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INTERACTOME OF STARGAZIN: RELEVANCE IN NEUROPSYCHIATRIC DISORDERS

Dissertação de Mestrado em Biologia Celular e Molecular, orientada pela Professora Doutora Ana Luísa Monteiro de Carvalho e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Julho 2016

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UNIVERSIDADE DE COIMBRA

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob orientação da Mestre Gladys Tarcila Lima Caldeira (Centro de Neurociências e Biologia Celular, Universidade de Coimbra) e da Professora Doutora Ana Luísa Monteiro de Carvalho (Departamento de Ciência da Vida, Universidade de Coimbra)

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Cover image: Part of the master piece “Vision Quest For Ethel” by Alisa Hughes

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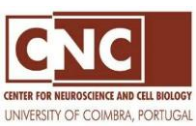

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TABLE OF CONTENTS

Agradecimentos 5

Table Of Contents 7

Resumo 1

Abstract 2

Abbreviations 3

Introduction 7

1. Glutamatergic Synapse 9

1.1. AMPA Receptors 10

1.1.1. Transmembrane AMPAR Regulatory Proteins 13

1.1.2. Stargazin 13

1.2. NMDA Receptors 14

1.3. Metabotropic Glutamate Receptors 14

2. Neuroplasticity 15

2.1. Hebbian Plasticity 15

2.2. Homeostatic Plasticity 17

3. Neuropsychiatric Disorders - General Overview 18

3.1. Schizophrenia 19

3.2. Intellectual Disability 21

3.3. Stargazin And Neuropsychiatric Disorders 22

4. Interaction Candidates 23

4.1. The M-Channel Subunit Kv 7.2 24

4.2. The Metabotropic Glutamate Receptor 5 26

5. Supporting Data 27

5.1. Kv 7.2 And mGluR5 As Possible New Partners In Stargazin Interactome 27

5.2. Analysis Of mGluR5-Stargazin Interaction 29

5.2.1. Class I Metabotropic Glutamate Receptor 5 Binds To Stargazin 29

5.2.2. mGluR5 And Stargazin Co-Localize Strongly At Synaptic Sites 30

6. Objectives 31

Materials And Methods 33

Results 41

1. Analysis Of The Kv 7.2-Stargazin Interaction 43

1.1. M-Channel Subunit Kv 7.2 Binds To Stargazin 43

1.2. Kv 7.2 And Stargazin Appear As Side By Side Partners 44

1.3. Stargazin Increases Kv 7.2-Mediated Currents 46

1.4. Intellectual Disability And Schizophrenia-Associated Mutations In Stargazin Impair Its Function In Regulating Kv 7.2 46

2. Analysis Of mGluR5-Stargazin Interaction. 48

2.1. Class I Metabotropic Glutamate Receptors Activation Increases Glua1 Levels In Cortical Neurons. 48

2.1.1 mGluR5 Modulation Of GluA1 Levels Is Dependent On Stargazin 49

Discussion 51

Closing Remarks and future directions 60

References 63

RESUMO

As doenças neuropsiquiátricas têm uma prevalência de 10% na população adulta, deste modo muitos esforços são actualmente direccionados à investigação das causas destas patologias. No entanto, os mecanismos sinápticos e os circuitos responsáveis pela fisiopatologia das doenças psiquiátricas são ainda pouco conhecidos. Várias mutações em genes que codificam proteinas sinápticas foram associadas a doenças cognitivas como Esquizofrenia e Deficiência Intelectual, reforçando a importância da disfunção sináptica na génese destas patologias.

A Stargazina é uma proteína sináptica conhecida pela sua função na regulação do tráfego e na inserção das subunidades dos receptores AMPA na sinapse. A Stargazina está também associada a importantes fenómenos de plasticidade neuronal Hebbianos e homeostáticos[1,2].

Foi descrita no nosso laboratório uma mutação pontual no gene *CACNG2,* que codifica a Stargazina, a partir de uma amostra proveniente de um doente com Esquizofrenia. Estudos preliminares mostraram que esta mutação afecta a função da Stargazina associada ao trafego e inserção de GluA1 na membrana sináptica. Recentemente, no mesmo domínio da Stargazina, também tinha sido identificada uma outra mutação num paciente com Deficiência Intelectual[3].

O foco deste projecto foi perceber como é que estas mutações na Stargazina podiam afectar a sua função. Com recurso à técnica de espectrometria de massa conseguimos identificar novas proteínas que possivelmente integram o interatoma da Stargazina. De entre a lista obtida estudámos o receptor metabotrópico do glutamato 5, mGluR5, cuja função está associada ao trafego e inserção de subunidades dos receptores AMPA na sinapse. Desta lista também foi estudada a subunidade Kv 7.2 dos canais de potássio dependentes de voltagem, os canais-M. Estes canais quando formados pelas subunidades Kv 7.2 e Kv 7.3 têm um papel preponderante na génese da correntes-M dependentes de actividade e cuja função é controlar a repolarização e a hiperpolarização da membrana após um potencial de acção[4].

Aqui demonstramos pela primeira vez a interação de Stargazina com Kv 7.2 através de co-imunoprecipitação. Ainda, provamos que as mutações na proteína Stargazina associadas com Esquizofrenia e Deficiência Intelectual levam à diminuição dos níveis de expressão do Kv 7.2 e são incapazes de potenciar as correntes mediadas por canais formados por Kv 7.2, ao contrário do que acontece com a forma WT da Stargazina. Em relação ao mGluR5 demonstrámos que, no córtex, a activação destes receptores aumenta os níveis de GluA1 e que este processo é mediado por Stargazina.

As evidências aqui apresentadas suportam o papel das interacções de Stargazina com Kv 7.2 e mGluR5 na regulação da excitabilidade neuronal e da função sináptica. Particularmente, estes aspectos da função da Stargazina podem estar alterados em doentes que apresentem mutações no gene da Stargazina.

Palavras-chave: Interactoma da Stargazina, mGluR5, Kv7.2, Esquizofrenia, Deficiência Intelectual, Recetores AMPA, correntes M.

ABSTRACT

Neuropsychiatric disorders have a point prevalence of 10% in the general adult population, and significant effort is being devoted to better understand their pathogenesis. However, the synaptic and circuit mechanisms responsible for the pathophysiology of mental disorders are poorly understood. Several mutations associated with cognitive disorders such as schizophrenia and intellectual disability are in genes which encode for synaptic proteins, thus reinforcing the importance of synapse dysfunction to the etiology of these disorders.

The *CACNG2* gene is a risk gene for Schizophrenia and Intellectual Disability, and encodes Stargazin, an auxiliary subunit of glutamate receptors of the AMPA type (AMPAR), essential for their trafficking and insertion at the synapse. Furthermore Stargazin is required for Hebbian and homeostatic plasticity mechanisms[1,2]. Interestingly, our laboratory has recently identified a new mutation in *CACNG2* in a schizophrenia patient and this variant was found to impair the trafficking of AMPAR to the cell surface; a different mutation in the Stargazin gene was described in an intellectual disability patient[3]. We are interested in understanding how these mutations in Stargazin affect its function.

Using mass spectrometry analysis of immunoprecipitated Stargazin, we identified new interactors for Stargazin, among which is the class I metabotropic glutamate receptor mGluR5, which regulates AMPAR synaptic insertion and trafficking, and Kv7.2, a subunit of the potassium voltage-gated channels, M-channels. M-channels formed by Kv7.2 and Kv7.3 are required for the activity-dependent-generation of the M-currents that control membrane repolarization and hyperpolarization after an action potential[4].

In this work, we have characterized the interactions between Stargazin and Kv 7.2 or mGluR5, and their functional consequences. We found that Kv7.2 co-immunoprecipites with Stargazin, and that the two proteins partially co-localize in dendrites in cultured cortical neurons. Silencing of Stargazin expression disrupts the dendritic localization of Kv7.2, an effect which cannot be rescued by the Schizophrenia- and Intellectual Disability-associated variants of Stargazin. Regarding the functional relevance of the Stargazin-mGluR5 interaction, our results show that the mGluR5-modulation of synaptic GluA1-AMPARs in cortical neurons is dependent on Stargazin.

The evidence herein presented supports a role for the interaction of Stargazin with Kv7.2 and mGluR5 in regulating neuronal excitability and synaptic function. These aspects of Stargazin function may be impaired in neuropsychiatric patients presenting mutations in the Stargazin gene.

Keywords: Stargazin interactome, mGluR5, Kv7.2, Schizophrenia, Intellectual Disability, AMPAR, M-currents

ABBREVIATIONS

3TM –third transmembrane domain

Acaa2 – 3-ketoacyl-CoA thiolase, mitochondrial

ADHD – Attention-Deficit-Hyperactivity Disorder

AMPAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor for glutamate

APPL1 – DCC-interacting protein 13-alpha

APV – (2R)-amino-5-phosphonovaleric acid

Arvcf – Armadillo repeat protein

ASD – Autism Spectrum Disorders

BD – Bipolar Disorder

CACNG2 – calcium channel, voltage-dependent, gamma subunit 2 - Stargazin coding gene

CaM – Calmodulin

CaMKII – Ca^{2+}-Calmodulin-dependent kinase II

CaMKIV - Ca^{2+}-Calmodulin-dependent kinase IV

Cars – Cysteine-tRNA ligase, cytoplasmic

cGKII – cGMP-dependent protein kinase II

CHO – Chinese hamster ovary cell line

CHPG – (*RS*)-2-Chloro-5-hydroxyphenylglycine

Clasp2 – CLIP-associating protein 2

CNQX – 6-cyano-7-nitroquinoxaline-2,3-dione, AMPAR/KAR antagonist

CNS – central nervous system

CNVs – copy-number variations

Co-IP – co-immunoprecipitation

CTX – cortex

D2 or 3– dopamine receptor type 2 or 3

Dhx9 – ATP-dependent RNA helicase A

DIV- days in vitro

Eef1b – Elongation factor 1-beta

Eef1g – Elongation factor 1-gamma

Eph4A – EPH receptor A4

EPSCs – excitatory post-synaptic currents

Erc1 – ELKS/Rab6-interacting/CAST family member 1

FMRP – Fragile-X mental retardation protein

GABA - gamma-Aminobutyric acid

GKRs – GPCRs kinases

GluA1-4 – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor for glutamate subunits 1-4

GluN1-3 – N-methyl-D-aspartate receptor for glutamate subunits 1-3

GPC II – glutamate carboxypeptidase II

GPCRs – G-protein coupled receptors

GRIA1-4 – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor for glutamate subunits 1-4 genes

Grm5 – Metabotropic glutamate receptor 5 gene

GTP – guanosine-3-phosphate

GWAS – Genome-wide association studies

HA – hemagglutinin

Hdac2 – Histone deacetylase 2

HEK293T – human embryonic kidney 293T cell line

Homer1a – Homer protein homolog 1a

Hsd3b1 – 3 beta-hydroxysteroid dehydrogenase/Delta 5-4-isomerase type 1 gene

E/I – Excitation/Inhibition ratio

ID – Intellectual Disability

Ikbkg – NF-kappa-B essential modulator

IP – immunoprecipitation

IP3 – phosphatidylinositol-(3)-phosphate

Iqsec2 – IQ motif and SEC7 domain-containing protein 2

Irs1 – Insulin receptor substrate 1

KAR – kainate receptor

Kcnq2 – Potassium voltage-gated channel subfamily KQT member 2

Kif21a – Kinesin-like protein KIF21A

Kv 7.2 – 7.5 – Voltage-gated potassium channel subunit 7.2 – 7.5

LTD – long-term depression

LTP – long-term potentiation

Luzp2 – Leucine zipper protein 2

Map 2 – microtubule-associated protein 2

mGluR1-8 – metabotropic glutamate receptors 1-8

mGluR5 – metabotropic glutamate receptor 5

mGluRs – metabotropic glutamate receptors

MPEP – 2-methyl-6-(phenylethynyl)-pyridine

mPFC –medial Pre-frontal cortex

MRI – magnetic resonance imaging

NAAG – neuropeptide N-acetylaspartyl-glutamate

Nars – Asparagine-tRNA ligase, cytoplasmic

NatA – N-alpha-acetyltransferase protein complex

NF-H – neurofilament H

NMDAR – N-methyl-D-aspartate receptor for glutamate

NRGN – neuroligin

P2 – membrane extracts

PET – positron emission tomography

PFC – prefrontal cortex

PICK1 – Protein Interacting with C Kinase

PIP2 – phosphatidylinositol-(4,5)-bisphosphate

PIP5K2A – phosphatidylinositol-5-phosphate 4-kinase, type II, alpha

Pitpnm2 – Membrane-associated phosphatidylinositol transfer protein 2

PKA – protein kinase A

PKC – protein kinase C

PLC – phospholipase C

Plxna1 – Plexin-A1 - semaphorin receptor

PP1 – protein phosphatase 1

PP2A – protein phosphatase 2A

PSD – post-synaptic density

PSD 95 – post-synaptic density protein 95

PTX – picrotoxin

Pygb – Glycogen phosphorylase, brain form

Qki – Quaking Protein

Rpl24 – 60S ribosomal protein L24

Rpl7a – 60S ribosomal protein L7a

Rps2 – 40S ribosomal protein S2

SCZ - Schizophrenia

Sipa1l1 – Signal-induced proliferation-associated 1-like protein 1

SNP –single nucleotide polymorphisms

SPECT – single photon emission computed tomography

SR-SIM – Superressolution Structured Illumination microscopy

Stg KD – shRNA targeting Stargazin

StgID – Stargazin mutated in V143L, associated with Intellectual Disability

StgOV – Stargazin overexpression

StgSCZ – Stargazin mutated in S148N, associated with Schizophrenia

StgWT – Stargazin wildtype, shRNA resistant

SYNGAP1 - Synaptic Ras GTPase-activating protein 1

TARPs – Transmembrane AMPAR regulatory protein

TARP-ϒ2 – Stargazin

Tcp1 – T-complex protein 1 subunit alpha

Top2b – DNA topoisomerase 2-beta

TTX – tetrodotoxin

Tubb3 – Tubulin beta-3 chain

Vars – Valine-tRNA ligase

vGluT – vesicular glutamate transporter

Chapter 1

INTRODUCTION

1. GLUTAMATERGIC SYNAPSE

Neurons in the central nervous system communicate almost exclusively by the production and release of a series of molecules known as neurotransmitters. Neuronal projections intersect each other and communicate through a structure named synapse, which is the place where the release of neurotransmitters occur. Three most common neurotransmitters are glutamate, GABA and acetylcholine. In this work, we will focus on glutamate and the glutamatergic synapse.

Glutamate is the major excitatory neurotransmitter. There are two types of glutamate receptors, the ionotropic and the metabotropic receptors. Ionotropic receptors integrate an ion channel part whereas metabotropic are coupled with the activation of G-proteins. Ionotropic receptors are highly clustered at the synapse, more precisely in the postsynaptic density (PSD), a region enriched in proteins, such as scaffolds, kinases, phosphatases associated with each other and with the cytoskeleton[5,6] (Fig:1). Concerning metabotropic glutamate receptors, these are mainly located at the periphery of the PSD but still in the perisynaptical region[7]. The ionotropic glutamate receptors are named according to their preferential agonists, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), the N-methyl-D-aspartate receptor (NMDAR) and the kainate receptor (KAR).

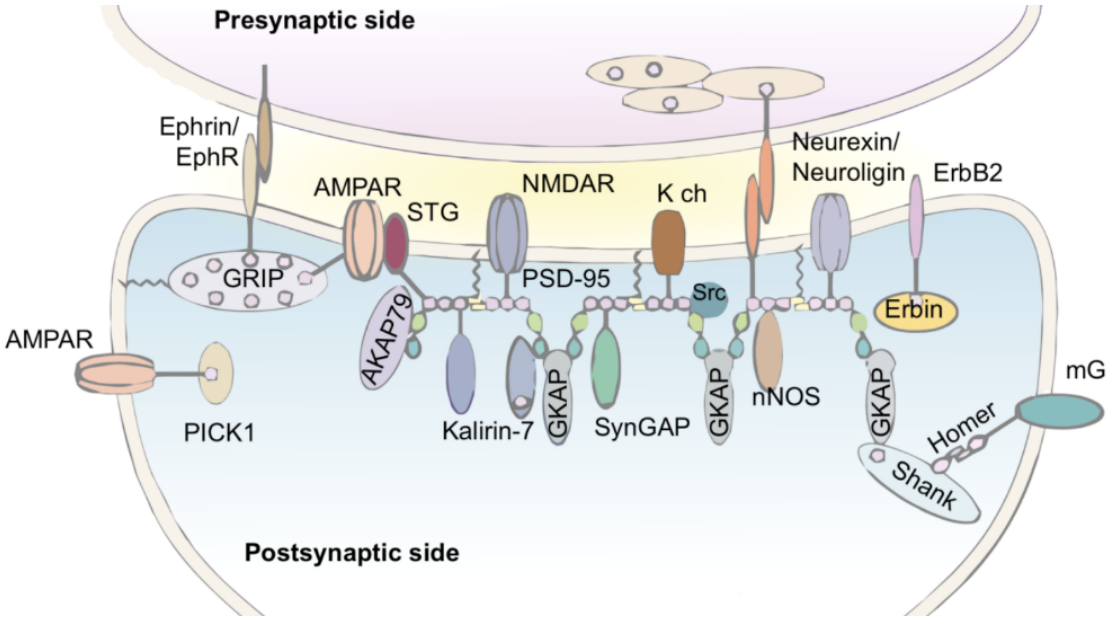

Figure 1: Glutamatergic post-synapse. The main PDZ-containing proteins of a glutamatergic synapse are shown. PDZ domains are represented by light-pink circles. Grey lines represent cytoplasmic C-termini of membrane proteins. Specific protein–protein interactions are indicated by the overlap of proteins. Only a subset of known protein interactions is represented. Crooked lines indicate palmitoylation of PSD-95 and GRIP. AKAP79 – A-kinase anchor protein 79; EphR – Ephrin receptor; ErbB2 – EGF related peptide receptor; GKAP – guanylate kinase-associated protein; GRIP – glutamate-receptor-interacting protein; K ch – potassium channel; mG – metabotropic glutamate receptor; nNOS – neuronal nitric oxide synthase; PICK1 – protein interacting with C kinase 1; PSD-95 – postsynaptic density protein 95; Shank – SH3 and ankyrin repeat-containing protein; SynGAP – synaptic Ras GTPase-activating protein. Adapted from[128]

1.1. AMPA RECEPTORS

The AMPAR is the ionotropic receptor for glutamate in the CNS[8,9] that is responsible for fast excitatory postsynaptic responses[10]. AMPAR are composed of (Fig: 2) four subunits (GluA1-GluA4) of about 105 kDa, encoded by different, yet closely related, genes (GRIA1-GRIA4). AMPAR subunit diversity is further increased by mRNA processing, by mRNA editing and alternative splicing, and by protein phosphorylation. The different subunits present high homology in the extracellular domain with the main differences in the intracellular region ultimately making them more prone to interact with different postsynaptic proteins. GluA1 and GluA4 have long C-termini tails, while GluA2 and GluA3 have short tails[11]. GluA1-GluA3 are more present in the outer layers of the cerebellar cortex, hippocampus, olfactory regions, basal ganglia, lateral septum and amygdala, whereas GluA4 is more abundant in the reticular thalamic nuclei and cerebellum (reviewed in[9]). The subunits are formed by four transmembrane domains (M1-M4), an extracellular N-terminus and intracellular C-terminus with a PDZ binding domain. The M2 is the pore forming domain and it does not span the phospholipidic bilayer[8,10,12](Fig: 3).

