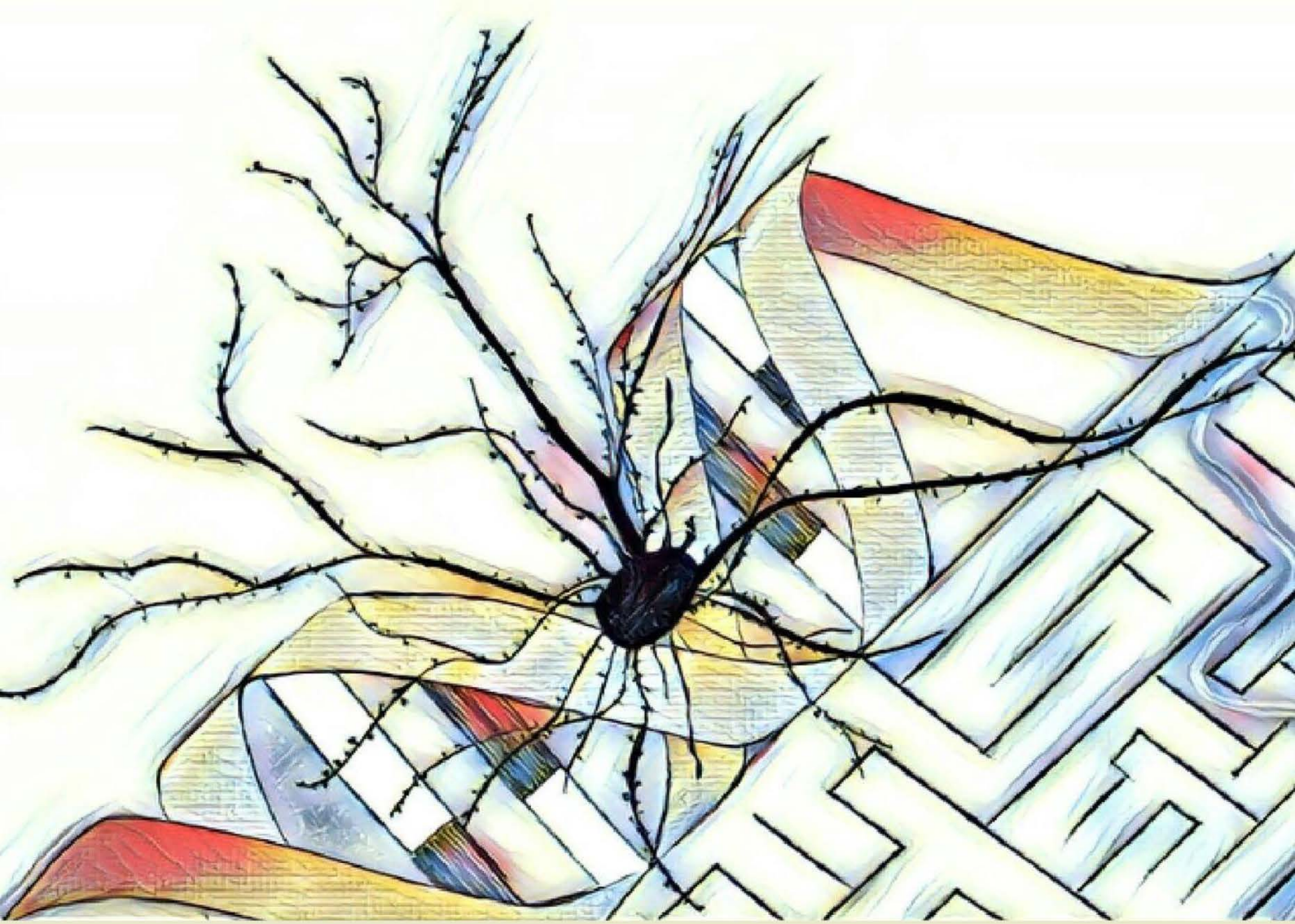

Gladys Tarcila Lima Caldeira

THE ROLE OF STARGAZIN IN THE PATHOGENESIS OF PSYCHIATRIC DISORDERS

Tese de doutoramento em Biologia Experimental e Biomedicina, ramo de Neurociências e Doença,
orientada pela Professora Doutora Ana Luísa Carvalho e pelo Investigador Doutor João Miguel Peça Lima Novo Silvestre,
apresentada ao Instituto de Investigação Interdisciplinar da Universidade de Coimbra.

Junho 2017

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UNIVERSIDADE DE COIMBRA

Gladys Tarcila Lima Caldeira

THE ROLE OF STARGAZIN IN THE PATHOGENESIS OF PSYCHIATRIC DISORDERS

PhD thesis presented to the Institute for Interdisciplinary Research from the University of Coimbra (IIIUC) for the fulfillment of the requirements for a Doctoral degree in Experimental Biology and Biomedicine, specialization in Neuroscience and Disease, under the supervision of Doctor Ana Luísa Monteiro de Carvalho (Life Science Department) and co-supervision of Doctor João Miguel Peça Lima Novo Silvestre (IIIUC).

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Gladys Tarcila Lima Caldeira

O PAPEL DA STARGAZINA NA PATOGÉNESE DE DOENÇAS PSIQUIÁTRICAS

Tese de doutoramento apresentada ao Instituto para a Investigação Interdisciplinar da Universidade de Coimbra (IIIUC) para a prestação de provas de Doutoramento em Biologia Experimental e Biomedicina na especialidade de Neurociências e Doença, sob a orientação da Professora Doutora Ana Luísa Monteiro de Carvalho (Departamento de Ciências da Vida) e co-orientação do Investigador Doutor João Miguel Peça Lima Novo Silvestre (IIIUC)

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Cover note

The image on the cover of this thesis is a representation of a cortical neuron over a DNA double helix.

"Her brother has a disease, an illness with the shape and sound of a snake. It slithers through the branches of our family tree. It must have broken her heart to know that I was next."

Nathan Filer in "The Shock of the Fall"

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ABBREVIATIONS

AAC	Anterior cingulate cortex
AMCA	Aminomethylcoumarin acetate
AMPA	Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	AMPA receptor
ANOVA	Analysis of variance
ARC	Activity-regulated cytoskeleton-associated protein
ASD	Autism spectrum disorder
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CACNG2	Calcium voltage-gated channel auxiliary subunit gamma 2
CaMKII	Calcium/calmodulin-dependent protein kinase II
CLAP	Chymostatin, leupeptin, antipain and pepstatin
CTD	Carboxy terminal domain
CTR	Control
DDM	n-Dodecyl β-D-maltoside
DIV	Days *in vitro*
DLPFC	Dorsolateral prefrontal cortex
DNA	Deoxyribonucleic acid
DS	Dorsal striatum
DTT	Dithiothreitol
ECF	Enhanced chemifluorescence
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
EPSC	Excitatory post synaptic currents
ER	Endoplasmic reticulum
GABA	Gamma aminobutyric acid
GABAR	GABA receptors
GFP	Green fluorescent protein
GluA	Glutamate receptor, ionotropic, AMPA
HA	Hemaglutinin
HBSS	Hank's balanced salt solution
HEK	Human embryonic kidney
HEPES	N-(2-hydroxyethyl)-1-piperazine-N''-(2-ethanesulfonic acid)
ID	Intellectual disability
IgG	Immunoglobulin G
IP	Immunoprecipitation
KAR	Kainate receptors
KD	Knockdown
kDa	Kilodalton
KO	Knock-out
LBD	Ligand binding domain

LTD	Long term depression
LTP	Long term potentiation
M	Membrane domain
MAGUK	Membrane-associated guanylate kinase
MAP2	Microtubule associated protein 2
Mecp2	Methyl CpG binding protein
MEM	Minimum essential medium eagle
mGluR	Metabotropic glutamate receptor
mRNA	Messenger RNA
NA	Nucleus accumbens
NMDA	N-methyl-D-aspartate
NMDAR	NMDA Receptors
NTD	N-terminal domain
NTD	Amino terminal domain
PBS	Phosphate buffered saline
PCP	Phenylcyclidine
PCR	Polymerase chain reaction
PDZ	Postsynaptic density 95-discs large-zona occludens
PFC	Prefrontal cortex
PIC	Portuguese island collection
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PSD	Postsynaptic density
PSD95	Postsynaptic density protein 95
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RNAi	Interference RNA
RT	Room temperature
SCZ	Schizophrenia
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-poliacrylamide gel electrophoresis
SEM	Standard error of the mean
shRNA	Short-hairpin Ribonucleic Acid
Stg	Stargazin
TARP	Transmembrane AMPAR-regulatory protein
TBS	Tris-buffered saline
TM	Transmembrane domain
TTX	Tetrodotoxin
VGAT	Vesicular GABA transporter
VGLUT	Vesicular glutamate transporter 1
VS	Ventral striatum
VTA	Ventral tegmental area
GWAS	Genome-wide association studies

KEYWORDS

Stargazin

AMPAR

Schizophrenia

Intellectual Disability

PALAVRAS-CHAVE

Stargazina

Recetores AMPA

Esquizofrenia

Défice Intelectual

ABSTRACT

Psychiatric disorders including intellectual disabilities and mood and affective disorders affect around one quarter of the general population. Schizophrenia and intellectual disability have been consistently associated to both cognitive and social impairment as a putative consequence of impaired synaptic function. However, although numerous genes have been suggested to predispose for these disorders, the mechanistic alterations at the basis of their pathophysiology remain obscure.

Synaptic networks are growingly recognized as major players in the development of schizophrenia, intellectual disability and other psychiatric disorders. In fact, recent reports accredited a major importance for *de novo* mutations in genes that encode glutamatergic postsynaptic proteins with roles in the modulation of synaptic strength. Importantly, these studies reinforce the notion that genes affected by mutations in schizophrenia partially overlap those mutated in autism and intellectual disability (Fromer et al., 2014; Hamdan et al., 2011; Kirov et al., 2012; Krumm et al., 2014; Network and Pathway Analysis Subgroup of Psychiatric Genomics, 2015; Purcell et al., 2014; Schizophrenia Working Group of the Psychiatric Genomics, 2014), suggesting that these disorders may share a common underlying feature: synaptic dysfunction. Thus, studying rare and highly penetrant risk mutations using animal models harboring these mutations may largely contribute to the understanding of the molecular mechanisms and neuronal circuit dysregulation that are implicated in psychiatric disorders.

The *CACNG2* gene, which codes for stargazin, has been pointed out as a susceptibility gene for psychiatric disorders (Liu et al., 2008; Nissen et al., 2012; Wilson et al., 2006; Yang et al., 2013). Stargazin is an auxiliary subunit for glutamate receptors of the AMPA type (AMPAR), which modulates AMPAR function, and is required for AMPAR trafficking to the surface and to synapses and for the homeostatic synaptic scaling of AMPAR. Using whole-genome sequence analysis, we identified a new *CACNG2* variant in a patient afflicted with schizophrenia (Stg^{SCZ}). We found that Stg^{SCZ}, and a previously identified stargazin variant associated to intellectual disability (Stg^{ID}), present altered cell surface trafficking properties, and fail to deliver AMPAR to synapses. Furthermore, Stg^{ID} fails to mediate homeostatic plasticity whereas Stg^{SCZ} affects the number of inhibitory synapses and neuronal dendritic arborization in cortical neurons. Our results show that different mutations in the *CACNG2* gene originate distinct effects on protein function, thus contributing to different cellular and eventually behavior phenotypes.

To further study the effects of the intellectual disability-associated mutation on stargazin function and on the development of disease-associated phenotypes, we designed and characterized a novel

knock-in mouse line carrying the Intellectual disability-associated mutation on the *CACNG2* gene, V143L. Assessment of locomotor activity, anxiety-like behaviors and cognitive performance showed that homozygous stargazin V143L *knock-in* mice present increased locomotor activity, deficits in spontaneous alternation, in motor learning and in nesting behavior.

Overall, the effects of disease-associated *CACNG2* mutations in cellular processes and in the behavioral phenotype of stargazin V143L *knock-in* mice suggest that stargazin plays an important role in the regulation of mechanisms underlying neuropsychiatric disorders.

RESUMO

As doenças psiquiátricas, incluindo défice intelectual e distúrbios de humor e do foro afetivo, afetam cerca de um quarto da população mundial. A esquizofrenia e o défice intelectual têm sido consistentemente associados a défices cognitivos e sociais, putativamente consequentes de alterações na função sináptica. No entanto, apesar da identificação de inúmeros genes predisponentes para doenças psiquiátricas, as alterações subjacentes a estas doenças são ainda pouco claras.

As redes sinápticas têm sido cada vez mais reconhecidas como importantes intervenientes no desenvolvimento da esquizofrenia, défice intelectual e outras doenças psiquiátricas. De facto, dados recentes atribuem uma grande relevância a mutações *de novo* em genes codificantes de proteínas constituintes da sinapse glutamatérgica, com funções importantes na modulação da força sináptica. Estes estudos reforçam a idéia de que genes afetados por mutações em esquizofrenia coincidem parcialmente com os afetados em autismo e défice intelectual (Fromer et al., 2014; Hamdan et al., 2011; Kirov et al., 2012; Krumm et al., 2014; Network and Pathway Analysis Subgroup of Psychiatric Genomics, 2015; Purcell et al., 2014; Schizophrenia Working Group of the Psychiatric Genomics, 2014), sugerindo que estas doenças possam partilhar uma causa: a disfunção sináptica. Assim, o estudo de mutações raras e com alta penetrância, através de animais modelo que são portadores dessas mutações, pode contribuir grandemente para a compreensão dos mecanismos moleculares e alterações nos circuitos neuronais que estão implicados nestas doenças.

O gene *CACNG2* que codifica para a stargazina foi apontado como potencial gene de susceptibilidade para doenças psiquiátricas (Liu et al., 2008; Nissen et al., 2012; Wilson et al., 2006; Yang et al., 2013). A stargazina é uma subunidade auxiliar dos receptors AMPA que modula a sua função e é essencial para o tráfego destes para a superfície celular assim como para o escalamento de receptores AMPA mediado por bloqueio da actividade. Com recurso a sequenciação de genoma inteiro identificámos uma variante do gene *CACNG2* num doente esquizofrénico (Stg^{SCZ}). Observámos que a variante Stg^{SCZ}, e também outra variante, previamente identificada num doente com défice intelectual (Stg^{ID}) apresentam alterações nas propriedades de difusão na membrana e mostraram-se deficientes na mediação do tráfego de receptores AMPA para as sinapses. Além disto, a variante Stg^{ID} impede a mediação de alterações homeostáticas enquanto que a variante Stg^{SCZ} influencia o número de sinapses inibitórias e a complexidade da arborização dendrítica em neurónios corticais. Os nosso resultados mostram que diferentes mutações no gene *CACNG2* dão origem a efeitos distintos na função da proteína, contribuindo para diferentes fenótipos celulares e possivelmente comportamentais.

De modo a aprofundar o estudo dos efeitos da mutação associada a défice intelectual na função da stargazina e também no desenvolvimento de fenótipos correlacionados com doença, gerámos e caracterizámos uma linhagem de murganhos portadores da mutação associada a défice intelectual no gene *CACNG2*, V143L. A avaliação da actividade locomotora, ansiedade e desempenho cognitivo mostrou que os murganhos portadores da mutação em homozigotia apresentam uma maior actividade locomotora, défices na alternação espontânea, na aprendizagem motora e na elaboração de ninhos.

No geral, o efeito destas mutações no gene *CACNG2* nos mecanismos celulares e fenótipo comportamental dos animais que são portadores da mutação V143L sugere um papel para a stargazina na regulação de mecanismos subjacentes ao desenvolvimento de doenças psiquiátricas.

CHAPTER 1

Introduction

1.1. Psychiatric disorders

Mental disorders affect approximately one quarter of the worldwide population and thus represent a major social and economic burden for most countries (Trautmann *et al.*, 2016). As chronic disorders and often shrouded by stigma and prejudice, they severely impact the lives of patients and their families. Psychiatric disorders such as depression, bipolar disorder, intellectual disability (ID), schizophrenia (SCZ) and autism spectrum disorders (ASD) are often associated to the presentation of abnormal thoughts, impaired memory and learning, social and self-care deficits.

Although a growing number of studies focus on understanding their etiology and on pinpointing the mechanisms that stem these disorders, these aspects are still obscure. These difficulties originate mainly from the fact that neuropsychiatric disorders are complex and heterogeneous, presenting variable neurological symptoms that often overlap between different disorders (Kirov *et al.*, 2008), as well as variable onset and pharmacological response. Furthermore, many of them are characterized by a strong genetic component that rarely relies on major effects from a small set of genes but rather on the sum of modest effects from a pool of genes that ultimately characterize the vulnerability to develop these disorders (Purcell *et al.*, 2014).

Recent evidence suggests that changes in genes encoding synaptic proteins that intervene or regulate neuronal transmission might participate in the pathology of these disorders. Furthermore, many of these alterations are common to different psychiatric disorders suggesting that they might share etiologic mechanisms (Fromer *et al.*, 2014; Purcell *et al.*, 2014).

1.1.1. Schizophrenia

SCZ is a devastating disorder that affects about 1% of the worldwide population. Studies including twins and adoption cases have demonstrated that this disorder shows a high heritability (about 80% in monozygotic twins) (Sullivan *et al.*, 2003), emphasizing the importance of genetics on its etiology. However, environmental factors also play an important role as several factors have been described to influence the onset and severity of symptoms (van Os *et al.*, 2010).

SCZ symptoms are divided in three main categories: positive (delusions, hallucinations, disorganized speech), negative (social withdrawal, affective flattening and anhedonia) and cognitive symptoms (lack of prioritization, focus or motivation, decreased verbal fluency). According to the last edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), for a SCZ diagnosis, two of these symptoms must be present for at least six months and include at least one month of active symptoms (American Psychiatry Association, 2013). Usually the first psychotic symptoms occurring in the late adolescence or early adulthood account for the first reference for a consultation, but a

decline in social and cognitive functioning is often observed much earlier (<10 years) in the prodromal phase of the disease (Gourzis *et al.*, 2002; Kahn *et al.*, 2015). Due to smoking, unhealthy diet and lifestyle, and also to the use of antipsychotic drugs, patients with SCZ usually have increased risk for obesity, diabetes and for the development of cardiovascular problems, which, along with the increased suicide attempt rate, combine to reduce life expectancy by approximately 20 years [reviewed in (Laursen *et al.*, 2014)].

The risk for onset and relapse of psychotic disorders such as SCZ is thought to be increased by psychological stress. Interestingly, the onset of SCZ has been highly correlated with stressful life events (Day *et al.*, 1987), suggesting an inability to handle changes and adapt. In agreement, a possible explanation for the late onset of SCZ is that cells and circuits are able to compensate for a certain amount of stimuli during a given period, before a threshold is reached and the homeostatic capacity of the network is exhausted (Ramocki and Zoghbi, 2008). An initial study involving around 40 patients with psychosis, relatives and controls showed that psychotic patients have greater sensitivity to everyday minor stress situations than controls, as an increase in psychosis intensity was associated with the occurrence of minor stressors in daily life (Myin-Germeys *et al.*, 2005). These results have recently been corroborated in larger studies (Klippel *et al.*, 2017). Accordingly, another study focusing on understanding how SCZ patients respond to life events during the first phases of the disease showed that SCZ patients recognize positive and negative life events as less controllable and poorly handled, comparing to controls (Horan *et al.*, 2005).

Disruption of neuronal homeostasis at a molecular level has been associated with neuropsychiatric phenotypes (Ramocki and Zoghbi, 2008). In fact, MECP2, a transcription factor that was shown to be indispensable for homeostatic plasticity (Zhong *et al.*, 2012), when disrupted can lead to the development of Rett syndrome, characterized by intellectual disability, anxiety and behavioral problems (Ramocki and Zoghbi, 2008). Also, several susceptibility genes for SCZ are required for homeostatic plasticity, including genes encoding ARC (Beique *et al.*, 2011), CaMKII (Groth *et al.*, 2011) and dysbindin (Dickman and Davis, 2009). Accordingly, an imbalance in the ratio of excitatory to inhibitory activity (E/I imbalance) has been suggested to be at the basis of SCZ. Either the hyperexcitability of pyramidal neurons or the hypoactivity of inhibitory neurons could elevate the E/I ratio leading to the behavior and cognitive symptoms of SCZ, including social dysfunction (Lisman, 2012). Environmental factors play a pivotal role in the etiology of SCZ. In fact, males have about 1.4 times greater probability of developing SCZ than females. Pre- and perinatal events including infections and malnutrition, obstetric complications, low birth weight, late fatherhood and maternal drug abuse have been described to influence the vulnerability to develop SCZ. [reviewed in (Kahn *et al.*, 2015)]. Socioeconomic factors have recently been associated to SCZ, and include childhood adversity, urban life and immigration. In fact, migrant individuals have an increased risk

of about 4.6 of developing SCZ (McGrath *et al.*, 2004). The observation of cognitive and social problems in children who later developed SCZ supports the neurodevelopmental theory of SCZ (Howes and Kapur, 2014). Interestingly, some of the risk factors for SCZ, such as in utero inflammation, caesarean section and social isolation have been correlated with increased concentration of dopamine in the striatum (Howes and Kapur, 2014).

Hypoxia prevails in the embryonic and fetal brain and is important for the regulation of the vascular growth induction in the embryo through the hypoxia induced factor 1 (HIF-1). Furthermore, around 1-2% of all genes are regulated by hypoxia. Thus, it has been hypothesized that hypoxia in prenatal and perinatal phases contributes to neurodevelopmental changes that later in life culminates in SCZ (Schmidt-Kastner *et al.*, 2006). In agreement with this theory is the implication of a large number of genes, regulated by hypoxia, in the etiology of SCZ. Among these genes are the *BDNF*, *COMT*, *DNTBP1*, *NRG1*, *RELN*, *TNF-alpha* and *RGS4* [reviewed in (Lang *et al.*, 2007; Schmidt-Kastner *et al.*, 2006)].

1.1.1.1. The dopamine hypothesis in SCZ

Several hypotheses regarding the pathogenesis of SCZ have been postulated. Most of them were proposed based on the observation of effects of therapeutic agents in the development of symptoms. The dopamine hypothesis of SCZ arose from the observations that administration of amphetamine and other compounds that increase dopamine concentration can induce psychotic symptoms, similar to those characteristic of SCZ. This hypothesis gained strength with the observation that antipsychotics drugs that are effective for some SCZ symptoms antagonize dopamine D2/D3 receptors (Creese *et al.*, 1976; Seeman *et al.*, 1976). Several genes that are closely related to the dopaminergic system have been implicated in SCZ, suggesting that alterations in the pathways where they participate might underlie the development of the disorder. The association between SCZ and the *DRD1* (Cichon *et al.*, 1996), *DRD2* (He *et al.*, 2016; Jonsson *et al.*, 2003), *DRD3* (Jonsson *et al.*, 2004), *DRD4* (Lung *et al.*, 2002) and *DRD5* (Muir *et al.*, 2001) genes, which code for dopamine receptors, has been addressed multiple times. Furthermore, polymorphisms in the gene encoding tyrosine hydroxylase, the rate-limiting enzyme for the biosynthesis of catecholamines, including dopamine and noradrenaline, have been associated with SCZ (Horiguchi *et al.*, 2014; Pae *et al.*, 2003). The catechol-O-methyltransferase (*COMT*) gene is localized in a chromosome region strongly implicated in SCZ, the 22q11 region. COMT is involved in the degradation of dopamine and is thus crucial for the regulation of its levels. One of the most studied polymorphisms in SCZ is the V158M polymorphism in the *COMT* gene, since it leads to increased catabolism of dopamine in the prefrontal cortex (PFC), impairing cognition and slightly increasing the risk for SCZ (Egan *et al.*,

2001). In addition, this polymorphism has been shown to predispose male schizophrenic carriers to aggressive behavior (Singh *et al.*, 2012). Polymorphisms in genes encoding the vesicular monoamine transporters 1 and 2 (VMAT 1 and 2) (Chu and Liu, 2010; Lohoff *et al.*, 2014; Lohoff *et al.*, 2008), the dopamine transporter (DAT) (Cordeiro *et al.*, 2010) and DARPP32 (Hu *et al.*, 2007) were also associated with increased risk for the disorder. Hyperactivity of the mesolimbic pathway, accompanied by hypoactivity in the mesocortical pathway are believed to account for the symptoms of SCZ (Brisch *et al.*, 2014). However, it has been proposed that the effects on the dopaminergic pathways might be a consequence from decreased GABAergic activity primarily in the limbic area and in the PFC resulting from the hypofunction of glutamate receptors of the N-methyl-D-aspartate (NMDA) type, NMDAR, occurring preferentially in these neurons (Adams *et al.*, 2013; Stahl, 2013). A representation of the main dopaminergic pathways implicated in SCZ is shown in

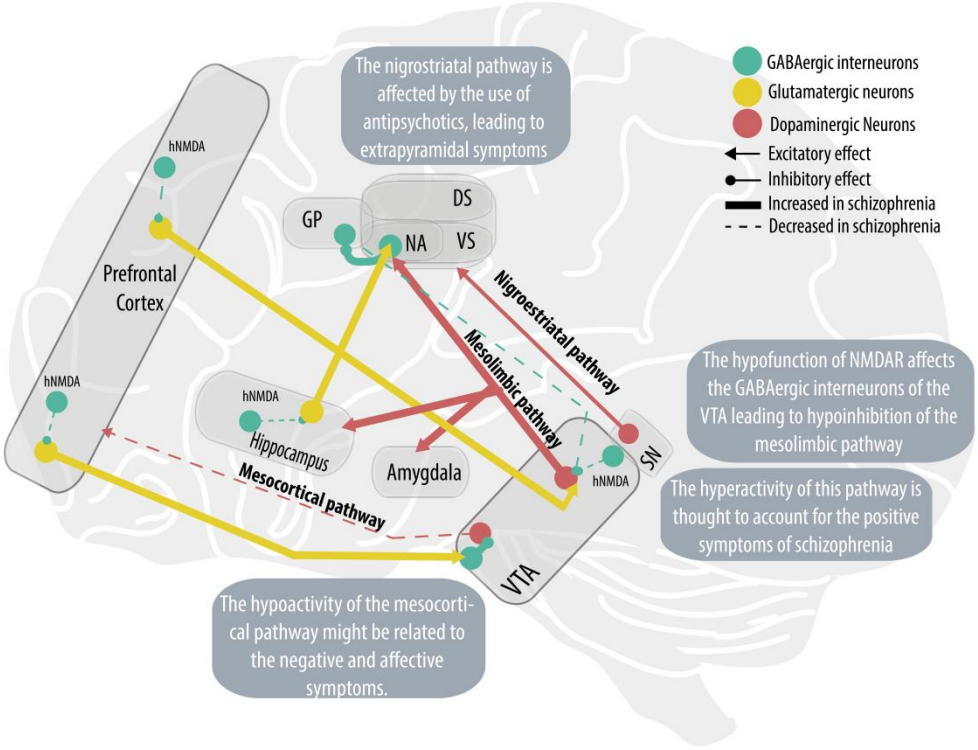


Figure 1.1.

Figure 1.1. The dopamine circuits of SCZ. Hyperactivity of the mesolimbic pathway, which projects from the VTA to the limbic structures, is thought to account for the positive symptoms of SCZ. Selective hypofunction of the NMDAR of the GABAergic neurons ultimately leads to decreased inhibition of the glutamatergic neurons that regulate the activity of the mesolimbic pathway and increased inhibition of the mesocortical pathway, which projects from the VTA to the PFC. The nigrostriatal pathway, which projects from the SN to the striatum, is affected by antipsychotic drugs, which leads to extrapyramidal symptoms such as dyskinesia and parkinsonism. VTA – ventral tegmental area; NA – nucleus accumbens; PFC – prefrontal cortex; GP – globus pallidus; SN – substantia nigra; DS – dorsal striatum; VS – ventral striatum; hNMDA – NMDAR hypofunction. Adapted from (Adams *et al.*, 2013; Stahl, 2013).

1.1.1.2. The glutamate hypothesis in SCZ

Phencyclidine (PCP), ketamine and MK-801, which are potent antagonists of the NMDAR, lead to the development of SCZ-like symptoms in healthy controls, exacerbate the symptoms in SCZ patients and increase the release of dopamine (Allen and Young, 1978; Barbon *et al.*, 2007; Coyle *et al.*, 2003; Lahti *et al.*, 2005). These observations have contributed to the postulation of the NMDAR hypofunction hypothesis of SCZ. According to this line of thought, the defective functioning of the NMDAR might underlie some clinical symptoms of SCZ and be at the basis of the observed dopaminergic dysfunction (Figure 1.1). In agreement with the NMDAR hypofunction hypothesis is the observation of an amelioration of SCZ symptoms with the use of activators of glycine and D-serine, which are NMDAR co-agonists (Heresco-Levy and Javitt, 2004), and with supplementation with D-serine (Fujita *et al.*, 2016; Kantrowitz *et al.*, 2010).

Evidence shows that NMDAR hypofunction affects preferentially GABAergic interneurons. A reduction of 73% in GABAergic neurons expressing the GluN2A subunit of NMDAR was observed in *postmortem* anterior cingulate cortex samples from SCZ patients (Woo *et al.*, 2004). The inhibition of NMDAR with ketamine resulted in a reduction of GAD67 expression in cultured cortical neurons and of the inhibitory synaptic transmission in prefrontal cortex slices (Cohen *et al.*, 2015). Furthermore, the ablation of NMDAR from cortical and hippocampal interneurons in mice, resulted in disinhibition of cortical excitatory neurons, and reduced neuronal synchrony. Also, SCZ-like phenotypes were noted, including hyperlocomotion, mating and nesting deficits, anxiety-like behaviors and impairments in social and working memory (Belforte *et al.*, 2010). These observations corroborate the hypothesis that NMDAR hypofunction in interneurons might contribute to SCZ pathology. However, what leads to NMDAR hypofunction remains elusive. Impaired NMDAR activity could result from a primary defect on NMDAR or be secondary to defects on synaptic proteins and receptors that regulate NMDAR-mediated signaling.

NMDAR are ionotropic glutamate receptors, composed of two obligatory GluN1 subunits and two GluN2 (GluN2A, GluN2B, and GluN2C) and/or GluN3 (GluN3A and GluN3B) subunits. Interestingly, the expression of some of these subunits has been found altered in samples from SCZ patients brains [see (Beneyto and Meador-Woodruff, 2008; Weickert *et al.*, 2013) for evidence of decreased expression of GluN1, GluN2A and GluN2C and (Mueller and Meador-Woodruff, 2004) for increased expression of GluN3A]. Furthermore, modifications in genes that encode the subunits of NMDAR have also been implicated in the disorder (Itokawa *et al.*, 2003; Iwayama-Shigeno *et al.*, 2005; Liu *et al.*, 2015; Ohtsuki *et al.*, 2001; Qin *et al.*, 2005; Tang *et al.*, 2006).

In agreement with a specificity for NMDAR hypofunction in inhibitory neurons is the observation that synaptic targeting of NMDAR in hippocampus pyramidal neurons and in excitatory synapses of interneurons relies on different mechanisms. Interestingly, in interneurons, NMDAR synaptic

targeting is dependent on both AMPAR containing the GluA2 subunit and on the AMPAR auxiliary protein stargazin (Mi *et al.*, 2004).

The implication of AMPAR altered function, expression or localization as a possible trigger of NMDA hypofunction has been proposed; however, the results regarding the alteration of AMPAR expression and function in SCZ *postmortem* brain samples are still inconclusive and often contradictory. Regarding the prefrontal cortex, a brain region implicated in SCZ, some studies reported no changes in the mRNA levels of these receptors (Healy *et al.*, 1998), whereas others describe decreased (Beneyto and Meador-Woodruff, 2006) or increased (Dracheva *et al.*, 2005) expression. The alterations in the protein levels of these subunits are not consensual either, and both decrease (Eastwood *et al.*, 1997), increase (Tucholski *et al.*, 2013), and no changes (Breese *et al.*, 1995; Hammond *et al.*, 2010) have been observed. These discrepancies, however, may come from differences in sample processing, detection methods and brain regions analyzed.

