A**). To validate that the phosphorylation response of eEF2 is indeed resulting from NMDAR stimulation, further, the synaptoneurosomes were pre-treated with a potent antagonist of NMDAR, AP-5. The NMDAR- mediated increase in the phosphorylation of eEF2 was not observed on AP-5 pre-treatment, showing the specificity of the assay (**Fig 3-10B).**

3.12. NMDAR-mediated translation response is altered in *Syngap1*$^{+/-}$

Further evaluation of the protein synthesis response on NMDAR activation was done during development in *Syngap1*$^{+/-}$ and WT mice. An increase in the p-eEF2 under basal condition was observed in *Syngap1*$^{+/-}$ condition at both the age groups, postnatal day 14-16 (WT=0.84±0.11; *Syngap1*$^{+/-}$=1.6±0.22%; $p<0.05$) and postnatal day 21-23 (WT=0.22±0.01%; *Syngap1*$^{+/-}$=0.9±0.11%; $p<0.05$; **Fig 3-9B, 3-9C)**. NMDAR-mediated increase in phosphorylation of eEF2 was not observed in synaptoneurosomes obtained from *Syngap1*$^{+/-}$ mice (stimulated/basal; WT=1.57±0.2; *Syngap1*$^{+/-}$ =0.7±0.09; $p<0.01$) at postnatal day 14-16 (**Fig 3-9B, 3-10C)**. Normalising the phospho/total-eEF2 level on stimulation in *Syngap1*$^{+/-}$ to WT showed a significant reduction in phospho/total eEF2 level on NMDAR stimulation (stimulated/basal; WT=1.00±0.12; *Syngap1*$^{+/-}$=0.45±0.06; **Fig 3-9B, 3-10C)**.

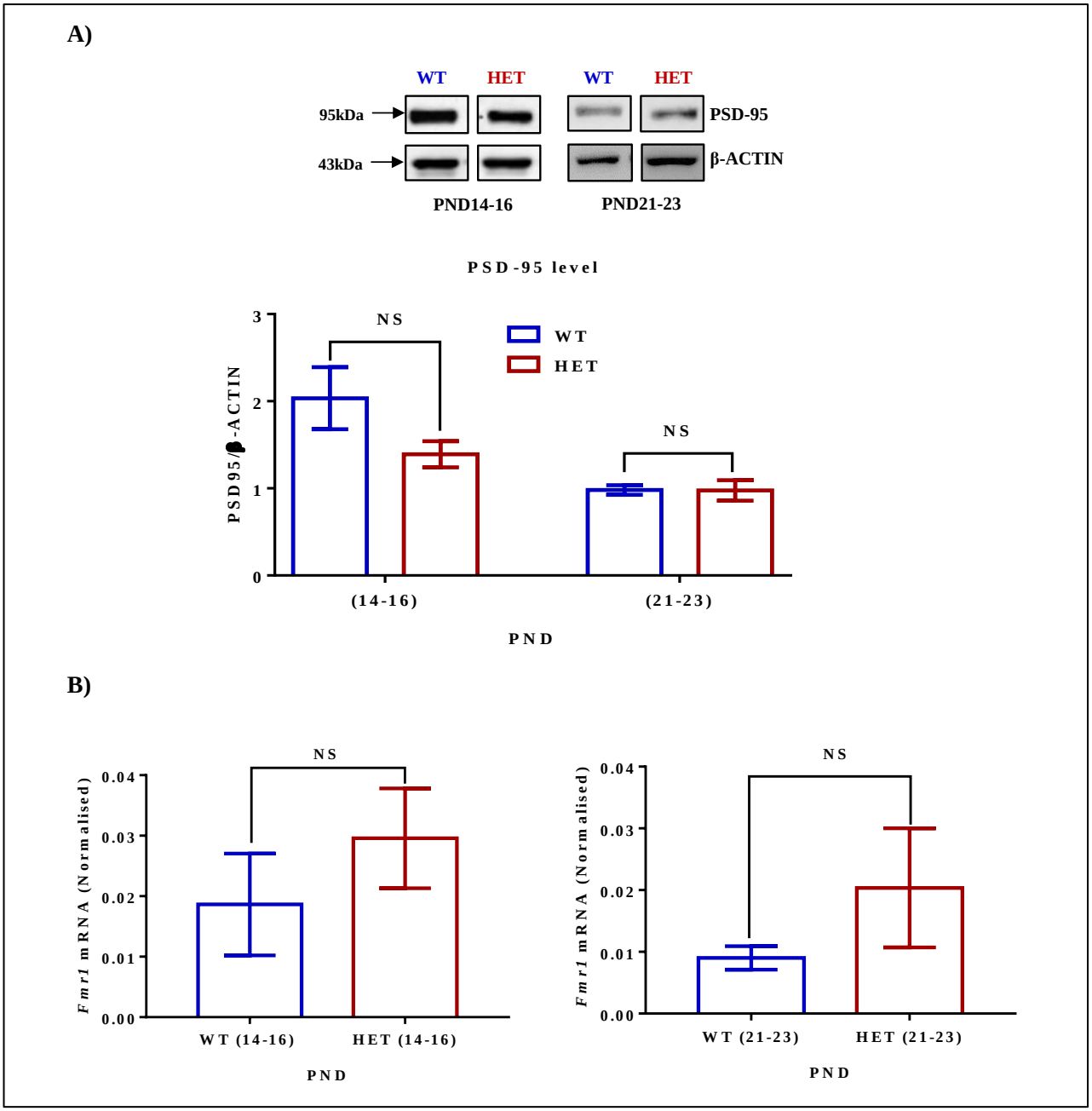

Figure 3-8: *Fmr1* mRNA level is unaltered in HET.

A. Representative immunoblots for PSD-95 normalised to β-ACTIN in the hippocampus during PND14-16 and PND21-23 in WT and HET. *Below* Bar graph showing a no significant difference in the level of PSD-95 at PND14-16 (WT: N=6; HET: N=6) and PND21-23 (WT: N=7; HET: N=4) between WT and HET; NS= not significant. Unpaired Student's *t*-test. **B.** Bar graph depicting relative *Fmr1* mRNA normalised to *β-actin* from total hippocampal lysate at PND14-16 (*left,* WT: N=3; HET: N=3) and PND21-23 (*right,* WT: N=3; HET: N=3); NS= not significant. Unpaired Student's *t*-test.

