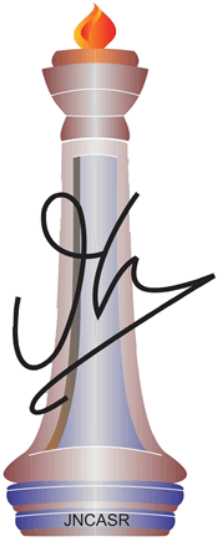
Investigating the Effect of *Syngap1* Heterozygous Mutation on Astrocytes

A thesis submitted for the partial fulfilment of the

MASTER OF SCIENCE

degree as part of the Integrated Ph. D. programme

(Biological Sciences)



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June 2018

DECLARATION

I hereby declare that the thesis entitled "**Investigating the effect of *Syngap1* heterozygous mutation on astrocytes**" is a result of investigations carried out by myself under the guidance of Dr. James P. Clement Chelliah at Chelliah Laboratory, Neuroscience Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bengaluru, India. This work has not been submitted elsewhere for the award of any other degree.

In keeping with the norm of reporting scientific observations, due to acknowledgements have been made wherever the work described has been based on other investigators. Any omission, which might have occurred by oversight or misjudgement, is regretted.

9th June 2018 Anjali Amrapali Vishwanath

CERTIFICATE

This is to certify that the work described in this thesis titled "**Investigating the effect of *Syngap1* heterozygous mutation on astrocytes**" is the result of investigations carried out by Ms. Anjali Amrapali Vishwanath in Chelliah Laboratory, Neuroscience Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bengaluru, India, under my guidance.

Dr. James Chelliah

(Research Supervisor)

Date: 09 June 2018

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LIST OF ABBREVIATIONS

µg: microgram

µL: Microlitre

ABAT: 4-aminobutyrate aminotransferase

ALDH1L1: Aldehyde dehydrogenase 1 family L1

AMPA: α-amino-3-hydoxy-5-methyl-4-isoxazolepropionic acid

AMPA-R: AMPA receptor

Anti-Anti: Antibacterial-antimycotic

AQP4: Aquaporin 4

BBB: Blood brain barrier

bp: Base pairs

BSA: Bovine serum albumin

CAMKII: Ca^{2+}/Calmodulin-dependent protein kinase II

CC: coiled-coil

CCD: Charge-coupled device

CNS: Central nervous system

CNV: Copy number variations

CPCSEA: Committee for the purpose of control and supervision of experimental animals

CT: Chamber Temperature

DHPG: Dihydrocyphenyl glycine

DIV: Days *in-vitro*

DMEM: Dulbecco's minimum essential medium

dNTP: Deoxynucleotide triphosphate

DSM-5: Diagnostic and Statistical Manual of Mental Disorders, fifth edition

E/I: Excitation/Inhibition

EAAT: Excitatory amino acid transporter

ECL: Enhanced chemiluminescence

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

EPM: Elevated plus maze

EPSP: Excitatory post-synaptic potential

ERK: Extracellular signal-regulated kinases

FABP7: Fatty-acid-binding protein 7

FBS: Fetal bovine serum

FGF: Fibroblast growth factor

FMRP: Fragile X mental retardation protein

FXS: Fragile X syndrome

GABA: γ- amino butyric acid

GAP: GTPase activating protein

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GEF: Guanine nucleotide exchange factors

GFAP: Glial fibrillary acidic protein

GLAST: Glutamate-aspartate transporter

GLT1: Glutamate transporter 1

GLUT1: Glucose transporter 1

GPCR: G-protein coupled receptors

GSTM1: Glutathione-S-transferase μ1

H_2O_2: Hydrogen peroxide

HBSS: Hank's balanced salt solution

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Het or Syn-Het: *Syngap1*$^{+/-}$ or *Syngap1* heterozygous

HRP: Horse radish peroxidase

ID: Intellectual disability

IPSP: Inhibitory post-synaptic potential

IQ: Intelligence quotient

kDa: Kilodalton

LIF: Leukaemia inhibitory factor

LIMK: LIM kinase

LRRTMs: Leucine rich repeat transmembrane proteins

LTD: Long-term depression

LTP: Long-term potentiation

MAPK: Mitogen-activated protein kinases

MECP2: Methyl-CpG-binding protein 2

MEGF10: Multiple EGF-Like-Domains 10

mEPSCs: miniature excitatory post-synaptic currents

MERTK: Tyrosine protein-kinase Mer receptor

mg: milligram

mIPSCs: miniature inhibitory post-synaptic currents

mM: millimolar

mPFC: medial prefrontal cortex

MRI: Magnetic resonance imaging

mRNA: Messenger RNA

ms: millisecond

mTOR: Mammalian target of rapamycin

mV: millivolt

NaOH: Sodium hydroxide

NGS: Normal Goat Serum

NMDA: N-methyl D-aspartate

NMDA-R: NMDA receptor

NO: Nitric Oxide

NPC: Neural progenitor cells

NSID: Non-syndromic intellectual disability

OCT: Optimal cutting temperature compound

OT: Object Temperature

P or PND: Post-natal day

PAGE: Polyacrylamide gel electrophoresis

PAK: p21-activated kinase

PB: Phosphate buffer

PBS: Phosphate buffered saline

PBST: Phosphate buffered saline with 0.1% Tween 20

PCR: Polymerase chain reaction

PFA: Paraformaldehyde

PGE: Prostaglandins

PH: Pleckstrin homology

PSD: Post-synaptic density

PVDF: polyvinylidene fluoride

RAC: RAS related C3 botulinum toxin substrate

RHEB: RAS homolog enriched in brain

RNA: Ribonucleic acid

ROI: Region of interest

rpm: Rotations per minute

SAP90: Synapse associated protein 90

SDS: sodium dodecyl sulphate

SH3: SRC homology 3

SID: Syndromic intellectual disability

SPARC: Secreted protein, acidic, rich in cysteine

SYNGAP1: Synaptic RAS GTPase activating protein 1

SYST-A: System A

SYST-N: System N

TAE: Tris acetate EDTA

TARPs: Transmembrane AMPA-R regulating proteins

TSP: Thrombospondin

TTX: tetrodotoxin

V: Volts

WHO: World health organization

WT: Wild-type (*Syngap1*$^{+/+}$)

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Chapter 1 Introduction

Proper functioning of complex systems depends on the precise and apt coordination of its components at multiple levels. A failure in this can lead to aberrant systemic functioning. One such complex system which requires co-ordinated function in our body is the brain that is central to the nervous system. It consists of 86 billion and 85 billion non-neuronal cells (Herculano-Houzel and Lent 2005). Neurons are cells specialised in conducting electrical impulses. They are the fundamental computational units of the brain and are highly interconnected. Other than the soma or cell body, neurons have highly differentiated processes such as axons to transmit signals, and dendrites to receive signals from other neurons. This way, neurons form a highly complex and intricate network whose properties, functioning, and dynamics determine the behaviour of an organism (Kandel 2013). The behaviour of an organism enables it to sense, process or understand, and appropriately respond to environmental stimuli. Therefore, any abnormalities in processes regulating neuronal network properties can lead to brain and behavioural disorders. Resultantly, such an organism cannot sense or respond to its environment in a normal manner, which can be detrimental to its survival.

1.1 Neuronal Plasticity

Dynamism is a distinctive, consistent, and essential property of neuronal networks that are responsible for its development and function. This ability of the network to change is called plasticity. Since the brain is a complex multi-level system, modulations can be brought about in any of these components at different levels and times, leading to diverse scales and degrees of plasticity that affect different network properties and behaviour in consequence.

1.1.1 Postnatal developmental Plasticity

The assembly of a neuronal network across the development of an organism depends upon changes brought about by the amalgamation of extrinsic environmental factors and intrinsic genetic factors. At birth, there is an increase in neurogenesis (production of neurons) and migration of neurons (from the zone of production to respective anatomical location) occurs (Stiles and Jernigan 2010). However, very few rudimentary neuronal connections exist. As the offspring age, there is a widespread surge in the number of connections, quantitatively almost twice as much as the adult number (Stiles and Jernigan 2010). Upon environmental experience, regressive development takes place, i.e., most of the synapses are pruned (eliminated) leaving

behind non-random functional circuits. This phenomenon is analogous to when a sculptor chips away unnecessary pieces to form a sensible object. In case of neuronal networks, it is an experience that 'shapes' the circuit into its final yet flexible topology (Kolb and Gibb 2011).

1.1.2 Critical periods during development

This period of surge and moulding regression till maturity is characterised by an enhanced level of plasticity. Neuronal networks are most susceptible to changes during this period, called the critical period (Hensch 2004). A critical period is a limited time window of development during which environmental changes have maximum and permanent modifying impact on networks responsible for a phenotype or behaviour (Meredith 2015). Input-dependent modifications that occur during this period, and not before or after, are important for the proper development of the phenotype. Critical periods are unique and specific for different behaviours such as sensory processes (e.g. vision), motor skills (e.g. walking), linguistic skills (e.g. speaking), and higher cognitive processes (e.g. mathematical or critical thinking). Any significant alterations in the environmental input – such as delay, abnormality, or absence of stimuli can lead to impaired behavioural phenotype. An example for this is an elegant experiment by Wiesel and Hubel demonstrating the critical period for visual cortex development in kittens. In this study, suturing close one of the eyelids of young kittens (3 months old) led to blindness (amblyopia) despite the eye and related structures were otherwise normal. However, similar treatment in adult cats did not have the same effect (Wiesel and Hubel 1963). This study shows that normal visual development is dependent on input during a limited time window early during the development of cats. Thereafter, many studies have demonstrated the existence of critical periods for various phenotypes across different organisms (Schaefer et al. 2017; Michel and Tyler 2005). In humans, these critical periods in developing children are observed to be phases during which one can more easily learn behaviours such as language as compared to adults (Bardin 2012).

1.1.3 Synapses and plasticity

Network properties are not solely dependent on external environmental stimuli. Intrinsic factors such as neuronal communication also play an important role in neuronal functioning and plasticity.

1.1.3 (a) Synaptic Transmission

Synapses are inter-neuronal and neuromuscular junctions essential for communication. They consist of the pre-synapse that is the terminal of the signal relaying neuron and a post-synapse,

which is the receptive terminal. Based on the mode of signal transmission, synapses in the brain are of two types: electrical and chemical synapses. In electrical synapses, the pre- and post-synaptic compartments are coupled by gap junctions (Robertson, Bodenheimer, and Stage 1963). Gap junctions are channels connecting the protoplasm of two cells, allowing for bidirectional diffusion of ions and molecules across it. Thus, when a action potential arrives at an electrical pre-synapse, the signal is relayed onto the post-synaptic cell mediated by ionic diffusion across the gap-junction (Bennett et al. 1963; Furshpan 1964; Pereda et al. 2013). Whereas in chemical synapses, the pre-synapse converts electrical impulses (membrane potentials) into chemical signals (neurotransmitters) which are then released into the synaptic space. Specific receptors then receive the neurotransmitters on the juxtaposed post-synapse, which is part of the receiving neuron (Kandel 2013). The pre- and post-synapses in this case are held-together by ligand molecules and the extracellular matrix. Although electrical synaptic transmission is faster, chemical synaptic transmission allows for regulation and signal modulation at various steps which are underlined below (Kandel 2013).

1.1.3 (a-i) Pre-synapse

The pre-synapse is a differentiated axonal terminal having a bulbous structure. It consists of synaptic vesicles filled with neurotransmitter molecules. Some of them are primed at the synaptic membrane interface ready to be released into the synaptic cavity (Murthy and De Camilli 2003). Upon the arrival of an action potential (a wave membrane potential difference), voltage-sensitive Ca^{2+} channels are activated. This leads to the influx of Ca^{2+}. The entry of Ca^{2+} ions leads to exocytosis or Ca^{2+}-dependent fusion of neurotransmitter containing vesicles to the membrane and emptying of contents, i.e., neurotransmitters (Augustine 2001; Kandel 2013).

1.1.3 (a-ii) Neurotransmitters

Neurotransmitters released into the synapse can be of several types, each having a different effect on the post-synapse. However, every neuron release only one type of neurotransmitter. Based on the effect the neurotransmitter can have on the post-synapse, they can be majorly classified into two categories – excitatory or inhibitory. Excitatory neurotransmitters bind to receptors leading to the generation of a small positive change in membrane potential making the neuron more likely to fire. This is called an excitatory post-synaptic potential (EPSP). Glutamate is one example of a common excitatory neurotransmitter. On the other hand, binding of inhibitory neurotransmitters to the receptors leads to depression or a small negative change

in membrane potential, making the neuron less likely to fire an action potential. This is termed as inhibitory post-synaptic potential (IPSP). γ- aminobutyric acid (GABA) is a major type of inhibitory neurotransmitter present in the central nervous system (Kandel 2013; Purves 2012).

1.1.3 (a-iii) Post-synapse

Post-synapses are specialised protrusions on dendrites of the receiving neuron called dendritic spines. They consist of a cluster of tightly packed proteins forming the 'post-synaptic density'. The density includes receptors and downstream signalling molecules important for sensing and relay of the intermediate neurotransmitter signal (Boeckers 2006).

Post-synaptic receptors can be of two types – ionotropic and metabotropic. Ionotropic receptors are ion channels whose opening depends on neurotransmitter binding. The opening thereby induced, leads to the influx of specific ions into the cell, leading to ion-specific effects. Metabotropic receptors are a type of G-protein coupled receptors (GPCR's) that are not conducive to ions. Upon ligand (neurotransmitter) binding, metabotropic receptors undergo conformational changes activating intracellular G-protein dependent signalling pathways (Silbering and Benton 2010; Kandel 2013).

Ion influx and activation of signalling molecules can lead to (a) short-term post-synaptic electrical potentials completing the transmission process and (b) long-term chemical and structural changes that mediate plasticity. Post-synaptic potentials alone are too small (sub-threshold) to generate an action potential. The probability of the generation an action potential is in fact dictated by the spatial and temporal summation of the post-synaptic potentials at that instant (Purves 2012).

Overall, this is the process through which electrical impulses are transmitted from one neuron to another across a synaptic junction (**Figure 1-1**).

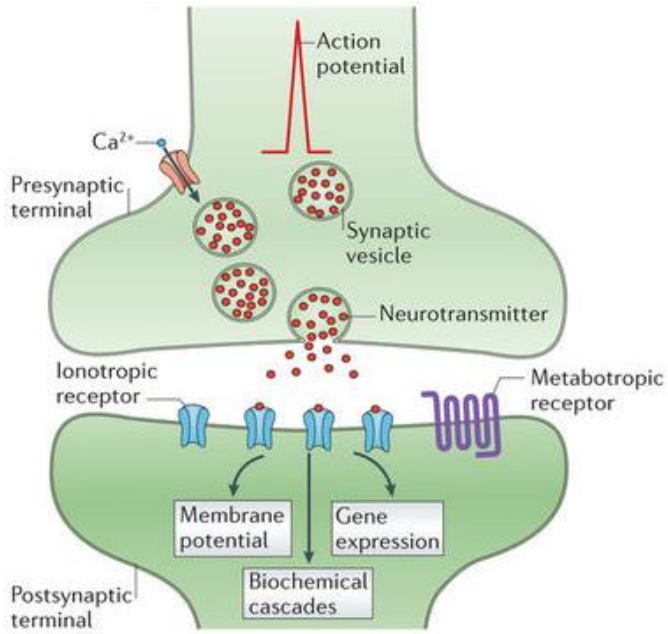

Figure 1-1 Synaptic transmission

When an action potential arrives at the axon terminal, Ca^{2+}-gated ion-channels are activated. Ca^{2+} influx leads to the release of neurotransmitters into the synaptic space. The neurotransmitters bind to the post-synaptic receptors, which then depolarise the post-synaptic membrane (Pereda 2014) (Used with permission from *Nature Reviews Neuroscience*; see **APPENDIX**)

1.1.3 (b) Synaptic Plasticity

Although the immediate effect of synaptic transmission determines neuronal firing, it can have long-term modifying effects on synaptic properties. Synapses are the smallest yet central neuronal structural unit capable of undergoing plasticity (Castren and Hen 2013). This can occur on various time-scales: short-term and long-term plasticity and consequently have short-lived or long-lasting effects. Among this, long-term synaptic plasticity has been the most widely studied form of plasticity as it is the process considered to be cardinal for the conversion of external experience into long-lasting traces of memory (Citri and Malenka 2008).

1.1.3 (b-i) Long-Term Plasticity

The human brain consists of around 0.15 quadrillion (in the neocortex alone) synapses (Pakkenberg et al. 2003), not all of which are active simultaneously or randomly. At a given time, specific configurations of neurons and synapses are activated, giving rise to functional

firing patterns that determine the behaviour of an organism. For a pattern to emerge from among the multitude of connections, the activation of specific connections needs to be favoured, while those of others suppressed. This kind of selection and modification of synapses is mediated by synaptic plasticity (Citri and Malenka 2008). This property of neurons was first theorised by Santiago Ramon y Cajal in the 19th century (Azmitia 2007). In 1949, Donald Hebb further postulated that plasticity occurs depending on the type of neuronal activity. He proposed that for two connected neurons, if the firing of one neuron persistently leads to the activation of the second, then some modification takes place such that the connections between them are strengthened. The connections are weakened if the neurons show uncorrelated firing (Hebb 1949). Thus, the pattern of neuronal activity leads to differences in synaptic weights. Synaptic weight is defined as the strength or efficacy of neurotransmission or in other words how much the firing of one neuron affects that of the receiving neuron. The strength of a synapse determines the size of the post-synaptic response (Voglis and Tavernarakis 2006).

Glutamate binding receptor NMDA (N-methyl D-aspartate) receptor mediated plasticity is one of the most widely studied forms of plasticity. It involves the action of both AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA receptors (Traynelis et al. 2010). AMPA receptors are ion channels that open upon binding of glutamate. NMDA receptors also are dependent on glutamate binding. However, Mg^{2+} blocks the ion pore at voltages less than zero. When glutamate is released, it binds to AMPA receptors, which then opens, allowing the influx of Na^{+} ions. This increases the membrane voltage to above 0 mV and depolarises the cell. This leads to the removal of the Mg^{2+} block from the NMDA receptor ion channel (Mayer, Westbrook, and Guthrie 1984; Nowak et al. 1984). Ca^{2+} ions then enter the cell, activating signalling pathways mediating long-term modifications (Willard and Koochekpour 2013).

