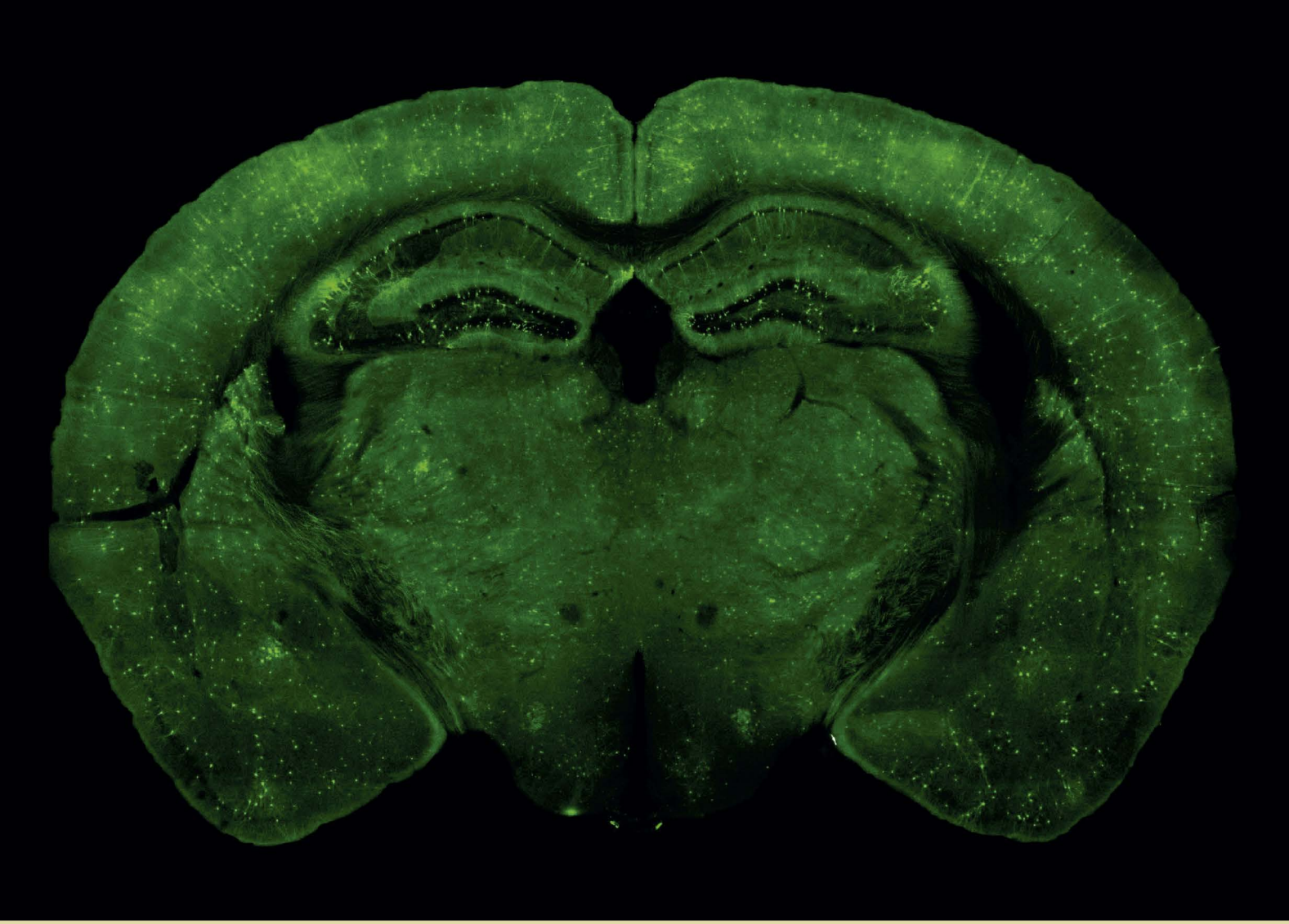

Tiago Batista Abrantes Rondão

Behavioral and Biochemical Characterization of Stargazin *knock-in* mice expressing an Intellectual disability-linked mutation

Dissertação de mestrado em Biologia Celular e Molecular com especialização em Neurobiologia,
orientada pela Professora Doutora Ana Luísa Carvalho e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Agosto 2017

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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 com especialização em Neurobiologia, realizada sob orientação da Professora Doutora Ana Luísa Monteiro de Carvalho (Departamento de Ciências da Vida, Universidade de Coimbra) e de Gladys Tarcila Lima Caldeira (Centro de Neurociências e Biologia Celular, Universidade de Coimbra).

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The beautiful thing about learning
is that no one can take it away
from you.

B. B. King

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ABBREVIATIONS

3CT	Three chamber test
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA receptor
AP-4	Activating enhancer binding protein 4
ASD	Autism spectrum disorder
BDNF	Brain-derived neurotrophic factor
BiP	Ig binding protein
BL	Brain lysate
CaMKII	Calcium/calmodulin-dependent protein kinase I
Caspr2	Contactin associated protein-like 2
CFP	Cyan fluorescent protein
CGCs	Cerebellar granule cells
CLAP	Chymostatin, leupeptin, antipain and pepstatin
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CTD	Carboxyterminal domain
DNA	Deoxyribonucleic acid
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
EPSCs	Excitatory postsynaptic currents
ER	Endoplasmatic reticulum
FRET	Fluorescence resonance energy transfer
FST	Forced swimming test
GCs	Granule cells
GFP	Green fluorescent protein
GnRHR	Gonadotropin-releasing hormone receptor
GRC	Glutamate receptor complex
GRIA3	Glutamate receptor 3 precursor
GRIK2	Glutamate receptor ionotropic, kainate 2 precursor
GRIN2A	Glutamate ionotropic receptor NMDA type subunit 2A
GRIN2B	Glutamate ionotropic receptor NMDA type subunit 2B
GRIP	Glutamate receptor interacting protein
HEPES	N-(2-hydroxyethyl)-1-piperazine-N"-(2-ethanesulfonic acid)
ID	Intellectual disability
iGluR	Ionotropic glutamate receptors
IPSCs	Inhibitory postsynaptic currents
IQ	Intelligence quotient
KA	Kainate
KAR	Kainate receptor
kDa	Kilodalton
KI	*Knock-in*

KO	*Knock-out*
LBD	Ligand binding domain
LTD	Long-term depression
LTP	Lon-term potentiation
MAGI 2	Membrane-associated guanylate kinase
MAP1 LC2	Microtubule-associated protein 1 light chain 2
mGluR	Metabotropic glutamate receptors
mRNA	Messenger RNA
MSD	*Mean square displacement*
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
nPIST	Neuronal isoform of protein-interacting specifically with TC10
NSID	Nonsyndromic ID
NTD	Amino terminal domain
OCD	Obsessive-compulsive disorder
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PP1	Protein phosphatase 1
PSD	Post synaptic density
PSD95	Postsynaptic density protein 95
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
SAP97	Synapse-associated protein 97
SCZ	Schizophrenia
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-poliacrylamide gel electrophoresis
SEM	Standard error of the mean
shRNA	Short-hairpin Ribonucleic Acid
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
SNPs	Single-nucleotide polymorphism
SPM	Synaptic plasma membrane
Stg	Stargazin
TARP	Transmembrane AMPAR-regulatory protein
TBS	Tris-buffered saline
TGN	Trans-Golgi network
TTX	Tetrodotoxin
VGCCs	Voltage-gated calcium channels
YFP	Yellow fluorescent protein

ABSTRACT

The plasticity of excitatory synapses is an essential brain process involved in cognitive functions and necessary for the proper development of intellectual capacities. Glutamate, the major excitatory neurotransmitter in the central nervous system, acts on ionotropic and metabotropic receptors. Together and distinctly, these receptors modulate neuronal circuits that underlie aspects of cognitive function. Therefore, glutamatergic synaptic transmission is a key player in learning and memory formation processes in brain areas such as the hippocampus, the prefrontal cortex and the cerebellum.

Within glutamatergic synapses AMPA receptors (AMPARs) mediate most of the rapid excitatory neurotransmission and undergo activity-dependent changes in their trafficking and surface expression, which have been proven to be fundamental mechanisms for synaptic plasticity processes such as long-term potentiation (LTP). An increase in the post-synaptic response to a stimulus is achieved either through elevating the number of AMPARs at the post-synaptic surface or by increasing the single channel conductance of these receptors. The ability to control the surface expression and activity of AMPARs is also essential for homeostatic plasticity, a set of mechanisms that act in order to stabilize neuronal and circuit activity by counterbalancing some of the plastic challenges faced by neurons. AMPARs directly interact with transmembrane AMPA receptor regulator proteins (TARPs), which act as their auxiliary subunits. TARPs influence AMPAR synaptic targeting, synapse expression and function by different mechanisms. Among the TARPs, stargazin (γ-2) is one of the most important and abundant members of this family. This protein regulates AMPA receptor function and stabilizes AMPARs in the synaptic membrane due to a strong interaction with PSD-95; consequently, it plays a crucial role in synaptic plasticity. Stargazin is encoded by the human *CACNG2* gene, and mice in which the homologous gene is disrupted (stargazer mice) show a dramatic loss of AMPA receptor activity in brain regions where stargazin is highly expressed.

A significant amount of previous studies suggest that the dysfunction of glutamatergic synapses is strongly implicated in several neurodevelopmental disorders, such as Intellectual disability (ID), autism spectrum disorders and schizophrenia. The disruption of glutamatergic synapses is often related with the dysfunction of synaptic scaffold proteins or TARPs. In this study, we looked into a *de novo* mutation (p. Val143Leu) in the *CACNG2* gene that was identified in a male with moderate ID. A previous study showed that this mutation significantly decreases stargazin's ability to bind to AMPARs and reduces cell surface expression of the GluA1 AMPAR subunit in transfected hippocampal neurons and HEK293 cells. In order to elucidate how this mutation affects protein function and contributes to the development of disease-associated phenotypes our laboratory

generated a *knock-in* mouse harboring the human mutation in the stargazin-encoding gene. In this project, we characterized the synaptic biochemical composition, hippocampal neuronal morphology and social behavior featured by these mice, to address the role of stargazin in normal neuronal development and to determine causality between a disease-associated mutation in the CACNG2 gene and the generation of ID-like behavior in mice.

Here, we found that mutant stargazin levels are decreased at whole-brain derived postsynaptic densities, but found no evidence for alterations in AMPARs subunits synaptic expression. Remarkably, neuronal morphology analyses revealed that stargazin V143L$^{+/+}$ *knock-in* mice CA1 pyramidal neurons exhibit decreased dendritic arborization complexity and a decrease in the total length of dendrites. Finally, stargazin V143L$^{+/+}$ *knock-in* mice displayed abnormal social behavior in the three chamber test and impairment in the perseverative species-typical burying behavior.

In conclusion, this study revealed that the altered form of stargazin, resultant from the V143L mutation in the *CACNG2* gene, is indeed causative of morphological and behavioral abnormalities in mice. Further analyses are needed to complete the characterization of this mouse model and to better understand to what extent the ID-associated mutation affects stargazin function, and leads to neuronal circuits alteration that underlie the behavior impairments reported in this study. Ultimately, we hope that the knowledge resulting from the study of these mice gives us valuable insights to understand the big picture of the stargazin-associated disorders and other similar conditions.

Keywords: Stargazin; AMPAR; Glutamatergic transmission; Intellectual Disability; *Knock-in* mice

RESUMO

A plasticidade sináptica que ocorre ao nível das sinapses excitatórias é um dos mecanismos cerebrais mais importantes para um normal desenvolvimento da nossa capacidade intelectual, sendo essencial para todas as funções cognitivas. O glutamato é o neurotransmissor mais abundante no sistema nervoso central e o principal mediador da neurotransmissão excitatória. A ação deste neurotransmissor é dependente da sua ligação a recetores na membrana pós-sináptica, podendo atuar em dois tipos de recetores: inotrópicos, que quando ativos permitem a passagem de iões como Na^+, K^+ e Ca^{2+}; e metabotrópicos, cuja função é ativar cascatas de proteínas intracelulares. A ação conjunta destes recetores modula o funcionamento de grande parte dos circuitos neuronais essenciais para as funções cognitivas. Assim, todos os constituintes do complexo sistema que forma as sinapses gluatamatérgicas desempenham um papel essencial nas regiões cerebrais que maioritariamente regulam a capacidade de aprendizagem e formação de memórias, tais como o hipocampo, o córtex pré-frontal e o cerebelo.

Os recetores ionotrópicos de glutamato do tipo AMPA são os principais mediadores da neurotransmissão excitatória rápida. A eficiência da transmissão glutamatérgica é dependente do padrão de atividade neuronal, podendo ser reforçada ou enfraquecida dependendo da quantidade de recetores na membrana celular sináptica, fenómeno designado de plasticidade sináptica. Os recetores AMPA interagem com várias proteínas que influenciam a sua funcionalidade. Entre estas destaca-se a família de proteínas chamadas TARP (proteínas transmembranares associadas aos recetores AMPA). Estas proteínas regulam a atividade dos recetores a vários níveis, tais como modulando o seu tráfego sináptico e as propriedades biofísicas do canal iónico dos recetores. Dentro desta família de proteínas, a stargazina é a mais conhecida e uma das mais abundantes. Esta proteína tem como principal função estabilizar os recetores na membrana sináptica, de forma dependente da sua interação com a PSD95. Tendo em conta o seu importante papel na regulação da atividade dos recetores AMPA, a stargazina é essencial para o correto funcionamento dos processos de plasticidade sináptica. Esta proteína é codificada pelo gene *CACNG2*, e murganhos que possuem uma forma corrompida deste gene (murganhos stargazer) têm uma redução dramática na atividade neuronal dependente dos recetores AMPA nas regiões cerebrais onde a proteína é mais abundante.

Vários estudos anteriores têm sugerido que a disfunção das sinapses glutamatérgicas está implicada em vários distúrbios psiquiátricos e do desenvolvimento, tais como o défice cognitivo, distúrbios do espectro autista e esquizofrenia. A anormal funcionalidade destas sinapses é frequentemente associada a anormalidades nas proteínas sinápticas como as TARPs. Este projeto

teve como objetivo estudar uma mutação (p. Val143Leu) no gene *CACNG2* que foi identificada numa criança com défice cognitivo. Um estudo anterior revelou que esta mutação diminui na capacidade da stargazina para interagir com os recetores AMPA, levando a uma diminuição dos níveis dos recetores na sinapse em neurónios transfetados para expressarem esta forma mutada. Foi criado pelo nosso laboratório um modelo animal de murganho geneticamente modificado que expressa a stargazina mutada (stargazina V143L). O estudo aqui apresentado descreve a caracterização deste animal modelo, ao nível da composição bioquímica das sinapses, morfologia dos neurónios do hipocampo e comportamento social, realizada com o intuito de tentar perceber de que forma a mutação na stargazina altera a capacidade funcional da proteína e como pode levar a um fenótipo característico de distúrbios como o défice cognitivo. Descobrimos que os neurónios da região CA1 do hipocampo destes animais têm uma arborização dendrítica de complexidade reduzida, bem como uma redução no tamanho das suas dendrites. Para além disso observámos que a forma mutada da stargazina se encontra menos expressa na sinapse. Finalmente, verificamos que estes animais exibem um comportamento social anormal e alterações no comportamento perseverante de ocultação de objetos, típico dos murganhos.

Concluindo, este estudo demonstra uma relação de causalidade entre a mutação V143L na stargazina, detetada num doente com défice intelectual, e alterações na morfologia neuronal no hipocampo e no comportamento social de murganhos. Contudo, é necessária uma análise mais aprofundada de modo a perceber em que medida é que esta mutação afeta a função da stargazina, levando consequentemente a alterações nos circuitos neuronais que regulam os comportamentos afetados nos animais. Com os resultados provenientes destas análises esperamos perceber melhor como alterações genéticas desta ordem levam a este tipo distúrbios cognitivos.

PALAVRAS-CHAVE: Stargazina; recetores AMPA; transmissão glutamatérgica; défice intelectual; murganhos geneticamente modificados

Chapter I

INTRODUCTION

1.1. GLUTAMATERGIC TRANSMISSION AS THE MAIN MECHANISM UNDERLYING COGNITIVE FUNCTION

1.1.1. Glutamatergic synapse

Glutamatergic synapses convey most excitatory neurotransmission in the mammalian central nervous system (CNS; Fig. 1.1). Plasticity of these synapses is an essential brain process involved in sensory processing cognitive functions, and so crucial for the proper development of intellectual capacities. Most excitatory synaptic transmission is mediated by glutamate, the main excitatory neurotransmitter, which acts on two classes of glutamate-gated ion channels, termed ionotropic glutamate receptors (iGluRs), and glutamate-activated G protein–coupled receptors - metabotropic glutamate receptors (mGluRs). iGluRs include N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (KA) receptors (NMDARs, AMPARs, and KARs, respectively) based on their affinity for the glutamate analogues NMDA, AMPA, and KA. All iGluRs are tetrameric complexes of integral membrane proteins with an extracellular N-terminus and an intracellular C-terminus, and the ligand binding domain is made up from N-terminal regions S1 and S2 (Fig. 1.2). These receptors are assembled from different subunits that contribute to unique channel conductance properties, signaling, localization, and interaction partners. Together, glutamate receptors modulate neuronal circuits that underlie aspects of cognitive function. Therefore, glutamatergic synaptic transmission becomes a key player in learning and memory formation processes in brain areas such as the hippocampus, the prefrontal cortex and the cerebellum. Hence, any nonfatal aberrations in the development or function of glutamatergic synapses have the potential to profoundly impair the cognitive abilities of the human brain.

The functionally mature glutamatergic synapse is a complex assembly where pre and postsynaptic compartments communicate by using a vast repertoire of molecules and signaling cascades. Following contact, transmembrane cell adhesion molecules, supported by their association with intracellular scaffolds, further facilitate the maturation of a functional synapse (Fig. 1.1). Subsequent sensory experience refines circuitry through a combination of activity-dependent synapse maturation steps [1]. Continuous synapse formation and elimination occurs throughout development, with synaptogenesis dominating early in development, followed by a period dominated by synapse pruning, which lasts roughly through adolescence. The synaptic structure is constituted by several distinct proteins which have different functions and act together for the successful and controlled neuronal transmission.

The presynaptic compartment is enriched with glutamate-filled synaptic vesicles and specialized active zones that support the release of glutamate from the nerve terminal. A wide array of proteins

works for the transport of glutamate into synaptic vesicles, localize filled vesicles to the active zone, and dock and prime vesicles for release (Fig. 1.1). Scaffolding proteins coordinate voltage-gated calcium channels (VGCCs) and intracellular calcium sensors to orchestrate the rapid release of glutamate in response to a calcium influx triggered by action potentials[2]. In addition, glutamate reuptake machinery in both neurons and neighboring astrocytes regulates the concentration of glutamate in the synaptic cleft.

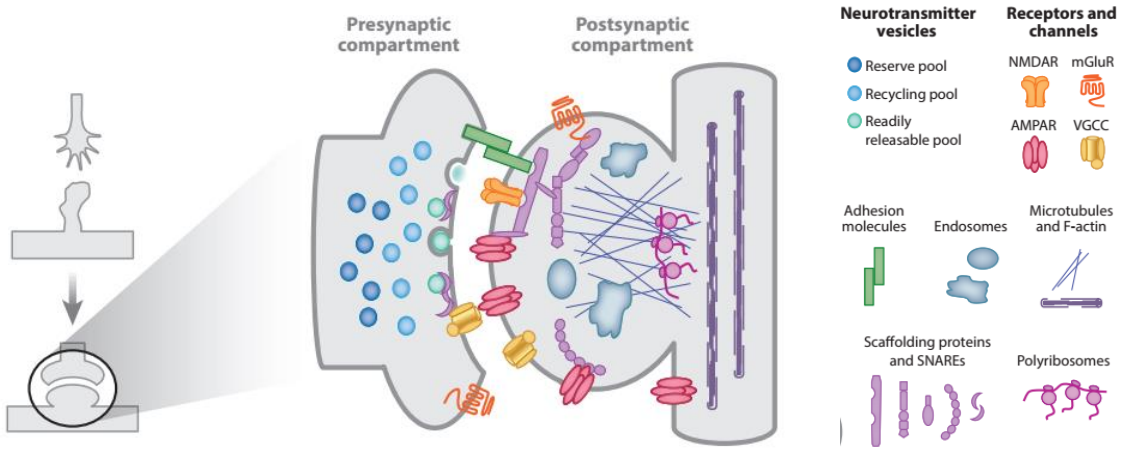

Figure 1.1. Simple schematic of glutamatergic synapse development and composition.
Synapse formation involving interaction and stabilization of an axonal growth cone and dendritic filopodia followed by structural and molecular maturation of both pre- and postsynaptic compartments; Typical mature glutamate synapse, composed of a presynaptic compartment packed with membrane proteins, neurotransmitter-filled synaptic vesicles, and SNARE protein complexes for vesicle fusion and glutamate release opposed to the a postsynaptic spine containing membrane proteins [e.g., glutamate receptors, voltage-gated ion channels, cell adhesion molecules], extensive scaffolding proteins, endosomes, and local protein translation machinery [3].

The postsynaptic compartment, found at specialized, actin-rich, dendritic protrusions called spines, is made of a compact network of scaffolding proteins, receptors, and signaling molecules that form what is known as the post synaptic density (PSD) in the spine head (Fig. 1.1). This structures often change their morphology and size with synaptic strength, a process that is termed as synaptic plasticity. In a general way, neuroplasticity refers to the ability of this neuronal circuits and associated connections to functionally and structurally adapt their activity in response to internal and salient environmental stimuli.