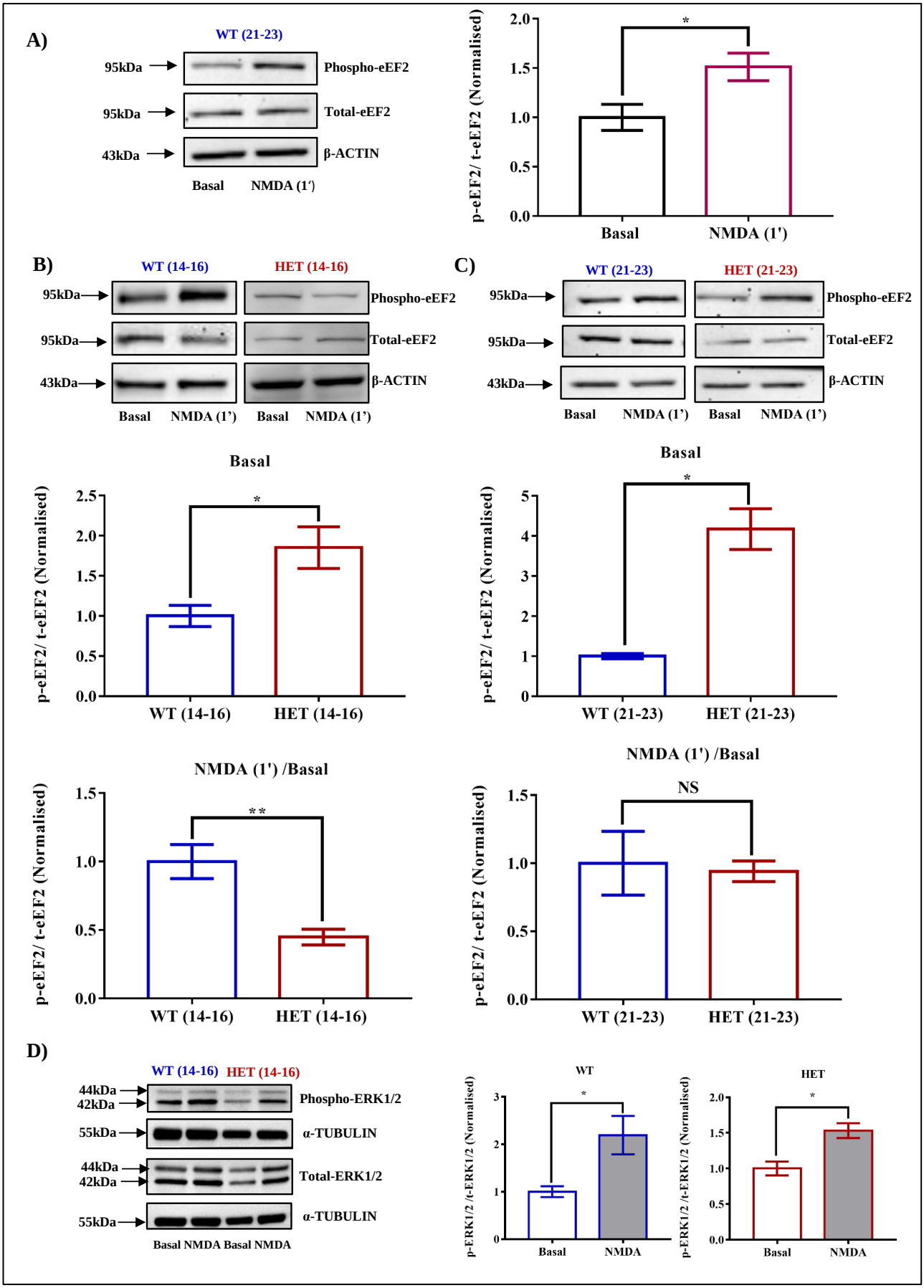
A)
WT (21-23)
95kDa
Phospho-eEF2
95kDa
Total-eEF2
43kDa
β-ACTIN
Basal
NMDA (1′)
p-eEF2/ t-eEF2 (Normalised)
Basal
NMDA (1')
B)
WT (14-16)
HET (14-16)
Phospho-eEF2
Total-eEF2
β-ACTIN
Basal
NMDA (1')
C)
WT (21-23)
HET (21-23)
Basal
NMDA (1')
Basal
WT (14-16)
HET (14-16)
WT (21-23)
HET (21-23)
NMDA (1') /Basal
NS
D)
WT (14-16)
HET (14-16)
44kDa
42kDa
Phospho-ERK1/2
55kDa
α-TUBULIN
Total-ERK1/2
Basal NMDA Basal NMDA
WT
HET
p-ERK1/2 /t-ERK1/2 (Normalised)

Figure 3-9: Dysregulated NMDAR-mediated translation response is recovered during PND21-23 in HET.

A. Representative immunoblot for phospho-eEF2 and Total-eEF2 showing increased phosphorylation on NMDAR stimulation for 1-minute in synaptoneurosomes from WT during PND21-23 (*left*). Pooled data of the same represented in the bar graph (*right,* Basal: N=4; Stimulated: N=4); *p<0.05; Unpaired Student's *t*-test. **B.** Representative immunoblots of phospho- and total-eEF2 normalised to β-ACTIN during PND14-16 in WT and HET (*top*). The bar graph shows increased phosphorylation of eEF2 at basal conditions in synaptoneurosome obtained from the hippocampus of HET as compared to WT during PND14-16 in the (*middle,* WT: N=4; HET: N=3). Bar graph showing decreased phosphorylation of eEF2 in HET on NMDAR stimulation as compared to WT in PND14-16 (*below,* WT: N=4; HET: N=4). *p<0.05, **p<0.01; Unpaired Student's *t*-test; WT and HET samples were run on the same gel; 1-minute stimulation blots were cropped and showed here. **C.** Representative immunoblots for phospho-eEF2 and total-eEF2 normalised to β-ACTIN during PND21-23 in WT and HET (*top*). Increased phosphorylation of eEF2 at the basal condition in HET synaptoneurosome as compared to WT during PND21-23 (*middle,* WT: N=3; HET: N=3). Bar graph showing the extent of phosphorylation in HET is similar to WT during PND21-23 (*bottom,* WT: N=3; HET: N=4). *p<0.05, NS= not significant; Unpaired Student's *t*-test. **D.** Representative immunoblot for Phospho-ERK1/2 and Total-ERK1/2 showing increased phosphorylation on NMDAR stimulation for 5-minute in synaptoneurosomes from WT and HET during PND14-16 (*left*). Pooled data of the same represented in the bar graphs (*right,* Basal: N=4; Stimulated: N=4); *p<0.05; Unpaired Student's *t*-test.

The possible reason behind the failure to respond to the NMDAR activation could be a basal level increase in the phosphorylation of eEF2 in *Syngap1*$^{+/-}$ mice. However, NMDAR-mediated increase in phosphorylated-eEF2 was recovered in *Syngap1*$^{+/-}$ to WT level at postnatal day 21-23, even though there was a significant increase in the phosphorylation of eEF2 under basal condition (stimulated/basal; WT=1.81±0.14; *Syngap1*$^{+/-}$=1.92±0.45; p=0.8233; **Fig 3-9C, 3-10D**). A similar pattern was observed on 2-minute stimulation (**Fig 3-10C, 3-10D**).