Based on this, the strength of a synapse can either be increased (a given signal leads to a greater post-synaptic response) or decreased (a given signal leads to a reduced post-synaptic response) (**Figure 1-2**). The direction of this modification is governed by spike timing dependent plasticity (Caporale and Dan 2008). If the presynaptic neuron fires within 20 ms before the firing of the post-synaptic cell, the released glutamate can activate numerous AMPA receptor (AMPAR) and NMDA receptors, causing a large Ca^{2+} influx. A large Ca^{2+} influx activates many protein kinases, that in turn activate many downstream pathways that bring about modifications of the post-synapse. AMPA receptor trafficking is one such pathway, where there is increased exocytosis of the AMPA receptors onto the post-synaptic membrane. Due to

the increased number of receptors, a given pre-synaptic signal will give rise to an increased post-synaptic response (Markram et al. 1997; Bi and Poo 1998). Cytoskeletal pathways are also activated leading to the enlargement of the synapse. This type of plasticity is called long-term potentiation (LTP) (Bliss and Lomo 1973; Citri and Malenka 2008) (**Figure 1-2**).

On the other hand, if the pre-synaptic cell fires 20 ms after the post-synaptic cell has fired, then it leads to long-term depression (LTD) or decrease in post-synaptic response (Markram et al. 1997; Debanne, Gahwiler, and Thompson 1994) (**Figure 1-2**). This happens because the post-synaptic cell has just fired, the membrane is now in the repolarising phase. The lower voltage in this period causes most of the NMDA receptors to be deactivated. Thus, when glutamate is released, only a few NMDA receptors are activated resulting in a reduced Ca^{2+} influx. The abated Ca^{2+} influx activates phosphatases, not kinases, that leads to the endocytosis or removal of AMPA receptors from the post-synapse. The post-synaptic response is therefore decreased for a given signal (Bi and Poo 1998). Additionally, the post-synapse structure reduces in size. Thus, the connection between the given pre-synapse and post-synapse is weakened (Citri and Malenka 2008).

LTP and LTD have received widespread attention as they are considered to be the cellular correlate of learning and memory (Bliss and Collingridge 1993; Citri and Malenka 2008; Nabavi et al. 2014). Memory is the recollection /reoccurrence of a pattern when a similar stimulus is provided. When memory is being formed, persistent stimulus/activity reinforce certain pathways and suppress others by the mechanism of LTP and LTD, creating a designated network pattern. Later, when the same or similar stimulus is provided, the same pattern is regenerated due to the previous reinforcement.

The precise functioning of a many (especially synaptic) proteins is required for non-erroneous synaptic transmission and plasticity. Therefore, a mutation in any of the genes encoding these proteins can lead to mental disorders (Blanpied and Ehlers 2004).

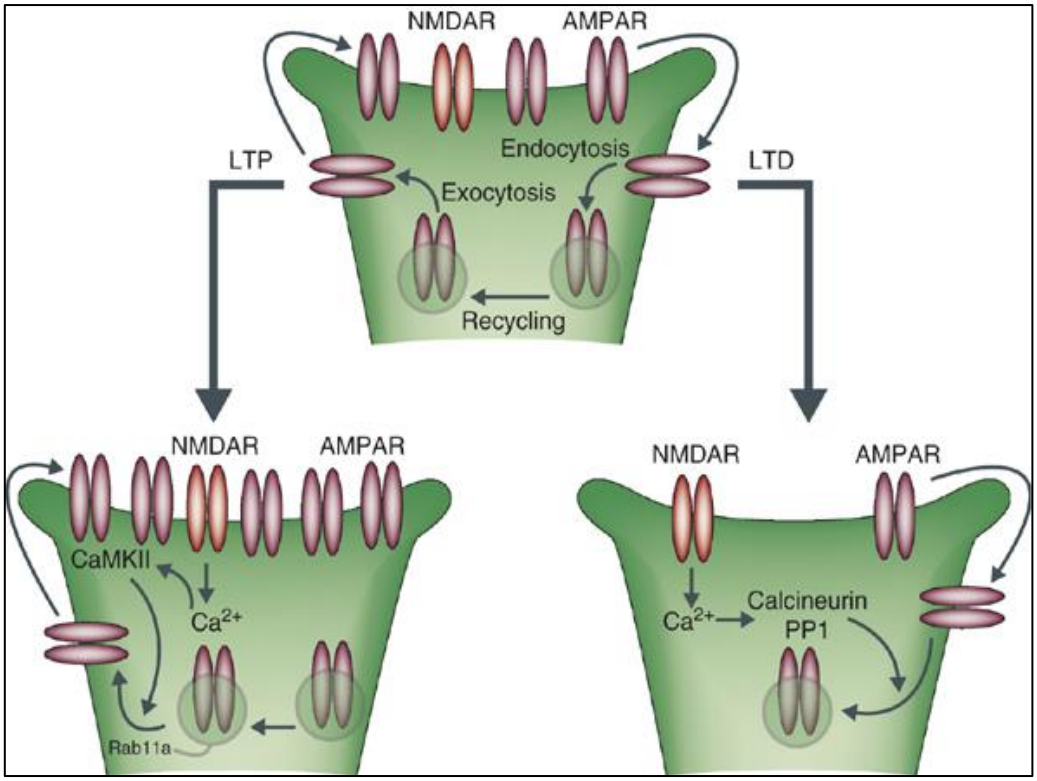

Figure 1-2 Long-Term plasticity

The efficacy or strength of the synapses as altered based on the nature of the previous signalling. Strong and persistent signalling leads to strengthening of the synapse or Long-term potentiation. On the other hand, weak or absence of signalling leads to weakening of the synapse or Long-term depression (Citri and Malenka 2008). (Used with permission from *Neuropsychopharmacology*; see **APPENDIX**)

1.2 Intellectual Disability

Intellectual Disability (ID; previously known as 'Mental Retardation') is one of the most commonly occurring complex neurodevelopmental disorder affecting 1-3% of the world population (van Bokhoven 2011). It is defined by the WHO as 'a significantly reduced ability to understand new and complex information (impaired intelligence). This results in a reduced ability to cope independently (reduced social functioning), and begins before adulthood, with a long-lasting effect on development" (Katz and Lazcano-Ponce 2008).

1.2.1 Diagnosis of ID

Intellectual Disability is diagnosed using standardised tests as a measure of intelligence. Individuals with an Intelligence Quotient (IQ) of less than 70 (which is two standard deviations

below the population mean of 100) are considered to fall within this category. According to the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders, fifth edition), proper diagnosis of Intellectual Disability should meet the following three criteria (Medicine, National Academies of Sciences, and Medicine 2015):

“

1. Deficits in intellectual functioning - reasoning, problem-solving, planning, abstract thinking, judgment, academic learning, and learning from experience”—confirmed by clinical evaluation and individualised standard IQ testing.
2. Deficits in adaptive functioning - that significantly hamper conforming to developmental and sociocultural standards for the individual's independence and ability to meet their social responsibility; and
3. The onset of these deficits during childhood.

”

1.2.2 Classification of ID

Intellectual Disability is classified based on two main criteria – (a) Severity (b) Symptoms

1.2.2 (a) Classification of ID based on the severity

Based on severity, ID is classified into five types: Mild, moderate, severe, profound and ‘unable to classify’ (Katz and Lazcano-Ponce 2008).

Initial studies performed a simple classification into these categories based on IQ score as shown in **Table 1-1**.

Severity	**IQ score**
Mild	50-69
Moderate	35-49
Severe	20-34
Profound	<20

Table 1-1 IQ based classification of Intellectual disability

However, according to DSM-5, the severity is no longer determined based on IQ, but on adaptive capabilities and the level of support required by the individual. This is assessed by

evaluating needs across 49 activities falling under six categories including home life, community life, life-long learning, employment, health and safety, and social life.

1.2.2 (b) Classification of ID based on symptoms

Based on the symptoms, Intellectual Disability can be classified into Syndromic (SID) and Non-Syndromic Intellectual Disability (NSID) (Kaufman, Ayub, and Vincent 2010).

1.2.2 (b-i) Syndromic ID

Patients with SID present with other evident morphological and metabolic abnormalities along with ID. Fragile X Syndrome is a type of SID. It is caused by a mutation in the gene encoding Fragile X Mental Retardation Protein (FMRP). Other than having severe cognitive deficits, the patients present with other abnormalities such as the elongated face, broad forehead, scoliosis, macrocephaly, etc.

1.2.2 (b-ii) Non-syndromic ID

In NSID, however, the patients present with cognitive impairment as the primary symptom. However, this definition is being challenged as these patients do exhibit other less obvious neurological abnormalities and defects. ID caused by mutations in the SYNGAP1 gene is an example of this subtype.

1.2.3 Causes of Intellectual Disability

Intellectual Disability is a complex disorder and can be caused by a wide range of factors (multifactorial). However, the cause for about 60% of the cases has not been identified. It is evident that most of these factors cause disruptions during more than one stage of brain development.

Factors causing Intellectual Disability can be broadly classified into three types: (a) Non-inheritable genetic factors (b) hereditary factors (c) acquired factors, and (d) environmental and sociocultural factors (Katz and Lazcano-Ponce 2008).

1.2.3 (a) Non-inheritable genetic factors

This includes disorders caused by genetic defects that were not inherited from the parent. This can be due to large-scale genomic changes such as chromosomal aberrations, copy number variations or mutations in single genes.

1.2.3 (a-i) Chromosomal Aberrations

In this, whole or large parts of chromosomes are either deleted, translocated, or duplicated. About 15% of ID cases can be attributed to chromosomal aberrations, two-thirds of which can be explained by the trisomy of the 21st chromosome or Down's Syndrome (47, XX +21 or 47, XY +21). Other examples include Klinefelter's Syndrome (47, XXY), Edward's Syndrome (Trisomy 18), Cri-du chat syndrome (deletion in chromosome 5), etc (Katz and Lazcano-Ponce 2008)

1.2.3 (a-ii) Copy number variations (CNVs)

Copy number variations are genomic insertions or deletions more than 1000bp (>1 kb) in size. Increasing number of studies have associated pathogenic CNVs with ID. Currently, about 20% of cases with ID can be attributed to copy-number variations (Katz and Lazcano-Ponce 2008; Pinto et al. 2010; Weldon et al. 2018).

1.2.3 (a-iii) Monogenic causes

ID is also known to be caused by damaging mutations in single genes (Katz and Lazcano-Ponce 2008). Examples include Fragile X Syndrome (mutations in *FMR1*) (Santoro, Bray, and Warren 2012), *SYNGAP1*-related NSID (Hamdan et al. 2011), Rett's Syndrome (mutations in *MECP2*) (Woodyatt and Ozanne 1997), etc. It must be noted that monogenic mutations can also lead to visible chromosomal abnormalities. For example, in Fragile X Syndrome, constrictions are observed near the tips of the X-chromosome (Butler 1998).

1.2.3 (b) Hereditary factors

ID causing hereditary factors are those that are inherited from the parent. These include metabolic disorders such phenylketonuria, Tay-Sachs disease, glycogen deposit disease, etc. (Reichenberg et al. 2016; Katz and Lazcano-Ponce 2008)

1.2.3 (c) Acquired factors

Non-genetic exogenous factors acquired during development can also cause brain disruptions leading to Intellectual Disability. These factors can occur either during the prenatal, peri-natal and post-natal developmental stages. Complications of any nature (e.g., Pre-natal: malnutrition, peri-natal: neonatal anoxia, post-natal: meningeal infection) during these phases can lead to ID. Complications can be caused by metabolic disruptions, exposure to toxins (e.g., foetal alcohol syndrome) or infections (e.g., syphilis, rubella) (Katz and Lazcano-Ponce 2008).

1.2.3 (d) Environmental and sociocultural factors

Environmental circumstances and socio-economic status can play a large role in development. Studies have indeed demonstrated that a greater fraction of the ID cases has been reported in developing countries with an increased level of poverty. This can be explained by the fact that such circumstances can have multifactorial effects (problems such as poor nutrition, hygiene, familial instability affecting all periods of development) (Reichenberg et al. 2016; Katz and Lazcano-Ponce 2008).

1.2.4 Treatment for ID

There is no known cure for intellectual disability. However, there exist many treatments that target different symptoms to relatively improve the quality of life (Kaufman, Ayub, and Vincent 2010). The type of treatment also depends on the time of diagnosis. If the disease is diagnosed early, specialised developmental training can be provided along with pharmacological treatments. Pharmacological treatments aim to correct for functional deficits caused by the causal factors (primarily genetic). This calls for a need to understand functions and mechanism of actions of factors causing ID, to develop effective therapeutic strategies (Harris 2006).

1.3 *SYNGAP1*-related Intellectual Disability

1.3.1 Properties of the SYNGAP1 protein

SYNGAP1 is a 140 kDa protein first identified by Chen et al., as one of the targets of phosphorylation by Ca^{2+}/Calmodulin-dependent protein kinase II (CAMKII) in the PSD (Post-synaptic Density) in the rat brain (Chen et al. 1998; Jeyabalan and Clement 2016) (**Figure 1-3**). CAMKII is one of the kinases activated by high-level of NMDA receptor mediated Ca^{2+} influx during glutamatergic synaptic transmission. In 1998, Kim et al., isolated the same protein, again in rat brain, as an interacting partner of the PDZ domains of PSD-95/SAP90 (Synapse associated protein 90) protein family using yeast two-hybrid studies (Kim et al. 1998).

1.3.1 (a) SYNGAP1 expression pattern

In studies investigating the tissue-specific expression of SYNGAP1 it was observed that SYNGAP1 expression is restricted and most highly expressed in the brain, especially in the cortex, hippocampus, and olfactory bulb regions (Kim et al. 1998; Chen et al. 1998). The SYNGAP1 expression was also studied on several types of cells (Kim et al. 1998; Chen et al. 1998; Zhang et al. 1999). They observed that SYNGAP1 expression was specific to only

excitatory glutamatergic cells (Kim et al. 1998; Chen et al. 1998; Zhang et al. 1999). Only SYNGAP1-β expression was found in inhibitory GABA expressing cells (Moon et al. 2008). In neurons, SYNGAP1 co-localized with NMDA and AMPA receptors at synapses. SYNGAP1- α1 localised to both synapses and cell body. However, SYNAGP1-β localisation was restricted to the spines. Further, it was shown that the localisation of SYNGAP1 is independent of the presence of the T/SXV PSD-95 binding motif (Vazquez et al. 2004). The localization of SYNGAP1 shows an activity dependent pattern. It was demonstrated that depolarisation of the post-synapse led to the removal of SYNGAP1 from the post-synaptic density towards the cytosolic side of the spine, near the region of increased CAMKII concentration (Yang et al. 2011). This could result from the fact that, removal of SYNGAP1 (α1), could free up PDZ 'slots' on PSD-95, where other plasticity proteins such as NEUROLOGIN-1, TARPs (Transmembrane AMPA-R regulating proteins), LRRTMs (Leucine rich repeat transmembrane proteins) are now free to bind (Walkup et al. 2016).

1.3.1 (b) SYNGAP1 domains

Studies carried have identified three major domains – a Pleckstrin homology (PH) domain, a C2 domain, and a RAS-GAP domain (Kim et al. 1998; Chen et al. 1998). Two additional motifs were identified – SH3 binding motif and a T/SXV motif. Later, the SYNGAP1 protein was also reported to contain a coiled-coil (CC) motif (Rumbaugh et al. 2006). Each of the motifs with respective amino acid positions (according to SYNGAP1 α1 isoform) and function are specified below:

1.3.1 (b-i) PH domain (Position 150-251) –

The PH (pleckstrin homology) domain near the N-terminus, binds to membrane phospholipids, helping recruitment of the protein to the membrane

1.3.1 (b-ii) C2 domain (Position: 263 to 362) –

The C2 domain is known to play a role in Ca2+ dependent phospholipid binding and membrane targeting processes. The C2 domain of SYNGAP1 was also found to be essential for its RAP-GAP activity (Pena et al. 2008).

1.3.1 (b-iii) RAS- GAP domain (Position: 392 to 729) –

The RAS-GAP activity of SYNGAP1 was first demonstrated by Chen et al., in 1998 (Chen et al. 1998). RAS-GAP stands for RAS-GTPase activating protein. RAS belongs to a family of proteins termed as small GTPases. RAS controls a myriad of signalling pathways and is

activated in its GTP-bound form. The GDP-bound RAS is its inactive form. The activation of RAS activity, by conversion of RAS-GDP to RAS-GTP, is mediated by Guanine nucleotide Exchange Factors (GEFs). RAS protein has low intrinsic GTPase activity, resulting in the very slow conversion of RAS-GDP to RAS-GTP. GTPase activating proteins (GAPs) catalyse this process and help in rapid inactivation of RAS. Thus, SYNGAP1 is a negative regulator of RAS activity (Kim et al. 1998).

1.3.1 (b-iv) SH3-binding motif (Position: 785 to 815) –

It was identified as a proline-rich sequence mediating interaction with SH3 (SRC homology 3) motif-containing proteins (Chen et al. 1998).

1.3.1 (b-v) CC motif (Position: 1189 to 1262) –

The coiled-coil motif in SYNGAP1 was found to responsible for trimeric assembly of SYNGAP1 proteins (Zeng et al. 2016).

1.3.1 (b-vi) T/SXV motif (Position: last four amino acids)-

T/SXV is a consensus sequence for the motif where either Threonine (T)/Serine (S) can be present in the first position. Any amino acid (X) can be present in the second position. Valine (V) must be present in the last (third) position. This motif is required for interaction with PDZ domain-containing proteins such as PSD-95. This enables its recruitment into the post-synaptic density and more specifically onto the NMDA receptor-signalling complex. IN SYNGAP1, this motif is represented by the last four C-terminal amino acids QTRV (Glutamine, Threonine, Arginine and Valine) (Chen et al. 1998).

1.3.1 (c) SYNGAP1 isoforms

SYNGAP1 has many isoforms arising due to differential start sites and alternative splicing. Though the existence of SYNGAP1 isoforms was first identified by in 1998 (Chen et al. 1998), a detailed characterization and analysis of these isoforms was carried out by in 2012. (McMahon et al. 2012). SYNGAP1 has many isoforms differing in their N- and C- terminal.

1.3.1 (c-i) N-terminally varying SYNGAP1 isoforms

Study by McMohan et al., have shown that 4 N-terminal differing isoforms, SYNGAP1-A, SYNGAP1-B, SYNGAP1-C, and SYNGAP1-E, result from different transcription start sites (TSS). The TSS for SYNGAP1-A is 10 kbp upstream to those of B and C. The SYNGAP1-A, SYNGAP1-B, and SYNGAP1-E have complete PH domains whereas SYNGAP1-C is shorter and lacks the PH Domain. These isoforms have different properties. Upon treatment with

bicuculline (inhibits GABA activity leading to increased excitation), SYNGAP1-B and C isoforms were upregulated, whereas SYNGAP1-A was downregulated. Addition of tetrodotoxin (TTX) blocked these changes, showing that the responses were activity-dependent. Regarding expression, SYNGAP1-A and B isoforms showed peak levels of expression at P14 (Post-natal Day 14) and thereafter there was a downregulation of expression (McMahon et al. 2012). Peak expression of these isoforms during developmental stages and down-regulation upon neuronal maturation point towards that SYNGAP1 is essential for normal brain development and mutations in this gene can be causal to disorders such as Intellectual Disability, Autism and Epilepsy (Berryer et al. 2013) which have onset during development.