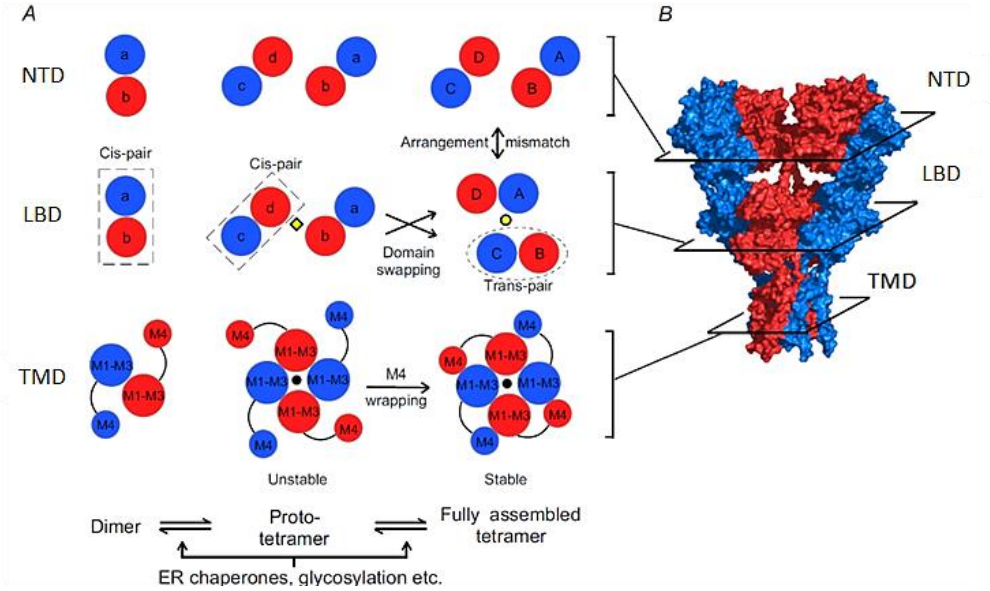

Figure 2: Scheme depicting AMPAR assembly – Dimer of Dimers. **A)** NTDs associate forming the dimers (left panels). At this stage the LBDs within the dimer form a cis-LBD pair (dashed rectangles). Dimers are unstable intermediates (proto-tetramer). In the proto-tetramer, the M1 and M3 helices, as well as the M2 loops, form the ion channel core (middle panels). The black dot denotes the ion channel pore. The cis-LBD pair adopts the same arrangement as the NTD dimers. The yellow square highlights the interface between the pairs, the B and D subunits. Recently, Gan and co-workers[13] proposed a mechanism for the assembly (right panels): 1) at the level of the TMD, M4 associates with M1–M3 of an adjacent subunit (M4 wrapping); 2) at the level of the LBD, cis-pairs dissociate altering the subunit arrangement and forming trans-pairs (domain swapping)(dotted ellipse). After the domain swapping, the dimer–dimer interface (yellow circle) is between the A and C subunits. These interactions stabilise the tetramer and the assembly could be subject to control by Endoplasmatic Reticulum chaperones and glycosylation, for instance. **B)** Crystal structure of homomeric GluA2 receptor found by Sobolevsky and co-workers[129]. The colour code is the same as in **A**. Subunit arrangement at each domain (planar sections) is shown in the right panels of **A**. NTD – N-terminus domain; LBD – ligand-binding domain; TMD – transmembrane domain; M1-M4 – transmembrane domains. Adapted from[13].

The assembly diversity, homomeric or heteromeric, and the processing by RNA editing, alternative splicing and phosphorylation give rise to differences in sensitisation/re-sensitisation kinetics, conductance, trafficking and insertion at the synapse[9,13]. The GluA2-containing receptors are the most predominant in most brain regions. This subunit is a constitutive target of mRNA glutamine/arginine (Q/R) editing at the pore domain (M2). The positively charged arginine decreases permeability to cations, thus the GluA2-containing receptors are impermeable to Ca^{2+}. Moreover, polyamines are no longer able to block the pore during depolarization, since they have positive charge, thus GluA2-containing receptors are non-rectifying channels, whereas GluA2-lacking receptors are inward rectifying. This edition is protective since it prevents glutamate-mediated excitotoxicity by decreasing neuronal excitability[14,15].

GluA subunits can also undergo alternative splicing at S2 region assuming two possible forms, flip or flop. The flip-flop cassette has only a few different amino acids, nonetheless this is enough to induce different desensitization and Endoplasmatic Reticulum (ER) export kinetics. Flip isoform desensitizes more slowly and is expressed in early stages of development, whereas Flop isoform desensitizes faster and its expression increases with maturation[16]. Flop form is arrested at the ER, whereas Flip form undergoes glycosylation reaching the surface at higher levels than Flop form[17].

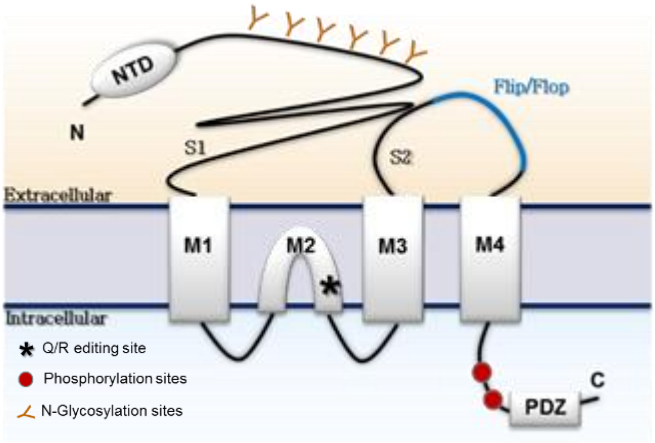

Figure 3: Scheme illustrating the structure of GluA subunits. The N-terminus is extracellular and can be glycosylated, whereas the carboxylic terminal is intracellular and can be phosphorylated. The C-terminus interacts directly with proteins of the postsynaptic density that regulate AMPAR insertion and trafficking. The M2 is the only intramembrane domain, resembling a hairpin loop. It forms the pore region and encompasses the Q/R editing point of GluA2. S1 interacts with S2 forming the ligand-biding domain that binds glutamate.

Besides RNA editing and splicing, the AMPAR subunits are regulated by phosphorylation of the C-terminus. This post-translational modification is a key mechanism in regulating AMPAR trafficking, insertion and function. It was shown that AMPAR phosphorylation enhances AMPAR-mediated currents whereas dephosphorylation has the opposite effect (reviewed in [9,12]). As reviewed by Santos and colleagues[9], the intracellular C-terminus is substrate of Protein Kinase A (PKA), Protein Kinase C (PKC), Ca^{2+}-Calmodulin-dependent Kinase (CaMKII) and cGMP-dependent protein kinase II (cGKII) (Fig: 4).

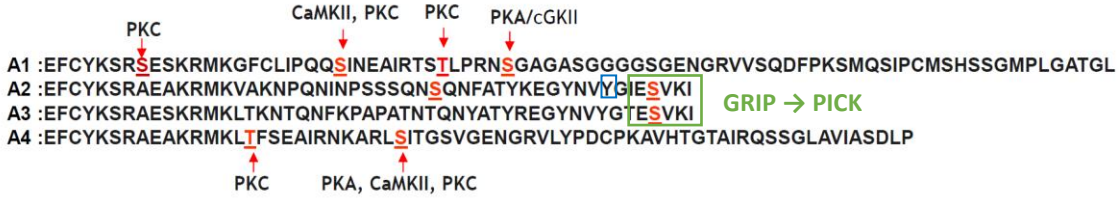

Figure 4: Representation of the C-terminus phosphorylation sites of each AMPAR subunit by different kinases (reviewed in[9]). GluA1-S845 (serine 845) phosphorylation by PKA and cGKII increases the opening probability and is required for synaptic insertion, whereas S831 phosphorylation by CaMKII and PKC increases conductance being also required for synaptic insertion. Only S845 mutations impair GluA1 delivery at the synapse. Both S residues are necessary for insertion but not sufficient. GluA1-T840 (threonine 840) and S818 are substrate for PKC, being T840 important for turnover and S818 critical for LTP-mediated insertion. GluA2-S880 phosphorylation (within the PDZ domain) by PKC induces fast internalization by reducing interaction with GRIP1 and recruiting PICK1, thus being critical for LTD induction. GluA2-Y876 (tyrosine 876) (in blue box) phosphorylation by Src family protein tyrosine kinases has similar effect to the S880, but without affecting PICK1 binding, thus facilitating AMPAR internalization. GluA4-S842 phosphorylation by PKA, CaMKII and PKC is necessary and enough for synaptic insertion of GluA4-containing receptors. This is modulated by metabotropic glutamate receptors. Adapted from [9]

Furthermore, all subunits can undergo palmitoylation in two cysteine residues at M2 and C-terminus. At M2, palmitoylation increases accumulation in Golgi and at C-terminus it induces internalization by blocking the interaction with stabilisers, such as 4.1N [18]. Thus, palmitoylation of the subunits is intimately related with AMPAR internalisation. Due to high regulation and control of AMPAR, these receptors have been observed as potential intervenient in mechanisms of neuropsychiatric disorders. In fact, it was described that the forward trafficking of AMPAR in frontal cortex of SCZ patients might be abnormal[19]. Additionally, GluA subunits and proteins from the postsynaptic density interacting with glutamate receptors, namely Stargazin[3] and shank3[20] have been found to be linked to neuropsychiatric disorders[21].

1.1.1. TRANSMEMBRANE AMPAR REGULATORY PROTEINS

Transmembrane AMPAR regulatory subunits (TARPs) are proteins from the postsynaptic density that interact with AMPAR. They modulate trafficking and insertion at the synapse and can regulate functional properties of AMPAR[22]. TARPs family includes Stargazin (ϒ2), ϒ3, ϒ4, ϒ5, ϒ7 and ϒ8, and all share homology to the skeletal muscle voltage-gated Ca^{2+} channel auxiliary subunits, ϒ1 and ϒ6. TAPRs may be subdivided according to their PDZ-domain type. Type I ϒ2, ϒ3, ϒ4 and ϒ8 TARPS have typical PDZ-domain and ϒ5 and ϒ7 have atypical PDZ-domain. Both types are found in both neurons and glia and display complex cell-type-specific expression that changes during development. Concerning the TARPs expression throughout the brain, the hippocampus is highly enriched in ϒ8, the cerebellum expresses mainly ϒ2 in cerebellar granule neurons, whereas ϒ3 seems to play an important role specifically in cerebellar Golgi cells. In neocortex and thalamus, ϒ2 is the prevalent TARP (reviewed in [23]).

1.1.2. STARGAZIN

Stargazin (also known as TARP-ϒ2) is a type I TARP encoded by the gene *CACNG2* (22q12.3). Initially, interest in this protein was triggered by the identification of *Stargazer*, a spontaneous mutant mouse which does not express Stargazin and displays cortical excitability alterations, absence seizures and the distinctive behaviour that justified its name, constantly tipping back the head to the top, "gazing the stars" [24]. Stargazin sequence presents moderate homology to the regulatory ϒ subunit of skeletal muscle voltage-gated Ca^{2+} channels and at first, it was suggested to be a new ϒ subunit for neuronal Ca^{2+} channels and able to reduce their availability at resting membrane potential[25].

TARP-ϒ2 is a 37kDa protein, highly expressed in cerebellum and cortex[23,26] and has a critical role in AMPAR trafficking and insertion[27]. Stargazin structure consists of four transmembrane domains and intracellular C and N-termini. At the C-terminus it has a PDZ binding domain allowing the interaction with PSD95, a key player in the stabilisation of AMPAR at the synaptic surface[26,27].

Recently, Stargazin was found to be regulated by phosphorylation at the C-terminus, where nine serine (S) residues are present. In fact, it was shown that phosphorylation and dephosphorylation of these residues was required for LTP and LTD, respectively[1]. Stargazin is phosphorylated by CaMKII and PKC in a NMDAR-dependent manner and its dephosphorylation occurs via PP1 activation[1]. Studies with phosphomimetic Stargazin (S9D) corroborate the above mentioned, increasing AMPAR clustering at the synapse and preventing LTD[1]. Additionally, quantum-dots analysis showed Stargazin phosphorylation by CaMKII induces AMPAR accumulation at the synapse[28]. Besides its role in Hebbian plasticity, a recent work from our lab described that Stargazin phosphorylation is required for homeostatic upscaling and experience-dependent plasticity of the retinogeniculate synapse[2]. In addition to the trafficking function of Stargazin, the ectodomain was found to interfere with AMPAR kinetics increasing the rate of channel opening[29].

1.2. NMDA RECEPTORS

NMDAR play a critical role in excitatory synaptic transmission in the CNS. NMDARs present voltage-sensitive blockade by extracellular Mg^{2+}, a high permeability to Ca^{2+} and slow activation/deactivation kinetics. NMDARs can also respond to a wide range of endogenous ligands and modulators present close to the synapse. Glycine is a co-agonist that must bind to the receptors prior their activation. Additionally, D-serine occurs at high levels in the brain, where it is an endogenous co-agonist at the "glycine site" of NMDAR[30]. Studies revealed that NMDARs are assembled as tetramers that differ in subunit composition. There are three subfamilies of NMDAR subunits GluN1, GluN2 (A, B, C and D) and GluN3 (A and B). GluN1 arises from only one gene but occurs in 8 different isoforms due to RNA splicing, GluN2 subunits are encoded by 4 different genes and GluN3 arise from two separate genes[31–33].The length of the subunits varies from 900 to over 1480 due to differences in the length of the intracellular C-terminus, which is the region involved in receptors trafficking and signalling pathways[31] Through the C-terminus, NMDAR subunits interact with multiple intracellular scaffolding, anchoring and signalling molecules including PDZ-domain containing proteins, and similarly to AMPAR, trafficking and insertion at the synapse is also regulated by phosphorylation[34]. Moreover, NMDAR are also key players in synaptic plasticity.

1.3. METABOTROPIC GLUTAMATE RECEPTORS

The Metabotropic Glutamate receptors (mGluRs) are G-Protein Coupled Receptors (GPCRs), thus having GTP-binding proteins as downstream effectors that modulate synaptic transmission in a slow manner. Regarding their function, they work as modulators by mediating excitatory and inhibitory transmission, regulating calcium and potassium channels, impacting neurotransmitter release and mediating mechanisms of neuroplasticity[7]. There are eight different forms (mGluR1-8) and the multiplicity of mGluRs is due to existence of splice variants. These receptors are widely expressed throughout the brain and may be divided into three groups, Class I, II and III, on the basis of their sequence similarities, pharmacological properties, and intracellular signal transduction mechanisms. Class I encompasses mGluR1 and 5 that are coupled with the activation of $G_{q\text{-}11}$ associated with phosphatidylinositol-(3)-phosphate (IP3)/Ca^{2+} signal through Phospholipase C (PLC) activating PKC. Class II, mGluR2, mGluR3, and Class III, mGluR4 and mGluR6-8, coupled to G_i-G_o and inhibit adenylyl cyclase [7,35]. Once activated, these receptors desensitize and undergo subsequent endocytosis that is crucial for either the re-sensitization or down-regulation of the receptor [36,37]. This desensitization, and consequent internalization, is mediated by GPCRs kinases (GRKs) and β-arrestins. GRKs are targeted to the plasma membrane and phosphorylate serine and threonine residues within the third loop of the mGluR C-terminus uncoupling the GPCR/G-

protein signalling[37]. CaM and PKC are also described to be involved in mGluR desensitization[38]. In order to be re-sensitized mGluRs are dependent upon receptor and β-arrestins dephosphorylation in endosomes[37].

Concerning their localization, Class I is mainly somatodentric, Class II is both somatodendritic and axonal, whereas Class III occurs mostly at axonal terminals, in the pre-synaptic compartment[7]. These receptors have been reported to be important for Hebbian and Homeostatic forms of plasticity and this will be under scope in the following section.

2. NEUROPLASTICITY

According to Hebb[39], simultaneous activation of pre and postsynaptic neurons induces synaptic strengthening. This involves plastic events at cellular and molecular level and it is thought to underlie memory and learning. The word plasticity arises from the modulations, regulations and alterations within pre- postsynaptic compartments. Postsynaptically, this can be either by stoichiometry changes, insertion and internalization, activation or inactivation of receptors and proteins of the postsynaptic density of glutamatergic synapses. Hebbian plasticity encompasses long-term potentiation (LTP) and long-term depression (LTD) events, whereas homeostatic plasticity consists of compensatory alterations within the network in consequence of periods of prolonged high or low activity (Fig: 5). Glutamatergic synapses can be influenced by activity and this depends on AMPAR and NMDAR function and regulation. A series of events, such as phosphorylation of the AMPAR subunits, or the interaction with proteins and regulators (mGluRs), promote insertion or internalization of the AMPAR. Hence, glutamate receptors are required players for neuroplasticity to occur.

2.1. HEBBIAN PLASTICITY

Hebbian plasticity, comprising LTP and LTD, is the most studied mechanism of synaptic adaptation induced by activity. LTP and LTD are feedforward processes induced by short periods of activity, yet they can last for long periods of time. Previous studies described the properties of these events highlighting their impact in physiological and pathological processes[40].

During LTP there is an increase of synaptic transmission upon a period of high-frequency stimulation. However, LTP can also be induced by pharmacological approach (chemical LTP).This transmission outlasts the stimulus and synapses remain potentiated. The opposite occurs with LTD where upon a stimulus there is a period with lower activity in the synapse[9].