Interestingly, it has been shown that the N-glycosylation of AMPAR GluA2 subunits is impaired in SCZ, suggesting abnormal trafficking of AMPAR from the endoplasmic reticulum (ER) to the cell surface (Tucholski *et al.*, 2013). Immature glycosylation of GluA2 subunits has also been previously described in *stargazer* mice, which lack stargazin (Tomita *et al.*, 2003). Furthermore, an increase in AMPAR GluA1 subunit expression in endosomes was observed in the brains of schizophrenics, compatible with increased endocytosis of these receptors (Hammond *et al.*, 2010).

Metabotropic glutamate receptors have also been implicated in SCZ; in fact, a selective agonist for metabotropic glutamate receptors 2/3 was the first drug for SCZ not acting as a dopamine antagonist to be developed, and besides ameliorating the positive and negative symptoms in SCZ, it does not show some adverse effects typically present with the conventional antipsychotics (Patil *et al.*, 2007). Accordingly, altered mRNA and protein levels of these receptors have been described in SCZ (Ghose *et al.*, 2009; Gupta *et al.*, 2005; Volk *et al.*, 2010).

Many other proteins that play a major role in glutamatergic transmission show altered expression levels in *postmortem* brain tissue from SCZ patients, supporting the hypothesis of an important role for glutamate in the pathogenesis of this disorder. Some of these proteins are indicated in Table 1.1.

1.1.1.3. The synaptic hypothesis in SCZ

As previously mentioned, SCZ is a highly complex disorder with a strong genetic component. Genetic studies mainly rely on linkage and association studies. Linkage studies consist on the evaluation of large pedigree samples aiming at identifying chromosomal regions that are likely to contain genes associated to a given disorder. Association studies search for genetic polymorphisms

that putatively affect the expression and/or function of a gene associated to the disorder (Lang *et al.*, 2007).

Table 1.1. Proteins implicated in glutamatergic transmission displaying altered expression in SCZ patients *postmortem* tissue

Protein	Alteration	Brain Region	Molecule	Reference
DAAO	↑	Cerebellum	mRNA	(Kapoor *et al.*, 2006; Verrall *et al.*, 2007)
		PFC and hippocampus	Protein	(Bendikov *et al.*, 2007)
Dysbindin	↓	DLPFC	mRNA	(Weickert *et al.*, 2004)
G72	↑	DLPFC	mRNA	(Korostishevsky *et al.*, 2004)
GRIP	↑	PFC, occipital cortex	mRNA	(Dracheva *et al.*, 2005)
		DLPFC	Protein	(Hammond *et al.*, 2010)
SAP197	↑	DLPFC	Protein	(Hammond *et al.*, 2010)
Serine racemase	↑	Hippocampus	Protein	(Steffek *et al.*, 2006)
	↓	PFC and hippocampus	Protein	(Bendikov *et al.*, 2007)
Vglut1	↓	PFC and hippocampus	mRNA	(Eastwood and Harrison, 2005)
	↑	ACC	mRNA	(Oni-Orisan *et al.*, 2008)
	↓	ACC	Protein	(Oni-Orisan *et al.*, 2008)

Upward or downward oriented arrow represents increased or decreased expression, respectively (Column 2) mRNA – messenger RNA, DLPFC – dorsal lateral prefrontal cortex, ACC – anterior cingulate cortex, PFC – prefrontal cortex.

Growing evidence from genetic studies supports a role for synaptic proteins in the etiology of SCZ. In fact, a genome-wide association study (GWAS) including data from over 60.000 participants implicated synaptic genes in the pathogenesis of SCZ (Network and Pathway Analysis Subgroup of Psychiatric Genomics, 2015). Furthermore, recent reports attributed a major importance to *de novo* mutations in genes that encode glutamatergic postsynaptic proteins with roles in the modulation of synaptic strength. In the study conducted by Purcell and colleagues, mutations are enriched in genes of the voltage-gated calcium ion channel, genes involved in the signaling complex of the activity-regulated cytoskeleton-associated scaffold protein (ARC) post-synaptic density, genes of the PSD95 signaling complex, as well as targets of the FMRP protein, implicated in fragile X (Purcell *et al.*, 2014). Fromer and colleagues corroborated these observations by showing that *de novo* mutations are overrepresented in glutamatergic synapse genes including genes related to ARC signaling, targets of the FMRP complex and also genes coding proteins in the NMDAR complex. Importantly, many of the genes with *de novo* mutations identified in this study overlap those affected by *de novo* mutations in other psychiatric disorders such as ASD and ID (Figure 1.2) (Fromer *et al.*, 2014). Table 1.2 shows some genes implicated in SCZ.

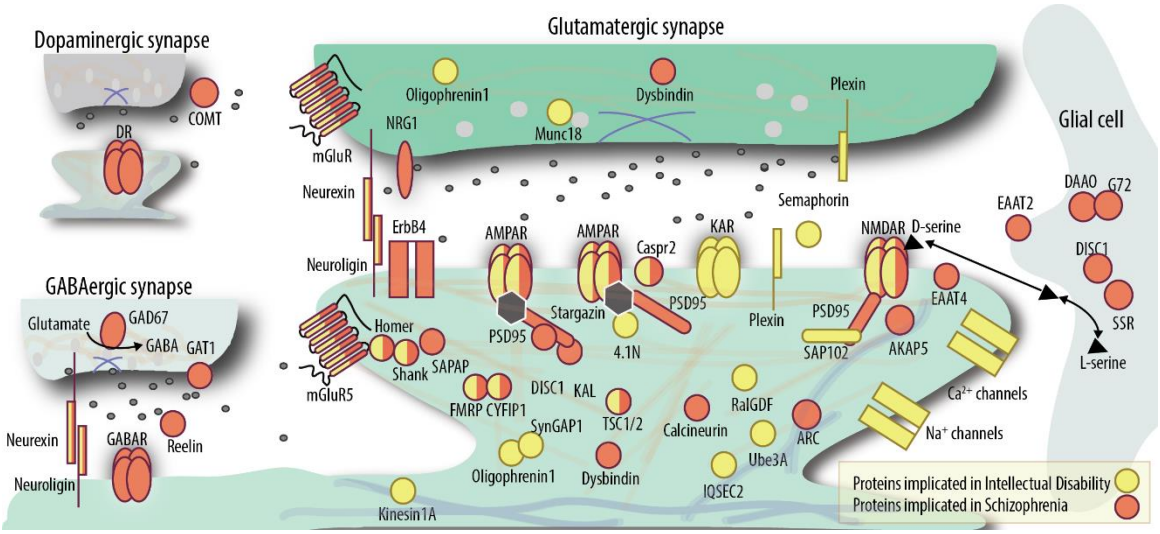


Figure 1.2. Synaptic proteins involved in SCZ and ID. Genes encoding for glutamatergic synapse proteins have been implicated in the development of SCZ. Many of those genes overlap in different psychiatric disorders, including ASD and ID. The figure represents a dopaminergic, a GABAergic and a glutamatergic synapse where some proteins implicated in SCZ (red) and ID (yellow) are depicted. DR – dopamine receptor, COMT – Catechol-O-methyltransferase, mGluR – metabotropic receptor, GABAR – GABA receptor, AMPAR – AMPA receptor, NMDAR – NMDA receptor, KAR – Kainate receptor, NRG1 – Neuregulin 1, ERBB4 – Erb-B2 Receptor tyrosine kinase 4, DISC1 – disrupted in schizophrenia 1, TSC – tuberous sclerosis, IQSEC2 – IQ motif and sec7 domain 2, FMRP – fragile X mental retardation protein, KAL – kalirin, CYFIP1 – cytoplasmic FMRP interacting protein 1, SSR – serine racemase, Ube3A – ubiquitin protein ligase E3A, EAAT – excitatory amino acid transporter, ARC – activity regulated cytoskeleton protein, DAAO – D-amino acid oxidase, RalGEF - Ral guanine nucleotide dissociation stimulator, AKAP5 – A-kinase anchoring protein 5.

The identification of a common pathway in SCZ and especially between psychiatric disorders is the utopia of genetic studies. Such discovery would enable the establishment of therapeutic strategies suiting patients with classical to complex manifestations of these disorders. However, despite the immensurable efforts being made in the field, key issues regarding these disorders remain obscure. Although numerous genes have been implicated in these disorders, different kinds of genetic studies have contributed to their identification. While many common SNPs and CNVs have been identified for a number of genes, other genes were associated to these disorders by linkage studies or even by the identification of rare *de novo* variants, urging the assessment of the net effect of these variants, or even of the involved pathways, for the resultant disorder (Hall *et al.*, 2015).

Although growing evidence ascertains a pivotal role for synaptic plasticity genes in psychiatric disorders, the specific mechanisms impacted or the degree of the alterations in the synaptic function in individuals carrying different mutations are questions that need to be answered before therapeutic strategies can be implemented. The identification of rare but penetrant variants poses a good chance into tackling these issues (Hall *et al.*, 2015). The generation of animal models bearing these mutations contributes largely to the elucidation of mechanisms and circuits implicated in

disease as these animal models allow studying the correlation between changes in mutated proteins function and phenotypes that are associated to psychiatric disorders.

Table 1.2. Genes implicated in SCZ

Gene	Protein	Function	Alteration	Reference
AKAP5	A-kinase anchoring protein 5.	Binds to and regulates PKA	CNV	(Wilson *et al.*, 2006)
ARC	Arg 3.1	Activity Regulated Cytoskeleton Associated Protein. Regulates activity-dependent endocytosis of AMPAR and homeostatic plasticity	SNP	(Huentelman *et al.*, 2015)
CACNA1C	Calcium Voltage-Gated Channel Subunit Alpha1 C.	Involved in calcium-dependent processes namely neurotransmitter release.	SNP	(Cross-Disorder Group of the Psychiatric Genomics, 2013)
CACNB2	Calcium Voltage-Gated Channel Auxiliary Subunit Beta 2	Member of the voltage-gated calcium channel superfamily	SNP	(Cross-Disorder Group of the Psychiatric Genomics, 2013)
CNTNAP2	Caspr2	Neurexin-like; cell adhesion molecule	CNV	(Friedman *et al.*, 2008)
COMT	Catechol-O-methyltransferase	Participates in catecholamine degradation	SNP	(Straub *et al.*, 2007)
CYFIP1	Cytoplasmic FMRP interacting protein 1	Mediates translation repression	CNV	(Stefansson *et al.*, 2008)
DAAO	D-amino acid oxidase	Degrades D-serine	SNP	(Yang *et al.*, 2013)
DISC1	Disrupted in SCZ 1	Neurite outgrowth and cortical development	Translocation	(Millar *et al.*, 2000)
DLG2	PSD93	Post-synaptic scaffold protein	CNV	(Walsh *et al.*, 2008)
DLGAP2	SAPAP2	Synapse organization and signaling	SNP	(Li *et al.*, 2014)
DTNBP1	Dysbindin	Organelle biogenesis	SNP	(Funke *et al.*, 2004)
ERBB4	Erb-B2 Receptor Tyrosine Kinase 4	Member of tyr protein kinase family	CNV	(Walsh *et al.*, 2008)
G72	D-Amino Acid Oxidase Activator	DAAO oxidase activator – D-serine degradation.	SNP	(Korostishevsky *et al.*, 2004)
GABRA1	GABARα1	$GABA_AR$ subunit	SNP	(Petryshen *et al.*, 2005)
GABRA6	GABARα6	$GABA_AR$ subunit	SNP	(Petryshen *et al.*, 2005)
GABRP	GABARπ	GABAR subunit	SNP	(Petryshen *et al.*, 2005)
GAD1	GAD67	Glutamic acid decarboxylase	SNP	(Straub *et al.*, 2007)
KALRN	Kalirin	Rho-GEF kinase	SNP	(Kushima *et al.*, 2012) (Russell *et al.*, 2014)
NRG1	Neuregulin 1	Mediates cell-cell signaling	Missense mutation	(Walss-Bass *et al.*, 2006)
NRXN1	Neurexin 1	Cell surface receptors that bind neuroligins	CNV	(Kirov *et al.*, 2008) (Rujescu *et al.*, 2009)
PPP3CC	Calcineurin	Phosphatase	SNP	(Liu *et al.*, 2007)

RELN	Reelin	Extracellular matrix protein that controls cell-cell interaction	SNP Missense mutation	(Ovadia and Shifman, 2011) (Zhou *et al.*, 2016b)
SHANK3	Shank3	Post-synaptic scaffold protein	Nonsense mutation	(Zhou *et al.*, 2016a)
SLC1A2	EAAT2	Glutamate transporter	SNP	(Deng *et al.*, 2004)
SLC1A6	EAAT4	Glutamate transporter	SNP	(Deng *et al.*, 2007)
SSR	Serine racemase	Conversion of L-serine to D-serine	SNP	(Morita *et al.*, 2007)

The name and function of the proteins is in accordance with the Genecard Human gene database (http://www.genecards.org/). CNV – copy number variation, SNP – single nucleotide polymorphism.

1.1.2. Intellectual disability

Intellectual disability (ID) is the most common neurodevelopmental disorder and affects 1 to 3% of the worldwide population. It is much more prevalent in males than females partially due to the fact that some of the syndromic forms of ID are X-linked. ID is an early-onset disorder that comprises a large variety of non-syndromic and syndromic conditions that present impairments in adaptive function in three domains: conceptual (language, reading, writing and memory, among others), social (empathy, interpersonal communication skills, etc.) and practical (personal care, job responsibilities and organization). Thus, the diagnosis is based on the assessment of adaptive function and also takes into consideration the IQ, which is considered low when the score is approximately two standard deviations below the population, that is, about 70 (American Psychiatry Association, 2013). An IQ below 50 is presented by moderate to severe ID patients (Chelly *et al.*, 2006).

The etiology of most cases of ID is unknown; however, advances in microarray and genome sequencing techniques allowed the identification of chromosomal abnormalities and point mutations in about 25% of the ID patients. Around 15% of the cases are estimated to be due to environmental insults such as prenatal malnutrition, premature birth, neurotoxicity, brain ischemia, infections and fetal alcohol syndrome (Chelly *et al.*, 2006).

Non-syndromic ID patients display deficits in cognitive function without presenting other symptoms, whereas for syndromic ID other features such as autism, epilepsy and dysmorphism are concomitant with intellectual deficit (Kaufman *et al.*, 2010; Volk *et al.*, 2015). Syndromic forms of ID are widely studied disorders and the alterations underlying the pathophysiology of most of them have been unraveled. Importantly, the recognition of these processes has contributed to the identification of mechanisms that play important roles in cognition. The following syndromes are highly incident in the general population and present ID as a major symptom:

Fragile X syndrome (FXS) is the most common form of inherited ID. It is characterized by macroorchidism in males, large ears and hands and asymmetric facial features. As an X-linked condition, it affects more severely males, who present hyperactivity, anxiety and autistic-like phenotypes (Zoghbi and Bear, 2012). This syndrome is caused by the loss of function of the FMRP protein as a consequence of *FMR1* gene silencing through promoter hypermethylation. FMRP interacts with mRNAs encoding important synaptic proteins, including PSD95, CaMKII, AMPAR subunits, ARC and mGluR5,and serves as a negative regulator of translation of several mRNA transcripts. It has been suggested that the lack of repression of mGluR5 signaling by FMRP, deregulates protein synthesis downstream of mGluR5 leading to FXS. A common feature of FXS is the increased density of immature spines suggesting a role for FMRP in both pruning and maturation of synapses (Volk *et al.*, 2015; Zoghbi and Bear, 2012).

Angelman syndrome patients display balance problems, behavior abnormalities, hyperactivity and dysmorphic features. The majority of the cases are a consequence of maternal deletion of a locus containing the ubiquitin protein ligase E3A (UBE3A) gene. This gene encodes for a ubiquitin ligase that is pivotal in activity-dependent proteasomal protein degradation (Volk *et al.*, 2015; Zoghbi and Bear, 2012). On the other hand, a common CNV found in autism patients that results in the duplication or triplication of a chromosome region including the UBE3A gene has been shown to affect the glutamatergic transmission in mice and to induce autism-related behavioral traits (Smith *et al.*, 2011).

Tuberous sclerosis is characterized by the presence of benign tumors and epilepsy. It is caused by mutations in the TSC1 and TSC2 genes, which encode for harmartin and tuberin, respectively. These proteins form a complex that negatively regulates the mTOR pathway (Zoghbi and Bear, 2012).

Rett syndrome is an X-linked form of ID characterized by autistic-like behaviors, motor impairments, epilepsy and cognitive deficits. It is caused by mutations in the *MECP2* gene, which encodes a transcription factor that regulates transcription.

The advances in sequencing technology enabled the identification of many genes associated with non-syndromic ID. Among those genes are 1) the *SYNGAP1* gene, which encodes a Ras-GAP protein that localizes to the dendritic spines where it suppresses NMDAR-mediated synaptic plasticity and AMPAR trafficking (Clement *et al.*, 2013); 2) *OPHN1* which encodes a Rho-GAP (oligophrenin1) that inhibits RhoA, Rac1 and Cdc42 (Khelfaoui *et al.*, 2007) and regulates AMPAR trafficking by interacting with components of the clathrin-mediated endocytosis machinery. The loss of oligophrenin1 impairs synapse and spine maturation (Nadif Kasri *et al.*, 2009); 3) *STXBP1* which encodes for Munc18, a protein that regulates vesicle release; genetic ablation of *STXBP1* results in blockade of neurotransmitter release (Toonen *et al.*, 2006).

Many other genes implicated in syndromic and non-syndromic ID encode proteins with major roles in synaptic transmission (Table 1.3).

Table 1.3. Genes implicated in ID

Gene	Protein	Function	Alteration	Reference
CACNA1G	Cav3.1c	Low voltage-gated Ca^{2+} channel	SNP	(Najmabadi *et al.*, 2011)
CNTNAP2	Caspr2	Neurexin-like; cell adhesion mlecule	SNP	(Zweier, 2012)
DGL3	SAP-102	Membrane-associated guanylate kinase (MAGUK); regulates NMDAR		(Tarpey *et al.*, 2004)
EPB41L1	4.1N	Neuronal cytoskeleton - regulation of AMPAR	SNP	(Hamdan *et al.*, 2011)
GRIA3	GluA3	AMPAR subunit	Translocation Microdeletion SNP	(Wu *et al.*, 2007) (Gecz *et al.*, 1999)
GRIK2	GluK6	Kainate receptor subunit	Deletion	(Motazacker *et al.*, 2007)
GRIN1	GluN1	NMDAR subunit	SNP	(Hamdan *et al.*, 2011)
GRIN2A	GluN2A	NMDAR subunit	SNP	(Endele *et al.*, 2010)
GRIN2B	GluN2B	NMDAR subunit	SNP	(Endele *et al.*, 2010)
IQSEC2	IQ Motif And Sec7 Domain 2	guanine nucleotide exchange factor for the ARF family of small GTP-binding proteins	SNP	(Shoubridge *et al.*, 2010)
KIAA2022	KIAA2022	Neuronal development	SNP	(Athanasakis *et al.*, 2014)
NRXN1	Neurexin1	Neurexin-neuroligin complexes – synaptic contacts	SNP	(Camacho-Garcia *et al.*, 2012; Zweier *et al.*, 2009)
OPHN1	Oligophrenin 1	Rho-GTPase activating protein	Deletion	(Bergmann *et al.*, 2003)
PLXNA3	Plexin A3	Receptor for Semaphorins	SNP	(Athanasakis *et al.*, 2014)
PLXNB3	Plexin B3	Receptor for Semaphorins	SNP	(Athanasakis *et al.*, 2014)
RALGDS	RalGEF	Effector of Ras-related RalA and RalB GTPase	SNP	(Najmabadi *et al.*, 2011)
SCN2A	Na(V)1.2	Voltage-gated Na^+ channel	SNP	(Rauch *et al.*, 2012)
SEMA5A	Semaphorin 5A	Bifunctional axonal guidance cue	Microdeletion	(Mosca-Boidron *et al.*, 2016)
SHANK2	Shank2	Scaffold protein in glutamatergic synapses	CNV, SNP	(Berkel *et al.*, 2010)
SHANK3	Shank3	Scaffold protein in glutamatergic synapses	SNP	(Hamdan *et al.*, 2011)
STXBP1	Munc18	Regulation of SNARE complex	SNP	(Hamdan *et al.*, 2011; Hamdan *et al.*, 2009b; Rauch *et al.*, 2012)
SYNGAP1	SynGAP	Ras GTPase activating protein	SNP	(Hamdan *et al.*, 2009a; Rauch *et al.*, 2012)
TET1	Methylcytosine dioxygenase	DNA methylation and gene activation	SNP	(Harripaul *et al.*, 2017)

The name and function of the proteins is in accordance with the Genecard Human gene database (http://www.genecards.org/). CNV – copy number variation, SNP – single nucleotide polymorphism.

1.1.3. Shared genetics of psychiatric disorders

Presently, the diagnosis of psychiatric disorders is based solely in the symptoms displayed by patients (American Psychiatry Association, 2013); however, there is considerable heterogeneity within diagnostic categories and patients often display clinical features from more than one disorder. Furthermore, the symptoms presented by an individual at the date of the diagnosis may vary substantially along time and follow completely distinct routes for different patients (O'Donovan and Owen, 2016).

The observation of increased risk for developing a psychiatric disorder for the relatives of affected individuals is in agreement with the reports that many genetic associations overlap among different psychiatric disorders, pointing for biologic pleiotropy (Fanous *et al.*, 2012; Fromer *et al.*, 2014; Porteous, 2008; Zhou *et al.*, 2016a). One study involving around 20 000 SCZ patients where ID CNVs were analyzed showed that a large proportion of the ID loci are probable risk factors for SCZ (Rees *et al.*, 2016). One possibility to overcome the difficulties in the diagnosis of patients with a complex pattern of symptoms, likely reflecting the presence of a pool of genetic alterations that are common among different disorders, is the establishment of a stratification strategy based on endophenotypes. It could be useful to define endophenotypes that cluster groups of clinical symptoms or other biomarkers, such as measures of cognition, brain structure, electrophysiology and biochemical characteristics, among others. This stratification would likely contribute to the development of more personalized and thus efficient therapeutic strategies (Figure 1.3). This pleiotropy also poses a problem for the study of cellular or animal models and requires awareness and caution concerning the correlation between the observed phenotypes and specific disorders. The next section focuses on the importance of mouse models for the study of psychiatric disorders.

1.1.4. Mouse models of psychiatric disorders

Given the complexity of most psychiatric disorders, finding a single disease model that recapitulates all the symptoms and particularities of a disorder is almost utopic. Nonetheless, animal models for these and other types of disorders have had an immensurable value in the elucidation of the mechanisms and players that intervene in the etiology of these disorders. Animal models have allowed the unravelling of protein function, the correlation between circuits and specific phenotypes and the validation of therapeutic approaches.

There are countless different models for psychiatry disorders. From systems biology tools to cell cultures and animal models, each offers different approaches and advantages that suit specific goals. With the advance of genetic tools, the generation of mouse models harboring mutations in

a specific gene or set of genes has become routine. As complex organisms, mouse models enable the study of synapses, circuits and behaviors, providing valuable information regarding protein function, regulatory mechanisms and for the design of therapeutic strategies (Mitchell *et al.*, 2011).

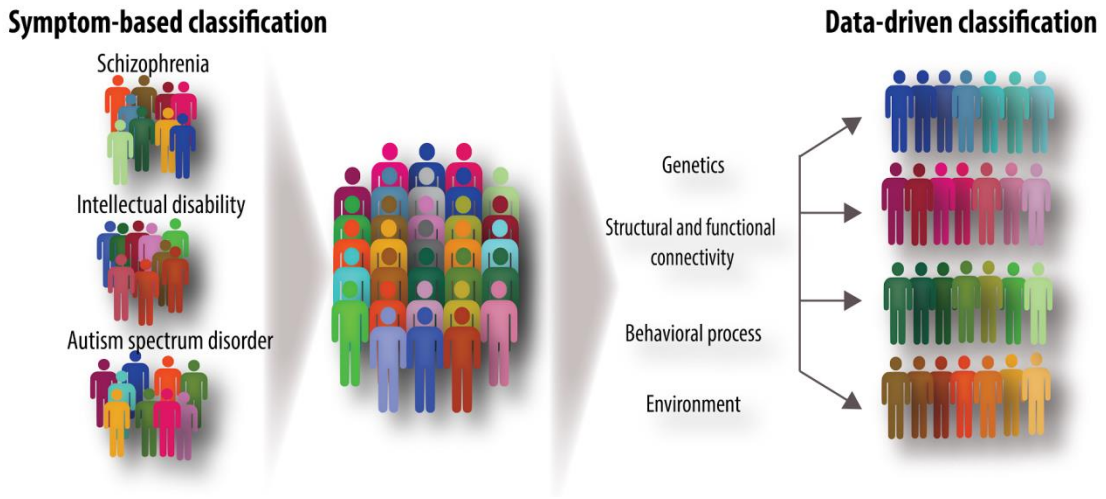


Figure 1.3. Data driven classification. The diagnosis of psychiatric disorders currently relies solely on the symptoms displayed by the patients. However, recent evidence suggest a significant genetic overlap between disorders. The consideration of other factors for diagnosis, such as genetics, molecular and circuitry alterations and also the life experience of each patient would contribute to the establishment of a more personalized stratification, allowing the development of more personalized and efficient therapies (Insel and Cuthbert, 2015)

Mouse models for SCZ fit generally into four categories: 1) induced by changes during development, 2) induced by drugs, 3) induced by lesions in specific brain regions and 4) induced by genetic manipulation (Jones *et al.*, 2011). One example of a neurodevelopmental model is the gestational MAM (methylazoximethanol) model. This antimitotic drug, when administered to pregnant rats affects brain development in a manner that is dependent on the gestational day. MAM animals present hyperactivity, reduced social interaction and deficits in the prepulse inhibition test (PPI) (Jones *et al.*, 2011). The prepulse inhibition is a natural response in which a prestimulus (weaker) diminishes the startle reflex to a subsequent, stronger stimulus. This adaptation is impaired in SCZ patients and the PPI is therefore a common test used in animal models of SCZ (Braff *et al.*, 1978).
Another developmental model is the post-weaning isolation model in which the animals are separated from their littermates after weaning, for long periods. Given the importance of social hierarchy and interaction for normal development, social deprivation leads to changes in neuronal development. These animals show deficits in the novel object recognition (NOR) test, spatial learning and increased social interaction (Jones *et al.*, 2011).

Post-development administration of drugs such as amphetamines or PCP (NMDAR antagonist) recapitulates some features of psychiatric disorders such as psychosis and cognitive impairment, which corroborates the involvement of both dopaminergic and glutamatergic systems in SCZ. Besides PPI impairment, these animals also display deficits in NOR and T-maze delayed alternation (Jones *et al.*, 2011).

Lesion models have largely contributed to the elucidation of the role of different brain regions in specific functions (Alstott *et al.*, 2009). The most common lesion model is the neonatal ventral hippocampal lesion by the injection of ibotenic acid, developed as a correlate for the changes in hippocampus observed in first-episode SCZ patients (Jones *et al.*, 2011).

The animal models for ID also include models generated by changes during development, induced by brain lesions and genetic models. ID models can be generated by changes induced during development through administration of MAM or maternal exposure to teratogenics such as alcohol. Brain lesions at early development are usually induced by hypoxia or by status epilepticus, leading to prolonged epileptogenesis, behavioral deficits and hyperexcitability of hippocampal networks (Scorza and Cavalheiro, 2011).

Finally, genetic models represent the vast majority of the models for ID and SCZ. Numerous genes implicated in these disorders have been modified in order to recapitulate missense or nonsense mutations, identified in patients or families. Furthermore, the deletion or overexpression of genes allow the inference of a gene product function, and implication in disease-related mechanisms and behaviors. Some genetic mouse models for synaptic genes implicated in SCZ and ID and referred in Tables 1.2 and 1.3, are indicated in Table 1.4.

Table 1.4. Genetic mouse models of ID and SCZ

Gene	Type of modification	Phenotype	Reference
CAMK2A	Missense mutation KI	Increased dendritic arborization, decreased spine density and excitatory synaptic transmission. Hyperactivity, impaired social interaction, increased repetitive behaviors	(Stephenson *et al.*, 2017)
DISC1	Deletion – truncation	Impaired working memory	(Koike *et al.*, 2006)
DTNBP1	Spontaneous deletion mutation	Deregulated spine formation and retraction, decreased PPI, Hyperactivity, spatial memory deficits Impaired motor learning and T-maze forced alternation test	(Jia *et al.*, 2014) (Talbot, 2009) (Takao *et al.*, 2008)
FMR1	KO	Hyperactivity, learning deficits	(The Dutch-Belgian Fragile X Consortium, 1994)
KALRN	KO	Reduced neuropil volume	(Russell *et al.*, 2014)

NRG1	Membrane domain mutant , KO ctoNRG1	Hyperactivity Reduced PPI. Hyperactivity. Hyperactivity, Impaired PPI, impaired social activity. Impaired reference spatial memory.	(Karl *et al.*, 2007) (Stefansson *et al.*, 2002)
NRLG1	KO	Deficits in spatial learning and memory, stereotyped behavior, reduced NMDA/AMPA ratio at corticostriatal synapses	(Blundell *et al.*, 2010)
NRXN1	KO Transgenic (OV)	Decrease in mEPSC frequency in hippocampus, impaired PPI, increased grooming behaviors, impaired nest behavior, improved motor learning Hyperactivity, reduced PPI, reduced social interaction, deficient spatial working and reference memory. Reduced mEPSC frequency in hippocampus and reduced mIPSC in the forebrain	(Etherton *et al.*, 2009) (Yin *et al.*, 2013)
OPHN1	KO	Deficits in spatial memory, impaired social behavior and hyperactivity. Reduced paired-pulse facilitation in hippocampus.	(Khelfaoui *et al.*, 2007)
PPP3CC	KO	Impaired working memory, increased locomotor activity, impaired social interaction, decreased PPI.	(Miyakawa *et al.*, 2003)
SHANK2	KO	Reduced social interaction. Decreased NMDAR	(Won *et al.*, 2012)
SHANK3	Insertion Nonsense mutation KI	Striatal synaptic transmission defects. Impaired social interaction Synaptic defects in PFC and social dominance behavior	(Zhou *et al.*, 2016a)
STXBP1	KO	Complete loss of neurotransmitter secretion	(Verhage *et al.*, 2000)
SYNGAP	KO	Excess mushroom-shaped spines, impaired transient NMDAR-dependent regulation of spine cytoskeleton; Accelerated synapse maturation – E/I balance disruption	(Carlisle *et al.*, 2008) (Clement *et al.*, 2012)
TSC2	Inactivating mutation	Deficits in learning and memory	(Ehninger *et al.*, 2008)
UBE3A	KO	Impaired neocortical experience-dependent plasticity	(Yashiro *et al.*, 2009)

KI – *knock-in*, KO – *knock-out*, OV – overexpression, cto – conditional overexpression

Rodent models are indisputable key tools in the translation of genetic findings regarding psychiatric disorders. The elucidation of mechanisms through which risk genes lead to molecular and behavior changes is crucial for the identification of the central pathways affected in these disorders. A limitation attributed to the use of animal models comes from the observed genetic overlap between different psychiatric disorders, which hinders the discrimination between phenotypes correlated to one or other disorder. Furthermore, the complexity of these disorders makes it unlikely that a full range of symptoms could be modelled in non-human animals (Davis and Isles, 2014).

In order to circumvent these limitations, recent studies on animals base their characterization not only in animal behavior but also on electrophysiological recordings, on the characterization of circuitry using optogenetics and imaging studies.