1.1.2. The role of AMPAR in synaptic plasticity

Within glutamatergic synapses α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid glutamate receptors (AMPARs) are responsible for the bulk of fast excitatory synaptic transmission. Native AMPA receptors are heterotetramers and can be composed by 4 distinct pore-forming subunits: GluA1–4 [4] (Fig. 1.2). Like the other ionotropic glutamate receptors, AMPARs are nonselective cation channels, allowing the passage of Na^+ (in), K^+ (out) and produce excitatory postsynaptic responses. All subunits contain three transmembrane domains (M1, M3, and M4), a channel pore loop (M2), a relatively long amino terminal domain (NTD) and a short carboxyterminal domain (CTD). Each subunit can be expressed as either "flip" or "flop" isoform (flip/flop module marked in green), with "flip" isoforms dominating early in development and "flop" isoforms being more abundant in adolescence and after. AMPA receptors containing "flop" subunits desensitize faster than those containing "flip" isoforms. About 80–90% of GluA2 and -A3 subunit and about 50% of GluA4 subunit undergo RNA-editing at the flip/flop exon junction resulting in an amino acid change from an arginine (R) to a glycine (G) that shortens the recovery from desensitization in receptors containing the affected subunits. Another editing event only affects the GluA2 subunit in the pore loop. Almost all GluA2 subunits are edited to contain an arginine (R) instead of a glutamine (Q), resulting in a linear I/V relationship and low Ca^{2+}-permeability of affected receptor assemblies. Through their CTD, most AMPA receptor subunit isoforms interact with different proteins of the postsynaptic density and/or can be phosphorylated by different protein kinases affecting the electrophysiological and biochemical properties of the receptor. GluA2 and -A4 can be expressed as long or short isoforms (with preferential expression of GluA2-short and GluA4-long) affecting the length of the CTD and possible protein interactions anzzzzd phosphorylation sites.

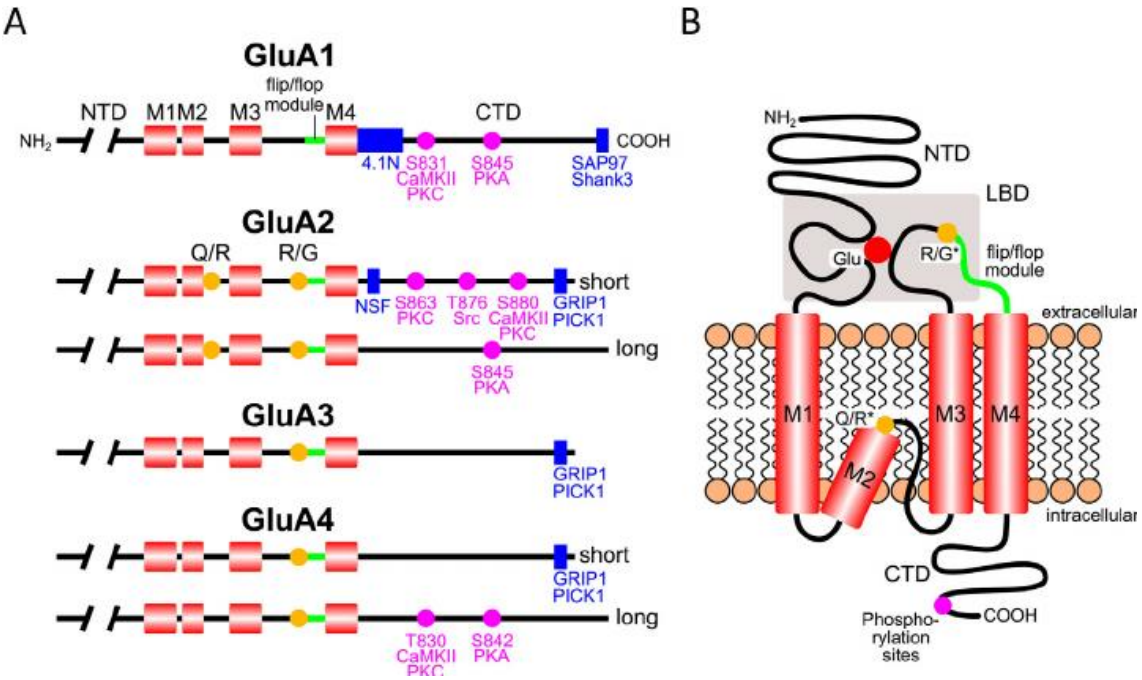

Figure 1.2. Structure and organization of AMPA receptor subunits. (A) Domain structure and splice variants of all AMPA receptor subunits (GluA1 to -A4). Flip/flop modules are marked in green, while RNA-editing sites are indicated by an orange dot and phosphorylation sites are indicated in pink. Some of the protein interacting sites are showed in blue. (B) Secondary structure of an AMPA receptor subunit in the plasma membrane showing the three transmembrane domains (M1, M3, and M4), and the channel pore loop (M2), the relatively long amino terminal domain (NTD) and the short carboxyterminal domain (CTD). (Adapted from Freudenberg et al., 2015)[5]

AMPARs undergo activity-dependent changes in their biophysical properties, trafficking and surface expression in response to specific patterns of neuronal activity [6, 7]. This modulation changes synaptic strength and is the ultimate mechanism that underlies much of the plasticity of excitatory transmission that is expressed in the brain. Increasing the post-synaptic response to a stimulus is achieved either through increasing the number of AMPA receptors at the post-synaptic surface (Fig. 1.3) or by increasing the single channel conductance of the receptors expressed. This was shown to be the basis of experience-dependent synaptic plasticity mechanisms such as long-term potentiation (LTP), contributing to cellular mechanisms of learning and memory. In contrast to AMPARs, NMDARs and mGluRs generally serve as sensors of specific activity patterns and initiate the signaling cascades to induce plasticity. Depending on the pattern of activity, persistent increases [long-term potentiation (LTP)] or decreases [long-term depression (LTD)] in synapse strength can be induced in an input-specific manner, thus altering the relative contribution of the stimulus-relevant subset of synapses to information processing.

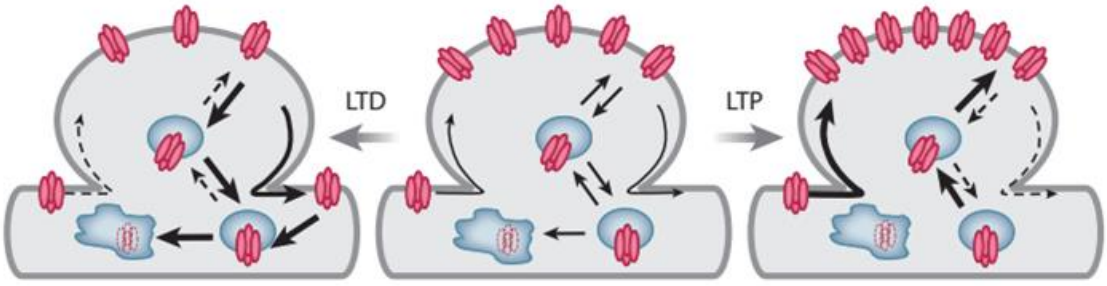

Figure 1.3. Simplified schematic of neuroplasticity processes. Activity-dependent, long-term changes in synaptic strength at postsynaptic sites are determined predominately by the number of AMPARs at a synapse. (Adapted from Volk et al., 2015) [3]

Synaptic plasticity can persist for days, weeks, or even years and is currently considered the most likely cellular substrate for adaptive cognitive processes [8]. *De novo* protein synthesis, which can occur locally in dendrites as well as in the soma, is also essential for maintaining both long-lasting synaptic plasticity and memory. Importantly, many proteins critical for glutamate receptor trafficking, along with some subunits of glutamate receptors themselves, can be synthesized at synapses in an activity-dependent fashion. Pre and postsynaptic cell adhesion molecules have also been implicated in the regulation of AMPAR trafficking[9]. Additionally, long-lasting changes in presynaptic release probability underlie synaptic plasticity at a subset of glutamatergic synapses.

During learning and development, neural circuits must maintain stable function in the face of many plastic challenges, including changes in synapse number and strength. Recent work has shown that, in addition to the rapidly induced (on the scale of seconds to minutes), input-specific synaptic plasticity, the ability to control the surface expression and activity of AMPARs is also essential to counterbalance the accumulation of the destabilizing influences in neuronal firing rate and stabilize neuronal and circuit activity, which is achieved due to the so called homeostatic plasticity mechanisms. Homeostatic plasticity is generally a slow (on the scale of hours to days) response and affects many or all synapses on a neuron and functions to maintain neuronal firing rates within a range favorable to information processing[10]. Much like rapid, input-specific synaptic plasticity, homeostatic plasticity can be expressed via changes in AMPAR expression, presynaptic function, or both. One such mechanism is synaptic scaling, which is a form of synaptic plasticity that adjusts the strength of all of a neuron's excitatory synapses up or down to stabilize firing[11]. Current evidence suggests that neurons detect changes in their own firing rates through a set of calcium-dependent sensors that then regulate receptor trafficking to increase or decrease the accumulation of glutamate receptors at synaptic sites. Additional mechanisms may allow local or network-wide changes in activity to be sensed through parallel pathways, generating a nested set of homeostatic mechanisms that operate over different temporal and spatial scales [12].

Since they play such an important role in fast synaptic changes, AMPA receptors cycle rapidly in and out of the plasma membrane in an activity-dependent manner (Fig. 1.3), which requires assembly with auxiliary proteins from the family of the transmembrane AMPA receptor-regulatory protein (TARPs). These different forms of plasticity that enable the mature brain to adapt and respond to a constantly changing external environment are essential both for the activity-dependent refinement of synapses and circuits in the developing CNS and for adaptive cognitive processes such as learning and memory. A large number of mutations in genes encoding proteins related to the previously mentioned mechanisms that regulate synaptic function are associated with neurodevelopmental diseases like intellectual disability (ID), autism spectrum disorder (ASD), and schizophrenia (SCZ) [3].

1.1.3. The TARPs family

AMPAR interact directly with protein members of the TARP family, as it has been reported that they co-immunoprecipitate with AMPAR in extracts from the brain regions where they are highly expressed [13](Fig. 1.4A). These proteins act as auxiliary subunits for mature receptors, modulating AMPAR surface expression, synaptic targeting and recycling as well as some of the receptors' biochemical properties [14, 15]. Thus, they are an integral part of the AMPA glutamate receptor complex (GRC)[16].

The critical importance of TARPs in synaptic transmission was first revealed when a neuronal-subunit (ɣ-2 or stargazin) was identified at the affected locus of the naturally occurring mouse mutant stargazer[10]. These mice were initially described to be lacking the prototypic TARP stargazin, present in granule cells (GCs) from wild-type animals, and consequently lack synaptic transmission at the mossy fiber-to-granule cell synapse due to a dramatic reduction in surface AMPARs in cerebellar GCs [11]. Co-expression of stargazin (ɣ2) protein with AMPAR subunits in heterologous systems markedly increases AMPAR surface expression [11, 12]. Hence, stargazin function was based on its ability to rescue the surface expression of AMPARs and restore AMPA receptor-mediated currents in stargazer cerebellar granule cells [13]. Subsequent work has identified three closely related isoforms (ɣ3, ɣ4, and ɣ8), which show distinct patterns of expression throughout the brain (Fig. 1.4B). Two other related molecules ɣ5 and ɣ7 also act as TARPs, but with different trafficking properties [17]. They show a reduced ability to deliver AMPARs to the cell membrane, possibly because their shorter C-tails confer protein interactions that differ from those of other TARPs [17]. TARPs family comprise then a total of six proteins named after their homology with ɣ1, a voltage gated calcium channel gamma subunit (*CACNG-1*) [15] – ɣ2, ɣ3, ɣ4, ɣ5, ɣ7, ɣ8. According to their PDZ-binding motifs in their COOH terminus, they are functionally classified as Type I and Type II TARPs. Type I TARPs

contain typical binding motif (–TTPV) and include ɣ2, ɣ3, ɣ4 and ɣ8, while Type II have atypical binding motif (–S/TTPC) and comprises ɣ5, ɣ7 (Fig. 1.5). Class I and II members also differ in their first extracellular domain (EX1) [14]. These structural diferences between the two classes members dictate their different pharmacological and gating effects on AMPA receptors. Moreover, the TARP-mediated stabilization of AMPAR in the membrane is depedent on the interaction with PSD95. For TARP:PSD95 interaction it is essencial that the C-terminal domain of the TARP becomes phosphorylated by Ca^{2+}-dependente kinases such as CaMKII and PKC.

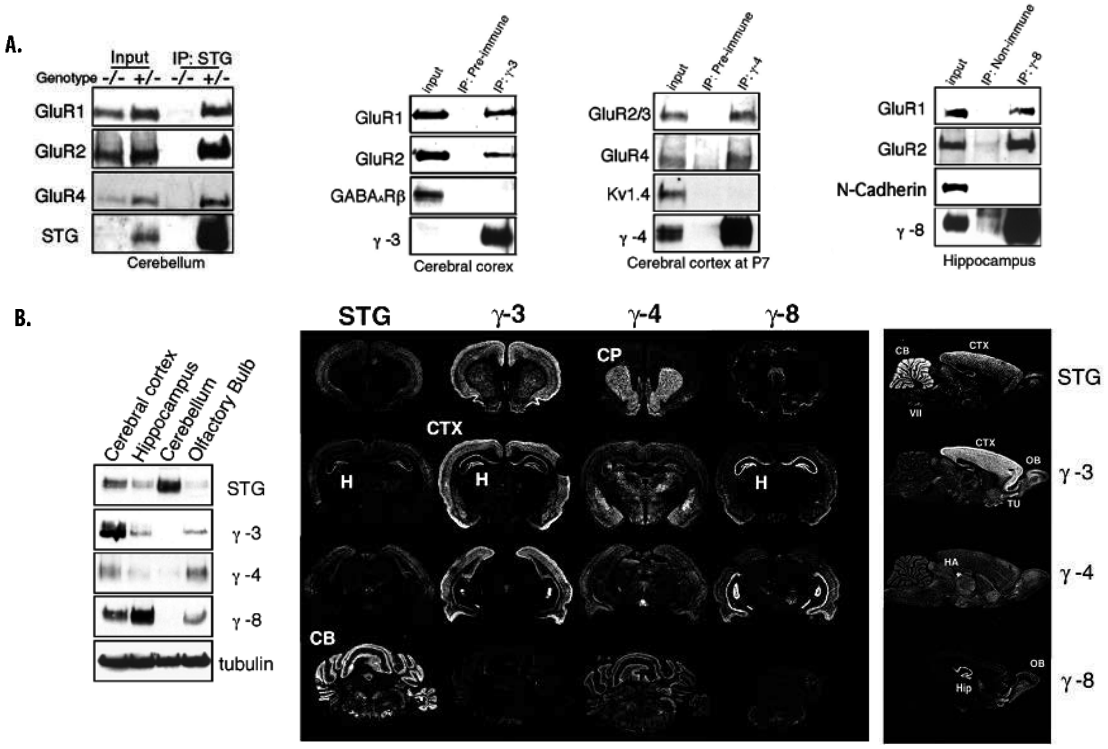

Figure 1.4. TARPS interact with AMPAR subunits and occur differently trough out the brain. (A) CoIP analyses reflecting Type I TARPs interactions with AMPAR. (B) TARPs distribution across the brain, showing a highly enrichment of stargazin in cerebellum, y3 in cerebral cortex, y8 in hippocampus, whereas y4 is modestly diffused trough out the brain with increased levels at the olfactory bulb. (Adapted from Tomita et al., 2003)

Overall, TARPs display a complex cell-type-specific expression, being present in both neurons and glia [13, 18]. Individual TARPs expression levels also diverge with development and throughout the brain. For instance, ɣ4 has been shown to have its expression peak in an early developmental stage, dicreasing in a later stage and contrasting with ɣ2, and ɣ8 which expression is low in neonatal stages but increases in adulthood. Across the brain, TARPs exhibit widespread and extensively overlapping expression patterns (Fig. 1.4-B); while stargazin occurs at highest levels in cerebellum and is modestly enriched in hippocampus and cerebral cortex, ɣ3 is highly expressed in cerebral cortex, ɣ8 in hippocampus and ɣ4 occurs diffusely in the brain. However, it has become evident that the

expression of individual TARPs in different brain regions does not necessarly directly correlate to their functional activity given their broad functional redundancy, particulary in the hippocampus. For instance, significant evidence suggests that hippocampal CA1 neurons can use any TARP to traffic AMPA receptors, relying more on γ-8 than on the other family members [19]. Biochemical and anatomical evidence shows that γ8 and stargazin are present in separate but overlapping subcellular compartments in hippocampal neurons [20]. Specifically stargazin and γ8 are localized to biochemically distinct compartments at the plasma membrane of this cells and each compartment contains a different set of PDZ-containing proteins, suggesting that the differential expression of both TARPs across the synapse is dependent on their different PDZ-binding domains and consequently on their interaction with different PDZ-containing scaffold proteins [20]. The separation pattern of PSD-95 and GRIP was similar to that of stargazin, whereas the separation pattern of SAP97 was similar to that of γ8. Accordingly, *knock-out* animal models addresing the absence of individual TARPs also demonstrated the functional redundancy of this protein family. For instance, *knock-out* mice for γ8 exhibited a modest reduction of 35% in synaptic AMPAR levels when compared to the extrasynaptic AMPAR, which were the decreased about 90%. Moreover, the alterations in AMPARs synaptic expression found in γ3, γ4, γ8 triple KO mice were similar to those of $\gamma8^{-/-}$, whereas $\gamma2^{-/-}$, $\gamma8^{-/-}$ KO mice exhibited a most severe reduction [19].

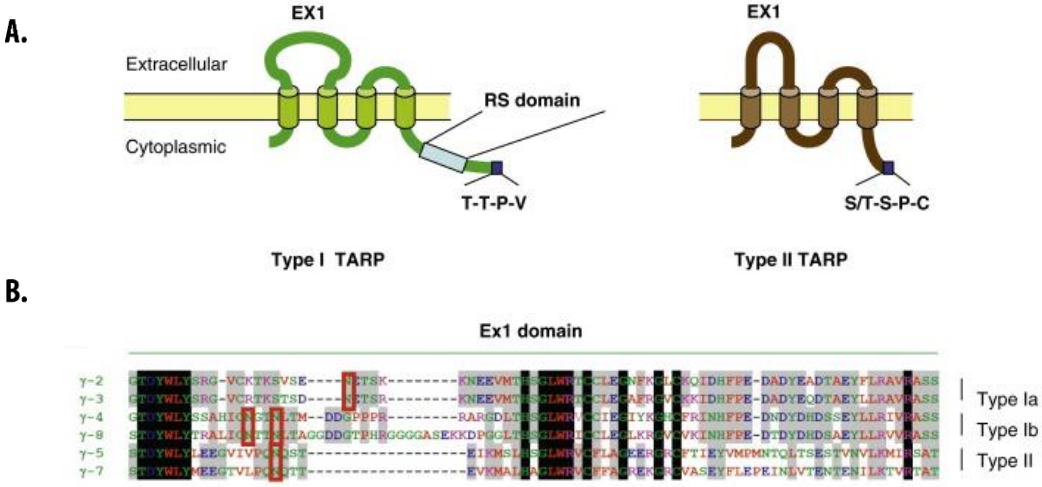

Figure 1.5. Schematic representation of Type I and Type II TARPs. (A) Type I TARPs have a larger first extracellular domain (EX1), which dictates pharmacological and gating effects on AMPA receptors. Type I TARPs have a canonical PDZ-binding domain (T-T-P-V), which localizes AMPA receptors at synapses. Type II TARPs have an atypical PDZ binding domain (S/T-S-P-C). Type I, but not Type II TARPs, have an extensively phosphorylated arginine/serine-rich (RS) cytoplasmic region, which mediates synaptic plasticity. (B) Sequence alignment of TARP EX1 domains. Black boxes indicate the amino acids conserved in all six TARPs. Common amino acids in type-specific TARPs are shown in gray. Red boxes show putative sites for N-glycosylation. (Adapted from Kato et al., 2010)

With such an imperative role in AMPAR functionality, TARPs are important mediators for synaptic plasticity. Indeed, there is a strong of evidence that TARP-associated AMPARs far outnumber those AMPARs that are combined with other protein partners [21]. Thus, a complete clarification of the properties of the AMPA receptor interactor proteins is crucial for the understanding of the global molecular machinery that regulates glutamate receptors-dependent excitatory synaptic transmission.

1.1.4. Stargazin (γ2 TARP)

Stargazin (γ2 or Stg) is a 37 kDa membrane protein, found mostly at the post synaptic density where it acts as an auxiliary subunit for AMPA receptors, modulating several aspects of the receptors function as discussed below. Beside its interaction with AMPA receptor subunits, stargazin interacts with synaptic PDZ proteins, such as PSD-95. The interaction of stargazin with AMPA receptor subunits is essential for delivering functional receptors to the surface membrane of granule cells, whereas its binding with PSD-95 through a carboxy-terminal PDZ-binding domain was found to be required for targeting the AMPA receptor to synapses [22]. Stargazin structure comprises an intracellular N-terminal segment, four transmembrane domains (TM 1 to 4), two extracellular loops (EX1 and EX2) and a C-terminal domain that includes a PDZ domain binding motif and nine serine residues, which are phosphorylated by CaMKII and PKC and are required for synaptic plasticity mechanisms (Fig. 1.6A). Different domains of stargazin regulate the interaction and regulation of AMPAR (Fig. 1.6A). For instance, stargazin physical interaction with AMPAR is also dependent on the first extracellular domain (EX1) and on the C-terminal domain[23]. In addition, it was recently shown that the third and fourth transmembrane domains (TM3 and TM4) interact with transmembrane domains of the AMPAR subunits, including the pore helices, and the extracellular domains are also involved, modulating the AMPAR ligand binding domain (LBD) closure and consequently affecting the conformation of the LBD layer that is associated with the activation and desensitization of the receptor [24, 25]. For Stg/AMPAR interaction, stargazin is positioned below the ligand-binding domain of the AMPA receptor (Fig. 1.6B).

In addition to modulating trafficking and stabilization of AMPAR at synapses, stargazin was shown to be required for the activity-dependent switch from GluA2-lacking to calcium impermeable, GluA2-containing AMPAR, a form of synaptic plasticity displayed by parallel fiber to stellate cell synapses in the cerebellum [26].

The spontaneous recessive mutant mouse stargazer (stg) lacks stargazin expression and displays an ataxic and epileptic phenotype, and a severe impairment in the acquisition of classical eyeblink conditioning in adulthood [27]. In the first characterization of this animal mouse model, these

abnormalities have been attributed to the specific reduction in brain-derived neurotrophic factor (BDNF) and the subsequent defect in TrkB receptor signaling in cerebellar granule cells (GCs). In the stg mutant cerebellum, EPSCs at mossy fiber (MF) to GC synapses are devoid of the fast component mediated by AMPA-type glutamate receptor and their GCs sensitivity to exogenously applied AMPA is greatly reduced [27].