LTP involves increasing and clustering of AMPAR at the synapse, particularly GluA1-containing receptors that are permeable to Ca^{2+} and can be blocked by polyamines due to absence of Q/R edition. Truthfully, knock-down of GluA1 in mice impairs LTP generation[9]. This insertion and trapping of AMPAR at the synapse is NMDAR-dependent meaning that LTP requires both neurotransmitter release and electrical stimulation allowing the removal of Mg^{2+} from blocking NMDAR[41].AMPARs are inserted in a rapid manner because recycling endosomes contain a pool of receptors ready for insertion, however they can also diffuse from the extrasynaptic surface towards the synaptic region[42–44]. In fact, even under basal conditions, AMPAR trafficking is very dynamic, nonetheless only GluA1-lacking receptors are passively incorporated into synapses[9,43,45]. Levels of Ca^{2+} entering determine whether the synapse undergo LTP or LTD. Both require NMDAR activity and increase in the intracellular Ca^{2+} [9,42]. However in LTP, the entrance of this cation is higher[9,42] thus activating kinase-cascades, such as CaMKIIα/β[46], PKA or PKC[43], rather than phosphatases. Indeed, CaMKII inhibitors and CaMKII knockdown blocked LTP, whereas expression of a constitutively active CaMKII was reported to occlude LTP (reviewed in[42]). TARPs and GluA subunits are known substrates of kinases[1,2,9,12] and, in fact, phosphorylation of AMPAR is required for their insertion at the synapse[9,12]. Once activated, CaMKII migrates towards the postsynaptic density and phosphorylates GluA1 at S831 enhancing channel conductance. TARP-ϒ2 (Stargazin) is a substrate of CaMKII and PKC, inducing its interaction with PSD95, an anchoring protein member of MAGUK family that stabilises AMPARs clusters at the synapse increasing their number[43].

Regarding LTD, it can be NMDAR-dependent or mGluR-dependent. In the NMDAR-dependent pathway the difference lays on the influx of Ca^{2+}. Due to low activity, NMDAR activation is decreased allowing only a small amount of Ca^{2+} to enter the cell, thus mainly the phosphatases are able to respond. Nevertheless, both forms include phosphatases and PKC activation. GluA1-S845 dephosphorylation drives endocytosis of the GluA1-containg receptors and Stargazin dephosphorylation decreases its interaction with PSD95 thus AMPARs are no longer stabilised at synaptic surface. Moreover, S880, in the PDZ domain of GluA2, is targeted by PKC inducing its internalization by interfering with the interaction with GRIP1[9](described in AMPAR section, see Fig: 4).

The mGluR-dependent LTD is associated with Class I metabotropic receptors that are coupled to G_{q-11}. Activation of PLC converts phosphatidylinositol-(4,5)-bisphosphate (PIP2) into IP3 that can activate PKC and ryanodine receptors from intracellular Ca^{2+} reservoirs. The Ca^{2+} influx activates phosphatases that, together with PKC and Protein Interacting with C Kinase (PICK1) , destabilize AMPARs in the synaptic surface[47]. In Fragile-X context, excess of mGluR5 activation facilitates LTD. This happens due to the lack of a protein, Fragile-X Mental Retardation Protein (FMRP), that exerts negative feedback over mGluR5 signalling, hence in its absence internalization of AMPAR is amplified[48].

2.2. HOMEOSTATIC PLASTICITY

If Hebbian plasticity was the only plastic mechanism occurring, synapses would scale up or down unconstrainedly causing abnormal functioning of the neuronal network. Hence, in order to compensate the feedforward processes, neurons have feedback processes, namely homeostatic plasticity.

Homeostatic plasticity occurs when there are long periods of high or low activity in order to re-stabilise the balance, inducing alterations in pre and postsynaptic sides. This form of neuroplasticity was reported to occur in a global manner, affecting all synapses of a neuron, but also locally operating on individual or small groups of synapses. Additionally, it can occur through pre and postsynaptic changes[49]. The main goal is to maintain the stability of the activity of the neuron or neuronal circuits[50] (Fig:5).

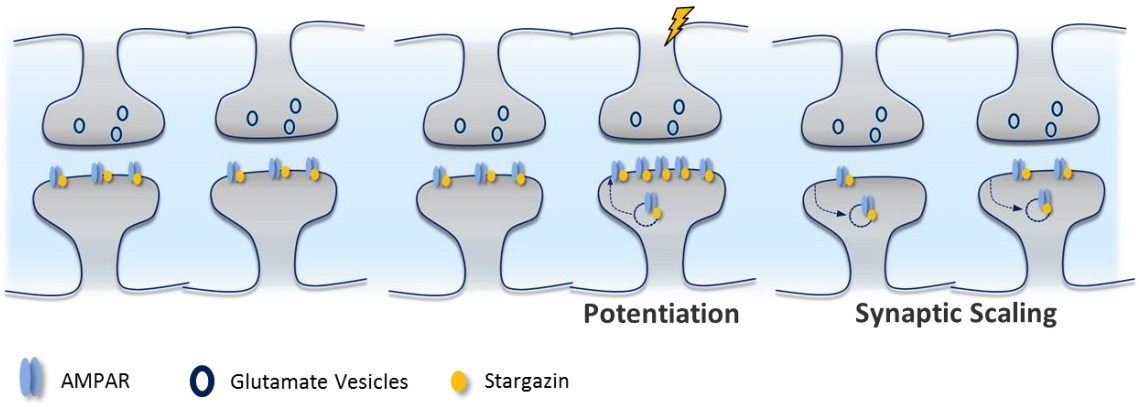

Figure 5: Neuroplasticity. Initially the synapses are balanced (left). Then, a period with neurotransmitter release and firing occurs locally at the second synapse. This induces LTP at the second synapse that gets potentiated by AMPAR insertion (centre). Finally, there is a down scaling of the potentiated synapse to re-stabilise the network, however the differences between the two spines must be kept and thus the first synapse is also down scaled.

Many are the mechanisms by which the neuron constrains excitability, such as activity-dependent regulation of intrinsic neuronal firing properties, pre and postsynaptic forms of excitatory synaptic plasticity and synaptic scaling. Synaptic scaling adjusts the strength of all excitatory synapses of a neuron by changing the number of available receptors (AMPAR and NMDAR) and modifying the synapses number (reviewed in[51]). The feedback process was first described in cultured neocortical neurons where perturbing the network activity generated compensatory changes in the synaptic strength returning the firing rates back to the basal (reviewed in[50]). Indeed, studies of chronical blockade of the inhibitory transmission with picrotoxin in hippocampal, spinal and cortical neurons leads to decrease in AMPARs at the surface, whereas chronic application of glutamate receptors antagonists (CNQX and APV) increased AMPARs at the surface (reviewed in[9]). In adult cortex, a reduction of inhibitory transmission towards excitatory cells usually precedes excitatory plasticity modifying the excitation and inhibition ratio[52]. *In vivo* studies of visual cortex have shown that

different types of synaptic inhibition deprivation (focal retinal, binocular retinal lesions and monocular deprivation) seem to be permissive for excitatory reorganization[53]. Focusing at excitatory synapse, pharmacological manipulations can induce bidirectional modifications inducing increase of VGlut in presynaptic compartment. In addition, both AMPAR and NMDAR were described to be regulated upon homeostatic plasticity protocols[49]. Furthermore, it seems homeostatic plasticity is cell-autonomous. To further test this hypothesis, selective blocking on individual neurons soma by microinjection of tetrodotoxin was found to scale up synaptic strength as it does when the network activity is blocked. Also, when blocking postsynaptic firing in neocortical neurons the scaling occurs by a reduced CaMKIV activation, thus requiring a decrease in the Ca^{2+} influx. AMPARS accumulate in clusters at the synapse translating in an increase of mini-EPSCs (Excitatory post-synaptic currents) amplitude. Scaling down is also cell-autonomous involving increase in Ca^{2+} influx and thus activating CaMKIV. Other calcium-dependent pathways have been identified, one involving Homer1a, an immediate early gene required for scaling down through a pathway that requires agonist-independent regulation of mGluRs. The second involves Eph4A that acts by regulating the non-canonical ubiquitin pathway in order to control AMPAR degradation. Scaling is totally blocked upon postsynaptic GluA2 knock-down and blocking presynaptic activity-induced GluA1 insertion at the synapse (reviewed in[49]). Additionally, recent works have shown Stargazin[2] and mGluR5[54] play important roles in homeostatic plasticity. In the work of Louros and co-workers, the Stargazin *knockdown* totally blocked the homeostatic increase of GluA1 in neurons treated with tetrodotoxin (TTX), a well-known activity blocker[2]. Regarding mGluR1/5, it was shown that inverse agonists of class I mGluRs impair AMPAR synaptic scaling upon a TTX stimulus[54]

Since homeostatic plasticity acts in order to maintain the *status-quo* in the network and neuropsychiatric disorders are accompanied by a disruption of this balance, mechanisms of homeostatic plasticity may represent an important component to study. Additionally, these mechanisms involve proteins found to be altered in psychiatric context.

3. NEUROPSYCHIATRIC DISORDERS - GENERAL OVERVIEW

The incidence of neuropsychiatric disorders has increased in the past decades and little is known about their etiology leading to an increased need for research in this field. Most of the neuropsychiatric disorders are also considered neurodevelopmental disorders, due to the existence of developmental risk factors that are related with their pathophysiology. Although diseases such as Autism Spectrum Disorders (ASD), Attention-Deficit-Hyperactivity Disorder (ADHD), Bipolar Disorder (BD), Intellectual Disability (ID) or Schizophrenia (SCZ) are examples of well-known neuropsychiatric disorders, their pathogenesis is poorly characterized. Furthermore, the variability and intensity of symptoms commonly postpone the correct diagnosis.

One of the main concerns regarding these disorders is the lack of targeted and specific therapeutic strategies, to cover the highly variable symptoms presented by the patients. In Europe, these disorders are definitely the major contributor to chronic conditions (WHO), accompanied by a strong life-disability component leading the governments to develop financial concerns.

Although distinguishable, most of the psychiatric disorders present common features such as impairment of cognition and social skills, strong hereditary component and similar origin. These disorders share cytoarchitectonical, connectional and functional features, affecting medial Prefrontal Cortex (mPFC), intralimbic, prelimbic and cingulate areas, which are associated with cognitive deficits[55]. Furthermore, alterations in mPFC development, in the proliferation of neuronal progenitor cells, in the migration of cortical neurons and in synaptogenesis are common features of these disorders[55]. Genome-wide association studies (GWAS) of neuropsychiatric patients have revealed mutations in genes encoding synaptic genes[56,57], showing that the mechanisms that affect neurotransmission might be impaired in these disorders. Indeed, evidence has emerged in the last years, indicating synaptic dysfunction as ultimately leading to an overall disruption of neurotransmission in neuropsychiatric context due to an unbalance between inhibition and excitation[58].

3.1. SCHIZOPHRENIA

Schizophrenia (SCZ) is one of the most debilitating psychiatric illnesses that affects higher brain function presenting severe symptoms that can be divided in positive, negative and cognitive symptoms. The diagnosis of Schizophrenia is only based on the patients' symptoms. The onset of this disorder occurs in young adulthood and persists throughout life, having a lifetime risk of about 1% and heritability within a range of 64-80%[59,60]. The positive symptoms include hallucinations, delusions whereas the negative symptoms are related to decrease of emotional reactivity, loss of motivation and anhedonia. These symptoms are accompanied also by cognitive impairment, with the individuals often displaying reduction of the IQ, attention deficit, delayed word recall or disorganized cognitive processes. Much has been done in order to determine susceptibility gene mutations for SCZ. Currently, it is accepted that, besides the environmental factors, the genetic risk for developing SCZ is polygenic, involving multiple alleles with small effect[61]. Moreover, this risk component was shown to overlap with BD, ASD and ID, but not with other non-neuropsychiatric disorders[61,57,55], which is in accordance with the above stated. In fact, BD may present a subset of psychotic symptoms similar to SCZ, although the set of symptoms and incidence is distinct[62].

Neuroimaging techniques such as magnetic resonance (MR), single photon emission computed tomography (SPECT) and positron emission tomography (PET) have provided important information about morphological and functional changes occurring in these patients brains. Studies in schizophrenic patients show brain structural abnormalities,

particularly increased volume of the ventricles due to shrinkage of the gray matter. This might be due to decreased spine density and altered dendritic arborisation in amygdala, parahippocampal gyrus, temporal lobe, thalamus, hippocampus and basal ganglia, being the prefrontal cortex (PFC), cortex (CX) and, ultimately, hippocampus the most affected regions (reviewed in [63–65]). In PFC, there is a high packaging of pyramidal neurons somas due to reduced soma size and reduced neuropil. In fact, schizophrenic patients present shorter and less branched dendrites, as well as lower spine density[63].

In a close look at synapses, the discovery that effective drugs were exerting their effect by blockage of dopamine receptor type 2 (D2) led to the formulation of the dopamine hypothesis claiming that the trigger of the disorder was an excess in dopaminergic transmission[64,66]. Dopaminergic hyperactivity, mainly in mesolimbic pathway, has been associated with SCZ and all currently known antipsychotic medications target this system[67,68]. Recently, tyrosine hydroxylase, the rate-limiting enzyme involved in the synthesis of dopamine, was found significantly increased in the substantia nigra of schizophrenic patients compared with patients with depression and healthy controls[69]. Regarding dopamine receptors, recent meta-analysis[70] found increased D2/3 receptor density in patients with SCZ, however in drug naïve patients there were not found any differences. PET imaging studies described increased D2 receptor availability in the group that had experienced long-term drug treatment, consistent with the idea that there is D2 upregulation with antipsychotic treatment[71].

Nonetheless, targeting dopaminergic system does not treat all SCZ associated symptoms, indeed D2 blocking is not capable of ameliorate cognitive deficits and large percentage of the patients suffers from diverse and adverse side effects due to this drug approach[55]. Later on, serotonin receptors were also found to be an important target in SCZ therapy context, giving strength to a new hypothesis that, besides dopamine, other neurotransmitters might be related to the etiology of the disease[62,72]. This was especially interesting since cognitive symptoms are not treated by targeting dopaminergic pathways, which may suggest it does not play a role in the generation of these symptoms.

The first evidence of the implication of the glutamatergic transmission in SCZ emerged in the 60s when phencyclidine (PCP), an antagonist of the NMDAR was capable of inducing positives, negatives and, strikingly, cognitive symptoms in healthy subjects and exacerbate these symptoms in schizophrenia patients.

The convergence between the dopamine and the glutamate hypothesis lays on NMDAR-dependent activation of GABAergic interneurons that modulate the release of dopamine in striatum[73,74]. Less NMDAR activity in the midbrain glutamatergic projections leads to striatum GABAergic interneurons hypoactivation, thus releasing dopaminergic neurons from the inhibition of these GABAergic interneurons. Hence, it is possible to consider dopaminergic hyperactivity to be a secondary event in SCZ, since the glutamatergic dysfunction may be underlying the dopamine dysfunction. Post-mortem studies have shown reduced mRNA and enzymatic activity of glutamate carboxypeptidase II (GCP II). This enzyme is responsible for the degradation the neuropeptide N-acetylaspartyl-glutamate (NAAG), which consists of a reversible antagonist of NMDAR[75]. Targeting NMDAR in order to increase its mediated activity seems

promising since it could ameliorate the positive and negative symptoms by regulating dopaminergic pathway and possibly target cognitive symptoms that remain untreatable. Therefore, studies have been trying to modulate class I mGluRs since they signal via Phospholipase C potentiating the NMDAR currents[76].

Currently, it is accepted that despite the origin, the cause behind the neuropsychiatric disorders could be the imbalance between the excitation and inhibition in the neuronal circuitry[58], giving rise to the new hypothesis where synapse dysfunction is underlying neuropsychiatric disorders. Recently, data from several studies have shown a strong correlation between synaptic dysfunction and SCZ. Genome-wide studies have reported strong association between psychiatric disorders, such as SCZ, major depression and BD, and histone methylation processes, neuronal signalling pathways and mutations in proteins known to co-localize with the post-synaptic density[77]. In fact, NOTCH, PLXNA2, NRGN are examples of synaptic proteins found to have single nucleotide polymorphisms (SNPs) in schizophrenic context[56]. Therefore, it is possible to consider summation of the low-effect SNPs found in many synaptic genes as a possible cause of the alterations of the disorder. Additionally, mutations and DNA copy number in the *CACNG2* gene encoding Stargazin, the main protein under the scope of this work, were associated with SCZ without causing deficits in executive function[78], thus emphasising the importance of glutamatergic transmission in the SCZ context. Moreover, DNA microarrays have shown a strong decrease in the transcription of glutamatergic and GABAergic neurotransmission-related genes[79] which further supports the hypothesis of synaptic dysfunction in neuropsychiatric disorders, including schizophrenia.

3.2. INTELLECTUAL DISABILITY

Previously named mental retardation, intellectual disability (ID) is a disability rather than a disease that involves impairment of the daily basis routine of an individual. ID affects almost 3% of the general population, among which there is an excess of 30-50% of males over females[80]. Compared to other pathologies, ID is less studied although it seems to be more prevalent than many others. Typical symptoms are decreased language, reading, writing, math and reasoning skills that ultimately lead to decreased IQ, usually lower than 70, and impaired memory[81]. Also, poor social skills is a characteristic of ID symptomology[81]. It is also common to find patients that are simultaneously affected by other neuropsychiatric disorders, such as ADHD or ASD. There is no determined age for the onset of ID, however the diagnosis occurs usually during adolescence[82]. It can be divided in two forms, syndromic and non-syndromic. The syndromic form is associated with clinical, morphologic, metabolic, radiological or biological abnormalities, whereas the non-syndromic is characterised by the absence of these features presenting solely the cognitive impairment as symptom[80]. The cause is usually multifactorial involving environmental and genetic factors, nonetheless up to 60% of the cases have a non-identifiable cause. In the last decade, chromosome aberrations causing Down Syndrome, Prader-

Willi and Angelman, Williams, Smith-Magenis, Miller-Dieker or DiGeorge Syndromes were found in 15% of the patients with severe syndromic ID[83]. Regarding non-syndromic form of ID, approximately 25% of the cases may be due to autosomal recessive inheritance[84]. However, *de novo* mutations in X-linked genes were recently described in non-syndromic ID patient samples. Using whole genome sequencing, this study showed mutations in N-alpha-acetyltransferase 10, a catalytic subunit of the N-alpha-acetyltransferase protein complex (NatA), which is highly expressed in developing brains and shows lower, yet ubiquitous, expression in adulthood[85]. NatA is responsible for acetylation of new proteins at the time of translation and non-ribosomal posttranslational acetylation processes[85]. Moreover, X-linked mutations in small GTPases, as Rho and Ras that have a key role in development and function of synapses, were found in ID patients, both syndromic and non-syndromic[83]. Both, X-linked and autosomal non-syndromic ID patients were shown to have mutations that lead to changes in the stoichiometry of synaptic protein complexes, whether by truncation, CNVs or dominant-negative effect (reviewed in[83]). Lately, Hamdan and co-workers described SNPs and truncating mutations in genes encoding synaptic proteins, such as Stargazin[3] and SYNGAP1[86]. This proteins co-localise at the synapse, more precisely at the glutamatergic postsynaptic compartment and directly mediate AMPAR trafficking and insertion, or in the case of SYNGAP1, indirectly affecting AMPAR trafficking by interacting with NMDAR as its downstream effector[86].

Altogether, evidence points towards the synaptic dysfunction theory in ID and highlights the importance of future studies focusing on the synaptic compartment, particularly the glutamatergic synapse where many genes were shown to be altered in neuropsychiatric context.