1.3.1 (c-ii) C-terminally varying SYNGAP1 isoforms

There exist seven SYNGAP1 isoforms that have different C-terminals as a consequence of alternative splicing- SYNGAP1-α1, SYNGAP1-α2, SYNGAP1-β1, SYNGAP1-β2, SYNGAP1-β3, SYNGAP1-β4, and SYNGAP1-γ. The SYNGAP1-α1 has been the most characterised isoform of SYNGAP1. It consists of an intact T/SXV motif and localises to the post-synaptic density due to its interactions with PDZ domains. Whereas the other SYNGAP1 isoforms lack this motif. However, these isoforms still localise to the spines. These isoforms two have a differing mode of action. SYNGAP1- α1 negatively regulates, synaptic strength, whereas SYNGAP1- α2 positively regulates it. Notably, this regulation is also dependent on the N-terminal identity of the SYNGAP1 protein (McMahon et al. 2012). Thus, different SYNGAP1 C-terminal isoforms differentially modulate neuronal synaptic response.

1.3.1 (d) SYNGAP1 interactions

Studies interactive partners of SYNGAP1 have helped decipher additional function of SYNGAP1. SYNGAP1 has been shown to interact with two PDZ domain-containing scaffolding post-synaptic proteins: PSD-95 and SAP102 (Kim et al. 1998). SYNGAP1 is indirectly associated with the GRIN2B (Glutamate receptor ionotropic, 2B) subunit of the NMDA receptor; it binds to PSD-95 which in turn binds to GRIN2B (**Figure 1-3**). The PDZ binding motif also enables the interaction of SYNGAP1 with MUPP1 (Multi-PDZ domain protein 1), which is part of the NMDA receptor signalling complex (Krapivinsky et al. 2004). SYNGAP1-β, which lacks the T/SXV motif, binds to the α-subunit of CAMKII (Li et al. 2001). A yeast two-hybrid screen performs in extracts from P6 mouse cerebellum, showed that the C-terminals of UNC51.1 and UNC51.2 interact with α1 and α2 isoforms of SYNGAP1

respectively, with a higher affinity for the α2 isoform. UNC51.1 and UNC51.2 are proteins localised to axonal shafts and growth cones. They are serine/threonine kinases important for the process of axon elongation (Tomoda et al. 2004). Functions uncovered from such interactions have been covered in section 1.3.4.

1.3.2 *SYNGAP1* mutations in neurodevelopmental disorders

The *SYNGAP1* gene is considered a high-risk factor gene for neurodevelopmental disorders as pathogenic mutations in this gene have been reported to be associated with many diseases (Kilinc et al. 2018).

1.3.2 (a) *SYNGAP1* related NSID

SYNGAP1 mutations are known to be causal for ~2-8% of unexplained cases of ID. Mutations in *SYNGAP1* were first linked to ID in studies carried out by Hamdan et al., in 2009 (Hamdan et al. 2009). In this study, 94 patients presented with NSID symptoms – global developmental delay, delayed motor development, hypotonia, severe language impairment, and moderate to severe mental retardation without dysmorphic features were screened. Three of these 94 probands had mutations in the SYNGAP1 gene (K138X, R579X and L813RFSX22). Two (K138X, R579X) were nonsense mutations, and one (L813RFSX22) was a frameshift leading to truncation. In all 3, the mutations were heterozygous and de-novo (absent from parental DNA). The mutations K138X, R579X and L813RFSX22, affected the N-terminal, RAS-GAP and SH3-binding domains respectively. Following this initial study, multiple reports have implicated mutations in SYNGAP1 in ID (Krepischi et al. 2010; Zollino et al. 2011; Writzl and Knegt 2013; Parker et al. 2015; Rauch et al. 2012; Deciphering Developmental Disorders 2017; Jeyabalan and Clement 2016).

1.3.2 (b) SYNGAP1 mutations in other neuropsychiatric disorders

Other than NSID, SYNGAP1 mutations have been linked to other neurodevelopmental brain disorders. SYNGAP1 related ID is highly co-morbid with autism and epilepsy. In one study, two patients with NSID also exhibited seizures and macrocephaly (Hamdan et al. 2011). One NSID patient also showed autistic features, but seizures were absent. After this, multiple studies have linked SYNGAP1 mutations to autism and epilepsy (Carvill et al. 2013; von Stulpnagel et al. 2015). SYNGAP1 mutations have also been linked to schizophrenia (Jeyabalan and Clement 2016; Purcell et al. 2014).

Mutations in *SYNGAP1* being common to the pathogenesis of multiple neurodevelopmental disorders demonstrates the fact that it plays and central and crucial role in the normal development of the brain and understanding the role of SYNGAP1 in the monogenic SYNGAP1-related NSID, may help shed light on more complex disorders such as autism and epilepsy and schizophrenia.

1.3.3 Mouse models of *SYNGAP1*-related NSID (*Syngap1*$^{+/-}$ *mice)*

Due to ethical and technical reasons, human studies limit the ability to explore SYNGAP1 function by experimental manipulation. Thus, mouse models were developed to understand the biological function and importance of SYNGAP1 in brain development, along with the aetiology of *SYNGAP1*-related NSID. In humans, *SYNGAP1* gene is present on chromosome 6 (Krepischi et al. 2010; Jeyabalan and Clement 2016), whereas, in mouse, the *Syngap1* gene is present on chromosome 17 (Kim et al. 2003; Jeyabalan and Clement 2016). Since, in all patients, heterozygous mutations have been found to be sufficient to produce the phenotype, *Syngap1*$^{+/-}$ mouse models were developed. Also, complete loss of SYNGAP1 in *Syngap1*$^{-/-}$ leads to early post-natal death, once again showing its crucial developmental role (Kim et al. 2003).

Over the past few years, multiple mouse models have been developed for SYNGAP1-related NSID. For a transgenic mouse to qualify as a model for a disease, it must satisfy three criteria- face validity, construct validity and predictive validity. The mouse model must exhibit same/corresponding phenotypes as seen in human patients (face validity). The method in which the construct is created must give rise to similar molecular and cellular phenomena (construct validity). And lastly, the model should be able to be fit for treatments that can be extrapolated onto humans (predictive validity) (Willner 1986).

Multiple valid *Syngap1*$^{+/-}$ (*Syngap1* heterozygous) mouse models have been developed to study the effect of *Syngap1* haploinsufficiency. The first mouse model was developed by Kim et al., in 2003 by inserting a neomycin cassette at the site of exon 7 and 8, leading to deletion of C2 domain and introduction of a premature stop codon (Kim et al. 2003). A similar neomycin strategy was followed in the development of another mouse model in which the C2 and GAP domain were deleted (Komiyama et al. 2002). An alternative study also used neomycin cassette insertion into intron3 flanked by loxP sites. An additional lox P site at intron 9, then lead to the deletion of exons 4-9 in the presence of Cre-recombinase (Vazquez et al. 2004). In a recent

study, a conditional knockout mouse model was developed, where the *Syngap1* gene chad flanking *flox* sites in one of the alleles. Thus, the knockdown of *Syngap1* was CRE-dependent (Clement et al. 2012) and could be restricted to selected desired cells or time points exhibiting CRE expression, to further dissect the role of SYNGAP1 in the brain.

1.3.4 Investigations into the function of SYNGAP1

1.3.4 (a) SYNGAP1 is a PSD-95 binding RAS-GAP downstream of NMDA receptor and CAMKII

Initial studies (Chen et al. 1998; Kim et al. 1998) characterising the SYNGAP1 molecule, demonstrated that SYNGAP1 is a post-synaptic protein that forms a complex with NMDA receptors and post-synaptic scaffolding protein PSD-95. This implicated its function in synaptic signalling. SYNGAP1 was also shown to contain a RAS-GAP domain like the other to RAS-GAPs known at the time – NEUROFIBROMIN-1 and p120-RAS-GAP (Chen et al. 1998). The RAS-GAP activity was also proved using *in-vitro* enzymatic assays, wherein SYNGAP1 was incubated with recombinant H-RAS, and % GDP was measured using thin layer chromatography. In this study, it was also observed that SYNGAP1 consists of 29 consensus sites (RXXS/T) for phosphorylation by CAMKII. P^{32}-labelling experiments implicated that SYNGAP1 is a substrate of CAMKII and using afore-mentioned *in-vitro* assays, it was shown that CAMKII negatively regulates the RAS-GAP activity of SYNGAP1 (Chen et al. 1998). Further studies confirmed that SYNGAP1 is indeed phosphorylated by CAMKII (Oh, Manzerra, and Kennedy 2004), mediated by SYNGAP1-CAMKII complexing with MUPP1 (Krapivinsky et al. 2004). It is important to note that, the pattern/sites of phosphorylation of SYNGAP1 by CAMKII determine the direction of change (increase/decrease) in RAS-GAP activity. Majorly, phosphorylation by has been shown to increase (not decrease) the RAS-GAP activity of SYNGAP1 by 70-95% (Oh, Manzerra, and Kennedy 2004) (**Figure 1-3**).

1.3.4 (b) SYNGAP1 regulates multiple RAS-like small GTPases

Other than RAS, SYNGAP1 has also been shown to regulate multiple RAS-like small GTPases.

1.3.4 (b-i) RAP-GTPase:

Experiments have demonstrated the RAP-GAP activity of SYNGAP1 (Walkup et al. 2015) (**Figure 1-3**). In fact, SYNGAP1 is a more potent RAP inactivator as compared to RAS

(Krapivinsky et al. 2004). The C2 and GAP domain interaction of SYNGAP1 was found to be necessary for its RAP-GAP activity (Pena et al. 2008). It is the differential phosphorylation of SYNGAP1 that alters its affinity towards the different small G-proteins (RAS-/RAP-). Phosphorylation of SYNGAP1 by CAMKII increases its RAP-GAP activity by 76% and its RAS-GAP activity only by 25%. Whereas, when phosphorylated by CDK5, its RAS-GAP activity is increased by 98% and RAP-GAP activity by 20%.

1.3.4 (b-ii) RAB5-GTPase:

RAB5 is another small GTPase like RAS known to be essential for endocytic membrane fusion and trafficking. It has been demonstrated that, the GAP domain negatively regulates RAB5 activity in a complex with UNC51.1, scaffolded by SYNTENIN. This signalling complex regulates endocytosis, important for axon formation in developing neurons (Tomoda et al. 2004).

1.3.4 (c) SYNGAP1 regulates MAPK signalling

1.3.4 (c-i) ERK/MAPK regulation: Until a study done by Komiyama et al., in 2002, the pathways downstream to SYNGAP1 were not known. In this paper, they report that SYNGAP1 negatively regulates the phosphorylation of ERK (Extracellular signal-regulated kinases)/MAPK (Mitogen-activated protein kinases) through RAS using immunoblotting. As compared to WT mice, SYNGAP1 heterozygous mice had increased levels of phospho-ERK (Komiyama et al. 2002). This was further confirmed by using SYNGAP1 inhibition and overexpression studies (Rumbaugh et al. 2006; Kim et al. 2005) (**Figure 1-3**).

1.3.4 (c-ii) P38 MAPK regulation: In contrast to ERK/MAPK regulation, it was observed that SYNGAP1 positively regulates the phosphorylation of P38-MAPK kinase, however in an activity-independent manner (Rumbaugh et al. 2006).

1.3.4 (d) SYNGAP1 regulates spine-localised protein expression

ERK/MAPK regulated mTOR (mammalian target of rapamycin) activity has been known to regulate local protein synthesis in dendritic spines (Costa-Mattioli et al. 2009). Subsequently, it was demonstrated that SYNGAP1 regulates local protein synthesis mediated by ERK/MAPK signalling (Wang, Held, and Hall 2013). ERK/MAPK regulates RHEB (RAS homolog enriched in braon), which then activates mTOR. mTOR further regulates dendritic local protein synthesis. Since, SYNGAP1 negatively regulates translation, *Syngap1*$^{+/-}$ mice have increased

levels of protein synthesis as shown by different studies (Wang, Held, and Hall 2013; Barnes et al. 2015)

1.3.4 (e) SYNGAP1 regulates post-synaptic cytoskeletal changes

Other than the ERK, SYNGAP1 also regulates synaptic cytoskeletal proteins. RAS-GTP binds to a RAC-GEF (RAC- guanine nucleotide exchange factor) TIAM1 (T-lymphoma invasion and metastasis-inducing protein 1), which activates RAC (RAS related C3 botulinum toxin substrate). Activated RAC, initiates PAK (p21-activated kinase) to phosphorylate LIMK (LIM domain kinase). Phospho-LIMK, in turn, phosphorylates COFILIN, which is a regulator of synaptic actin dynamics. Phospho-COFILIN catalyses actin polymerisation. Thus, as expected, in *Syngap1*$^{+/-}$ mice, increased RAS activity leads to increased actin polymerisation (Carlisle et al. 2008) **(Figure 1-3**).

1.3.4 (f) SYNGAP1 regulates AMPA receptor trafficking

NMDA-R mediated signalling has been known to regulate the trafficking of AMPA receptors to the post-synaptic membrane, leading to modulation of synaptic response or in other words – plasticity (Morris et al. 1986; Dudek and Bear 1992). Since, SYNGAP1 is associated with NMDA-R mediated signalling, the role of SYNGAP1 in AMPA-R trafficking was probed. For the first time, Kim et., al showed using immunocytochemistry that the number of AMPA receptors was increased in *Syngap1*$^{+/-}$ mice (Kim et al. 2003). The number of NMDA receptors, however, remained unchanged. Upon overexpression of SYNGAP1, the surface AMPA receptors were reduced (Kim et al. 2005). (Rumbaugh et al. 2006) showed that the increase/decrease in surface AMPA receptors was due to altered trafficking in primary neuronal cultures. Thus, SYNGAP1-regulates NMDAR-mediated AMPA-R trafficking and plasticity, and mutations in this gene can cause impairments in these phenomenon (discussed in section 1.3.5 (d)) (**Figure 1-3**).

1.3.5 Neuronal and synaptic phenotypes of mice with *Syngap1* mutations

1.3.5 (a) Increased neuronal apoptosis in *Syngap1*$^{-/-}$ mice

Alterations at the molecular signalling level manifest into cellular abnormalities. *Syngap1*$^{-/-}$ mice die within the first postnatal week (Kim et al. 2003). Upon investigation, it was found that this was due excessive neuronal apoptosis, due to increased activation of caspase-3 in these mice (Knuesel et al. 2005). This might be under the regulation of Ras, as Ras is a known

mediator of cellular proliferation (Duronio and Xiong 2013). Thus, SYNGAP1 is an essential protein regulating neuronal survival and proliferation during development.

1.3.5 (b) *Syngap1*$^{+/-}$ neurons show adult neuron-like morphology at an early age

Looking into the other neuronal phenotypes, it has been demonstrated that there are no changes in neuronal branching of hippocampal pyramidal neurons. However, a reduction in neuronal spatial volume was observed (Clement et al. 2012). On the other hand, somatosensory pyramidal neurons in *Syngap1*$^{+/-}$ mice exhibit adult like characteristics -increased arbour complexity, total neurite length and occupational volume at P21 itself. This difference is abolished in adult (~P60) mice. This indicates that neurons in *Syngap1*$^{+/-}$ mice undergo early neuronal maturation (Aceti et al. 2015).

1.3.5 (c) *Syngap1*$^{+/-}$ mice have increased number of mature spines

As compared to those from Wild-type mice, hippocampal neurons cultured from *Syngap1*$^{+/-}$ mice have spines which are mushroom-shaped and larger in size (a hallmark of mature spines) 10 DIV. In cultures, this difference is maintained until 21 DIV, when all synapses normally reach maturation (Vazquez et al. 2004). Other than this, increased clusters of post-synaptic proteins were also observed. The same observation (increased mushroom-shaped spines) was seen in adult hippocampal neurons *in-vivo* (Carlisle et al. 2008).

SYNGAP1 is a synaptic protein with a developmentally regulated expression pattern. SYNGAP1 expression increases during development peaks at PND 14 and stays at a slightly reduced level after this (Clement et al. 2012). In a normal developmental trajectory, immature filopodial shaped spines are converted into mature mushroom-shaped spines, over a time period of 3 weeks, postnatally. Upon inspection of spine sizes at various developmental time points, it was observed that in *Syngap1*$^{+/-}$ mice, no difference in the number of mushroom-shaped spines (hippocampal neurons) was seen in the first week of development. AT PND14, *Syngap1*$^{+/-}$ mice have increased number of mushroom-shaped spines as compared to WT controls. The levels of mushroom-shaped spines in *Syngap1*$^{+/-}$ mice at PND14 is comparable to adult levels of the same. This increase in spine size was attributed to an increased number of AMPA receptors in the post-synapse and due to cytoskeletal mediated changes (Carlisle et al. 2008). The similarity of P14 spine size to that of adult spine size, indicates that in *Syngap1*$^{+/-}$ neurons there is the accelerated maturation of dendritic spines (Clement et al. 2012) (**Figure 1-4**).

Thus, both *in-vitro* and *in-vivo* neurons showed phenotypes consistent with early maturation. Development of the brain consists of a series of temporally arranged processes. Therefore, such changes in the maturation time trajectory of neurons, can lead to incomplete development or aberrations in neurons and neuronal circuits properties such critical-period plasticity (discussed in section 1.3.5 (e)), giving rise to phenotypes corresponding to neurodevelopmental disorders (Washbourne 2015).

1.3.5 (d) Synaptic transmission and plasticity in *Syngap1*$^{+/-}$ mice

Since SYNGAP1 is a post-synaptic molecule, a part of the NMDA receptor mediated signalling pathway, mutations in this gene can lead to abnormalities in synaptic transmission and plasticity.

1.3.5 (d-i) Syngap1 haploinsufficiency alters basal synaptic transmission during development

Across development, there is a gradual maturation of dendritic spines (filopodial to mushroom shaped), which involves increased insertion of AMPA receptors leading to unsilencing of synapses by increasing the basal synaptic transmission (Sheng and Lee 2001). No changes were found in the basal synaptic transmission or paired-pulse ratio in adult *Syngap1*$^{+/-}$ mice (Komiyama et al. 2002). However, demonstrated that basal synaptic transmission is increased in P14 mice but remains unchanged in young (<P14) and adult mice (Clement et al. 2012). This early increase in synaptic transmission further supports the concept of accelerated maturation of neuronal dendritic spines.