Furthermore, to validate the loss in the NMDAR-mediated responses on eEF2 phosphorylation observed at postnatal day 14-16 in *Syngap1*$^{+/-}$ is not due to unhealthy degraded synaptoneurosomes, we assessed the phosphorylation pattern of ERK which is another well-known marker for NMDAR-mediated signalling pathway. We found an increase in the ERK phosphorylation on NMDAR stimulation in both WT and *Syngap1*$^{+/-}$ at postnatal day 14-16 (**Fig 3-9D**), indicating that the synaptoneurosomes were healthy as well as responsive to the stimulation assay.

We further investigated if the recovery of NMDAR-mediated signalling observed at postnatal day 21-23 in *Syngap1*$^{+/-}$ persists till adulthood. We performed similar synaptoneurosome stimulation experiments at a later age group (postnatal day> 60). Our data showed that NMDAR-mediated response on eEF2 phosphorylation was indeed lost in the *Syngap1*$^{+/-}$ at postnatal day>60 **(Fig 3-10E).** In conclusion, rescue in NMDAR-mediated phosphorylation of eEF2 was transient and only present at a specific age window when FMRP was downregulated in *Syngap1*$^{+/-}$.

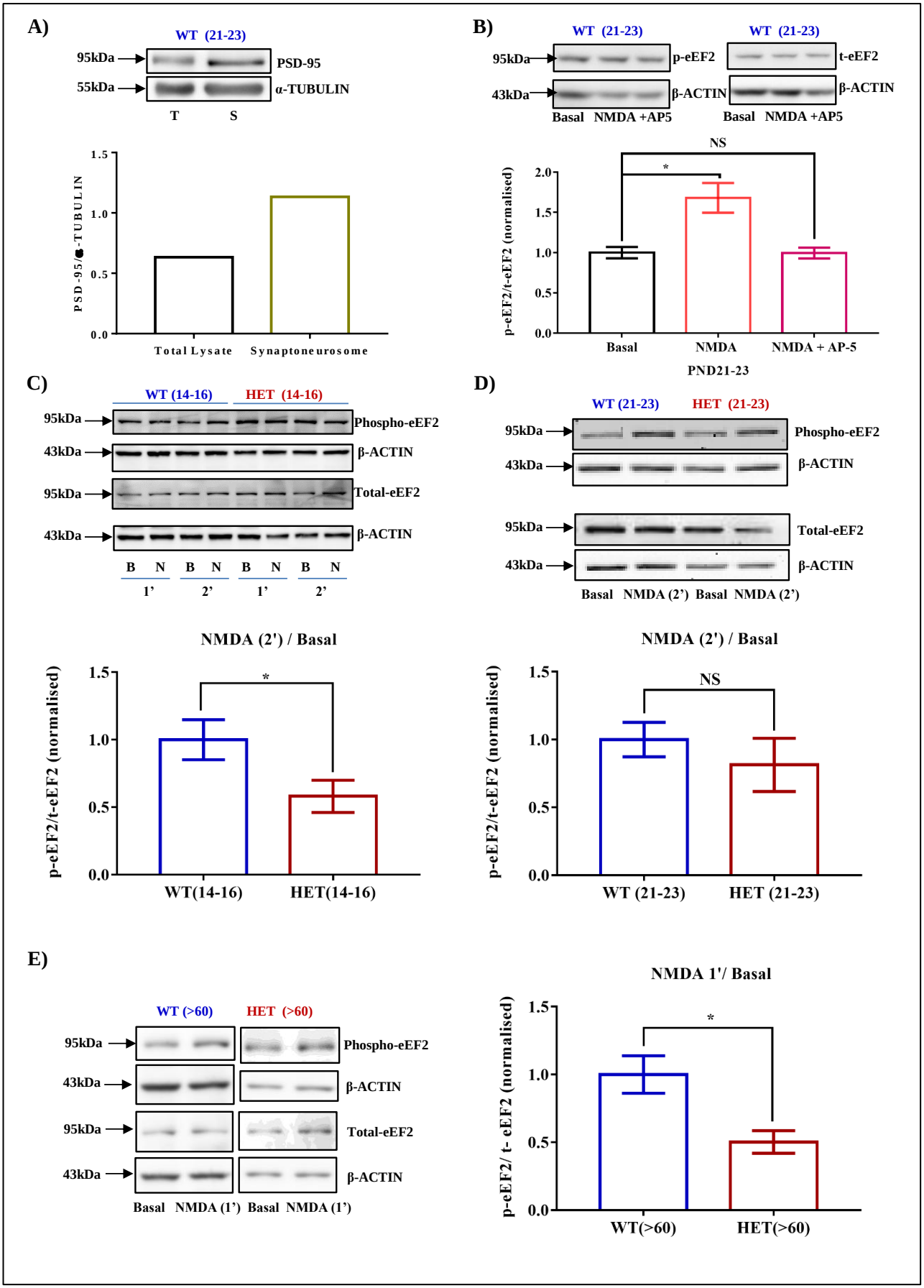
A)
WT (21-23)
95kDa
PSD-95
55kDa
α-TUBULIN
T
S
PSD-95/α-TUBULIN
Total Lysate
Synaptoneurosome
B)
WT (21-23)
WT (21-23)
95kDa
p-eEF2
t-eEF2
43kDa
β-ACTIN
β-ACTIN
Basal NMDA +AP5
Basal NMDA +AP5
NS
*
p-eEF2/t-eEF2 (normalised)
Basal
NMDA
NMDA + AP-5
PND21-23
C)
WT (14-16)
HET (14-16)
95kDa
Phospho-eEF2
43kDa
β-ACTIN
95kDa
Total-eEF2
43kDa
β-ACTIN
B N B N B N B N
1' 2' 1' 2'
NMDA (2') / Basal
*
p-eEF2/t-eEF2 (normalised)
WT(14-16)
HET(14-16)
D)
WT (21-23)
HET (21-23)
95kDa
Phospho-eEF2
43kDa
β-ACTIN
95kDa
Total-eEF2
43kDa
β-ACTIN
Basal NMDA (2') Basal NMDA (2')
NMDA (2') / Basal
NS
p-eEF2/t-eEF2 (normalised)
WT (21-23)
HET (21-23)
E)
WT (>60)
HET (>60)
95kDa
Phospho-eEF2
43kDa
β-ACTIN
95kDa
Total-eEF2
43kDa
β-ACTIN
Basal NMDA (1') Basal NMDA (1')
NMDA 1'/ Basal
*
p-eEF2/ t- eEF2 (normalised)
WT(>60)
HET(>60)

Figure 3-10: NMDAR-mediated translation response impaired during adulthood in HET.