Besides stargazin enrichment in cerebellum where it plays a crucial role, it has been shown that this protein is expressed diffusely across the brain and it is important for AMPAR-dependent synaptic plasticity in several brain regions. Expression of a mutant stargazin lacking the PDZ-binding domain in hippocampal pyramidal cells disrupts synaptic AMPA receptors, indicating that stargazin-like mechanisms for targeting AMPA receptors may be widespread in the central nervous system [11]. Moreover, at the hippocampal synapses, LTP and LTD are shown to require stargazin/γ-2 phosphorylation and dephosphorylation, respectively [28].

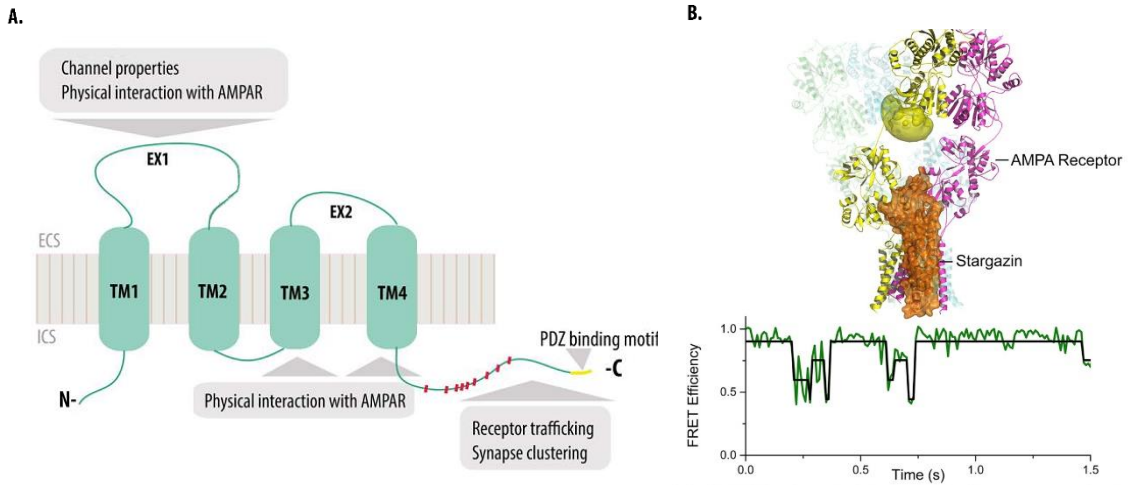

Figure 1.6. Stargazin two-dimensional structure and interaction sites. (A) Stargazin two-dimensional structure and disposition across the membrane, evidencing its interaction points and correspondent roles. (B) Model of AMPAR/Stargazin complex determined by lanthanide resonance energy transfer nanopositioning in live cells (top); single molecule FRET trajectory revealing stargazin-dependent conformational changes in full-length AMPA receptors. (Adapted from Shaikh et al., 2016)[29]

1.2. STARGAZIN REGULATES AMPAR FUNCTION AND SYNAPTIC EXPRESSION BY DIFFERENT MECHANISMS

1.2.1. Stargazin is involved in biosynthetic AMPA receptor transport

Although many AMPAR assemblies can exit the endoplasmic reticulum (ER) membrane and reach the cell surface in the absence of a TARP, there is compelling evidence, derived from a variety of experimental approaches, to support the view that the initial interaction between TARPs and AMPARs occurs at the level of ER membrane.

Upon association in the ER, stargazin enhances surface expression of AMPA receptors that otherwise appear to be trapped in the endoplasmic reticulum (ER) [30]. For instance, homomeric Q/R edited GluA2 (GluA2(R)) largely remains within the ER. The positively charged arginine residue introduced by editing, crucial in conferring calcium-impermeability to GluA2-containing AMPARs, appears to act as an ER retention signal[31]. Thus, GluA2 subunits not co-assembled with unedited (glutamine, at the Q/R site) subunits, are held within the ER [32, 33]. Co-expression of GluA2(R) with stargazin appears to reduce the barrier to ER export, allowing a significant increase in the expression of homomeric GluA2 receptors at the cell surface [34]. Stargazin similarly enhances the trafficking and expression of flop AMPAR isoforms, which are less normally exported from the ER than the flip variants [35]. Additional FRET studies have shown that YFP tagged AMPARs quench the fluorescence of CFP tagged stargazin molecules within the ER [30]. Moreover, the ER chaperone BiP (Ig binding protein), appears to be involved in the interaction between AMPARs subunits and stargazin [36] reinforcing the view that stargazin associate with AMPARs in the ER membrane.

As AMPARs mature and pass from the ER through the trans-Golgi network (TGN), they undergo additional posttranslational modifications – predominantly glycosylation and phosphorylation – before being packaged into vesicles for cell surface delivery. TGN processing and posttranslational modification commonly make significant additional contributions to the molecular weight of the mature protein. Thus, when these modifications are removed, the residual change in molecular weight can be used as an indicator of AMPAR maturation [32].

Co-expression of stargazin in cerebellar GCs from stargazer mice, increases the proportion of AMPARs that display the heavy molecular weight of the mature glycosylated protein, supporting the view that TARPs assist AMPAR trafficking through the Golgi apparatus, and ultimately, to the plasma membrane [13].

A variety of secondary proteins, which interact with stargazin, have been implicated as mediators of this Golgi and membrane trafficking. These include nPIST (neuronal isoform of protein-interacting specifically with TC10) [37]), MAP1 LC2 (microtubule-associated protein 1 light chain 2) [38], MAGI 2

(membrane-associated guanylate kinase, WW and PDZ domain containing 2) [39] and the adapter protein complex AP-4 [40].

Previous studies showed that the C-terminal domain of stargazin is necessary for its trafficking effects, including efficient trafficking through the ER and cis-Golgi compartments [23]. In order to understand whether stargazin masks ER retention signals on AMPA receptors or act as an ER export signal, the C-terminus of stargazin (amino acids 203–323) was fused with the C-terminus of GluA1 and continued in-frame with YFP [41]. This construct (R1i46cSTG-YFP) was expressed heterologously, and, by using confocal microscopy to assess the trafficking pattern, it was possible to see that R1i46cSTG-YFP was effectively trafficked from the ER to the plasma membrane as determined by identifying cells in which the surface expression was much greater than the cytosolic expression. This was contrasting with GluA1 in the absence of stargazin and more comparable with GluRA1 co-expressed with stargazin. Thus, the results suggest that C-terminal of the TARP protein mediates the interactions that underlie AMPAR exit from the ER, and fusion of the stargazin C-terminus to the AMPAR facilitates membrane trafficking [41]. In addition, the same C-terminal domain of stargazin was fused to the Gonadotropin-releasing hormone receptor (GnRHR) – GnRHRcSTG-GFP – to test whether the reduced ER retention was related to a GluA1-specific interaction. This protein, a member of the G-protein-coupled receptor superfamily, is also inefficiently trafficked to the plasma membrane and, like AMPA receptors, is largely retained in the ER in both homologous and heterologous cells [21]. Strikingly, the C-terminal fusion of stargazin dramatically enhanced GnRHR transport to the cell surface, with nearly every cell demonstrating robust surface expression. The results indicate that the C-terminus of stargazin encodes an intrinsic and transferable membrane sorting signal that can operate independently of the rest of the stargazin protein.

1.2.2. Stargazin regulates synaptic targeting of AMPARs

TARPs assist AMPAR delivery specifically to the synaptic membrane [11, 22]. For instance, AMPARs that are co-assembled with stargazin can diffuse rapidly and freely in the cell surface. TARPs immobilize and anchor the AMPAR at the synapse due to the interaction between the PDZ binding domains of stargazin with PSD95 in the postsynaptic density (Fig. 1.7), leading to the accumulation AMPARs at synaptic sites [22]. Truncated stargazin that lacks this anchoring region (Stargazin C) fails to rescue synaptic AMPAR responses in stargazer GCs [11]. About 100 amino acids upstream of the C-terminal PDZ-binding motif, the stargazin C-tail harbours a stretch of seven arginines interleaved by nine serines (RS domain), so that the overall charge of this domain is highly positive. It has been shown that the serine residues present in the RS domain are phosphorylated in an activity-dependent manner by CaMKII and PKC [12], so that the RS domain changes from highly positive to

negative upon phosphorylation. Biochemical and functional studies have demonstrated that phosphorylation of the RS domain is crucial for the enhanced synaptic targeting of AMPARs mediated by stargazin, since it facilitates the interaction between stargazin and PSD-95 (Fig. 1.7), which can be suppressed by de-phosphorylation via protein phosphatase 1 PP1 [28]. Contrarily, TARP phosphorylation by PKA facilitate AMPAR removal from synapses by disrupting the PDZ-binding site of the TARPs and perturbing their interaction with PSD95 [42].

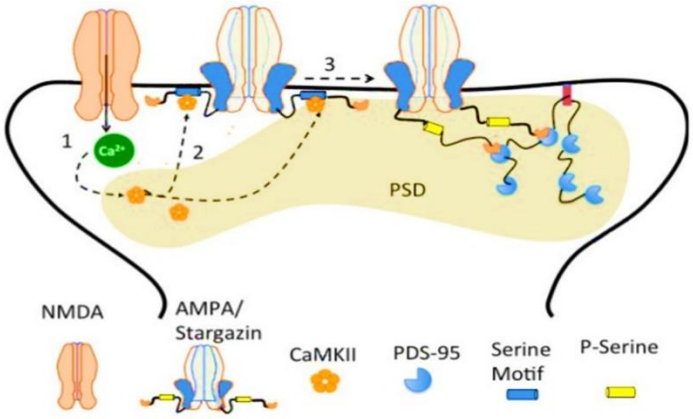

Figure 1.7. AMPAR synaptic insertion mediated by phosphorylated stargazin. The influx of calcium (1) into the spine via NMDA receptores activates CaMKII (2), which phosphorylates serine (P-serine) residues on the cytoplasmic terminal of stargazin. This activity disrupts the interaction between the positively-charged regions in the stargazin CTD and the membrane phospholipids, facilitating the binding of stargazin to PSD95 complexes, thereby trapping AMPAR at the PSD. (From Rudy, Jerry W. 'The Neurobiology of Learning and Memory', Sinauer Associates, Inc. Publishers)[43]

Therefore, these post-translational modifications of stargazin contribute to its ability to modulate synaptic strength. When the amplitudes of AMPAR-mediated EPSCs at cerebellar mossy fiber-granule cell synapses were compared between generated ɣ-2 phospho-mimic (all nine phosphorylatable serine residues to aspartic acid) and nonphospho-mimic (all nine phosphorylatable serine residues to alanine) knockin mice [44], larger amplitudes were observed in ɣ-2 phospho-mimic KI mice (stargazinSD) than in ɣ-2 nonphospho-mimic KI mice (stargazinSA). In primary cultured neurons, phospho-mimic ɣ-2 is more stable than non-phospho-mimic ɣ-2 [45, 46].
It was also shown that membrane phospholipids play a role in the modulation of synaptic localization of the AMPAR/stargazin complex. It was observed that stargazin phosphorylation inhibits its ability to interact with lipids, and is accompanied by an increase in the number of the AMPAR/stargazin complexes in the synapse [44]. With these studies, the authors conclude that negatively-charged lipids of lipid bilayers interact with positively-charged regions in the stargazin cytoplasmic domain and inhibit the binding to PSD-95. The previously mentioned serine phosphorylation neutralizes the charge on nearby arginine residues, decreasing their interaction

with negatively charged membrane phospholipids and thus increasing AMPAR membrane mobility [28].

Most recently, a study addressed the extension and retraction of stargazin C-tails as an important factor to regulate binding to PSD-95. Using phospho-mimetic mutations they artificially increased the apparent length of stargazin C-tail and found that it was sufficient to potentiate binding to PSD95, AMPAR anchoring, and synaptic transmission. In conclusion, they showed that the phosphorylation of Stargazin facilitates binding to PSD-95 by increasing the length of Stargazin C-terminal and thus preferentially engaging interaction with the farthest located PDZ domains regarding to the plasma membrane, which present a greater affinity for the stargazin PDZ-domain-binding motif [47] (Fig. 1.7).

1.2.3. Stargazin influences AMPAR channel properties and kinetics of deactivation and desensitization

Additionally to its role on the modulation of AMPAR trafficking, stargazin regulates the channel properties of AMPARs while they remain bound in the membrane. For instance, stargazin has been shown to increase the single-channel conductance of AMPARs by between 40 and 130% [12, 48, 49] (Fig. 1.8A). Stargazin slow the kinetics of AMPAR deactivation (channel closure upon glutamate removal) and desensitization (channel closure upon glutamate binding) [12, 48, 49] and greatly enhances charge transfer through AMPAR channels (Fig. 1.8B). Stargazer mice (γ2 $^{stg/stg}$) show no AMPAR activity at cerebellar mossy fiber–granule cell synapses [11], and no miniature EPSCs (mEPSCs) was observed in primary cerebellar granule cell cultures [27]. Overexpression of type I TARPs (stargazin, γ3, γ4, and γ8) restores mEPSCs in primary cerebellar granule cells cultures from stargazer [11, 13]. However, the decay kinetics of mEPSCs in neurons expressing type I TARPs (stargazin and γ3) are faster than those in neurons expressing type II TARPs (γ4 and γ8) [50, 51]. This isoform-specific modulation of AMPAR kinetics is also observed in heterologous cells [50, 51]. In addition, stargazin also modulate ion permeability of AMPARs. The presence of stargazin reduces the rectification of AMPARs, rendering them more calcium permeable [26, 50-53]. Stargazin is also involved in CaMKII-mediated modulation of AMPAR conductance [54].

In addition, stargazin modifies some of the basic pharmacological properties of AMPARs. It increases the apparent glutamate affinity of AMPARs by roughly three-fold (11; 44) (Fig. 8C). Moreover, it alters the affinity and efficacy of AMPAR potentiators (a class of reagents that increase AMPAR signaling [12, 55] by blocking channel closure) such as cyclothiazide, PEPA, and CX546 [56, 57]. Furthermore, the presence of stargazin modifies the action of CNQX which is a classical competitive AMPAR antagonist on "TARPless" AMPARs, but is converted to a partial agonist when AMPARs are

co-assembled with a TARP [58]. Intriguingly, kainate, cyclothiazide, and CNQX bind to the extracellular domain of AMPARs; this binding localization suggests that the modulatory effects of stargazin may also occur at the extracellular domains of AMPARs. In support of this concept, the extracellular loop 1 of stargazin has been shown to be both necessary and sufficient for the modulation of AMPAR kinetics [23, 50]. On the contrary, stargazin cytoplasmic domain was shown to mediate surface expression of AMPAR/stg complex [23], and other studies have also recently shown that the cytoplasmic domains of some TARP isoforms modulate AMPAR properties (48,49).

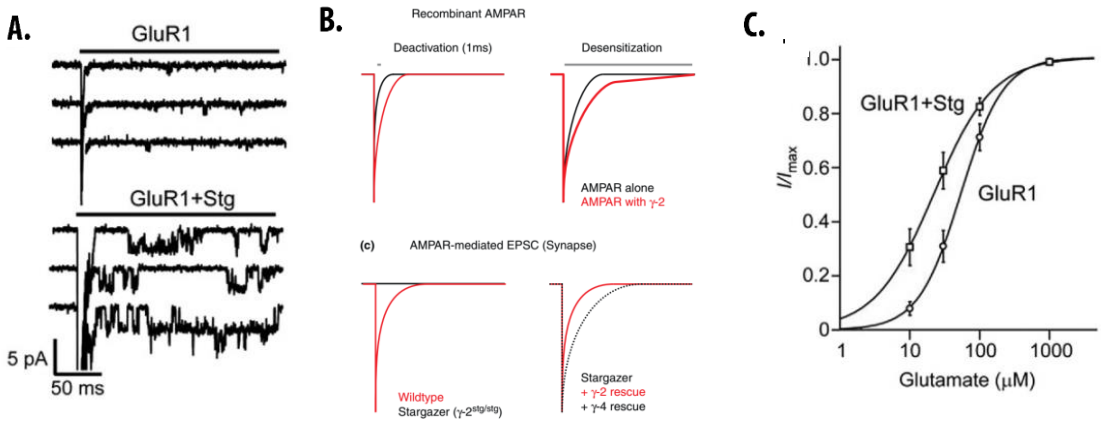

Figure 1.8. Effect of Stargazin in channel properties and kinetics of AMPAR. (a) Single channel currents from GluA1-containing AMPARs expressed in the absence and presence of Stg, illustrating an increase the single-channel conductance mediated by stargazin; (b) Modulation of channel kinetics showing that Stg slow both deactivation and desensitization of AMPAR and overexpression of various TARPs rescued loss of mEPSCs in stargazer granule cells; (c) dose-response curves of peak AMPAR current amplitude show increased glutamate affinity of GluA1-AMPAR in the presence of stargazin.

Stargazin has been shown to functionally uncouple from AMPARs upon AMPAR desensitization, which auto-inactivates the AMPAR/Stg complex and modulates short-term plasticity [59]. Interestingly, however, since this modulation is reversible, AMPAR/stg is unlikely to be physically dissociated; rather it is functionally decoupled without dissociation [60]. These finding clearly suggest that mechanisms that cause dissociation of stargazin from AMPARs can trigger a rapid change in AMPAR properties. Indeed, it has recently been demonstrated that transmitter activation may instigate dissociation of AMPARs from stargazin, and lead to AMPAR endocytosis [59, 60].

This evidence points to an involvement of stargazin in synaptic plasticity, specifically in the bidirectional control of hippocampal LTP. This interaction was shown to be necessary for synaptic AMPAR function by measuring AMPAR-mediated EPSCs following PDZ-domain mutation [61], and by acute disruption of the interaction between TARPs and PSD-95 using biomimetic divalent ligands [62].

1.2.4. Stargazin in is required for synaptic plasticity

AMPAR-dependent synaptic plasticity has been shown to be dependent on stargazin phosphorylation. As previously said, the ability of stargazin to stabilize AMPAR at the synapse is dependent on its phosphorylation at a set of conserved serine residues in its cytoplasmic C-terminal tail, which is dynamically regulated by synaptic activity such that activation of CaMKII and PKC induces phosphorylation, whereas activation of PP1 and PP2 dephosphorylates these sites. Evidence taken from measurements of NMDA-induce AMPAR currents potentiation revealed that LTP requires stargazin phosphorylation by CaMKII/PKC whereas dephosphorylation of stargazin mediates LTD [12].

Additionally, it has been shown that Stargazin is essential for homeostatic plasticity in the cortex. Cortical neurons transfected with a sh-RNA (shRNA4) to knock down stargazin expression showed an impaired scaling-up of AMPAR after blockage of neuronal activity with TTX, suggesting that stargazin indeed mediates synaptic scaling (Fig. 1.9). Importantly, it was also shown that phosphorylation of stargazin is required for this effect [63].

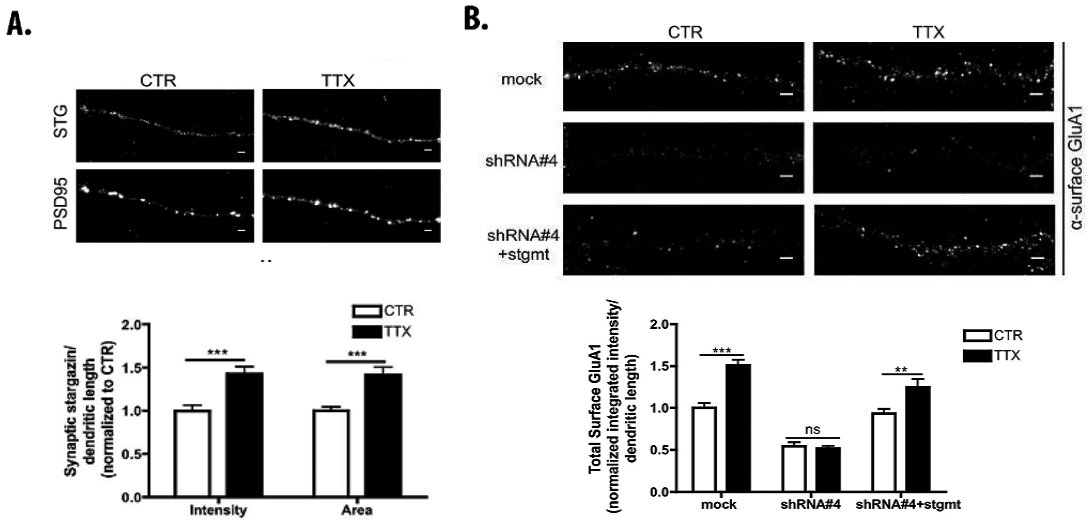

Figure 1.9. Stargazin is involved in homeostatic synaptic scaling. (A) Quantification and comparison of synaptic stargazin expression upon neuronal activity blockage by TTX. Stargazin becomes more expressed at the synapse after neuronal activity blockage. (B) Quantification of total surface intensity of GluA1 clusters by immunocytochemistry in neurons transfected with shRNA#4 to knock-down stargazin, or neurons transfected with shRNA#4 and a stargazin mutant refractory to this shRNA. Control neurons or neurons expressing the stargazin rescue construct responded to prolonged TTX incubation by upregulating GluA1 levels. Stargazin knock-down impaired the homeostatic plasticity response of scaling-up [63].

1.2.5. Stargazin promotes dendritic growth and increases dendritic complexity

To test the role of TARPs in regulating dendritic growth, individual TARPs were overexpressed at different neuronal developmental stages in pyramidal neurons in organotypical cortical slices [64]. At 10 DIV type I TARP γ8 promoted apical dendritic elongation and branching in pyramidal cells in layers II/III and layers V/VI, while γ2, γ3 and γ4 failed to increase apical length. Moreover, at DIV 10, there was no effect on basal dendritic growth.