1.3.5 (d-ii) Glutamate receptor current in *Syngap1*-mutant mice

As SYNGAP1 takes part in post-synaptic signalling, the post-synaptic excitatory currents were studied. No change in NMDA receptor mediated currents was observed in adult *Syngap1*$^{+/-}$ mice (Komiyama et al. 2002). However, cultured neurons from *Syngap1*$^{-/-}$ mice showed an increase in both the frequency and amplitude of the mEPSCs (miniature excitatory post-synaptic currents)(Vazquez et al. 2004). This increase in frequency indicates an increase in the number of functional synapses, and the increase in the amplitude suggests an increased response from a given post-synapse. Since there is no change in NMDA receptor current, this change might result from an increase in the number of AMPA receptors and AMPA-mediated current. In *Syngap1*$^{+/-}$ mice, no change in mEPSC frequency or amplitude was seen in any age group other than at P14. A corresponding increase in AMPA/NMDA ratio was also observed only at P14 (Clement et al. 2012). This demonstrates that at an early age of P14, *Syngap1*$^{+/-}$

mice has elevated AMPA-mediated currents and an increased number of active synapses. This corroborates with an increased number of mature, enlarged mushroom-shaped spines (**Figure 1-4**) and increased synaptic AMPA-receptor cluster in *Syngap1*$^{+/-}$ mice at an earlier developmental age (Clement et al. 2012; Aceti et al. 2015). This phenomenon is not restricted to the hippocampus. A study performed in Layer 2/3 neurons of the mPFC (medial prefrontal cortex) in the mouse forebrain area, shown to be majorly responsible for the phenotypes observed in *Syngap1*$^{+/-}$ mice, also show increased glutamatergic activity – these neurons exhibit mEPSC amplitude and frequency (Ozkan et al. 2014).

1.3.5 (d-iii) LTP is reduced in *Syngap1*$^{+/-}$ mice

NMDA-R mediated plasticity is one of the most important and widespread forms of plasticity in the brain (Citri and Malenka 2008). Since, SYNGAP1 participates in NMDAR-mediated signalling that subsequently gives rise to long-term plasticity (Komiyama et al. 2002), mutations in *Syngap1* can lead to impaired plasticity. Recordings from slices obtained from *Syngap1*$^{+/-}$ mice, show an impairment in LTP as predicted. Although potentiation was successful in *Syngap1*$^{+/-}$ mice, there was no stabilisation of LTP (Komiyama et al. 2002; Kim et al. 2003; Ozkan et al. 2014). The RAS-mediated activation of ERK is essential for maintenance of LTP. *Syngap1*$^{+/-}$ mice showed no change in levels of phospho-ERK upon induction of LTP. However, basal levels of ERK was already high in *Syngap1*$^{+/-}$ mice (Komiyama et al. 2002; Ozkan et al. 2014). Since the basal NMDAR-mediated signalling was already saturated in *Syngap1*$^{+/-}$ mice, induction of LTP fails to elicit a further rise in this signalling pathways, leading to an impairment in LTP. However, the genetic rescue of *Syngap1* is able to restore SYNGAP1 signalling in adult *Syngap1*$^{+/-}$ mice, rescuing LTP (Ozkan et al. 2014). Thus, SYNGAP1 regulates NMDAR-mediated plasticity.

1.3.5 (d-iv) LTD in *Syngap1*$^{+/-}$ mice

Depending upon the nature of stimulus, NMDA-R mediated signalling can also lead to Long-term synaptic depression (Luscher and Malenka 2012). LTD is a lesser studied form of synaptic plasticity, especially in *Syngap1*$^{+/-}$ mice. Kim et al., showed that LTD induced by Paired-pulse low-frequency stimulation is unchanged in *Syngap1*$^{+/-}$ mice (Kim et al. 2003). However, LTD induced by acute application of NMDA is impaired in *Syngap1*$^{+/-}$ mice (Carlisle et al. 2008). Thus, NMDA receptor-mediated LTD is impaired in *Syngap1*$^{+/-}$ mice. LTD can also be induced by the activation of metabotropic glutamate receptors (mGluRs). Upon mGluR stimulation using DHPG (Dihydrocyphenyl glycine, an mGluR agonist), the induced LTD is enhanced in

Syngap1$^{+/-}$ mice (Barnes et al. 2015). They demonstrate that LTD in *Syngap1*$^{+/-}$ mice is independent of protein synthesis. They speculate the cause of this to be the overabundance of proteins required for the maintenance of LTD resulting from increased protein synthesis in *Syngap1*$^{+/-}$ mice (Barnes et al. 2015). As a result of SYNGAP1 being downstream of NMDA-R, it mediates bi-directional plasticity (LTP and LTD) depending on the type of stimulus.

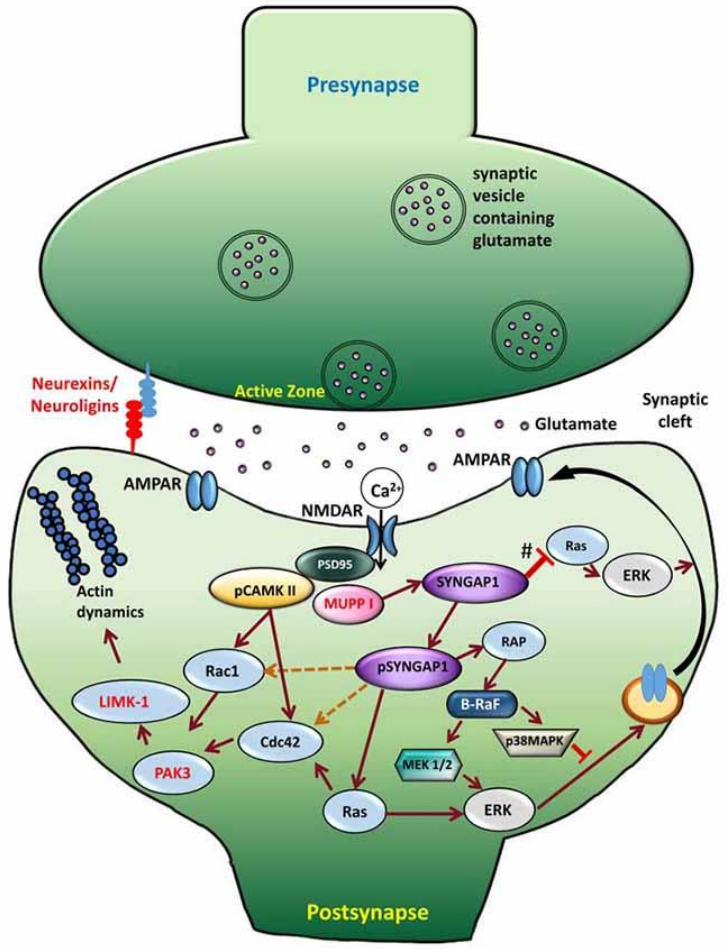

Figure 1-3 Role of SYNGAP1 in neurons

When glutamate is released from the pre-synapse, it binds and activate AMPA receptors that leads to Na^+ ion influx and increase in membrane potential. This removes the Mg^{2+} block from the NMDA receptors leading to Ca^{2+} influx. This then leads to phosphorylation of CAMKII, which then phosphorylates SYNGAP1. pSYNGAP1 then inhibits RAS and RAC mediated signalling pathways. These pathways control AMPA receptor insertion and post-synaptic cytoskeletal dynamics respectively. In case of SYNGAP1 haploinsufficiency, the inhibition of these pathways is lifted leading to increased AMPA receptor insertion and enlarged spines (Jeyabalan and Clement 2016).

1.3.5 (e) Neuronal circuit properties in *Syngap1*$^{+/-}$ mice

SYNGAP1 has been shown to play a crucial role in the brain at a synaptic and cellular level where mutations in *Syngap1* leads to neuronal abnormalities. Since neuronal circuit dynamics depend on the underlying synaptic and neuronal properties, mutations in *Syngap1* might also translate to altered or abnormal neuronal circuit properties.

1.3.5 (e-i) *Syngap1*$^{+/-}$ mice have increased excitation

Increased excitatory current in *Syngap1*$^{+/-}$ mice, can lead to alterations in the excitation/inhibition (E/I) ratio in the brain. A balanced E/I is crucial for proper synaptic and circuit dynamics and eventual emergent brain functions. Therefore, an altered E/I ration can lead to synaptic, circuit and behavioural abnormalities. In line with this theory, *Syngap1*$^{+/-}$ mice were found to have increased neuronal and network excitation (**Figure 1-4**). In the mPFC of *Syngap1*$^{+/-}$ mice, neurons were shown to develop increased mEPSC activity (frequency and amplitude) by adulthood. A decrease in mIPSC was also observed, thus shifting the E/I balance towards increased excitation (Ozkan et al. 2014). *Syngap1*$^{+/-}$ mice correspondingly exhibit seizure phenotype and reduced threshold to flurothyl-induced seizures (Clement et al. 2012). Additionally, EEG recordings show epileptiform discharges in *Syngap1*$^{+/-}$ mice, which are absent in their Wild Type counterparts. (Ozkan et al. 2014). This phenomenon may underlie the epileptic phenotype commonly observed in human patients with ID.

1.3.5 (e-ii) Critical periods are altered in *Syngap1*$^{+/-}$ mice

Critical periods are developmental time windows, during which experience-dependent changes in network patterns can shape and have a long-lasting effect on organismal behaviour. Activity-dependent reorganisation of circuits takes place when the critical period is open and is reduced when it is closed (Bardin 2012; Meredith 2015). E/I balance has been shown to one of the major factors that regulate the duration and amount critical period plasticity that shape ultimate circuit and behavioural phenotypes (Hensch and Bilimoria 2012; Hensch 2004; Clement et al. 2012). A disrupted E/I balance without homeostatic compensation has been implicated in many neurodevelopmental disorders (Meredith 2015; LeBlanc and Fagiolini 2011). Thus the E/I imbalance (Clement et al. 2012; Ozkan et al. 2014) and abnormal homeostatic plasticity (Wang, Held, and Hall 2013) might lead to alterations in critical period windows in *Syngap1*$^{+/-}$ mice. Indeed, studies performed in the thalamocortical region of *Syngap1*$^{+/-}$ mice brain, showed that high-frequency stimulation failed to elicit LTP in P4 and P7 mice in this region. This difficulty in eliciting LTP (reduced experience plasticity) in this region usually arises only by the end of

the first postnatal week in WT mice marking the maturation of these synapses (Crair and Malenka 1995). Thus, *Syngap1*$^{+/-}$ mice demonstrate early maturation of synapses and circuits, further confirmed by reduced spine motility and increased spine size at early stages in these mice (Clement et al. 2013; Clement et al. 2012; Aceti et al. 2015). Early maturation and hyperactivity in these circuits lead to premature activation of synapses from silent to functional. This process requires the NMDA receptor mediated AMPA receptor insertion involving SYNGAP1. Thus, a dysfunctional *Syngap1* resulting from mutations, leads to the precocious unsilencing of synapses, reducing their ability for experience dependent plasticity, thereby altering the critical period (Jeyabalan and Clement 2016) (**Figure 1-4**). This phenomenon is not restricted to the thalamocortical region but also stands true for other areas of the brain (Clement et al. 2013).

1.3.6 Morphological and anatomical phenotypes of *Syngap1*$^{+/-}$ mice

Syngap1$^{+/-}$ mice do not have any gross anatomical alterations in the brain (Komiyama et al. 2002; Aceti et al. 2015). However systematic analysis of brain volume using MRI, has revealed that *Syngap1*$^{+/-}$ mice have reduced brain volume (Kilinc et al. 2018). This corresponds to reports of microcephaly in ID patients with *SYNGAP1* mutations (Hamdan et al. 2011).

At the microscale level, *Syngap1*$^{+/-}$ mice show the disrupted organisation of cortical circuits (Aceti et al. 2015). *Syngap1*$^{+/-}$ mice also show aberrant pattern formation of barrels in the somatosensory cortex (Barnett et al. 2006).

1.3.7 Behavioural phenotypes of *Syngap1*$^{+/-}$ mice

Precise and coordinated functioning of synapses is important for proper network properties, which in turn are responsible for behavioural endophenotypes of organisms. With respect to *Syngap1*$^{+/-}$ mice, network hyperactivity can lead to altered synaptic dynamics and abnormal connectivity, giving rise to a range of behavioural abnormalities. *Syngap1*$^{+/-}$ mice breed and groom normally as compared to their Wild-type counterparts. They are similar in size to WT mice. Detailed behavioural analysis revealed multiple differences of different nature.

In open field analysis, *Syngap1*$^{+/-}$ mice show increased locomotor activity during both young and adult stages (Guo et al. 2009; Clement et al. 2012; Muhia et al. 2010). They show increased exploration at the central zone as compared to Wild-type indicating that these mice are hyperactive and might have abnormal levels of anxiety.

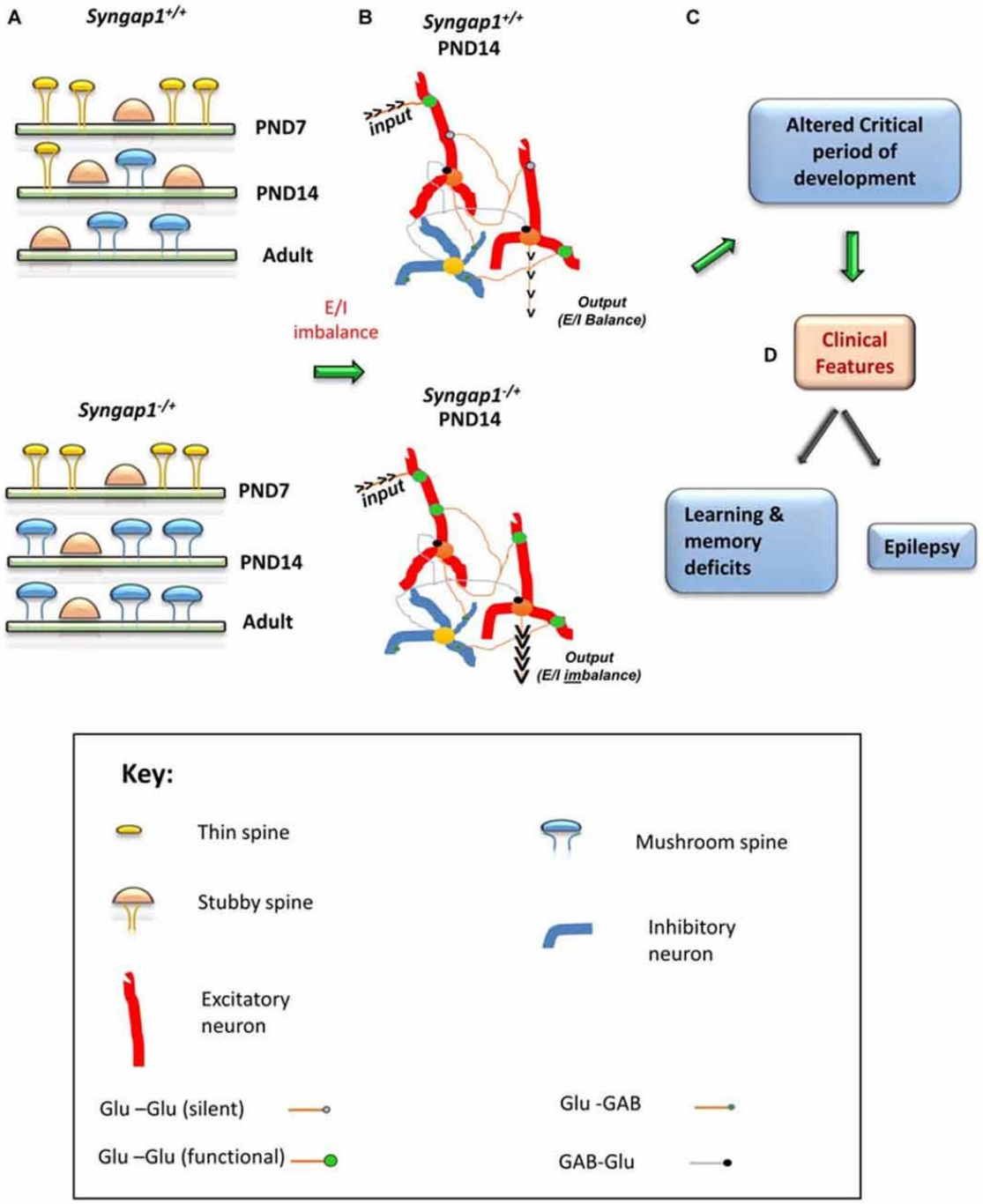

Figure 1-4 Impact of SYNGAP1 haploinsufficiency on neuronal circuit function

SYNGAP1 haploinsufficiency leads to dysregulated post-synaptic signalling causing increased AMPA receptor insertion, spine size and density. The abnormal upregulation of signalling leads to accelerated maturation of spines. It also creates an imbalance in excitation to inhibition ratio leading to abnormal circuit dynamics and altered critical period (Jeyabalan and Clement 2016).

Using the elevated plus maze (EPM) test, *Syngap1*$^{+/-}$ mice spent more time in the open arm which is otherwise not preferred and avoided by WT mice (Muhia et al. 2010). This demonstrated that *Syngap1*$^{+/-}$ mice exhibit reduced levels of anxiety. In-depth analysis performed by (Kilinc et al. 2018), demonstrated that this abnormal behaviour in *Syngap1*$^{+/-}$ mice arises from a disrupted innate response and not due to altered associative learning. In addition to EPM, cliff avoidance test revealed that *Syngap1*$^{+/-}$ mice are more prone to explore and jump off edges as compared to WT mice, demonstrating the increased risk-taking behaviour. This is in keeping with similar behaviour observed in patients with *SYNGAP1*-related NSID (Weldon et al. 2018).

A variety of behavioural tests showed that *Syngap1*$^{+/-}$ mice demonstrate reduced learning and memory. Although no change was observed in contextual fear conditioning, *Syngap1*$^{+/-}$ mice showed decreased freezing behaviour after initial training with foot shock following an audiogenic cue. Proper response during cued fear conditioning involves proper functioning of emotional circuits and associative learning, which might be impaired in *Syngap1*$^{+/-}$ mice (Guo et al. 2009). In the Morris water maze test, *Syngap1*$^{+/-}$ mice took more time to find the hidden but known platform (due to previous experience), as compared to WT mice. When the location of the platform is altered, *Syngap1*$^{+/-}$ mice show impaired spatial memory (Muhia et al. 2010; Komiyama et al. 2002). Other than this, radial arm maze test revealed that *Syngap1*$^{+/-}$ mice also have impaired working and reference memory (Muhia et al. 2010).

With respect to social behaviour, *Syngap1*$^{+/-}$ mice failed to distinguish between familiar and unfamiliar conspecifics and preferred to spend time in isolation rather than socialising, which is a behaviour important for intra- and inter-group interactions to form social structures such as hierarchies (Guo et al. 2009).