Furthermore, for some genes different mouse models have been developed aiming at addressing different questions. This is the case of the NRG1 gene. Transmembrane domain *knock-out* mice in which neuregulin1 is not present at the membranes and thus lacks functionality, presented both hyperactivity and reduced PPI, which are phenotypes often observed in animal models of psychiatric disorders (Stefansson *et al.*, 2002). On the other hand, mice that conditionally overexpress neuregulin1 (ctoNrg1) were developed to mimic the high levels of neuregulin1 observed in the forebrains of SCZ patients. These animals exhibited hyperactivity, impaired PPI, social activity and spatial reference memory and present both glutamatergic and GABAergic transmission deficits. These deficits were abrogated in adulthood upon cessation of overexpression, which suggests that these alterations are reversible and opens a window of opportunity for therapeutic treatment (Yin *et al.*, 2013).

Interestingly, hyperactivity and PPI deficits presented by both models were reverted by clozapine, suggesting that this drug might be effective in the amelioration of the features correlated to these phenotypes, in patients harboring mutations in the *NRG1* gene (Stefansson *et al.*, 2002; Yin *et al.*, 2013). This observation is a good example of the validity and utility of animal models in the testing and development of therapies.

Most animal models developed so far are *knock-out* mice, which lack the expression of a particular protein of interest. *Knock-out* mice are a reliable tool not only to study the function of proteins and specific mechanisms in which they participate, but also allow the correlation between absence of a functional protein and specific processes and/or behaviors that correlate to disease. More recently, *knock-in* models expressing disease-associated human mutations have been developed. These models show increased human validity, by allowing the expression of protein variants identified in humans, and present cellular, circuits and behavior phenotypes that more closely relate to patients symptoms.

Knock-in mice for an ASD-associated *de novo* mutation in the gene encoding CaMKII were recently generated. This mutation decreased the interaction between CaMKII and Shank3 and NMDAR and affected the excitatory synaptic transmission in cultured neurons. Mice harboring this mutation presented hyperactivity, deficient social interaction and increased repetitive behaviors (Stephenson *et al.*, 2017).

In a recent work from Zhou and colleagues, two lines of *knock-in* mice were developed for two variants of Shank3, one carrying a mutation identified in an ASD patient and the other expressing a Shank3 variant identified in a SCZ patient. Interestingly, the effect of the two variants was distinct. Whereas the ASD-linked mutation in Shank3 affected striatal synaptic transmission and impaired social interaction, the SCZ-linked mutation induced profound synaptic defects in the PFC and affected social dominance behavior (Zhou *et al.*, 2016a). These data demonstrate that mutations in the same gene may have distinct phenotypic outcomes. The study of the effect of different mutations in the same gene, associated to specific disorders, allows the establishment of a variant/phenotype correlation, shedding light on the specific mechanisms and phenotypes associated to a given disorder.

1.2. Glutamatergic synapses

Glutamate is the main excitatory neurotransmitter in the brain and is thus able to influence virtually any circuit in the brain. Normal glutamatergic signaling is vital for the functioning and maintenance of neuronal circuits, behaviors and responses to environmental stimuli (Iacobucci and Popescu, 2017). For this reason, glutamate receptors have been intensively studied in the past years (Shepherd and Huganir, 2007). Numerous proteins, including metabotropic and ionotropic receptors, compose the postsynaptic compartment of glutamatergic synapses.

Glutamate metabotropic receptors (mGluRs) are G-protein coupled receptors that are important for synaptic transmission modulation. Upon glutamate binding, these receptors transmit signals through the receptor protein to intracellular signaling partners (Niswender and Conn, 2010).

The ionotropic receptors consist in three distinct types, the kainate receptors (KAR), NMDAR and AMPAR, named based on their affinity for the glutamate analogues, kainate, NMDA and AMPA, respectively. Despite their similar structure, ionotropic receptors present different current kinetics. Glutamate binding to AMPAR generate a fast influx of cations that rapidly subsides. These currents determine the onset and maximal amplitude of the excitatory postsynaptic currents (EPSC) following a synaptic stimulus. Upon AMPAR-mediated membrane depolarization, NMDAR are released from the magnesium blockade, allowing the entry of calcium. NMDAR-mediated currents, in contrary to AMPAR-mediated ones, rise and decline more slowly and contribute to the decay phase of the EPSC (Iacobucci and Popescu, 2017). Both types of receptors have been implicated in neuropsychiatric disorders and their modulation as a therapeutic strategy has been proposed several times [reviewed in (Chang *et al.*, 2014; McCullumsmith *et al.*, 2012; Ward *et al.*, 2015)]

1.2.1. Metabotropic receptors

mGluRs couple to G-proteins to modulate synaptic transmission through the activation of intracellular signaling pathways. The eight different subtypes of mGluRs (mGlu1-8) are divided in three subgroups according to their distinct molecular and pharmacological properties. Group I mGluRs includes mGlu1 and mGlu5, which couple to G_q-proteins and stimulate phospholipase C (PLC), and the synthesis of IP_3 and diacylglycerol, leading to an increase in the intracellular calcium concentration and PKC activation. Importantly, group I mGluRs bind to Homer proteins which are linked to IP_3 receptors and Shank proteins that associate with PSD95 and NMDAR (Uematsu *et al.*, 2015). Group II includes mGlu2 and mGlu3 and group III includes mGlu 4, 6, 7, and 8. Groups II and III are negatively coupled to adenylate cyclase and thus inhibit cyclic AMP formation (Cartmell and Schoepp, 2000). The mGluRs topology includes an extracellular N-terminal domain (NTD), three extracellular and three intracellular loops, and an intracellular C-terminal domain (CTD). The intracellular domains are necessary for the interaction with G-proteins to modulate transmission through intracellular second messengers (Ferraguti and Shigemoto, 2006). Depending on the receptor subtype, mGluRs localize in the presynaptic or postsynaptic compartments allowing the modulation of synaptic transmission by distinct mechanisms. Group I receptors were shown to localize away from active zones but restricted to postsynaptic sites, whereas Group II receptors localize perisynaptically, both in post- and presynaptic terminals. Group III receptors, on the other hand, localize in the presynaptic compartment, near the active zones (Cartmell and Schoepp, 2000). These receptors have important roles in both Hebbian and homeostatic plasticity mechanisms (Gladding *et al.*, 2009; Hu *et al.*, 2010) and particularly mGluR5 has been extensively implicated in neuropsychiatric disorders, namely Fragile X and other autism spectrum disorders (Dolen and Bear, 2008).

1.2.2. NMDA receptors

NMDAR are highly permeable to calcium but ion influx is impacted by voltage-sensitive magnesium blockade. Thus, their activation requires previous depolarization of the plasma membrane. Furthermore, the activation of these receptors is dependent on the binding of a co-agonist, usually D-serine or glycine, to the obligatory subunit, GluN1 (Paoletti *et al.*, 2013).

NMDAR are heterotetrameric receptors composed by seven possible subunits, one GluN1, four GluN2 and two GluN3 subunits. Functional NMDAR contain at least one GluN1 subunit, to where the co-agonist binds, and one GluN2 subunit which provides the site for glutamate binding (Iacobucci and Popescu, 2017). Differences in the C-tail length, due to alternative splicing, account

for subunits of different size, ranging from 900 to 1480 aminoacids. Importantly, these differences influence the trafficking of NMDAR. NMDAR contain an NTD, involved in the assembly of subunits; the ligand-binding domain (LBD), which binds glycine or D-serine in the case of GluN1 and GluN3 and glutamate for GluN2; the transmembrane domain (TMD), which includes three membrane spanning domains (M1, M3 and M4) and one reentrant pore loop (M2), and the CTD involved in receptor trafficking and binding to cytoplasmatic proteins (Paoletti *et al.*, 2013).

Although the GluN1 subunit is widely expressed from embryonic stage to adulthood, expression of the GluN2 variants varies throughout development and across brain regions. During embryonic stage only GluN2B and GluN2D subunits are expressed, and the expression of GluN2A starts after birth whereas the expression of GluN2D drops. GluN2C is expressed later in development, around the postnatal day 10. GluN3A expression peaks in early postnatal stage whereas GluN3B expression increases constantly; in adulthood GluN3B is expressed mainly in motor neurons. These differences in expression pattern suggest that different subunits have different roles in cellular processes such as synaptogenesis and synaptic maturation in the case of GluN2B, GluN2D and GluN3A (expressed early during development), and synaptic plasticity in the case of GluN2A and GluN2B, which are the predominant subunits in the hippocampus and cortex of the adult brain (Paoletti *et al.*, 2013).

Many important forms of plasticity that underlie memory and learning require NMDAR. In fact, both long-term potentiation (LTP) and long-term depression (LTD) depend on calcium influx through NMDAR ion channels. Since the direction of plasticity depends on the magnitude and time of calcium increase, which is influenced by NMDAR composition, it has been speculated that receptor composition dictates whether potentiation or a depression occurs (Paoletti *et al.*, 2013). Accordingly, mice lacking GluN2A subunit present deficient LTP (Sakimura *et al.*, 1995), whereas specific GluN2B blockers affect LTD (Massey *et al.*, 2004).

Although the excess of calcium through NMDAR is excitotoxic and leads to neuronal death, the hypofunction of these receptors has also been linked to numerous disorders. Thus, both agonists and antagonists have been proposed as potential therapeutic strategies for different disorders (Paoletti *et al.*, 2013).

1.2.3. AMPA receptors

AMPAR are ionotropic glutamate receptors that mediate fast excitatory synaptic transmission. AMPAR undergo rapid activity-regulated recycling at the plasma membrane and also dynamic AMPAR membrane diffusion between synaptic and extrasynaptic sites.

Four closely related genes, *GRIA1-4* encode the GluA1, GluA2, GluA3 or GluA4 subunits of AMPAR that assemble in homomeric or heteromeric tetramers (dimers of dimers) (Hollmann and Heinemann, 1994). The protein sequences of GluA subunits usually comprises around 900 aminoacids resulting in a molecular weight of about 105 kDa (Santos *et al.*, 2009).

AMPAR subunits consist in the NTD, the LBD and the TMD. The TMD consists of four hydrophobic domains, three of which span the membrane (M1, M3 and M4), and one that is a reentrant loop (M2), that forms the channel pore. Furthermore, AMPAR present a short intracellular CTD that is a substrate for post-translational modifications (such as phosphorylation) and protein interactions with intracellular partners. The NTD and LBD assemble in two-fold symmetric dimers of dimers and the TMD assemble in four-fold symmetry. In consequence, there is a different dimerization between subunits, according to the domain layer (Greger *et al.*, 2017; Santos *et al.*, 2009; Zhao *et al.*, 2016). (Figure 1.4).

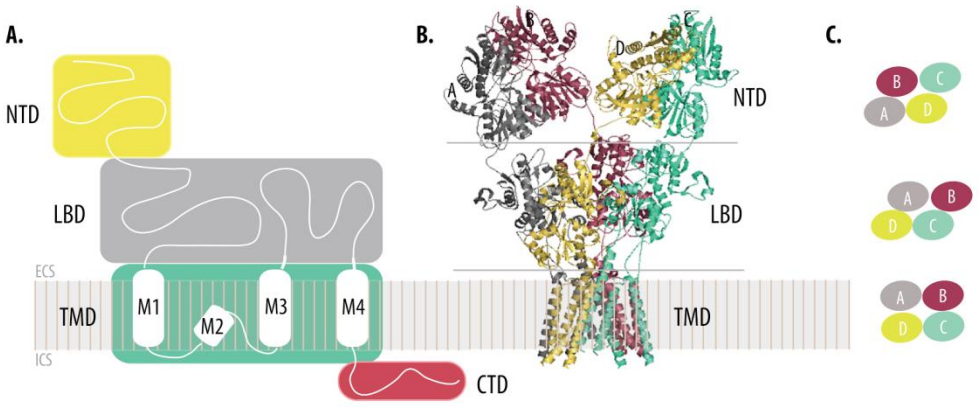


Figure 1.4. AMPAR membrane topology and structure. Representation of an AMPAR subunit domain structure (**A)** Structure of a GluA2 homomeric receptor based on the model 3KG2 (Protein DataBank) and designed in PyMol software. Individual subunits are colored differently (**B**). The dimerization between subunits occurs differently in each domain layer (**C**). NTD – N-terminal domain, LBD – Ligand-binding domain, TMD – Transmembrane domain, M1-M4 – Membrane domains 1-4, CTD – C-terminal domain, ECS – Extracellular space, ICS – Intracellular space. Adapted from (Greger *et al.*, 2017; Nakagawa *et al.*, 2006).

Subunit composition varies according to brain region, cell type and developmental stage (Song and Huganir, 2002). GluA1, GluA2 and GluA3 are expressed in the cortex, hippocampus, olfactory regions, basal ganglia and amygdala, whereas GluA4 is more expressed in the cerebellum and reticular thalamic nuclei (Santos *et al.*, 2009). Typically, AMPARs are GluA1:GluA2 or GluA2:GluA3 heteromers (Wenthold *et al.*, 1996).

Synaptic strength is tightly regulated by the number of synaptic AMPAR and, in many synapses, the increase and decrease in the number of these receptors at the post synapse underlie LTP and LTD, respectively (Anggono and Huganir, 2012).

AMPAR subunits can undergo several post-translational modifications, such as phosphorylation (Rosenmund *et al.*, 1994; Tan *et al.*, 1994; Wang *et al.*, 1994), palmitoylation (Hayashi *et al.*, 2005) and ubiquitination (Widagdo *et al.*, 2015). Post-transcriptional editing (Krampfl *et al.*, 2002) also occurs, with significant impact in the case of the GluA2 subunit, and alternative splicing results in flip/ flop variants (Sommer *et al.*, 1990) or in receptor subunit variants with different cytoplasmic tail length (Kohler *et al.*, 1994). All these variations lead to the differential modulation of AMPAR function, interactions and pharmacological or kinetic properties. In fact, the GluA2 mRNA is edited in >99% cases, resulting in the switch of a glutamine for an arginine (Q/R) in the M2 domain of GluA2 and rendering AMPAR impermeable to calcium. GluA2-containing AMPAR also present linear current-voltage relationship whereas receptors lacking the GluA2 subunit are permeable to calcium and present an inwardly rectifying current-voltage relationship (Anggono and Huganir, 2012; Wright and Vissel, 2012)].

Several kinases phosphorylate AMPAR, which is crucial for the regulation of their trafficking and function. GluA1 is phosphorylated by CaMKII and PKC in serine 831, which leads to increased single channel conductance, and by PKA at serine 845, which enhances the mean open probability (Banke *et al.*, 2000; Barria *et al.*, 1997; Roche *et al.*, 1996) . Interestingly, the combined but not the individual elimination of the phosphorylation of these sites prevents LTP, suggesting that the phosphorylation of one site might compensate for the other (Lee *et al.*, 2010). LTD, however, is dependent on serine 845 phosphorylation (Lee *et al.*, 2010). GluA2 is phosphorylated in serine 880 by PKC and in tyrosine 867 by Src. Phosphorylation of these residues is thought to be important for AMPAR internalization (Chung *et al.*, 2000; Hayashi and Huganir, 2004).

Furthermore, AMPAR interact with proteins that function as auxiliary subunits and regulate their trafficking, surface expression, subcellular localization and gating. Examples of these proteins are the transmembrane AMPAR regulatory proteins (TARPs), cornichons (CNIH), CKAMP44 (shisa9), PORCN, GSG1L, SynDIG1 and Caspr1. These proteins are differentially expressed throughout the brain, interact with specific AMPAR subunits and present different subcellular localizations, influencing AMPAR differently.

As will be further discussed in this chapter, stargazin, a member of the TARP family, is required for LTP and LTD in a phosphorylation-dependent manner (Tomita *et al.*, 2005b). Some CNIH proteins and GSG1L are also required for synaptic plasticity, since the deletion of both CNIH2 and 3, which specifically interact with the GluA1 subunit, impacts LTP (Herring *et al.*, 2013). Accordingly, the deletion of GSG1L, which affects the effect of the CNIHs in AMPAR, increases LTP (Gu *et al.*, 2016). Although GSG1L is important for the regulation of synaptic strength in hippocampal neurons, it does not affect AMPAR desensitization and deactivation kinetics (Mao *et al.*, 2017).

CKAMP44 modulates short-term plasticity by affecting the AMPAR desensitization rate and by decreasing paired-pulse ratio of AMPAR currents in hippocampal neurons (von Engelhardt *et al.*, 2010). PORCN and ABHD6 were recently identified as AMPAR interactors and both were described to increase GluA1 levels in heterologous cells while decreasing the glutamate-evoked whole cell currents. The PORCN-mediated effect on AMPAR is due to the retention of AMPAR intracellularly (Erlenhardt *et al.*, 2016) and overexpression of ABHD6 also led to a drastic reduction in AMPAR-mediated excitatory neurotransmission in neurons (Wei *et al.*, 2016).

SynDIG1 is a transmembrane protein that interacts with AMPAR and regulates their expression in nascent synapses in an activity-dependent manner, which suggests a role for this interaction in synaptic plasticity (Kalashnikova *et al.*, 2010).

Recently, Caspr1 was shown to interact with AMPAR and regulate their surface and synaptic expression, and the amplitude of glutamate-evoked AMPAR-mediated currents in an hetereologous system (Santos *et al.*, 2012).

Similarly to stargazin, Shisa6 contains a PDZ binding site that binds to PSD95 PDZ domains, and consequently stabilizes AMPAR at synapses. Furthermore, Shisa6 also protects AMPAR from full desensitization (Klaassen *et al.*, 2016).

Besides these auxiliary subunits, AMPAR interacts with intracellular proteins that participate in AMPAR regulation, namely SAP97, a MAGUK that was suggested to affect AMPAR maturation and trafficking, 4.1N, an actin cytoskeleton protein that binds to GluA1 and regulates its surface expression, GRIP that regulates the trafficking and synaptic targeting of AMPAR and plasticity, PICK1, which mediates GluA2 synaptic removal upon LTD induction, NSF, a component of the SNARE complex required for the insertion of GluA2 into the membrane and synapses and AP2, which interacts with GluA2 and participates in the NMDA-induced internalization of AMPAR (Anggono and Huganir, 2012).

1.2.4. Stargazin, an auxiliary subunit for AMPAR

Stargazer mice, which lack stargazin protein expression, arose in the Jackson Laboratory as a result of a spontaneous mutation in chromosome 15. *Stargazer* mice present with ataxic gait, eye-blink conditioning impairment, absence seizures and a distinctive head tossing phenotype, the star gazing phenotype (Letts *et al.*, 1998; Noebels *et al.*, 1990).

Stargazin is a 37kDa membrane spanning protein with a cytosolic amino terminus, four transmembrane domains and a cytosolic carboxy terminus (Figure 1.4). Taking into consideration its modest sequence similarity (around 25%) with the γ (γ1) subunit of the skeletal muscle voltage-

gated Ca^{2+}channel (coded by the *CACNG1* gene), and the shared phenotypes between the *stargazer* mice and the tottering, ducky and lethargic mouse models, which present a defect in the Ca^{2+} channel subunit, stargazin was suggested as the γ2 subunit for these channels (Letts, 2005).

Defective Ca^{2+} entry was first proposed to account for the observed decrease in the mRNA levels of BDNF, with consequent decrease in the expression of $GABA_A$ receptor α6 and ß3 subunits in cerebellar granule cells (Thompson *et al.*, 1998), thus contributing to ataxia and eye-blink conditioning impairment in *stargazer* mice (Letts *et al.*, 1998; Qiao *et al.*, 1998). However, Hashimoto and colleagues described an impairment of AMPAR function in *stargazer* and suggested this impairment was the most likely responsible for the cerebellar dysfunction observed in *stargazer*. Their data showed that EPSCs at mossy fibers to granule cells synapses were devoid of the fast component mediated by AMPAR whereas the slow component, mediated by NMDARs, was unchanged (Hashimoto *et al.*, 1999). Furthermore, this alteration was not accompanied by a decrease in the protein levels of AMPARs and was not observed in the hippocampus of *stargazer* or the cerebellum of BDNF *knock-out* mice (Hashimoto *et al.*, 1999). Later on, it was suggested that the decreased levels of BDNF mRNA in the cerebellum of *stargazer* mice could be attributed to an abnormal response to the glutamate receptor-mediated upregulation of BDNF mRNA, which did not respond to AMPAR stimulation but showed intact NMDA modulation-mediated response (Meng *et al.*, 2007). Additionally, it was proposed that the expression and assembly of $GABA_AR$ is regulated by AMPAR-mediated excitability or is a downstream consequence of AMPAR activity loss (Payne *et al.*, 2007).

In 2000, Chen and colleagues showed that calcium currents are normal in *stargazer* mice corroborating the previous hypothesis (Hashimoto *et al.*, 1999) that altered calcium channel function was unlikely the primary cause for the defect in cerebellum granule cells in *stargazer* mice. Evidence for an AMPAR defect emerged from the observation of lack of AMPAR-mediated currents in *stargazer* cerebellum granule cell cultures, whilst the NMDAR-mediated currents were not significantly different from the control (Chen *et al.*, 2000). Furthermore, AMPAR subunits GluA2, GluA3 and GluA4 were virtually inexistent at granule cells synapses whereas modest labeling at the cytoplasm indicated the expression of AMPAR in these cells. Their data also showed that stargazin physically interacts with GluA1, GluA2 and GluA4 subunits, as well as with several PDZ domain-containing proteins such as PSD95, SAP-97, SAP-102 and PSD93. Although the PDZ binding domain of stargazin does not interfere with the binding to AMPAR, it is required for stargazin clustering at the synapse.

1.2.5. The TARP family.

Besides stargazin and γ1, six other γ-subunit-related genes have been identified. Due to their role in the regulation of AMPAR, the proteins encoded by these genes comprise the transmembrane AMPAR regulatory proteins (TARP) family. TARPs share a high homology with each other, (around 60%) and present lower homology with γ1 and claudin-1, an adhesion protein (23% of sequence similarity) (Tomita *et al.*, 2003). Based on sequence similarity and functional properties, TARPs are divided in subgroups. Based on their roles on the modulation of the desensitization decays of AMPAR, stargazin and γ3 represent the Type Ia TARPs, whereas γ4 and γ8 are Type Ib TARPs (Cho *et al.*, 2007). The remaining TARPs, γ5 and γ7, represent the type II. γ5 specifically regulates GluA2-containing AMPAR and, along with γ7, show distinct properties comparing to Type I TARPs, as, for instance, the requirement of GluA2 mRNA edition for the regulation of GluA2-containing AMPARs (Kato *et al.*, 2008; Kato *et al.*, 2007). TARPs characteristics are summarized in the Table 1.5.

Table 1.5. Classification of the type I and type II TARPS.

	Pharmacology		Channel properties			Trafficking	PDZ-binding motif
	Kainate efficacy	Glutamate efficacy	Deactivation and desensitization rates	Steady-state current	Rectification		
Type Ia (γ2 and γ3)	++	+	--	++	++	+	Typical (TTPV)
Type Ib (γ4 and γ8)	++	++	---	+++	++	+	Typical (TTPV)
Type II (γ5)	↔	-*	+	-*	+	↔	Atypical (SSPC)
Type II (γ7)	+	↔	-	++	+	↔	Atypical (TSPC)

* - specific to GluA2, (+) – positive regulation, (-) – negative regulation, (↔) – neutral regulation. Adapted from (Kato *et al.*, 2010a).

TARPs are differently expressed throughout the brain; stargazin is enriched in the cerebellum, cortex, hippocampus and thalamus (both in pyramidal cells and interneurons), whereas γ3 is more expressed in the cortex, γ4 in the olfactory bulb and γ8 in the hippocampus. Both stargazin, γ3 and γ8 expression is low in neonatal stages but increases in adulthood. In contrast, γ4 expression peaks at postnatal day 6 and then decreases in later development. All four TARPs co-immunoprecipitate with AMPAR in extracts from the brain regions where they are highly expressed (Tomita et al., 2003). Type II TARP γ7 is expressed in the cerebellum whereas γ5 is expressed both in the cerebellum and hippocampus (Kato et al., 2008; Kato et al., 2007).

The C-terminal of the different types of TARPs presents distinct features that affect AMPAR regulation. In fact, the C-terminal of Type I but not Type II TARPs is necessary for the trafficking of AMPAR to the surface (Tomita *et al.*, 2005a). Although both types of TARPs contain a PDZ binding domain in their C-terminal, the sequences are different, leading to a weaker binding to PSD95 in

the case of type II TARPs. Furthermore, the C-terminal of type I but not type II TARPs presents a set of serine residues that contribute largely to the phosphorylation state of the TARP (Kato *et al.*, 2010b), and whose importance in synaptic plasticity has been described (Louros *et al.*, 2014; Tomita *et al.*, 2005b). In addition, Type I TARPs also increase AMPAR glutamate affinity, and decrease deactivation and desensitization rates in a much greater extent than type II TARPS [reviewed in (Kato *et al.*, 2010b)]. Type I TARPs affect dendritic growth of neocortical pyramidal neurons in an NMDAR-dependent manner, whereas type II TARPs fail to do so (Hamad *et al.*, 2014).

Despite belonging to the same subfamily, stargazin and γ8 are distributed differently at the synapse in hippocampal neurons; whereas stargazin localizes mainly to the synaptic area, γ8 is distributed both in the synaptic and extrasynaptic area. Furthermore, these proteins are regulated by different sets of kinases and phosphatases in cortical neurons, suggesting that their function is correlated to the activation of different intracellular signaling pathways (Inamura *et al.*, 2006).

1.2.6. Stargazin regulates AMPAR trafficking, synaptic stabilization and kinetics

Stargazin is essential for the regulation of AMPAR surface expression and function, by several distinct mechanisms. Along with other TARPs, stargazin masks ER retention signals facilitating AMPAR export from the ER (Vandenberghe *et al.*, 2005b). In fact, the C-terminus of stargazin contains a membrane sorting signal that pulls AMPAR to the surface through the interaction with stargazin in a region that is not the cytoplasmic tail. This ability is not receptor-dependent, as the C-terminal domain of stargazin, when coupled to other proteins, also mediates their trafficking to the basolateral membrane in Madin-Darby canine kidney cells, where basolateral sorting may reflect somatodendritic sorting in neurons, suggesting that stargazin sorting signal either directs sorting vesicles to the basolateral membranes or allows enhanced endocytosis from the apical membrane (Bedoukian *et al.*, 2008). In addition, nPIST, a protein enriched in the Golgi, interacts with the C-tail of stargazin helping AMPAR exit the Golgi (Cuadra *et al.*, 2004), where they become fully glycosylated (Shepherd and Rosell, 2007).

The C-terminal of stargazin contains nine serine residues that are phosphorylated by PKC and CaMKII (Tomita *et al.*, 2005b). CaMKII activation leads to the rapid recruitment and stabilization of AMPAR at the synapse by decreasing their mobility at synaptic sites. This effect does not require the phosphorylation of AMPAR subunits, but is dependent on the CaMKII-dependent phosphorylation of stargazin and consequent binding to PSD95 (Opazo *et al.*, 2010). Recently, stargazin was shown to interact with negatively charged lipid bilayers in a phosphorylation-dependent manner due to the presence of several positively charged aminoacids (arginine residues)

near the phosphorylatable serine residues in the C-tail of stargazin. Importantly, this interaction with the lipids prevents the interaction with PSD95, which is resumed after the phosphorylation of stargazin (Sumioka *et al.*, 2010). On the other hand, the phosphorylation of threonine 321 in the PDZ domain of stargazin by PKA disrupts the interaction with PSD95 (Chetkovich *et al.*, 2002; Choi *et al.*, 2002).

Besides enhancing the trafficking of AMPAR to the cell surface, stargazin is important for the stabilization of these receptors at the synapse, and the interaction with PSD95 is essential for this mechanism (Chen *et al.*, 2000). This interaction regulates the number of AMPAR at the synapse and although the increase in the expression of stargazin increases the number of AMPAR at the cell surface, the synaptic currents do not change if the PSD95 levels are kept constant. On the other hand, increasing the number of PSD95 slots recruits new AMPAR to the synapse without changing the number of surface AMPAR (Schnell *et al.*, 2002).

Tianeptine is a widely used antidepressant structurally similar to tricyclic antidepressants but with particular pharmacological properties, since unlike most of the antidepressant drugs it fails to modulate the monoaminergic system [reviewed in (McEwen *et al.*, 2010)]. Although its mode of action remains elusive, tianeptine has been shown to prevent and reverse stress-induced remodeling of hippocampal neurons (Magarinos *et al.*, 1999) and stress-induced diminished performance in the eight-arm radial- and Y-mazes (Conrad *et al.*, 1996; Luine *et al.*, 1994). Furthermore, it enhances spontaneous alternation and left-right discrimination in the T-maze (Jaffard *et al.*, 1991), thus resulting in a beneficial effect in memory and learning. Recent evidence show that tianeptine regulates AMPAR function. In fact, it leads to an increase in the phosphorylation of Serine 831 and Serine 845 of GluA1 subunits in the hippocampus (Svenningsson *et al.*, 2007) and potentiates AMPA-elicited firing activity in a CaMKII- and PKC-dependent manner (Szegedi *et al.*, 2011). More recently it was found to be a potent modulator of AMPAR surface diffusion through the CaMKII-dependent phosphorylation of stargazin, favoring its binding to PSD95 (Zhang *et al.*, 2013).

Stargazin not only regulates the trafficking and stabilization of AMPAR but also enhances their activity (Yamazaki *et al.*, 2004). It was shown to reduce AMPAR channel desensitization (Priel *et al.*, 2005; Turetsky *et al.*, 2005), to slow deactivation in response to glutamate and to accelerate the recovery from desensitization (Priel *et al.*, 2005). In *Xenopus oocytes,* stargazin increased glutamate-evoked currents to a much greater extent than it increased the surface expression of GluA1, suggesting that stargazin enhances GluA1 function independently from its effect on receptor trafficking. Single AMPAR channel activity recordings showed that stargazin increases the frequency of channel opening (Tomita *et al.*, 2005a). Interestingly, stargazin enhances the effect of AMPAR potentiators that slow the deactivation and desensitization of these receptors, abolishing, in

addition, the preference for flip or flop alternatively spliced versions of AMPAR subunits (Tomita *et al.*, 2006).

AMPAR lacking the edited version of the GluA2 subunit are calcium-permeable and are subject to voltage-dependent blockade by polyamines. Stargazin was shown to reduce the polyamine blockade of calcium permeable AMPAR, increasing their single-channel conductance (Soto *et al.*, 2007). Interestingly, the threshold of glutamate concentration at which AMPAR autoinactivate is increased when stargazin is present (Morimoto-Tomita *et al.*, 2009). The desensitization of AMPAR leads to the dissociation between stargazin and AMPAR in a few milliseconds, contributing to a short-term modulation of synaptic strength (Morimoto-Tomita *et al.*, 2009). More recently, this activity-mediated dissociation of AMPAR and stargazin was shown to induce an increased surface diffusion of the receptors, allowing the exchange of desensitized receptors by naïve ones (Constals *et al.*, 2015).

1.2.7. Stargazin interaction with AMPAR

Distinct domains of stargazin are responsible for stargazin's effects on AMPAR trafficking and activity (Figure 1.5). Whereas the cytoplasmic tail of stargazin determines receptor trafficking, the first extracellular domain controls the channel properties; the disruption of the latter interaction alters the amplitude and shape of synaptic responses (Bedoukian *et al.*, 2008; Tomita *et al.*, 2005a). Also, kainate sensitivity requires the first extracellular and the second transmembrane domain of stargazin (Tomita *et al.*, 2005a). In 2007, Tomita et al. showed that stargazin-dependent regulation of AMPAR requires the glutamate-binding module, since a mutation equivalent to the Lurcher mutation in GluAδ2 (which corresponds to a residue in the extracellular region of GluA1) prevented stargazin-mediated AMPAR trafficking and gating (Tomita *et al.*, 2007).