3.3. STARGAZIN AND NEUROPSYCHIATRIC DISORDERS

In neuropsychiatric context, , CACNG2 was reported as a susceptibility locus for neuropsychiatric disorders such as BD, ID and SCZ[78,87].

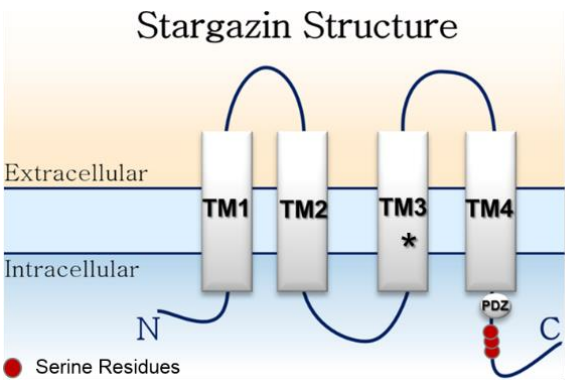

Figure 6: Scheme of Stargazin structure. Stargazin consists of four transmembrane domains (TM1-TM4) and intracellular C and N-termini. C-terminus has a PDZ biding-domain and nine consecutive serine residues that can be phosphorylated by CaMKII and PKC. This region is responsible form interactions with AMPAR and PSD95, thus it is of utmost importance for AMPAR trafficking, insertion and modulation. At TM3 were found two mutations, V143L in ID and S148N in SCZ (black asterisk).

Studies have reported that Stargazin transcripts were increased in prefrontal cortex of SCZ patients[88] and in anterior cingulate cortex other TARPs were found disregulated[89].

Recently, a single-nucleotide mutation (V143L) was found in a ID patient and it was proved to impair the interaction with AMPAR[3] (Fig:6). Moreover, our lab identified a single-nucleotide mutation in the same domain (S148N) (Fig:6) in a SCZ patient (unpublished data).

Predictions point to a protein function damaging probability close to 97%, for both mutations (Fig:7), and studies in this lab have shown that both variants present impaired membrane and synaptic insertion of AMPAR (unpublished data). Thus, it is possible that the interactome of these variants is different from the wild type form.

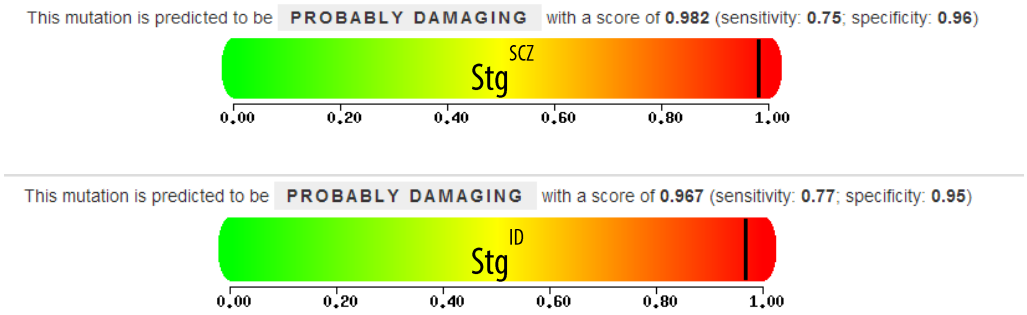

Figure 7: The identified mutations in the 3TM of Stargazin (V143L in ID and S148N in SCZ) were predicted to be probably damaging with a score of 98.2% for StgSCZ and 96.7% for StgID. The prediction was calculated with poly-phen 2 prediction toll[130].

4. INTERACTION CANDIDATES

Our lab performed Stargazin immunoprecipitation assays from cortical neuron samples and, in order to evaluate the interactome of Stargazin, Mass Spectrometry analysis was undertaken in collaboration with Dr. Ka-wan Li from the Vrei University of Amsterdam. Interestingly, among many other potential new interactors, we found mGluR5 and Kv 7.2. mGluR5 is a Class I Metabotropic Glutamate Receptor [7] and Kv 7.2 is a subunit of the voltage-gated potassium channel, M-channel[4]. We chose these candidates to be under the scope of the present study because of their known association with neuropsychiatric disorders and because of their importance for neuronal transmission. Moreover, mGluR5 activation is known to decrease the currents induced by M-channels suggesting a possible link between the three proteins.

4.1. THE M-CHANNEL SUBUNIT Kv 7.2

The M-channels are sensitive to depolarization (-60 mV), thus opening the pore and generating an outward K^+ current (M-currents) to repolarise the neuron during an action potential. The neuronal M-current was first described in 1980 as a slowly activating, non-inactivating K^+ current. Hence, M-channels set the resting potential, control synchronous population firing activity and modulate excitability of neurons[4]. The M-channel is an heteromeric channel, composed by Kv7.2 and Kv7.3 subunits, and it is the most relevant channel for the generation of the M-currents[90,91]. These channels locate mainly at the axonal initial segment and node of Ranvier, but also at the somatodendritic compartment[92,93]. It was shown that M-channels can co-localise with Na_v voltage-gated Na^+ channels that are directly involved in action potential generation[92,94]. Besides M-channels, Kv 7 family members (Kv 7.1-Kv 7.5) may assemble as homomers or heteromers.

The KCNQ2 gene (20q13.33) encodes for the voltage-gated potassium channel subunit Kv7.2. There are four homologous subunits (Kv7.2-7.5) that assemble as tetramers to form the M-channels[93,95]. Each subunit consists of six transmembrane domains and intracellular C and N-termini. The region encompassing the transmembrane segments S1-S4 forms the voltage sensing domain and S5-S6 forms the pore through which the K^+ ions flow. (Fig: 8). Kv7 subunits have a long C-terminus with regions determining heteromeric and homomeric assembly, interaction with regulatory proteins, subcellular localization, and binding of accessory proteins. In this region, there are four α-helices (A to D) conserved among Kv 7 family[96], being A and B Calmodulin- (CaM) binding regions[97]. In the work of Nájera and colleagues, CaM was immunoprecipitated in the absence and presence of Ca^{2+}, and for Kv 7.2, Ca^{2+}decreases the binding to CaM[96]. The function of this interaction relates to Kv 7 maturation, trafficking and insertion in the membrane[93]. Syntaxin 1A was also described to interact specifically with the helix-A of Kv 7.2[93]. in CHO cell line, co-expression of Syntaxin 1A decrease significantly Kv 7.2 mediated currents[98]. It has been shown that Kv 7.2 and Kv 7.3 are regulated by the levels of PIP2. PIP2 decreases when $G_{q\text{-}11}$-coupled metabotropic receptors, as mGluR5, are activated, which leads to a decrease in the opening pore probability of these channels, thus decreasing the currents[91,93].

Also, Kv 7.2 C-terminus is a target of phosphorylation by PKC, known downstream effector of mGlur5, however it remains unclear how it interferes with the regulations mediated by CaM and PIP2. Nevertheless, it is known that removing two putative PKC phosphorylation sites in *helix* B prevents the effect of $G_{q\text{-}11}$-coupled receptors activation upon Kv 7.2-mediated currents[99]. Finally, only in the distal end of Kv7.2 and Kv7.3 C-termini exists a domain for the interaction with the adaptor protein Ankyrin G, allowing the co-localization at the axonal initial segment and Ranvier nodes, crucial sites for action potential initiation and propagation[100].

Regarding neuropsychiatric disorders, Kv7.2 has been associated with mild and severe forms of epilepsy. Kv 7.2 mutations has been identified in Benign Neonatal Epilepsy[98]. Studies showed Kv7.2 knockout is lethal and Kv7.3 is not able to compensate the phenotype; furthermore, a mouse model of Kv7.2 dominant-negative effect displays hyper-

excitability and seizures[101]. Currently, the M-channels are being studied as potential therapeutic targets for epilepsy[102]. In schizophrenia, mutations in PIP5K2A interfere with activation of the M-channels, thus affecting M-currents leading to loss of dopaminergic balance[103,104]. Moreover, dysfunction of the PP2A-βϒ subunit regulation pathway is associated with BD, since dephosphorylation of Kv 7.2 increases the currents, whereas phosphorylation decreases Kv 7.2 activity[105].

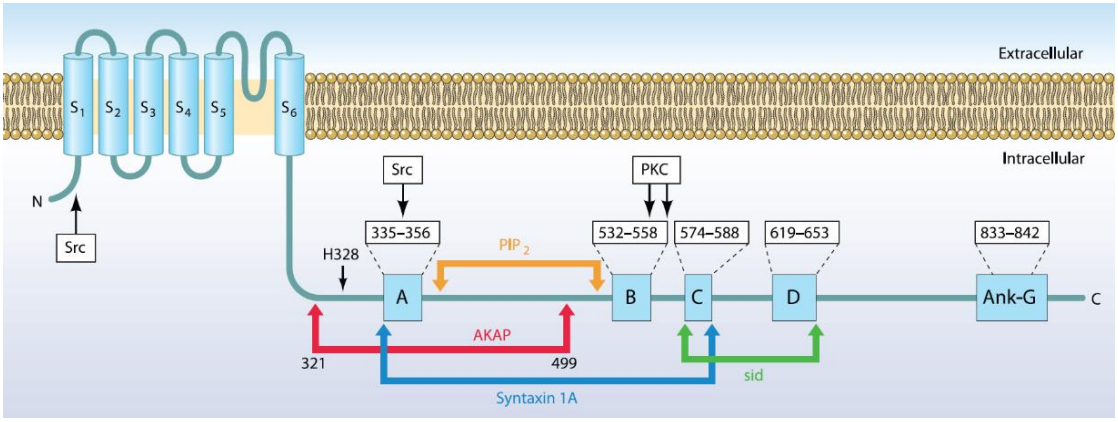

Figure 8: Schematic representation of Kv 7.2 subunit structure. Six transmembrane domains (S1-S6) and intracellular termini. S1-S4 are the voltage sensing domain that induces the opening of the channel. S5-S6 and loop in between are responsible by the formation of the pore[95]. A, B, C, and D correspond to the four α-helices identified; sid - Subunit interaction domain; CaM – calmodulin; AKAP – A-kinase-anchoring protein; PKC – protein kinase C; Ank-G –ankyrin-G; PIP2 – phosphatidylinositol-(4,5)-bisphosphate. Adapted from[93]

4.2. THE METABOTROPIC GLUTAMATE RECEPTOR 5

The class I metabotropic glutamate receptor 5 (mGluR5) is encoded by the GRM5 gene (11q14.3) and its expression encompasses the entire brain having a distribution complementary to that of mGluR1. mGluR5 expression increases in the first post-natal week reaching a peak at the second week, decreasing thereafter[7]. There are two isoforms, mGluR5a is mostly expressed at birth and decreases gradually being replaced by mGluR5b, the predominant form in adulthood. Moreover, in perinatal stages, it is more expressed in axons and during development it tends to be inserted perisynaptically in the somatodendritic compartment being more widely distributed in dendritic spines[7]. Generally, mGluRs have a large extracellular N-terminus, 7 helical transmembrane domains and intracellular C-terminus. These receptors are GPCRs and particularly, the class I is G_{q-11}-coupled inducing phosphoinositide hydrolysis by PLC having PKC as downstream effector [106]. As reviewed above, activation of these receptors induces its desensitization and internalization[37] in a CaM and PKC dependent manner[38].

In hippocampus, as in cortex, mGluR5 has been associated with LTP[107–109] and LTD[110]. However, the most-studied mechanism dependent on mGluR5 is the induction of LTD in the hippocampus. In this context, mGluR5 was associated with Fragile-X syndrome where the absence of FMRP leads to excessive mGluR5-dependent AMPAR internalisation[111]. Nonetheless, opposite effects have been recently described in cortex, where loss of postsynaptic mGluR5 decreases AMPAR-mediated currents[112]. Furthermore, FMRP knockout mice showed impaired LTP in layer V visual neocortex, similar to the results in the same region of wild-type mice slices treated with the selective mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP)[109], thus suggesting that LTP in neocortex is mGluR5-dependent. Also, alterations in dendritic arbour and increased intrinsic membrane excitability were described[112]. Regarding this last observation, mGluR1 and mGluR5 activity was shown to induce M-channels closure through PLC pathway, thus decreasing the M-currents which translates in increased excitability[90]. Homeostatic scaling was also found to require class I mGluRs in a Homer1a-dependent manner[54]. Finally, a recent paper highlighted the importance of mGluR5 activity in neuropsychiatric diseases such as ASD, anxiety disorders and SCZ[113,114]. Moreover, allosteric modulators are now being studied as potential therapeutical approaches in SCZ to facilitate mGluR5 activation by endogenous glutamate and having positive effects in NMDAR-dependent and independent cognitive impairment[115,116]. Furthermore, it was shown that mGluR5 activation is able to improve NMDAR currents through PLC[74] and it is independent from mGluR1[76].

5. SUPPORTING DATA

As mentioned, previous work from our lab described an important function for Stargazin in homeostatic plasticity[2]. Several distinct and important functions were attributed to Stargazin and interestingly, evidence suggests a possible role for Stargazin in the pathogenesis of psychiatric disorders, namely schizophrenia and intellectual disability. In fact, our lab has recently described a mutation in the CACNG2 gene in a schizophrenia patient and this mutation was shown to affect known and novel functions of Stargazin.

Although many binding partners of this protein were already described, to our knowledge, no thorough evaluation of the Stargazin interactome has been performed. Recently, in order to clarify the mechanisms in which Stargazin plays a role, immunoprecipitation of Stargazin from cortical neurons was performed, followed by mass spectrometry analysis. In this analysis several previously unknown binding partners were identified, and in the present work we studied the role of Stargazin interaction with mGluR5 and Kv 7.2. In order to elucidate the data behind this project, in this section we describe unpublished data obtained in our lab that support that studies that are presented in this thesis.

5.1. Kv 7.2 AND mGLUR5 AS POSSIBLE NEW PARTNERS IN STARGAZIN INTERACTOME

Previous work from Dr Susana Louros and Gladys Caldeira in our laboratory identified new Stargazin interaction partners. Total and membrane (P2) extracts of 11 DIV cortical neurons were used to immunoprecipitate Stargazin, and the immunoprecipitates were separated by SDS-PAGE (as shown in Fig: 10A with Silver staining protocol) and stained with colloidal Coomassie blue. The gel was divided by lanes and each lane was cut in 5 parts. The samples were analysed by Orbitrap-Liquid chromatography-Mass Spectrometry (in collaboration with Dr Ka Wan Li, Vrei University of Amsterdam). The obtained list of proteins (Fig: 10B) was then analysed with ©STRING[117], a database of known and predicted protein-protein interactions, producing the following predicted network of interaction (Fig: 10C). During the present work, we have focused our attention on mGluR5 and Kv 7.2, two novel Stargazin interactors identified in these experiments. As shown in Fig: 10D, mGluR5 is predicted to co-localize with Stargazin whereas Kv 7.2 is predicted to be co-expressed and to be a possible interactor.

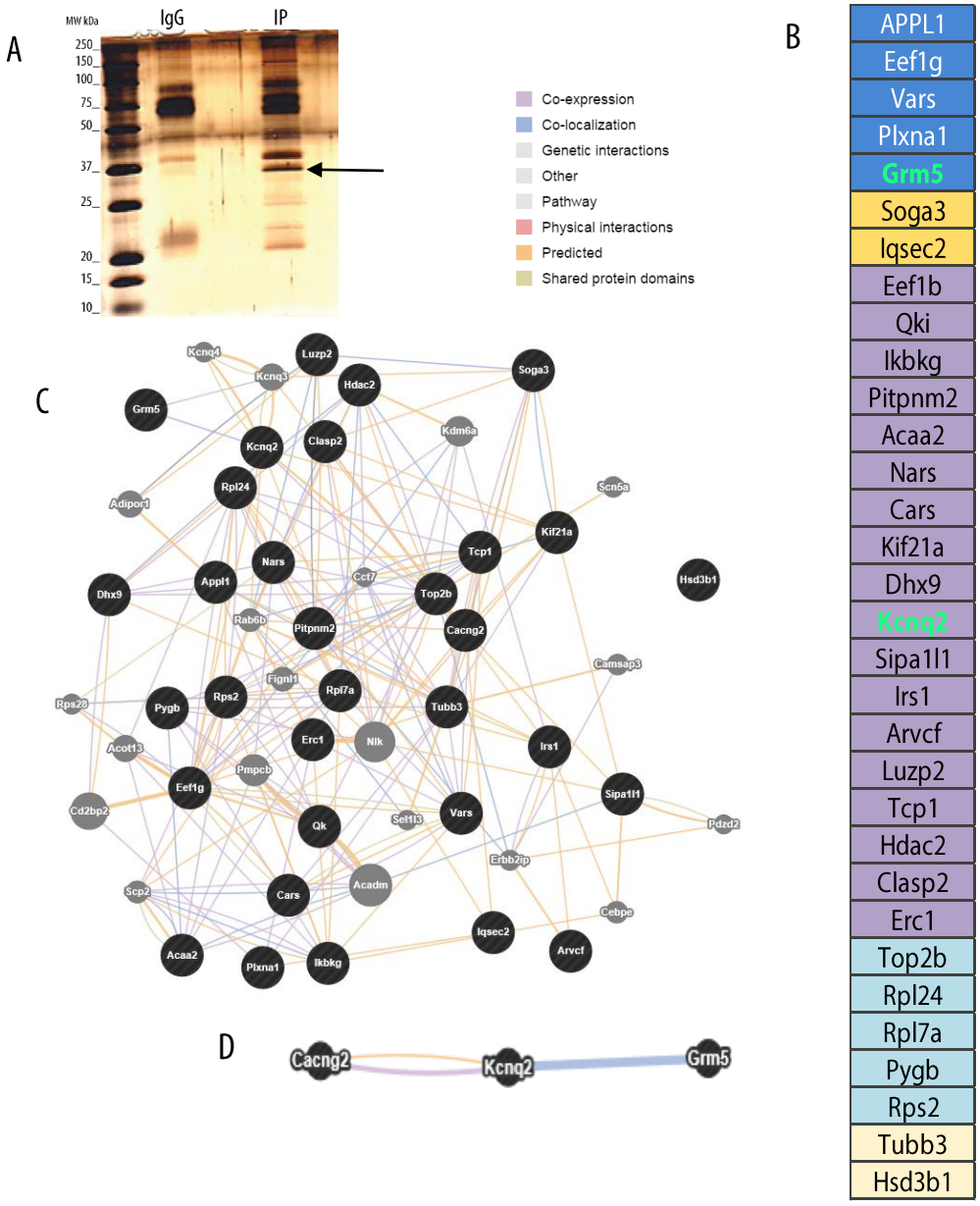

Figure 10: The interactome of Stargazin. (A) Stargazin immunoprecipitates (IP) from cortical neurons were separated by SDS-PAGE and the gel was silver-stained. Non-immune IgGs were used as the immunoprecipitation negative control. The presence of the 37 kDa band in the IP lane, and its absence in IgG lane, indicates Stargazin was successfully immunoprecipitated (black arrow). **(B)** List of proteins identified through Mass Spectrometry analysis of Stargazin immunoprecipitates from cortical neurons. Rows in dark blue refer to proteins present in all Ns of total and P2 extracts, rows in dark yellow refer to proteins present in all Ns of total extracts and 1N of P2 extracts, rows in light purple refer to proteins present in all Ns of total extracts, rows in light blue refer to proteins present in 3 Ns of total extracts, and rows in light yellow refer to proteins present in all Ns of P2 extracts. MGluR5 and Kcnq2 are the focus of the present work. **(C)** The proteins in this list were analysed with ©STRING, which resulted in a predicted map of interactions. **(D)** Magnification of mGluR5, Kv 7.2 and Stargazin connections. mGluR5 is predicted to co-localize with Stargazin (blue line) whereas Kv 7.2 is predicted to be co-expressed (purple line) and to be a possible interactor (orange line). Data kindly provided by Gladys Caldeira obtained in collaboration with Dr. Ka Wan Li.