The expression of AMPARs in neocortical neurons reaches adult levels by the second to third postnatal week [65]. Therefore, the effect of TARPs was then tested between 10 and 15 DIV. Like in the earlier time window, the overexpression of γ8 promoted growth of apical dendrites of layer II/III pyramidal neurons, and the effect was stronger at 15 DIV than [66, 67] at 10 DIV. Surprisingly, the overexpression of γ2 and γ3 strongly increased apical dendritic growth of pyramidal cell in layers II/III, but not in layers V/VI. Type II TARPs failed to induce dendritic growth at 10-15 DIV. This suggests that the mechanisms by which type II TARPs modulate AMPARs function are either too weak to become translated into a dendritic growth response, or that they modulate AMPARs that contain subunits not involved in dendritic growth. Taken together, the results suggest that pyramidal cell apical dendrite growth is stimulated only by TARPs that increase trafficking and expression of synaptic AMPARs. Additionally, these observations suggest that the localization of a TARP and the associated AMPAR in dendritic spines is a key factor for pyramidal cell dendritic growth and confirms the previous finding that AMPARs regulate apical dendritic growth (53) in agreement with their preferential distribution in apical dendrites (54). Sholl analysis was performed to address the dendritic complexity of neurons overexpressing TARPs and confirm the effect of the type I TARPs on dendritic growth at 10 DIV and 15 DIV. An increase in apical dendritic complexity proximal to the soma of γ8-overexpressing pyramidal cells in both layers at 10 DIV was observed. Furthermore, the total number of apical dendritic intersections in both layers was significantly higher in γ8-transfected pyramidal cells. The Sholl analysis of basal dendrites confirmed the negative effect of γ8 on basal dendrites at 10 DIV. Moreover, γ2, γ3 and γ4 failed to modulate dendrites at 10 DIV. However, when overexpressed between 10 and 15 DIV, γ2, γ3 and γ8 increased apical dendritic complexity between 100 and 200 µm from the soma. Basal dendritic complexity was not altered. These results suggest that the action of γ2, γ3 and γ8 shift with age to more distal apical dendritic zones.

1.3. NEURODEVELOPMENTAL AND PSYCHIATRIC DISEASES ARE ASSOCIATED WITH DYSFUNCTION OF GLUTAMATERGIC SYNAPSES

Recent evidence supports the idea that abnormal excitatory synapse function is a major cause of cognitive disorder [3]. Additionally, activity-dependent plasticity of AMPA receptor signaling not only underlies aspects of learning and memory but can also lead to persistent changes associated with drug addiction [14]. Besides, glutamate receptor-induced excitotoxicity also plays a key role in neurodegenerative disorders [14]. Up to date, dysfunction of glutamatergic synapses constituents has been linked to common neurodevelopmental diseases, like Intellectual disability (ID), autism spectrum disorders (ASD), and several psychiatric diseases such as schizophrenia (SCZ) (Figure 1.10). Consequently, several promising experimental compounds that target glutamate receptors have emerged as potential therapies for psychiatric disorders [68]. Since TARPs have such an important role for the maintenance of functional synapses, it is expected that dysfunction of these proteins can be involved in some forms of these diseases. In fact, emerging human genetic evidence suggests that TARPs may play a role in the etiology of disorders such as the ones previously mentioned.

Stargazin is encoded by the *CACNG2* gene, which localizes to a region of chromosome 22 – 22q13.1 – implicated in neuropsychiatric disorders [69]. *CACNG2* is associated with a subgroup of schizophrenia patients [70], and analysis of a consanguineous family exhibiting a high frequency of epilepsy, schizophrenia and hearing loss identified a link to a region within 22q13.1, which contains the *CACNG2* locus [66]. Plus, alterations in the DNA copy number in *CACNG2* [71], and in the levels of stargazin mRNA [72] were found in post-mortem schizophrenic brains. Quantitative expression analysis showed increased levels of stargazin in dorsolateral prefrontal cortex in patients with bipolar disorder, a psychiatric disorder which shares several psychotic features with schizophrenia [67]. Moreover, a *de novo* missense mutation in *CACNG2* has been found in a non-syndromic ID patient with moderate ID severity [73]. The human γ-3 gene (*CACNG3*) has also been implicated as a susceptibility locus in a subpopulation of patients suffering from childhood absence epilepsy (CAE). Taken together, the emerging data from these genetic studies support a role for TARPs in psychiatric disorder pathogenesis in the CNS, pointing these proteins as possible novel pharmacological targets and/or markers for a variety of diseases.

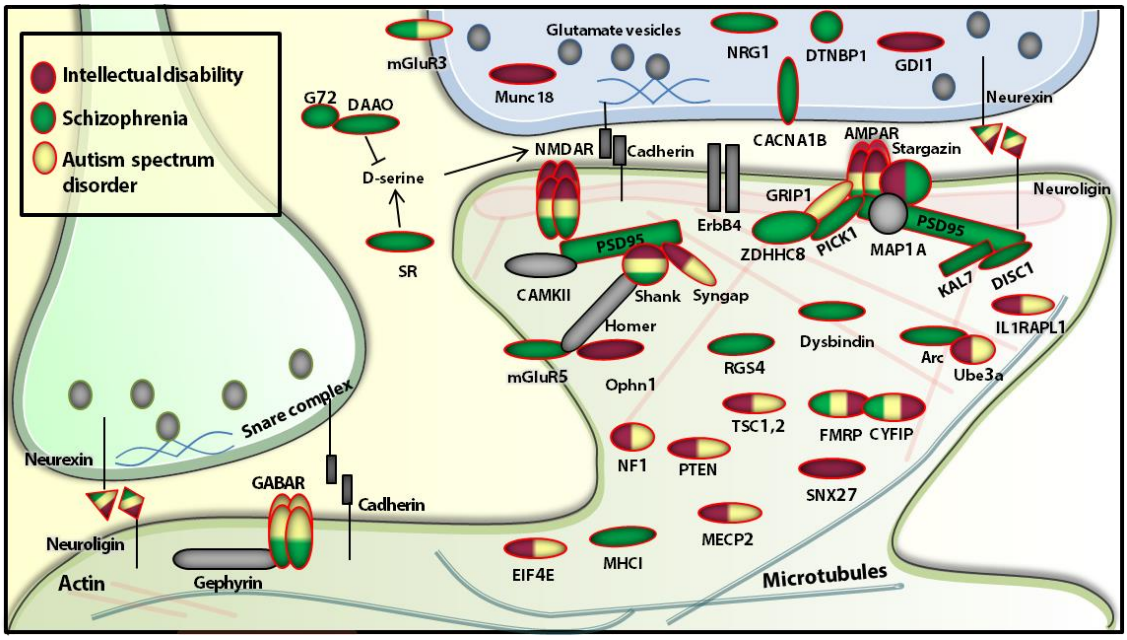

Figure 1.10. Synaptic proteins implicated in ID, ASD and SCZ. Genes encoding for a variety of proteins of both glutamatergic and GABAergic synapses have been linked to neurodevelopmental and psychiatric diseases such as ID, ASD and SCZ. Adapted from Volk et al., 2015.

1.3.1. Intellectual Disability

Intellectual disability (ID) is a neurodevelopmental disorder characterized by significant limitations in intellectual functioning and adaptive behavior, with onset before the age of 18. It is the most common neurodevelopmental disorder, affecting 1–3% of the general population, and can be broadly divided into the nonsyndromic ID (NSID) forms, in which intellectual deficit is the only clinical feature, and the syndromic forms that are specifically associated with additional abnormalities such as dysmorphic features, metabolic anomalies, autism or epilepsy. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), experts classify intellectual disability severity according to the types of cognitive impairment, dividing it into four categories: mild, moderate, severe and profound intellectual disability. The diagnosis is based on the degree of impairment regarding three main skill areas: conceptual (i.e. learning, memory and speech), social (i.e. social engagement, conversation complexity, empathy, etc) and practical life (self-care, organization, and safety). The IQ score is also assessed for the diagnosis, which is considered low below 70, the minimal threshold to fit the mild category. However, mental health professionals place less emphasis on the IQ scores and more on the independence capacity of the individual, taking into account the amount and type of intervention needed [74].

Genetic abnormalities identified in ID are heterogeneous, ranging from chromosomal aneuploidy to single gene point mutations, and currently account for approximately 25% of ID cases. Extrinsic factors such as premature birth and environmental insult are estimated to account for an additional 15%, leaving approximately 60% of ID cases with unknown etiology, although unidentified genetic factors undoubtedly contribute significantly to this population [75]. To date, researchers have identified more than 450 genes in association with ID [76]. However, since most of these genes are related to syndromic ID, little is known about the genetics of NSIDs. In addition to genetic information gleaned from syndromic ID, recent technological advances have facilitated the identification of many genes, primarily *de novo* mutations, associated with nonsyndromic ID. The majority of the genes identified so far in NSID are either X-linked or autosomal recessive [3]. Although an autosomal-dominant mode of inheritance is not frequently observed in NSID because of the reduced reproductive fitness associated with this condition, autosomal-dominant mutations arising *de novo* may explain a large fraction of cases.

Functional categorization of these genes has revealed a significant enrichment of genes affecting glutamatergic synapse structure and function, coding for proteins involved in synaptic plasticity, learning and memory. The importance of the glutamatergic pathways in such conditions is highlighted by the fact that pathogenic mutations have been identified in genes encoding for all classes of iGluRs in patients with ID [49] - GRIA3 (MIM 305915), GRIK2 (MIM 138244), GRIN2A (MIM 138253), and GRIN2B (MIM 138252) -, reflecting the critical role for iGluRs in mediating information flow throughout the brain. Accordingly, the proteins that constitute the synaptic glutamate receptor complexes, such as TARPs, constitute attractive candidate genes for neurocognitive diseases such as ID.

1.3.2. The identification of a stargazin variant – stgV143L – associated with intellectual disability

Recently, a study addressed the hypothesis that *de novo* mutations (DNMs) in genes encoding members of the GRC could explain an important fraction of the sporadic NSID cases [77]. To explore this, this study involved the sequencing of 197 genes encoding glutamate receptors and a large subset of their known interacting proteins in sporadic cases of NSID. DNMs were found in 11% of the patients. Strikingly, genetic and functional observations indicate that the majority of the identified mutations affect protein function and may be pathogenic. Specifically, a mutation in the *CACNG2* gene, which encodes for stargazin, was identified in a male with moderate ID. This mutation leads to replacement of valine143 for a leucine residue in stargazin (p.V143L) (Fig. 1.11A),

a highly evolutionarily conserved residue (Figure 1.11B), thereby suggesting that this site might be important for the function of stargazin. Accordingly, V143L mutation was predicted to be damaging to the function of stargazin by PolyPhen-2 [78, 79], a tool designed to predict functional effects of human Single-nucleotide polymorphism (SNPs) (Fig. 1.11C).

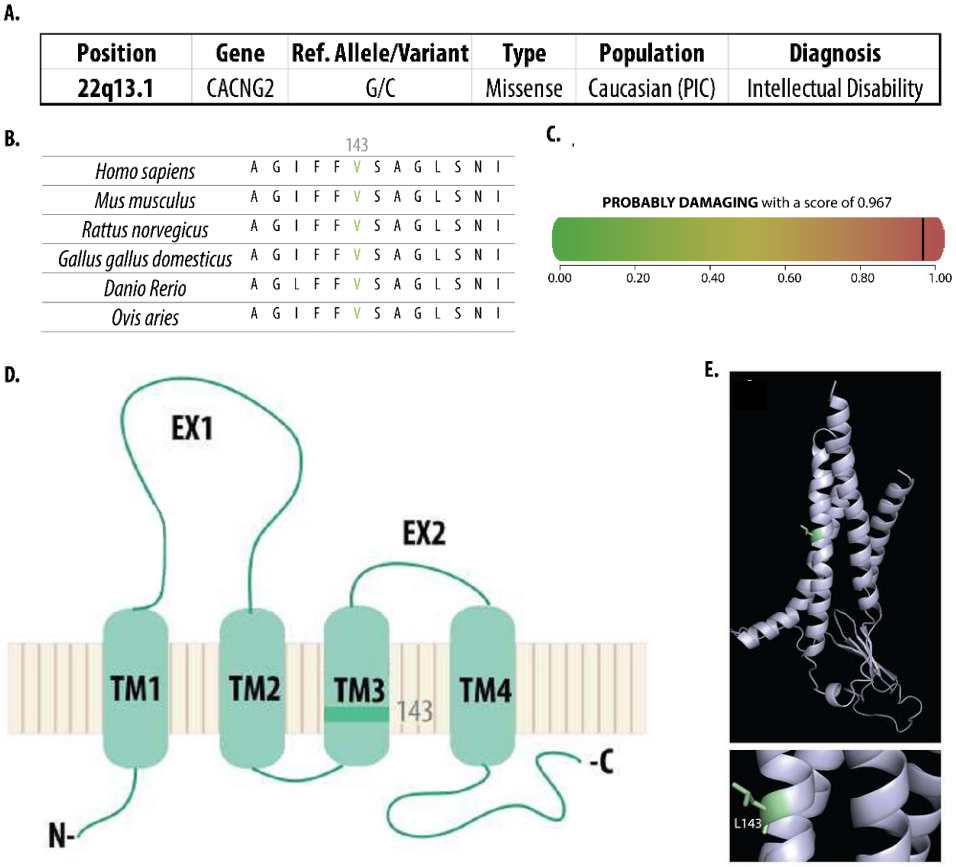

Figure 1.11. Characterization and location of the V143L missense mutation. (A) Characterization and location of the newly found mutation in exon 3 of *CACNG2* gene. This missense mutation lead to the substitution of valine 143, a highly conserved residue among species. Consequently, it is predictably damaging to protein functionality according to the PolyPhen-2 prediction tool [78, 79]. (D-E) The ID-associated mutation in stargazin structure is situated in the TM3, where it interacts with transmembrane domains of the AMPAR subunits. This figure is the courtesy of Gladys Caldeira.

Hamdan and colleagues [73] evaluated the impact of the DNM p.V143L mutation on stargazin function. Co-immunoprecipitation analysis showed that the mutation significantly decreases stargazin's ability to bind to GluA1 or GluA2 AMPAR subunits in a transfected cell line (Fig. 1.12A). In addition, cell surface expression of GluA1 was reduced in transfected hippocampal neurons and HEK293 cells producing mutant stargazin, as compared to cells producing the wild-type protein (Fig. 1.12B). Consistent with these findings, expression of stargazin-V143L mutant decreased both

miniEPSC amplitude and frequency in transfected hippocampal neurons, suggesting that the p.V143L in stargazin caused a reduction in glutamatergic transmission (Fig. 1.12C).

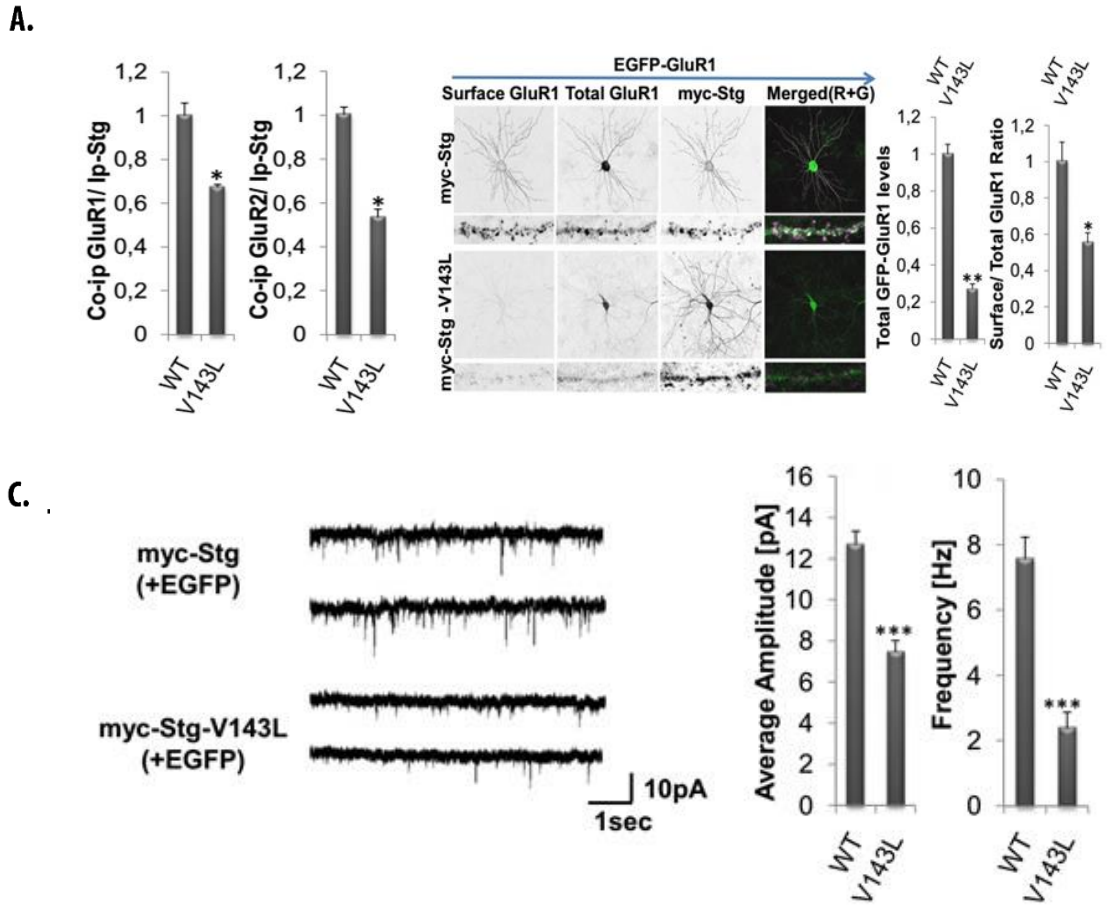

Figure 1.12. The impact of Stg^{ID} mutation in protein function. (A) Co-immunoprecipitation of AMPARs (both GluR1 and GluR2, a.k.a. GluA1 and GluA2, respectively) with Stg was reduced in the Stg-V143L transfected HEK293 cells. (B) Surface and total GluR1 expression levels were reduced in hippocampal neurons expressing Stg-V143L; (C) Voltage-clamp whole-cell recordings in the presence of an extracellular solution containing TTX. Both mEPSCs amplitude and frequency were decreased in Stg-V143L expressing neurons. (From Hamdan et al., 2011)

1.4. CONSTRUCTION AND CHARACTERIZATION OF StgV143L EXPRESSING *KNOCK-IN* MICE

1.4.1. Animal models as an approach for studying TARPs

Despite the identification of numerous ID-associated mutations in genes encoding proteins relevant for glutamatergic synaptic function, detailed biochemical, electrophysiological and behavioral assessments are still needed to assess causality between disruption of a gene and development of an ID-like phenotype, and to evaluate the impact of these mutations on neuronal physiology, circuit-level activity, and cognitive function.

Given the apparent functional redundancy of TARPs, questions remain regarding why are there so many TARP family members and why do some cell types express one single TARP subtype while others express a multitude. For instance, although the hippocampus has a selective enrichment of ɣ8, CA1 pyramidal neurons are known to express multiple TARP family members, including stargazin, ɣ3, ɣ4, ɣ7. A useful way to tackle these questions is to consider TARPs differential expression patterns and complex effects on AMPAR in well-characterized cell types in the hippocampus, cerebellum, cerebral cortex, and thalamus. Besides, more can be learned about subtype-specific TARPs by examining the effects following genetic manipulation, using genetically modified animal models.

Undoubtedly, the use of appropriate animal models has led to a great deal of the knowledge that has improved our understanding of several pathologies. They have become an extremely valuable tool for the elucidation of the mechanisms and players that intervene in the etiology of all kinds of disorders, as they allow us to translate genetic alterations into specific molecular and behavioral changes. Besides, they have been extremely useful for the design and validation of therapeutic approaches. For studying neurological disorders, it is essential to examine disease-associated alterations in integral neuronal networks, which is compromised in *in vitro* studies. For that reason, rodents are the most used animal models in the field, as they are complex organisms which turn it possible to correlate specific genetic changes with biochemical and physiological alterations, neuronal circuits and behavioral phenotypes. Besides the genetic modified ones, animal models can also be created by induced brain lesions, drug-induced modifications or simply conditional environmental exposures that lead to neuronal modifications. However, in the context of ID and psychiatric disorders, genetic modelling has been the mostly used approach, given the high genetic component of these disorders.

Within the genetical approach, *knock-out* mice represent the majority of animal models developed so far. This approach results in the lack of expression of a particular protein of interest, and the analysis is based on examining the effects resulting of its absence. Although *knock-out* mice might be a very powerful tool to evaluate some mechanisms related or dependent on the functionality of a specific protein, in a biomedical context the obtained results cannot always can be translated to the human outcome, since in most of cases the medical condition affected by that protein is not a consequence of its total absence. Thus, more recently another strategy has emerged and revealed promising results – the *knock-in* animal models. This model allows the expression of protein variants identified in human diseases so the outcoming phenotypes regarding cellular, circuitry and behavioral alterations have an increased translational validity. A limitation that has been attributed to animal models is the overlap between different disorders, regarding both their genetic features and behavioral phenotypes. To overcome these limitations, recent animal models-based studies

often complete their characterization with electrophysiological studies, imaging, optogenetic a chemogenetics studies. The latters allow the observation of cell-specific circuitry alterations by conditioning the genetic pool in localized brain regions.

As previously said, TARP family members have been implicated in several neurodevelopmental and psychiatric diseases. Moreover, due to their role in modulation of AMPAR they are serious candidates to assess disease associated-phenotypes. Consequently, regarding the specific DNM p.Val143Leu in the *CACNG2* gene, the present work reports a pioneer biochemical and behavioral characterization of a *knock-in* mouse expressing this mutant form of stargazin generated in our laboratory (Fig. 1.13), in order to elucidate how this mutation affects the stargazin function and how it can be related with the development of disease-associated phenotypes.

The StgID *knock-in* mice expressing the STGV143L mutant form was generated prior to the beginning of this project, using a gene targeting strategy (Starkey and Elaswarapu, 2010). For the generation of the mouse model, a target vector was designed to comprise a middle arm (MA) containing the modification to be inserted in the mouse genome flanked by two homology arms, a long (LA) and a short one (SA), that allowed the homologous recombination with the genomic DNA of mouse embryonic stem (ES) cells (Fig. 1.13A). The construct also included a random sequence, non-homologous to the mouse genome, in the SA/MA junction in order to allow the genotyping of the animals. The genotyping primers were designed specifically for this region, allowing the discrimination between WT and StgV143L alleles (Fig. 1.13B). The vector was then electroporated into R1 embryonic stem (ES) cells, derived from the crossing of 129/Sv and 129/SvJ substrains. Their DNA was isolated and a third selection method was performed based on a PCR reaction where positive amplification only occurs if homologous recombination takes place. ES cell colonies were expanded and microinjected into blastocysts of C57/B6 mice. The resulting chimeras were crossed with WT C57/B6 mice for four generations before biochemical and behavioral analyses.