Thus *Syngap1*$^{+/-}$ mice show defects in learning and memory, social behaviours, reduced anxiety levels and increased risk-taking behaviours demonstrating the fitness of this model for *SYNGAP1*-related NSID. (Ozkan et al. 2014) showed that most of these abnormalities could be accounted for by isolated damage caused to forebrain neuronal networks caused by *Syngap1* haploinsufficiency. Genetic rescue of *SYNGAP1* in adult phenotypes fails to rescue these behavioural abnormalities confirming the developmental nature of this disorder.

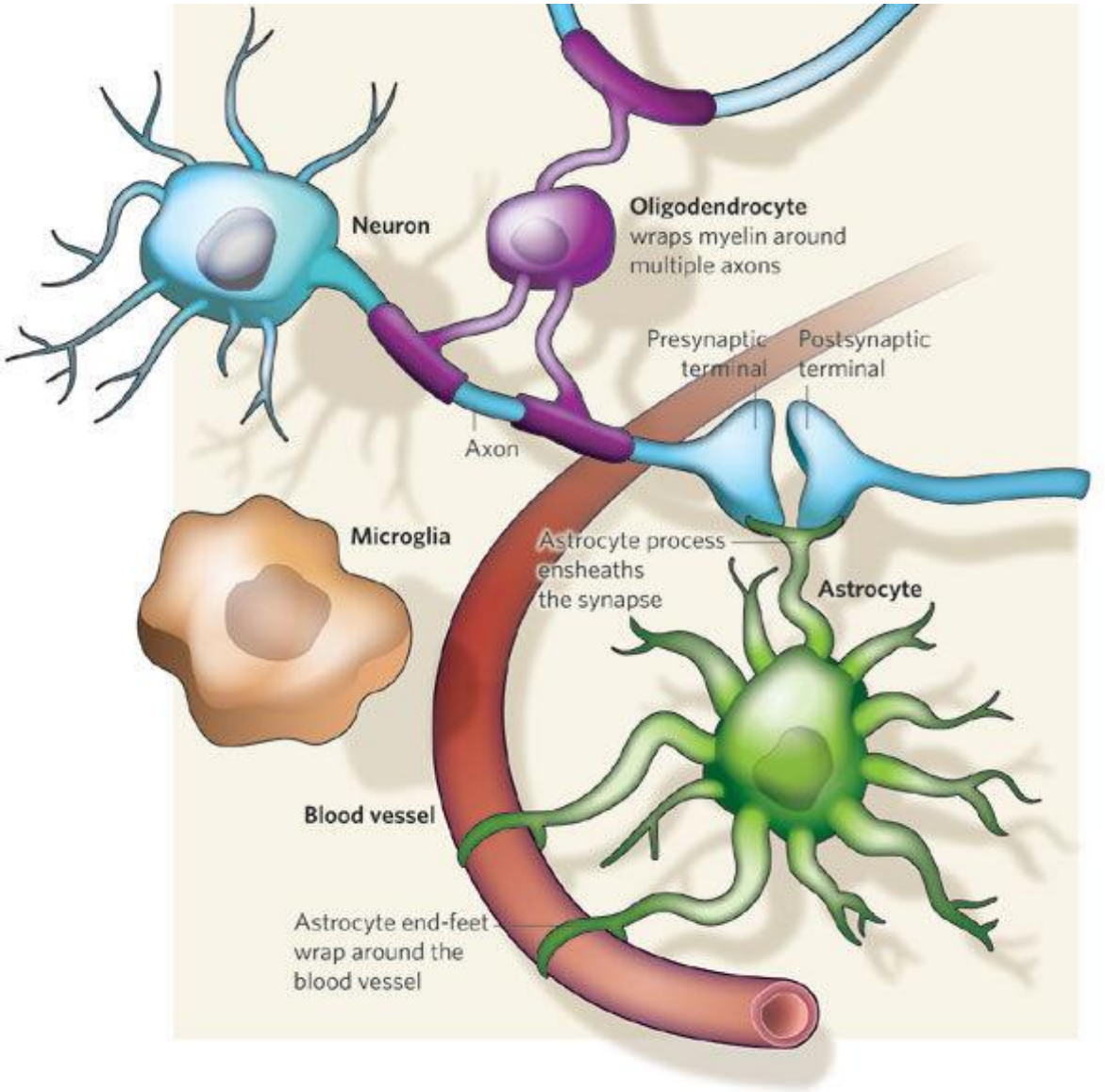

Figure 1-5 Types of glial cells in the brain

The brain consists of many non-neuronal cells called glia. Oligodendrocytes, microglia and astrocytes are the most common types of glia found in the brain (Allen and Barres 2009). (Used with permission from *Nature*; see **APPENDIX**)

1.4 Astrocytes

Other than electrical impulse conductive neurons, the brain consists of other types of cells termed as glia. This category commonly includes cells such as oligodendrocytes (responsible for myelination of neurons), microglia (immune cells of the brain) and astrocytes (**Figure 1-5**).

Astrocytes ("star cells") are glial cells present in the central nervous system. As the name suggests, these cells are star-shaped having many branches and sub-branches. These astrocytes are the most common glial cell type found in the brain. The types and properties of astrocytes varies with species, brain region, and time.

1.4.1 Types of Astrocytes

Astrocytes can be classified based on two criteria:

1.4.1 (a) Based on developmental lineage (Montgomery 1994)

Astrocytes can arise from two different types of precursor cells. These astrocytes derived from different lineages have distinct morphological properties.

1.4.1 (a-i) Type I: These develop from the precursor cells committed solely to the astrocyte lineage. The presence of these have been shown *in-vivo*. *In-vitro,* these cells have a firbroblast-like phenotype (Raff et al. 1983).

1.4.1 (a-ii) Type II: These develop from precursor cells that are bi-potential O2A cells (oligodendrocyte, type 2 astrocyte precursor). This type of precursor can thus give rise to either oligodendrocytes or astrocytes. Though these types of cells have been extensively studies in culture, little experimental evidence suggest the presence of such type II astrocytes *in-vivo* (Tabata 2015).

1.4.1 (b) Based on the rough location (Sofroniew and Vinters 2010) (**Figure 1-6**)

1.4.1 (b-i) Protoplasmic: These are found in grey matter. These are highly branched, and the ends of the astrocytic processes ensheath neuronal synapses. Their end feet also contact blood vessels forming the outer layer of the blood brain barrier.

1.4.1 (b-ii) Fibrous: These are found in the white matter. They have long fibre-like processes and are branched to a lesser degree as compared to their protoplasmic counter-part. The ends of the astrocytic processes ensheath nodes of Ranvier in this case.

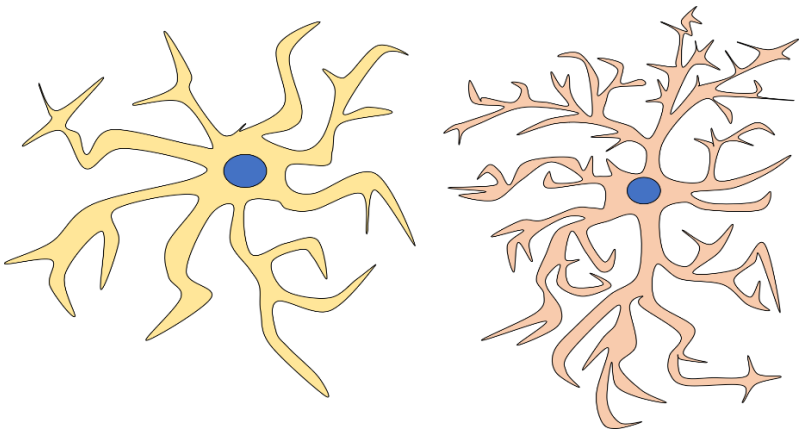

Figure 1-6 Types of astrocytes

Astrocytes can be protoplasmic (right; highly branched, found in grey matter) or fibrous (left; less branched, found in white matter).

1.4.2 Anatomical Organization

In the developing brain, newly generated astrocytes encompass overlapping domains, whereas in the adult brain, mature astrocytes have independent, non-overlapping domains (Robertson 2013; Bushong, Martone, and Ellisman 2003). They only contact other astrocytes at their distal tips in the form of gap junctions, and thereby form a continual syncytium (Montgomery 1994). In the adult brain, a single astrocyte is estimated to contact about 300-600 dendrites and more than 10^5 synapses (Halassa et al. 2007).

1.4.3 Molecular Markers for Astrocytes

Many molecules have been found that selectively label astrocytes (Sofroniew and Vinters 2010).

Glial Fibrillary Acidic Protein (GFAP) is a widely used marker to stain astrocytes. It is a protein found in intermediate filaments of astrocytes. GFAP stains the main stem branches of the astrocyte, which represent only ~15% of the total volume of the astrocyte. It does not label the fine branches of the astrocytes, which interact with neurons. Also, GFAP is not exclusive to astrocytes; it is expressed in many other cell types such as ependymal cells. Additionally, GFAP is expressed by only a fraction of the astrocytic population. Another drawback of GFAP as a marker is that GFAP expression cannot be detected in the cortex after two weeks postnatally. Thus, GFAP may not the best choice to label astrocytes, calling for the need for

better markers. Recently, other astrocyte-specific markers have been used such as ALDH1L1 (aldehyde dehydrogenase 1 family L1), AQP4 (aquaporin 4), glutamine synthetase, and S100β.

1.4.4 Role of astrocytes in brain function

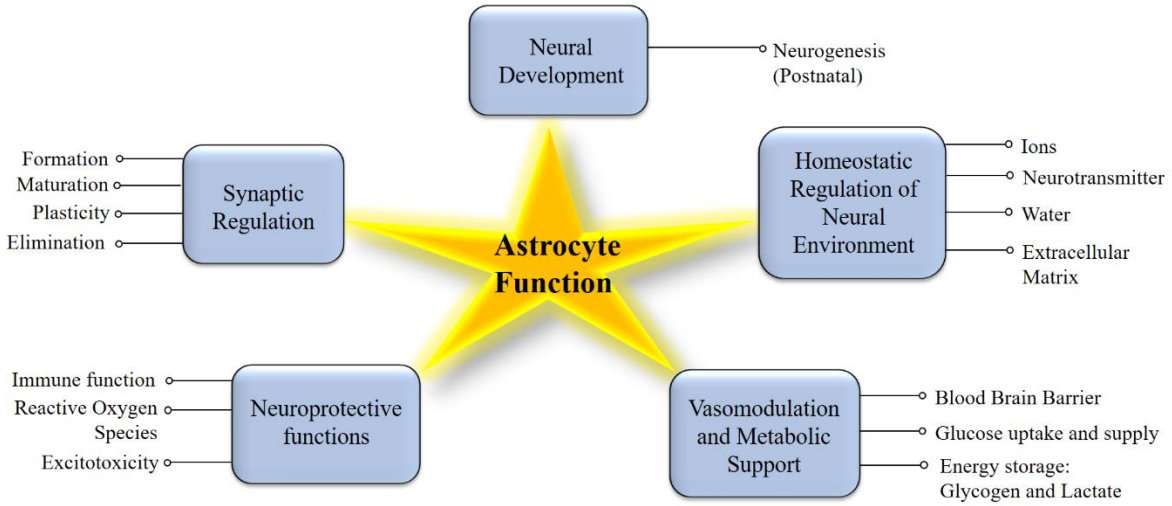

Figure 1-7 Function of astrocytes

Astrocytes, initially thought of only as supporting cell, have multifunctional and essential roles in the brain. Based on (Allen 2014; Sofroniew and Vinters 2010; Clarke and Barres 2013).

Astrocytes have been shown to play diverse and crucial role in the development and functioning of the brain as outlined henceforth. Astrocytes:

1.4.4 (a) Guide neurons during developmental migration:

In the developing brain, the lengthy processes of the radial glia act as a scaffold for the migration of neurons to take up their respective places in the brain. Once migration is over, these radial glia differentiate into astrocytes (Molofsky et al. 2012).

1.4.4 (b) Provide nutrition to neurons:

Astrocyte processes have end-feet. Of these, some end-feet contact blood vessels and are an integral part of the blood-brain barrier to take up nutrients from blood vessels and furnish neurons with energy metabolites (Sofroniew and Vinters 2010; Stobart and Anderson 2013; Elsayed and Magistretti 2015). For example, astrocytes take up glucose through GLUT1 (glucose transporter 1) and supply glucose to neurons.

Astrocytes also have glycogen reservoirs and carry out glycogenesis to produce lactate which is then supplied to neurons, during periods of intense neuronal activity (Stobart and Anderson 2013).

Since the blood-brain barrier is impermeable to blood-borne lipid-containing lipoproteins, lipid synthesis takes place *de novo* in the brain. Astrocytes are the sites of major lipid synthesis, which are then supplied to neurons. Astrocytes also synthesise cholesterol and release it complexed with Apolipoprotein E. The role of cholesterol in synapse function is disucussed later (Pfrieger and Ungerer 2011; Allen 2014).

1.4.4 (c) Reuptake and recycling neurotransmitters

During synapse function, neurotransmitters are released into the synaptic space. Some of it binds to postsynaptic receptors and rest remains in the space. This has to be rapidly cleared to prevent any residual stimulation. Astrocytes have receptors and transporters for of these neurotransmitters present in the synaptic space. These neurotransmitters are taken up by astrocytes, metabolised and transported to neurons for re-synthesis of neurotransmitters.

For example, in glutamatergic synapses, glutamine is taken up by astrocyte through means of transporters such as EAATs (excitatory amino acid transporters), GLT-1 (Glutamate Transporter 1), and GLAST (glutamate-aspartate transporter). This is then broken down into synaptically inactive glutamine and released back into the pre-synapse through SYST-N (system N). This is then taken up by neurons through a transporter SYST-A (system A). This can be converted back to glutamate or even GABA by neurons (Allen 2014).

1.4.4 (d) Secrete ECM proteins and adhesion molecules

Astrocytes secrete molecules that make up the extracellular matrix in the brain. They also secrete adhesion molecules. These are important for contact-mediated signalling in neurons for survival and sensing the environment (Montgomery 1994).

1.4.4 (e) Secrete neurotrophic molecules and neurite-promoting factors

Astrocytes secrete neurotrophic factors such as pyruvate that ensure neuron survival. They express neurite-promoting factors which are substrate-bound matrix proteins such as laminin. Astrocytes also express nerve growth factors (Montgomery 1994).

1.4.4 (f) Maintain homeostasis in the neural microenvironment

Astrocytes ensure homeostasis of the following things in the neuron micro-environment (Montgomery 1994; Sofroniew and Vinters 2010):

1.4.4 (f-i) Fluid:

Astrocytic processes are rich in water channels such as AQP4 (aquaporin 4) that ensure fluid homeostasis.

1.4.4 (f-ii) Ion:

Astrocytes expresses various ion channels and ion transporters. During synaptic transmission, ions, most commonly K^+, are released into the extracellular space. This must be cleared within a short duration to prevent neuronal depolarisation due to extracellular K^+ accumulation. This is one of the major functions of astrocytes.

1.4.4 (f-iii) pH:

Astrocytes have various transporters such as proton shuttles, Na^+/H^+ exchanger, bicarbonate transporters, etc. that helps in maintaining physiological pH.

1.4.4 (g) Control angiogenesis and vasomodulation

Astrocytes are responsible for the induction of angiogenesis, which is necessary for proper blood supply to the brain. With respect to the Blood Brain Barrier (BBB), astrocyte not only induces the formation of BBB during development but also regulate the properties of BBB in the adult brain. Astrocytes regulate global blood flow by increasing/decreasing blood vessel diameter by signalling through molecules such as prostaglandins (PGE) and Nitric Oxide (NO) (Sofroniew and Vinters 2010).

1.4.4 (h) Have neuroprotective functions

Astrocytes prevent neural toxicity by carrying out various protective functions. Previously described ion and transmitter uptake help prevent excitotoxicity (neuronal death due to excessive excitation). Astrocytes produce glutathione that helps in removal of damaging oxidative reactive species (Sofroniew and Vinters 2010). In case of damage/infection, astrocytes become reactive and increase in size and cell number and signal other cells such as microglia about the damage. This helps in repair of the damaged region (Montgomery 1994; Sofroniew and Vinters 2010). Astrocytes also phagocytose CNS debris and infectious agents that help in the protection of neurons (Montgomery 1994). Since astrocytes are connected by

gap junctions, any toxic substance can be easily dissipated across the continuum to reduce damage.

1.4.4 (i) Promote the myelinating activity of oligodendrocytes

Astrocytes secrete leukaemia inhibitory factor (LIF) that promotes myelination by oligodendrocytes (Molofsky et al. 2012).

1.4.4 (j) Have neuroendocrine functions

Astrocytes play a role in the secretion of neuronal hormones (Montgomery 1994).

1.4.5 Role of astrocytes with respect to synapses

It was initially thought that the role of astrocyte in synapse formations and function is secondary to previously described astrocyte support functions. However, this was disproved, and various lines of evidences show that astrocytes are actively involved in synapse formation and function. According to 'the tripartite synapse' hypothesis, other than pre-synaptic and post-synaptic terminal, astrocytic ends that ensheath the synapse is also an integral, active and functional part of the synapse (Perea, Navarrete, and Araque 2009) (**Figure 1-8**).

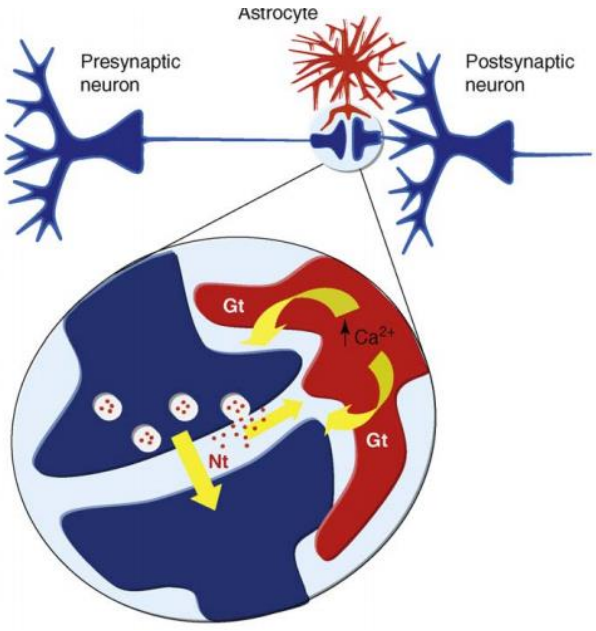

Figure 1-8 The tripartite synapse

The synapse initially thought to only consist of the pre- and post-synapse contains a third member – the end feet of astrocytes. This way astrocytes take part and regulate synaptic signalling (Perea, Navarrete, and Araque 2009). (Used with permission from *Trends in Neurosciences*; see **APPENDIX**)

1.4.5 (a) Development Role

Astrocytes have a very crucial role in neuronal development. During development, it has been observed that the timing of excitatory synapse formation coincides with the maturation of astrocytes in the brain (Clarke and Barres 2013). This suggests astrocyte requirement for synapse formation. Astrocytes signal to neurons through both secreted factors and membrane-bound factors (contact-mediated signalling). These factors help in synapse induction, formation, maturation, function, and elimination.