5.2. ANALYSIS OF mGLUR5-STARGAZIN INTERACTION

5.2.1. CLASS I METABOTROPIC GLUTAMATE RECEPTOR 5 BINDS TO STARGAZIN

Regarding the possibility of mGluR5 being a binding partner of Stargazin, an immunoprecipitation assay for Stargazin using cortical total or membrane (P2) extracts was previously performed in our lab. Following the immunoprecipitation, samples were processed by SDS-PAGE and the Western Blot membranes were stained with a specific antibody against mGluR5. The specific 150 kDa band of mGluR5 is present in Stargazin immunoprecipitation samples (Fig: 11A and B), indicative of mGluR5 binding to Stargazin.

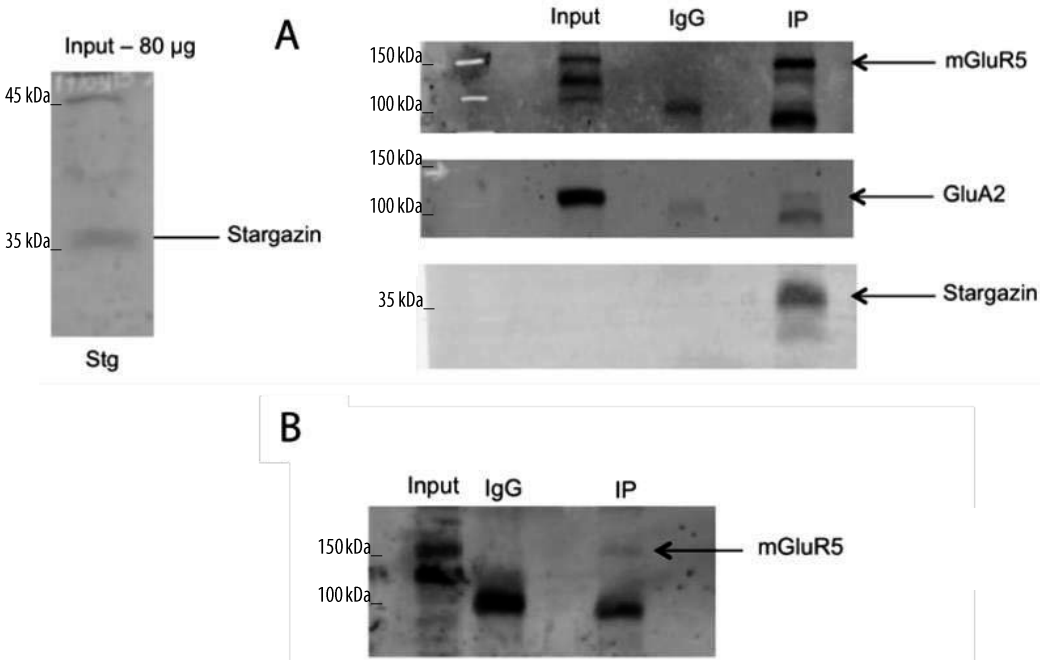

Figure 11: Co-immunoprecipitation of Stargazin and mGluR5 from isolated P2 fraction (A) or total neuronal extracts (B). Stargazin (37 kDa) was immunoprecipitated with a Stargazin-specific antibody, and its immunoprecipitation was confirmed by Western blot. Co-precipitation of mGluR5 (150 kDa) was detected both in the immunoprecipitation samples obtained from P2 fractions (A) and from total neuronal extracts (B). Co-precipitation of GluA2 (a well-known Stargazin interactor) was also detected. Data kindly provided by Gladys Caldeira and Laura Oliveira.

5.2.2. mGLUR5 AND STARGAZIN CO-LOCALIZE STRONGLY AT SYNAPTIC SITES

mGluR5 is found in dendrites, both peri- and post-synaptically, and it plays a key role in Hebbian plasticity, namely in mGluR-dependent LTD [118]. After testing for the interaction between mGluR5 and Stargazin, we evaluated the dendritic localization of mGluR5 and its co-localization with Stargazin in cortical neurons.

Through immunocytochemistry, previous data from our lab showed that mGluR5 indeed co-localizes with Stargazin (Fig: 12A-D). Moreover, this co-localization is not only higher in synaptic sites, identified by the presence of vGlut1 puncta, but also increases with neuronal development (Fig: 12B, C and D). Indeed, in mature neurons, > 50% of the synaptic Stargazin clusters co-localize with mGluR5, and > 50% of the synaptic mGluR5 clusters co-localize with Stargazin (Fig: 12C, D).

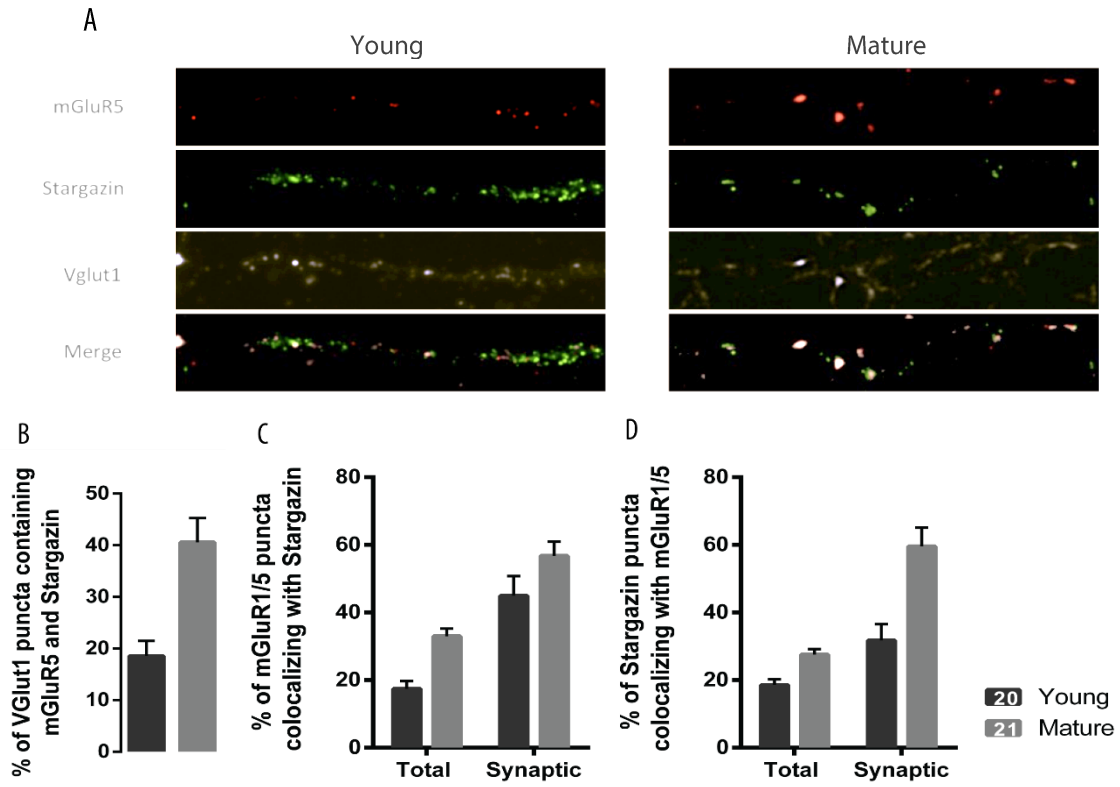

Figure 12: Co-localization between Stargazin and mGluR5 (A) Immunostaining for mGluR5, Stargazin and vGlut1 was performed in young (14-15 DIV) and mature (19-20 DIV) dissociated cortical neurons. mGluR5 and Stargazin puncta were quantified **(B, C and D)** from n≥20 neurons imaged from two different experiments. Data kindly provided by Laura Oliveira.

OBJECTIVES

Stargazin was the first transmembrane protein found to interact with AMPAR[119]. Stargazin-AMPAR interaction is well-known to regulate receptor trafficking and synaptic insertion[26,27], and to change AMPAR kinetics[29]. Hence, Stargazin is a major player in synaptic transmission, especially in the post-synaptic compartment. Therefore, further knowledge about Stargazin's interacting partners is of great importance. As presented in the above section, the study of Stargazin interactome was performed and led to the design of this project. Thereafter, the possible binding partners of Stargazin, mGluR5 and Kv 7.2 were chosen to be further evaluated. Both proteins are known to be associated with neuropsychiatric disorders and are important regulators of neuronal transmission, similarly to Stargazin.

Therefore, the main goals of this project were the following:

1. To confirm the interaction between Stargazin and the identified putative binding partner Kv 7.2.

2. To study the localization of Kv 7.2 relatively to Stargazin in dendrites of cortical neurons and in different stages of development.

3. To study the functional impact of the Stargazin-Kv7.2 interaction in the function and neuronal localization of Kv 7.2.

4. To determine whether the Intellectual Disability and Schizophrenia-associated point mutations in Stargazin impair the Stargazin-mediated regulation of Kv 7.2.

5. To evaluate if Stargazin is implicated in the mGluR-mediated regulation of synaptic AMPA receptors in cortical neurons, given the interaction between mGluR5 and Stargazin described in our laboratory.

Chapter 2

MATERIALS AND METHODS

ANTIBODIES

Primary Antibodies (host species)	Application (dilution)	Brand
GluA1 N-terminal (mouse)	ICC (1:100)	Millipore (MA, USA)
HA (mouse)	IP, WB (1:150, 1:1000)	InvivoGen (CA, USA)
Normal Mouse IgG		Millipore (MA, USA)
KCNQ2 (rabbit)	ICC, WB (1:500, 1:1000)	Abcam (Cambridge, UK)
MAP2 (chicken)	ICC (1:5000)	Abcam (Cambridge, UK)
mGluR5 (mouse)	ICC, WB (1:500, 1:1000)	Neuromab (CA, USA)
PSD 95 (mouse)	ICC (1:200)	Thermo Scientific (MA, USA)
PSD 95 (rabbit)	ICC (1:200)	Cell Signalling (MA, USA)
Stargazin (rabbit)	ICC, WB (1:500, 1:1000)	Millipore (MA, USA)
vGlut1 (guinea pig)	ICC (1:5000)	Millipore (MA, USA)

Secondary Antibodies	Application (dilution)	Brand
Alexa 488-conjugated anti-rabbit	ICC (1:500)	Molecular Probes (Leiden, The Netherlands)
Alexa 568-conjugated anti-mouse	ICC (1:500)	Molecular Probes (Leiden, The Netherlands)
Alexa 647-conjugated anti-guinea pig	ICC (1:500)	Molecular Probes (Leiden, The Netherlands)
Alexa 647-conjugated anti-mouse IgG2a	ICC (1:500)	Molecular Probes (Leiden, The Netherlands)
Alexa 647-conjugated anti-rabbit	ICC (1:500)	Molecular Probes (Leiden, The Netherlands)
Alkaline phosphatase-conjugated anti-mouse	WB (1:10000)	GE Healthcare (Carnaxide, Portugal)
Alkaline phosphatase-conjugated anti-rabbit	WB (1:10000)	GE Healthcare (Carnaxide, Portugal)
AMCA-conjugated anti-chicken	ICC (1:200)	Jackson ImmunoResearch (Pennsylvania, USA)

NEURONAL CULTURES (CORTICAL HIGH- AND LOW-DENSITY CULTURES)

Rat cortical cultures were prepared from cortices of E17-18 Wistar rat embryos. After dissection, cortices were incubated for 10 min at 37ºC with 2mg/ml trypsin, in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS: 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). Cortical cells were washed 6 times with HBSS followed by mechanical dissociation with a glass pipette. The cells were counted and plated in neuronal plating medium (MEM supplemented with 10% horse serum, 0.6% glucose, 1 mM pyruvic acid and 44mM $NaHCO_3$ at pH 7.2) in 6 well plates ($0.885x10^6$ cells/cm^2, high-density), coated with poly-D-lysine (0.1 mg/ml). After 2-3h the medium was replaced by fresh Neurobasal medium (GIBCO Invitrogen) supplemented with SM1 (1:50), 0.5 mM glutamine and 0.12 mg/ml gentamycin. High-density cultures were fed once a week.

For imaging purposes, low-density-cultures were plated in 60 mm culture dishes containing 6 poly-D-lysine-coated coverslips ($0.3x10^6$cells/dish) in neuronal plating medium. After 2-3 h, coverslips were flipped over an astroglial feeder layer in Neurobasal medium (GIBCO Invitrogen) supplemented with SM1 (1:50), 0.5 mM glutamine and 0.12 mg/ml gentamycin. These neurons grew facing the feeder layer but were kept separate from the glia by wax dots on the neuronal side of the coverslips. To prevent the overgrowth of the glial cells, cortical cultures were treated with 5 µM cytosine arabinoside after 2-3 days in vitro (DIV). Cells were maintained at 37°C in a humidified incubator of 5% CO_2, for up to 3 weeks and fed twice a week.

For proteomic studies, cortical high-density cultures were used after 11 days in vitro (DIV), thus for imaging purposes low-density cortical neurons were used with the same age. To assess possible impact of neurodevelopment in our studies, experiments were also performed in 14 to 15 DIV neurons (Young) and 19 to 21 DIV neurons (mature).

In the experiments that required mGluR5 specific stimulation or mGlur1/5 stimulation, cortical low-density cultures were treated with the specific mGluR5 agonist (*RS*)-2-Chloro-5-hydroxyphenylglycine (CHPG) (Tocris Bioscience) or with (*S*)-3,5-Dihydroxyphenylglycine (DHPG) (Tocris Bioscience). Each coverslip was incubated with 200 µM CHPG or 100 µM DHPG, in conditioned medium, at 37°C in 5% CO_2 for 30 min. After the incubation period, the medium was removed and the neurons were fixed for 15 min in 4% sucrose/4%paraformaldehyde in PBS at room temperature followed by immunocytochemistry.

CONSTRUCTS FOR TRANSFECTION

The short-interfering RNA construct were generated by Dr. Susana Louros [2]. Briefly, for pll3.7shRNA, the following DNA oligonucleotides:

5'-TGAAGAACGAGGAAGTTATGTTCAAGAGACATAACTTCCTCGTTCTTCTTTTTT-3' and

5'-ACCTTCAATACTGGGTAAGGAAGTTCTCTCCTTACCCAGTATTGAAGGAAAAAA-3'

were annealed and subcloned into Xho1/Hpa1 sites of the pII3.7 vector. Stargazin shRNA-resistant construct was produced by site-directed mutagenesis, using the HA-tagged WT Stargazin construct, kindly provided by Dr. Daniel Choquet (Bordeaux, France).Two silent mutations were introduced: GCGAGCAAAAAGAATGAGGAAGTCATGACCCATTC. For the generation of the Stargazin mutants V143L and S148N we introduced single point mutations: GCCGGCATCTTCTTCCTGTCTGCAGGTCTGAG and CGTGTCTGCAGGTCTGAATAACATC ATTGGCATCA, respectively.

The control shRNA targets firefly luciferase (Flavell et al., 2006). Kv 7.2 wildtype plasmid was a kind gift from Dr. Maurizio Taglialatela (University of Naples Federico II, IT).

CORTICAL NEURONS TRANSFECTION – CALCIUM PHOSPHATE METHOD

Constructs were recombinantly expressed in primary low-density cultures of cortical neurons using the calcium phosphate transfection protocol [adapted from (Jiang et al. 2004)]. Briefly, a $CaCl_2$ solution (2.5 M in 10 mM HEPES) was added, drop-wise, to plasmid DNA to a final concentration of 250 mM $CaCl_2$. The mix was then added to an equivalent volume of HEPES-buffered transfection solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose, and 42 mM HEPES, pH 7.2), and gently vortexed and incubated in the dark at room temperature for 30 min to allow the development of the precipitates . The precipitated DNA was added drop-wise to the coverslips, previously transferred into a 12-well plate containing 200µl of conditioned medium, and the cultures were incubated at 37°C in 5% CO_2 for 2 h in the presence of kynurenic acid (2 mM). To dissolve DNA precipitates, the medium of each well was replaced for new culture medium with kynurenic acid (2 mM), slighltly acidified with HCl (5 mM final concentration), and the plate was incubated at 37°C in 5% CO_2 for 15 min. Coverslips were then transferred back into the original astroglial plate and the plasmids were allowed to express for 4 DIV.

HEK293T CELL LINE CULTURE AND TRANSFECTION – CALCIUM PHOSPHATE METHOD

Human Embryonic Kidney 293T cells (HEK293T) were maintained at 37°C in 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM, GIBCO Invitrogen), supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 44mM $NaHCO_3$ at pH 7.2, to a sub-confluence of 50-70% and diluted 1:20 when 90-100% confluence was reached. Constructs were transiently expressed in 50-70% confluent HEK293T cell line cultures using a calcium phosphate transfection protocol. A 2 M $CaCl_2$ solution was added, drop-wise, to plasmid DNA to a final concentration of 200 mM $CaCl_2$. The mix was then added to an equivalent volume of HEPES-buffered saline (280 mM NaCl, 50 mM HEPES and 1.5 mM Na_2HPO_4, pH 7.1). The mixture was gently vortexed and added drop-wise to the wells and incubated at 37°C in 5%

CO_2 for 5 h. To remove the precipitates, the medium was replaced by fresh DMEM (GIBCO Invitrogen), supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 44mM $NaHCO_3$ at pH 7.2. The plasmids were allowed to express for 48 h.

CORTICAL CULTURE EXTRACTS (TOTAL AND MEMBRANE EXTRACTS)

To obtain total extracts, high-density cortical cultures were washed twice with ice-cold PBS pH 7.4 and were then scraped on ice with TEEN buffer (25 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl and 1% Triton X-100) supplemented with protease and phosphates inhibitors as follows, 50 mM sodium fluoride (NaF), 1.5 mM sodium orthovanadate (Na_3VO_4) and the cocktail of protease inhibitors. Cell lysates were incubated on ice for 10 min and then subjected to 5 cycles of sonication, (each cycle concerns 5 seconds of sonication followed by 5 seconds of pause), for 50 seconds total. After centrifugation at 16.100xg for 10 min at 4ºC, protein in the supernatants was quantified using the Bicinchoninic acid protein assay kit (BCA, Pierce Biotechnology).