A. Representative immunoblot depicting the enrichment of PSD-95 in synaptoneurosome (S) compared to Total hippocampal lysate (T; *top*). A bar graph is showing quantified data normalised to α-TUBULIN (*below*). **B.** Representative immunoblot for Phospho-eEF2, Total-eEF2, and β-ACTIN in synaptoneurosomes after 1-minute stimulation with NMDA, AP-5, and NMDA+AP-5 (*top*). Quantified data as histogram shows increased phosphorylation of eEF2 on NMDA treatment is lost when co-treated with AP-5 (*below*). *p<0.05, One-way ANOVA followed by Dunnett's multiple comparison tests. **C.** Representative immunoblot images for Phospho-eEF2, Total-eEF2, and β-ACTIN in synaptoneurosomes after 1-minute and 2-minute NMDAR stimulation during PND14-16 (*top*). All samples (both WT and HET) were run on the same gel (B: Basal; N: NMDA stimulation). Bar graph showing that a 2-minute activation of NMDAR alters phosphorylation of eEF2 in HET (N=3) compared to WT (N=3) in PND14-16 (*below*). *p<0.05, NS= not significant. Unpaired Student's *t*-test. **D.** Representative immunoblot for Phospho- and Total-eEF2 normalised to β-ACTIN synaptoneurosomes stimulated with NMDA for 2-minute in WT and HET during PND21-23 (*top*). Bar graph depicting unaltered phosphorylation of eEF2 in HET (N=3) compared to WT (N=3) post-2-minute activation of NMDAR. NS= not significant. Unpaired Student's *t*-test. **E.** Representative immunoblots of phospho- and total-eEF2 normalised to β-ACTIN during PND>60 in WT and HET (*left*). Bar graph showing decreased phosphorylation of eEF2 in HET on NMDAR stimulation as compared to WT in PND>60 (*right*, WT: N=4; HET: N=4). *p<0.05; Unpaired Student's *t*-test.

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CHAPTER - 4 Discussion

Activity-mediated protein synthesis in neurons regulates many synaptic plasticity mechanisms (Klann, Antion et al. 2004, Pfeiffer and Huber 2006). The translation is tightly regulated in the synapses. Many protein synthesis regulators are involved in such crucial regulation. Fragile X Mental Retardation Protein (FMRP; encoded by *FMR1* gene) is one of the known regulators of protein synthesis in the brain (Huber, Gallagher et al. 2002). Mutation in the *FMR1* gene leading to the absence of FMRP causes Intellectual Disability (ID) in human, similar to *SYNGAP1*$^{+/-}$ mutation (Garber, Visootsak et al. 2008, Hamdan, Gauthier et al. 2009).

An earlier study has shown that mGluR-mediated LTD was enhanced and became independent of protein synthesis in the mouse model of Fragile X Syndrome (Huber, Gallagher et al. 2002). Therefore, the dysregulation in the protein synthesis, in turn, disrupt the mGluR-LTD in the *Fmr1* KO mice. Few studies further showed that the protein synthesis is enhanced in the *Syngap1*$^{+/-}$ mouse model of ID similar to *Fmr1* KO (Wang, Held et al. 2013, Barnes, Wijetunge et al. 2015). Further investigation by Barnes *et al.* showed that *Syngap1*$^{+/-}$ mice of the age of postnatal day 25-32 manifested enhanced mGluR-mediated LTD which is a hallmark of Fragile X Syndrome (Huber, Gallagher et al. 2002, Barnes, Wijetunge et al. 2015). In the present study of this thesis, investigation on a similar aspect showed that increased mGluR-mediated LTD was persisted till adulthood in *Syngap1*$^{+/-}$ mice. Overall, it was hypothesised that upon activation of Group I mGluRs protein synthesis happens rapidly. Many of these proteins synthesised due to the Group I mGluR activation facilitate the induction of LTD by regulating AMPAR endocytosis. Also, FMRP controls a subset of these proteins expressed downstream to Group I mGluR activation. Therefore, it is an interesting question to understand how Group I mGluR-mediated LTD is regulated by SYNGAP1. Enhanced mGluR-mediated LTD in *Syngap1*$^{+/-}$ mice further raise the possibility of SYNGAP1 being associated with the functions of FMRP.

Two such common pathophysiologies, excessive protein synthesis and exaggerated mGluR-mediated LTD, observed in both *Fmr1* KO and *Syngap1*$^{+/-}$ mice indicated that there could be a probable cross-talk between FMRP and SYNGAP1. However, to date, only one study has investigated the inter-relation between these two proteins; FMRP and SYNGAP1 (Barnes, Wijetunge et al. 2015). Harlow *et al.* have shown that *Fmr1* KO mice manifested delayed maturation of synapses, in contrary to the *Syngap1*$^{+/-}$ mice (Harlow, Till et al. 2010). *Syngap1*$^{+/-}$ mice exhibited early maturation of the synapses (Clement, Aceti et al. 2012, Clement, Ozkan et al. 2013). Based on these results, Barnes *et al.*

proposed that the mutation in *Fmr1* and *Syngap1* belongs to two opposite spectra of the synaptic pathophysiology. Therefore, crossing both the mutations could be an attractive approach to improve the synaptic deficits and pathophysiology observed in Fragile X Syndrome and *Syngap1*$^{+/-}$ mutation. However, crossing *Fmr1* KO with *Syngap1*$^{+/-}$ mice failed to show any improvement. Double-crossed mice exhibited exaggerated mGluR-mediated LTD, similar to the *Fmr1* KO and *Syngap1*$^{+/-}$ mice (Barnes, Wijetunge et al. 2015). Therefore, crossing *Fmr1* KO and *Syngap1*$^{+/-}$ mice is not a practical approach to ameliorate the pathophysiology associated with these mutations. The possible reason behind this failure to improve synaptic pathology in the double-crossed mice is unclear. However, the results listed in this thesis could be helpful to predict that chronic depletion of both these genes *Fmr1* and *Syngap1* may not be an effective strategy as both FMRP and SYNGAP1 are essential for healthy brain development.

Since SYNGAP1 is known to regulate synaptic maturation during a specific developmental window (Clement, Aceti et al. 2012, Clement, Ozkan et al. 2013), it was hypothesised that the role of FMRP in *Syngap1*$^{+/-}$ could also be developmentally regulated. Hence, the developmental expression profile of FMRP in the hippocampus of *Syngap1*$^{+/-}$ mice was investigated. Quantitative immunoblotting analysis showed a reduced level of FMRP at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice. Also, the FMRP expression level decreased as age increased in WT, consistent with earlier studies and further reiterated the fact that the function of FMRP is particularly crucial during the early developmental age window, similar to that of SYNGAP1. However, the reason behind reduction in the FMRP level only at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice is unclear. It was speculated that FMRP level was reduced at postnatal day 21-23 as a compensatory effect of synaptic dysregulation observed at postnatal day 14-16. *Syngap1*$^{+/-}$ mice expresses a decreased level of SYNGAP1 in the brain. Such a reduced level of SYNGAP1 was sufficient enough to cause dysfunction in synapse and in turn, learning and memory deficits. Therefore, it was inevitable to think that reduced level of FMRP might have some role to compensate for the reduced level of SYNGAP1. As FMRP is a well-known regulator of protein synthesis, it was obvious to ask if FMRP negatively regulates *Syngap1* mRNA translation. However, still, the exact mechanism of speculated compensation is unknown.