Role of Secreted Factors:

During development, astrocytes secrete numerous factors. Some factors help in the induction of synapses:

1.4.5 (a-i) Thrombospondins: Thrombospondins (TSPs) are extracellular matrix molecules that help in excitatory synapse induction and formation. It helps to increase the number of synapses. Thrombospondins act through α2γ1-Gabapentin receptor (a subunit of the voltage-gated Ca^{2+} ion channels). Binding of TSPs activates Ca^{2+} signalling which leads to recruitment of synaptic adhesion and scaffolding molecules to synaptic sites. TSPs also interacts with other molecules such as Neuroligins and Integrins. Thrombospondins signal through integrins leads to down-regulation of AMPA receptor levels in the synapse. Thus, synapses induced by thrombospondins are structurally normal but functionally inactive and leads to formation of silent synapses. Thrombospondins-1 and -2 are expressed during development but not in adulthood. An analogue- thrombospondin-4 may carry out similar functions in the adult brain (Clarke and Barres 2013; Allen 2014).

1.4.5 (a-ii) Hevins:

Hevins can also induce the formation of synapses, but unlike TSPs, their expression is seen even in adulthood. Hevins bind to Neuroligins and Neurexins in synapses leading to clustering of trans-synaptic adhesion molecules (Clarke and Barres 2013; Allen 2014). SPARC (secreted protein, acidic, rich in cysteine) SPARC is the antagonist of HEVIN and also works through Neurexins and Neuroligins. SPARC also signals through integrins, leading to down-regulation of membrane AMPA receptors (Kucukdereli et al. 2011).Other secreted factors help in the formation of functional synapses i.e. synaptic maturation

1.4.5 (a-iii) Glypicans:

Glypicans are molecules of the heparin sulphate proteoglycan family. These are initially membrane-bound but are then cleaved to form active signalling molecule.

Glypicans help in the maturation of synapses by increasing surface expression and clustering of surface AMPA receptors leading to an increase in frequency and amplitude of excitatory synaptic events (Clarke and Barres 2013).

1.4.5 (a-iv) Cholesterol:

Cholesterol complexed with Apo-lipoprotein-E released by astrocytes are taken up by the neuronal Apo-lipoprotein receptor. Cholesterol increases the content of transmitters in pre-synaptic vesicles, and also increases the efficacy of synaptic release (Allen 2014).

1.4.5 (a-v) ADNF: (activity-dependent neurotrophic factor):

These increase the number of NMDA receptors in the post-synapse (Allen 2014).

Astrocytes also influence formation of inhibitory GABAergic synapses, although very less is known about the molecular players in this case. However, it is known that astrocytes use separate signalling molecules for inhibitory synapse formation such as (Clarke and Barres 2013).

Astrocytes also play a role in synapse elimination either directly or indirectly. Directly, astrocytes target phagocytic receptors: MEGF10 (Multiple EGF-Like-Domains 10) and MERTK (tyrosine protein-kinase Mer receptor), which interact with weak synapses and phagocytose them, each through different signalling pathways. Indirectly, astrocytes (immature) induce expression of C1q complement protein on weak synapses, which marks them for opsonisation by microglia. In this way, astrocyte help in synaptic pruning process required for correct circuit formation (Allen 2014).

Contact-mediated signalling

During development, the dendritic spines and axonal growth cones as well as astrocytic processes are highly dynamic. Spontaneous synapses keep forming and disappearing. However, their stabilisation happens through astrocytic contact. Contact by astrocytes increases the number of and strength of synapses. γ-Protocadherins form homophilic adhesions and induce synaptogenesis (Garrett and Weiner 2009). Astrocytic contact influences both pre-synaptic axons and post-synaptic dendrites. Axons do not have the ability to receive synapses.

This is induced by astrocytes by altering the localisation of neurexins. Astrocytes also can contact dendritic spines alone and regulate their morphology (Clarke and Barres 2013). However, it has also been reported that astrocytic contact inhibits synapse formation. In the adult brain, astrocytes monitor and modulate synaptic activity.

1.4.5 (b) Astrocytes respond to synaptic activity

Astrocytes have receptors for neurotransmitters. Therefore, when the synapses are activated, the transmitters excite not only post-synaptic neuron, but also the surrounding astrocyte. Binding of transmitters to the receptors results in a change(spike) in Ca^{2+} levels in the astrocytic processes immediately and much later in the cell body.

Conversely, astrocytes also affect synaptic activity. They release transmitters and modulators –called gliotransmitters. These include ATP, adenosine, glutamate, and D-serine (Allen 2014; Araque, Carmignoto, and Haydon 2001). These gliotransmitters helps astrocytes to mediate three types of synaptic plasticity:

1.4.5 (b-i) Short-term plasticity:

Astrocytes mediate short-term plasticity (changes in vesicle release probability) by signalling through ATP. ATP is converted to adenosine in the extracellular space, and this affects different synapses differently based on the receptor-type. Through A1 receptors, the probability of vesicle release decreases, whereas through A2A receptors, the probability of vesicle release increases (Allen 2014).

1.4.5 (b-ii) Long-term plasticity:

Astrocytes can induce LTP by the Ca^{2+}-dependent release of NMDA receptor co-agonist D-serine in the presence of glutamate. Astrocytes can also release glutamate, increasing post-synaptic stimulation (Allen 2014).

1.4.5 (b-iii) Homoeostatic plasticity (synaptic scaling):

The abovementioned types of plasticity are synapse-specific. However, another kind of plasticity exists where there is a global change in the strength of all the synapses of a neuron in the correct direction. This is activity-dependent and if the neuron is continually excited, the synapses are de-sensitised, and if the stimuli is absent, the sensitivity of synapses increases. This happens for excitatory synapses, and the opposite takes place for inhibitory synapses. This opposing force is required to prevent over-strengthening of synapses (which can lead to epilepsy) due to continuous stimulation or under-function of synapses to preserve connections.

Astrocytes mediate this homeostatic plasticity in neurons by secreting TNFα that has a differing effect on different types of synapses (Allen 2014).

1.4.5 (c) Synapse ensheathment by astrocytes

Not all synapses are ensheathed by astrocytic processes. The percentage of synapses ensheathed by astrocytes differs from brain region to region. Also, the degree of synapses covered, and proximity of astrocyte to synapses can also vary. This is mediated by Eph (EPHRIN) signalling. Eph signals astrocyte process retraction. Thus, depending on if and how the synapse is ensheathed, the properties of synapse varies. In synapses that is not ensheathed by astrocytes, transmitter spill-over takes place and can affect other naked synapses. This mechanism may help coordinate or synchronize the activity of a group of closely localized and functionally isolated neurons. On the other hand, ensheathed synapses are independent and unaffected by these spill-overs (Allen 2014).

1.4.6 Astrocytes in the pathophysiology of neurodevelopmental disorders

Many neurodevelopmental disorders such as Fragile X Syndrome (FXS) and Rett's Syndrome (RS) are considered to be diseases of the synapse. In these cases, the major problem is synaptic dysfunction. Since astrocytes play such essential and active role in synapse formation and function, it is vital to study the contribution of astrocyte and its function/dysfunction to disease pathology of such disorders. Indeed, many pieces of evidence suggest that it is indeed so.

In case of both Rett's Syndrome (resulting due to loss of function of methyl-CpG-binding protein 2: MeCP2) and Fragile X Syndrome (resulting from loss of function of Fragile X mental Retardation protein: FMRP), it was found that growing Wild-type astrocyte with diseased neurons ablated the disease phenotype (stunted dendrites in RS and very elongated spines in FXS). On the other hand, growing wild-type neurons with diseased astrocytes led to the development of disease phenotype (Jacobs and Doering 2010; Jacobs, Nathwani, and Doering 2010; Ballas et al. 2009). In case of Down syndrome (resulting from the trisomy of the chromosome 21), when neurons were grown in the presence of Down's syndrome astrocytes, there was abnormal spine development. The number of spines and spine activity was reduced. It was then found that TSP-1 (which plays a role in controlling synapse number and activity), a astrocyte-secreted regulator of synaptogenesis is markedly downregulated in Down's syndrome astrocytes (Garcia et al. 2010), causing abnormal synaptic phenotype.

Further, many genes such as 4-aminobutyrate aminotransferase (ABAT), glutathione-S-transferase μ1 (GSTM1), fatty-acid-binding protein 7(FABP7), thought to be associated with autism show a high level of expression and enrichment in astrocytes (Clarke and Barres 2013).In all the above disorders, restoring the respective protein to normal levels in the diseased astrocytes led to the rescue of the disease phenotype. This raises the question whether targeting astrocytes can help in therapy of these disorders.

1.5 Hypothesis, Aim and Significance of this thesis

As described previously, astrocytes have shown to play a prominent role in the pathology of many neurodevelopmental disorders presenting with ID. We hypothesise that in *SYNGAP1*-related NSID astrocytes show abnormal phenotypes and contribute to the pathology of this disorder. In view of this, we aim to elucidate the mechanism of involvement of astrocytes in phenomenon reported to be disrupted in *Synagp1*$^{+/-}$ mice. Understanding this aspect can help to design new therapeutic strategies targeting astrocyte-mediated rescue of abnormal phenotypes observed in this disorder. This is much-needed as the genetic rescue of *Syngap1* in adult *Synagp1*$^{+/-}$ mice fails to bring about the rescue of developmentally caused abnormalities.

Chapter 2 Materials and Methods

2.1 Animal maintenance

All experimental animals were bred and maintained in the Animal house at JNCASR under 12-hour dark and light cycle. Food and water were available ad libitum. All the experiments were performed in accordance with guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and Institutional Animal Ethics Committee (IAEC)**.** B6;129-*SYNGAP1*^{tm1Rlh}/J strain of mice heterozygous for *Syngap1* knockout allele (*Syngap1*$^{+/-}$ or Syn-Het) were obtained from The Jackson laboratory, USA, and used as animal models for *SYNGAP1*-related NSID.

2.1.1 Mouse model Genetics

In these mice, exon 7 (common to all splice variants) as well as exon 8 (encodes part of the C2 domain) are replaced by a neomycin cassette in the opposite direction, creating a knockout allele (Kim et al. 2003).

2.2 Genotyping

2.2.1 DNA Isolation

Mice were scuffed, tagged by Monel ear tags procured from Kent Scientific (#INS1005-1Z). 2-5 mm of mouse tail was collected during weaning. Using a scalpel blade, the tail tissue was cut into small pieces. The pieces were suspended in 180 µL of 50 mM NaOH (#106482, Merck) lysis solution and vortexed thoroughly (#3020, Tarsons). This was then heated at 95 °C for 10 minutes in a thermomixer (#88871003, Thermo Scientific) to facilitate lysis. 20 µL of 1 M Tris-Cl (pH 8.0) was added to neutralize the solution. The tubes were then centrifuged at 12,000 rpm (#5424, Eppendorf) at room temperature for 10 minutes to sediment the debris. The supernatant containing the genomic DNA was collected into fresh microcentrifuge tubes (#500000, Tarsons).

2.2.2 Polymerase Chain Reaction (PCR)

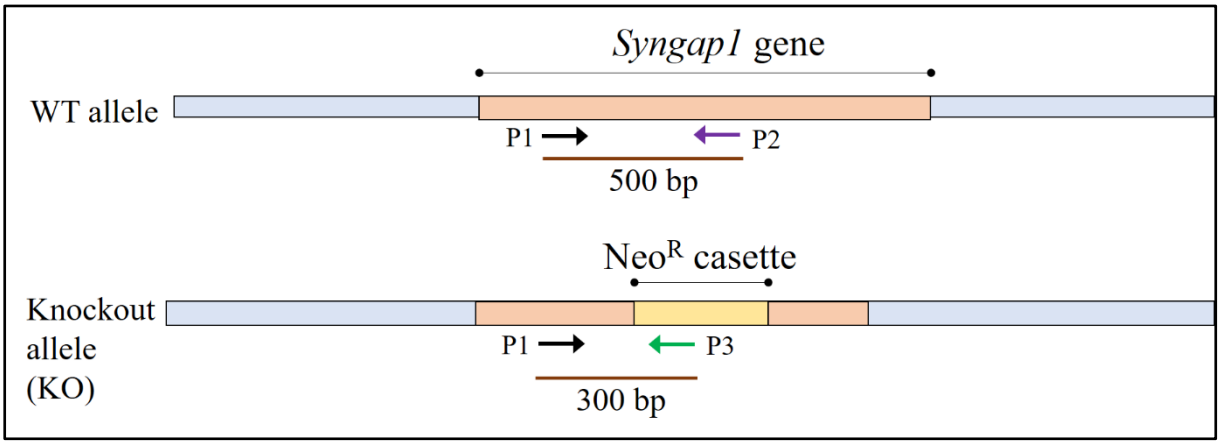

Figure 2-1 Mice Genotyping Strategy.

Primers 1 and 2 detect the WT allele and generate a 500 bp amplicon. Primers 1 and 3 detect the knockout allele and generate a 300 bp amplicon.

2.2.2 (a) Strategy

PCR was used to detect the presence of neomycin resistance cassette. The following three primers (Sigma Aldrich, India) were used:

Primer 1: 5'- ACCTCAAATCCACACTCCTCTCCAG-3',

Primer 2: 5'- AGGGAACATAAGTCTTGGCTCTGTC-3',

Primer 3: 5'- ATGCTCCAGACTGCCTTGGGAAAAG-3'.

Primers 1 and 2 are forward and reverse primers specific to the *Syngap1* gene. Specifically, Primer 1 binds to the Intron 6 region, and Primer 2 binds to the Intron 7 region of the *Syngap1* gene. Primer 3 is a reverse primer that binds to the neomycin resistance cassette.

Primer 1 and 2, therefore, detects the wild-type allele and produces an amplicon of approximately 500bp. In case of the mutant allele, Primer 2 fails to bind due to the loss of Intron 7. Thus, Primer 3 binds to the neomycin resistance cassette and produces a 300 bp amplicon with Primer 1. Consequently, genotyping mice will produce 3 different types of bands: a) a 500 bp band for *Syngap1*$^{+/+}$ mice b) both 500 bp and 300 bp band for a *Syngap1*$^{+/-}$ mice and c) a 300 bp band for *Syngap1*$^{-/-}$ mice.

2.2.2 (b) Reaction Setup

Following DNA isolation, the PCR reaction was setup in PCR tubes (#AB0620, Abgene) as follows: 1X Taq Buffer (#KK1015, Kapa Biosystems); 0.2 mM dNTP (#IBS-786-442, G-Biosciences); 1 µM each of Primer 1, 2 and 3; 1.5 U Taq polymerase (#KK1015, Kapa Biosystems), 1.5 µL gDNA and autoclaved Milli-Q (#ZRX003IN, Merck Millipore) up to 20 µL. The tubes were briefly centrifuged (#1000, Tarsons) for 5s after which they were placed in the thermocycler (Mastercycler nexus GX2, Eppendorf). PCR was carried out as follows: Initial denaturation at 95 ºC for 3 min; 35 cycles of 3 steps – 30s denaturation at 95 ºC, 45s annealing at 61.9 ºC, and 35s extension at 72 ºC; final extension of 2 minutes and holding at 4 ºC.

2.2.3 Agarose Gel electrophoresis

1% agarose (#50004, Lonza) gel was prepared in 1X Tris Acetate EDTA (TAE) Buffer (#R023, G-Biosciences) with Ethidium Bromide (EtBr) (#MB071, HiMedia) to a final concentration of 0.1 µg/mL. 2 µL of 6X DNA electrophoresis dye (30% glycerol (#0854, Amresco), 0.25% bromophenol blue (39121, Fisher Scientific), 0.25% xylene cyanol (#0819, Amresco) was added per 10 µL of the PCR product. 10 µL of this mixture was then loaded and electrophoresed (custom made, IISc) at 5 V/cm until the dye-front has reached 3/4th the length of the gel. DNA bands were visualized and imaged using Bio-Rad Gel Doc XR Imaging System.

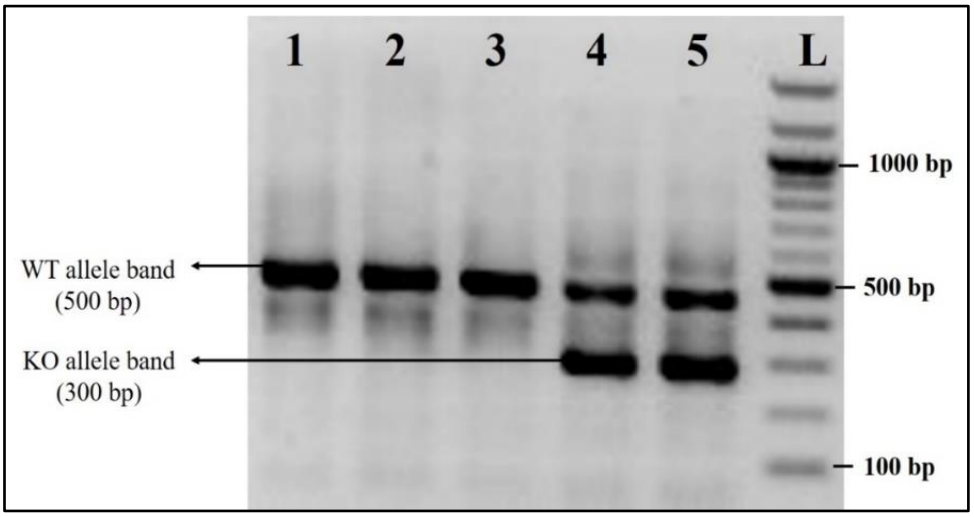

Figure 2-2 : **Electropherogram of genotyping PCR products.**

Lanes: 1-5: PCR products resulting from gDNA extracted from tail samples of different mice; L-Ladder. The 500 bp band corresponds to the wild type (WT) SYNGAP1 allele and the 300 bp band corresponds to the knockout (KO) Synagp1 allele.