Total extracts were used for immunoprecipitation (see next section) or immediately run. To do so, the extracts were denaturated with 5× concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM Na_3VO_4, 3.33% (v/v) β-mercaptoethanol, and 0.01% bromophenol blue) and left at room temperature for 15 min and then processed by SDS-PAGE. The samples were not boiled to avoid protein aggregation.

IMMUNOPRECIPITATIONS

After protein quantification, 800µg of protein were used per condition. Samples were diluted to 2 µg/µl final concentration in supplemented TEEN Buffer. Each condition underwent a clearing step, where each was incubated with 30 µL of 2x Protein A Sepharose CL-4B (GE Healthcare) for 1h, at 4º C, in rotation. Clearing the samples helps reducing non-specific binding and background, thus helping when a contaminating protein is interfering with visualization of the protein of interest. Subsequently, samples were centrifuged 1 min at 1.500 xg rpm at 4º C. The supernatant was incubated overnight, at 4º C, in rotation, with 2 µg of antibody or IgG. In the next day, 80 µL of 2x Protein A Sepharose CL-4B was added to each condition and left in rotation for 2h at 4º C. Samples were centrifuged 1 min at 1000 rpm at 4º C and 30 µL of supernatant (flow-through) was collected and processed by SDS-PAGE and Western blot to confirm the efficiency of the immunoprecipitation Afterwards, samples were washed 6 times by centrifugation (1 min, 1500 xg at 4ºC) with Teen buffer supplemented as follows : (1) complete TEEN buffer, (2) complete TEEN buffer + 1% Triton X-100, (3) complete TEEN buffer, (4) complete TEEN buffer + 150 mM NaCl, (5) complete TEEN buffer + 1% Triton X-100

and (6) complete TEEN buffer). After the last wash, samples were further centrifuged to remove the exceeding buffer. Beads were then denatured with 2x SDS-PAGE Sample Loading Buffer (Nzytech).

GEL ELECTROPHORESIS AND WESTERN BLOT

Samples were resolved by SDS-PAGE in 11% or 8% polyacrylamide gels. For western blot analysis, transference was performed overnight at 40 V, at 4ºC, onto a PVDF membrane (Millipore, Madrid, Spain). The membranes were blocked for 1 hour with 5% (w/v) Blocking agent (GE Healthcare) and 0.1% Tween 20 in TBS (20 mM Tris, 137 mM NaCl, pH 7.6) (TBS-T), and probed during 1 h 30, at room temperature, or overnight, at 4ºC, with the primary antibody in 3% (w/v) blocking solution. Following several washes with TBS-T for a period of 30 min, membranes were incubated for 1h, at room temperature, with alkaline phosphatase-conjugated IgG secondary antibody (anti-mouse or anti-rabbit, depending on the primary antibody host-species) in 5% blocking solution. Lastly, after washed, the membranes were developed by the enhanced chemifluorescence method (ECF) on a Storm 860 Gel and Blot Imaging System (GE Healthcare, Carnaxide, Portugal) or ChemiDoc™ Touch Imaging System (Bio-Rad).

IMMUNOCYTOCHEMISTRY, IMAGING AND QUANTITATIVE FLUORESCENCE ANALYSIS

Neurons were fixed for 15 min in 4% sucrose/4%paraformaldehyde in PBS at room temperature, and permeabilized with ice-cold PBS + 0.25% Triton X-100 for 5 min. Then, the coverslips were incubated in 10% BSA in PBS for 30 min at 37ºC to block nonspecific staining, and incubated with the indicated primary antibodies diluted in 3% BSA in PBS (overnight, 4ºC). After washing in PBS, cells were incubated with the secondary antibody diluted in 3% BSA in PBS (45 min, 37ºC). After several washes in PBS, the coverslips were mounted using fluorescent mounting medium from DAKO (Glostrup, Denmark).

For labelling surface GluA1-containing receptors, neurons were fixed as described followed by blocking with 10% HS in PBS for 30 min at 37º C. Coverslips were then incubated with GluA1 N-terminal primary antibody in 5% HS in PBS. Each coverslip was then efficiently rinsed in PBS and incubated with the specific secondary antibody. Neurons were again well washed in PBS and the following steps were as described after fixation, except for the usage of HS. Imaging was performed on a Zeiss Axiovert 200 M microscope, using a 63x-1.4 NA oil objective. For quantification, sets of cells were cultured and stained simultaneously, and imaged using the exact settings. Images were quantified using image analysis software (ImageJ). The region of interest was randomly selected and the dendritic length was measured using the MAP2 staining. For GluA1, Kv 7.2 and mGluR5 signals quantification, the fields were chosen by the GFP channel in

experiments involving transfection, GFP-positive neurons. For experiments without transfection, fields of imaging were obtained by looking at the protein of interest. Images were thresholded so that recognizable clusters were included in the analysis. The Image J function "analyze particles" permitted the calculation of cluster intensity, number, and area for each region. Co-localization of two proteins was determined by their overlap with thresholded PSD95, ANK-G or STG signals. A minimum of two independent preparations were made per experiment, and at least 6 cells per each condition of each preparation had to be possible to analyse, so that it could be considered. Image analyser was blinded for all conditions.

STATISTICAL ANALYSIS

The immunoreactivity obtained was calculated as a percentage of the control. Data are presented as mean ± SEM of at least two different and independent experiments Statistical analysis of the results was performed using Unpaired Mann-Whitney test, Kruskal-Wallis or two-way ANOVA analysis followed by either Dunn's or Bonferroni post-hoc tests: n.s. non significant, ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$.

Chapter 3

RESULTS

Recently, in our lab, immunoprecipitation of Stargazin was performed, followed by mass spectrometry analysis. In this analysis several unknown binding partners were identified and in the present work we studied the relevance of Stargazin interaction with mGluR5 and Kv 7.2 (Fig: 10, supporting data). In order to pursue our objectives, we started by evaluating Stargazin-Kv 7.2 interaction as follows.

1. ANALYSIS OF THE Kv 7.2-STARGAZIN INTERACTION

1.1. M-CHANNEL SUBUNIT Kv 7.2 BINDS TO STARGAZIN

To test the hypothesis that Stargazin and Kv 7.2 are indeed binding partners we co-transfected HEK293T with Kv 7.2WT and StgWT plasmids. Using a heterologous system allows us to test whether the two proteins interact directly. Taking advantage of the HA-tagged Stargazing construct, we used an immunoprecipitation assay (IP) to pull-down Stargazin followed by SDS-PAGE and Western Blot, where we confirmed the co-immunoprecipitation of Kv 7.2 with Stargazin (Fig: 13A). Kv 7.2, when expressed alone in HEK293T, cannot be immunoprecipitated with anti-HA antibody (Kv 7.2WT, IP). However, the immunoprecipitation is incomplete as a faint band remains in the flow-through (FT) of the IP condition (Fig: 13B).

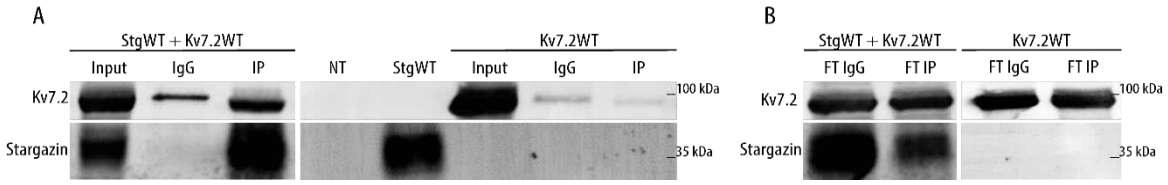

Figure 13: Co-immunoprecipitation of Stargazin and Kv 7.2 from co-transfected HEK293T cells. HEK293T were transfected with wild-type HA-tagged Stargazin and wild-type Kv7.2 (StgWT + Kv7.2WT) or with either StgWT or Kv7.2WT alone. All immunoprecipitation (IP) assays were performed with the anti-HA antibody to target the HA tag only present in the Stargazin construct; non-immune IgGs were used as the immunoprecipitation negative control. NT – non-transfected cells. **(A)** Kv 7.2 co-precipitates with Stargazin as seen by the occurrence of a 96 kDa band in the StgWT + Kv7.2WT/IP lane. Kv 7.2 does not bind to normal IgG. Non-immune mouse IgG used for the negative control is polyclonal and produces an unspecific and faint band ~100kDa. **(B)** Representative blot of flow-through (FT) show incomplete IP of HA-tagged Stargazin as well as incomplete co-IP of Kv 7.2. This experiment was performed once.

1.2. Kv 7.2 AND STARGAZIN APPEAR AS SIDE BY SIDE PARTNERS

Since Kv 7.2 is expressed in axons and in somatodendritic compartment[92,93], and taking into account the striking relevance of Stargazin in the post-synapse, we started by analysing the co-localization of Stargazin and Kv 7.2 in dendrites of cultured cortical neurons through immunocytochemistry using specific antibodies against Kv 7.2 and Stargazin. In order to address if, during development, there are alterations in the expression levels of Kv 7.2 and its co-localization with Stargazin, we performed immunocytochemistry in young (14-15 DIV) and mature (20-21 DIV) neurons. Our data showed that the expression levels of Kv 7.2 increase during development (Fig: 14D, E and F). Kv 7.2 and Stargazin co-localization in dendrites is apparently low at both stages of development (Fig: 14B and C). However, there is a slight increase of co-localization in mature neurons (Fig: 14B and C). Although both interactors seem to have low co-localization, the intensity, area and puncta per length tend to increase within the puncta simultaneously containing Stargazin and Kv 7.2 (Fig: 14G, H and I).

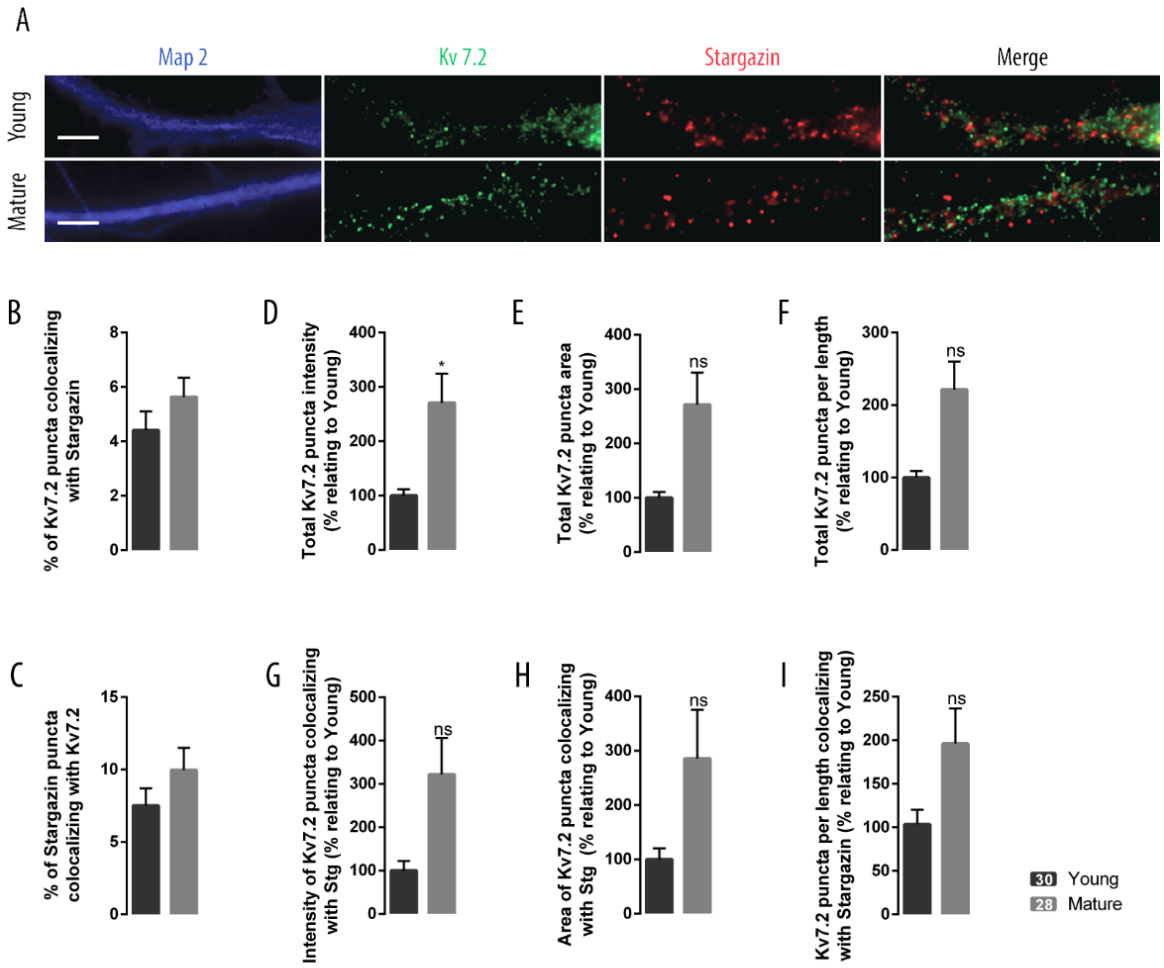

Figure 14: Co-localization between Stargazin and Kv 7.2 in dendrites, at two different developmental stages. (A) Immunostaining for Kv 7.2, Stargazin and MAP2 (to label dendrites) was performed in young (14-15 DIV) and mature (20-21 DIV) dissociated cortical neurons. Kv 7.2 and Stargazin puncta were quantified **(D to I)** from n≥28 neurons for each age group, imaged from three different experiments. Young and mature neurons were obtained in parallel from the same cortical isolations, and were stained and acquired in parallel. Results are presented as means ± S.E.M. Unpaired Mann-Whitney test, ns $p \geq 0.05$ and * $p < 0.05$. Scale bar 4 µm.

In collaboration with Dr. Luísa Cortes, we looked at the localization of Stargazin and Kv7.2 in dendrites of cultured cortical neurons using Superresolution Structured Illumination Microscopy (SR-SIM) (Fig: 15). Low overlap of the Stargazin and Kv7.2 signal is detected, but Stargazin and Kv7.2 puncta are often found in juxta-position.

The analysis of Stargazin and Kv7.2 co-localization will be further analysed in axons, where both proteins are known to be present.

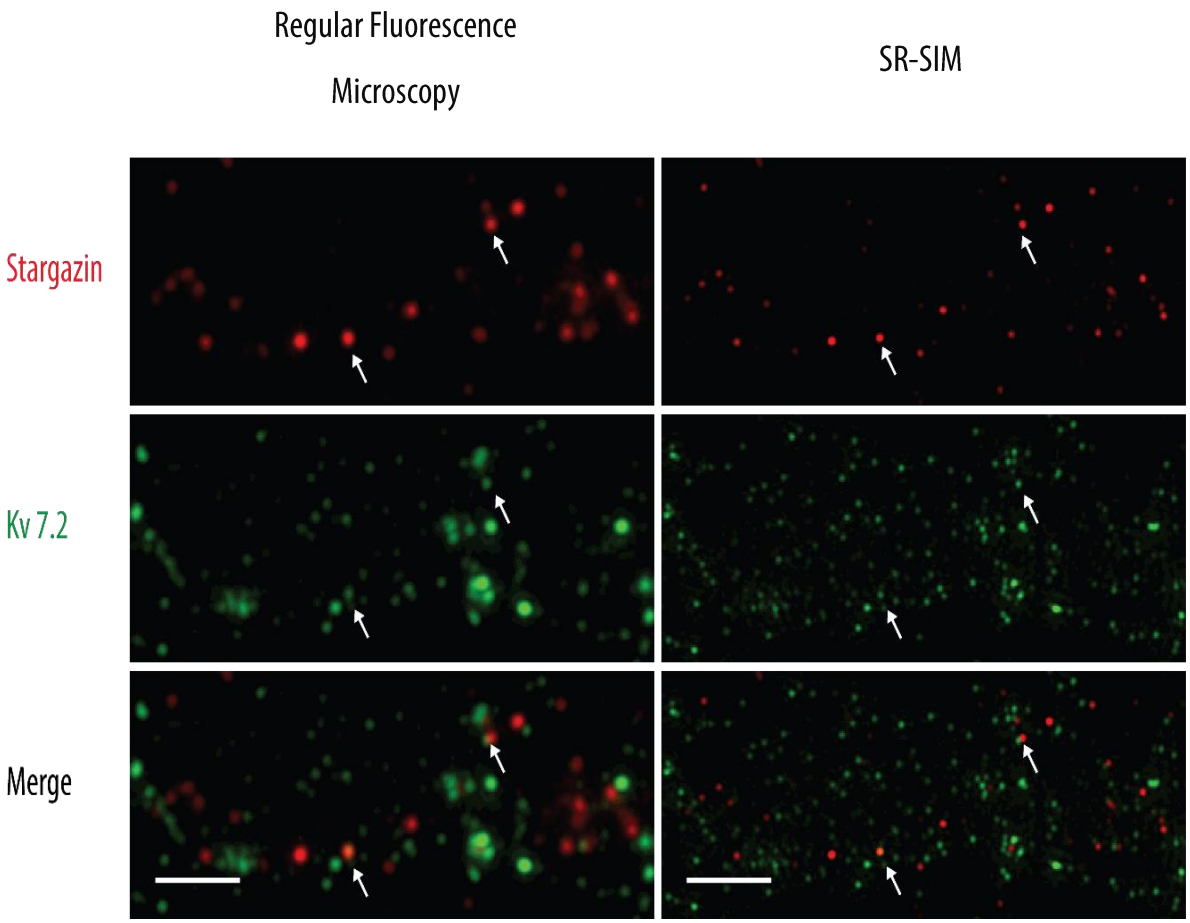

Figure 15: Juxta-position of Kv 7.2 and Stargazin. Staining of Kv 7.2 and Stargazin was performed in young (14-15DIV) dissociated cortical neurons. The same dendrite was acquired with regular fluorescence microscopy (left) and Superresolution Structured Illumination Microscopy (SR-SIM) (right). The white arrows indicate co-localizing or juxta-positioned puncta. Images kindly acquired by Dr Luísa Cortes. Scale bar 2 µm.

1.3. STARGAZIN INCREASES Kv 7.2-MEDIATED CURRENTS

M-channels are voltage-gated outward potassium channels, which set and regulate the membrane potential and thus excitability and firing rate of neurons[4]. Kv 7.2 are subunits of these channels and given their interaction with Stargazin we tested whether Stargazin can modulate Kv 7.2-mediated currents. In collaboration with Dr. Maurizio Tagialatela (University of Naples Federico II), a set of experiments were performed in CHO cells, which were transfected with Kv7.2 alone, or co-transfected with Kv 7.2WT and increasing amount of StgWT plasmids (1:1, 1:5 and 1:10). Kv7.2-mediated currents were recorded using the whole-cell configuration of the patch-clamp technique, as previously described [120]. Dr. Taglialatela and colleagues found that co-expression of Stargazin with Kv7.2 significantly increases the amplitude of Kv 7.2-mediated currents, without changing their voltage-dependent gating (data not shown). These data strongly support a functional role for the Stargazin-Kv7.2 interaction.