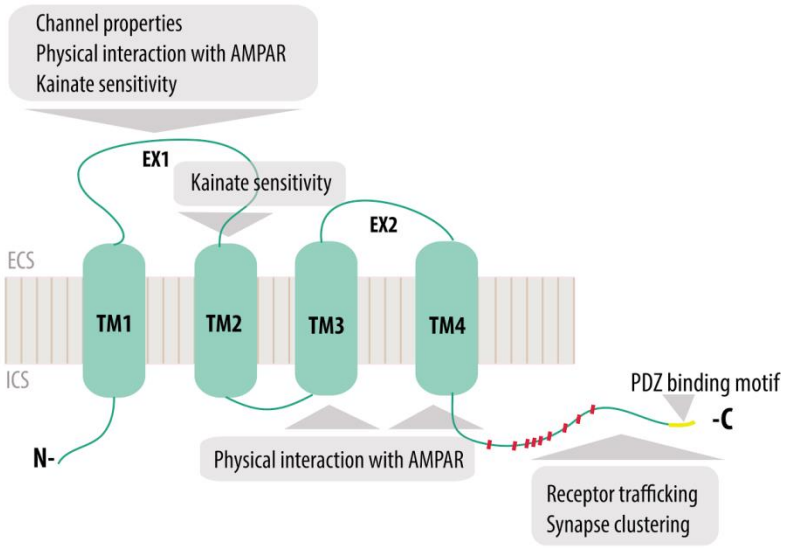


Figure 1.5. Stargazin domain structure. Stargazin contains an intracellular N-terminal segment, four transmembrane domains, two extracellular loops and a C-terminal domain that includes a PDZ domain binding motif (yellow line) and nine serine residues (red dots), which are phosphorylated by CaMKII and PKC and are required for synaptic plasticity mechanisms. Different domains of stargazin regulate the interaction and regulation of AMPAR (gray boxes). ECS – extracellular space, ICS – intracellular space, EX – extracellular domain, TM – transmembrane domain. Adapted from (Price *et al.*, 2005).

By swapping the domains of stargazin by the correspondent domains from γ5, a TARP that does not regulate the trafficking of AMPAR, Tomita and colleagues proposed that the first extracellular domain and the intracellular tail of stargazin are indispensable for stargazin binding to AMPAR. They showed that the inclusion of an HA-tag in the end of the first extracellular domain but not in the middle prevented the interaction with AMPAR, suggesting a role for this region in the interaction. Additionally, deletion analysis of the C-terminal of stargazin identified a critical role for residues 212 to 268 in this interaction (Tomita *et al.*, 2004).

The importance of the first extracellular domain (Ex1) of stargazin for the interaction with AMPAR was further corroborated by Cais and colleagues, who additionally showed that Ex1 binds to both the N-terminal domain (NTD) an the ligand binding domain (LBD) of GluA2, and that this interaction is implicated in aspects of AMPAR gating (Cais *et al.*, 2014).

Recent cryo-electron microscopy studies focusing on the interaction of GluA2-AMPAR with stargazin showed that besides the extracellular domains of stargazin, which modulate the LBD closure and affect conformational rearrangements of the LBD associated with AMPAR activation and desensitization, also the transmembrane domains of stargazin (TM) interact with AMPAR and buttress the ion channel pore (Zhao *et al.*, 2016). The latter interaction is mediated by an interface between the TM3 and TM4 of stargazin and the membrane domains 1 and 2 of one GluA2 subunit and the membrane domain 4 of the adjacent GluA2 subunit (Twomey *et al.*, 2016; Zhao *et al.*, 2016). Additionally, a two-steps mechanism for this interaction was recently proposed, based on systematic mutagenesis and electrophysiology data (Ben-Yaacov *et al.*, 2017). According to this model, the binding of stargazin to AMPAR is sufficient to destabilize the channel closed-state, which promotes channel opening upon binding of the agonist. In turn, agonist binding leads to conformational changes that allow the interaction between the extracellular parts of stargazin and AMPAR, stabilizing the open state (Ben-Yaacov *et al.*, 2017). This model is in agreement with previous data on the role of stargazin on the stabilization of the open state of AMPAR (Shaikh *et al.*, 2016).

The stoichiometry of the AMPAR-stargazin complex remains elusive. It has been proposed that AMPAR assemble with a variable number of TARP molecules, depending on their availability (Hastie *et al.*, 2013; Shi *et al.*, 2009). Neurons with high TARP expression, such as hippocampal pyramidal

neurons, would present a higher TARP/AMPAR stoichiometry, comparing to neurons expressing lower levels of TARPs, such as dentate gyrus granule neurons, providing a mechanism for cell-specific regulation of AMPAR (Shi *et al.*, 2009). Twomey and colleagues suggested that the preferred stoichiometry of the GluA2-stargazin interaction is one or two stargazin molecules for each tetramer of GluA2 (Twomey *et al.*, 2016), whereas others proposed that one to four stargazin molecules assemble with each receptor (Hastie *et al.*, 2013; Zhao *et al.*, 2016). In addition, the stoichiometry between AMPAR and different members of the TARP family vary; the maximum of four γ3 molecules interact with each AMPAR whereas for γ4 this number rarely goes above two (Hastie *et al.*, 2013). The existence of multiple stoichiometry states that depend on TARPs levels attributes an important role to the expression level of these proteins on AMPAR function.

1.2.8. Other Stargazin interactors

Stargazin has been shown to interact with several proteins apart from AMPAR; however, the purport of some of these interactions has not been fully elucidated. Following is a list of the interaction partners reported to this date:

- Besides PSD95, stargazin associates to other synaptic PDZ proteins such as PSD93, SAP-97, SAP-102 (Chen *et al.*, 2000), MUPP1 and OMP-25. However, whereas all these proteins interact with stargazin *in vitro*, only PSD93 and PSD95 were shown to interact with stargazin in the brain (Dakoji *et al.*, 2003).
- MAGI-2, a scaffolding protein that interacts with several ligands, including NMDAR, interacts with stargazin in the cortex, in a manner dependent on the C-terminal domain of stargazin (Deng *et al.*, 2006).
- nPIST, a Golgi-enriched PDZ protein, interacts with stargazin in the hippocampus and might be implicated in the stargazin-mediated trafficking of AMPAR (Cuadra *et al.*, 2004).
- Stargazin interacts with Erbin, a PDZ domain-containing protein that is specifically localized at excitatory synapses in cortical inhibitory neurons and that has been shown to be required for the stabilization of stargazin, whose levels decrease in the absence of Erbin, and for the surface expression of AMPAR at these synapses (Tao *et al.*, 2013).
- The light chain (LC2) of the microtubule-associated protein 1A (MAP1A) is a protein that regulates neuronal differentiation and microtubule dynamics. This protein forms a complex with stargazin and GluA2 and the interaction is believed to play a role in the trafficking of AMPAR to the cell surface, in cerebellar neurons (Ives *et al.*, 2004)

- Adaptor protein (AP)-2, AP-3 and AP-4, members of the adaptor protein complexes which regulate vesicular trafficking of membrane proteins, were shown to interact with stargazin. The interaction with AP-4 was suggested to be necessary for the proper somatodendritic localization of AMPAR and regulation of autophagic pathways in neurons (Matsuda *et al.*, 2008). AP-2 and AP-3 form a complex with stargazin and, whereas the AP-2 interaction is required for NMDA-induced AMPAR endocytosis, the AP-3 interaction regulates the late endosomal/lysosomal trafficking of AMPAR and consequently controls the recycling of AMPAR to the cell surface (Matsuda *et al.*, 2013)
- Stargazin was recently shown to interact with serine racemase, an enzyme involved in the conversion of L-serine to D-serine, a co-agonist of NMDAR. By binding to stargazin and PSD95, serine racemase localization at the membrane is facilitated, rendering the enzyme inactive. The activation of AMPAR leads to serine racemase internalization and thus disrupts its interaction with stargazin. In turn, more D-serine is produced, which potentially promotes NMDAR activation. These observations suggest that serine racemase might bridge AMPAR and NMDAR functions (Ma *et al.*, 2014).
- Finally, ARC, a protein implicated in SCZ and required for memory and learning, was shown to interact with stargazin; the interaction was found to be necessary for ARC-mediated AMPAR downregulation through decrease of surface GluA1 (Zhang *et al.*, 2015).

1.3. Synaptic plasticity

Synaptic plasticity is a process through which specific changes in activity culminate in changes in synaptic strength that persist even when the triggering stimulus has ceded (Martin *et al.*, 2000). Synaptic plasticity is believed to represent a cellular foundation for memory and learning. Cellular and molecular mechanisms underlying synaptic plasticity have been widely studied since the first model for synaptic plasticity was formulated in 1949, by Donald Hebb. The Hebbian forms of plasticity, LTP and LTD are rapidly induced, long-lasting processes, which in many synapses are inextricably linked to the modulation of AMPAR.

LTP and LTD are induced by distinct patterns of activity. LTP requires the simultaneous activation of pre and postsynaptic neurons, which allows the maximization of calcium influx through NMDAR following activity-dependent depolarization of the membrane with consequent release of magnesium block. On the other hand, LTD results from the repeated activation of the presynaptic neurons at low frequencies in the absence of activation of the postsynaptic neuron, which due to the incomplete blockade of NMDAR, is sufficient for the entry of calcium. Experimentally, LTP can

be induced in hippocampal slices by the high frequency (usually, 50-100Hz for 1 sec) stimulation of Schaffer collaterals which results in the large depolarization of the postsynaptic CA1 neurons and consequent calcium entry. LTD is induced by low frequency stimulation of the presynaptic neurons (usually 1-3Hz for 5-15 min) which causes a modest depolarization of the postsynaptic neuron and an equally modest entry of calcium through the NMDAR (Luscher and Malenka, 2012). LTP can be induced chemically in cultured neurons by different protocols, and shares key properties of LTP induced by high frequency stimulation in hippocampal slices. These protocols include stimulation with glutamate, with forskolin which leads to a rise in the cAMP levels, with APV, a NMDAR blocker which leads to a homeostatic increase in NMDAR levels, enabling a subsequent potentiation upon membrane depolarization, with glycine, a NMDAR cofactor, or KCl, which induces the depolarization of the membrane [reviewed in (Molnar, 2011)]. As further mentioned below, the activation of the type I mGluRs is able to induce LTD and the stimulation with DHPG, an agonist of these receptors, is commonly used for chemical LTD induction (Naie *et al.*, 2007).

The GluA1 subunit of AMPAR was extensively correlated to LTP. In fact, this subunit and CaMKII, which is responsible for its phosphorylation in serine 831, were both shown to be indispensable for LTP, as in mutant mice lacking their expression LTP induction was abolished (Barria *et al.*, 1997; Meng *et al.*, 2003; Pettit *et al.*, 1994; Silva *et al.*, 1992). The CaMKII-dependent phosphorylation of GluA1 is essential for activity-dependent recruitment of GluA1-containing AMPAR to the synapse, whereas their stabilization at the post synaptic density (PSD) requires the intervention of PDZ-binding proteins. Furthermore, many of the auxiliary subunits that decorate AMPAR have been implicated in both LTP and LTD, suggesting that they not only modulate AMPAR under basal conditions but are also important for the changes occurring in these receptors in response to changes in activity. A widely accepted model for LTP postulates that, under basal conditions, AMPAR are retained in nanodomains (slots) containing PSD95, through the interaction between TARPs and PSD95. Due to the remodeling of actin filaments, to which PSD95 nanodomains are anchored, AMPAR are continually repositioned in the PSD. Thus, the number of AMPAR at the synapses is maintained. When LTP is induced, the CaMKII-mediated phosphorylation of TARPs enhances their binding to PSD95 and consequently increases the retaining capacity of the slots for AMPAR, mediating synaptic potentiation [reviewed in (Henley and Wilkinson, 2016)].

LTD, on the other hand, results from the endocytosis of AMPAR; however, despite extensive study, the precise molecular pathways underlying LTD remain obscure. One hypothesis is that, in the hippocampus, calcium signaling plays a bidirectional role in plasticity (Malenka and Bear, 2004), as NMDAR-dependent entry of high levels of calcium would activate CaMKII, which has low affinity for calcium, whereas low levels of calcium would selectively activate calcineurin, a phosphatase with high affinity for Ca^{2+}. The activation of calcineurin leads, in turn, to the dephosphorylation of several

proteins at the PSD, namely GluA1(Lee *et al.*, 1998). However, recent evidence defied this hypothesis by demonstrating that although APV, a competitive antagonist of NMDAR indeed blocks LTD, this process was not prevented by MK801, a non-competitive antagonist of these receptors. This suggests that LTD induction is not dependent on the ion flux through NMDAR but rather on NMDAR-dependent intracellular signaling pathways (Mayford *et al.*, 1995; Nabavi *et al.*, 2013). These results have been discussed and different observations have been described in terms of MK-801 effectiveness in blocking LTD in the hippocampus. More studies are necessary to tackle the specific role of NMDAR in LTD (Babiec *et al.*, 2014). Nevertheless, there is a general consensus with regard to the loss of synaptic AMPAR during LTD.

The activation of group I mGluRs is also able to induce LTD; however the mechanisms of mGluR-dependent LTD vary among brain regions. In the cerebellum, LTD induction requires the activation of phospholipase C (PLCβ), generation of inositol trisphosphate (IP_3), release of calcium from intracellular stores and the activation of PKC, whereas in the hippocampus mGluR-dependent LTD is independent of the increase in calcium, PLCβ or PKC [reviewed in (Luscher and Huber, 2010)] but instead involves Homer 1b/c, which couples mGluR5 to the calcium-independent ERK signaling cascade. Furthermore, evidence suggest that mGluR-LTD is dependent on rapid dendritic protein synthesis which is regulated by the interaction of Homer with mGluRs. Protein synthesis in though to promote AMPAR endocytosis and presynaptic neurotransmitter release (Gladding *et al.*, 2009).

Changes in the number of synapses are also observed following activity stimuli triggering long-term plasticity (Hasegawa *et al.*, 2015). During LTP, spines were shown to increase in volume before the accumulation of AMPAR (Kopec *et al.*, 2006). Furthermore, in hippocampus, theta burst stimulation led to the formation of new spines (Nagerl *et al.*, 2004).

Oppositely, LTD was shown to decrease the lifetime of synapses and to lead to the selective removal of synapses, putatively altering connectivity (Wiegert and Oertner, 2013). Specifically, mGluR-mediated LTD leads to robust spine shrinkage and elimination that lasts for up to 24h (Ramiro-Cortes and Israely, 2013).

When a synapse is potentiated, it becomes more excitable and more prone to further potentiation. When one synapse undergoes LTD, further synaptic depression is facilitated. If unimpeded, these processes pose a problem for the neuronal networks stability. Nonetheless, homeostatic mechanisms that compensate for these changes enable the maintenance of stable function.

Currently, the best-studied form of homeostatic plasticity is synaptic scaling, which is displayed by central glutamatergic neurons.

Synaptic scaling was first described in cultured cortical neurons, where the blockade of activity with the sodium channel blocker tetrodotoxin (TTX), or blocking of GABA-mediated inhibition with bicuculline, generated compensatory changes in synaptic strength that respectively increased or

decreased mEPSC amplitudes. Furthermore, these changes were shown to be independent form NMDAR-mediated signaling (Turrigiano *et al.*, 1998). Similar results were obtained in cultured spinal neurons, where, the authors found this modulation to be regulated by the accumulation of AMPAR at synapses (O'Brien *et al.*, 1998). Both Ca^{2+}-permeable AMPAR (Maghsoodi *et al.*, 2008; Sutton *et al.*, 2006) and GluA2-containing AMPAR (Gainey *et al.*, 2009; O'Brien *et al.*, 1998) have been implicated in homeostatic plasticity, but evidence suggests that their function is assigned to distinct mechanisms. Whereas more global synaptic scaling, triggered by the inhibition of neuronal firing alone, regulates both GluA1 and GluA2-containing receptors, the simultaneous blockade of action potentials and NMDARs is accounted for by homeostatic mechanisms that specifically increase GluA1 levels, through local dendritic synthesis [reviewed in (Fernandes and Carvalho, 2016)].

Numerous proteins play a role in homeostatic plasticity, namely PICK1 and ARC, which regulate AMPAR endocytosis (Anggono *et al.*, 2011; Shepherd *et al.*, 2006), PSD95 and PSD93 (Sun and Turrigiano, 2011), CaMKII (Groth *et al.*, 2011) and calcineurin (Arendt *et al.*, 2015), which, respectively, phosphorylate and dephosphorylate GluA1 and stargazin (Louros *et al.*, 2014).

1.3.1. The role of stargazin in synaptic plasticity

Amongst a vast range of synaptic proteins, stargazin has also been implicated in both LTP and LTD. The NMDAR-induced activation of PKC and CaMKII, with consequent phosphorylation of stargazin, is required for LTP; in fact, expression of a non-phosphorylatable version of stargazin was sufficient to prevent LTP in hippocampal slices. Conversely, the dephosphorylation of stargazin, mediated by calcineurin, results in the formation of a ternary complex between stargazin and the adaptor proteins AP2 and AP3, allowing the NMDAR-induced endocytosis of AMPAR (Matsuda *et al.*, 2013), indispensable for LTD (Nomura *et al.*, 2012; Tomita *et al.*, 2005b). Furthermore, the phosphorylation of stargazin by PKA, at the threonine residue 321, disrupts stargazin interaction with PSD95, preventing activity-dependent clustering of stargazin, and consequently AMPAR, at synapses (Stein and Chetkovich, 2010).

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 (Jackson and Nicoll, 2011).

Stargazin also plays a role in short-term plasticity, since upon desensitization after glutamate binding, AMPAR dissociate from stargazin and thus are less stable at the synapses. This mechanism is thought to allow rapid replacement of desensitized AMPAR by naïve receptors, allowing faster recovery from desensitization-mediated synaptic depression. In fact, when the glutamate binding-

dependent loss of the AMPAR: stargazin interaction is prevented, the glutamate-induced increase in AMPAR mobility is lost, with effects on short-term plasticity (Constals *et al.*, 2015).

A recent study from our laboratory showed that stargazin controls AMPAR synaptic accumulation during homeostatic scaling. In the absence of stargazin, synaptic scaling is impaired in cortical neurons (Louros et al., 2014). Accordingly, stargazin is indispensable for experience-dependent plasticity at the retinogeniculate synapse. Refinement of this synapse is specifically disrupted during the experience-dependent phase in *stargazer* mice lacking stargazin expression (Louros *et al.*, 2014).

1.4. The *CACNG2* gene as a susceptibility gene for psychiatric disorders

Growing evidence points towards the *CACNG2* gene, which encodes for stargazin, as a potential susceptibility gene for psychiatric disorders, namely SCZ, bipolar disorder and ID. In fact, the *CACNG2* gene is located in the 22q chromosome region, which has been strongly implicated in the pathogenesis of SCZ (DeLisi *et al.*, 2002; Gill *et al.*, 1996; Wilson *et al.*, 2006), and risk-conferring single nucleotide polymorphisms (SNP) were identified in the *CACNG2* gene of a subgroup of SCZ (Liu *et al.*, 2008; Yang *et al.*, 2013), and bipolar disorder (Nissen *et al.*, 2012) patients. Additionally, copy number variations (CNVs) of the *CACNG2* gene (Wilson *et al.*, 2006) and altered stargazin expression levels have been described in *postmortem* brain samples from SCZ (Beneyto and Meador-Woodruff, 2006) and bipolar disorder patients (Silberberg *et al.*, 2008). Recently, a missense mutation was identified in the *CACNG2* gene in an 8-year-old non-syndromic intellectual disability patient and was suggested to affect the function of the protein (Hamdan *et al.*, 2011).

In addition, changes in the mRNA and protein levels of other TARPs, namely γ3, γ4, γ5, γ7 and γ8 have been detected in the anterior cingulate cortex (ACC) cortex of schizophrenic patients (Drummond *et al.*, 2013).

As reviewed in this chapter, stargazin plays a pivotal role in the regulation of AMPAR and synaptic plasticity, thus changes in its function likely contribute to alterations in neuronal transmission and possibly to disruption of synaptic homeostasis. Taken together, we propose to study the role of stargazin in the development of psychiatric disorders, namely SCZ and ID.

OBJECTIVES

Genetic studies indicate that proteins of the glutamatergic synapse play a pivotal role in the etiology of psychiatric disorders (Fromer *et al.*, 2014; Hamdan *et al.*, 2011; Kirov *et al.*, 2012; Krumm *et al.*, 2014; Network and Pathway Analysis Subgroup of Psychiatric Genomics, 2015; Purcell *et al.*, 2014; Schizophrenia Working Group of the Psychiatric Genomics, 2014). Stargazin is a postsynaptic AMPAR auxiliary subunit that regulates AMPAR trafficking and function. Importantly, stargazin is implicated in LTP and LTD, and recent data from our laboratory showed that stargazin is required for AMPAR synaptic scaling in a phosphorylation-dependent manner (Louros *et al.*, 2014; Tomita *et al.*, 2005b). Emerging evidence suggests that the *CACNG2* gene, which codes for stargazin, is a susceptibility gene for neuropsychiatric disorders (Hamdan *et al.*, 2011; Liu *et al.*, 2008; Wilson *et al.*, 2006; Yang *et al.*, 2013).

Given the crucial role of stargazin in excitatory synapse function and plasticity, and the recent implication of the stargazin-encoding gene in neuropsychiatric disorders, the main goal of this work was to elucidate the role of stargazin in these disorders, focusing on SCZ and intellectual disability since mutations in *CACNG2* were found in association to these two diseases. Therefore, we pursued the following objectives:

1) We searched for exonic mutations in the *CACNG2* gene in psychiatric disorder patients from the Portuguese Island Collection (PIC). We used both whole-genome and exon sequencing data. This task was performed in collaboration with Dr. Carlos Pato, Dr. Ayman Fanous and Dr. Célia Carvalho.
2) We characterized the cellular phenotypes originated by expression of two stargazin variants, one described in an intellectual disability patient (Hamdan *et al.*, 2011) and the other one identified by us in a SCZ patient. We employed a molecular replacement approach to examine the cellular impact of stargazin mutations. Endogenous stargazin expression in cortical neurons was depleted by shRNA-mediated *knock-down* and wild-type (WT) or disease-associated variants of stargazin were expressed. We characterized alterations in the cell surface mobility of stargazin, in AMPAR traffic and in synaptic scaling triggered by expression of the stargazin variants. We further evaluated effects in the neuritic tree and in synapse density in neurons expressing the disease-associated stargazin variants.
3) We searched for new stargazin interactors. Using co-immunoprecipitation assays followed by mass spectrometry, we identified new stargazin interaction partners, some of which have been implicated in psychiatric disorders.

4) We generated *knock-in* mice for the stargazin mutation associated with intellectual disability, Stg^{ID}, and performed the behavioral characterization of these mice. Stg^{ID} mice, generated through gene targeting, were submitted to behavior tests to evaluate locomotor activity, social behavior, anxiety-related phenotypes and cognition.

Herein we describe the first missense mutation in the *CACNG2* gene associated to SCZ. ID- and SCZ-associated mutations in *CACNG2* showed disruptive effects on stargazin surface mobility, basal and activity-dependent AMPAR trafficking, synapse number and neuritic network. The animal model that we produced expressing mutated stargazin showed an ID-like phenotype, including defective working memory and motor learning, and altered social behavior. Our findings support a role for stargazin in the pathogenesis of cognitive disorders such as SCZ and intellectual disability.

Data presented on Chapter 2 and 3 is under preparation for submission as a research paper:

Caldeira GL, Louros S, Rondão, T, Rodrigues, MV, Pato, C, Chen, C, Peça J, Cavalho AL. Human mutations in the *CACNG2* gene result in cellular and behavior phenotypes related to psychiatric disorders (in preparation).

CHAPTER 2

Identification and *in vitro* characterization of disease-associated variants of the *CACNG2* gene

2.1. Summary

Stargazin is a postsynaptic protein that physically interacts with and regulates AMPAR expression and function. Stargazin is required for several mechanisms of synaptic plasticity, such as long-term potentiation and long-term depression, and for homeostatic scaling of AMPARs at the synapse. Interestingly, single nucleotide polymorphism (SNP) analyses provide evidence associating the *CACNG2* gene encoding stargazin with SCZ and bipolar disorder, and the *CACNG2* gene is located in a region of chromosome 22 strongly implicated in these disorders. A recent study identified a missense mutation in the *CACNG2* gene in an intellectual disability (ID) patient. Given the crucial role for stargazin in excitatory transmission and synaptic plasticity, and the genetic data suggesting that the stargazin gene may be a susceptibility gene for cognitive disorders, herein we explore a role for stargazin in these disorders.

Using whole-genome sequencing data from patients from the Portuguese Island Collection, we identified a missense mutation in the *CACNG2* gene in a SCZ patient. To investigate the mutational effects on protein function, we studied neuronal alterations elicited by expression of this variant of stargazin (Stg^{SCZ}), or by expression of the previously described ID-linked stargazin variant (Stg^{ID}). We show that the two mutations result in shared and distinct effects on stargazin function. Whereas both mutations affect the cell surface diffusion of stargazin and its role in the trafficking of AMPARs, the ID-linked stargazin variant fails to homeostatically scale AMPARs upon blockade of neuronal activity, or to enhance the synaptic expression of AMPAR upon exposure to the anti-depressant and cognitive enhancer drug tianeptine. Furthermore, the expression of Stg^{SCZ} (but not of Stg^{ID}) affects the number of inhibitory synapses and dendritic arborization in dissociated rat cortical neurons. These results suggest that different disease-associated mutations in the *CACNG2* gene originate both common and disparate cellular phenotypes, which are likely related to their role in disease pathogenesis. Our data provide new mechanistic insight into how genetic deficits in *CACNG2* gene can lead to alterations in AMPAR traffic and neuronal network formation, and corroborate recent evidence that attributes a major role to synaptic network components in psychiatric disorders.

2.2. Introduction

SCZ is a devastating disorder that affects around 1% of the population worldwide. It is characterized by a heterogeneous set of clinical manifestations that include positive symptoms such as delusion and hallucinations, negative symptoms such as anhedonia and blunted affect, and also cognitive symptoms. Despite countless efforts, the mechanisms implicated in the development of SCZ have not yet been fully elucidated, hindering the attempts to develop effective therapy.

It is well known that genetics plays an important role in the development of most psychiatric disorders, and several genes implicated in a wide range of pathways have been associated to these disorders. Recent evidence from genome-wide association studies implicated genes encoding synaptic proteins in the pathogenesis of SCZ (Fromer *et al.*, 2014; Network and Pathway Analysis Subgroup of Psychiatric Genomics, 2015; Purcell *et al.*, 2014). Synaptic networks have been growingly recognized as major players in the development of SCZ; in fact, recent reports attributed a major importance to *de novo* mutations in genes that encode glutamatergic postsynaptic proteins with major roles in the modulation of synaptic strength. Importantly, these studies reinforce the notion that genes affected by mutations in SCZ overlap those mutated in autism and intellectual disability (Fromer *et al.*, 2014; Kirov *et al.*, 2012; Purcell *et al.*, 2014).

Emerging evidence suggests that stargazin, a synaptic protein which is an auxiliary subunit of AMPAR, may play a role in the development of these disorders. In fact, SNPs and CNVs in the *CACNG2* gene, which codes for stargazin, were associated with SCZ and bipolar disorder (Liu *et al.*, 2008; Wilson *et al.*, 2006; Yang *et al.*, 2013). The mRNA and protein levels of stargazin were shown to be altered in the dorsolateral prefrontal cortex of SCZ patients (Beneyto and Meador-Woodruff, 2006) but not in the anterior cingulate cortex (Drummond *et al.*, 2013). Furthermore, a missense mutation in the *CACNG2* gene was found in an intellectual disability patient and was suggested to affect stargazin function (Hamdan *et al.*, 2011).

Stargazin is a 37 kDa protein that is highly expressed in the cortex, cerebellum and hippocampus (Tomita *et al.*, 2003). It was the first identified TARP and it plays an important role in the expression, trafficking and kinetic modulation of AMPARs (Bedoukian *et al.*, 2006; Chen *et al.*, 2000; Tomita *et al.*, 2005a; Vandenberghe *et al.*, 2005b). Phosphorylation and dephosphorylation of stargazin were shown to be required for LTP and LTD, respectively (Tomita *et al.*, 2005b), and recently our laboratory described the implication of stargazin in homeostatic plasticity and experience-dependent plasticity in the retinogeniculate synapse (Louros *et al.*, 2014).

Herein we describe a missense mutation in the third exon of the *CACNG2* gene in a SCZ patient from the Portuguese Island Collection. This variant, Stg^{SCZ}, as well as the one previously identified by Hamdan and colleagues (Hamdan *et al.*, 2011) in an intellectual disability patient, Stg^{ID}, present

increased cell surface mobility, and impact the delivery of AMPAR to the synapse in rat cortical neurons. Expression of Stg^{ID} also impairs synaptic scaling, whereas Stg^{SCZ} expression affects dendritic arborization and the number of inhibitory synapses in cortical neurons, which correlates with E/I imbalance in SCZ patients [reviewed in (Anticevic and Lisman, 2017)]. Taken together, our results suggest that stargazin might be a player in mechanisms that are disrupted in psychiatric disorders, and corroborate the hypothesis that mutations in the same gene can culminate in different cellular phenotype which may be associated with different symptoms, characterizing distinct disorders.

2.3. Results

2.3.1. SCZ-associated mutation, S148N, is predicted to affect the function of stargazin

In order to study a possible relevant role for stargazin in the etiology of psychiatric disorders, we searched for mutations in the *CACNG2* gene in patients from the Portuguese Island Collection (PIC). The PIC, which is a part of the Genomic Psychiatry Cohort (GPC) (Pato *et al.*, 2013), includes individuals and multiplex families with SCZ and/or bipolar disorder from the Portuguese islands, Madeira and Azores, and mainland (Pato *et al.*, 1997; Pato *et al.*, 2004). The population from the Portuguese Islands is a geographically isolated and genetically homogeneous population, where several families present more than one affected individual; it is therefore an interesting population for genetic studies (Pato *et al.*, 1997). After diagnosis and selection of subjects to be included in this group, their genomic DNA was extracted from whole blood as described previously (Pato *et al.*, 2004; Pato *et al.*, 2013; Sklar *et al.*, 2004) and whole genome sequencing was performed. The information regarding the *CACNG2* gene sequence from 35 control individuals, 19 bipolar disorder, 11 SCZ and 2 depression patients was kindly provided by Dr. Carlos Pato and Dr. Ayman Fanous. In collaboration with Dr. Célia Carvalho from the University of Azores, we increased the number of individuals to be included in this study. To do so, we extracted genomic DNA from whole blood from 15 control indiviuals, 5 SCZ and 3 bipolar disorder patients and sequenced the four exons of the *CACNG2* gene using Sanger sequencing (Figure 2.1A).

We identified a new mutation in the *CACNG2* gene, c.725G>A, in a SCZ patient. This mutation leads to the replacement of a serine residue for an asparagine residue in the 148th position of the protein sequence (p.S148N) (Figure 2.1B). This variant was not previously described in any of the databases collecting sequencing variants for the general population that we used, at the date of our search (May 2017): Exome Variant Server (http://evs.gs.washington.edu/EVS), SwissVar (http://swissvar.expasy.org), dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP) or Exome Aggregation Consortium (ExAc; http://exac.broadinstitute.org).

Sequence analysis of the four exons of *CACNG2* was also performed for the parents and one of the siblings of the proband (a deceased sibling, with a SCZ diagnosis, was not included in this study since the DNA was not available for analysis). This analysis showed that the mother, with major depression diagnosis, is also a carrier of the mutation (Figure 2.1 C,D), whereas the father and a healthy sibling do not carry this variant.