2.3 Primary Cell Culture

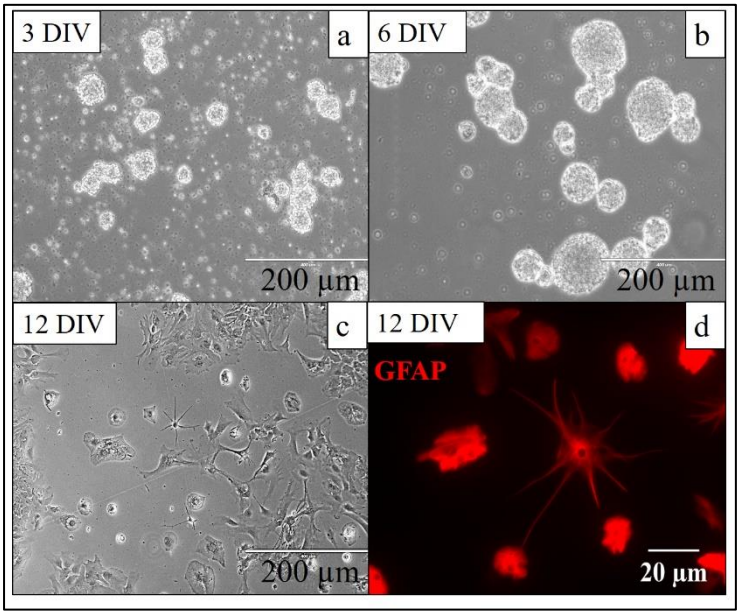

Figure 2-3 Neuron progenitor cell culture and differentiation into astrocytes.

(a-b) Phase contrast images of neural progenitor cells (NPCs) at (a) 3 DIV (b) after 1st passage – 6 DIV. (c) Phase contrast image of astrocytes differentiated from NPCs – 12 DIV (d) Immunocytochemistry marking for astrocytes (GFAP in red).

2.3.1 Isolation and suspension culture of Neural Progenitor Cells (NPCs)

Brains were dissected from post-natal day (P) 0-0.5 mice and immersed in ice-cold Hibernate-A (#A12475-01, Gibco) supplemented with 1X antibacterial-antimycotic (#15240062, Invitrogen), 1X glutamax (#350350-061, Gibco) and 1X B27 (#17504044, Gibco). Under a stereomicroscope (#MZ6, Leica), cortex and hippocampus were dissected out and meninges was removed. The tissue was then chopped into fine pieces. Tissue pieces were transferred to 0.7 mL (per brain) 0.01% trypsin digest solution (0.01% Trypsin-EDTA (#150500065, Invitrogen), 0.2% BSA (#GRM105, HiMedia), in 1X HBSS solution), triturated 10-15 times and incubated at 37 °C for 15 minutes. Trypsin was inactivated by the addition of equal volume of 10% FBS solution (10% FBS (#RM10434, HiMedia), 1X glutamax, 0.5% glucose (#G8270, Sigma) in DMEM low glucose (#11885084, Gibco)). Debris were allowed to settle for 10 minutes. The supernatant was then separated and added to 1 mL Dissociation media (0.1% sodium bicarbonate (#S6014, Sigma), 15 mM HEPES (#5310, Merck Millipore), 0.5% glucose in 1X HBSS solution). Cells were pelleted by centrifugation at 1600 RPM for 10 min. For washing, cells were resuspended in dissociation media and re-pelleted. The washing step was repeated, and the cells were resuspended in 5 mL (per brain) NPC growth media (5:3 DMEM low glucose: Neurobasal medium (#10888022, Gibco) supplemented with 1X Glutamax, 1X anti-anti, 1% N2 (#17502048, Gibco), 2% B-27, 20 ng/mL EGF (#315091000, Peprotech) and 20 ng/mL FGF (#10018B100, Peprotech)). This was plated onto 60 mm cell culture dishes (#30701119, Eppendorf). For passaging NPC's, the solution was collected from the plates and

neurospheres pelleted by centrifugation at 1600 RPM for 10 minutes. This was resuspended in trypsin digest solution and the afore-mentioned protocol was repeated.

2.3.2 NPC to astrocyte differentiation

Astrocyte cell culture was obtained by differentiation of NPC's. For this, neurospheres were pelleted by centrifugation and trypsinized (as described previously) at 37 °C for 30 minutes. Debris were removed, and supernatant washed once with dissociation media. The cells were finally suspended in astrocyte growth media (5:3 DMEM low glucose: Neurobasal medium supplemented with 10% FBS and 1% N2) and plated onto poly-lysine (#P2636, Sigma) coated 60 mm dishes (#B0341201H, Eppendorf). The media was changed every 3-4 days. Cells show GFAP expression by 4 DIV.

2.3.3 Primary neuron culture

P0-P1 mice brain was dissected as described previously. The tissue pieces were transferred to 1 mL (per brain) digestion buffer (hibernate solution containing 50 U/mL papain (#LS003127, Worthington Biochemical), 1X anti-anti, 1X glutamax and 2% B27). This was incubated at 37 °C for 30 minutes. The tissue pieces were allowed to settle down, supernatant was discarded and, tissue pieces were resuspended in fresh complete hibernate media (Hibernate-A medium containing 1X anti-anti, 1X glutamax and 2% B27). Tissue pieces were triturated 10-20 times using a small-bore pipette tip. Debris was allowed to settle for 10 minutes and supernatant centrifuged (#5810R, Eppendorf) at 80 x *g* for 5 minutes to pellet cells. For washing, cells were resuspended in complete hibernate medium and re-pelleted. Cells were washed again and finally resuspended in Neuronal Growth Medium (Neurobasal-A medium supplemented with 1X anti-anti, 1X glutamax and 2% B-27).

2.3.4 Astrocyte-neuron co-culture

Coverslips were treated with polylysine and ~200,000 astrocytes (7 DIV) were plated on each. Astrocyte feeder layer was grown till confluent to minimize well-well differences. On the day of plating neurons, media was removed from the wells, and astrocyte layer was washed with 1X Phosphate Buffered Saline (PBS) (137 mM NaCl (#GRM031, Hi-Media), 2.7 mM KCl (#13305, Fischer Scientifc), 10 mM Na_2HPO_4 (#GRM6365, Hi-Media), 1.8 mM KH_2PO_4 (#GRM1188, Hi-Media)). Complete neurobasal medium (Neurobasal-A medium supplemented with 1X anti-anti, 1X glutamax and 2% B-27) was added to all the wells prior to starting procedure for neuron isolation. Neurons were then isolated as described above and

30,000 cells were plated onto the astrocyte feeder layer. This was allowed to grow for 24 hours *in-vitro*, after which cells were fixed and stained for further analysis.

2.4 Immunoblotting

2.4.1 Lysate Preparation

2.4.1 (a) Preparation of Mouse Brain Tissue Lysate

Mice were euthanized by cervical dislocation and brains were dissected out and placed in ice-cold PBS. The cortical and hippocampal regions were dissected and tissue pieces were then homogenized in 10 volumes of RIPA buffer containing the following: 150 mM NaCl (#G15915, Fischer Scientific), 50 mM Tris-Cl (pH 7.4), 0.1% Triton-X (#RM845, Hi-Media), 0.25% sodium deoxycholate (#D6750, Sigma), 5m M EDTA (#RM1195, HiMedia), 0.1% SDS (#1610302, Bio-Rad), 1X Protease Inhibitor Cocktail (#11836170001, Roche), 1X Phosphatase Inhibitor Cocktail (#P2850, #P0044, #P5726; Sigma). Tissues were homogenized on ice in a Dounce homogenizer (#D9063, #D9938; Sigma). Debris were separated by centrifugation at 16,000 g (#5920R, Eppendorf) for 30 minutes at 4 °C. The supernatant was aliquoted and stored at -80 °C.

2.4.1 (b) Preparation of Astrocyte Cell culture Lysate

Cultures astrocytes grown on an 80mm dish were scraped and pelleted. The pellet was then resuspended in 200 µL RIPA buffer. The sample was repeatedly pipetted to lyse the cells until a smooth-flowing solution was obtained. All processing was carried out on ice.

2.4.2 Protein Estimation by Bradford Method

Bovine Serum Albumin (BSA) was used as the standard to estimate the protein concentration in each of the lysates. The following solutions (100 µL each) were prepared in a 96-well plate: blank - 100 µL autoclaved Milli-Q; BSA standards (in µg/mL)-5, 10, 20, 40, 60, 80, 100; unknowns (1 µL of protein lysate added to 99 µL of autoclaved Milli-Q). 200 µL of 1X Bradford reagent (#5000006, Bio-Rad) was added to each well. The plate was incubated in dark for 5-60 minutes. Absorbance was measured using a ELISA plate reader (Versamax, Molecular Devices) at 595 nm and values were used to plot a standard curve. This was then used to calculate the protein concentration of the unknown samples.

2.4.3 SDS-Poly Acrylamide Gel Electrophoresis (PAGE) and Blotting

30 µg of each lysate's volume was made up to 20 µL. 4 µL of denaturing 5X SDS dye was added and the samples were heated at 95 °C for 10 minutes in a thermomixer. Denatured samples were loaded onto 10% polyacrylamide gels (0.375 M Tris pH 8.8, 10% Acrylamide (#161056, Bio-Rad), 1% SDS, 1% APS (#GRM1094, Hi-Media), 0.045 TEMED (#1610801, Bio-Rad)). Electrophoresis was performed at 80V for 3 hours. Proteins were transferred at 4°C onto PVDF membrane (#1620177, Bio-Rad) at 80 V for 3 hours. Ponceau S (#RM977, Vasa) staining was done to check the quality of individual blots. The membrane was blocked using 5% skim milk (#M530, Hi-Media) for 1 hour at room temperature. Blots were incubated with primary antibodies overnight at 4 °C (monoclonal mouse anti-GFAP- 1:1000 (#MA515086, Thermo Scientific), polyclonal rabbit anti-GAPDH- 1:5000 (#G9545, Sigma), polyclonal rabbit anti-SYNGAP1- 1:1000 (#PA1016, Thermo Scientific). Blots were then washed 3 times (10 minutes each) in 1X PBST (0.1% Tween-20 (#28599, Sisco Research Laboratories) in 1X PBS). After washing, blots were incubated with secondary antibody solution (HRP- tagged anti-mouse (#1706516, Bio-Rad)/anti-rabbit (#1721019, Bio-Rad) secondary antibodies in 5% skim milk) for 1 hour at room temperature. 3 washes in 1X PBST was given and blots were stored at 4 °C until further processing. 100 µL of ECL (Luminol + H_2O_2) (#1705060, Bio-Rad) was added and spread evenly on the blots. Bands were visualized using a CCD-Imaging System (Versadoc MP 4000, Bio-rad). For quantification, ImageJ software was used. Briefly, bands were selected individually and converted into intensity curves. The tails of the curves coinciding with background was removed and the remaining area measured. Area of bands of protein of interest was divided by that of bands of control protein for normalization.

2.5 Tissue processing

2.5.1 Transcardial Perfusion

Mice were anaesthetised with halothane (#30039034, Neon). The chest cavity of the mice was cut opened, and a butterfly needle (0.55 x 19mm, Scalp Van) was inserted into the right ventricle of the heart. A nick was made at the right atrium. Blood was drained by passing ice cold PBS. Whole body fixation was performed by passing ice-cold paraformaldehyde (#158127, Sigma) solution (4% in 0.1 M PB). The brain was then dissected out and immersed in 4% Paraformaldehyde (PFA) (#158127, Sigma) solution overnight at 4°C for post-fixation.

Brains were then shifted to 30% sucrose (#TC048, HiMedia) solution (in 1X PBS) and incubated until complete penetration.

2.5.2 Cryosectioning

Sucrose-treated brain was embedded in OCT (#DIG46181, Tissue-Tek) on chucks and placed inside cryostat (#CM3050s, Leica) chamber. Prior to this, chamber temperature (CT) was maintained at -20 °C and object temperature (OT) at -22 °C. After 20 minutes, once the OCT has solidified, 20 µm brain sections were collected in 1X PBS solution in 24-well plates.

2.6 Immunofluorescence

2.6.1 Immunohistochemistry

20 µm sections were immersed in 10 mM sodium citrate (#27625, Fischer Scientific) (pH 6.0) (with 0.1% Tween-20) and heated at 70-90 °C for 30 minutes for antigen retrieval. The sections were washed 2 times in 1X PBS solution. Sections were then placed in blocking solution (5% NGS (#50062Z, Thermo Scientific), 5% BSA, 0.1% Triton-X in 1X PBS). After this, sections were incubated in primary antibody solution (mouse anti-GFAP diluted 1:1000 in blocking solution) overnight at 4 °C. Sections were washed 5 times (10 min each) in 0.1% PBST solution and incubated in secondary antibody solution (Alexa Fluor anti-mouse 488 (#A11001, Thermo Scientific) diluted 1:500 in blocking solution). After 4 washes with 0.1% PBST, sections were counterstained with hoechst (#H3570, Thermo Scientific) solution (1:1000 in 1X PBS). After 2 washes in 1X PBS (5 min each), sections were mounted onto glass slides. (Mountant: 90% glycerol, 10% 1 M Tris-Cl (pH 8.0), 0.005% (w/v) n-propyl gallate (#164878, Sisco Research Laboratories). Imaging was done using Zeiss 510 meta Confocal Microscope.

2.6.2 Immunocytochemistry

Media was removed from cells and then seeded onto poly-lysine coated coverslips, and the cells were washed once with 1X PBS. 0.25 mL of fixing solution (4% PFA+4% sucrose in 1X PBS solution was added to each of the wells and incubated on ice for 10 minutes. Cells were washed twice with 1X PBS and placed in 200 µL of blocking/permeabilization solution (10% NGS,0.1% Triton-X in 1X PBS) for 30 minutes at room temperature on a shaker. Cells were then incubated in a primary antibody solution (diluted in blocking solution: chicken anti-GFAP 1:1000 (#GFAP, Aves), mouse anti-βIII-TUBULIN 1:1000 (#T8660, Sigma) and rabbit anti-SYNGAP1 1:500) for 45 minutes at room temperature on a shaker. Cells were washed 3 times (2 min each) with 1X PBS and incubated with respective secondary antibody solutions for 20-

30 minutes at room temperature on a shaker (#3060, Tarsons). Cells were washed again as above and mounted onto glass slides. Imaging was done using an Epifluorescence microscope (Nikon Eclipse 80i).

2.7 Image Analysis

2.7.1 Quantification of number of astrocytes

3 fields (40x) covering the CA1 hippocampal region were imaged per section. Images were processed using Fiji software. A square region of interest (ROI) was drawn per field, and number of astrocytes were quantified for each ROI. ROI size was kept constant across fields and sections.

2.7.2 Sholl Analysis

3 fields (40x) covering the CA1 hippocampal region were imaged per section. Images were processed using Fiji software. Processing and Sholl analysis was performed using Fiji software. In each image, individual astrocytes were delineated and cropped out. The cropped images of single astrocytes were smoothened and thresholded such that processes were highlighted. Thresholds were kept constant per image. Thresholded images were then skeletonized to generate traces of astrocyte processes. A line was drawn from the centre of the cell to the edge of the largest astrocyte process. The Sholl analysis plugin was then used to generate cell-wise graphs (Ferreira et al. 2014).

2.8 Statistics

All graphs were plotted in Microsoft Excel 2016. Results were expressed as ±Standard Error of Mean (SEM). The significance between Wild Type and *Syngap1*$^{+/-}$ from different age group were assessed by unpaired Student's *t-test*, unless otherwise mentioned. For Sholl Analysis, 2-way ANOVA was used to test the effect of genotype with post-hoc Bonferroni multiple comparisons test.

Chapter 3 Results

3.1 Expression of SYNGAP1 in astrocytes

A mutation in the *Syngap1* gene can affect astrocytic function in a non-cell autonomous or cell autonomous manner. In the former scenario, since *Syngap1* is expressed in neurons, a mutation in this gene can cause neuronal abnormalities, which might then indirectly affect astrocytic properties. In the latter case, if *Syngap1* is expressed in astrocytes, then mutations in this gene can directly affect astrocytes. So far, no studies have specifically investigated the expression of *Syngap1* in astrocytes. However, in one study in which a large-scale transcriptome analysis was carried out in mouse astrocytes, *Syngap1* mRNA is reported to be expressed in astrocytes (Cahoy et al. 2008). In the present study, for the first time we investigate the expression of SYNGAP1 protein in astrocytes using immunoblotting and immunocytochemistry.

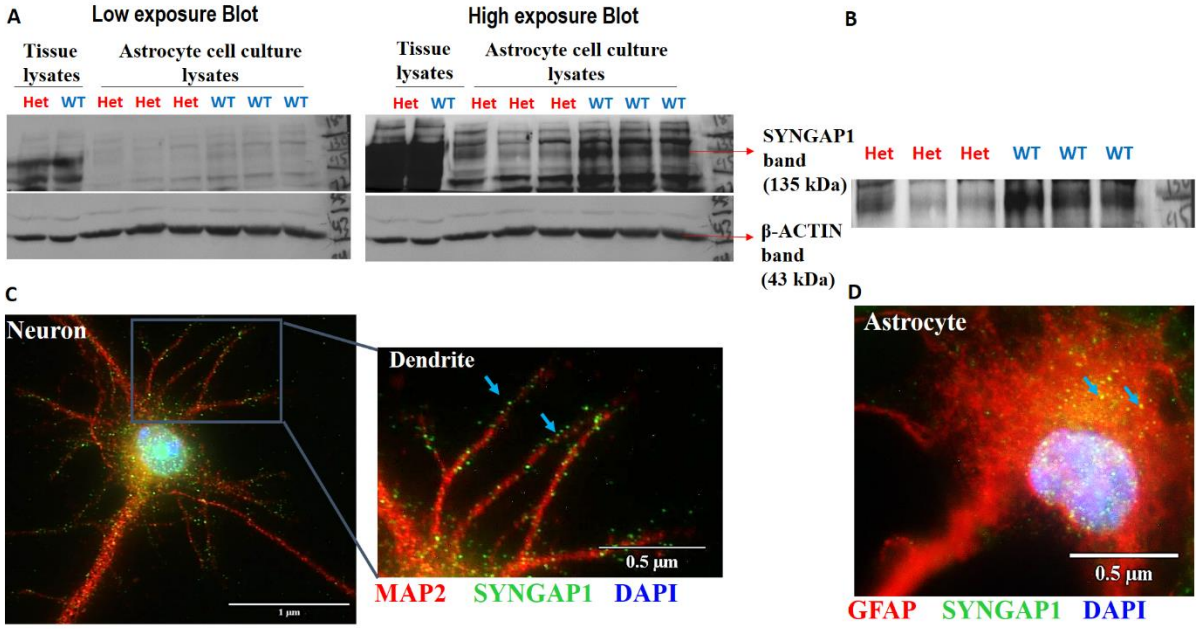

Figure 3-1 SYNGAP1 expression in astrocytes

A. Immunoblot against SYNGAP1 in astrocyte cell culture lysates. Brain tissue lysates were used as a positive control. (WT – Wild-type or *Syngap1*$^{+/+}$; Het – Syn-Het or *Syngap1*$^{+/-}$) β-ACTIN was used as the loading control. **B.** Magnified image showing a band corresponding to the expected molecular weight of SYNGAP1 (~135 kDa). **C.** Cultured neurons stained for SYNGAP1 as a positive control. (Left) 5 DIV neurons showing synaptic localization of SYNGAP1 as expected. **D.** Cultured astrocytes showing punctate SYNGAP1 expression pattern.