1.4. INTELLECTUAL DISABILITY AND SCHIZOPHRENIA-ASSOCIATED MUTATIONS IN STARGAZIN IMPAIR ITS FUNCTION IN REGULATING Kv 7.2

The work presented to this point introduces Kv 7.2 as new interactor of Stargazin, a synaptic protein known for its role in AMPA receptors trafficking and insertion in the synapse[27]. A recent genetic study identified a point mutation in the CACNG2 gene encoding Stargazin in a patient with Intellectual Disability (ID), which results in an amino acid change in the third Transmembrane Domain (3TM) of Stargazin [3]. Unpublished data from our group suggests that this mutation impacts GluA1 trafficking and insertion at synaptic sites. Another point mutation in the same domain, TM3, has been described by our group, this time in a schizophrenia patient (SCZ) (reviewed in introduction). Taking this into account, we inquired whether these point mutations in Stargazin could affect the expression of Kv 7.2.

To address this question, we silenced Stargazin expression in young cortical neurons by transfecting Stargazin shRNA (Stg KD) or control shRNA (CTR), and immunostained transfected neurons for Kv7.2 (Fig: 16A). Stargazin knock-down in fact caused a big, although not significant, decrease in the synaptic presence of Kv 7.2 (Fig: 16C). To evaluate whether the disease-associated variants of Stargazin can perform wildtype Stargazin role in regulating Kv7.2 levels, neurons were co-transfected with Stargazin shRNA and either wild-type shRNA resistant Stargazin (Stg KD + Stg WT) or with Stargazin shRNA and the shRNA resistant disease-associated mutants of Stargazin (Stg KD + StgID or Stg KD + StgSCZ). When wild-type Stargazin was expressed in neurons where the endogenous Stargazin was silenced (Stg KD + Stg WT), the levels of Kv7.2 were restored to values similar to control. However, in neurons expressing the mutant forms of Stargazin (StgID or StgSCZ) the rescue was incomplete (Fig: 16C) suggesting that the mutations in Stargazin impair its role in regulating the levels of this protein.

In fact, recent data obtained in collaboration with Dr. Taglialatela showed that, unlike wild-type Stargazin, the variants of Stargazin associated with Intellectual Disability and Schizophrenia are unable to modulate Kv7.2-mediated currents when co-expressed with Kv7.2 in a cell line (data not shown).

Altogether these results suggest that the disease-associated mutations in Stargazin either impair its interaction with Kv7.2 or its functional impact on this interactor.

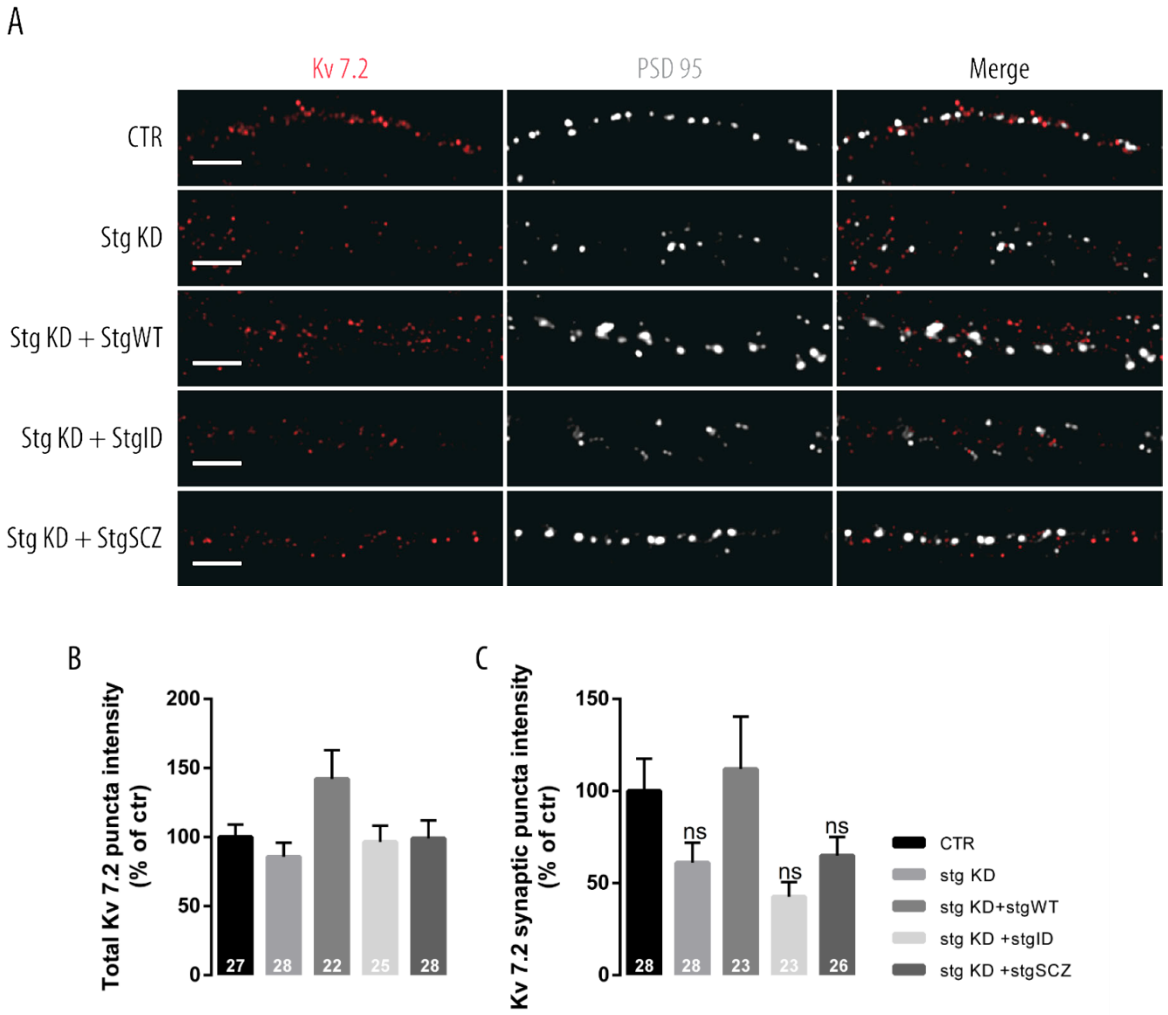

Figure 16: Kv 7.2 expression is affected by Stargazin knock-down and expression of disease-related Stargazin mutants. (A) Representative crops of analysed dendrites CTR-control, stg KD - Stargazin knock-down, stg KD+stgWT - Stargazin knock-down and rescue with wild-type form of Stargazin, stg KD+stgID - Stargazin knock-down and rescue with intellectual disability mutant form of Stargazin, stg KD+stgSCZ - Stargazin knock-down and rescue with schizophrenia mutant form of Stargazin. Staining of Kv 7.2 and PSD 95 was done in young dissociated cortical neurons. Kv 7.2 and PSD 95 puncta were quantified from n≥22 neurons imaged from three different experiments. Transfected neurons were identified by GFP expression from the bicistronic shRNA plasmid. Results are presented as means ± S.E.M. Kruskal-Wallis and post-hoc Bonferroni test, ns p≥0.05. Scale bar 4 µm.

With the list of putative binding partners of Stargazin (Fig: 10B, supporting data), Kv 7.2 and mGluR5 were chosen as focus of this work. Previously in our lab, immunoprecipitation of Stargazin was performed, in order to test interaction with mGluR5 (Fig: 11, supporting data). Once demonstrated the interaction between Stargazin and mGluR5 in cortical neurons, co-localization studies were performed. Results showed high co-localization between Stargazin and mGluR5, mainly at the synapse (Fig: 12, supporting data). Altogether, the data guided us to assess the function of Stargazin-mGluR5 interaction in mGluR5 function.

2. ANALYSIS OF mGLUR5-STARGAZIN INTERACTION

2.1. CLASS I METABOTROPIC GLUTAMATE RECEPTORS ACTIVATION INCREASES GLUA1 LEVELS IN CORTICAL NEURONS

Class I metabotropic glutamate receptors activation is described to alter AMPAR trafficking and anchoring at synaptic sites [110]. On the other hand, Stargazin is a TARP also known as a mediator of AMPAR trafficking and insertion at synapses, playing a pivotal role in Hebbian and Homeostatic plasticity mechanisms[22,26]. Therefore, we tested whether the activation of mGluR5 in cortical neurons changes GluA1 synaptic content. As reviewed previously in this work, mGluR activation in hippocampal neurons induces internalization and degradation of AMPAR, seen by the decrease in GluA1 content at the surface and particularly, at synapses. This effect is mechanistically linked to mGluR-dependent LTD [118].
To assess the impact of class I mGluR activation, and particularly mGluR5, on GluA1 synaptic levels in cortical neurons, we stimulated young (11 DIV) cortical neurons for 30 min with 100 µM DHPG, to activate mGluR1 and 5, or with 200 µM CHPG, a specific agonist of mGluR5. We assessed the cell surface content of GluA1 by immunocytochemistry, incubating fixed non-permeabilized cells with an antibody against an extracellular epitope in GluA1. The synaptic GluA1 puncta were identified by their co-localization with the postsynaptic scaffold protein, PSD95 (Fig. 17A).
We found that cortical neurons respond to mGluR5 activation by CHPG by increasing both surface and synaptic levels of GluA1 (Fig: 17B-G). All parameters that were evaluated [GluA1 puncta intensity (Fig: 17E and B), area of puncta (Fig: 17F and C) and the number of puncta (Fig: 17G and D,)] were increased in cortical neurons following the CHPG stimulation. These results indicate that mGluR1 activation in cortical neurons affects GluA1 differently from the effects in hippocampal neurons [121], which is in agreement with previous studies using cortical neurons [109].
Surprisingly, the DHPG stimulus that activates both mGluR1 and 5, caused smaller changes in the GluA1 surface and synaptic levels in cortical neurons (Fig: 17).

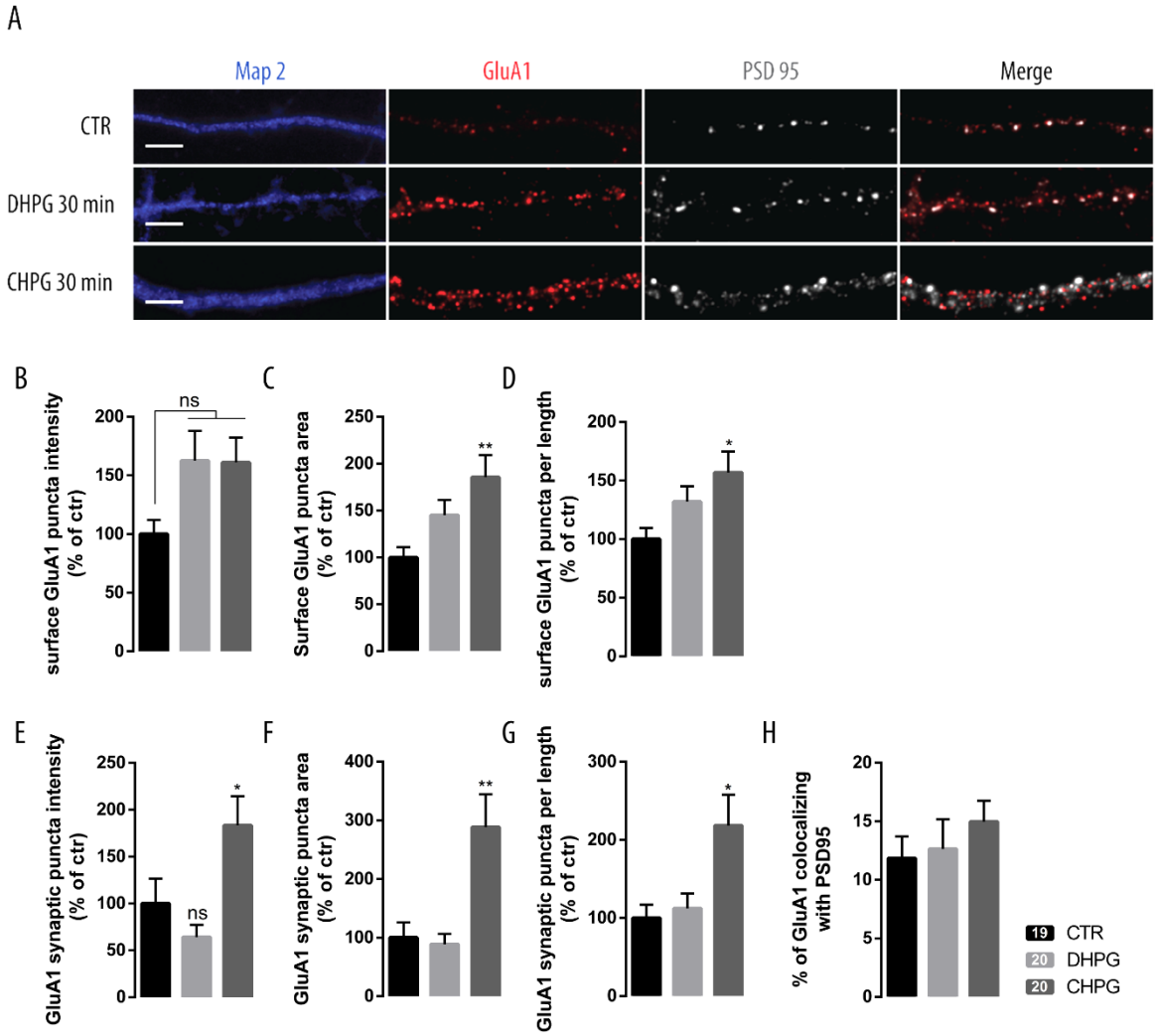

Figure 17: mGluR 1/5 and mGluR5 activation increase GluA1 cell surface and synaptic levels in cortical neurons. (A) Representative images of analysed dendrites. **(B to G)** mGluR5 specific activation with 200 µM CHPG (30 min) increases total (B-D) and synaptic (E-H) GluA1 levels. Activation of both mGluR1 and 5 (with 100 µM DHPG, 30 min incubation) tends to increase total GluA1 levels but not synaptic GluA1 levels. **(B to H)** Staining of GluA1 and PSD 95 was performed in young (11-12 DIV) dissociated cortical neurons. GluA1 and PSD 95 puncta were quantified from n≥19 neurons for each experimental condition, imaged from two different experiments. Results are presented as means ± S.E.M. Kruskal-Wallis test and post-hoc Dunn's test, ns $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$. Scale bar 4 µm.

2.1.1 mGLUR5 MODULATION OF GLUA1 LEVELS IS DEPENDENT ON STARGAZIN

Given the interaction between mGluR5 and Stargazin, we hypothesized that Stargazin could be an important player in mediating the increase of GluA1 upon mGluR5 activation with CHPG. To test this possibility, we silenced Stargazin expression in cortical neurons by transfecting with a shRNA specific for Stargazin (Stg KD) previously validated in our laboratory [2], or with control shRNA (CTR). To exclude the contribution from potential off-target effects of the Stargazin shRNA, neurons were co-transfected with Stargazin shRNA and a shRNA resistant form of wild-type Stargazin (Stg KD

+ StgWT). Transfected neurons were incubated in CHPG, and alterations in GluA1 levels were evaluated as described before. Given the well-known role of Stargazin in the traffic of AMPA receptors, the results from this experiment have a complex interpretation. However, we found that the effect of CHGP on GluA1 synaptic levels is blocked when Stargazin is silenced (Fig: 18).

In a different approach, we overexpressed Stargazin in cortical neurons (StgOV), which were co-transfected with CTR shRNA for direct comparison with the previous experiments. As expected based on many previous studies[27,122] Stargazin overexpression leads to an increase on synaptic GluA1. Significantly, the effect of CHPG on GluA1 synaptic levels is dramatically exacerbated by Stargazin overexpression (Fig: 18), suggesting that Stargazin availability facilitates the mGluR5-mediated effect on GluA1 synaptic levels in cortical neurons.

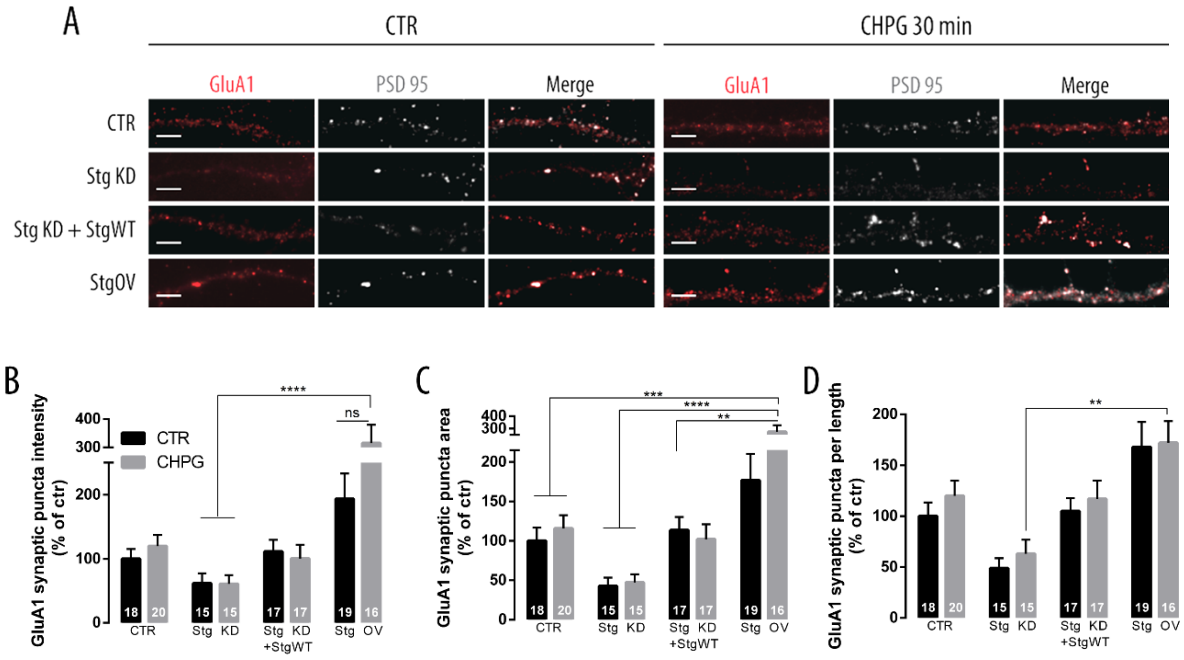

Figure 18: mGluR5-induced GluA1 increase in cortical neurons is dependent on Stargazin levels. Cortical neurons were transfected with control shRNA (CTR), Stargazin shRNA (Stg KD), or co-transfected with Stargazin shRNA and an shRNA resistant form of Stargazin (Stg KD + Stg WT), or co-transfected with control shRNA and Stargazin WT (Stg OV), to overexpress Stargazin. When indicated, neurons were incubated with 200 µM CHPG for 30 min. GluA1 cell surface synaptic levels were evaluated by immunocytochemistry. **(A)** Representative crops of analysed dendrites. **(B to D)** mGluR5 activation with CHPG tends to increase synaptic GluA1 levels. This effect is absent in the Stg KD condition, and exacerbated by Stargazin overexpression. Immunostaining for GluA1 and PSD 95 was done in young (11-12 DIV) dissociated cortical neurons. GluA1 and PSD 95 puncta were quantified from n≥15 neurons for each condition imaged from two different experiments. Results are presented as means ± S.E.M. Two-Way ANOVA test and post-hoc Bonferroni's test, ** p<0.01, ***p<0.001 and **** p<0.0001. Scale bar 4 µm.