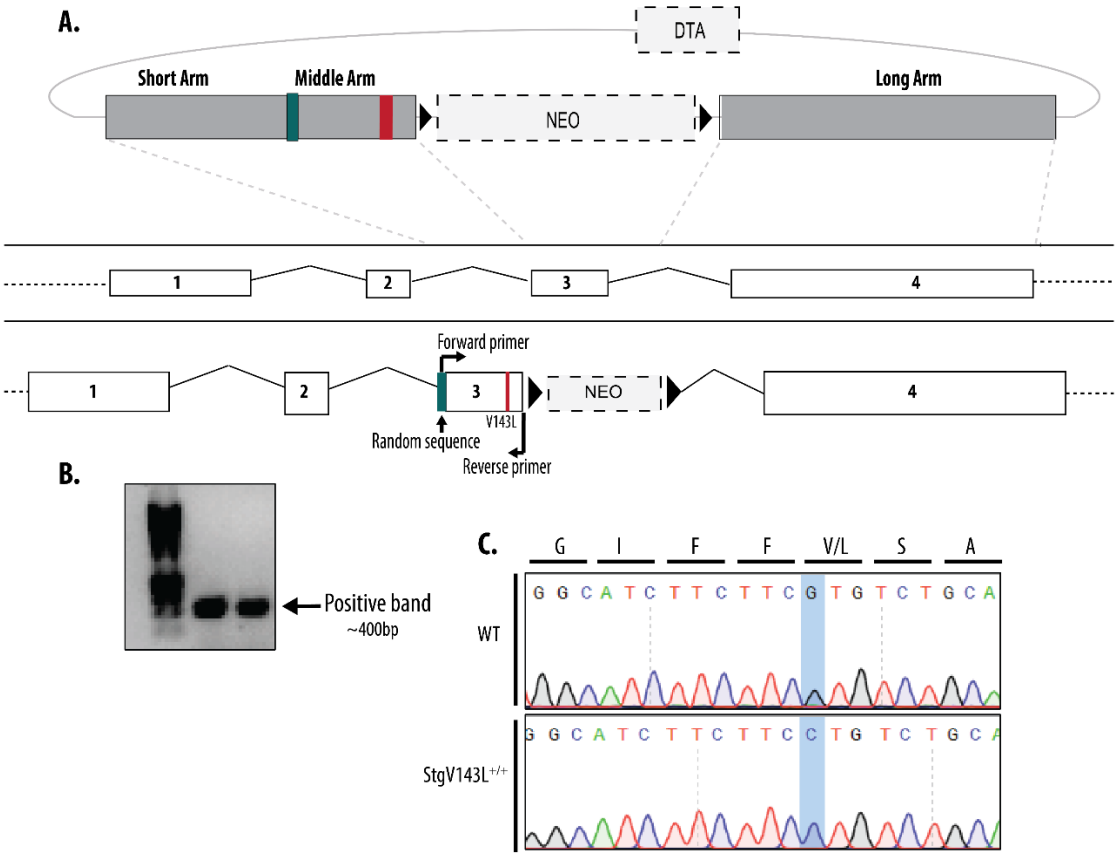

Figure 1.13. Generation and genotyping of Stg^{ID} *knock-in* mice. (A) Vector construction, including the three homology arms and the two selection markers, Neo and DTA. A forward primer designed against a synthetic random sequence (non-existing in the mouse genome – green bar), inserted upstream the middle arm, allows the specific targeting of the mutated allele. (B) A 400bp band, amplified by PCR, indicates the presence of at least one mutated allele. (C) Confirmation of the correct targeting was performed by sequencing the third exon of the *CACNG2* gene in WT and homozygous *knock-in* (StgV143L$^{+/+}$) mice. The vector was designed by Gladys Caldeira. This figure is courtesy of Gladys Caldeira.

1.4.2. Supporting Data

Unpublished work from our laboratory has revealed evidence that the V143L variant of stargazin presents increased cell surface diffusion in neurons, suggesting a reduced ability to stabilize AMPAR in the membrane (Fig. 1.14). Additionally, it was shown that AMPAR synaptic targeting was compromised in neurons expressing the V143L variant of stargazin (Fig. 1.15).

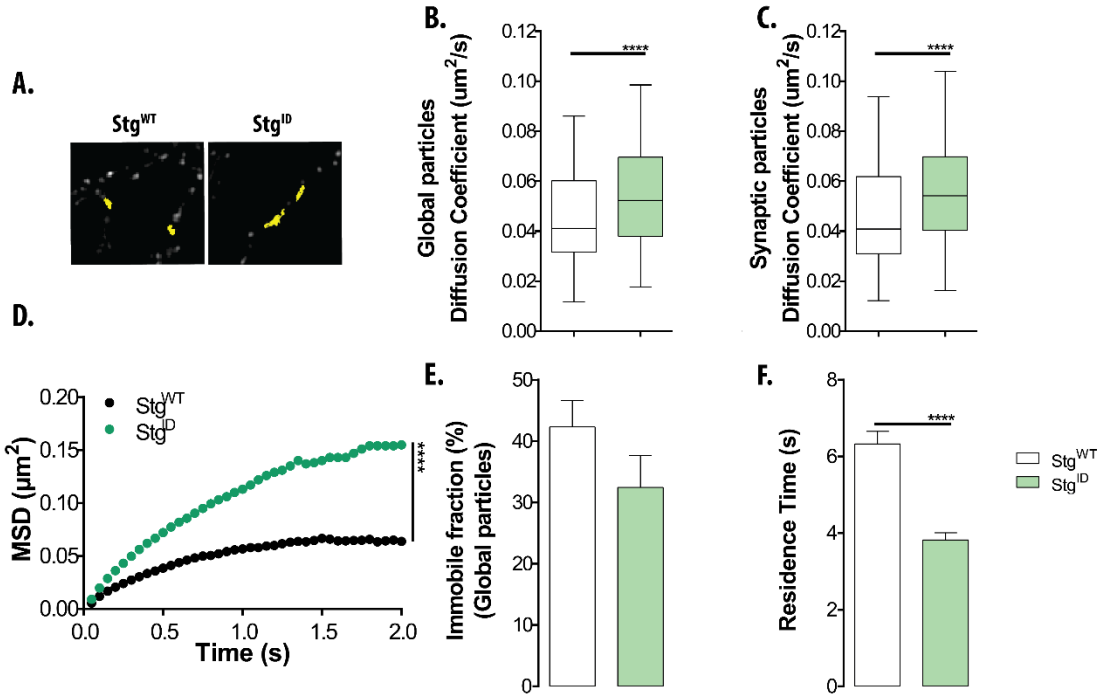

Figure 1.14. StgID variant has altered surface diffusion properties. (A – F) The StgID variant (V143L) shows increased cell surface diffusion in neurons, as revealed by single particle tracking using quantum dots. The mean square displacement (MSD) - indicating the surface area covered by StgID – and the diffusion coefficient of StgID are increased, and its synaptic residence time is decreased. Experiments performed by Gladys Caldeira.

Preliminary characterization of StgID mice revealed several behavioral changes in these animals when compared to wild-type mice. Whereas anxiety-like behavior (assessed using the open field test and the elevated plus maze) and motor abilities (in the rotarod) were undistinguishable between StgID and wild-type mice, StgID animals displayed impaired motor learning, weakened working memory (assessed in the T-maze) and apparent defective sense of self-care (nest building). These observations suggest that the ID-associated mutation in stargazin results in ID-like behavioral alterations, and prompt a thorough analysis of StgID mice behavior, synaptic and morphological alterations.

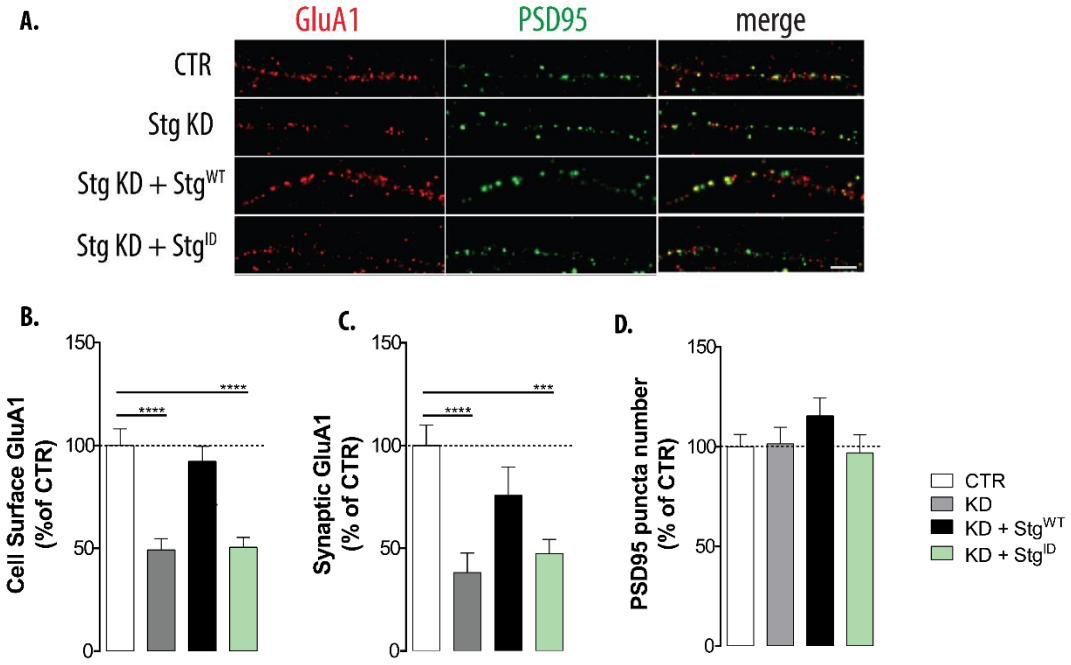

Figure 1.15. Functional impact of Stg^{ID} mutant in aspects underling AMPAR trafficking. (A-D) GluA1 containing AMPAR surface expression is reduced in neurons expressing the Stg^{ID} mutant form synaptic trafficking of AMPAR is remarkably affected in neurons expressing the Stg^{ID} variant, similarly to what occurs upon stargazin knock-down. Experiments performed by Gladys Caldeira

OBJECTIVES

This study was designed to characterize the biochemical and behavioral features displayed by a *knock-in* mouse model generated to express a mutant form of stargazin identified in a patient with moderate ID. Stargazin is a well-known synaptic protein which act as an auxiliary subunit for AMPA receptors, thereby modulating several properties of these receptors and consequently the AMPAR-mediated synaptic plasticity, the main mechanism underlying the potentiation of glutamatergic synapses. With such an important role for glutamatergic synaptic transmission, we decided to address if this mutant form of stargazin affects the function of AMPARs and the synaptic plasticity mechanism that rely on these receptors. To do so, we assessed several aspects of this genetically modified mice, such as biochemical composition of the synapses, neuronal morphology and behavior.

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

I. Determine if StgV143L mutation is affecting the function of stargazin and its cellular localization.

II. Understand if this mutation alters AMPAR function and synaptic targeting.

III. Assess whether this mutated form influences post synaptic density composition.

IV. Evaluate if the altered form of stargazin affects dendritic growth and arbor complexity.

V. Evaluate at what extent this mutation might lead to a disease-associated phenotypic behavior

Chapter II

MATERIALS AND METHODS

2.1. Animal maintenance and manipulation

Mice were housed in a temperature-controlled room with a 12-hour light/dark cycle with free access to food and water. For the behavior analysis, mice were maintained on a 12-hour dark/light cycle at 22°C to 25°C and tested at 2 months of age. For all of the experiments *knock-in* and control animals were sex and age matched. All the procedures were reviewed and approved by the Portuguese Authority for Animal Health (DGAV) (DL 113/2013, artigo 44º). All efforts were made to minimize animal suffering.

2.2. Purification of postsynaptic density fractions and Western Blot analysis

Postsynaptic densities (PSDs) were isolated/purified from the whole brain of P60 C57BL/6 mice. Mice were anesthetized with isoflurane and euthanized by cervical dislocation. The brains were then collected and homogeneized in an HEPES-buffered sucrose solution [4mM HEPES (pH=7.4), 0.32M Sucrose, supplemented with protease and phosphatase inhibitors (0.2mM PMSF, 0.1mM sodium orthovanadate, 50mM sodium fluoride, 200mM DTT, CLAP)] in a motor driven glass-Teflon homogenizer at 900 rpm (40 strokes). The homogenate was collected and centrifuged at 700 x g for 15 min to obtain the non-nuclear fraction (S1). A fraction of the S1, which corresponds to the brain lysate, was recovered in 2% SDS and 9M urea and stored at -20ºC. The remaining S1 was again centrifuged at 700 x g and the resultant supernatant was further centrifuged for 15 min at 18000 x g to yield the crude synaptossomal pellet (P2). P2 was resuspended in HEPES-buffered sucrose solution and lysed in a glass-Teflon homogenizer at 900 rpm (10 strokes), and incubated for 1h with orbital rotation at 4ºC. The lysate was then centrifuged at 25000 x g for 20 min to yield the lysed synaptossomal membrane fraction (P3). P3 was resuspended in a HEPES/EDTA-buffered solution [50mM HEPES (pH=7.4), 2mM EDTA, 0.2mM PMSF, 0.1mM sodium orthovanadate] and solubilized in 0.5% TritonX-100. A fraction of this synaptic plasma membrane (SPM) lysate was recovered in 20% SDS and stored at -20ºC. The remaining P3 fraction was incubated, in rotation, at 4ºC for 15 min and then centrifuged at 32,000 x g for 20 min. The resulting pellet (PSD-1P) was resuspended in HEPES/EDTA-buffered solution with 0.5% TritonX-100, incubated with orbital rotation for 15 min at 4ºC and centrifuged at 200,000 x g for 20 min. The resulting pellet, corresponding to the purified PSDs, was resuspended in HEPES/EDTA-buffered solution and recovered in 20% SDS and 9M urea. The purity of the extracted "crude" PSD sample was verified by Western Blot analysis, which tested for highly enriched levels of the major PSD scaffold protein PSD95 and a depletion of the presynaptic protein Synaptophysin.

Protein concentration was determined by the Bio-Rad method. Protein samples were denatured with x loading buffer (NZYTech 5x Loading buffer) and separated by SDS-PAGE in 11% polyacrylamide gels for posterior Western Blot analysis. Proteins were transferred overnight at 40V to a PVDF membrane (Millipore). After blocking with 5% blocking solution (GE Healthcare), in 0.1% Tween-20 supplemented TBS (20mM Tris, 137 mM NaCl, pH 7.6) (TBS-T), membranes were incubated with the primary antibody (in 5% blocking solution in TBS-T) for 2h at RT. Following three 15 min washes with TBS-T, membranes were incubated with alkaline phosphatase-conjugated secondary antibody (in 5% blocking solution in TBS-T) for 45 min at RT and then washed three times with TBS-T. The membranes were developed with the alkaline phosphatase substrate ECF (GE Healthcare), and the fluorescent signal was acquired in a Storm 860 Gel and Blot system (GE Healthcare). Primary and Secondary antibodies for Western Blot analysis are shown in Table 1. Normalization to the loading control was performed using either tubulin or Ponceau S staining.

Table 1. Antibodies used for Western Blot analysis in the present study.

Primary antibody (host species)	**Dilution**	**Brand**
CaMKII (Mouse)	1:1000	Sigma
Caspr2	1:250	Abcam (Cambrige, UK)
GluA1 C-terminal (Rabbit)	1:1000	Millipore (MA,USA)
GluN1 C-terminal (Mouse)	1:1000	Neuromab (CA, USA)
PSD-95 (Mouse)	1:1000	Thermo Scientific
Stargazin (Rabbit)	1:750	Millipore (MA,USA)
Synaptophysin (Rabbit)	1:1000	Abcam (Cambrige, UK)
α-tubulin	1:1000	Sigma
Secondary antibody (host species)	**Dilution**	**Brand**
Alkaline Phosphatase-conjugated anti-mouse	1:10000	Jackson ImmunoResearch (Pennsylvania, USA)
Alkaline Phosphatase-conjugated anti-rabbit	1:10000	Jackson ImmunoResearch (Pennsylvania, USA)

2.3. Imaging and Sholl Analysis

P30 mice from each genotype group were subjected to tail vein injection with the adeno-associated virus AAV9.hSyn.HI.eGFP.WPRE.bGH (from Penn Vector Core) (5ul virus/95 ul saline) to express green fluorescent protein (GFP) under the regulation of the synapsin-1 promoter. AAVs with serotype 9 cross the blood-brain barrier [80], and the human synapsin-1 promoter drives the expression of EGFP in neurons.

Mice were perfused at P60, after being subjected to a battery of behavioral tests. For perfusion, mice under isoflurane anesthesia were rapidly dissected to expose the heart. Next, 20 mL of ice-cold 1x phosphate-buffered saline (PBS) (10x PBS - 87,6 g of NaCl (Acros); 32,5 g of Na2HPO4.7H2O (Fisher); 4 g of KH2PO4 (Fisher) was perfused through the left ventricle. A cut in the atrium was done in order to allow the blood to exit circulation and eventually sacrifice the anesthetized animal by blood loss. Afterwards, 40 mL of fresh ice-cold 4% paraformaldehyde (PFA) (4% PFA - 4 g of PFA (Fisher); in 1x PBS) was perfused to fix the tissue. Dissection and removal of the brain was carried out and brain samples were then immersed in ice-cold 4 % PFA solution and left overnight at 4ºC. Samples were changed to a 4 % PFA solution containing 30 % sucrose (30 g sucrose (Fisher); 100 mL of 4 % PFA) at 4 ºC, to further fix and preserve the tissue through an osmotic exchange. Brain samples were then sliced in the vibratome (Leica, Wetzlar, Germany) to generate 100 um thick slices. Slides were mounted using Vectashield antifade mounting medium (Vector Labs, USA) and let dry in the dark at room temperature for four hours.

Images were acquired in the Zeiss Confocal LSM 710, with a 63x objective using the Z-stack and tile scan functions. Each Z-stack image was captured at a resolution of 1024×1024 and the stack enabled the acquisition of ~50 images per field, 0.80 µm apart. The laser and focus settings were adjusted to each slice as they varied significantly.

Sholl analysis was performed to measure arbor complexity of CA1 pyramidal neurons. Briefly, the neurons were first drawn by manual tracing using Neurolucida © software. Then, the quantification was automatically performed by scoring the number of dendrite intersections with concentric circles of gradually increasing radius (10 µm radius increase per circle), centered at the cell body. Data was processed separately for basal and apical dendrites.

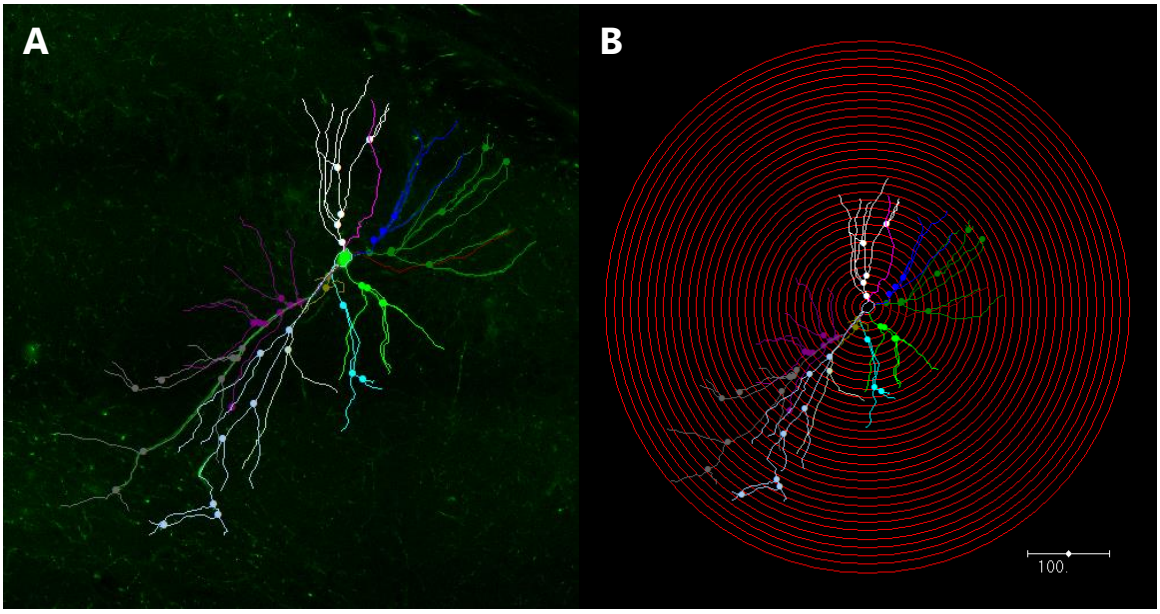

Figure 2.1. Representative images of neuronal arbor complexity analysis. (A) Manual tracing of a GFP-expressing CA1 pyramidal neuron. (B) Sholl analysis display in Neurolucida © software.

2.4. Three Chamber Social Interaction Test

The 3 Chamber Test (3CT) was performed using P45 C57BL/6 mice from 4 different cohorts. The test was divided in two different trials following a 20 minutes adaptation period in the empty arena (Fig. 2.2A). In both trials, mice were released into the middle chamber and allowed to explore the other two compartments, containing wired cages (Fig. 2.2B). In the first trial an unknown stimulus male mouse was placed in a wired container – mouse compartment (left side), while in the adjacent compartment a similar container was empty – object compartment (right side). In the second trial, a new unknown stimulus male mouse was placed in the previously empty container. The time spent sniffing the cages, with or without the stranger stimulus mice, during the first 5 minutes of the trials, was evaluated with the Observer XT 12 (Noldus, Netherland).