In order to regulate the translation of *Syngap1* mRNA, FMRP should interact with the mRNA. An earlier study by Darnell *et al.* shed some light towards this angle. Darnell *et al.* identified *Syngap1* mRNA as a potential target of FMRP by a high-throughput assay (Darnell, Van Driesche et al. 2011). However, many of these identified mRNA targets were not validated further. Therefore, it was difficult to conclude if FMRP interacts with *Syngap1* mRNA to regulate its translation.

FMRP is known to interact with its target mRNA via G-quadruplex structures form by the mRNA. Hence, it was essential to identify if *Syngap1* mRNA can form a G-quadruplex structure. Bioinformatics analysis using QGRS Mapper online tool predicted the presence of putative G-quadruplex in the *Syngap1* mRNA. It made the point stronger that FMRP could interact with *Syngap1* mRNA via the G-quadruplex structures formed by the mRNA. However, it was crucial to validate such prediction by direct biochemical association study. Immunoprecipitation of FMRP from the hippocampal lysate showed the association of *Syngap1* mRNA with FMRP. In fact, this study is the first of its kind to establish and validate the interaction between FMRP and *Syngap1* mRNA.

Further, the knockdown of *FMR1* (leading to a reduced level of FMRP) by siRNA in HeLa cells showed that SYNGAP1 level was increased compared to scrambled siRNA treatment indicating that FMRP indeed regulate the expression of *Syngap1*. Therefore, in this thesis work, it was established that FMRP not only interacts with *Syngap1* mRNA but also regulates its translation.

As mentioned earlier section that both FMRP and SYNGAP1 expression were developmentally regulated and they play a crucial role in the early brain development, it was interesting to see if the interaction between FMRP and *Syngap1* mRNA also changes during the development. Immunoprecipitation study showed that FMRP-*Syngap1* mRNA interaction reduced in the hippocampus of *Syngap1*$^{+/-}$ mice at postnatal day 21-23. This reduced interaction between FMRP-*Syngap1* mRNA could be because of the already reduced level of FMRP being present at this age window. However, this reduction in the interaction could eventually have an effect on the translation of *Syngap1* mRNA.

In this thesis work, the translation status was evaluated by Polyribosome profiling assay. Polysome study did not show any change in the steady state protein synthesis rate indicated by RPLP0 (a ribosomal large subunit marker protein) distribution in *Syngap1*$^{+/-}$ mice. Further, polysome analysis showed *Syngap1* mRNA translation was reduced at postnatal day 14-16 in *Syngap1*$^{+/-}$ mice compared to their WT littermate controls. However, at postnatal day 21-23 *Syngap1* mRNA translation rate increased and reached to the WT level. This increase in the *Syngap1* mRNA translation might be because of decreased FMRP-*Syngap1* mRNA interaction due to the reduced level of FMRP expression at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice. As speculated earlier that the reduction of FMRP indeed has an effect on the *Syngap1* mRNA translation. Overall, the reduced level of FMRP led to increased translation of *Syngap1* mRNA at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice.

Studies have reported that *Syngap1*$^{+/-}$ mice exhibited dysregulated NMDAR-mediated signalling (Komiyama, Watabe et al. 2002, Rumbaugh, Adams et al. 2006, Carlisle, Manzerra et al. 2008). These

studies have also shown that SYNGAP1 further associates with NR2B (Rockliffe and Gawler 2006) and in turn, negatively regulates NMDAR-mediated activation of ERK (Kim, Dunah et al. 2005). Further, via the ERK-mediated signalling pathway, SYNGAP1 regulates the trafficking of AMPARs on to the postsynaptic membrane (Rumbaugh, Adams et al. 2006). Therefore, ERK-mediated pathway has been a major pathway affected due to the haploinsufficiency of *Syngap1*. Komiyama *et al.* further investigated the ERK activity in *Syngap1*$^{+/-}$ mice under basal condition. Authors have shown that the basal level of ERK phosphorylation was significantly increased in *Syngap1*$^{+/-}$ condition (Komiyama, Watabe et al. 2002). This enhanced activity of ERK under basal condition indicates the dysregulation of NMDAR-mediated signalling in *Syngap1*$^{+/-}$ condition. Strikingly, when NMDAR activity was induced in *Syngap1*$^{+/-}$ condition, ERK phosphorylation further increased (Komiyama, Watabe et al. 2002). However, this increase in ERK activity on NMDAR stimulation is not consistent with the deficits observed in the NMDAR-mediated LTP in *Syngap1*$^{+/-}$ mice (Komiyama, Watabe et al. 2002). Even though there was a robust increase in ERK phosphorylation still NMDAR-LTD was impaired in *Syngap1*$^{+/-}$ mice.

Thus, NMDAR-mediated translation repression was evaluated in this thesis work to understand the NMDAR-mediated signalling deficits in *Syngap1*$^{+/-}$ mice. A previous study has already reported that activation of NMDAR caused a robust reduction in global translation through phosphorylation of elongation factor eEF2 (Scheetz, Nairn et al. 2000). To understand the effect of NMDAR activation on global protein synthesis, NMDAR stimulation was done in the purified synapses (synaptoneurosomes) obtained from the hippocampus of both *Syngap1*$^{+/-}$ mice and their WT littermate controls. Immunoblotting analysis showed that phosphorylation of eEF2 was significantly increased in the synaptoneurosomes obtained from the *Syngap1*$^{+/-}$ mice under the basal condition at postnatal day 14-16, as well as at postnatal day 21-23. This increased eEF2 phosphorylation further confirms that the NMDAR-mediated signalling pathway is dysregulated in the *Syngap1*$^{+/-}$ condition, consistent with the earlier studies. However, the mechanism for the increased phosphorylation in *Syngap1*$^{+/-}$ mice under basal condition is yet to be answered. One probable mechanism could be the involvement of Ca^{2+}/Calmodulin kinases. Increased excitatory neuronal activity observed in *Syngap1*$^{+/-}$ mice might lead to an increase in intracellular Ca^{2+} levels and thereby, a subsequent increase in eEF2 phosphorylation via Ca^{2+}-Calmodulin kinase.

Further, stimulation of the synaptoneurosomes with NMDA, agonist of NMDAR showed that there was a significant increase in eEF2 phosphorylation in WT condition indicating a rapid repression of global protein synthesis on NMDAR activation, consistent with the earlier findings of Scheetz *et al.* However, the NMDAR activation in the synaptoneurosomes obtained from *Syngap1*$^{+/-}$ mice of

postnatal day 14-16 did not show any increase in the phosphorylation level of eEF2. As the phosphorylation was already high under the basal condition in *Syngap1*$^{+/-}$ mice at postnatal day 14-16, further stimulation failed to show any subsequent response. Hence, the failure to response to the NMDAR activation reiterates that NMDAR-mediated pathway is disrupted in *Syngap1*$^{+/-}$ mice at postnatal day 14-16.