A *de novo* missense mutation in the same gene was recently described in an 8 year-old patient with moderate, non-syndromic, intellectual disability (Hamdan *et al.*, 2011). This mutation (Figure 2.1 E) leads to substitution of valine143 for a leucine residue in stargazin (p.V143L).

Both mutations affect residues highly conserved among species (Figure 2.1F), suggesting that these sites may be important for the function of stargazin. Accordingly, S148N and V143L mutations were predicted to be damaging to the function of stargazin by the PolyPhen-2 (Adzhubei *et al.*, 2013) (Figure 2.1G,H, respectively for S148N and V143L), SIFT(Kumar *et al.*, 2009) and PROVEAN (Choi *et al.*, 2012) prediction tools.

A.

Controls	50
Schizophrenia	16
Bipolar disorder	22
Major depression	2

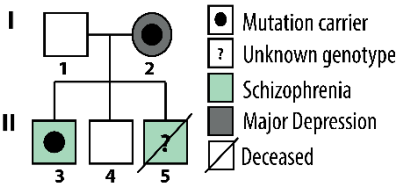
B.

Position	Gene	Ref. Allele/Variant	Type	Population	Diagnosis
22q13.1	CACNG2	G/A	Missense	Caucasian (PIC)	Schizophrenia

C.

Proband – 9007-27	C	A	G	G	T	C	T	G	A	A	T	A	A
Mother – 9007-26	C	A	G	G	T	C	T	G	A	A	T	A	A
Non-affected brother – 9007-28	C	A	G	G	T	C	T	G	A	G	T	A	A
Father – 9007-25	C	A	G	G	T	C	T	G	A	G	T	A	A

D.



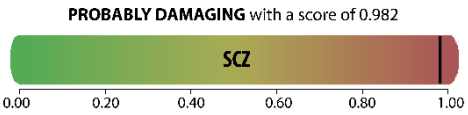
E.

Position	Gene	Ref. Allele/Variant	Type	Population	Diagnosis
22q13.1	CACNG2	G/C	Missense	Caucasian (PIC)	Intellectual Disability

F.

	143						148					
Homo sapiens	A	G	I	F	F	V	S	A	G	L	S	N	I
Mus musculus	A	G	I	F	F	V	S	A	G	L	S	N	I
Rattus norvegicus	A	G	I	F	F	V	S	A	G	L	S	N	I
Gallus gallus domesticus	A	G	I	F	F	V	S	A	G	L	S	N	I
Danio Rerio	A	G	L	F	F	V	S	A	G	L	S	N	I
Ovis aries	A	G	I	F	F	V	S	A	G	L	S	N	I

G.



H.

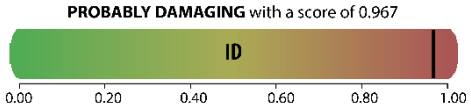


Figure 2.1. Identification of a new missense mutation in the *CACNG2* gene. Using whole-genome or exome sequencing data from patients from the Portuguese Island Collection (**A**), a new point mutation was identified in heterozygosity in exon 4 of the *CACNG2* gene in a SCZ patient (**B**). The point mutation results in the replacement of serine 148 for an asparagine. The mother of the proband, who suffers from major depression, is also a carrier of this missense mutation (**C**,**D**). Another mutation was previously found in exon 3 of the *CACNG2* gene in an 8-year-old patient with intellectual disability (**E**). Both mutations lead to the replacement of highly evolutionarily conserved residues (**F**) and were predicted to be damaging to the function of the protein. This prediction was performed using PolyPhen-2 (Adzhubei *et al.*, 2013), a tool designed for the prediction of functional effects of human SNPs (**G, H**).

2.3.2. Disease-associated stargazin variants, Stg^{SCZ} and Stg^{ID}, present altered surface diffusion properties

Stargazin has a function in stabilizing AMPAR at synaptic sites, through its binding to the postsynaptic scaffold protein PSD95, in a phosphorylation-dependent manner (Bats *et al.*, 2007; Chen *et al.*, 2000; Schnell *et al.*, 2002; Sumioka *et al.*, 2010). On the other hand, stimuli that promote the dephosphorylation of stargazin are described to mediate the trafficking of stargazin to extrasynaptic sites where it can be internalized (Matsuda *et al.*, 2013; Schnell *et al.*, 2002; Tomita *et al.*, 2005b). It was previously demonstrated that the immobilization of AMPAR at synaptic sites requires both the phosphorylation of stargazin and its binding to PSD95 in a CaMKII-dependent manner. Thereby, this interaction regulates the lateral diffusion of stargazin-associated AMPAR between extrasynaptic and synaptic sites (Bats *et al.*, 2007; Opazo *et al.*, 2010). Importantly, the lateral diffusion of AMPAR ensures high-frequency synaptic transmission by allowing the rapid exchange of desensitized and naïve receptors, highlighting the importance of this dynamics mediated by stargazin (Constals *et al.*, 2015; Heine *et al.*, 2008).

To explore the effect of the disease-associated mutations on the diffusion properties of cell surface stargazin, we co-transfected low-density cortical neurons with plasmids encoding Homer-GFP, for synapse identification, and HA-tagged *wildtype* (WT) stargazin (Stg^{WT}) or stargazin variants, Stg^{ID}or Stg^{SCZ}. We monitored individual stargazin complexes by single nanoparticle imaging of HA-stargazin using quantum dots (QDs) labeled for HA (Figure 2.2A).

We observed that Stg^{ID} and Stg^{SCZ} particles display increased global and synaptic diffusion coefficient (Figure 2.2 B,C) and a higher mean square displacement (Figure 2.2 D), suggesting that these particles are more mobile than the Stg^{WT} ones. In agreement, the fraction of immobile particles of the disease-associated variants is slightly decreased (Figure 2.2 E), and their synaptic residence time, or the time they spend at synapses, is decreased (Figure 2.2F).

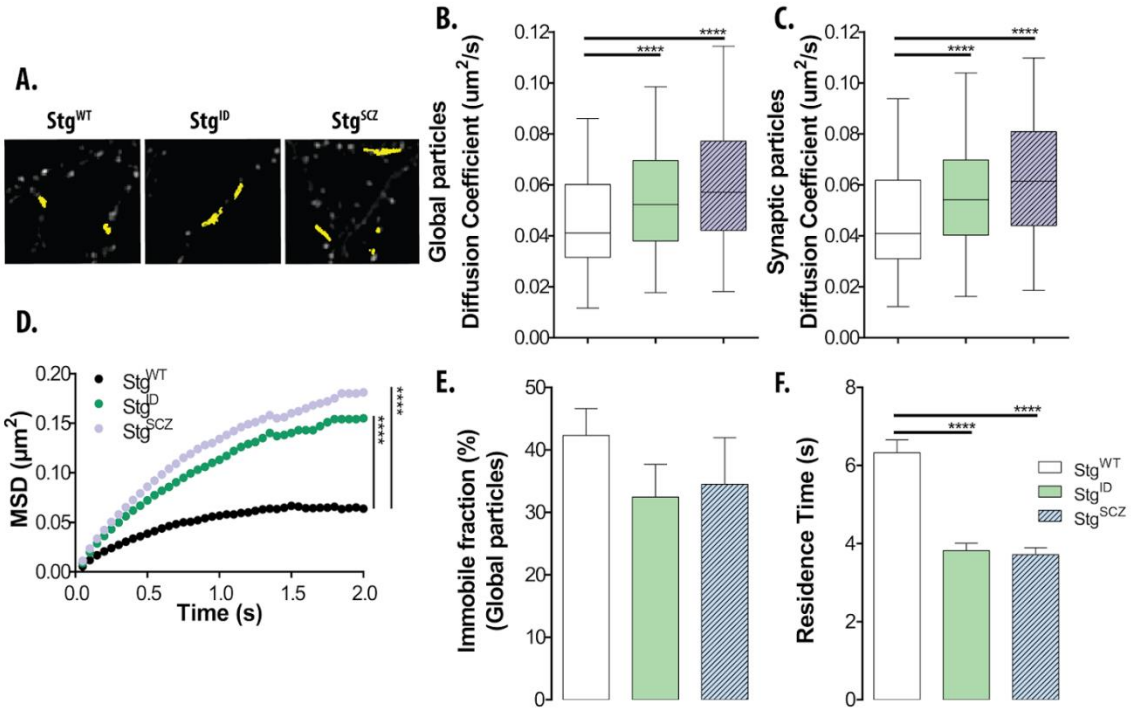


Figure 2.2. Disease-associated mutations affect stargazin surface diffusion properties. The trajectory of single particles of stargazin variants (Stg^{WT}, Stg^{ID} and Stg^{SCZ}) was assessed using QuantumDots coupled antibodies to target the extracellular HA-tag in stargazin (**A**). The diffusion coefficient, which correlates with the speed of the particles, was significantly increased both when considering all particles (global particles) and synaptic Stg^{ID} and Stg^{SCZ} particles, compared to Stg^{WT} (**B**,**C**). Evaluation of the Mean Square Displacement (MSD) showed that the surface area covered by stargazin disease-associated variant particles was larger than that covered by Stg^{WT} (**D**). Accordingly, the fraction of immobile particles was smaller for $Stg^{ID}$and Stg^{SCZ} than WT stargazin (**E**), and they showed less time spent at the synapses (**F**). ****p<0.0001, One-way ANOVA followed by Dunnet's multiple comparison test. Data from three independent experiments is presented as median ±25%-75% interquartile range. N(Stg^{WT})=825, N(Stg^{ID})=693, N(Stg^{SCZ})=685 particles.

2.3.3. Effects of mutations in stargazin on its expression, trafficking to the surface and ability to interact with AMPAR

Stargazin is a four transmembrane domain protein (Letts *et al.*, 1998) that contains a C-terminal intracellular region with nine phosphorylatable serine residues and a PDZ-binding motif, which allows its interaction with PSD95 and PSD93 (Dakoji *et al.*, 2003). Through co-immunoprecipitation assays, stargazin was shown to physically interact with AMPAR in a manner dependent on the first extracellular domain and on the C-terminal domain (Tomita *et al.*, 2004). Later, the second extracellular domain of stargazin was also implicated in this interaction (Cais *et al.*, 2014). More recently, cryo-EM structure of the homomeric GluA2 AMPAR saturated with stargazin molecules showed that the extracellular domains of stargazin are positioned to modulate the AMPAR ligand-binding domain (LBD) closure and to affect the conformation of the LBD layer that is associated with the activation and desensitization of the receptor. Furthermore, the third and fourth transmembrane domains (TM3 and TM4) interact with transmembrane domains of the AMPAR subunits, including the pore helices (Twomey *et al.*, 2016; Zhao *et al.*, 2016). Interestingly, both mutations, identified in SCZ and intellectual disability patients, are situated in TM3 (Figure 2.3). Considering the importance of this domain for the interaction with AMPAR it is plausible that a significant change in its structure could weaken stargazin's interaction with AMPAR. As mentioned before, the Polyphen-2 prediction tool, that takes into consideration evolutionary conservation and the structure of the protein, predicted the two mutations to be deleterious to the function of stargazin (Figure 2.1 G, H). We therefore evaluated the effect of V143L and S148N on the total and cell surface expression of stargazin in neurons, and on stargazin's interaction with AMPAR.

To study whether the disease-associated mutations in stargazin affect its expression levels, high-density cortical neurons were transduced with lentivirus expressing HA-tagged Stg^{WT}, Stg^{ID} or Stg^{SCZ} (Figure 2.4 A), and mRNA and protein levels of HA-tagged stargazin were evaluated. Both mRNA and protein levels (Figure 2.4 B, C) of the Stg^{SCZ} variant were slightly, albeit not significantly decreased, when compared to the levels of Stg^{WT}. To test whether Stg^{ID} and Stg^{SCZ} are trafficked to the neuronal surface similarly to Stg^{WT}, we transfected low-density cortical neurons with plasmids encoding HA-tagged Stg^{WT}, Stg^{ID} or Stg^{SCZ} (Figure 2.4 D). Cell surface and intracellular HA-stargazin pools were immunolabeled with an antibody against the HA tag (Figure 2.4 E), which is extracellularly located in the cell surface protein (Fig. 2.4 A). Both the cell surface (Figure 2.4 F) and the intracellular labeling (Figure 2.4 G) of HA-stargazin, as well as the ratio between cell surface and intracellular fractions (Figure 2.4 H), were evaluated. In cells transfected with the disease-associated variants, the cell surface HA-stargazin levels were lower than in cells expressing WT HA-stargazin, whereas intracellular HA-stargazin levels were similar in all conditions. The cell surface/intracellular

ratio of HA-stargazin was decreased for Stg^{ID} and Stg^{SCZ}, suggesting that these variants are less trafficked to the cell surface.

To investigate whether stargazin variants interact with AMPAR, we co-transfected HEK 293T cells with plasmids encoding the HA-tagged stargazin variants and GluA1 (Figure 2.4 I). We immunoprecipitated HA-stargazin, and evaluated co-immunoprecipitation of GluA1 (Figure 2.4 J). Non-immune IgGs were used for negative controls. GluA1 co-immunoprecipitated with both variants suggesting that the mutations do not significantly impact the interaction with GluA1 AMPAR subunit.

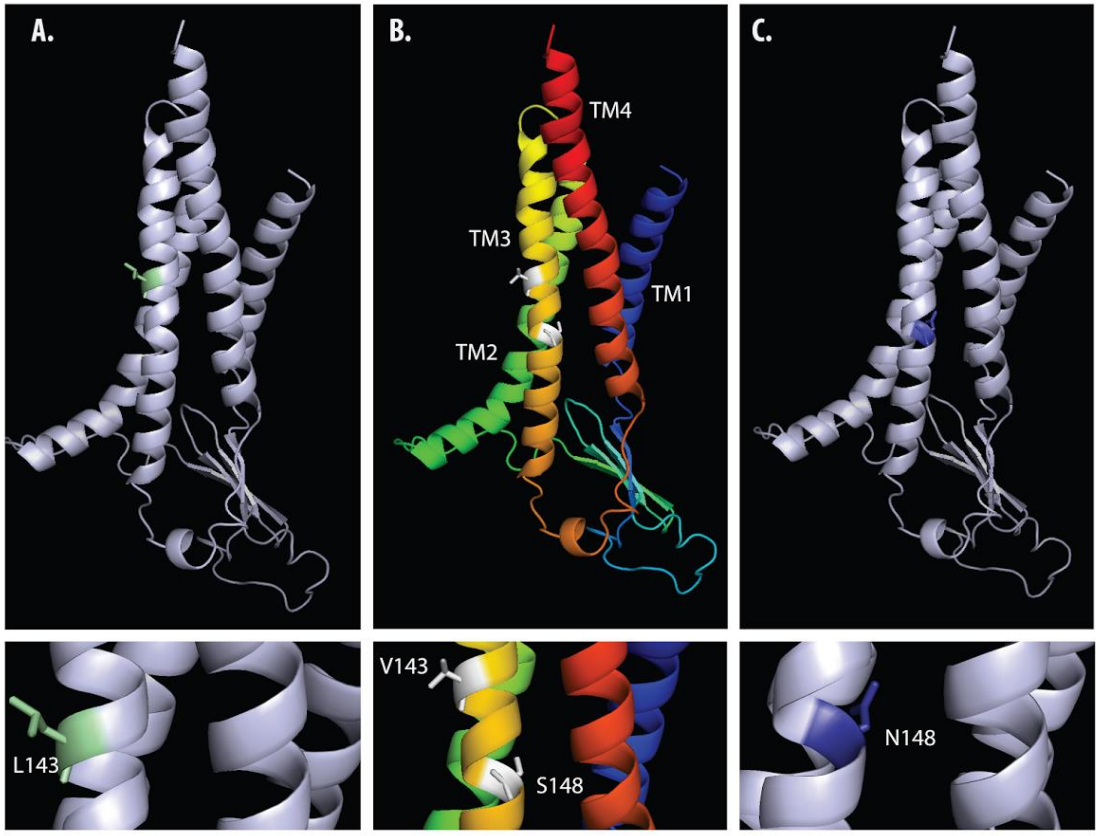


Figure 2.3. Protein structure of WT stargazin and localization of the disease-associated stargazin variants. Stargazin structure was modeled based on the 5kk2.1.E model, using the Swiss-model tool. Protein structure representations were obtained using PyMol software. In the left panel, residue leucine 143 (ID-associated variant) is labeled in green (**A**). In the middle panel the residues valine 143 (top) and serine 148 (bottom), which are present in WT stargazin, are labeled in white (**B**). The right panel shows asparagine 148 (SCZ-associated variant) in blue (**C**). Bottom panels are magnifications of the panels above. TM – transmembrane domain.

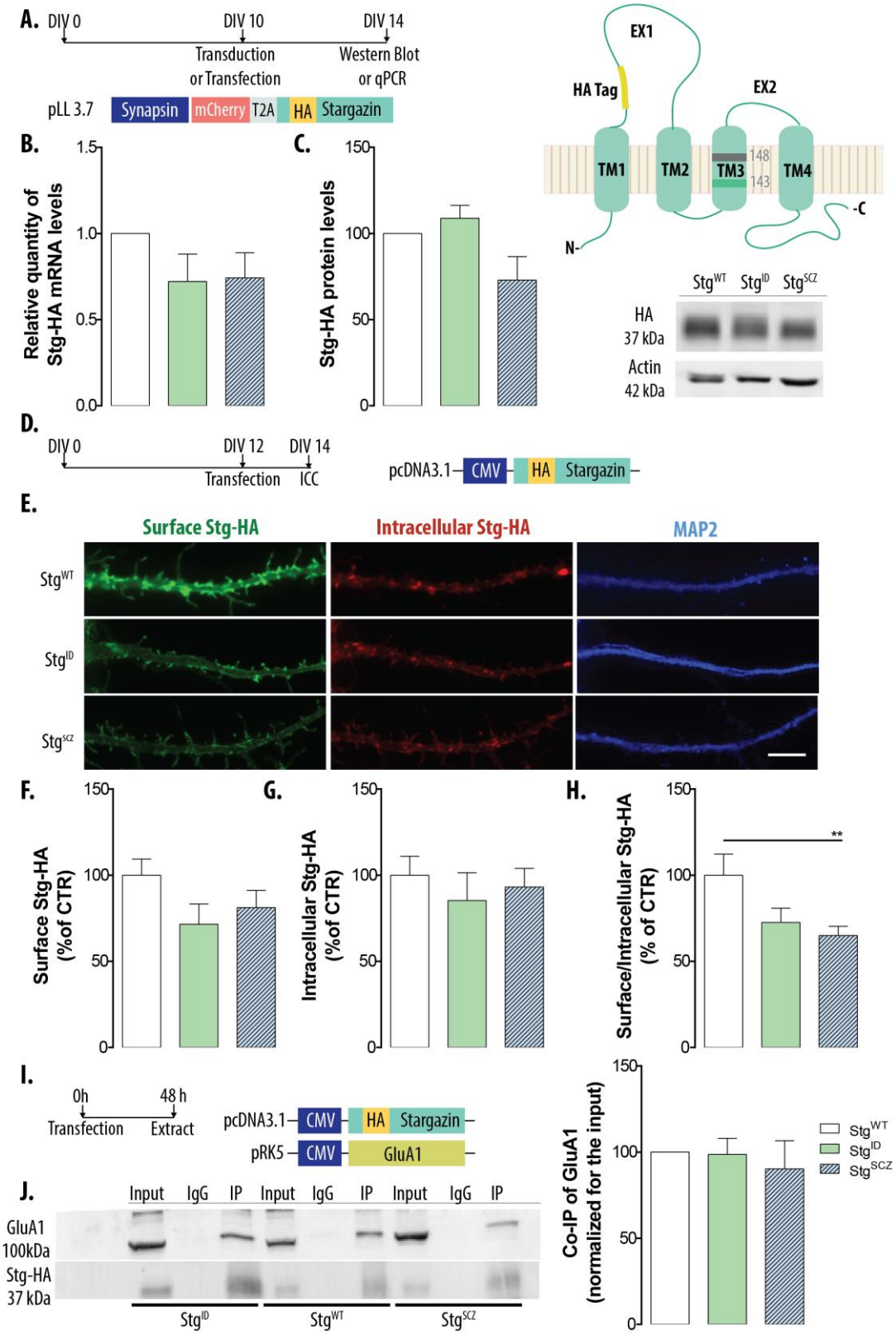

A.
DIV 0
DIV 10
DIV 14
Transduction or Transfection
Western Blot or qPCR
pLL 3.7
Synapsin
mCherry
T2A
HA
Stargazin
B.
Relative quantity of Stg-HA mRNA levels
C.
Stg-HA protein levels
EX1
HA Tag
EX2
TM1
TM2
TM3
TM4
148
143
N-
-C
Stg^WT
Stg^ID
Stg^SCZ
HA
37 kDa
Actin
42 kDa
D.
DIV 0
DIV 12
DIV 14
Transfection
ICC
pcDNA3.1
CMV
HA
Stargazin
E.
Surface Stg-HA
Intracellular Stg-HA
MAP2
Stg^WT
Stg^ID
Stg^SCZ
F.
Surface Stg-HA (%of CTR)
G.
Intracellular Stg-HA (%of CTR)
H.
Surface/Intracellular Stg-HA (% of CTR)
**
I.
0h
48 h
Transfection
Extract
pcDNA3.1
CMV
HA
Stargazin
pRK5
CMV
GluA1
J.
Input
IgG
IP
GluA1
100kDa
Stg-HA
37 kDa
Stg^ID
Stg^WT
Stg^SCZ
Co-IP of GluA1 (normalized for the input)
Stg^WT
Stg^ID
Stg^SCZ

Figure 2.4. Disease-associated stargazin variants expression and interaction with AMPAR. High-density cortical cultures were transfected or transduced at DIV 10 with lentivirus expressing HA-tagged Stg^{WT} or the stargazin variants Stg^{ID} or $Stg^{SCZ,}$ under the control of the synapsin promoter (**A**). A slight decrease in the mRNA (**B**) or protein levels (**C**) of the Stg^{SCZ} variant was observed, compared to Stg^{WT}. qPCR and immunolabeling of HA-stargazin were performed from total extracts from ≥ six independent experiments. Data are presented as mean±SEM. Low-density cortical neurons were transfected at DIV12 with plasmids encoding HA-stargazin variants followed by immunostaining for HA at DIV14 (**D**). Representative images of Stg-HA cell surface and intracellular distribution (**E**), and quantification of cell surface (**F**) and intracellular (**G**) intensity of Stg-HA clusters, showed a reduction in surface expression levels of Stg^{ID} and Stg^{SCZ} not accompanied by changes in the intracellular levels. Thus, the ratio between the two fraction showed a reduction for both disease-associated variants, compared to Stg^{WT}, which reached statistical significance for Stg^{SCZ} (**H**). Clusters were quantified from ≥30 cells from ≥ five independent experiments.**p<0.01; t-test. Data are presented as mean±SEM. Scale bar represents 5 µm. HEK 293T cells were transfected with expression plasmids for GluA1 and Stg-HA variants (**I**). Total protein extracts were used to immunoprecipitate HA-tagged stargazin (negative controls were performed with non-immune IgGs). Immunoprecipitated samples were probed by immunoblotting for HA-stargazin and GluA1. Both Stg^{ID} and Stg^{SCZ} variants interacted with GluA1-containing AMPAR (**J**). Right: quantification of GluA1 co-immunoprecipitation with stargazin from four independent experiments. Data are presented as mean±SEM.

2.3.4. Stargazin disease-associated variants impact AMPAR trafficking and stabilization at the synapse

Stargazin associates with AMPAR in the endoplasmic reticulum (ER) (Vandenberghe *et al.*, 2005a) during a late phase of AMPAR maturation (Shanks *et al.*, 2010), and mediates complexed AMPAR trafficking out of the ER, through a membrane sorting signal present in its C-terminal region (Bedoukian *et al.*, 2006; Bedoukian *et al.*, 2008). Stargazin then mediates AMPAR trafficking to the cell membrane and their stabilization at the synapse (Jackson and Nicoll, 2011; Opazo *et al.*, 2010). Receptor immobilization at synapses requires stargazin phosphorylation by CaMKII (Opazo *et al.*, 2010) and interaction with PSD95, through the PDZ binding motif present at the C-terminal domain (Bats *et al.*, 2007; Chen *et al.*, 2000; Schnell *et al.*, 2002; Sumioka *et al.*, 2010). Accordingly, *stargazer* mice, which lack stargazin, present decreased levels or even absence of AMPAR at the synapse in different brain regions (Barad *et al.*, 2012; Chen *et al.*, 2000; Shevtsova and Leitch, 2012). Importantly, stargazin also affects AMPAR kinetics, as it reduces receptor desensitization and slows receptor deactivation, in a manner dependent on its first extracellular domain (Priel *et al.*, 2005; Tomita *et al.*, 2005a; Turetsky *et al.*, 2005).

Besides this extensively documented functions, stargazin was also shown to regulate the sensitivity of AMPAR to antagonists (Cokic and Stein, 2008) and the pharmacology of AMPAR potentiators (Tomita *et al.*, 2006), the channel conductance and polyamine block of calcium-permeable AMPAR (Shelley *et al.*, 2012; Soto *et al.*, 2014; Soto *et al.*, 2007), and the AMPAR-dependent clustering of

NMDAR in hippocampal interneurons (Mi *et al.*, 2004). In addition, stargazin is involved in several synaptic plasticity mechanisms, such as LTP, LTD and homeostatic plasticity (Jackson and Nicoll, 2011; Louros *et al.*, 2014; Nomura *et al.*, 2012; Tomita *et al.*, 2005b), which depend on AMPAR traffic. To determine if the disease-associated mutations affect the stargazin-mediated trafficking of AMPAR to the cell membrane and their stabilization at the synapse, we utilized a molecular replacement strategy. We transfected low-density cortical neurons with control shRNA (CTR) or with a specific shRNA that depletes the levels of endogenous stargazin [KD; (Louros *et al.*, 2014)]. Stargazin shRNA is expressed from a bicistronic vector that leads to co-expression of GFP (Figure 2.5 A), thereby providing a way to identify transfected neurons. As previously described (Louros *et al.*, 2014), stargazin silencing led to a decrease on the cell surface and synaptic levels of GluA1-containing AMPAR (Figure 2.5. B-D), assessed by immunolabeling GluA1 using an antibody specific for the extracellular N-terminal region. AMPAR clusters were considered synaptic when colocalizing with PSD95, whose expression was not affected by stargazin silencing (Fig. 2.5 B, E). To test stargazin mutants, cells were co-transfected with stargazin shRNA and shRNA-refractory stargazin, either Stg^{WT} (KD + Stg^{WT}) or the disease-associated variants of stargazin (KD + Stg^{ID} or KD + Stg^{SCZ}) (Figure 2.5 A). Whereas WT stargazin rescues cell surface (Figure 2.5 B, C) and synaptic (Figure 2.5 B, D) AMPAR levels to control levels, both Stg^{ID} and Stg^{SCZ} failed to mediate AMPAR traffic to the cell surface and to the synapse. Neither variant affected PSD95 levels (Figure 2.5 E). Taken together, these data indicate that the disease-associated stargazin mutations affect the role of stargazin in trafficking GluA1-containing AMPAR to the surface and to synapses.

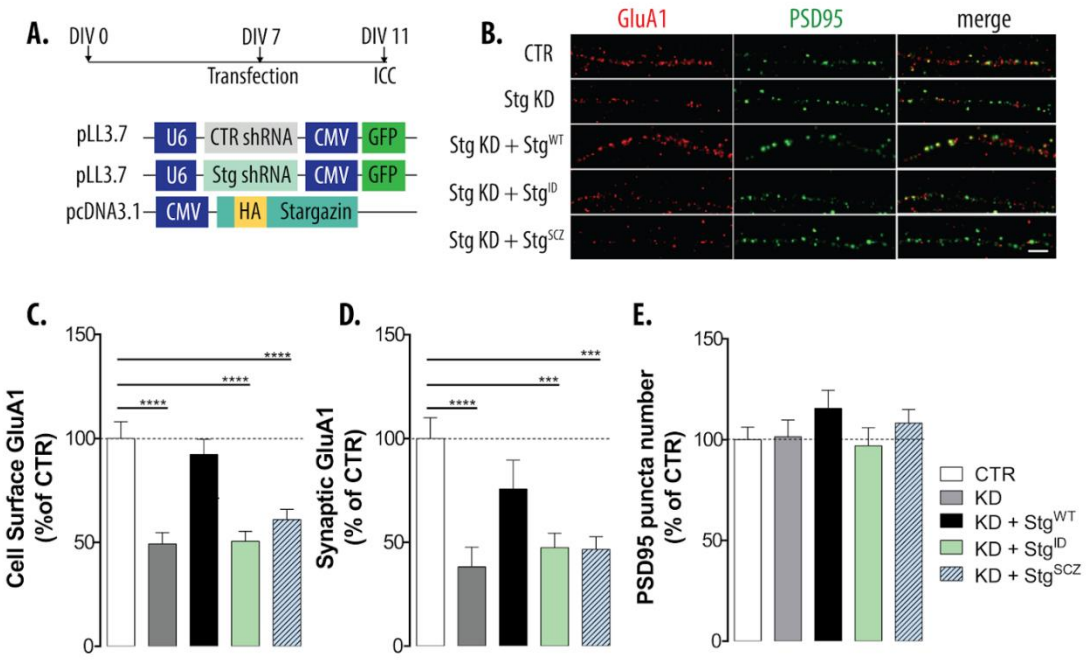


Figure 2.5. Disease-associated stargazin variants impact AMPAR trafficking and stabilization at synapses.
Low-density cortical neurons were transfected at 7 DIV with pLL-shRNA-CTR, pLL-shRNA-Stg, which down-regulates endogenous stargazin, or co-transfected with pLL-shRNA-KD and pcDNA-Stg-HA variants (**A**). Total surface and synaptic levels of GluA1 were analyzed by immunocytochemistry at DIV 11. Representative images of GluA1 distribution (**B**) and quantification of total and synaptic intensity of GluA1 clusters (**C**, **D**) show impaired trafficking of GluA1-containing AMPAR in neurons transfected with Stg^{ID} and Stg^{SCZ} variants. GluA1 accumulation at synaptic sites was assessed by the colocalization with PSD95 clusters, whose intensities are not significantly different between conditions (**E**). Clusters were quantified from n≥25 cells imaged from three independent experiments; ****p< 0.0001; ***p<0.001; one-way ANOVA. Data are presented as mean ± SEM. Scale bar represents 8 µm.

2.3.5. Intellectual disability-associated stargazin variant impairs homeostatic synaptic scaling

Normal cognition and behavior depend on tight neuronal homeostatic control mechanisms that are influenced by several molecular processes, whose dysregulation overlaps among several neuropsychiatric disorders (Ramocki and Zoghbi, 2008). Altered proteins that are required for homeostatic processes may lead to dysfunctional neuronal networks with weakened synaptic flexibility, eventually causing overlapping clinical phenotypes (Ramocki and Zoghbi, 2008). In agreement with this hypothesis is the observation that the same genes, particularly the ones involved in the modulation of synaptic plasticity, have been implicated in intellectual disability, ASD and several behavioral disorders such as bipolar disorder and SCZ (Cross-Disorder Group of the Psychiatric Genomics, 2013; Fromer *et al.*, 2014; Kirov *et al.*, 2012; Power *et al.*, 2017; Purcell *et al.*, 2014; Volk *et al.*, 2015).

Another important observation is that in many of these disorders, individuals present periods with normal development before disease onset, suggesting that neurons are able to compensate for slight changes over time before an accumulation of events causes system dysfunction (Ramocki and Zoghbi, 2008). In fact, in the case of SCZ the common onset stage is late adolescence or early adulthood, and is often associated with stressful events.