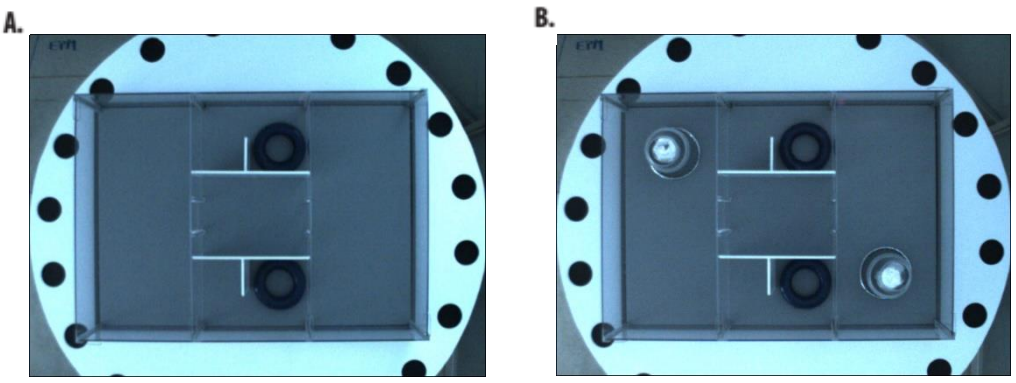

Figure 2.2. Set up for the three chamber test. Representative images of the arena settings for the habituation period (A) and for the trials (B), showing the two cages in the left and right chambers separated by a corridor, the middle chamber.

2.5. Marble Burying Test

The marble burying test was set up as previously described[1]. Each mouse was placed inside a clean home cage (20×26×13 cm) with 5 cm-thick fresh bedding, lightly tamped down to make a flat and even surface, covered with 24 glass marbles placed in a regular pattern (4x6) on the surface, evenly spaced, each about 3 cm apart (Fig. 2.3)**.** Mice were placed facing the cage corner and left for free exploration for 30min. All trials were videotaped and the number of buried marbles (2/3 their depth) was evaluated by a group of 6 observers blind to the genotype.

Figure 2.3. Set up for the marble burying test. Representative image showing the marbles placed in a regular pattern of 4x6. All animals were subjected to test using the same pattern of orientation and colors.

2.6. Forced Swim Test

The forced swimming test was performed to assess depressive-like behavior. Briefly, a 2 L glass beaker was filled with 1.5 L of water at 24 ± 1°C. Animals were introduced into the water and their behavior was videotaped for 10 min. Afterwards, the mice were removed and allowed to dry in a clean cage before returning to their home cage. The last 6 min of the test were scored for total time spent immobile using the Observer XT 12 (Noldus, Netherland). The immobility was defined as the lack of motion of the whole body, except for small movements necessary to keep the animal's head above the wate

Chapter III

RESULTS

3.1. ADRESSING THE BIOCHEMICAL COMPOSITION OF Stg^{ID} MICE POSTSYNAPTIC DENSITIES

3.1.1. Stargazin expression is decreased in postsynaptic densities isolated from StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice.

Stargazin is a synapse-enriched protein whose major know function is in the synaptic traffic of AMPA receptors. Therefore, for the biochemical characterization of Stg^{ID} mice, we isolated postsynaptic densities (PSDs) from whole brain extracts of Stg^{ID} *knock-in* mice as well as from heterozygous and wild-type mice, with the objective of determining whether the disease-associated mutation in stargazin affects the molecular composition of the PSD. To characterize the purified samples, PSD95 and synaptophysin were evaluated for their expression levels both at PSDs and whole brain, as enrichment in PSD95 expression and depletion of synaptophysin are hallmarks of purified PSDs. In fact, we found that the postsynaptic scaffold protein PSD95 is significantly increased in PSD fractions comparatively to whole brain lysate or to synaptic plasma membranes, and that the presynaptic protein synaptophysin is depleted from isolated PSDs (Fig. 3.1A), thus confirming successful PSDs isolation.

We compared PSD95 expression levels in PSDs isolated from Stg^{ID} *knock-in*, heterozygous and wild-type mice, since it is the main scaffold protein in the PSD and directly binds to stargazin PDZ binding domains, being a crucial player to assure the normal stargazin function as a regulator of AMPAR synaptic expression. We did not detect significant changes in the PSD95 expression between genotypes (Fig. 3.1B).

We found it essential to look at the expression levels of stargazin, the protein coded by the gene where the intellectual disability-associated mutation that we are studying occurs. We evaluated stargazin expression levels specifically in postsynaptic densities isolated from whole brain, as well as its expression levels in whole brain total extracts. We detected a significant decrease in stargazin levels in PSDs isolated from Stg^{ID} *knock-in* and heterozygous mice (Fig. 3.1C), whereas in whole brain extracts stargazin levels were not significantly changed, albeit a tendency for reduced expression of stargazin in heterozygous and *knock-in* mice was observed (Fig. 3.1D). These observations indicate that the mutation in stargazin impairs its synaptic traffic and/or protein stability.

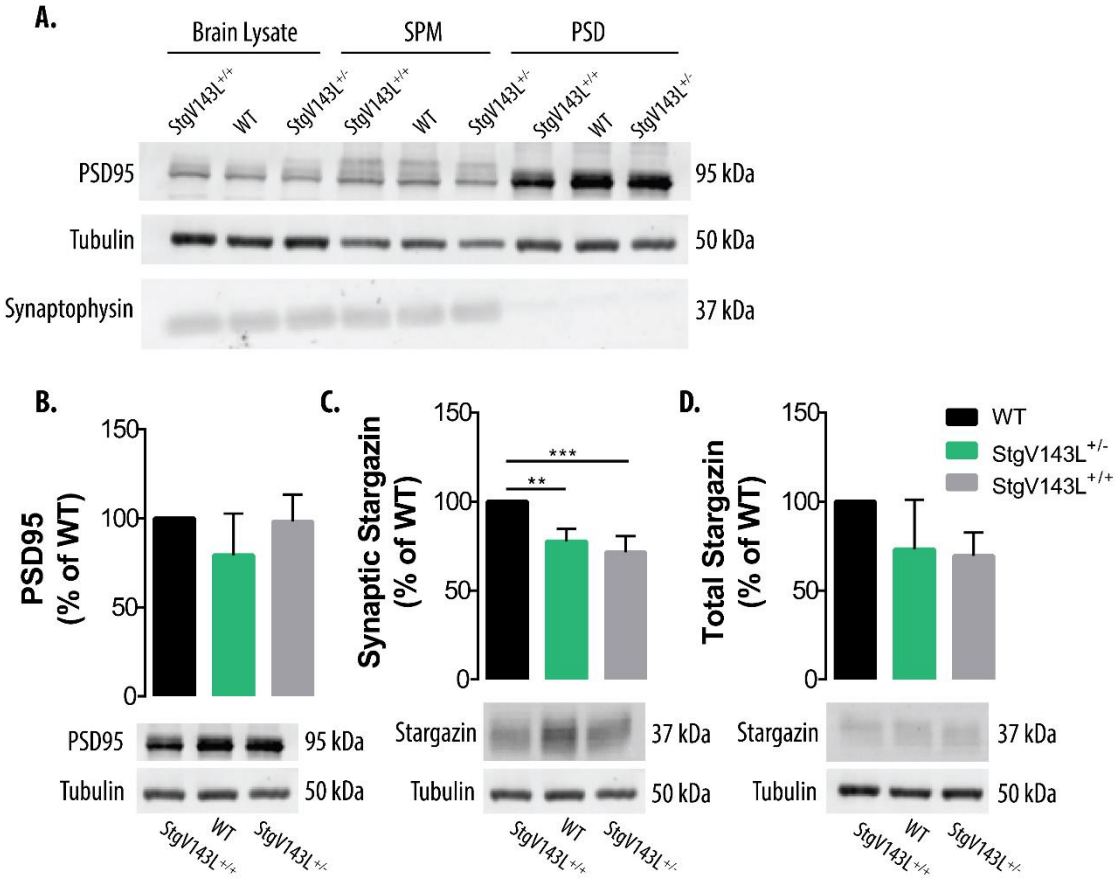

Figure 3.1. Diminished synaptic stargazin expression in StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice, compared to wild-type mice. (A) Protein expression in whole brain lysates, isolated synaptic plasma membranes (SPM) and post-synaptic densities (PSD) from WT, StgV143L$^{+/-}$ (HET) and StgV143L$^{+/+}$ (KI) mice. PSDs were successfully isolated from whole brain extracts from StgV143L$^{+/+}$, heterozygous or wild-type mice. (B) PSD95 expression levels in post-synaptic densities isolated from whole brain normalized to WT. (C) Stargazin expression levels in post-synaptic densities isolated from whole brain. (D) Stargazin expression levels in whole brain lysates. Data are presented as mean ± sem. Expression levels for target proteins were normalized for tubulin expression. ***p<0.001, **p<0.01, Mann–Whitney *U* test. N=6 for WT, N=7 for StgV143L$^{+/+}$ and N=5 for StgV143L$^{+/-}$ mice.

3.1.2. Expression levels of synaptic proteins at the PSD in Stg^{ID} mice.

Additionally, we found critical to evaluate the synaptic expression levels of GluA1 and GluA2 AMPAR subunits, since the main functions of stargazin include the regulation of processes like the trafficking of AMPAR to the cell surface, as well as the anchoring of receptors in the synaptic membrane [11]. Moreover, due to the recent evidence showing that stargazin might also have a role in NMDAR function [81], we considered the NMDAR GluN1 subunit as an important target for this biochemical analysis. We did not detect significant differences in the synaptic content of any of the receptor subunits in Stg^{ID} or heterozygous mice compared to wild-type mice (Fig. 3.2).

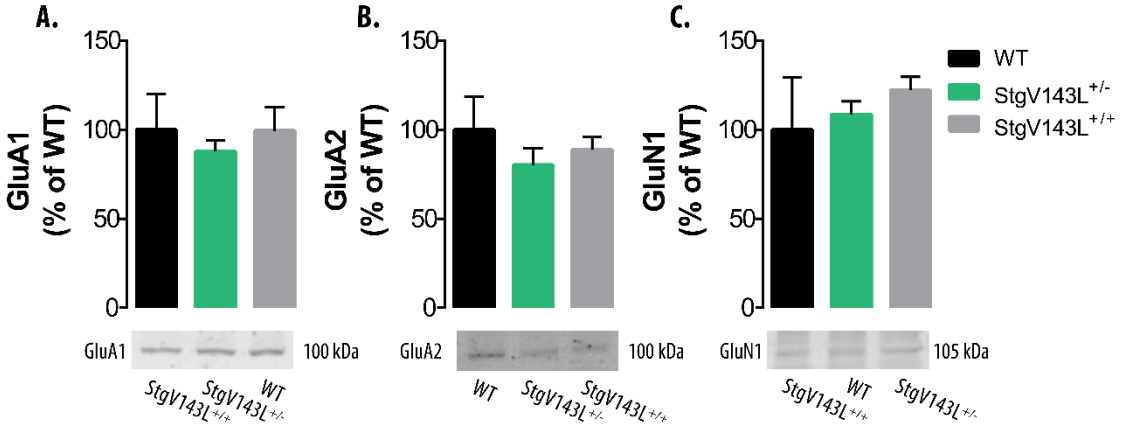

Figure 3.2. AMPA and NMDA glutamate receptors subunits GluA1, GluA2 and GluN1 expression shows no significant alterations in postsynaptic densities isolated from Stg^{ID} or heterozygous mice, when compared to wild-type mice. (A-C) Protein expression levels of AMPAR subunits GluA1 and GluA2, and NMDAR subunit GluN1 in isolated post-synaptic densities (PSD) from WT, StgV143L+/- (HET) and StgV143L+/+ (KI) mice. Data are presented as mean ± sem. Expression levels for target proteins were normalized using whole membrane Ponceau S staining. N≥5 for wild-type, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice.

Stargazin activity is dependent on the phosphorylation by CaMKII [28] and it was recently reported that *knock-in* mice expressing a disease-associated mutated form of this kinase exhibited behavioral and biochemical alterations similar to ones observed in this study [82]. Therefore, we addressed this kinase expression levels in PSDs, and found no differences between genotypes (Fig. 3.3B). Finally, we also looked at the contactin associated protein-like 2 (Caspr2) synaptic expression. Caspr2 is a transmembrane protein associated with an autoimmune form of encephalitis [83]. This protein is localized at the juxtaparanodes of myelinated axons, first described as a member of a protein complex that organize and stabilize ion channels at plasma the membrane, specifically the Kv1 potassium channels. Caspr2 has been recently reported to be present at dendrites and, similarly to stargazin, to have a role in regulating AMPAR function. Caspr2 knockout neurons displayed an overall impairment in synaptic transmission, including a significantly decrease in frequency of spontaneous miniature (m)EPCS and miniature (m)IPSCs, as well as impaired dendritic arborization and spine development [84]. Moreover, it was shown that CASPR2 knock-down affected the AMPAR trafficking and maturation of glutamatergic synapses by leading to the accumulation of abnormal cytosolic GluA1 aggregates [85]. Due to this demonstrated critical role of Caspr2 in synapse function, this protein has also been one of the main focuses of the lab. We did not detect significant alterations in the expression levels of Caspr2 in Stg^{ID} or heterozygous mice compared to wild-type mice, despite a consistent tendency for decreased expression (Fig. 3.3B).

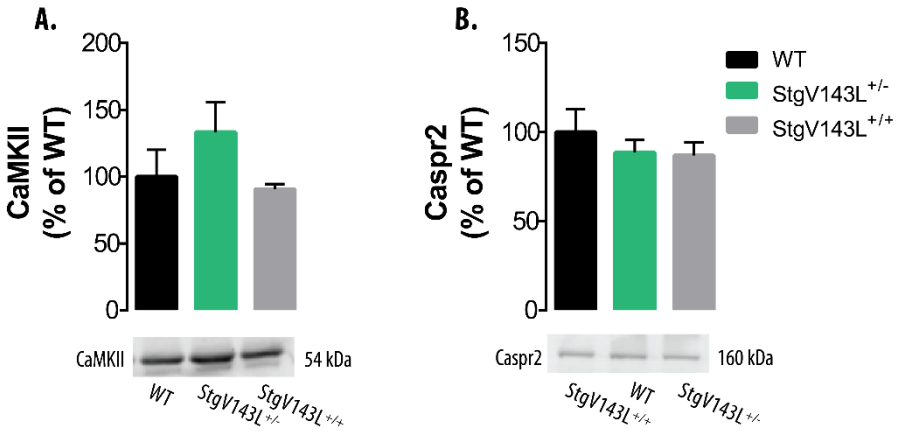

Figure 3.3. Expression levels of CaMKII and Caspr2 are unaltered at the PSD of Stg^{ID} mice. Protein expression levels of CaMKII (A) and Caspr2 (B) in isolated post-synaptic densities (PSD) from WT, StgV143L+/- (HET) and StgV143L+/+ (KI) mice. Data are presented as mean ± sem. Expression levels for target proteins were normalized using whole membrane Ponceau S staining. N≥4 or wild-type, StgV143L$^{+/-}$ and StgV143L$^{+/+}$. Mice.

Overall, the biochemical analysis of PSDs isolated from whole brain extracts of Stg^{ID} mice revealed decreased levels of stargazin, but did not show alterations in the postsynaptic scaffold protein PSD95, in AMPAR or NMDAR subunits, in the major PSD component CaMKII or in the cell adhesion molecule Caspr2.

3.2. HIPPOCAMPAL NEURONAL MORPHOLOGY OF Stg^{ID} MICE

Type I TARPs have been shown to have a significant role in dendritic growth (Hamad et al., 2014). Overall, the overexpression of γ2 (or stargazin), γ3 or γ8 leads to an increase in dendritic complexity in cortical pyramidal cells layers II/III and V/VI, but in a stage-specific manner. The reported data suggest that γ8 regulates pyramidal cell dendritic growth in an earlier time window, while γ2 and γ3 become efficient dendritic modulators at a later developmental stage. Moreover, it appears that the action of all the three TARPs shift with age to more distal dendritic zones. Given this evidence, we tested whether Stg^{ID} mice present alterations in dendritic morphology.

3.2.1. Dendritic arborization is affected in CA1 hippocampal pyramidal neurons of StgV143L$^{+/+}$ mice.

Here, we addressed the arbor complexity of hippocampal pyramidal neurons of Stg^{ID}, heterozygous and wild-type mice. As a strategy to visualize neuronal morphology, P30 mice were subjected to tail vein injection with adeno-associated virus (AAVs, serotype 9) expressing eGFP under the control of the synapsin 1 promoter. At P60, mice were perfused and brains were sliced. As previously reported [80], serotype 9 AAVs cross the blood-brain barrier and lead to sparse infection and neuronal expression of eGFP (Fig. 3.4) particularly in the cortex and hippocampus but also in other brain regions. In fact, we initially designed this analysis to assess the dendritic arbor of both hippocampal and cortical pyramidal neurons, given the expression of stargazin in both brain regions, and their relevance for ID-related phenotypes. However, due to the short time window to conclude this study and taking in consideration the significant amount of time needed for both the image acquisition and quantification, here we report data on the dendritic morphology of CA1 hippocampal neurons only. For that, and since we are working with *ex vivo* brain slices and not *in vitro* neurons, we took in consideration that this analysis can only be made if the neurons compared are from a region where the neurons size is homogeneous. Moreover, as previously mentioned the neuronal transduction approach used in this study allows only a sparse expression of the virus in the whole brain. Consequently, the transduced cortical neurons revealed to be too variable in their size. Thus, and although stargazin is more expressed in the cerebral cortex than in the hippocampus [86], we opted to address the impact of the stargazin mutation in hippocampal neuronal morphology as these neurons are less variable in size, and directed our analysis to neurons situated within a 300 µm range for all animals. Additionally, the fact that this analysis is directed to a brain region where the effects of stargazin on neuronal morphology have not been tested before (previous studies were performed in the cortex; [64]) gives the results presented here a character of novelty.

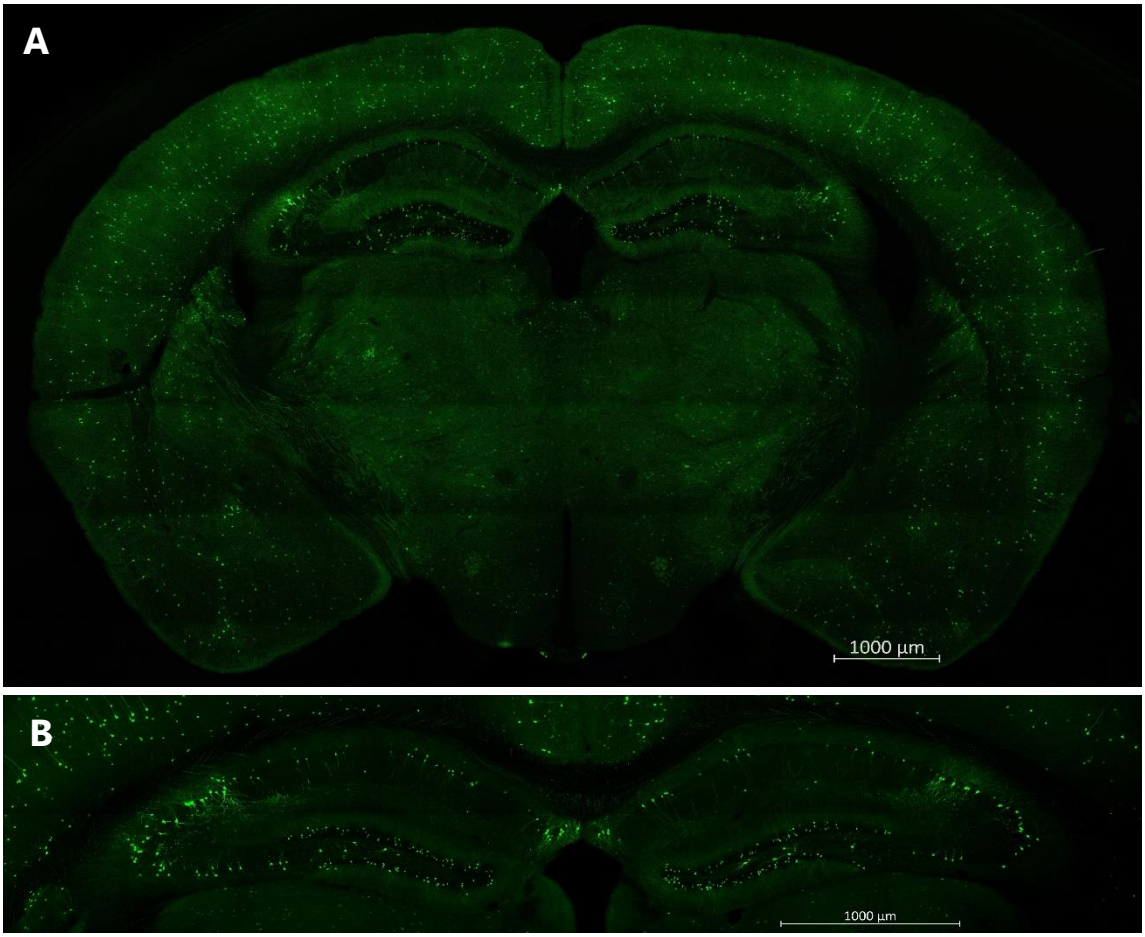

Figure 3.4. Representative images of neuronal GFP expression in the brain of tail-injected mice with virus (AAV9) to express GFP. (A) GFP-expressing neurons in a coronal slice obtained from the whole brain of mouse tail-injected with adenovirus to express GFP in neurons (AAV9.hSyn.HI.eGFP.WPRE.bGH) 4 weeks after injection. (B) High transduction levels obtained in the hippocampus.

We evaluated apical and basal dendrites in transduced neurons in the CA1 hippocampal region of Stg^{ID}, heterozygous and wild-type mice, after reconstructing the dendritic arbor (Fig. 3.5A). We found that basal dendrites in Stg^{ID} mice show decreased complexity, as evaluated by Sholl analysis which revealed dendritic contraction in Stg^{ID} neurons compared to neurons from heterozygous or wild-type animals (Fig. 3.5B). Apical dendrites showed smaller number of intersections in both Stg^{ID} and heterozygous mice, although in heterozygous CA1 hippocampal neurons we found a higher number of intersections for long (> 210 µm) distances from the soma than in wild-type mice or Stg^{ID} (Fig. 3.5C). Together, these data indicate that the stargazin mutation associated with ID, at least when expressed in homozygoty, affects hippocampal neuronal morphology towards a withered dendritic tree.