The synaptoneurosomes obtained from *Syngap1*$^{+/-}$ mice of postnatal day 21-23, however, responded to the NMDAR stimulation. There was an increase in phosphorylation of eEF2 on NMDAR activation in *Syngap1*$^{+/-}$ synaptoneurosomes. The extent of increment in phosphorylation of eEF2 was similar to the WT synaptoneurosomes. In conclusion, the extent of eEF2 phosphorylation in *Syngap1*$^{+/-}$ mice was similar to WT, which indicates that there could be a recovery of NMDAR-mediated signalling in the *Syngap1*$^{+/-}$ mice at postnatal day 21-23.

The investigation carried out as part of this thesis work showed that *Syngap1* mRNA translation was upregulated at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice. Therefore, it is possible that increased level of *Syngap1* mRNA translation led to increased SYNGAP1 level, which in turn effectively brought the dysregulated NMDAR-mediated signalling under check. As mentioned earlier, SYNGAP1 is a crucial regulator of NMDAR-mediated signalling; therefore, modulating the level of SYNGAP1 could eventually modulate the NMDAR-mediated signalling response in *Syngap1*$^{+/-}$ mice at postnatal day 21-23.

The work also has shown that FMRP interacts with *Syngap1* mRNA and regulates its translation. Further, the interaction between FMRP and *Syngap1* mRNA was significantly reduced at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice. Hence, reduced FMRP-*Syngap1* mRNA interaction suggests that an increased number of *Syngap1* mRNA might be accessible for the translation machinery to be translated further. In fact, the increased *Syngap1* mRNA translation at postnatal day 21-23 also indicates the same.

Understanding the mechanism behind the differential interaction of FMRP and *Syngap1* mRNA was crucial. The data presented in the results section clearly showed that *Fmr1* mRNA translation was downregulated at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice. Whereas, there was no alteration in the transcription of the *Fmr1* gene. It was not clear how the translation of *Fmr1* mRNA was downregulated in *Syngap1*$^{+/-}$ mice. It was speculated that increased neuronal excitation in *Syngap1*$^{+/-}$ condition could have some compensatory effect on the translation machinery. However, such speculation was not investigated further. Overall, due to the downregulated *Fmr1* mRNA translation, FMRP level also dropped significantly at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice. Reduced level of

FMRP might lead to reduced FMRP-*Syngap1* mRNA interaction, in turn, increased *Syngap1* translation.

The work of this thesis also showed that the recovery of the NMDAR-mediated signalling was transient and observed only at postnatal day 21-23, not at postnatal day 14-16 or in adulthood. Further analysis demonstrated that FMRP level was downregulated only at the age of postnatal day 21-23 in *Syngap1*$^{+/-}$ mice, which strongly suggests that FMRP-mediated translation regulation of *Syngap1* mRNA was the core mechanism behind the partial recovery of NMDAR-mediated signalling at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice.

The findings of this thesis further can be corroborated with the observations made by Clement *et al.* in which authors have shown that *Syngap1*$^{+/-}$ mice manifested increased synaptic transmission and increased AMPAR/NMDAR-mediated currents at postnatal day 14-16 but eventually returned to WT level in the later age (Clement, Aceti et al. 2012). Based on the results reported in this thesis, a model was proposed in which increased NMDAR-mediated response to protein synthesis is compensating for the loss of SYNGAP1 during development in *Syngap1*$^{+/-}$. In addition to that, a fine-tuned downregulation of *Fmr1* translation during a specific developmental window in *Syngap1*$^{+/-}$ mice might compensate for the dysregulation in NMDAR-mediated signalling. The schematic model is described in the Figure 4-1.

The work has been carried out as part of this thesis is particularly fascinating with respect to the critical period of maturation of the hippocampus in mice. Early maturation of hippocampal neurons has already been shown in *Syngap1*$^{+/-}$ at postnatal day 14-16, whereas WT neurons mature at 3-week of age, i.e. at postnatal day 21-23 (Clement, Aceti et al. 2012). The results presented in the earlier section indicate that these two age groups are crucial for any compensation to occur in the mammalian brain. Once the window of critical period of development is over, rescuing the pathophysiology caused by any pathological mutations becomes difficult.

This work has indicated that eEF2 phosphorylation on NMDAR activation was modulated due to the FMRP downregulation in *Syngap1*$^{+/-}$ at postnatal day 21-23. Earlier studies have demonstrated that *Fmr1* KO mice manifested dysregulation in the NMDAR-mediated signalling, suggesting that FMRP indeed play a vital role to regulate NMDAR-mediated signalling pathway (Toft, Lundbye et al. 2016). Further, NMDAR activation by sensory stimulation showed a rapid increase in the FMRP level, indicating FMRP's role in the NMDAR-mediated pathway in the brain (Todd, Malter et al. 2003, Gabel, Won et al. 2004). A more recent study by Chmielewska *et al.* has shown that FMRP indeed regulates the translation downstream to the NMDAR-mediated signalling pathway (Chmielewska,

Kuzniewska et al. 2018). Therefore, the NMDAR-mediated phosphorylation response of eEF2 observed in this thesis work is most likely to be regulated by the FMRP. Furthermore, the changes in the eEF2 phosphorylation coinciding with the changes in the FMRP expression level in *Syngap1*$^{+/-}$ mice at postnatal day 21-23, strongly suggests the same.