A study focusing on understanding how SCZ patients respond to life events during the first phases of the disease showed that SCZ patients recognize positive and negative life events as less controllable and poorly handled, comparing to controls (Horan *et al.*, 2005; Myin-Germeys *et al.*, 2001). Accordingly, the SCZ patient carrying the mutation in *CACNG2* identified in this work, as well as his mother, who suffers from major depression, demonstrated an exacerbated response to neutral stressors, comparing to the control population (Célia Carvalho, personal communication), when evaluated in a stress scale (Carvalho *et al.*, 2015). Interestingly, a recent study reported an increase in the stargazin levels in the anterior cingulate cortex of monkeys associated with stress

coping induced by social isolation (Lee *et al.*, 2016), suggesting that stargazin may be implicated in stress-related adaptive mechanisms.

Amongst a vast range of synaptic proteins, stargazin has been implicated in both LTP and LTD since its phosphorylation and dephosphorylation were shown to be required for the induction of these forms of plasticity, respectively (Matsuda *et al.*, 2013; Nomura *et al.*, 2012; Tomita *et al.*, 2005b). LTP and LTD are rapidly induced and long-lasting plasticity processes, which allow the strengthening or weakening of specific synapses, contributing to memory storage in the brain. However, the intrinsic positive feed-back nature of LTP and LTD (when a synapse undergoes LTP or LTD it becomes more or less excitable, respectively, and is further potentiated or depressed more easily), leads to a stability problem for neuronal circuits, which could easily be driven to runaway excitation or synaptic silencing. Homeostatic plasticity is a set of mechanisms that counteract these disturbances, maintaining neuronal activity within physiological range [reviewed in (Fernandes and Carvalho, 2016)]. These homeostatic mechanisms at the synaptic level participate in neuronal homeostasis, and may be compromised in neuropsychiatric disorders (Wondolowski and Dickman, 2013). Interestingly, a previous study from our laboratory showed that homeostatic scaling up of AMPAR upon chronic blockade of activity requires stargazin (Louros *et al.*, 2014).

Given the role of stargazin in homeostatic plasticity mechanisms (Louros *et al.*, 2014), and the implication of these mechanisms in the etiology of neuropsychiatric disorders (Ramocki and Zoghbi, 2008; Wondolowski and Dickman, 2013), we evaluated the effect of disease-associated stargazin mutations on stargazin-mediated homeostatic scaling of GluA1-containing AMPAR. To do so, we transfected low-density cortical neurons at DIV 7 with control shRNA (CTR), or a shRNA specific for stargazin (KD), to knock-down endogenous stargazin, or co-transfected neurons with the stargazin shRNA and the shRNA-insensitive stargazin variants (KD + Stg^{WT}, KD + Stg^{ID} KD + Stg^{SCZ}). At DIV 9, neurons were incubated with 1µM TTX for 48h, and surface GluA1-containing receptors and total PSD95 were immunolabeled (Figure 2.6 A, B). An increase in the surface and synaptic GluA1 expression after blockade of activity with TTX was observed in the control condition. This homeostatic upregulation of synaptic AMPAR in response to prolonged blockade of activity has been extensively reported in different systems [reviewed in (Fernandes and Carvalho, 2016)]. As previously reported (Louros *et al.*, 2014), synaptic upscaling of GluA1-containing AMPAR was prevented by the depletion of stargazin, and rescued by introduction of the shRNA-refractory WT form of stargazin (Stg^{WT}) (Fig. 2.6 B-D). Although to a smaller extent, expression of Stg^{SCZ} rescued the GluA1 levels at the cell surface upon incubation in TTX (Fig. 2.6 B-D), but expression of the Stg^{ID} variant did not result in increased levels of surface and synaptic GluA1 as a response to activity blockade (Fig. 2.6 B-D). These data suggest that the ID-associated stargazin mutation completely abolishes stargazin-mediated scaling of AMPAR whereas Stg^{scz}, although deficient in promoting the

trafficking of AMPAR to the surface under basal conditions, is able to respond to neuronal activity manipulations that trigger homeostatic plasticity. These results indicate that the two mutations in stargazin, although present in the same transmembrane domain, differentially affect the function of stargazin in mediating activity-dependent AMPAR traffic.

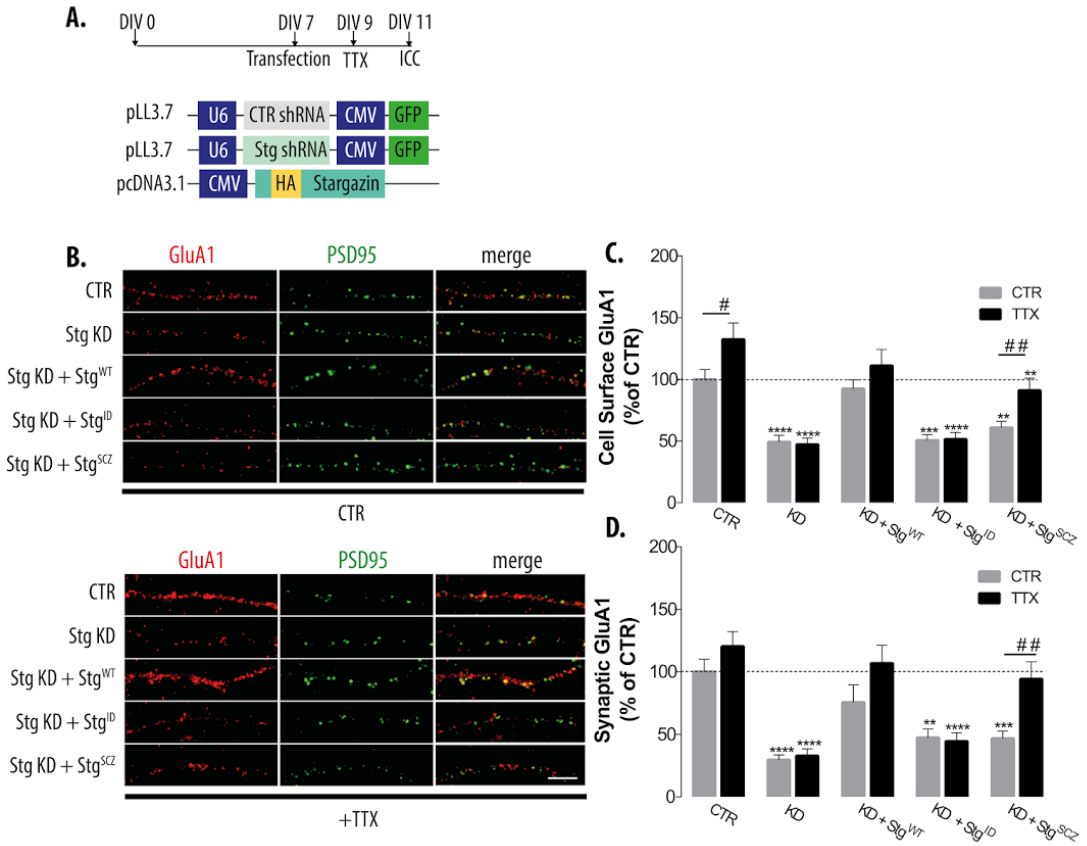


Figure 2.6. Intellectual disability-associated stargazin variant affects homeostatic scaling of AMPAR. Low-density cortical neurons were transfected at DIV7 with pLL-shRNA-CTR, pLL-shRNA-Stg, which down-regulates endogenous stargazin, or co-transfected with pLL-shRNA-Stg and pcDNA-Stg-HA variants. Transfected neurons were incubated with TTX (1μM) at DIV 9 and total surface and synaptic levels of GluA1 were analyzed by immunocytochemistry at DIV 11 (**A**). Representative images of GluA1 and PSD95 distribution (**B**) and quantification of total and synaptic intensity of GluA1 clusters (**C,D**) show that synaptic scaling was blocked in cortical neurons upon stargazin silencing, and could be rescued by the expression of Stg^{WT} or Stg^{SCZ}, but not by the Stg^{ID} variant. GluA1 accumulation at synaptic sites was assessed by the colocalization with PSD95 clusters. Clusters were quantified from n≥25 cells imaged from three independent experiments; ****p< 0.0001; ***p<0.001; **p<0.01; *p<0.05; #p<0.05; ##p<0.01 two-way ANOVA, multiple comparison tests. Data are presented as mean ± SEM. Scale bar represents 8 μm. The gray bars in graphs C and D represent previously presented content (Figure 2.5 C and D).

2.3.6. The cognitive enhancer tianeptine reverts the deficits induced by the S148N mutation in stargazin-mediated trafficking of AMPAR

Tianeptine is a widely used antidepressant structurally similar to tricyclic antidepressants but with particular pharmacological properties. This drug has drawn much attention since it challenges the monoamine hypothesis of depression. Unlike most of the antidepressant drugs, tianeptine does not modulate the monoamine axis but instead activates a cascade of events that culminate in cellular adaptations with antidepressant effects, which include the phosphorylation of AMPAR. In fact, tianeptine does not show affinity for kainate, AMPA, NMDA, GABA or monoamine neurotransmitter receptors and does not interfere with the reuptake of serotonin, a common target of antidepressant drugs [reviewed in (McEwen *et al.*, 2010)], but studies involving radioligand binding and cell-based functional assays identified tianeptine as a μ-opioid receptor agonist, implicating these receptors in the clinical effects of the drug (Gassaway *et al.*, 2014).

Tianeptine has been shown to prevent and reverse the stress-induced remodeling of hippocampal neurons (Magarinos *et al.*, 1999), and to prevent stress-induced effects on the performance in the eight-arm radial- and Y-mazes (Conrad *et al.*, 1996; Luine *et al.*, 1994). Furthermore, tianeptine was shown to have a beneficial effect on learning and working memory since it increased spontaneous alternation and left-right discrimination the in T-maze (Jaffard *et al.*, 1991).

Although tianeptine does not bind to AMPAR, it was shown to have an effect on AMPAR function. In fact, it leads to an increase in the phosphorylation of Ser831 and Ser845 of GluA1 subunits in the hippocampus (Svenningsson *et al.*, 2007) and potentiates AMPA-elicited firing activity in a CaMKII and PKC-dependent manner (Szegedi *et al.*, 2011). More recently it was found to be a potent modulator of AMPAR surface diffusion through the CaMKII-dependent phosphorylation of stargazin, which favors its binding to PSD95 (Zhang *et al.*, 2013).

Given the effects of tianeptine on stargazin, we tested whether it can revert the AMPAR trafficking defects related to the disease-associated mutations in stargazin (Stg^{ID} and Stg^{SCZ}). Cortical neurons where stargazin was silenced (Stg KD), or which express either WT stargazin or the stargazin variants (KD + Stg^{WT}, KD + Stg^{ID} KD + Stg^{SCZ}), were incubated with 100 μM of tianeptine for 30 minutes (Figure 2.7 A). Neurons were then fixed and stained for cell-surface GluA1 and PSD95 (Figure 2.7 B). In control neurons, exposure to tianeptine results in an increase in surface and synaptic levels of GluA1 that is abrogated when stargazin is absent (Stg KD), suggesting that the effect of tianeptine on AMPAR synaptic expression is through stargazin. Interestingly, tianeptine can partially rescue the defect in surface and synaptic AMPAR levels in neurons expressing Stg^{SCZ}, but not in neurons expressing Stg^{ID} (Figure 2.7 C, D).

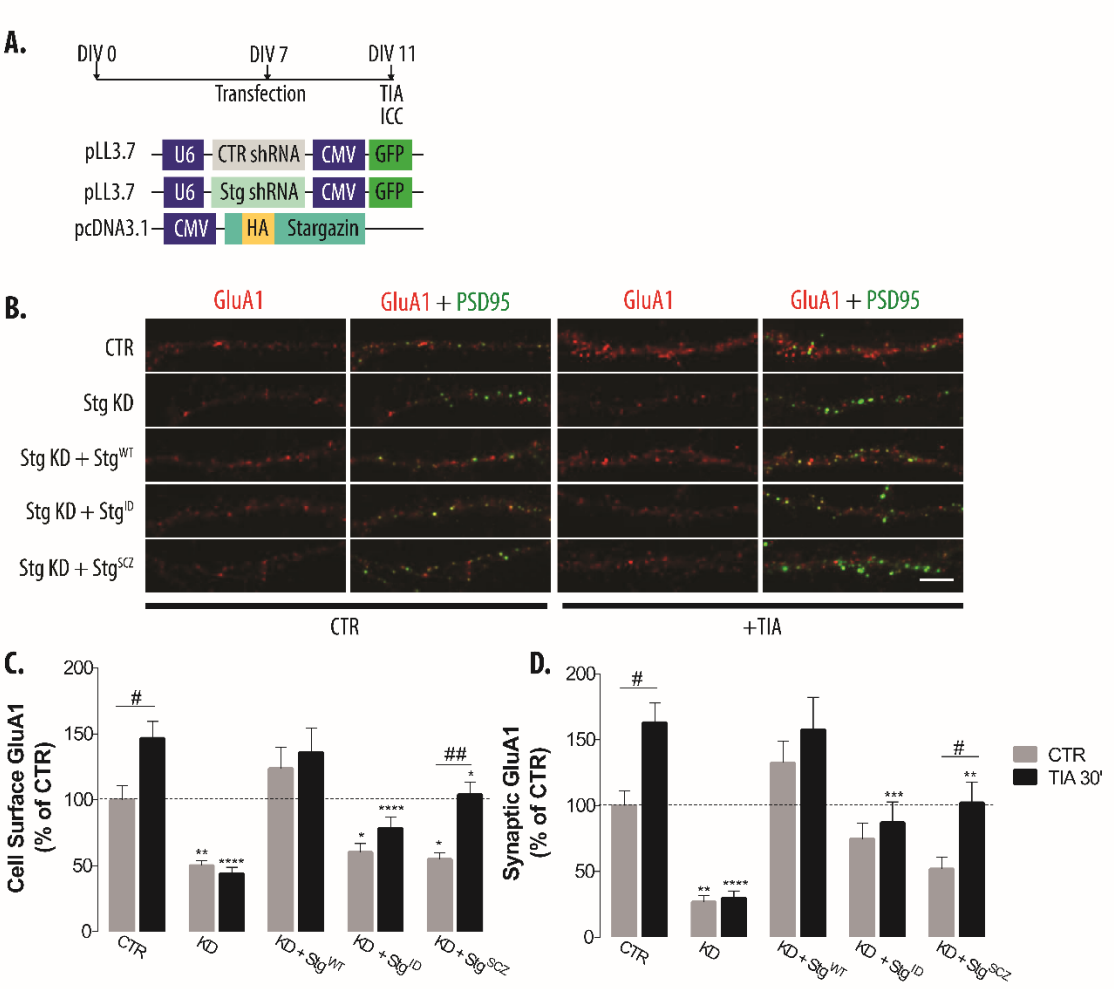


Figure 2.7. Tianeptine reverts the defects in AMPARtrafficking in neurons expressing Stg^{SCZ}. Low-density cortical neurons were transfected at DIV7 with pLL-shRNA-CTR, pLL-shRNA-Stg which downregulates endogenous stargazin, or co-transfected with pLL-shRNA-Stg and pcDNA-Stg-HA mutants. Transfected neurons were incubated in tianeptine (100 µM) for 30 min and total surface and synaptic GluA1 levels were analysed by immunocytochemistry (**A**). Representative images of GluA1 distribution (**B**) and quantification of total and synaptic intensity of GluA1 clusters (**C,D**) show that tianeptine increases the surface levels of GluA1, an effect that is abolished when stargazin is absent or when the Stg^{ID} variant is expressed. On the other hand, Stg^{SCZ} expressing neurons respond to the tianeptine stimulus by partially increasing the levels of surface and synaptic GluA1. GluA1 accumulation at synaptic sites was assessed by the colocalization with PSD95 clusters. Clusters were quantified from n≥20 cells imaged from three independent experiments; ****p< 0.0001; ***p<0.001; **p<0.01; *p<0.05; #p<0.05; ##p<0.01 two-way ANOVA, multiple comparison tests. Data are presented as mean ± SEM. Scale bar represents 8 µm. Data were kindly provided by Susana Louros.

2.3.7. The absence of stargazin, or the expression of the disease-associated variant Stg^{SCZ}, influence inhibitory synapse density and dendritic arborization

Behavioral deficits in SCZ, ASD and other psychiatric disorders have been hypothesized to be related to disturbances in the excitation/inhibition balance (Reviewed in (Lisman, 2012; Rogasch *et al.*, 2014))(Yizhar *et al.*, 2011). Accordingly, several studies described SCZ-associated changes in excitatory (Halim *et al.*, 2003; Kristiansen *et al.*, 2006; Toro and Deakin, 2005; Weickert *et al.*, 2004) and inhibitory synapse markers (Hoftman *et al.*, 2015; Volk *et al.*, 2002). A well-documented feature of SCZ is a reduction in the volume of cortical gray matter (Ellison-Wright and Bullmore, 2010; Fornito *et al.*, 2009), which has been calculated in around 5-8% in the prefrontal cortex (Bennett, 2011a). Possible explanations for this loss in cortical areas are a regression of synapses and/or alterations in the complexity of the dendritic arbor in subjects with SCZ. According to a quantitative evaluation of the contributions of cellular components to the volume of gray matter in the cortex, synapses represent around 6% of the total volume, whereas dendrites occupy around 30% of the volume of the cortex (Bennett, 2011a, b). Here, we evaluated how expression of the ID- and SCZ-associated stargazin variants affects the density of excitatory and inhibitory synapses, and cortical neurons dendritic arborization.

To explore the influence of stargazin and of disease-associated variants of stargazin in the regulation of synapse density, we silenced stargazin expression in low-density cortical neurons and introduced either WT stargazin or the ID- and SCZ-associated stargazin variants (KD + Stg^{WT}, KD + Stg^{ID} KD + Stg^{SCZ}) (Figure 2.8A). To evaluate excitatory and inhibitory synapses we labeled VGLUT and VGAT, respectively, by immunocytochemistry (Figure 2.8B). Quantification of the number and intensity of VGLUT clusters showed no significant variation between conditions (Figure 2.8 C), whereas the number and intensity of VGAT clusters were decreased in neurons depleted for stargazin or expressing Stg^{SCZ}, suggesting defects in inhibitory synapses in these situations (Figure 2.8 D).

Interestingly, stargazin overexpression in developing cortical neurons has been shown to result in increased dendritic arborization (Hamad *et al.*, 2014). We analyzed the dendritic arbor of young (DIV 11) and mature (DIV 14) cortical neurons after stargazin depletion and expression of either WT stargazin of the disease-associated stargazin variants (KD + Stg^{WT}, KD + Stg^{ID} KD + Stg^{SCZ}) (Figure 2.9 A). Sholl analysis showed a decreased number of intersections in young neurons where stargazin was absent or which expressed the variant associated with SCZ (Figure 2.9 B,C), but no significant differences in mature neurons expressing either of the constructs (Figure 2.9 B, D). These observations suggest that stargazin contributes to the dendritic tree complexity in an early stage of neuronal development, and that the SCZ-associated variant of stargazin is unable to mediate this

stargazin function. Interestingly, stargazin silencing or expression of Stg^{SCZ} in older neurons did not affect the neuronal dendritic arbor, indicating that the role of stargazin in this regard is restricted to an early stage of neuronal development.

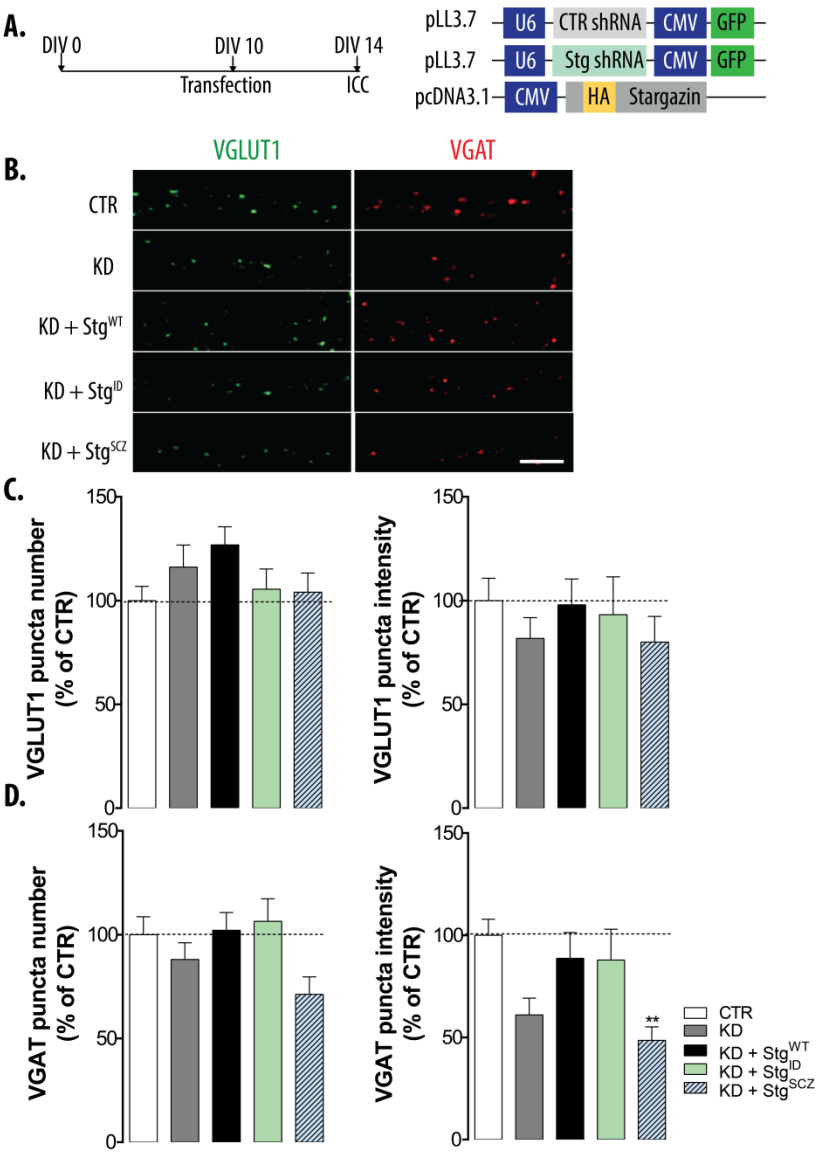


Figure 2.8. Neurons expressing the Stg^{SCZ} variant present decreased inhibitory synapse marker intensity and number. Low- density cortical neurons were transfected at DIV10 with pLL-shRNA-CTR or pLL-shRNA-Stg, which silences endogenous stargazin, or co-transfected with pLL-shRNA-Stg and pcDNA-Stg-HA variants. Immunocytochemistry for VGLUT1 and VGAT was performed at DIV 14 (**A**). Representative images of VGAT and VGLUT distribution (**B**) and quantification of the fluorescence intensity and number of clusters of VGLUT1 (**C**) and VGAT (**D**) showed decreased intensity of VGAT puncta in neurons depleted for stargazin or expressing the Stg^{SCZ} variant. Clusters were quantified from n≥39 cells imaged from five independent experiments; **p<0.01; one-way ANOVA, Bonferroni test. Data are presented as mean ± SEM. Scale bar represents 8 µm.

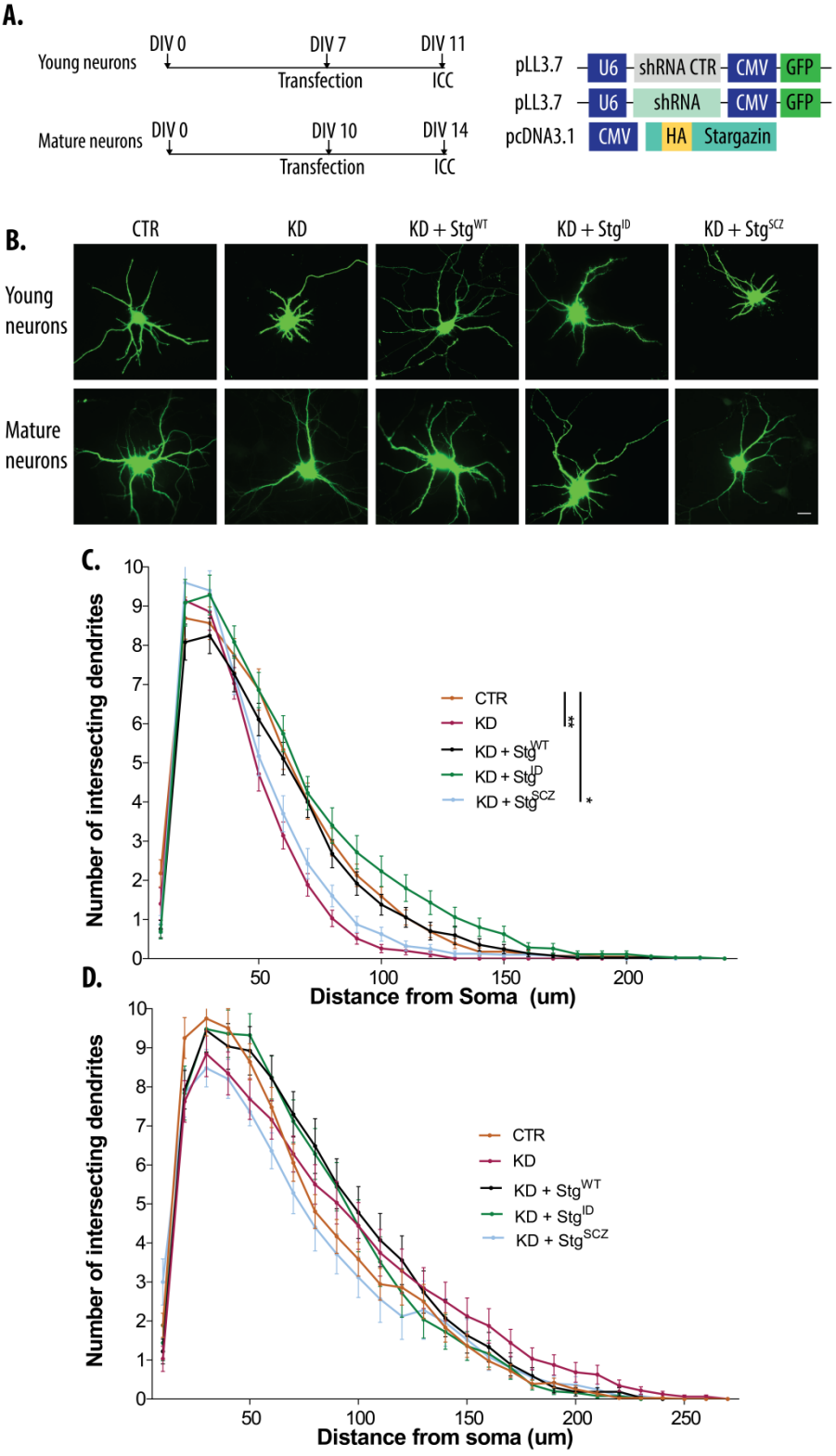

A.
Young neurons
DIV 0
DIV 7
DIV 11
Transfection
ICC
Mature neurons
DIV 0
DIV 10
DIV 14
Transfection
ICC
pLL3.7
U6
shRNA CTR
CMV
GFP
pLL3.7
U6
shRNA
CMV
GFP
pcDNA3.1
CMV
HA
Stargazin
B.
CTR
KD
KD + Stg^WT
KD + Stg^ID
KD + Stg^SCZ
Young neurons
Mature neurons
C.
Number of intersecting dendrites
Distance from Soma (um)
CTR
KD
KD + Stg^WT
KD + Stg^ID
KD + Stg^SCZ
**
*
D.
Number of intersecting dendrites
Distance from soma (um)
CTR
KD
KD + Stg^WT
KD + Stg^ID
KD + Stg^SCZ

Figure 2.9. Expression of the Stg^{SCZ} variant affects cortical neurons dendritic arbor. Low-density cortical neurons were transfected at DIV 7 or DIV 10 with pLL-shRNA-CTR, pLL-shRNA-Stg, which downregulates endogenous stargazin, or co-transfected with pLL-shRNA-Stg and pcDNA-Stg-HA variants. The dendritic arbor was analyzed at DIV 11 or DIV 14 (respectively for neurons transfected at 7 DIV or 10 DIV) by tracing GFP, co-expressed from the bicistronic pLL-shRNA Stg plasmid (**A**). The number of neurite intersections at different distances from the soma was analyzed with the Neurolucida software Sholl analysis tool. In young neurons *knock-down* of stargazin or expression of the Stg^{SCZ} variant led to significantly less intersections at long distances from soma, *i.e.* shorter dendrites (**B, C**), whereas in older neurons no changes were observed among the different conditions (**B**, **D**). Results were obtained from three independent experiments for mature neurons and from four independent experiments for young neurons;*p<0.05; **p<0.01; two-way ANOVA. Data are presented as mean ± SEM.

2.3.8. Newly identified stargazin interactors are implicated in psychiatric disorders

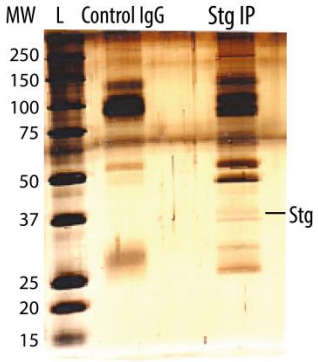
Besides AMPAR and the MAGUKs, stargazin was shown to interact with several other proteins, including AP-2, AP-3 and AP-4, involved in endocytosis (Matsuda *et al.*, 2013; Matsuda *et al.*, 2008), nPIST, a protein implicated in the trafficking of membrane proteins (Cuadra *et al.*, 2004), MAGI-2, a scaffold protein suggested to mediate the interaction of stargazin/AMPAR complexes with other postsynaptic molecules (Deng *et al.*, 2006), and Erbin, a protein that is specifically expressed in cortical interneurons, and is crucial for stargazin stability, among others.

The *in vitro* data obtained in the first part of this work suggest that besides its known role as an AMPAR auxiliary protein stargazin might play other cellular functions. We hypothesized that stargazin's yet undescribed interactors could explain its influence on the number of inhibitory synapses and on dendritic arborization, and also its putative role in psychiatric disorders.

To perform an unbiased characterization of the stargazin interactome, stargazin was immunoprecipitated from total or membrane-enriched cortical neurons extracts, and co-immunoprecipitated proteins were analyzed by silver-staining (Figure 2.10 A) and identified by mass spectrometry, in collaboration with Dr. Ka Wan Li (Vrei University, Amsterdam).

Mass spectrometry analysis identified 44 stargazin-interacting proteins (Figure 2.10 B,C), two of which, mGluR5 and Plexin1, we confirmed by western blot analysis given their importance for neuronal function (Figure 2.10 D).

A.



B.

Stargazin interaction partners (P2 fractions)

Plexin-A1	Plxna1
DCC-interacting protein 13-alpha	Appl1
Elongation factor 1-gamma	Eef1g
Metabotropic glutamate receptor 5	Grm5
Tubulin beta-3 chain	Tubb3
3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase type 1	Hsd3b1

C.