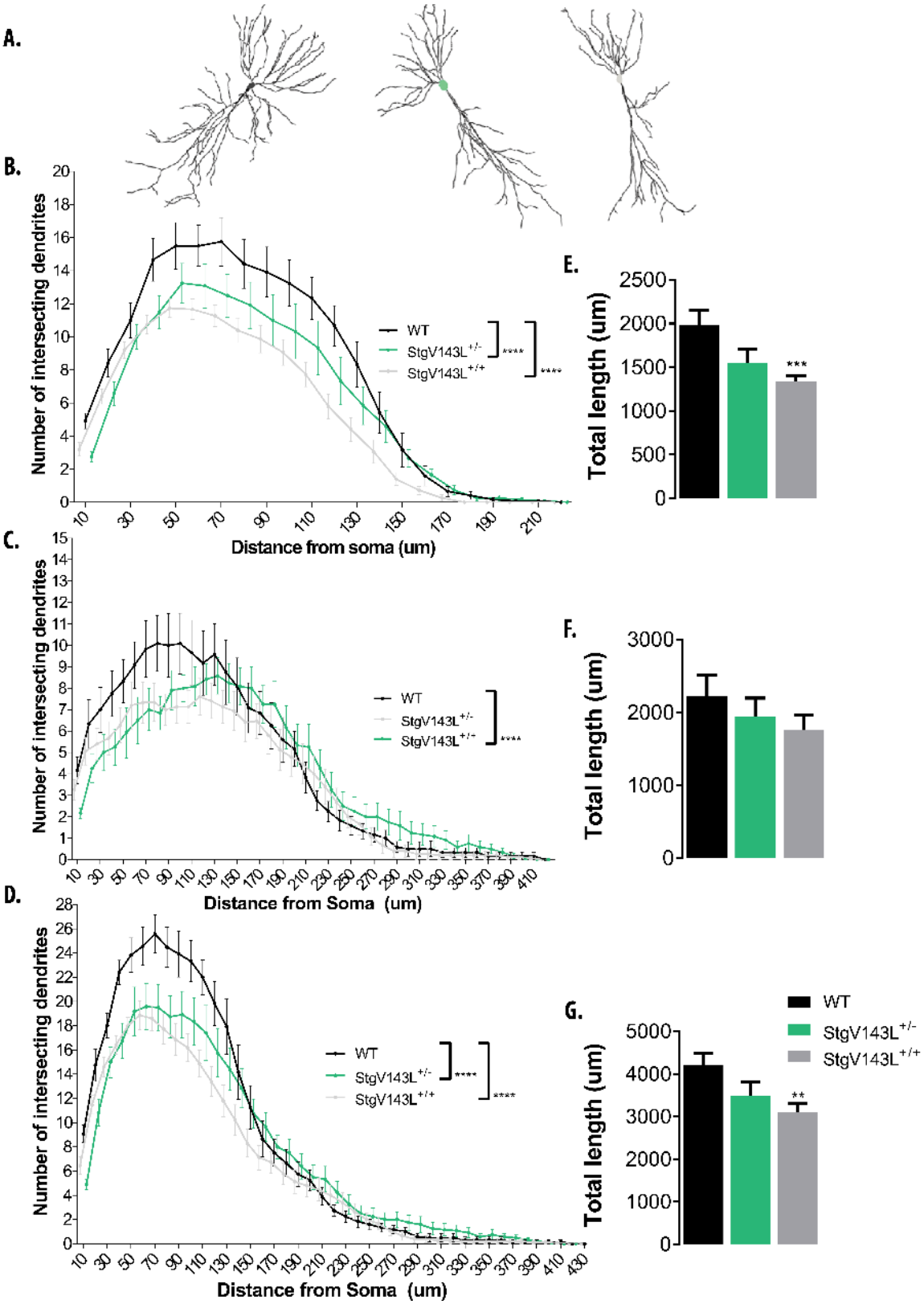
A.
B.
Number of intersecting dendrites
Distance from soma (um)
WT
StgV143L+/-
StgV143L+/+
****
****
E.
Total length (um)
***
C.
Number of intersecting dendrites
Distance from Soma (um)
WT
StgV143L+/-
StgV143L+/+
****
F.
Total length (um)
D.
Number of intersecting dendrites
Distance from Soma (um)
WT
StgV143L+/-
StgV143L+/+
****
****
G.
Total length (um)
**
WT
StgV143L+/-
StgV143L+/+

Figure 3.5. Arbor complexity of CA1 hippocampal pyramidal neurons of wild-type, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice. (A) Representative images for the neuronal morphology of CA1 hippocampal pyramidal neurons of *wild-type*, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice (A). Number of intersecting neurites for each soma-based concentric circle and total dendritic length from Basal (B,E), Apical dendrites (C,F) and both together (D,G). Basal dendritic complexity is reduced in CA1 neurons of StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice. Apical dendritic complexity is diminished in StgV143L$^{+/+,}$ mice and seems to be reduced in StgV143L$^{+/-}$ mice, although not significantly. The total length of basal dendrites is also diminished in StgV143L$^{+/+,}$ mice. Results are presented as mean ± SEM for each distance from soma. obtained from 13, 12 and 15 neurons of two wild-type, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice, respectively.

3.3. BEHAVIORAL CHARACTERIZATION OF Stg^{ID} MICE.

3.3.1. Stg^{ID} *knock-in* mice exhibit normal emotional response in forced swimming test.

Forced swimming test (FST) was initially described as a behavioral method for screening antidepressants with different mechanisms of action [87]. Since then, it has been also used to evaluate a depressive-like state in mouse models generated by either genetic or drug-induced alterations, since it reflects mental states such as negative mood and helplessness. Thus, FST has also been pointed as a reliable approach to assess self-care in mice, a feature that is affected in many ID patients. Moreover, we believe that for a complete phenotypic behavioral characterization depressive behavior must always be assessed, to determine if any other observed alteration might be a result of a depressive-like state. In our study, mice were subjected to the FST after all the other behavioral tests, which allowed us to know whether those tests induced stress in mice or not. We did not find differences between Stg^{ID}, heterozygous and wild-type mice in the forced swimming test (Fig. 3.6), as evaluated by measuring the time animals spent swimming during a 6 min period.

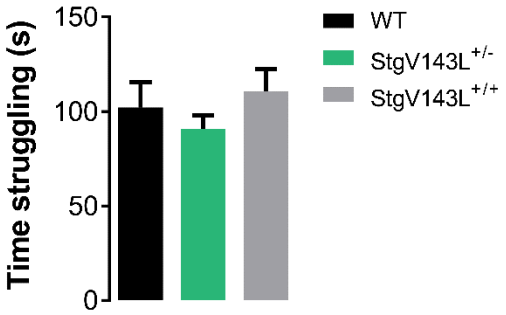

Figure 3.6. Forced swim test shows no signs of depressive-like behavior in Stg^{ID} *knock-in* mice. Forced swim test was performed to evaluate the emotional response of mice when placed into a glass beaker filled with 1.5 L of water. Performance of *wild-type*, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ was compared considering the time spent struggling/swimming in a total of 6 minutes. Data are represented as mean ± sem, N=9 for WT, 10 for StgV143L$^{+/-}$ and 12 for StgV143L$^{+/+}$ mice. No significant differences were found using Unpaired T-test.

3.3.2. Social behavior is affected in Stg^{ID} *knock-in* mice.

Alterations in social behavior are common symptoms of several neuropsychiatric and neurodevelopmental diseases, including ID. Typically, ID patients display deficits in several social skills, including the will to socially engage with other people. To address whether Stg V143L *knock-in* mice display social interaction deficits, we first examined social interaction in the three-chamber test, a commonly used method to measure social approach behavior in mice (Fig. 3.7). Briefly, taking the fact that mice are typically considered a very socializing species, the tendency to approach or avoid a compartment with a stimulus mouse provides a reliable measure of sociability. Another approach to evaluate sociability is to address social novelty by measuring the preference to socialize with a new unknown strange mouse over a familiar one. Wild-type mice tend to spend more time in the mouse compartment in comparison to the object compartment and more time in the compartment with a novel strange mouse than in the chamber with an already known/familiar mouse.

In fact, we found that as expected wild-type mice show preference for interacting with a stimulus mouse over an empty cage, a preference that was also displayed by heterozygous or Stg^{ID} mice (Fig. 3.7A-D). However, Stg^{ID} mice spent less time investigating the stimulus animal when compared to wild-type mice ($p=0.171$, Fig. 3.7C, D). This reduced interaction does not seem to be due to differences in physical activity, since the distance traveled by Stg^{ID} mice and their velocity are comparabe to those of wild-type mice (Fig. 3.8). To further explore whether Stg^{ID} mice display social impairment, we exposed mice to a second task in which the middle chamber is bordered by one chamber containing a familiar animal and the other chamber contains an unknown mouse (Fig. 26A, lower panel). Wild-type animals spent significantly more time exploring the unknown mouse than the familiar animal, as expected (Fig. 3.7B, E, F); however, neither Stg^{ID} nor heterozygous mice showed preference for interaction with the new mouse over the familiar animal (Fig. 3.7B, E, F). Thus, Stg^{ID} mice showed an impairment in social interaction.

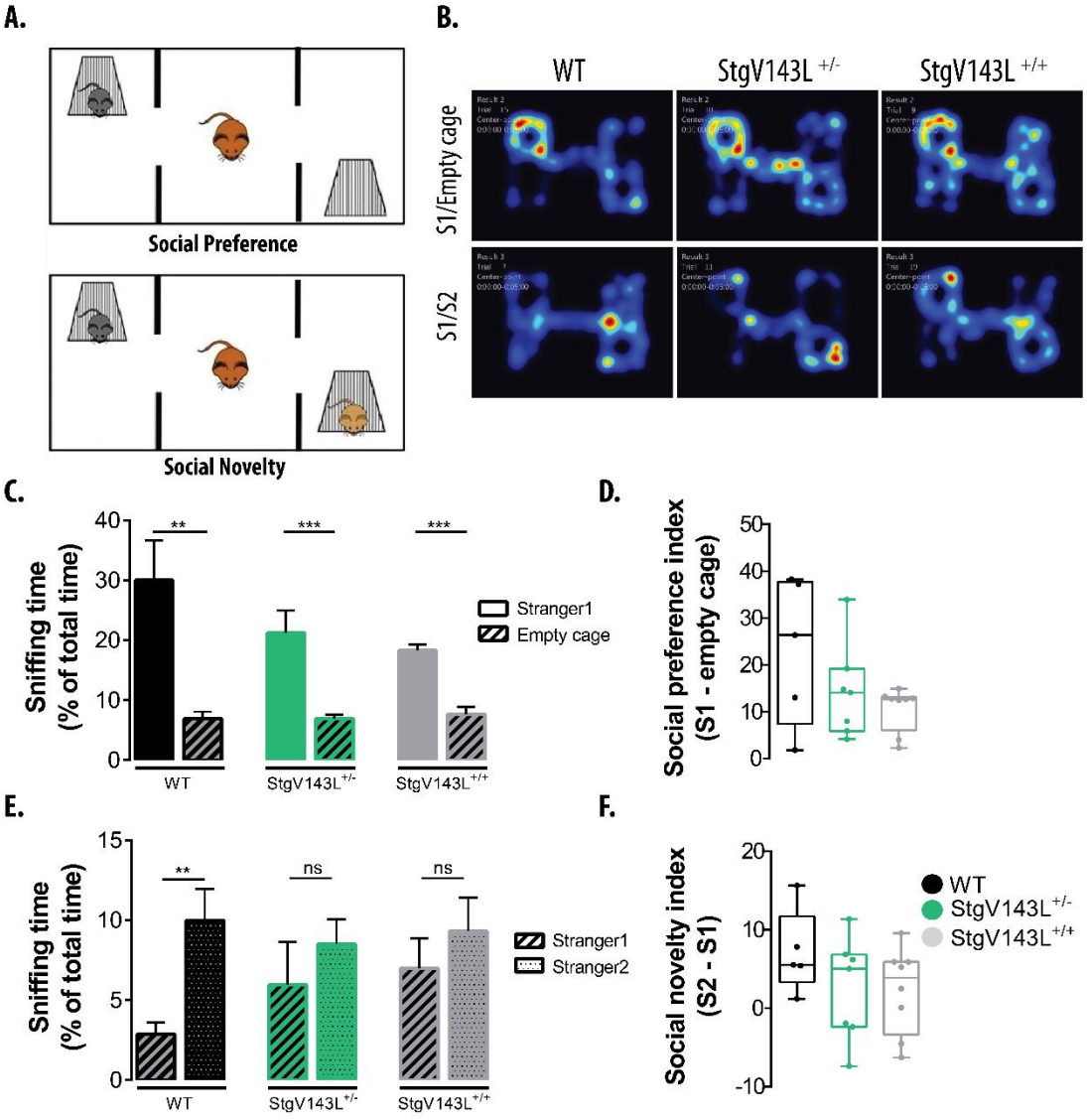

Figure 3.7. Altered social interaction in StgV143L$^{+/-}$ and StgV143L$^{+/-}$ mice. (A) The 3 chamber test was performed to assess social interaction in Stg^{ID} mice. Mice were left to explore a 3-chamber maze for 20 min with a strange animal in one chamber (Stranger 1) and an empty cage in the other, to evaluate social preference. Similarly, to assess social novelty, mice were allowed to explore the maze a second time for 10 min with a new strange animal (Stranger 2) in the second compartment instead of an empty cage (A). Mice social behavior was compared considering the time spent sniffing the wire cages in both chambers. (B) Representative images of *wild-type*, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice performances in both trials. (C) The time spent in close interaction with the stimulus mouse versus empty cage demonstrates that all three groups of mice display a social preference for the mouse-containing cage, but Stg^{ID} animals spent less time than wild-type animals interacting with the stimulus mouse. (D) Preference index reflecting the numerical difference between the time in close interaction with S1 and the empty cage. (E) The amount of time spent in close interaction with a novel social mouse versus a familiar mouse indicates that StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice did not descriminate between the familiar and new animal, and the totals for time spent with both animals were closer. This tendency is visible in the preference index for social novelty (F), derived from the numerical difference between the time in close interaction with S2 and S1. *p<0.05, ***p<0.001, **p<0.01, Mann–Whitney *U* test. Data are represented as mean ± sem for N=7 for wild-type and StgV143L$^{+/-}$, and N=8 for StgV143L$^{+/+}$ mice.

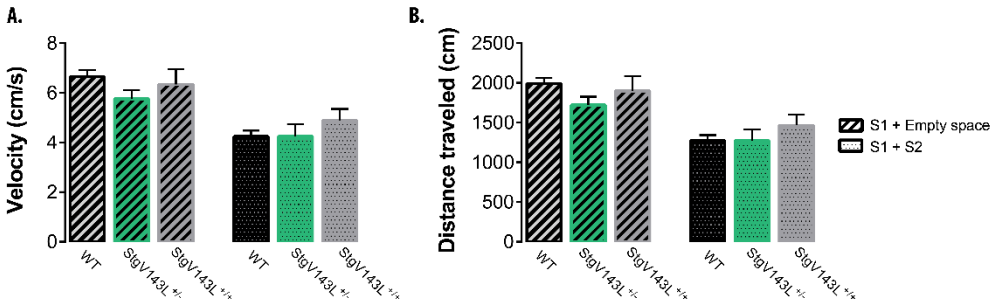

Figure 3.8. Stg^{ID} mice exhibit normal locomotor activity in the 3 Chamber Test. Median velocity (A) and distance traveled (B) by WT, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice during their performance in 3CT. Data are represented as mean ± sem for N=7 for wild-type and StgV143L$^{+/-}$, and N=8 for StgV143L$^{+/+}$ mice.

3.3.3. Natural burying behavior is impaired in Stg^{ID} *knock-in* mice.

Digging and burrowing are established as frequent typical behaviors in many rodents species. In the laboratory, burrowing reflects this naturally occurring behavior [87, 88] and it was which was shown to be an extremely sensitive test for a lot of different treatments and experimental-induced alterations in mice [89-91]. In addition, many rodents (including rats and mice) exhibit a burying behavior, commonly referred to as "defensive burying". Burying behavior in rodents usually refers to the displacement of bedding material using the snout and forepaws in an effort to cover an object [92]. Early reports showed that mice tend to bury both noxious objects such as shock probes [92] and non-noxious objects such as marbles and food [87]. According to that, another way of measuring digging behavior was developed and standardized as the "marble burying test" [93]. Marble burying behavior is sensitive to strain differences and it has been highly assessed to test the effects of a variety of drugs, such as anxiolytics and similars, commonly used to study and treat anxiety and obsessive-compulsive disorder (OCD) [94-97]. Besides, this behavior has been intensively assessed in many other different contexts that not only anxiety and OCD, as it was proven to be a reflect of a deeply perseverative species-typical behavior that responds to a lot, sometimes very small, alterations in both environment and genetics of mice [98] . Since these species-typical behaviors have been shown to be extremely sensitive to such a wide variety of treatments and factors, taking advantage of their simplicity and the fact that the target behaviors occur spontaneously in mice they have been particularly useful in assessing phenotypic changes in genetically modified mice.

We determined the number of glass marbles buried during a 30 min exploration period, out of 24 initially exposed marbles, by wild-type, heterozygous and Stg^{ID} animals. Stg^{ID} mice buried

significantly less marbles than wild-type animals (Fig. 3.9), suggesting either decreased perseverance or decreased anxiety-like behavior in animals expressing the mutant form of stargazin.

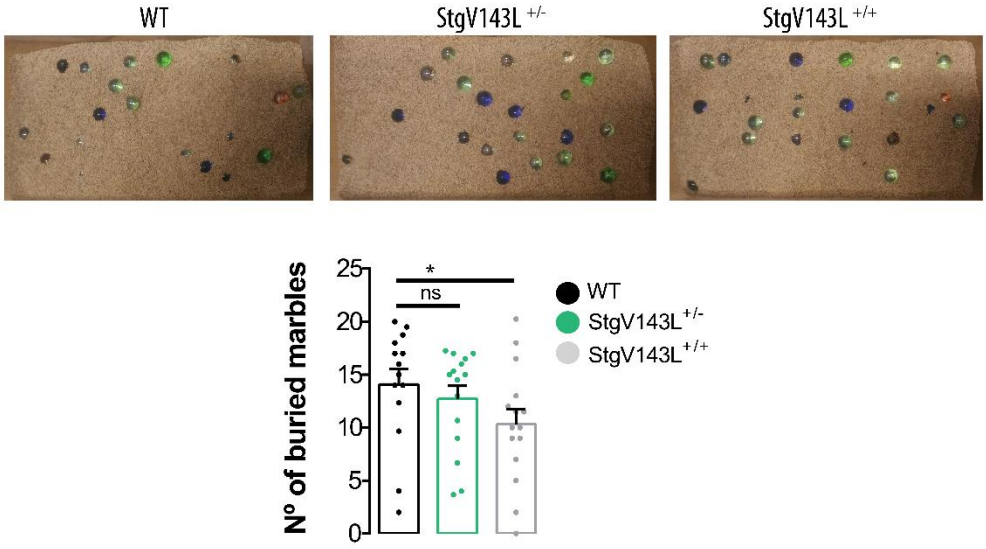

Figure 3.9. Marble burying test shows defective burying behavior in StgV143L$^{+/+}$ mice. The marble burying test was performed to analyze the mice species-typical perseverative behavior of burying both aversive and non-aversive objects. Mice were left alone and undisturbed for 30 min inside an home cage with 24 glass marbles under a flat surface of 5 cm-thick bedding and the number of buried marbles was compared between the three goups. *p<0.05, Mann–Whitney *U* test. Data are represented as mean ± sem for N=14, 15, 15 for *Wild-type*, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ animals, respectively.

Chapter IV

DISCUSSION

Recent advances have improved the understanding of neurodevelopmental disorders such as ID, and identified mutations in genes encoding synaptic proteins as frequently associated with these disorders. However, their etiology and pathogenic mechanisms are still unknown in many cases. In this study, we characterized *knock-in* mice expressing a mutation in the *CACNG2* gene encoding stargazin found in an ID-patient. The present findings shed light on the role of stargazin in regulating neuronal morphology and social interactions, and demonstrate a causal effect of an ID-associated *CACNG2* mutation in the development of disrupted social behavior.

Cerebellar granule cells (CGNs) from stargazer mice are virtually devoid of both synaptic and extrasynaptic AMPARs [11, 27], suggesting that stargazin accounts for the entirety of type I TARP function in this cell type. This is somewhat surprising given the central importance of TARPs in AMPAR function and that most cell types examined thus far express multiple, largely redundant TARP subtypes. Regarding TARPs role in glutamatergic synaptic transmission, animal models have been created using KO animals for all identified TARPs except for γ5. Despite the documented behavioral phenotypic outcome of stargazer mice, which includes dyskinesia, head-tossing, severe ataxia, seizures and low body weight no behavioral changes were observed in single KO-models for other TARPs [16]. In contrast, some of these models displayed alterations regarding AMPAR trafficking; while stargazer mice showed severe loss of synaptic and extrasynaptic AMPARs in cerebellar granule neurons, synaptic AMPARs loss in PC and thalamic nucleus reticularis neurons and reduction of synaptic AMPARs in CA1 pyramidal neurons, γ4 KO mice showed loss of synaptic AMPARs in striatal medium spiny neurons of neonates (P5–6) and normal levels in juveniles (P14-16) and adults [99, 100], and γ8 KO mice had modest reduction in synaptic AMPARs but severe loss of extrasynaptic AMPARs in CA1 hippocampal neurons [20, 101]. Moreover, double and triple KO animals for different combinations of TARPs were generated to deeply understand the functional overlap of TARPs. Among these, double KOs including stargazin silencing lead to more severe phenotypes than the one observed in stargazer mice and some animals failed to thrive, whereas all triple KO including stargazin were lethal. Strikingly, stargazin/ γ8 exhibited more severe reduction in synaptic AMPARs than y8 KO alone. This, together with the anatomical evidences (Fig.1.4), suggests that γ8 and stargazin may be present in separate but overlapping subcellular compartments in hippocampal neurons. More interestingly, all the double KO combinations without stargazin were viable and lead to a normal behavioral phenotype, and the same was for the triple *knock-out* combination without stargazin (γ3/ γ4/ γ8) which showed only a modest reduction in CA1 synaptic AMPARs expression, similar the loss of γ8 alone. Taking together, these results highlight the important role of TARPs in modulation of AMPAR in vivo, pointing out these proteins as serious candidates to assess disease associated-phenotypes. Moreover, the latter findings regarding the absence of significant defects in triple KO models for TARPs that excluded stargazin from the KO

combination attribute stargazin an extra relevance among TARPs, and one might speculate that the observed phenotypes are due to the fact that contrarily to other TARPs, stargazin absence is not compensated for its function when absent. Following the same thought, we can even speculate that in absence of other TARPs, stargazin rises as the main compensatory strategy in the brain for the synaptic targeting of AMPAR and consequently for AMPAR-dependent synaptic plasticity. This might be particularly striking in CA1 neurons, since the evidence regarding AMPARs synaptic levels found in stargazer, stargazin/ γ8 double KO and γ3/ γ4/ γ8 tripe *knock-out* mice can mean that despite stargazin being less expressed in the hippocampus it might be actually compensating γ8 function regarding the synaptic targeting of AMPARs and/or, in the presence of both, have a complementary role for the targeting/anchoring, whereas γ8 function might be more crucial in the previous steps.