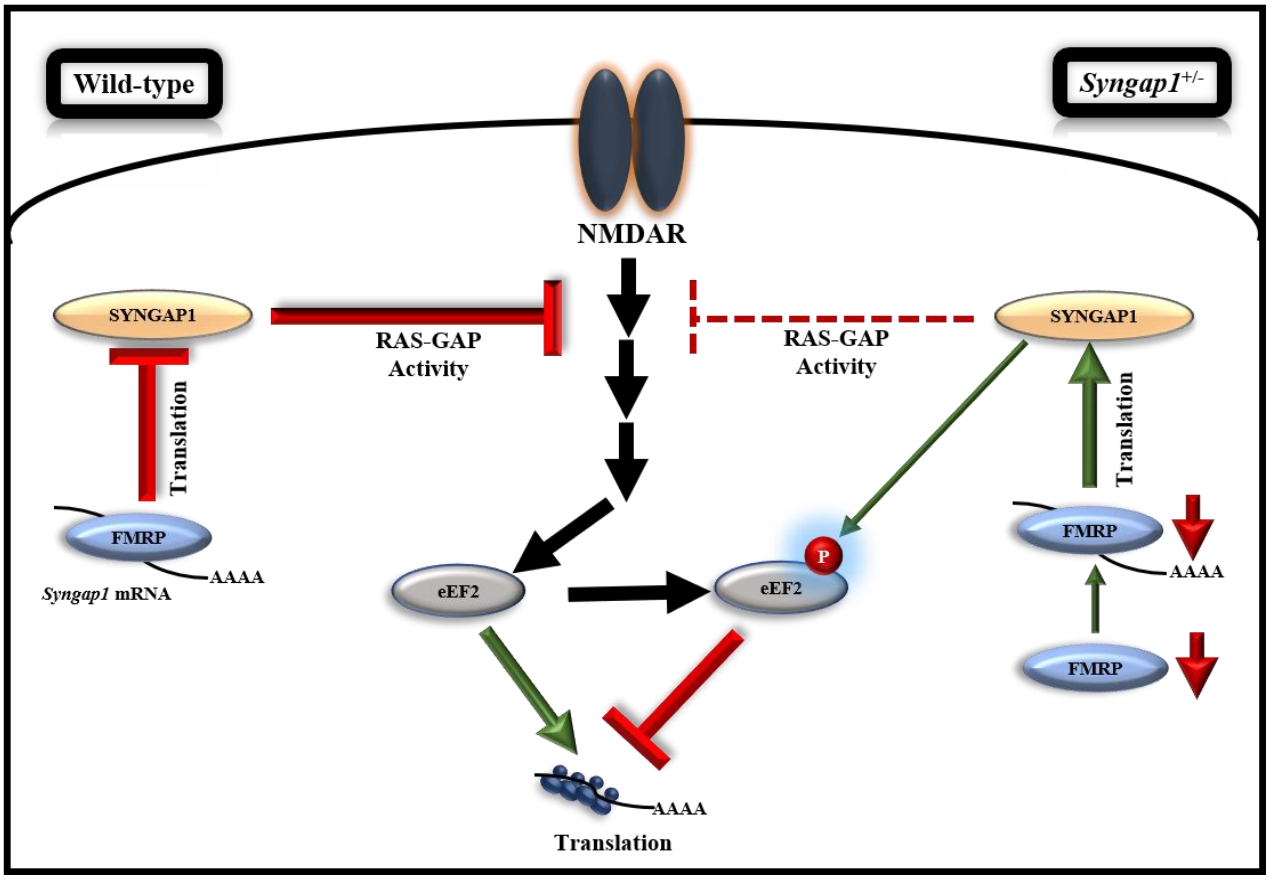

Figure 4-1: Schematic model is illustrating the FMRP-mediated translation of *Syngap1* and its impact on NMDAR-mediated signalling

This model shows that FMRP regulates *Syngap1* mRNA translation, which in turn regulates NMDAR-mediated signalling. In WT, NMDAR stimulation in synapse led to increased phosphorylation of eEF2, which resulted in global translation inhibition and the signalling was efficiently regulated by SYNGAP1. Whereas, in *Syngap1*$^{+/-}$ at PND14-16, NMDAR-mediated signalling was impaired as depicted by the loss of phosphorylation response to eEF2 due to a decreased level of SYNGAP1. At PND21-23 in *Syngap1*$^{+/-}$, FMRP level was low that increased translation of *Syngap1* mRNA leading to an increased SYNGAP1 level compared to PND14-16. Thus, an elevated level of SYNGAP1 might recover the NMDAR-mediated signalling via phosphorylation of eEF2.

In conclusion, this thesis work suggests that an altered response to activity-mediated protein synthesis during development is one of the major causes of abnormal neuronal function observed in the *Syngap1*$^{+/-}$ mouse model. However, chronic depletion of two genes with common core pathophysiology may not be a useful approach to rescue the deficits observed in either of these mutations. Hence, crossing *Fmr1*$^{-/y}$ and *Syngap1*$^{+/-}$ mice failed to show rescue (Barnes, Wijetunge et al. 2015). A most possible reason for this failure to rescue the deficits could be the essentiality of both these genes for healthy brain development. Therefore, modulating these proteins at a specific developmental window could be a potential therapeutic strategy for treating ID-related pathophysiology.

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CHAPTER - 5 Summary and Future directions

Patients with Intellectual Disability (ID) manifest learning and memory defects. *Syngap1*$^{+/-}$ mouse model of ID was used to study the effect of Synaptic RAS-GTPase Activating Protein (SYNGAP1) on protein synthesis, and its crosstalk with protein synthesis regulator Fragile X Mental Retardation Protein (FMRP). Electrophysiological recordings showed stimulation of Group I Metabotropic Glutamate Receptors (mGluR) led to increased Long-term depression (LTD) in the hippocampus of *Syngap1*$^{+/-}$ mice. The same pathophysiology was observed in Fragile X syndrome (*FMR1* mutation; depletion of FMRP) that led to the hypothesis that SYNGAP1 and FMRP might crosstalk to regulate protein synthesis in the brain. This work demonstrated that reduced level of *Fmr1* mRNA translation led to decreased FMRP level in the hippocampus of *Syngap1*$^{+/-}$ mice at postnatal day (PND) 21-23. The investigation reported here also showed that FMRP interacts with and regulates the translation of *Syngap1* mRNA. Besides, immunoprecipitation experiments showed that FMRP-*Syngap1* mRNA interaction was decreased at postnatal day 21-23, resulting in increased *Syngap1* mRNA translation. To understand the physiological relevance of the compensatory increase in *Syngap1* translation, NMDAR-mediated translation response was assessed by assaying phosphorylation of eEF2. NMDAR-mediated signalling was impaired in the hippocampal synaptoneurosomes obtained from *Syngap1*$^{+/-}$ mice at postnatal day 14-16. However, the impaired protein synthesis response was recovered at postnatal day 21-23, possibly because of the downregulation of protein synthesis regulator FMRP in the brain. Hence, the extensive investigations were done as part of this thesis, which proposes a model for the functional crosstalk between FMRP and SYNGAP1 in the brain.

The data presented in this thesis have shown that FMRP expression level reduced in *Syngap1*$^{+/-}$ mice, specifically at postnatal day 21-23. The synaptoneurosomes stimulation experiments also showed that NMDAR-mediated eEF2 phosphorylation came to normal level in *Syngap1*$^{+/-}$ mice at the same age window, indicating a link between FMRP and NMDAR-mediated signalling. Further, investigation demonstrated that reduced FMRP, in turn, upregulated *Syngap1* mRNA translation in *Syngap1*$^{+/-}$ mice at postnatal day 21-23. Therefore, the recovery in the eEF2 phosphorylation response observed in *Syngap1*$^{+/-}$ mice at postnatal day 21-23 could be because of the increased level of SYNGAP1. Thus, downregulation of FMRP expression led to upregulation of SYNGAP1 level, rescuing the NMDAR-mediated signalling. In conclusion, modulating FMRP level could be a practical approach to modulate the SYNGAP1 level in the brain.

A hallmark study by Clement *et al.* thoroughly investigated the synaptic pathophysiology associated with the *Syngap1*$^{+/-}$ mutation in the mouse model (Clement, Aceti et al. 2012). Authors have shown that *Syngap1*$^{+/-}$ mice manifested increased AMPA/NMDA ratio at postnatal day 14-16, which came back to the WT level at later age group like in adulthood. The similar pattern was observed for the spine morphology. Authors concluded that early maturation of dendritic occurred in *Syngap1*$^{+/-}$ mice (Clement, Aceti et al. 2012). Synaptic deficits occurred at postnatal day 14-16 in *Syngap1*$^{+/-}$ mice was the reason for the altered behavioural responses, and learning and memory defects in this mouse model (Guo, Wang et al. 2012). The behavioural deficits persisted to adulthood in *Syngap1*$^{+/-}$ mice (Guo, Wang et al. 2012).