Stargazin interaction partners (Total extracts)

DCC-interacting protein 13-alpha	APPL1
3-ketoacyl-CoA thiolase, mitochondrial	Acaa2
AP-2 complex subunit mu	Ap2m1
Armadillo repeat protein	Arvcf
Cysteine--tRNA ligase, cytoplasmic	Cars
CLIP-associating protein 2	Clasp2
Acyl-CoA-binding protein	Dbi
Dynactin subunit 1	Dctn1
ATP-dependent RNA helicase A	Dhx9
Elongation factor 1-beta	Eef1b
Elongation factor 1-gamma	Eef1g
ELKS/Rab6-interacting/CAST family member 1	Erc1
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	Gnb2
Metabotropic glutamate receptor 5	Grm5
Histone deacetylase 2	Hdac2
Stress-70 protein, mitochondrial	Hspa9
NF-kappa-B essential modulator	Ikbkg
IQ motif and SEC7 domain-containing protein 2	Iqsec2
Insulin receptor substrate 1	Irs1
Junction plakoglobin	Jup
Potassium voltage-gated channel subfamily KQT member 2	Kcnq2
Kinesin-like protein KIF21A	Kif21a
Leucine zipper protein 2	Luzp2
Myelin expression factor 2	Myef2
Asparagine--tRNA ligase, cytoplasmic	Nars
Nucleoside diphosphate kinase B	Nme2
Membrane-associated phosphatidylinositol transfer protein 2	Pitpnm2
Plexin-A1	Plxna1
Glycogen phosphorylase, brain form	Pygb
Quaking Protein	Qki
Ras-related protein Rab-10	Rab10
GTP-binding nuclear protein Ran	Ran
60S ribosomal protein L24	Rpl24
60S ribosomal protein L7a	Rpl7a
60S acidic ribosomal protein P0	Rplp0
40S ribosomal protein S2	Rps2
40S ribosomal protein S3	Rps3
Signal-induced proliferation-associated 1-like protein 1	Sipa1l1
Beta-synuclein	Sncb
SOGA-3	Soga3
T-complex protein 1 subunit alpha	Tcp1
DNA topoisomerase 2-beta	Top2b
Valine--tRNA ligase	Vars

D.

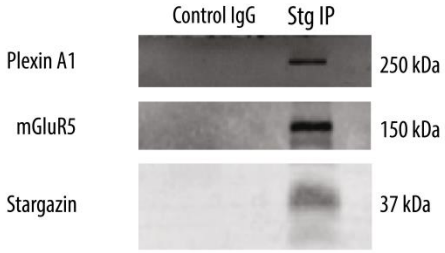


Figure 2.10. New stargazin interaction partners. Stargazin was immunoprecipitated from the membrane fraction or total extracts of 11 DIV high-density cortical neurons. The protein content of immunoprecipitated samples was characterized by mass spectrometry. Only proteins which were co-immunoprecipitated with stargazin and were not present in the control condition (immunoprecipitation using non-immune IgGs), in at least one duplicate of two independent experiments, were considered. Stargazin and co-immunoprecipitated proteins can be visualized in a silver nitrate stained gel (**A**). Stargazin binding parterns were identified through mass spectrometry analysis (**B**, **C**). The co-immunoprecipitation of mGluR5 and PlexinA1 with stargazin was confirmed by Western Blot (**D**).

Interestingly, several proteins identified in our screening have been implicated in SCZ and/or intellectual disability and others play pivotal roles in important cellular processes. A summary of those functions is depicted in Table 2.1.

Table 2.1. New interactors of stargazin

Protein	Function
μ subunit of AP-2	AP-2 is a protein involved in the endocytic pathway that was previously identified as an interactor of stargazin (Matsuda *et al.*, 2013).
Arcvf (Armadillo Repeat gene deleted in Velo-Cardio-Facial syndrome)	Member of the catenin family; associated with SCZ and also with learning and working memory deficits in mice (Mas *et al.*, 2009; Sanders *et al.*, 2005; Suzuki *et al.*, 2009).
CLASP2 (CLIP-associated protein 2)	CLASP2 is a nervous system enriched, microtubule plus end-tracking protein that has been shown to play a role in the regulation of dendritic development, since its *knock-down* results in a less complex dendritic tree in cortical neurons (Hur *et al.*, 2011).
Erc1	Rab6 interacting protein; associated with childhood apraxia of speech (Thevenon *et al.*, 2013).
Gnb2 (G protein subunit beta 2)	Associated with SCZ (Fromer *et al.*, 2014).
HDAC2 (histone deacetylase 2)	HDAC2 inhibitors prevent antipsychotic drugs-mediated downregulation of mGluR2, augmenting their therapeutic effect; these observations support HDAC2 as a promising target to improve SCZ treatment (Kurita *et al.*, 2012; Kurita *et al.*, 2013). Recent next-generation sequencing data showed *de novo* mutations in the HDAC2 gene as potentially causative of intellectual disability (Martinez *et al.*, 2017).
Hspa9	The *knock-down* of the heat shock protein family A, member 9, also known as mortalin, in the medial prefrontal cortex is associated with alterations in sensorimotor gating in rats, a common observation in SCZ (Gabriele *et al.*, 2010).
IQSEC2	Mutations in the *IQSEC2* gene, which codes for a guanine nucleotide exchange factor, have been reported to cause nonsyndromic intellectual disability (Shoubridge *et al.*, 2010) and X-linked intellectual disabilities (Gandomi *et al.*, 2014; Kalscheuer *et al.*, 2015; Tran Mau-Them *et al.*, 2014).
KCNQ2	KCNQ2, a member of the voltage-gated potassium channel family, has been recently implicated in ID with childhood onset seizures (Hewson *et al.*, 2017)
mGluR5	Extensively implicated in Fragile X syndrome [reviewed in (Dolen and Bear, 2008)] and SCZ [reviewed in (Matosin *et al.*, 2015)].
Plexin A1	Plexin A1 is a receptor for Semaphorin3A, which is known to act as a repulsive factor for axon guidance. Null mice for this factor present reductions in dendritic length and branching, suggesting a role for plexinA1-mediated signaling in dendritic arborization (Fenstermaker *et al.*, 2004).
Quaking	Quaking is an RNA binding protein, which is encoded by a gene located in a susceptibility locus for SCZ in chromosome 6 (Aberg *et al.*, 2006); its overexpression in hippocampal neurons was recently demonstrated to reduce dendritic arborization (Irie *et al.*, 2016).

2.4. Discussion

SCZ and intellectual disability are different neuropsychiatric disorders, with distinct clinical features, although significant genetic overlap between the two diseases has been found (e.g. (Carroll *et al.*, 2016; Cochoy *et al.*, 2015; de Sena Cortabitarte *et al.*, 2017; Fromer *et al.*, 2014; Li *et al.*, 2016; McCarthy *et al.*, 2014; Parente *et al.*, 2017; Rauch *et al.*, 2012; Sun *et al.*, 2011)), suggesting a possible shared genetic etiology. One major unanswered question is how different mutations in the same gene can contribute to different clinical phenotypes. Independent reports have suggested *CACNG2* as a susceptibility gene for psychiatric disorders, namely SCZ and bipolar disorder (Ament *et al.*, 2015; Beneyto and Meador-Woodruff, 2006; Drummond *et al.*, 2013; Liu *et al.*, 2008; Nissen *et al.*, 2012; Yang *et al.*, 2013), and identified a mutation in *CANCG2* linked to intellectual disability. Here, we identified a missense mutation in *CACNG2* in a SCZ patient, and describe the *in vitro* characterization of cellular phenotypes caused by the *CACNG2* mutations identified in SCZ and intellectual disability patients. The aim of this work was to elucidate the neuronal mechanisms involving stargazin that are affected by the two disease-associated mutations, with the goals of understanding the mutational effect on protein function and how different mutations in the same gene can lead to different cellular phenotypes, which may help explain the distinct clinical symptoms associated to these mutations. We show for the first time that ID- and SCZ-associated mutations in *CACNG2* lead to common and distinct effects on AMPAR traffic, homeostatic plasticity, neurite outgrowth and response to the antidepressant and cognitive enhancer drug tianeptine.

Commonality in cellular phenotypes associated with stargazin variants

Stargazin was the first TARP to be identified and shown to regulate AMPAR. Its importance in regulating AMPAR trafficking, gating and in synaptic plasticity has been extensively studied since the year of 2000, when their interaction was described for the first time (Chen *et al.*, 2000). Stargazin diffusion at the neuronal surface is regulated by interaction with intracellular proteins, namely the synaptic scaffold PSD95, and impacted by stargazin phosphorylation (Opazo *et al.*, 2010). Immobilization of stargazin at the synapse mediates synaptic trapping of AMPARs. The C-tail of stargazin is partially bound to the plasma membrane through electrostatic interactions and disruption of this interaction in a phosphorylation-dependent manner is necessary for binding to PSD95 (Sumioka *et al.*, 2010). Thus, this interaction controls stargazin stabilization at the synapse, and recent reports show that phosphorylation of the stargazin C-tail leads to its extension into the PSD, and facilitates binding to PSD95, thus increasing synaptic strength (Hafner *et al.*, 2015). This

evidence suggests that the stargazin-PSD95 interaction is highly controlled and rather sensitive to structural changes in either of the proteins.

Both ID- and SCZ-associated mutations in stargazin affect the cell surface lateral diffusion of the protein, increasing its mean square displacement and diffusion coefficient. This suggests that these mutations impact the mechanisms that contribute to stargazin lateral membrane diffusion, which besides being regulated by stargazin phosphorylation (Kristiansen *et al.*, 2006) could be partially determined by interactions mediated by the third transmembrane domain where the two mutations reside. Further studies are necessary to assess if disease-associated stargazin variants interact less with PSD95 and/or other proteins that might help stargazin stabilization at the synapse. Furthermore, in order to understand the effect of altered diffusion of stargazin in the stabilization of AMPAR at the synapses, it would be important to evaluate the cell surface mobility of single AMPAR particles in cells expressing these disease-associated variants.

Recent structural studies have characterized the AMPAR-stargazin complex by cryo-electron microscopy (Twomey *et al.*, 2016; Zhao *et al.*, 2016), and the protein-protein interfaces were further characterized using a combination of mutagenesis and electrophysiology (Ben-Yaacov *et al.*, 2017). These analyses have ascertained a function for transmembrane domain 3 in stargazin in AMPAR modulation. According to Zhao and colleagues, the TM3 and TM4 transmembrane domains form extensive hydrophobic interactions with M1 and M2 from one GluA2 subunit (the AMPAR subunit in the analyzed complex) and with the M4 of the adjacent subunit, enabling the modulation of AMPAR by stargazin. Furthermore, a conserved acidic region spanning 85-95 residues is present in the extracellular loops of stargazin, adjacent to the α-1 helix and interacts with positively charged lipids on the lower lobe of the LBD of the receptor. Stargazin is thus ideally positioned to modulate domain closure and efficacy of partial agonists (Zhao *et al.*, 2016). A two-steps mechanism was additionally proposed; firstly, the binding of stargazin to AMPAR membrane domains would be sufficient to induce conformational changes in these domains, destabilizing the channel closed state and thus enabling a more efficient opening upon agonist binding; in a second stage, the conformational changes induced by agonist binding would allow the interaction between extracellular parts of stargazin and the receptor, stabilizing the open state (Ben-Yaacov *et al.*, 2017). The two disease-associated mutations in stargazin that we studied are localized in the TM3, which suggests that they may interfere with stargazin's interaction with AMPAR subunits, given the prime role described for this domain in stargazin. In fact, a previous study that identified the V143L mutation in stargazin in an ID patient described a decrease in the interaction of the Stg^{ID} variant with GluA1 and GluA2 AMPAR subunits (Hamdan et al., 2011). However, we found that both Stg^{ID} and Stg^{SCZ} variants bind to the GluA1 subunit of AMPAR when co-expressed in a heterologous system, and could not detect changes in the interaction. It would be important to complement these

studies by testing the interaction between the stargazin variants and AMPAR in neurons. Additionally, it is possible that although no differences in the binding of stargazin variants to AMPAR GluA1 subunit can be detected in immunoprecipitation studies, the functional effect of stargazin on regulating efficacy of agonists or receptor kinetics is altered. These aspects deserve to be further investigated.

One striking common feature of Stg^{ID} and Stg^{SCZ} is that both variants affect basal AMPAR surface expression. In the case of Stg^{ID}, our results are in agreement to what was previously reported by Hamdan and colleagues, who found decreased total and surface expression of GFP-GluA1 and decreased amplitude and frequency of AMPAR-mediated mEPSCs upon overexpression of stargazin V143L in neurons (Hamdan et al., 2011). We now find, employing a molecular replacement approach, that depletion of endogenous stargazin and expression in cortical neurons of either stargazin V143L or S148N results in impaired AMPAR traffic. These result show that both variants of stargazin fail to mediate AMPAR traffic to the cell surface and to synapses, suggesting that they impact glutamatergic transmission.

Differences in cellular phenotypes generated by ID- and SCZ-associated stargazin variants

Besides common effects on the basal trafficking of AMPAR to the surface and to the synapse, Stg^{ID} and Stg^{SCZ} also display distinct cellular phenotypes. Notably, the Stg^{ID} mutation interfered with stargazin-dependent scaling of AMPAR upon blockade of neuronal activity, whereas in neurons expressing Stg^{SCZ} AMPAR synaptic upscaling was observed. Additionally, whereas Stg^{SCZ} expression resulted in increased AMPAR traffic to synapses induced by tianeptine, neurons expressing Stg^{ID} failed to show a tianeptine-induced increase in synaptic AMPARs. One possibility is that the Stg^{ID} mutation results in a structurally altered protein whose function cannot be modulated by mechanisms that increase its trafficking role. It is possible that Stg^{ID} is unable to interact with proteins implicated in plasticity mechanisms, and in the mechanisms induced by tianeptine. In fact, several proteins required for the synaptic scaling of AMPAR are binding partners of stargazin, namely CaMKII (Groth *et al.*, 2011; Opazo *et al.*, 2010; Tomita *et al.*, 2005b), ARC (Shepherd *et al.*, 2006; Zhang *et al.*, 2015), mGluR5 (Hu *et al.*, 2010) and PSD95 (Sun and Turrigiano, 2011).

Stargazin function is tightly regulated by posttranslation modifications which could be putatively affected by the disease-associated mutations. CaMKII and PKC-dependent phosphorylation, and calcineurin-dependent dephosphorylation, of the nine serines localized in the C-terminal of stargazin have been associated with homeostatic and long-term plasticity mechanisms (Louros *et al.*, 2014; Nomura *et al.*, 2012; Tomita *et al.*, 2005b), whereas phosphorylation of the threonine residue 321 by PKA regulates the interaction between stargazin and PSD95 and, in turn, the

anchoring of AMPAR at synapse (Choi *et al.*, 2002; Stein and Chetkovich, 2010). Although the Stg^{SCZ} variant presents a deleterious effect in basal trafficking of AMPAR, it is able to mediate homeostatic plasticity and to respond tianeptine, known to increase CaMKII-mediated stargazin phosphorylation and in turn its binding to AMPAR (Zhang *et al.*, 2013). This phosphorylation-dependent effect of tianeptine on the function of stargazin suggests that the opposite behaviors presented by the Stg^{SCZ} and Stg^{ID} variants could rely on differential phosphorylation. Further evaluation of the phosphorylation levels and affinity to interaction partners of these disease-associated stargazin variants would be helpful to understand how these mutations impact the trafficking of AMPAR and why two mutations in the same transmembrane domain originate different behaviors in terms of plasticity-induction.

Considering that the stabilization of AMPAR at the synapse is dependent on the CaMKII-mediated phosphorylation of stargazin, and given that both disease-associated variants present a higher diffusion coefficient concomitant with less time spent at the synapse, it would be interesting to test the effect of tianeptine in the diffusion properties of the variants. In addition, directed mutagenesis of the phosphorylatable serines in the C-terminal of stargazin variants in order to prevent or to mimic phosphorylation would inform on whether changes in the levels of phosphorylation underlie the effects of the mutations.

In accordance to reports describing changes in inhibitory synapse markers in SCZ (Hoftman *et al.*, 2015; Volk *et al.*, 2002), we report that stargazin depletion or expression of Stg^{SCZ} (but not Stg^{ID}) result in a decrease on the density of inhibitory synapses in cortical neurons, as measured by evaluating VGAT labeling. In agreement with our observations, a study involving 42 SCZ patients, described a reduction in VGAT levels in the prefrontal cortex (Hoftman *et al.*, 2015). However, another study did not find changes in the ratio between VGLUT1/VGAT in SCZ patients (Fung *et al.*, 2011), suggesting that these changes may be restricted to some SCZ endophenotypes.

The observed decrease in inhibitory synapse density upon stargazin silencing or Stg^{SCZ} expression suggest either a stargazin-dependent regulation of inhibitory synapses or a compensatory response to reduced AMPAR expression. According to this second possibility, it was recently demonstrated that the selective decrease of surface and synaptic GluA1 and GluA2-containing AMPAR by anti-AMPAR antibodies produced in auto-immune encephalitis, while not changing the density of excitatory synapses or affecting NMDARs, led to a decrease in VGAT staining and inhibitory synapses currents (Peng *et al.*, 2015). Given that VGAT is a presynaptic protein, its regulation by stargazin or AMPAR would require a trans-synaptic effect. Stargazin, on the other hand, was shown to mediate cell adhesion, which might be facilitated by stargazin localization at sites of close cellular contact (Price *et al.*, 2005). Our study revealed changes in inhibitory synapse density in neurons expressing the SCZ-associated stargazin mutation, but not the ID-associated variant, indicating that

these two mutations differentially affect the mechanisms through which stargazin impacts the formation/maintenance of inhibitory synapses. These mechanisms are currently not known.

We demonstrated that the dendritic arborization of young (DIV 11) cortical neurons was affected by the absence of stargazin or the expression of the Stg^{SCZ} variant, but not by expression of Stg^{ID}. Stargazin has been previously shown to affect dendritic arborization, as its overexpression led to increased complexity of the dendritic tree of neocortical pyramidal neurons (Hamad *et al.*, 2014). In addition, stargazin interacts with several proteins that are associated with dendritic arborization regulation, namely CaMKII (Stephenson *et al.*, 2017), CLASP2, PlexinA1 and quaking proteins. A decreased ability for Stg^{SCZ} to bind one of these or other proteins could be the basis for alterations in dendritic arborization in neurons expressing Stg^{SCZ}; thus, evaluation of the interaction between these proteins and stargazin variants would be relevant to address this question. Of note, both stargazin depletion and Stg^{SCZ} expression affected dendritic complexity of young neurons, but did not produce an effect in older neurons, suggesting a role for stargazin in a particular developmental stage, which cannot be carried out by Stg^{SCZ}.

One of the main observations in the brains of subjects with SCZ is the decrease in the volume of cortical grey matter (Bennett, 2011b), which could be explained by changes in the number of synapses and/or in the complexity of dendrites. Interestingly, the Stg^{SCZ} variant affected both processes in cortical neurons, further supporting a contribution for Stg^{SCZ} in the development of the disorder.

In conclusion, here we describe two different mutations in *CACNG2* that present different effects on the function of the protein and confer different sensitivity to treatment with tianeptine. Both mutations affect surface diffusion of stargazin, and one of its first and well-described functions, the trafficking and stabilization of AMPAR receptors in baseline conditions. However, whereas the V143L variant associated to ID does not mediate synaptic scaling of AMPAR upon blockade of activity, the SCZ-associated S148N mutation affects dendritic arborization and the density of inhibitory synapses in cortical neurons. Our observations are in agreement with several studies showing cross-disorder effects of gene variants (e.g. (Fanous *et al.*, 2012; Network and Pathway Analysis Subgroup of Psychiatric Genomics, 2015; Zhou *et al.*, 2016a)).

The finding of common effects for genetic variants of stargazin associated with different disorders may help explain the occurrence of shared clinical phenotypes. On the other hand, the identification of mutation-specific defects provides insight on how different mutations in stargazin can be associated with different clinical manifestations. Direct association of clinical symptoms with cellular defects is difficult at this point, but the mutation-specific cellular phenotypes that we identify could be harnessed for targeted therapy.

2.5. Material and Methods

Evidence shows that genetics plays a pivotal role in the development of most psychiatric disorders. Although for some of these disorders no single causative gene is known, many genes, particularly genes that encode synaptic proteins, were shown to be implicated, even if with a mild effect, in the generation of these disorders. The *CACNG2* gene encodes for stargazin, an important protein in the expression and regulation of AMPAR. Interestingly, existent data pointed *CACNG2* as a putative susceptibility gene for SCZ, bipolar disorder and intellectual disability. In order to deepen the knowledge about this protein in a context of disease we searched for exonic mutations in the DNA of SCZ and bipolar disorder patients from the Portuguese Island Cohort. To do so, we used whole genome sequencing data, kindly provided by Dr. Carlos Pato (SUNY Downstate Medical Center), and in addition, for a different group of patients, we sequenced the exons of the *CACNG2* gene, in collaboration of Dr. Célia Carvalho (University of Azores). In this study, we identified a new missense mutation in the *CACNG2* gene of a SCZ patient. Furthermore, a *de novo* missense mutation in the same gene was recently described in an 8 year-old patient with moderate, non-syndromic intellectual disability. Thus, in the first part of this work, we studied, *in vitro*, the impact of these mutations in the function of the protein and its possible role in the development of disease-related phenotypes.

We used overexpression and protein silencing through RNAi technology to study the impact of stargazin variants in several features of stargazin expression and function. Therefore, low-density cortical cultures cells were transfected with the calcium phosphate method. Low-density cortical cultures were used for imaging purposes, specifically to address the role of the mutations in the surface diffusion of stargazin, in the trafficking and synaptic scaling of AMPAR, in the number of excitatory, inhibitory and finally in the dendritic arborization of transfected neurons. Dense cortical cultures, transduced with lentiviruses, and HEK cell cultures transfected with the calcium phosphate method, were used to obtain total and P2 extracts for mRNA level quantification and for immunoprecipitation and/or Western Blot analysis.

2.5.1. Whole-genome sequencing data analysis

Sequencing data from the *CACNG2* gene was extracted from whole-genome sequencing data kindly provided by the group of Dr. Carlos Pato (SUNY Downstate Medical Center) specifically for this gene. The DNA of 35 controls, 19 bipolar disorder patients, 11 SCZ patients and 2 major depression patients was used for this purpose. Sequence analysis was performed using the IVG2.3 software (Broad Institute, MA, U.S.A.).

2.5.2. Sanger sequencing of the exons of the *CACNG2* gene

To increase the number of individuals in this study, and since we focused only in the coding regions of the *CACNG2* gene, we additionally sequenced through Sanger sequencing (GATC Biotech) the exons in *CACNG2* in 15 controls, 3 bipolar disorder patients and 5 SCZ patients. The DNA was extracted from whole blood collected in 8 ml EDTA tubes and kept refrigerated until utilization for the extraction, using the DNA Isolation Kit for Mammalian Blood from Roche. The primers used to amplify the *CACNG2* exons are shown in Table 2.2.

2.5.3. Prediction Tools

In order to predict the impact of *CACNG2* mutations in the function of stargazin, we used three different prediction tools.

The PolyPhen-2 prediction tool uses eight sequence-based and three structure-based predictive features selected automatically by an algorithm. PolyPhen-2 calculates the probability of a mutation to be damaging and estimates the chance of the result being a false (specificity) or a true (sensitivity) positive. The mutation is also qualitatively classified as benign, possibly damaging or probably damaging (Adzhubei *et al.*, 2013; Adzhubei *et al.*, 2010).

SIFT (Sorting intolerant from tolerant) algorithm predicts whether an aminoacid substitution affects the function of the protein based on the conservation of that residue throughout evolution (Kumar *et al.*, 2009). The PROVEAN (Protein variation effect analyzer) is an alignment-based score that predicts the damaging effects of variations by measuring the change in sequence similarity to a protein sequence homolog before and after the alteration (Choi *et al.*, 2012).

2.5.4. Protein structure representation

Protein structure representative images were obtained using PyMol, a system that allows the molecular visualization, analysis and creation of high quality images for protein structure (The PyMol Molecular Graphic System, Version 1.8 Schrodinger, LLC). Protein DataBank files, supported by PyMol software, were obtained from the Swiss Model repository. Swiss Model is a database of annotated 3D protein structures generated by a homology-modelling pipeline that also allows the interactive building of models if a sequence has not been modeled previously (Guex *et al.*, 2009).

2.5.5. Constructs and primers for neuronal transfection

For *knock-down* experiments we used a shRNA construct generated and validated previously in our laboratory (Louros *et al.*, 2014). For the generation of shRNA-resistant constructs we performed site directed mutagenesis using specific primers that introduced two silent mutations in HA-tagged WT stargazin inserted in a pcDNA3.1 plasmid (a gift from Dr. Daniel Choquet, IINS, Bordeux, France). Stargazin mutants, V43L and S148N, were generated by the introduction of single point mutations through site directed-mutagenesis. All mutations were confirmed by DNA sequencing.

Viral vectors were generated in our laboratory by cloning PCR-amplified HA-stargazin variants into the pLL 3.7 vector downstream of a segment containing the mCherry cDNA and a T2A self-cleaving peptide. Expression driven by the synapsin promoter allowed the expression of the mCherry fluorescent protein and the HA-tagged stargazin variants specifically in neurons.

Cloning and mutagenesis assays were performed using the PrimeStar HS from Takara. For enzymatic digestions, PCR products and plasmid DNAs were incubated for 2h at 37ºC. All the restriction enzymes were purchased from New England Biolabs. Mutagenesis and amplification primers are shown in Table 2.2.

2.5.6. Cortical neuron cultures (High-density and Banker cultures)

Primary cultures of rat cortical neurons were prepared from the cortices of E17 Wistar rat embryos. The cortices were incubated with trypsin (2mg/ml, 10 min, 37ºC, GIBCO Invitrogen) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS: 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red), washed 6 times with HBSS and then mechanically dissociated. After counted, the cells were plated in neuronal plating medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1 mM pyruvic acid) in poly-D-lysine (0.1 mg/ml) coated 6-well plates ($0.85x10^6$ cells per well). The medium was replaced, after 2 hours, by Neurobasal medium supplemented with SM1 (1:50), 0.5 mM glutamine and 0.12 mg/ml gentamycin. For imaging assays we used the Banker culture system. Cortical cells were plated at a low density ($0.3x10^6$ cells per dish) in 60 mm dishes containing five poly-D-lysine coated coverslips (18mm). As described before, the medium was exchanged by supplemented neurobasal medium 2 hours after plating. In this type of cultures, commonly named Banker cultures (Kaech and Banker, 2006), neurons grew facing a confluent feeder layer of astroglial cells but were kept apart from the glial cells by wax dots placed on the coverslips. The cultures were treated with 5 µM cytosine arabinoside, two days after plating, to prevent the

overgrowth of glial cells and were maintained in an incubator with 5% CO_2, at 37ºC. Conditioned medium was partially replaced by fresh supplemented neurobasal medium every 3 days.
Cells were stimulated with 1µM TTX for 48 hours at DIV 7 to induce up scaling of AMPAR and with 100µM tianeptine for 30 min at DIV 11 to promote stargazin phosphorylation.
Low-density cultures were used for imaging by immunocytochemistry or Quantum-dots analysis whereas dense cultures were used for mRNA extraction and total or P2 extracts protein extracts followed by immunoprecipitation and/or western blot. All animal procedures were reviewed and approved by ORBEA and DGAV Portugal.

2.5.7. Neuronal transfection with calcium phosphate

The constructs mentioned above were transfected into cortical neuron cultures using calcium phosphate-mediated transfection protocol (Jiang *et al.*, 2004). A $CaCl_2$ solution (2.5 M in 10 mM HEPES) was added, dropwise, to the DNA diluted in TE, to a final concentration of 250 mM $CaCl_2$. This solution was then added to the equivalent volume of HEPES-buffered transfection solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11mM dextrose and 42 mM HEPES, pH=7.2). The DNA precipitates (100 µl) were added, dropwise, to the coverslips in conditioned medium and 2 mM of kynurenic acid. The cultures were incubated for 2 h at 37ºC and 5% CO_2. The DNA precipitates were dissociated by adding acidic medium containing 2mM kynurenic acid and approximately 5 mM HCl for 15 minutes at 37ºC and 5% CO_2. Coverslips were then transferred to the original dish. Number of days expressing the constructs varied according to the experiment.

2.5.8. HEK 293T cells transfection with calcium phosphate

Human embryonic kidney (HEK) 293T cells plated in 6-well plates and and cultured to 70% confluency were transfected using the calcium phosphate method with 5 µg of pcDNA 3.1 GluA1 and 5 µg of pcDNA 3.1-HA-Stg^{WT}, pcDNA 3.1-HA-Stg^{ID} or pcDNA 3.1-HA-Stg^{SCZ}. To allow the formation of DNA precipitates, a solution of 2 M $CaCl_2$ was added dropwise to the diluted DNA. This mixture was then added to HEPES-buffered transfection solution (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.05) and incubated for 20 min, at room temperature (RT), in the dark. The precipitates were added to the cells and incubated at 37ºC, in 5%CO_2 for 5 hours. After this period, the medium was completely changed in order to remove the remaining precipitates and prevent toxicity. The cells were kept at 37ºC, in 5%CO_2, for 48h for expression.

2.5.9. Viral production and neuronal transduction

HEK293T cells plated in 100 mm dishes were co-transfected as described above, with 10 µg of pCMV-ΔR8.91 and 6µg of pMD (VSVG), to express structural viral proteins, and with 10 µg of the construct of interest to express control shRNA, stargazin shRNA (also expresses GFP) and the HA-tagged stargazin variants (also expresses mCherry). Cells were incubated with the precipitates at 37ºC, in 3%CO_2 for 5 hours. After this time the medium was completely replaced and the cells were incubated at 37ºC, in 5%CO_2, for 48h to express the plasmid content. The medium containing the viral particles was collected and filtered with a 45µm filter Stericup (Millipore) and centrifuged at 22000 rpm, for 2 hours at 4ºC (Beckman Coulter, OptimaTM L-100 XP ultracentrifuge, rotor 28i). After discarding the supernatant, 200µl of bovine serum albumin (BSA) 1% in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) were added to the pellet. After brief and gentle resuspension, the solution was aliquoted and stored at -80ºC.
The viral titers and the multiplicity of infection (MOI) were determined as below:

$$\text{Titer (TU/l)} = \frac{\text{Target cell number x \% of mCherry positive cells}}{\text{volume of viral supernatant (ml))/100}}$$

$$\text{MOI} = \frac{\text{Volume of viral supernatant(ml)x titer(TU/ml)}}{\text{Target cell number}}$$

Dense cortical cultures were transduced at DIV 10 with a MOI=5 (represents about 80% of neuronal infection) and maintained for four days.

2.5.10. Immunocytochemistry for morphology and quantitative fluorescence analysis

Cells were fixed for 15 min in 4%sucrose/4%paraformaldehyde in PBS at RT and permeabilized for 5 min with 0,25% Triton X-100, in PBS at 4ºC. Unspecific staining was blocked by incubation with 10% BSA in PBS for 30 min, at 37ºC. The neurons were then incubated with the primary antibody diluted in 3% BSA in PBS for 2h at 37ºC or overnight at 4ºC. Before and after incubating with the secondary antibody, also diluted in 3% BSA in PBS, for 45 min, 37 ºC, cells were washed 6 times with PBS. Coverslips were mounted in DAKO (Gostrup, Denmark) fluorescent mounting medium. In order to stain surface proteins, live cells were incubated with primary antibody diluted in conditioned medium for 10 minutes and then fixed and stained as described above. For the cellular localization assays, to allow the distinction between the surface and intracellular pools, cells were live stained with the primary antibody (diluted in conditioned medium), fixed and stained with the secondary antibody. After permeabilization and blocking, cells were stained for the second time with the same

primary antibody (diluted in 3%BSA in PBS) and then stained with a secondary antibody coupled to a different fluorophore.

The imaging was performed using a Zeiss Axiovert 200 M microscope and a 63X-1.4 NA oil objective. In each experiment, cells were treated simultaneously and stained with the same antibody preparations and images were acquired blind to condition with the exact same settings for all conditions. Blind to condition quantification was performed in Fiji analysis software, with a macro that automatized quantification steps. The region of interest (ROI) was chosen randomly, by using MAP2 and/or GFP staining to confirm that the selected dendrite was GFP-positive*, i. e.* from a transfected neuron. The threshold was defined to include detectable clusters and the intensity, area and number of particles of the selected area were analyzed. Synaptic puncta were defined by their colocalization with PSD95.