Overall, this gives stargazin a preeminent role in regulating physiological functions that are dependent on AMPAR-synaptic plasticity, such as cognitive function, memory formation, motor learning and maintenance of neuronal firing homeostasis.

In this study, western blot analysis revealed a significant decrease in stargazin expression at the PSDs of both heterozygous and Stg^{ID} *knock-in* mice and no differences regarding the protein expression in brain lysates. These findings suggest that the synaptic traffic or synaptic anchorage of the disease-associated stargazin variant are disrupted. This is consistent with previous results obtained in the lab in transfected neurons, which showed that this variant of stargazin has increased mobility and surface diffusion. Stargazin function is known to be dependent on the phosporylation by kinases such as CamKII. Acordingly, for a complete characterization of the disease-associated mutation STGV143L, the phosporylation of this mutant form must be assessed. To do so, we started by looking at the expression levels of this kinase at the PSDS. Our findings revealed no alterations in the expression levels of this protein, which we think is not unexpectable due to the fact that stargazin is not known to influence CaMKII function, but the opposite. However, AMPARs are also more effective when phosphorylated. Thus, we hypotethized that, for instance, an eventual increase in the levels of this kinase could mean either that the mutant form of stargazin is compromised regarding its phosporylation by CaMKII, cycling at a higher rate between the phosporylated and the non-phosphorylated state, or that if hypothetically this altered form has an impaired capacity to potentiate the AMPAR synaptic response, more phosphorylation could be happening to counterbalance the deficiency of stargazin as auxiliary protein for AMPAR improved functionality. Additionaly, to adress the phosphorylation state of stargazin as a possible affected mechanis by this missense mutation we further intend to assess the expression levels of the different possible p-Stargazin forms at the PSDs.

Regarding the synaptic expression of the other proteins tested we found no alterations in any of them. Among these, AMPAR levels are especially important to be taken into account. Despite the stargazin main function as AMPAR synaptic expression modulation and AMPAR-dependent synaptic transmission, no changes were found in their synaptic expression levels, which contrast to what we would expect. However, the expression levels assessed in this study were obtained only in PSDs isolated from whole brain samples. Given the fact that TARPs function is redundant regarding their overlapped expression across the brain, whole brain expression analyses may fail to reveal how the stargazin mutation affects synaptic composition in particular brain regions where stargazin is highly expressed compared to other TARPs. Thus, it is imperative to look at the expression levels of this proteins in specific brain regions, namely the ones where stargazin is expressed and has already been reported to have an impact, such as cerebral cortex, thalamus [102], hippocampus [11, 28], and finally the cerebellum, the region where this protein is most expressed and where it seems to be the main (eventually the only) functional TARP [13]. In fact, we have already started this region-specific observation but we are still in a very preliminary stage. So far, we have already accomplished to successful purify post synaptic density fractions from one cortex sample of each genotype (Supplementary data – Fig. 2).

Stg V143L causes alterations in neuronal morphology

Stargazin has been shown to modulate dendritic complexity as assessed by performing sholl analysis in neurons overexpressing this protein [64]. Specifically, the overexpression of γ2 strongly increased apical dendritic length and branching complexity of pyramidal cells in cortical layers II/III. To determine whether the expression of the STGV143L mutant form results in behavioral alterations due to changes in neuronal architecture, Sholl analysis was performed in CA1 hippocampal pyramidal neurons. According to the *in vitro* existing data, this study revealed impaired dendritic complexity in neurons expressing the ID-associated form of stargazin, which are promising results regarding stargazin modulation of dendritic arborization. Namely, we revealed that STGV143L$^{+/+}$ mice have a significantly reduced number of dendritic intersections with the concentric circles in Sholl analysis. Interestingly, STGV143L$^{+/-}$ mice appear to have a slight increase in the number of distal apical branches although the difference is not significant. Together, these data suggest that altered neuronal morphology may at least partially be a mediator for the dysfunctional mechanisms underlying ID. Indeed, altered neuronal morphology has been described in other mouse models of ID. A mouse model of X-linked ID associated with impaired removal of histone methylation (Kdm5c-KO) displayed defects in dendritic arborization and spine morphology in neurons of basolateral amygdala; neurons of Kdm5c-KO mice had significantly fewer intersections in outer concentric

circles, reduced dendritic length and significantly lower spine density [103]. Mouse models for Williams–Beuren syndrome exhibit decreased dendritic complexity and spine number in cortical neurons [104], and KO mice for SORBS2, a gene associated with ID, exhibit reduced dendritic complexity [105]. The ID-associated mouse model Katnal1 mutant mice also have fewer dendritic spines [106]. Plus, a diversity of mouse models for the most common syndromic form of ID – Down Syndrome – showed developmental abnormalities such as alterations in the structure of dendritic spines in cortical and hippocampal neurons and impaired hippocampal long-term potentiation (LTP) [107, 108].

Taking into account that several studies reported that TARPs absence lead to decreased numbers of dendritic spines, we definitely consider that this subject must be also assessed in StgID mice.

Social impairments in Stg V143L$^{+/+}$ mice

One of the most distinguishable features in ID patients is the impairment regarding their social skills, which is one of most important aspects assessed for the diagnosis and individual characterization of the patients. In fact, psychological studies addressing ID patients have shown that adults with intellectual disability are vulnerable to stressful social interactions [109]. The 8 years old male ID patient identified as having the STV143L mutation was diagnosed with a moderate form of ID. According to the DMS-5 classification, people with moderate ID have an IQ situated between 35 to 49, and display noticeable neurodevelopmental delays regarding their cognitive function, speech and motor skills. The moderate category includes patients with fair communication skills, who are able to communicate in basic, simple ways but not at complex levels. Also, they may have signs of physical impairment (i.e. thick tongue) and deficits in social skills, having difficulty in social situations and problems with social cues and judgment. Regarding their self-care ability, moderate ID patients can care for themselves and are able to learn basic health and safety skills, but might need more instruction and support than the typical person. Many can live in independent situations and travel alone to nearby and familiar places, but some still need the support of a group home. Taking this into account, and the fact this ID patient is heterozygous for this mutation, if there is a causal relationship between the mutation and the clinical phenotype it is expectable that *knock-in* mice expressing this altered form also display cognitive and social impairments with increased significance in the case of STGV143L $^{+/+}$ homozygous mice.

As previously said, ID is characterized as a pathology that affects a large spectrum of behaviors and skills, including cognitive, memory, practical and social skills. Among these, cognitive impairment is usually the most striking one and its prevalence is consistently attributed to be greatly dependent of hippocampus. Besides, the mentioned skills cannot be seen independently of each other. For

instance, practical and social skills can never be acquired without a proper cognitive and memory function, and much of what we learn is dependent on our social environment and social interactions. In fact, successful behavior requires actively acquiring and representing information about the environment and people, and manipulating and using those acquired representations flexibly to optimally act in and on the world. While frontal lobes have figured prominently in most accounts of flexible or goal-directed behavior, the hippocampus is known to play a critical role by forming and reconstructing relational memory representations that underlie flexible cognition and social behavior. There is mounting evidence that damage to the hippocampus can produce inflexible and maladaptive behavior when such behavior places high demands on the generation, recombination, and flexible use of information [110]. This is seen in abilities as diverse as memory, navigation, exploration, imagination, creativity, decision-making, character judgments, establishing and maintaining social bonds, empathy, social discourse, and language use. Thus, hippocampal abnormalities can produce profound deficits that can impair the adaptation to almost all real-world situations. Given this strong relation attributed to ID and hippocampal dysfunction, in this study we addressed this brain region with more detail, but not without considering that the hippocampus function of supporting the flexible use of information in general cannot occur effectively without its extensive interconnections with other neural systems.

The three chamber test was performed to assess social interaction in StgID mice. Here, in the social preference part of the test we observed all three groups of mice displaying a social preference for the mouse containing cage over the empty cage. However, for the Stg^{ID} *knock-in* group there is a decrease in the total time spent sniffing the animal containing cage when compared to control animals (although the difference is not statistically significant; p=0.171), and consequently leading to a tendency in the preference index to decrease, suggesting that WT mice have a greater preference for the animal cage compared to the *knock-in* group. These data suggest an alteration in social behavior in Stg^{ID} mice, which was confirmed in the second stage of the three chamber interaction test, which assesses social novelty. In the second test, unlike wild-type mice, which prefered an unfamiliar animal to a known stimulus animal, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice did not show this preference, and the totals for time spent with both animals were similar between genotypes, suggesting that StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice failed to recognize the social novelty of having a new stranger mouse in the arena. Consequently, the preference index shows a more pronounced preference for the new stranger animal by WT mice compared to heterozygous or Stg^{ID} mice. No significant differences regarding distance moved and velocity were found in either trials (Fig. 3.5), which lead us to suggest that the differences observed between groups in these behavioral tests were not conditioned by variances in locomotor activity. These data strongly support alterations in social behavior in Stg^{ID} mice.

Similarly, other genetic models of ID display alterations in social interactions. Kdm5c-KO mice exhibited abnormal social behavior, including aggression as well as impaired learning and memory and reduced anxiety [103]. Kdm5c-KO mice performance in the three chamber test revealed a reduced motivation and/or interest in social interaction. While WT mice spent significantly more time exploring the stimulus mouse than an inanimate object, Kdm5c-KO mice spent similar time between the two. This phenotype is consistent with the manifestation of autistic behaviors in some individuals with KDM5C mutations [111]. On the other hand, mouse models for Williams–Beuren syndrome exhibit hypersociability in the three chamber test [112]. Recently, using a very similar strategy to the one present in this study, a *knock-in* mouse model was generated harboring an ASD-associated de novo mutation in the gene encoding for CaMKII - Camk2a$^{E183V/E183V}$. Social behavior was assessed in these mice by performing the 3CT and the results revealed social deficits and decreased exploratory behavior by Camk2a$^{E183V/E183V}$ *knock-in* mice. *Knock-in* mice for this mutation also exhibited a very significant impairment in marble burying behavior when compared to WT. Beside the fact that this mutation is associated with ASD, these evidences are particularly interesting due to the similarities with the results presented in this study, specifically taking into the account that this kinase regulates stargazin activity. Moreover, ID-associated abnormal sociability is a feature shared with most ASDs. The vast majority of animal models for ASDs display impaired social recognition and lack of preference for social novelty in 3CT [113].

Mice frequently exhibit species-typical behaviors such as digging and burrowing. In the wild, they dig in the ground to find and hoard food, to create a refuge from predators and cold and to make a safe nursery area for the progeny. In the laboratory, early reports showed that mice will spontaneously dig when given a suitable substrate such as deep bedding [11,12,13] and burrow virtually any substrate[13]. Hence, burrowing in the laboratory truly does seem to reflect a natural behavior. Over the course of several years, it was shown that burrowing was an extremely sensitive test, detecting scrapie disease in mice long before there were any clinical signs [89], and being impaired by a variety of treatments such as the administration of lipopolysaccharide (LPS) [90] or interleukin-1-beta overproduction [91]. Moreover, mice are known to exhibit a burying behavior, such that they tend to bury both noxious objects such as shock probes[10] and non-noxious objects such as marbles and food[10,3]. Glass marbles are assumed to be non-aversive to mice as Njung'e and Handley confirmed by showing that they did not avoid the marble-containing side of a two-compartment box[5]. Marble burying behavior is sensitive to strain differences and drugs, and it has given valuable results when used to test the effects of anxiolytics and 5-HT-active compounds, including those used clinically for obsessive-compulsive disorder (OCD). Several reports showed that this behavior

is attenuated by low doses of anxiolytic benzodiazepine drugs [93, 95] or even inhibited by 5-HT-compounds systems, many of which known for attenuating anxiety, depression or OCD [94-97]. Therefore, this pharmacological evidence could lead to the conclusion that mice bury marbles because marbles evoked anxiety, or because mice are naturally obsessive-compulsive. In fact, this behavior has sometimes been considered as an index of anxiety. Also, in the context of ASD, between the numerous animal models for evaluating compulsive-like behaviors that have been developed over the past three decades, the animal behavior tests with the greatest validity and ease of use are perhaps the marble burying test and the nestlet shredding test. However, it has also become apparent that many agents, even psychostimulants, inhibit marble burying [98]. So, whether marble burying really models anxiety or OCD became quite controversial over the past decade, but later Thomas et al. (2009) addressed the relation between marble burying with several factors previously reported to be affecting this behavior, such as the genetic component of mice, anxiety-like traits and novelty-induced anxiety. As a result, they showed that marble burying is genetically regulated, varying across strain, not correlated with anxiety measures in other assays and not stimulated by novelty, as proven by conducting multiple tests to reduce the novelty of marbles, which failed to alter burying behavior. Thus, the present understanding of most scientists in the field is that marble burying may be more appropriately considered as an indicative measure of repetitive digging, reflecting a perseverative species-typical behavior highly responsive to many factors with little change across multiple exposures, more than novelty-induced anxiety or compulsive-like traits. Due to its sensitiveness and simplicity, marble burying was revealed to be a useful tool for the phenotypic characterization documented in this study.

Hence, and although marble burying behavior is mostly studied in the context of anxiety and OCD, whereas this study is focused on ID, we assessed this behavior since ID has been strongly related with these diseases, often sharing some of the features and symptoms known to be characteristic of these conditions. Moreover, there is evidence that digging and burrowing are at least partly dependent on hippocampal function [114]. Reports addressing burrowing behavior after excitotoxic hippocampal and prefrontal cortex lesions showed striking impairments in several "species typical" behaviors; hippocampal-lesioned mice made poorer nests, hoarded and burrowed less [114]than both non-lesioned and prefrontal cortex-lesioned mice. Therefore, one interpretation of these results might that marble burying will be affected by any agent affecting hippocampal function. Now, ID is reported to be consistently linked to hippocampal dysfunction [115, 116] since it is the main brain region responsible for learning and memory formation. Although stargazin is preferencially expressed in the cerebellum and cortex, it is also significantly expressed in hippocamus when compared to the other TARPS with the exception of ɣ8, the most expressed TARP in this region. Moreover, as previously said, ɣ-8 KO mice had only a modest reduction in synaptic AMPARs [101]

and in a lesser extent than what was observed in stargazin/ γ-8. Thus, in a ID context it might be seriously possible that the disease-associated phenotype indentified in the 8 years old male patient due to the StgV143L mutant form can be at least partly dependent on the effect of this altered form in the hippocampus.

Thus, we evaluateded marble burying behavior of StgID mice by comparing their performance with wild-type mice. The percentage of marbles buried by wild-type mice was 59%, significantly bigger than the 30% of StgV143L$^{+/+}$. We think that the fact that *knock-in* mice displayed an impairment in such a perseverative behavior suggests that this mice are indeed afected in a general way regarding their "normal" behavior. At a cellular level, it would make sense to address if this StgV143L mutation-mediated behavioral impairment is indeed a consequence of an hippocampal dysfunction. To look at that, biochemical analysis such as the ones performed and documented in this study must be applied to the different brain regions where stargazin is expressed in order to be able to correlate the eventual changes in those specific regions with the alterations in the behaviors already seen as being affected.

It is important to recall the fact the 8 year-old male identified as having the STV143L mutation is heteroziygous and his medical condition was labeled as a moderate form of ID, which includes significant deficits in both learning and social skills. Moreover, the differences in results in this study are significantly more pronounced between WT and STV143L $^{+/+}$ mice than between WT and STV143L $^{+/-}$ mice. These findings correspond to what was expected since heterozygous mice have both forms of stargazin whereas knocked-in mice have only the mutated form, so the normal brain function dependent on functional stargazin is predictable to be more affected in the STV143L$^{+/+}$ ones. Overall, the effects of StgV143L mutation observed both in cellular mechanisms and behavioral performance of stargazin V143L *knock-in* mice show that indeed this stargazin altered form is affecting the normal brain functions analyzed in this study, presenting stargazin as an important player in the regulation of mechanisms underlying the ID-associated phenotype. However, deeper analyses are needed so we can be report precisely at what level the biochemical changes are related with the disease-associated behavioral phenotype observed in these mice. Specifically, we think the next critical approach is to evaluate the synaptic expression levels of stargazin, stargazin phosphorylated forms, glutamate receptors and other synaptic proteins as well as the proteins known to interact directly with stargazin, such as CaMKII, in specific brain regions, namely the hippocampus, cortex, thalamus and cerebellum – where stargazin is mostly expressed comparing to the other TARPSs.

Functional analysis of hippocampal circuits – mEPSCs in CA1 neurons, LTP and LTD evaluation – must be performed as this is will enable us to conclude whether or not the disease associated phenotype is a consequence of impaired AMPAR-dependent synaptic plasticity.

Chapter V

CLOSING REMARKS AND FUTURE PERSPECTIVES

Overall, the results obtained with this study point out stargazin as a target for cognitive-impairment-associated diseases like ID. Taking together the preliminary characterization of the Stg V143L *knock-in* mice herein presented and the massive amount of data existent regarding stargazin and TARPs function, it becomes clear that it is worth further understanding how stargazin dysfunction underlies disease pathogenesis, not only because of the evident impact of this protein on cognitive impairment but also in psychiatric and degenerative conditions such as schizophrenia and epilepsy. Plus, taking the fact that very little is known about the etiology of non-syndromic forms of ID, partly due to the fact that a great majority of patients were never assessed for their genetic background, the identification and characterization of this stargazin variant is an incentive to persuade clinicians to investigate the patients genetic profile. Despite the significant findings about TARPs functional redundancy regarding both their cell and region-specific roles obtained over the last decade, no further advances have since come to light. This leaves a gap that we think deserves further investigation, mainly because most of the conclusions drawn about individual TARPs were derived from their expression levels across the brain, despite the fact that biochemical and electrophysiology studies with *knock-out* models for individual and combinations of individual TARPs revealed that their specific functions might be overlapped or even complementary within the same cell type or brain region. For instance, stargazin has been intensively studied and related with cerebellum and little with other brain regions, but the findings previously described in literature have strongly suggested that it might have a significant role in many other regions across the brain, namely in the hippocampus. Accordingly, the findings presented in this study corroborate the speculation that stargazin might be playing a more important role in hippocampus than it has been considered so far, namely the impaired dendritic arborization in CA1 pyramidal neurons, and the impaired social and burying behavior which have been reported to be dependent on hippocampal function. In fact, in the specific context of ID it is logical that hippocampal dysfunction underlies, at least partially, the disease-associated phenotypes. Thus, we think that new approaches should be designed to assess the individual TARPs function and, for instance, how they might counterbalance the lack of function of their homologous family members. That could ultimately give us significant insights to better understand how they might be useful to experimentally modulate their compensatory capacity in the absence of others. This would be particularly interesting and promising to develop pharmacological approaches to medical conditions such as the ones reported in this study associated to stargazin variants, both in ID and SCZ.

Darwin would probably say that the urge for knowledge gave us the fittest brain, which gave us the ability to investigate every single aspect of what is surrounding and inside us. Some, however, would disagree with this. Either way, we must keep using it to pursue the improvement of our fitness and adaptation to change. Thereby, the ambition for knowing more shall never be compromised.

APPENDIX

SUPPLEMENTARY DATA

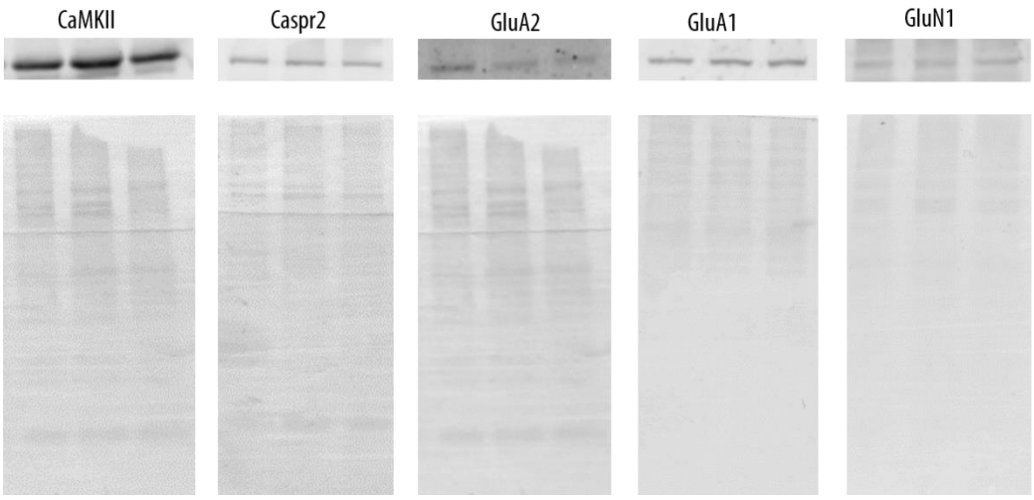

Figure 1. Loading controls for the second set of Western Blot analysis.

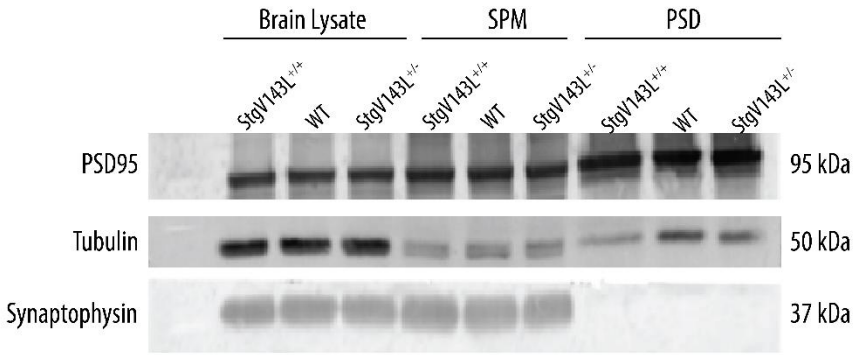

Figure 2. PSD purification from cerebral cortex of WT, StgV143L$^{+/+}$ and StgV143L$^{+/+}$.

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