As SYNGAP1 plays an essential role in spine maturation during the early development, its expression is also developmentally regulated. *Syngap1* mRNA level usually peaks at postnatal day 14-16, suggesting its essentiality at that time window (Clement, Aceti et al. 2012). Surprisingly, the synaptic deficits and the spine morphology defects were also prominent at postnatal day 14-16. Thus, the reduced level of SYNGAP1 expression at postnatal day 14-16 has the highest impact in *Syngap1*$^{+/-}$ mice. The work reported in this thesis speculated that reduced level of SYNGAP1 level at postnatal day 14-16 was compensated at a later age group, postnatal day 21-23, due to a reduction in FMRP leading to increased *Syngap1* mRNA translation.

Therefore, reducing the FMRP level at an earlier age window than postnatal day 14-16 could be a practical approach to ameliorate the pathophysiology associated with *Syngap1*$^{+/-}$ condition. Knocking down *Fmr1* at postnatal day 7-9 might upregulate the *Syngap1* mRNA translation at this time window. Thus, it is possible to have a sufficient level of SYNGAP1 at postnatal day 14-16 when its availability is crucial for the healthy development of the mammalian brain.

shRNA against *Fmr1* mRNA will be designed and packaged in lentiviral vector for the efficient delivery into the brain to knockdown *Fmr1*. The lentiviral packaged shRNA will be stereotaxically injected into the mouse brain. IHC and immunoblotting will estimate the efficiency of the knockdown for FMRP from the brain tissue (hippocampus). Further, SYNGAP1 level will be checked in the hippocampus upon knockdown of *Fmr1* as a proof of principle experiment. The proportion of the matured spines will be investigated at postnatal day 14-16 by two-photon imaging techniques. Clement *et al.* showed a proportion of mushroom-shaped matured spines were significantly high in *Syngap1*$^{+/-}$ mice due to the reduced SYNGAP1 level (Clement, Aceti et al. 2012). In the same study, authors also showed that the AMPA/NMDA ratio was increased in *Syngap1*$^{+/-}$ mice at postnatal day. Therefore, reducing FMRP level due to the knockdown at postnatal day 7-9 will upregulate

SYNGAP1 level in the brain at a later age group, presumably by postnatal day 14-16. To check the effect of such increased SYNGAP1 at postnatal day 14-16 in *Syngap1*$^{+/-}$ mice, electrophysiological experiments combined with two-photon imaging will be done. Whole cell patch clamp study will estimate the AMPA/NMDA ratio in the hippocampal neurons at postnatal day 14-16. Further, to assess the NMDAR-mediated translation response, eEF2 phosphorylation will be monitored upon NMDA stimulation in hippocampal synaptoneurosomes. Also, LTP will be investigated as *Syngap1*$^{+/-}$ mice manifested impaired LTP (Komiyama, Watabe et al. 2002). Finally, behavioural experiments will be employed to check cognitive and social efficacies. The schematic model of future experiments is described in the Figure 5-1.

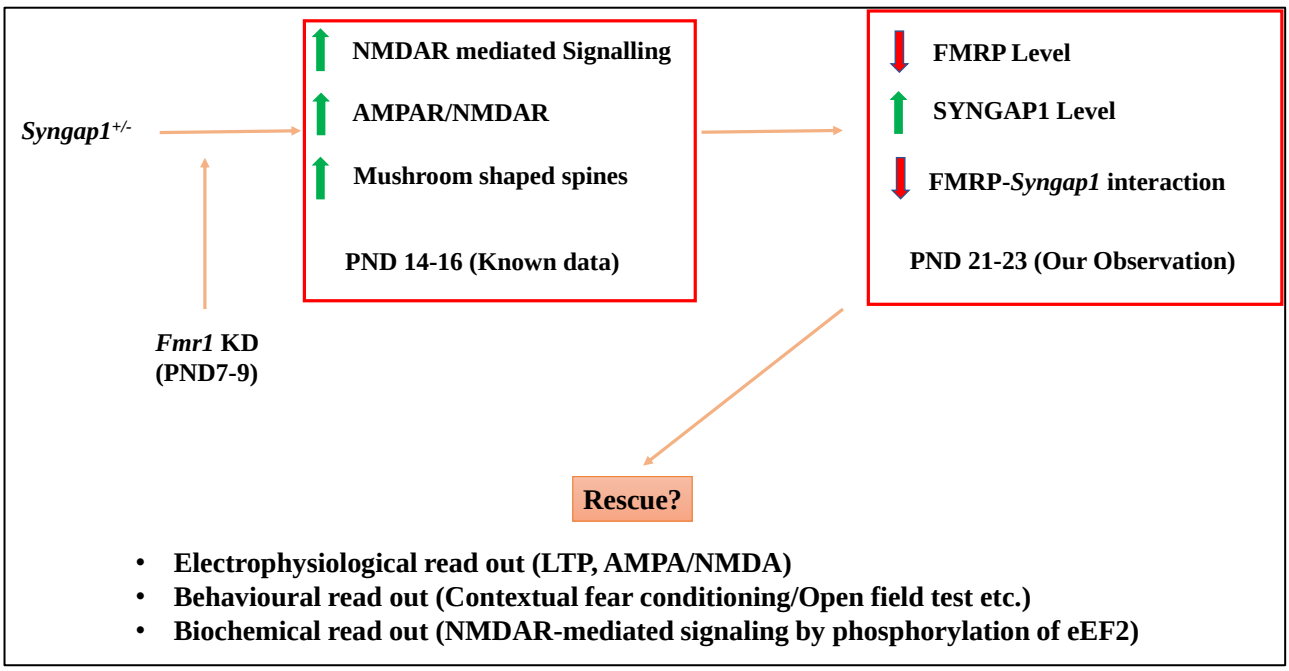

Figure 5-1: Outline of future experiments

The core synaptic pathophysiology observed at postnatal day 14-16 in *Syngap1*$^{+/-}$ mice. The work of this thesis showed that due to reduced FMRP level and reduced FMRP-*Syngap1* mRNA interaction, *Syngap1* mRNA translation was upregulated at postnatal day 21-23 in *Syngap1*$^{+/-}$ mice. Probably, due to increased SYNGAP1 level, NMDAR-mediated signalling was rescued at postnatal day 21-23. So, *Fmr1* KD at postnatal day 7-9 may increase the translation of *Syngap1* mRNA, leading to an increased level of SYNGAP1 at postnatal day. Different electrophysiological, biochemical, and behavioural assays will be done to evaluate the rescue of the *Syngap1*$^{+/-}$-related pathophysiology observed in the mouse model.

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