Chapter 4

DISCUSSION

With this study we unveiled a hidden face of Stargazin, presenting Stargazin as a potential player in other cellular processes besides the well-known regulation of AMPAR traffic. Following Stargazin immunoprecipitation from total and P2 cortical extracts, Stargazin interactors were identified through Orbitrap-Liquid chromatography-Mass Spectrometry. An Orbitrap analyser can collect high sensitivity data with high resolution[123]. Taking advantage of a database of known and predicted protein-protein interactions, ©STRING[117], we predicted the network (Fig: 10C) of Stargazin's interactions. Within the presented list containing multiple types of possible interactors, such as elongation factors (Eef1g), regulators of autophagy (Soga3), proteins associated with microtubule motor activity (Kif21a), lipid metabolism associated enzymes (Acaa2) or synaptic receptors (Plxna1), we chose to study mGluR5 and Kv 7.2 because of their strategic roles in synaptic transmission and already known importance in the context of neuropsychiatric disorders[105,124].

STARGAZIN-Kv 7.2 INTERACTION MAY PROTECT NEURONAL CIRCUITS FROM HYPER-EXCITABILITY

The Kv 7.2-Stargazin physical interaction (Fig: 13A) and co-expression as initially predicted in the obtained protein-protein interactions network was confirmed (Fig: 10C). Furthermore, by performing the co-immunoprecipitation assay in a non-neuronal heterologous system where many neuronal scaffolds or interaction facilitators are absent, we were able to confirm that, in fact, the interaction between the two proteins is probably direct. Although the representative blot (fig:13B) shows incomplete IP of Stargazin as well as incomplete co-IP of Kv 7.2, the co-immunoprecipitation of the two proteins is clear.

Very few Kv 7.2 interactors have been identified so far. As reviewed previously, CaM[96] and Syntaxin 1A[98] were reported as Kv 7.2 binding partners. The function of the Kv 7.2-CaM interaction is associated with channel trafficking, maturation and insertion in the plasma membrane[96]. Syntaxin 1A, on the other hand, has a Soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) motif and is known to be involved in pre-synaptic fusion of neurotransmitters-containing vesicles into the synaptic cleft. The Kv 7.2-Syntaxin 1A interaction is described to decrease Kv 7.2-mediated currents[125]. Kv 7.2 regulation largely impacts neuronal excitability, thus interactors function might be highly important to tune Kv 7.2 activity. Interestingly, both CaM and Syntaxin 1A, the known Kv7.2 interactors, have in common with Stargazin their role in synaptic transmission. Both, together with Stargazin, are major players in pre- or post-synaptic processes, or in both as it is the case of CaM. The articulation between Kv 7.2 interactors is probably crucial to allow a fine control of excitability, which in turn modulates synaptic transmission. Kv7.2 is known to act as a brake for transmission, and its interactors may be important in regulating the effectiveness of this process. Association/dissociation with different molecular partners is likely to enhance or alleviate the brake-effect of Kv 7.2 on transmission, and this tuning mechanism, if perturbed, may deviate local and, consequently global transmission

towards extreme conditions of hyper- or hipo-excitability, a well described feature of neuropsychiatric disorders[58]. Therefore, it is important to determine when and where Kv7.2 interactors have a role. In the case of the Stargazin interaction with Kv 7.2, these are questions that still remain to be answered, and that we would like to pursue in the future.

In the predicted network of interactions produced by STRING, Kv 7.2 was not predicted to co-localize with Stargazin. Indeed, Kv 7.2 and Stargazin have low co-localization in dendrites of cortical neurons, as determined with the methods that we used (fig: 14B and C). However, Stargazin silencing in cortical neurons led to a decrease on the dendritic levels of Kv7.2 (Fig. 16), pointing to a role for Stargazin in determining the localization of Kv7.2 channels.

To better evaluate the co-localization of the two proteins, we imaged labelled cortical neurons with Superresolution Structured Illumination Microscopy (SR-SIM) (fig: 15). SR-SIM is technique in fluorescence microscopy that can be used to generate superresolution images of a sample (e.g. cells, thin tissue cuts, etc.). The sample is illuminated with mathematically well-defined grating patterns which in turn are rotated and shifted over the specimen resulting in a number of raw patterned images. The final image is calculated from these raw images by recombining the information contained in the grating pattern. This process improves signal-to-noise, reduces the off-focus blur from the z axis and can achieve a doubling of the resolution in all three dimensions. With the help of Dr. Luísa Cortes we obtained SR-SIM images of cortical neurons co-stained for Kv7.2 and Stargazin, and were able to conclude that some of the co-localizing puncta (yellow dots) in the images obtained using regular fluorescence microscopy are in fact juxta-posed in the images acquired with SR-SIM (fig: 15). Since Kv 7.2 function in action potential propagation and the regulation of membrane potential is best known in axons, as subunits of the M-channels, it would be important to analyse the co-localization between Stargazin and Kv7.2 in axons.

As shown in previous works, Stargazin has a function in regulating AMPAR trafficking[2,22], both at the synapse as well as throughout the biosynthetic pathway. Stargazin is synthesised and processed in the endoplasmic reticulum together with other transmembrane proteins, such as GluA subunits of AMPARs. Afterwards, Stargazin is secreted in vesicles together with AMPAR subunits, to be inserted in the membrane[126]. Taking this well-known function for Stargazin, it could be in fact interacting directly with Kv 7.2 during its transport towards the neuronal surface, but only transiently in time and possibly not in the membrane. Nevertheless, a sorting function for Stargazin concerning other proteins besides AMPAR subunits has not been addressed before.

Interactors of Kv7.2 may exert a function in the functional regulation of these channels. To test whether this is the case for Stargazin, in collaboration with Dr. Maurizio Taglialatela at the University of Naples Federico II we addressed the putative role of Stargazin in the regulation of M-currents. In fact, the obtained results in transfected CHO cells show that co-expression of Stargazin potentiates Kv 7.2-mediated currents, without affecting channel gating properties. This suggests higher channel availability at the cell surface, probably due to trafficking improvement of Kv 7.2 by Stargazin, rather than alterations in Kv 7.2 channel-opening kinetics or voltage-dependence. Potentiation of the Kv 7.2-currents

decreases neuronal excitability. Recalling the brake-effect of Kv 7.2 channels on excitability mentioned above, these data showing that Stargazin interaction with Kv7.2 enhances channel currents suggest that Stargazin would protect the circuit from hyper-excitability. Thus, we could expect alterations in Kv 7.2-currents if Stargazin is mutated in regions necessary for the interaction.

Kv 7.2 FUNCTION IS AFFECTED BY SCHIZOPHRENIA AND INTELLECTUAL DISABILITY-ASSOCIATED VARIANTS

It was our goal to evaluate whether the interaction between Kv7.2 and Stargazin could be important in a context of disease, namely, in the context of neuropsychiatric disorders such as schizophrenia and intellectual disability. Thus, we studied how mutant forms of Stargazin associated to disease, V143L (Stg^{ID}) and S148N (Stg^{SCZ}), would impact Stargazin-mediated regulation of Kv 7.2. As presented before, both mutations are in the 3TM and have predicted highly damaging effect on Stargazin function (fig: 7).

Both Stg^{ID} and Stg^{SCZ} failed to rescue the levels of Kv 7.2 in cells where endogenous Stargazin was silenced. However, it is striking that the decrease in Kv 7.2 levels that occurs when Stargazin is silenced or when the Stargazin mutants are expressed is observed mainly concerning Kv7.2 localized in the proximity of excitatory synapses (fig: 16C). One possibility is that the signal that we detect in proximity to the synaptic marker may be axonal, from axonal bundles that normally form around dendrites. As discussed above, the dendritic vs axonal co-localization of Stargazin and Kv7.2 needs to be further investigated, as well as the impact of Stargazin mutations in the dendritic and axonal populations of Kv7.2. In particular it would be important to evaluate the impact of Stargazin's variants on Kv 7.2 levels in the axonal initial segment, where M-currents have a well described role [4].

Finally, to clarify the effect of each Stargazin mutant, the same electrophysiological assay used to assess Stargazin impact in Kv 7.2-mediated currents was performed with the Stargazin variants, in collaboration with Dr Taglialatela. In CHO cells, both variants were co-expressed with Kv 7.2 and, contrarily to what was observed upon Stargazin co-expression, the Stargazin-mediated potentiation of the currents was absent. Moreover, no gating changes were detected upon co-expression of the wild-type Stargazin or the variants. Altogether the IP and co-localization data, together with the electrophysiology data, may suggest that the functional impact of the Stargazin-Kv 7.2 interaction occurs due to an increase in trafficking efficacy, as it is the case for the Stargazin-AMPAR interaction. Furthermore, a recent work identified the 3TM domain of Stargazin as the one responsible domain for the interaction with GluAs[127], thus sustaining the functional impact of the mutations since both V143L (ID) and S148N (SCZ) are in the 3TM domain of Stargazin. Furthermore, this suggests that the third domain may not interact exclusively with AMPAR subunits, and that it could be required for the interaction with other proteins such as Kv7.2. Further experiments will test this possibility.

Given the immunocytochemistry and electrophysiology results above discussed, the functional impact of Stargazin interaction with Kv 7.2 may represent a breakthrough to better understand the epileptic features of the *Stargazer* mouse. The *Stargazer* mouse, which does not express Stargazin, displays typical features, particularly cortical excitability and absence seizures[24] (reviewed in introduction). The heteromeric M-channel is composed of Kv7.2-7.3 and is well-known to set the resting potential, control synchronous population firing activity and modulate excitability of neurons[4]. Mutations in the subunits of M-channels are largely associated with epilepsies[95,98,101,120]. Given the role of Stargazin in the regulation of Kv 7.2-mediated currents (data not shown) that we have unveiled, and the known function of the M-channels, it is tempting to suggest a correlation between the decrease of the hyperpolarizing currents mediated by Kv 7.2 that we would expect in the absence of Stargazin and the increased cortical excitability of the *Stargazer* mouse. It is possible that the lack of Stargazin in the *Stargazer* mouse decreases the levels of Kv 7.2 (as in Fig:.16.B and C), possibly limiting the assembly of the Kv 7.2 and 7.3 subunits to form M-channels. Insufficient number of M-channels would impair the generation of the hyperpolarizing M-currents leading the circuitry to enter a hyper-excitable state that would ultimately induce seizures. To summarize, we suggest that Stargazin mutations might be leading to an overall alteration in the network synaptic transmission that could ultimately result in excitation/inhibition imbalance, and consequently in disease development.

Altogether, these results are in agreement with the proposal put forward by Karl Deisseroth[58], that the imbalance between inhibition and excitation in the network is the link between all neuropsychiatric disorders.

mGLUR5 FUNCTION IN AMPAR REGULATION IS REGION-SPECIFIC AND STARGAZIN-DEPENDENT

In the present work we also explored the functional relevance of interaction between mGluR5 and Stargazin. Previous studies in the lab biochemically confirmed this interaction, and determined that Stargazin and mGluR5 co-localize extensively in dendrites of cortical neurons. Here, we focused on testing the impact of Stargazin on the modulation by group I mGluR of GluA1 synaptic levels in cortical neurons.

For the first time, similarly to Kv 7.2, mGluR5 was discovered to be a binding partner of Stargazin (fig: 11A and B). However, in order to assess if mGluR5 interacts directly with Stargazin, further studies must be implemented, namely evaluation of this interaction in a transfected cell line would inform about a potential direct interaction between the two proteins. On the other hand, mGluR5 and Stargazin have high co-localization, particularly at the synapse (fig: 12C and D) where more than 50% of the synaptic Stargazin puncta co-localize with mGluR5, and more than 50% of the synaptic mGluR5 puncta co-localize with Stargazin. These results are in agreement with the literature, since both Stargazin and mGluR5 are known to be present at glutamatergic synapses (reviewed in introduction).

Literature reports that Stargazin expression mediates GluA insertion at the synapse[1] and class I mGluR activation is well described to cause GluA1 internalization, inducing the mGluR-dependent LTD [108] in the hippocampus.

Strikingly, in cultured cortical neurons, class I mGluR activation increases GluA1 synaptic content (fig: 17). Moreover, mGluR5 plays a determinant role in the GluA1 increase, since specific mGluR5 activation with CHPG is sufficient to significantly increase GluA1, both total and synaptic levels. In order to investigate the role of Stargazin in the mGluR5-mediated increase of GluA1, we silenced (Stg KD) or overexpressed (StgOV) Stargazin and stimulated the cells with CHPG. Since synaptic GluA1 levels are highly decreased when we knockdown Stargazin[2], it is difficult to interpret the lack of effect on mGluR5 activation in this particular condition (Fig: 18B-D). However, overexpressing Stargazin increases GluA1 synaptic levels as previously documented[27,45], and upon mGluR5 activation neurons overexpressing Stargazin present an upsurge of GluA1 (Fig: 18B-D), suggesting a Stargazin-dependent mGluR5 function in AMPAR trafficking.

Summing up these results on GluA1 levels upon mGluR5 activation, it is clear that further studies must be developed to better understand what is happening in cortical neurons undergoing mGluR5 activation.

Some groups have shown data pointing towards a possible role for mGluR5 activation in LTP induction specifically in cortical regions, namely in the deep-layer visual neocortex[109], and in the increase of AMPAR-mediated currents in neocortical layer 2/3 pyramidal neurons[112]. Wilson and co-workers strikingly describe impaired LTP in FMRP knockout mice[109] in neocortex. In fact, their results show that FMRP knockout impairs LTP just like application of MPEP, a specific mGluR5 antagonist, thus proving that impaired LTP is due to decreased mGluR5 signalling. Their work indicates that whereas mGluR5 activation triggers LTD in the hippocampus, it leads to LTP in the cortex. Our results are in agreement with this cortical function of mGluR5, and further suggest that Stargazin and its interaction with mGluR5 may be implicated in this process.

With this, the remaining question is to determine the possible molecular mechanism underlying mGluR5-induced increase in GluA1 levels. mGluR5 was shown to be coupled with the activation of G_{q-11}proteins which leads to phosphoinositide hydrolysis, activating PKC (reviewed in Introduction). As reviewed previously, AMPAR subunits are substrate of many kinases, including PKC. Moreover, the specific phosphorylation of S831 residue is required for synaptic insertion and it is also reported to increase AMPAR conductance[9]. Also, Stargazin is itself described as a substrate of PKC at the C-terminus, and this phosphorylation increases AMPAR clustering at the synapse. To address if this mechanism is responsible for the GluA1 increase, we intend to measure the AMPAR-mediated currents in cortical neurons stimulated with CHPG, as well as to perform biochemical assays to measure PKC activity and specific phosphorylation of GluA1-831 serine residue upon mGluR5 activation in the cortex.

Overall, our data suggest that Stargazin affects the cortical function of mGluR5, which could be dependent on the interaction between the two proteins.

Chapter 5

CLOSING REMARKS

AND

FUTURE DIRECTIONS

In this work, Kv 7.2 and mGluR5 were described for the first time as Stargazin binding partners.

The direct interaction of Stargazin with Kv 7.2 seems to potentiate the hyperpolarizing Kv 7.2-mediated currents, possibly preventing the circuitry from entering hyper-excitable states. Moreover, Stargazin point mutations associated with SCZ and ID fail to promote this potentiation, and to secure the dendritic localization of Kv7.2, suggesting an impairment in the regulation of these currents in patients presenting mutations in Stargazin. Hence, the presented results are in accordance with the currently accepted hypothesis that the unbalance between the inhibition and excitation is one of the main causes of neuropsychiatric disorders.

Given the direct interaction between Stargazin and Kv 7.2 in transfected cells, it would be of major interest to test the interaction in brain tissue, and to understand which domains are mediating this interaction using, for example, deletion mutants of both proteins. To better understand the impact of this interaction, intracellular and surface levels of Kv 7.2 should be measured in cells where Stargazin is silenced or mutated. Possible approaches could be biotinylation assays or imaging studies using an antibody against an extracellular domain of the Kv7.2. Since Stargazin-Kv 7.2 co-localization is very low, it would be important to understand under which circumstances it is potentiated, or whether it is restricted to particular cellular localizations. Finally, a *knock-in* mouse model for intellectual disability, recently generated in our lab (expressing Stargazin V143L), represents a great opportunity to better understand the *in vivo* function of the Stargazin-Kv7.2 interaction, in particular its role in neuronal excitability.

Our results concerning the Stargazin-mGluR5 interaction suggest that the outcome of mGluR5 activation is brain-region specific. In fact, mGluR5 specific activation in the cortex induces opposing events to those described in hippocampus. An upsurge of GluA1 was shown to occur upon mGluR5 activation in cortex, in a Stargazin-dependent manner. It will be important to further characterize the effects of mGluR5 on AMPAR-mediated responses in the cortex, and to unravel the role of Stargazin in this mechanism.

The new Stargazin interactions described in this work and the functional impact of Stargazin point-mutations open an all-new range of possibilities and questions. First, it would be interesting to study the role of Stargazin on mGluR5-dependent LTD in the hippocampus. To do so, spine morphology and volume, as well as AMPAR-mediated currents could be evaluated using hippocampal organotypic slices. Although, an mGluR5-mediated increase in GluA1 is not necessarily accompanied by an increase in AMPAR-mediated currents, studying the AMPAR currents in cortical neurons in presence of CHPG would provide insight about what is occurring in cortex and importantly, if it is somehow dependent on Stargazin.

Furthermore, unpublished data from our lab showed impaired GluA1 trafficking to the surface in StgID-transfected neurons. Remaining questions are if this decrease is enough to impair AMPA-mediated synaptic transmission during activity periods and if mGluR5 activation would be able to prevent this effect in these cells.

Finally, another well described impact of mGluR5 activation is the decrease of the M-currents, thus increasing neuronal excitability. Given the presented hypothesis, that Kv 7.2 trafficking is Stargazin dependent, it would be interesting to understand if Stargazin is involved in the mGluR5-dependent regulation of M-currents.

Chapter 6

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