Upon probing for SYNGAP1 in lysates of cultured astrocytes, a band corresponding to the expected SYNGAP1 size was observed at about 135 kDa (**Figure 3-1 B**). This band matched the SYNGAP1 band in brain tissue lysate controls (**Figure 3-1 A**). However, this data is not conclusive as extra bands were observed and could be due to non-specific binding of the antibody. The immunoblotting needs to be further standardized to determine whether these bands are non-specific. In addition, SYNGAP1 immunocytochemistry was performed in cultured cells. As expected, in cultured neurons, SYNGAP1 showed punctate staining along dendrites, indicative of synaptic localization (**Figure 3-1 C**). In astrocytes, SYNGAP1 was detected and also showed a punctate expression pattern (**Figure 3-1 D**).

3.2 Number of astrocytes in *Syngap1*$^{+/-}$ mice

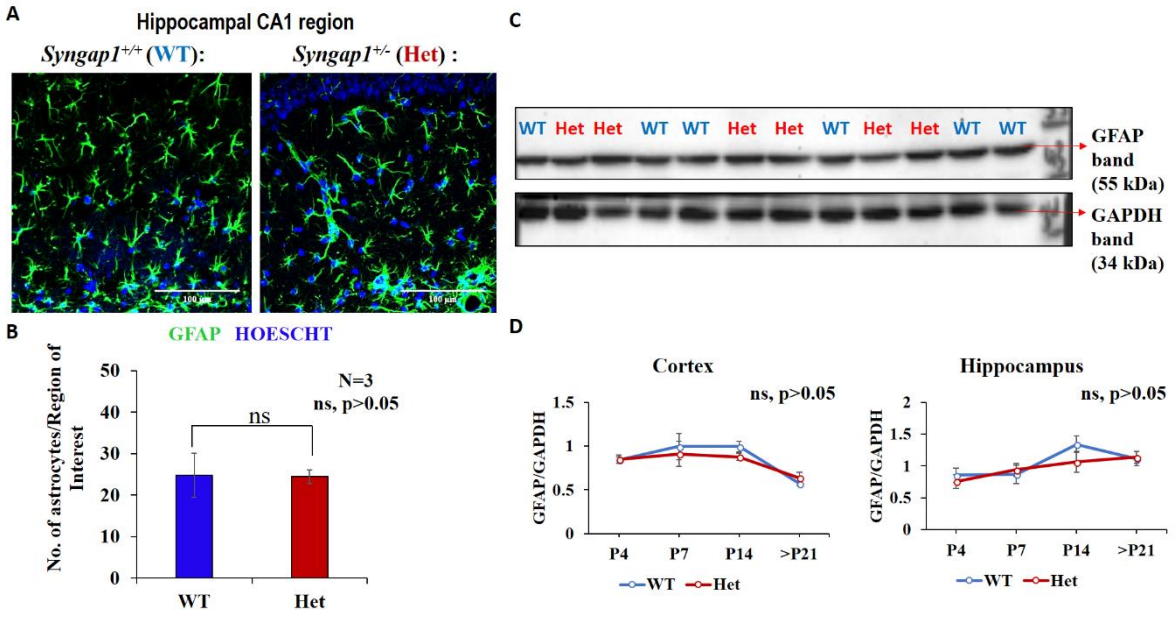

Figure 3-2 Number of astrocytes in Wild-type (*Syngap1*$^{+/-}$) and *Syngap1*$^{+/-}$ mice

A. Immunofluorescence for GFAP, highlighting astrocytes in the CA1 region of the hippocampus. **B.** Quantification of the number of astrocytes in the hippocampus of wild-type and Syn-Het mice (N=3 mice, p>0.05, Students t-test). **C.** Immunoblot for GFAP in WT and Syn-Het mice. **D.** Quantification of GFAP levels at different age groups (N numbers according to Age, WT, Syn-Het: Cortex P4- 8,6; P7- 6,6; P14- 7,6; Adult >P21- 6,6 and Hippocampus P4- 8,9; P7- 7,6; P14- 6,7; Adult >P21- 6,6) (p>0.05 per age group, Students t-test).

Next, we investigated if any properties of astrocytes were affected by a mutation in *Syngap1*. No change in the number of astrocytes was observed in the hippocampus of adult WT (*Syngap1*$^{+/+}$) and Syn-Het (*Syngap1*$^{+/-}$) mice (**Figure 3-2 A, B**). This was corroborated by checking for GFAP levels by immunoblotting. No change in GFAP levels were observed between WT and Syn-Het mice in either the cortex or hippocampus in adult mice or during any other developmental time point (**Figure 3-2 C, D**).

3.3 Astrocyte morphology in *Syngap1*$^{+/-}$ mice

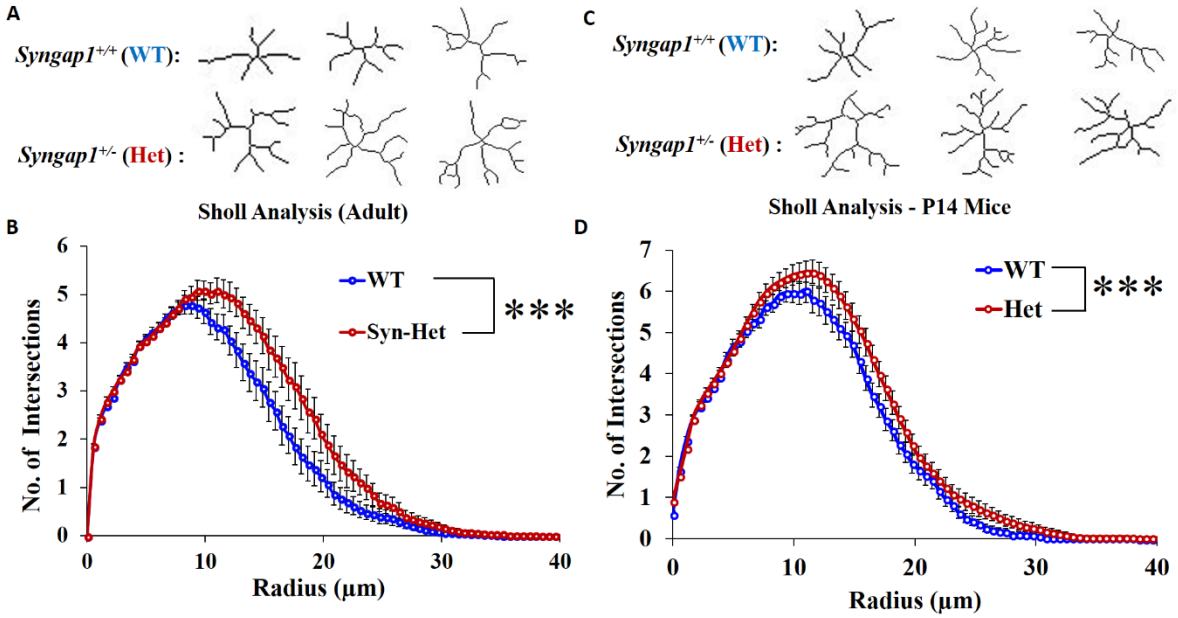

Figure 3-3 Astrocyte branching in WT and Syn-Het mice

A. Traces of astrocytes in the hippocampus of adult WT and Syn-Het mice. **B.** Sholl Analysis for astrocytes in the hippocampus of adult WT and Syn-Het mice (N=3 mice per genotype; WT n=106 cells, Syn-Het n=105 cells; $p<0.001$; 2-way ANOVA). **C.** Traces of astrocytes in hippocampus of P14 WT and Syn-Het mice **D.** Sholl Analysis for astrocytes in the hippocampus of adult WT and Syn-Het mice (N=1 mice per genotype; WT n=47 cells, Syn-Het n=48 cells; $p<0.001$; 2-way ANOVA).

Another property of astrocytes is that they have highly branched morphology. Since *Syngap1* mutation affects cytoskeleton and branching in neurons (Clement et al. 2012), we questioned if the same holds true for astrocytes. Sholl Analysis was used to analysis the degree of branching of astrocytes in the hippocampal CA1 region of WT and Syn-Het mice. Astrocytes

in Syn-Het mice had increased branching as compared to WT mice at both P14 and adult stages (**Figure 3-1 A-D**).

3.4 Astrocyte neuron co-culture

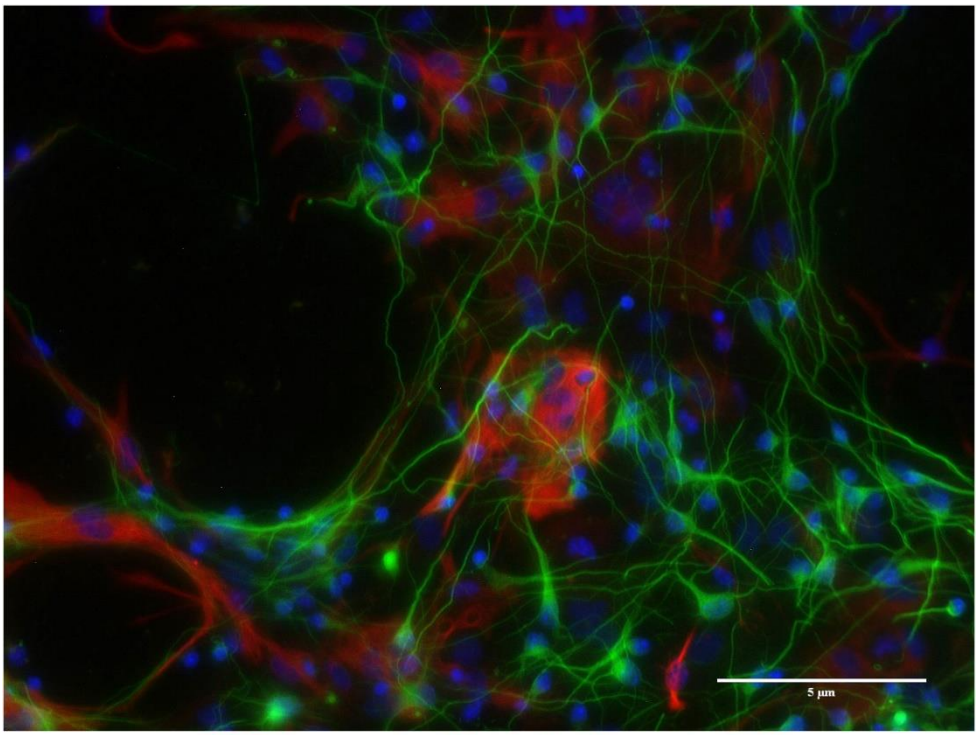

Figure 3-4 Co-culture of neurons and astrocytes

Neurons (Green: βIII-tubulin) plated on top of Astrocyte feeder layer (Red: GFAP). Nuclei are stained in blue (DAPI).

In order to study the effect of differential astrocyte properties (WT and Syn-Het) on neurons, there is need to dissect astrocytes and neurons with varying genotypes. Since, the mouse model used here has a non-conditional genomic knockdown of *Syngap1*, all cell types are *Syngap1*$^{+/-}$. Thus, one cannot dissect out the affect of astrocytic change in properties on neuronal function. One way to study this, is to perform *in-vitro* co-culture of astrocyte and neurons. This method allows us to culture neurons and astrocytes having different genotype (WT and Syn-Het) in various combinations. Similar co-culture studies were performed in case of other neurodevelopmental disorders such as Fragile X Syndrome (Jacobs and Doering 2010; Jacobs, Nathwani, and Doering 2010), Rett Syndrome (Ballas et al. 2009) and Down Syndrome (Garcia et al. 2010). Due to technical difficulties, the co-culture method (**Figure 3-4**) is currently under standardization.

Chapter 4 Discussion

Non-Syndromic Intellectual Disability is a neurodevelopmental disorder, one of the causes of which is a monoallelic mutation in *SYNGAP1* (Hamdan et al. 2009; Hamdan et al. 2011). Studies so far have been neuron-centric, concerning neuronal cellular and network phenotypes affected due to *Syngap1* haploinsufficiency. However, the brain consists of other non-neuronal cell types of cells that contribute to its functioning. Among these are astrocytes, which have increasingly garnered attention over the past few years due to their crucial role in almost all aspects of neuronal functioning (Sofroniew and Vinters 2010). How astrocytes are affected and contribute to the pathology of *SYNGAP1*-related intellectual disability remain an unexplored area, which we have attempted to study here.

In this disorder, astrocytes can be affected in a cell-autonomous manner if SYNGAP1 is expressed in astrocytes. Immunoblotting and immunocytochemistry studies in cultured astrocytes suggest that SYNGAP1 might be expressed in astrocytes (**Figure 3-1**). However, since other bands were observed in the immunoblot, this finding needs to be validated by technique optimization. Additionally, Real-Time PCR experiments have to be performed to demonstrate expression of *Syngap1* mRNA in astrocytes. SYNGAP1 expression in astrocytes also needs to be demonstrated *in vivo*.

Further, while studying the effect of *Syngap1* mutation on astrocytes, we observed that there is no change in the number of astrocytes (**Figure 3-2**). However, *Syngap1* haploinsufficiency leads to increased branching of astrocytes in the hippocampus of both P14 and adult mice (**Figure 3-3**). This was anticipated as *Syngap1* haploinsufficiency also affects cytoskeletal properties and branching in neurons (Clement et al. 2012). In neurons, SYNGAP1 regulates RAS activity, which in turn regulates RAC mediated dynamics of ACTIN, thereby affecting the cytoskeleton (Carlisle et al. 2008). We speculate that, in astrocytes also, SYNGAP1 might regulate RAS activity. In astrocytes, RAS activity has been shown to regulate RAC and ACTIN dynamics, that ultimately affect the astrocytic branching (Kalman et al. 1999) (**Figure 4-1**). RAS negatively regulates RAC activity and RAC in turn negatively regulates the extent of astrocyte branching (Kalman et al. 1999). Therefore, if SYNGAP1 negatively regulates RAS activity, then knockdown of SYNGAP1 will lead to increased RAS activity. Increased RAS activity will lead to greater inhibition of RAC. As a result of this, the inhibition on astrocytic morphology is reduced leading to increased branching in astrocytes (**Figure 4-1**).

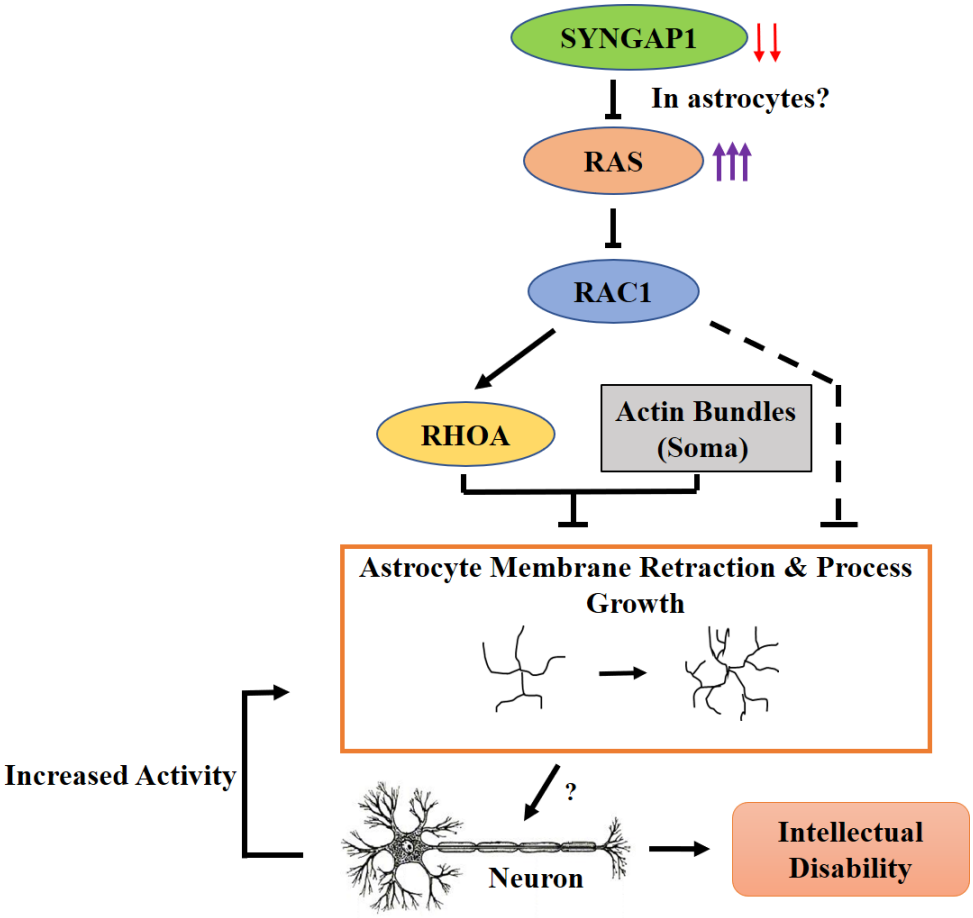

Figure 4-1 Working model for the effect of Syngap1 haploinsufficiency on astrocytes

SYNGAP1 might negatively regulate RAS activity in astrocytes, ultimately leading to increased branching of astrocytes in case of *Syngap1* knockdown. On the other hand, increased branching of astrocytes might also result from increased neuronal activity. However, the effect of increased astrocytic branching on neuronal function and pathology remains unknown.

On the other hand, cell extrinsic factors might also affect astrocyte branching. A recent study showed that astrocyte morphology depends on the level of neuronal activity (Stogsdill et al. 2017). Studies have also shown that astrocytes develop their final morphology around second week of post-natal development, which coincides with the time of synaptogenesis (Freeman 2010). Increased excitatory activity has been reported in case of *Syngap1*$^{+/-}$ neurons. Thus, this might also explain the increased branching observed in *Syngap1*$^{+/-}$ astrocytes at P14 and adult stages.

Finally, to study the effect of the distinctive types of astrocytes (WT and Syn-Het) on neurons also of varying genotypes (WT and Syn-Het), co-culture needs to be performed. This technique is still under optimization for analysis (**Figure 3-4**).

Aside from further validation of the current findings, in future we would like to elucidate the function of SYNGAP1 in astrocytes. Building upon the co-culture experiment, we would like to study the astrocyte specific effect of *Syngap1* haploinsufficiency in-vivo using CRE-induced conditional *Syngap1* knockdown under a *Gfap*-promoter. Our final aim will be to examine if targeting astrocytes has a therapeutic potential in intellectual disability.

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