For dendritic arborization analysis, images were acquired with an Objective Plan-Apochromat 20x/0.8 and the Sholl analysis was performed using the Neurolucida software. In Sholl analysis, circumferences with crescent and defined radius were designed from the soma. To evaluate the arborization of a neuron, the number of intersections to a given circumference was calculated. The further and more numerous the intersections are the more complex the arborization of the neuron is. Primary and secondary antibodies are indicated in Table 2.3.

2.5.11. Quantum dots labeling and imaging

Stargazin variants surface diffusion was evaluated by using quantum dots as fluorescent probes as described in (Bruchez, 2005). Low-density 12 DIV cells were co-transfected with plasmids encoding Homer-GFP, for synapse identification, and each of the HA-tagged stargazin variants. At DIV 14, cells were incubated for 10 min at 37ºC with anti-HA antibody (1:3000) diluted in conditioned medium. After one washing step, anti-rat IgG conjugated QD655 (diluted 1:10 in PBS) was diluted in conditioned medium with BSA 2% (1/2000) and incubated with cells for 5 min at 37°C. All washes were performed in ECS containing (in mM) NaCl 145, KCl 5, Glucose 10, Hepes 10, $CaCl_2$ 2 and $MgCl_2$ 2), supplemented with BSA 2% at 37°C. After washing, neurons were mounted in an open chamber (K.F. Technology SRL) and imaged in ECS. Single-particle tracking was performed as in (Opazo *et al.*, 2010). Cells were imaged at 37°C on an inverted microscope (AxioObserver Z1, Carl Zeiss) equipped with a Plan Apochromat 63× oil objective (NA = 1.4). Homer1C-GFP signal was detected by using a HXP fluorescence lamp (For QDs: excitation filter 425/50 and emission filters 655/30, Chroma). Fluorescent images from QDs were obtained with an integration time of 50 ms

with up to 600 consecutive frames. Signals were recorded with a digital CMOS camera (ORCA Flash 4.0, Hamamatsu). QD-labeled Stg-HA was imaged on randomly selected dendritic regions.

The tracking of single QDs was performed with a software, using the Matlab tool (Mathworks Inc., Natick, USA). Single QDs were identified by their diffraction limited signals and their blinking fluorescent emission. The trajectory of a QD tagged receptor could not be tracked continuously due to the random blinking events of the QDs. When the positions before and after the dark period were compatible with borders set for maximal position changes between consecutive frames and blinking rates, the subtrajectories of the same receptor were reconnected. The values were determined empirically: 2–3 pixels (0.32–0.48 μm) for maximal position change between two frames and maximal dark periods of 25 frames (1.25 s). MSD curves were calculated for reconnected trajectories of at least 20 frames. The QDs were considered synaptic if colocalized with Homer dendritic clusters for at least five frames. Diffusion coefficients were calculated by a linear fit of the first 4–8 points of the mean square displacement (MSD) plots versus time depending on the length of the trajectory within a certain compartment. The resolution limit for diffusion was 0.0075 μm2/s as determined by (Groc et al., 2004) whereas the resolution precision was ~40 nm.

2.5.12. Total protein and P2 extracts - cultured rat cortical neurons and HEK cells

For total extract preparation, cortical neurons or HEK cells were washed with ice-cold PBS and then incubated with Teen buffer (25 mM Tris pH 7.4, 1mM EDTA, 1 mM EGTA, 150 mM NaCl and 1% Triton X-100) supplemented with phosphatase (50 mM sodium fluoride, 1.5 mM sodium orthovanadate and 1mM okadaic acid) and protease (DTT, PMSF, Chymostatin, leupeptin, aprotinin and pepstatin) inhibitors. Cells were scraped and sonicated for 45s. Cellular extracts were then centrifuged at 16,100 x *g* for 10 min at 4ºC and the resultant pellet was discarded. Protein concentration was determined with the bicinchoninic acid (BCA) assay kit and the samples were denatured using denaturating loading buffer (NZYTech 5x Loading buffer).

To obtain P2 extracts, cortical neurons were washed with ice-cold PBS and then incubated with HEPES A (4mM HEPES pH 7.4 and 0.32 M sucrose) supplemented with protease and phosphatase inhibitors. Cells were scraped and homogenized with a potter, followed by further homogenization in a motor glass-teflon homogenizer at 900 rpm, at 4ºC. The samples were centrifuged at 900 x *g* for 15 min, 4ºC to remove the pellet (P1) containing the nuclear fraction. The supernatant 1 (S1) was centrifuged at 18000 x *g* for 15 min to yield a pellet containing washed crude synaptosomal fraction (P2). The resultant pellet was resuspended in 500 μl of supplemented Teen buffer + DDM (25 mM

Tris pH 7.4, 1mM EDTA, 1 mM EGTA, 150 mM NaCl and 1% DDM) and sonicated for 45 s. P2 samples were used for immunoprecipitation.

2.5.13. Immunoprecipitation

For immunoprecipitation assays, cellular extracts were diluted with supplemented Teen buffer to the concentration of 2 µg/µl. The extracts (about 1.6 mg of protein) were pre-cleared by incubation with 30 µl of sepharose A beads 2x (GE Healthcare) diluted in Teen buffer, for 1 h at 4ºC.
After centrifugation at 3000x *g* for 1 min, the supernatant was divided in two tubes, one to be incubated with the antibody and the other to be incubated with species-matched, non-specific IgGs, overnight, at 4ºC. 80 µl of beads were added to the extracts and incubated for 2 h at 4ºC. Following centrifugation at 3000x *g*, 1 min, to spin down the beads bound to the antibody-protein complexes, 30µl of the supernatant were recovered to evaluate the efficiency of the immunoprecipitation. The beads were washed six times: three times with Teen, twice with 1% Triton supplemented Teen and once with 150 mM NaCl supplemented Teen. After a final centrifugation at 3000 x *g* for 1 min, the supernatant was discarded and the proteins were eluted from the beads by 40 µl of loading buffer 2x (diluted from the NZYTech loading buffer 5x). Samples were processed by SDS-PAGE followed by Western Blot, silverstaining or coomassie blue staining and mass spectrometry analysis.

2.5.14. SDS-PAGE and Western Blot

Samples were processed by SDS-PAGE in 11% polyacrylamide gels. Proteins were transferred overnight at 40V or for 1h30min at 300 mA 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 3-5% blocking solution in TBS-T) for 2h at RT or overnight at 4ºC. 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, and the fluorescent signal was acquired in a Storm 860 Gel and Blot system (GE Healthcare). Primary and Secondary antibodies are shown in Table 2.3.

2.5.15. Silver staining for in gel protein detection

Silver staining was performed as previously described (O'Connell and Stults, 1997). Proteins were fixed for 30 min in a solution containing 25% methanol and 25% acetic acid. The gel was washed in 50% ethanol and 30% ethanol and then sensitized with $Na_2S_2O_3$(0.2 g/l) for 1 min. After washing twice with water, the gel was stained with $AgNO_3$ (2 g/l) for 20 min and developed in a solution containing 37% formaldehyde (0.7 ml/l), 30 g/l $Na_2CO_3$and 10 mg/l $Na_2S_2O_3$ until bands became visible (about 20 min). The staining was stopped with a solution containing Tris (50 g/l) and 2.5% acetic acid. Image acquisition was performed with a Canon LiDE 110 scanner.

2.5.16. Colloidal Coomassie blue staining

The colloidal coomassie blue staining was performed as previously described (Candiano *et al.*, 2004). The dye solution was prepared as follows: Phosphoric acid (to a final concentration of 10%) was added to 1/10 of the final volume of water, followed by ammonium sulfate (final concentration of 10%). After dissolution, Coomassie blue G-250 (final concentration 0.12%) was added with the help of a strainer. After the solids were dissolved, water was added up to 80% of the volume and under stirring, anhydrous methanol was added to complete the volume. Gels were stained for 2 h at RT, destained with water and cut with a clean scalpel.

2.5.17. Protein identification by liquid chromatography coupled to MS analysis (LC-MS/MS)

For MS analysis purposes, samples in 1x loading buffer were incubated with 8 µl of 30% acrylamide (Biorad) for 30 min to alkylate cysteinyl residues. After resolving the sample by SDS-PAGE, polyacrylamide gels were stained with colloidal Coomassie blue and cut in five slices, for each gel lane, and processed as described previously (Chen, 2011). Briefly, gel pieces were subjected to tryptic digestion. The resulting peptides were separated through HPLC using a capillary reverse phase C18 column. Protein identification was carried out on the Electrospray LTQ-Orbitrap (Thermo Fisher Scientific) with a stainless steel nano-bore emitter (Proxeon, 30 µm ID).
MS analysis was performed in collaboration with Dr. Ka Wan Li (Neuroproteomics laboratory, Center for Neurogenomics and Cognitive Research, Vrei University, Amsterdam).

Table 2.2 Oligonucleotide primer sequences.

Use	Section	Sequence
Amplification of the Exon 1 (human) (FW)	2.1.2.	5'CAAGCCTCTTCCCGTGTGATC3'
Amplification of the Exon 1 (human) (RV)	2.1.2.	5'GTGGTGGAAGGAGTGAACTAAG3'
Amplification of the Exon 2 (human) (FW)	2.1.2.	5'GATCCTCTCTGCAGTACTGCG3'
Amplification of the Exon 2 (human) (RV)	2.1.2.	5'CGGAAAGGAGAGAGGCTTAGC3'
Amplification of the Exon 3 (human) (FW)	2.1.2.	5'CCTCTCCTCTAAGGCTGTGTC3'
Amplification of the Exon 3 (human) (RV)	2.1.2.	5'CAACACGACCTAGTGCAAGTCTT3'
Amplification of the Exon 4 (human) (FW)	2.1.2.	5'GCGGATGTCCATCTACACC3'
Amplification of the Exon 4 (human) (RV)	2.1.2.	5'TGGTACATGGGAAATCATGC3'
Amplification of shRNA cDNA (FW)	2.1.5.	5'TGAAGAACGAGGAAGTTATGTTCAAGAGACATAACTTCCTCGTTCTTCTTTTTT-3'
Amplification of shRNA cDNA (RV)	2.1.5.	5'ACCTTCAATACTGGGTAAGGAAGTTCTCTCCTTACCCAGTATTGAAGGAAAAAA3'
Mutagenesis primer for the generation of shRNA resistant Stg-HA constructs (FW)	2.1.5.	5'GCGAGCAAAAAGAATGAGGAAGTCATGACCCATTC3'
Mutagenesis primer for the generation of shRNA resistant Stg-HA constructs (RV)	2.1.5.	5'GAATGGGTCATGACTTCCTCATTCTTTTTGCTCGC3'
Mutagenesis primer for the V143L mutation (FW)	2.1.5.	5'CCGGCATCTTCTTCCTGTCTGCAGGTCTG3'
Mutagenesis primer for the V143L mutation (RV)	2.1.5.	5'CAGACCTGCAGACAGGAAGAAGATGCCGG3'
Mutagenesis primer for the S148N mutation (FW)	2.1.5.	5'CGTGTCTGCAGGTCTGAATAACATCATTGGCATCA3'
Mutagenesis primer for the S148N mutation (RV)	2.1.5.	5'TGATGCCAATGATGTTATTCAGACCTGCAGACACG3'
Amplification primers for Stg-HA cDNA (FW)	2.1.5.	5'GCGGCGCGCCGATGGGGCTGTTTGATCGAGGTG3'
Amplification primers for Stg-HA cDNA (RV)	2.1.5.	5'GCGGATCCTTATACGGGGGTGGTCCGGC3'
Amplification Primers for T2A cDNA (FW)	2.1.5.	5'GCTGTACAAGTTAATTAAAGGTAGTGGAGAGGGCAGAGG3'
Amplification Primers for T2A cDNA (RV)	2.1.5.	5'GCGGCGCGCCTGGGCCAGGATTCTCCTCGACG3'

Table 2.3. Antibodies used in the present study.

Primary antibody (host species)	Application (Dilution)	Brand
GluA1 C-terminal (Rabbit)	WB (1:1000)	Millipore (MA,USA)
GluN1 N-terminal (Mouse, IgG1)	ICC (1:100)	Neuromab (CA, USA)
GluA1 N-terminal (Mouse, IgG2a)	ICC (1:100)	Millipore (MA, USA)
GluA1 N-terminal (Sheep)	ICC (1:200)	
HA (mouse)	ICC (1:200/1:500)	Invivogen CA, USA)
	Quantum dots (1:300000)	
	WB (1:1000)	
MAP2 (Chicken)	ICC (1:5000)	Abcam (Cambridge, UK)
Normal rabbit IgG	IP (1:200)	Millipore (MA, USA)
PSD95 (Mouse)	ICC (1:200)	Thermo Scientific
Stargazin (Rabbit)	WB (1:750)	Millipore (MA, USA)
	ICC (1:200)	
VGAT	ICC (1:1000)	Synaptic Systems (Gottingen, Germany)
VGLUT1 (Guinea pig)	ICC (1:5000)	Millipore (MA, USA)
Secondary antibody (host species)	**Application (Dilution)**	**Brand**
Alexa 488-conjugated anti-rabbit	ICC (1:5000)	Molecular Probes (Leiden, The Netherlands)
Alexa 488-conjugated anti-rat	ICC (1:5000)	Molecular Probes (Leiden, The Netherlands)
Alexa 568-conjugated anti-rabbit	ICC (1:5000)	Molecular Probes (Leiden, The Netherlands)
Alexa 568-conjugated anti-rat	ICC (1:5000)	Molecular Probes (Leiden, The Netherlands)
Alexa 568-conjugated anti-mouse	ICC (1:5000)	Molecular Probes (Leiden, The Netherlands)
Alexa 568-conjugated anti-sheep	ICC (1:5000)	Molecular Probes (Leiden, The Netherlands)
Alexa 647-conjugated anti-mouse	ICC (1:5000)	Molecular Probes (Leiden, The Netherlands)
Alexa 647-conjugated anti-guinea pig	ICC (1:5000)	Molecular Probes (Leiden, The Netherlands)
Alkaline Phosphatase-conjugated anti-rabbit	WB (1:10000)	Jackson ImmunoResearch (Pennsylvania, USA)
Alkaline Phosphatase-conjugated anti-mouse	WB (1:10000)	Jackson ImmunoResearch (Pennsylvania, USA)
AMCA-conjugated anti-chicken	ICC (1:200)	Jackson ImmunoResearch (Pennsylvania, USA)

CHAPTER 3

Generation and characterization of *knock-in* mice for the intellectual disability-associated stargazin mutation, V143L.

3.1. Summary

Two disease-associated mutations in the *CACNG2* gene affect the cellular function of the encoded protein, stargazin (Chapter 2). These mutations, described in an intellectual disability patient and in a SCZ patient, affect the surface diffusion properties of stargazin and impact the synaptic trafficking of AMPAR and homeostatic plasticity. These observations prompt the generation of animal models of disease, to further study the consequences of the disease-associated mutations in *CACNG2* on several aspects of synaptic transmission, synapse composition, neuronal morphology and on animal behavior.

Here, we generated *knock-in* mice carrying either hetero- or homozygous insertion of the ID-associated stargazin (Stg^{ID}) mutation, and sought to assess *in vivo* the impact of this stargazin mutation on animal behavior. Considering the expression pattern of stargazin and its putative role in psychiatric disorders such as intellectual disability and SCZ, we focused on behavior tasks that rely on the function of brain areas where stargazin is highly expressed and/or behavior paradigms that are relevant in a disease context.

We observed that Stg^{ID} *knock-in* mice display altered behavioral phenotypes, namely increased locomotor activity, impaired working memory and motor learning, and altered nesting behavior. Our results show that the intellectual disability-related V143L mutation in stargazin contributes to a disease-like behavioral phenotype, and suggest a potential role for stargazin in the pathophysiology of human cognitive disorders.

3.2. Introduction

The prevalence of intellectual disability (ID) ranges from 1% to 3% of the population, and the disease is characterized by impairments that affect adaptive functioning in three domains: conceptual (language, reading, writing and memory, among others), social (empathy, interpersonal communication skills) and practical (personal care, job responsibilities and organization). The ID diagnosis is based on clinical assessment of adaptive function and also takes into account the IQ, which is considered low when the score is approximately two standard deviations below the average population IQ, that is, about 70 or below (American Psychiatry Association, 2013).

Although the most severe forms of ID are associated with chromosomal abnormalities or gene defects, near 60% of the patients remain without a genetic diagnosis. Research on genetic causes has traditionally focused on the X-chromosome due to the significant higher incidence of ID in males (Athanasakis *et al.*, 2014). Indeed, many X-linked disorders such as fragile X and Rett syndrome present ID as a major symptom. The development of new sequencing techniques allowed the recognition of an endless number of *de novo* and/or X-linked mutations, putatively causative of ID, linking numerous genes to the pathogenesis of this disorder. Among those are genes that encode for proteins that play a pivotal role in synaptic transmission, including NMDAR, AMPAR and KAR subunits (Endele *et al.*, 2010; Hamdan *et al.*, 2009a; Hamdan *et al.*, 2009b; Motazacker *et al.*, 2007; Najmabadi *et al.*, 2011; Tarpey *et al.*, 2004; Wu *et al.*, 2007) [reviewed in (Kaufman *et al.*, 2010)].

A missense mutation (V143L) in the *CACNG2* gene, which codes for stargazin, was described in an intellectual disability patient (Hamdan *et al.*, 2011). In the previous chapter, we reported that this mutation affects the surface diffusion properties of stargazin, impacts AMPAR synaptic trafficking and homeostatic synaptic scaling. In an attempt to further elucidate the effect of the V143L mutation on the function of stargazin, and to appraise its causal role in the development of behavioral phenotypes that correlate with ID symptomatology, we generated a *knock-in* mouse line for the stargazin V143L mutation, and characterized some of their behavior features. To do so, we used a gene targeting approach, which allowed the directed modification of one nucleotide in the third exon of the *CACNG2* gene via homologous recombination (Figure 3.1). This technique enabled the expression of Stg^{ID} in all the cells that would naturally express stargazin.

Given the expression pattern of stargazin, the brain regions that closely relate to ID and the phenotypes/features most commonly observed in ID and other psychiatric disorder models, we submitted these mice to a battery of tests to allow the evaluation of motor ability, anxiety-like behavior and, most importantly, cognitive performance.

3.3. Results

3.3.1. Generation of the Stg^{ID}*knock-in* mice.

Gene targeting enables the introduction of directed modifications in the genome through homologous recombination and is a popular method to generate animal model that have greatly increased our understanding of neuropsychiatric disorders(Peca *et al.*, 2011; Peca and Feng, 2012). This technique makes use of mouse embryonic stem cells (ES cells), which are pluripotent cells from the inner cell mass of the blastocyst and can contribute to the formation of both somatic and germline tissues (Starkey and Elaswarapu, 2010).

To target the *CACNG2* gene and mediate the modification of the nucleotide in the third exon of the gene found to be mutated in an ID patient, we designed a targeting vector containing two homology arms, the long arm (LA) and the short arm (SA), and a third arm containing the modification to be inserted in the mouse genome, the middle arm (MA). In order to allow the genotyping of the animals, we included a random sequence, non-homologous to the mouse genome, in the SA/MA junction. The design of genotyping primers specific for this region allowed the discrimination between WT and Stg^{ID} *knock-in* (StgV143L^{+}) alleles (Figure 3.1 A, B).

The vector also contained two selection markers, a neomycin (NEO) cassette that confers resistance to geneticin, and a Diphtheria toxin (DTA) cassette, which sits outside the homology region. Whereas the first enables the selection of cells where the vector was successfully incorporated, the second allows the selection of cells where non-homologous insertion into the genomic DNA took place. However, because non-homologous insertion may occur in silent regions of the genome where DTA is not expressed, further screening is required to discriminate between these two situations. After electroporation of our vector into R1 ES cells, derived from the crossing of 129/Sv and 129/SvJ substrains, their DNA was isolated and we performed a third selection method, based on a PCR reaction where positive amplification only occurs if homologous recombination took place. Positive ES cell colonies were then expanded and microinjected into blastocysts of C57/B6 mice. The resulting chimeras were crossed with C57/B6 mice for at least four generations before biochemical and behavioral analyses.

Confirmation of the correct targeting was performed by sequencing the third exon of the *CACNG2* gene in WT and homozygous *knock-in* (StgV143L$^{+/+}$) mice (Figure 3.1C). Western blot analysis of whole brain lysated from WT, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice shows stargazin expression (Figure 3.1D)

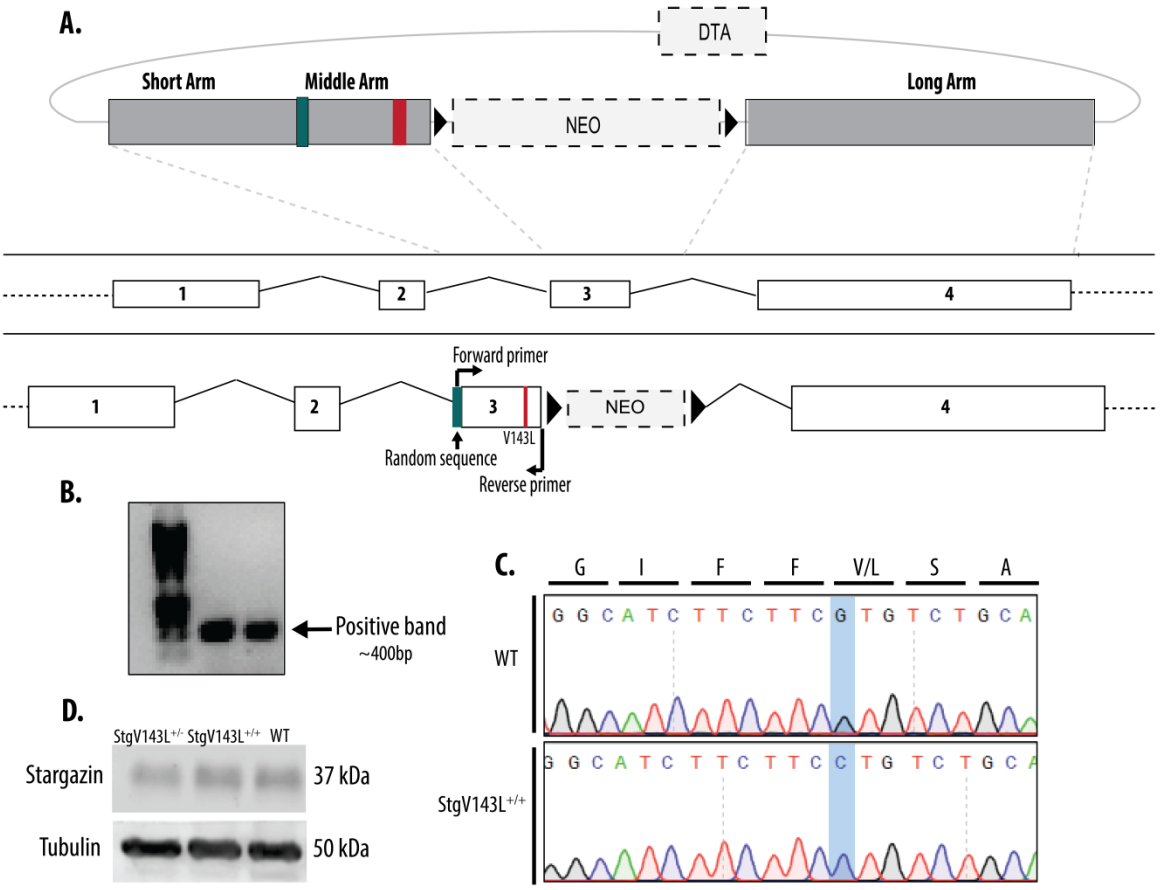


Figure 3.1. Stg^{ID}*knock-in* mice generation and genotyping. A vector containing two selection markers, Neo and DTA and three homology arms was constructed: the short and the long arm allowed homologous recombination with the genomic DNA of mouse embryonic stem (ES) cells, and the middle arm contained the ID-associated point mutation (V143L) (red bar) (**A**). 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. A 400bp band, amplified by PCR, indicates the presence of at least one mutated allele (**B**). The discrimination between homozygous and heterozygous animals requires a second PCR, using a forward primer directed against the original region replaced by the random sequence in the *knock-in*. The directed mutagenesis was confirmed by sequencing of the third exon in WT and StgV143L$^{+/+}$ animals, by Sanger sequencing (**C**). The expression of stargazin in WT, StgV143L$^{+/+}$ and StgV143L$^{+/-}$ mice in total brain lysates was confirmed by Western blot (**D**).

3.3.2. Homozygous Stg^{ID}*knock-in* (StgV143L$^{+/+}$) mice show increased locomotor activity and no distinct anxiety-like behaviors.

Psychiatric disorders are highly complex disorders, associated to numerous and diversified symptoms. The diagnosis of these disorders relies on the identification of a certain number and type of symptoms that must be present for a minimum amount of time. It is unlikely that a single patient displays all the symptoms associated with these disorders and likewise, it is a difficult task

to mimic all the symptoms of a disorder in animal models. On the other hand, there is significant symptom overlap among psychiatric disorders, and animal models for a given psychiatric disorder often display symptoms that are related to other psychiatric disorders (Yin *et al.*, 2013; Zhou *et al.*, 2016a). In the wake of these observations and taking into account the expression pattern of stargazin and its association to both ID and SCZ, we characterized Stg^{ID}*knock-in* mice behavior in cognition tests but also regarding other behavior phenotypes that have been previously found to be altered in models of these disorders, such as locomotor activity, anxiety-like, and nesting behaviors (Ehninger *et al.*, 2008; Miyakawa *et al.*, 2003; Moretti *et al.*, 2005; The Dutch-Belgian Fragile X Consortium, 1994; Zhou *et al.*, 2016a).

Given the early onset of ID, and the diagnosis of SCZ at early adulthood, we tested for behavior alterations in animals in early adulthood (6-8 weeks old). In order to assess the locomotor activity and anxiety-like behaviors, the animals were tested in the open field (OF) arena and in the elevated plus maze (EPM). The OF was first proposed by Calvin Hall in 1934 to measure emotionality in rats and from then on it has been widely used for different species and purposes. Numerous studies validated the observation that towards higher luminosity levels animals move less due the freezing phenotype associated to anxiety-like behaviors. In addition, anxious/fearful animals spend less time in the center and take longer to first explore that part of the arena (Gould, 2009; Walsh and Cummins, 1976). The EPM consists of four arms that are elevated from the ground; two of them are enclosed by walls whereas the other two are open and thus subjected to more illumination. Mice have a natural tendency to avoid open, unprotected spaces, a behavior that is magnified in animals showing anxiety-like phenotypes (Gould, 2009).

Representative trajectories performed by WT, heterozygous Stg^{ID}*knock-in* ($StgV143L^{+/-}$) and $StgV143L^{+/+}$ mice, left in the OF arena for 60 minutes, are shown in Figure 3.2 A. To evaluate locomotor activity, the total distance traveled and velocity were assessed and displayed separately for male (Figure 3.2 B-E) and female (Figure 3.2 F-I) mice, due to evident disparate results between genders. Although no significant changes were observed in the behavior of males with different genotypes, $StgV143L^{+/+}$ females showed increased locomotor activity, compared to WT females, characterized by increased distance traveled (Figure 3.2 F) and increased velocity (Figure 3.2 G). The time spent in the center and the latency to enter the center for the first time do not vary significantly among genotypes (Figure 3.2 D,E, H, I) or between genders (data not shown). These results are in agreement with similar anxiety-like levels between $StgV143L^{+/-}$, $StgV143L^{+/+}$ and WT animals.

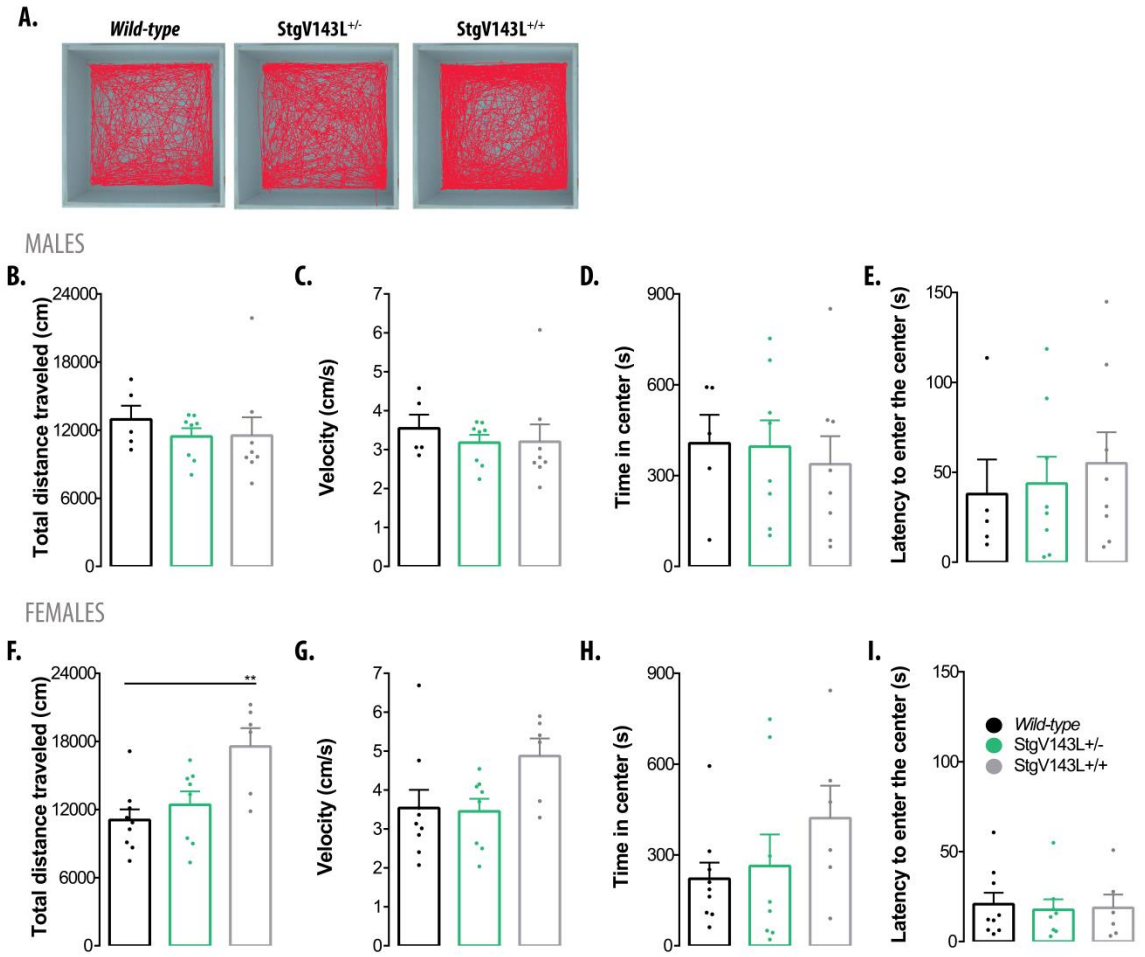


Figure 3.2. Female StgV143L$^{+/+}$ mice show hyperactivity. Locomotor activity was evaluated using the open field test. A representative trace of the total trajectory performed by an animal from each genotype is displayed in **A**. Both male (**B-E**) and female (**F-I**) mice were placed for 60 minutes in a 40 cm x 40 cm arena whose center was illuminated with 100 lux. Total distance traveled by the mice as well as their average velocity were increased in female StgV143L$^{+/+}$ mice (**F, G**), comparing to WT females, suggesting hyperactivity. Both the time spent in the center and the latency to enter the center for the first time were not significantly different between genotypes or genders, suggesting that these mice do not present anxiety-like behaviors. **p<0.01 One-way ANOVA followed by Dunnet's multiple comparison test. N=5,8,8 for WT, StgV143L$^{+/-}$, StgV143L$^{+/+}$male animals, respectively, and N=9,8,6 for WT, StgV143L$^{+/-}$, StgV143L$^{+/+}$ female mice, respectively.

Representative trajectories of WT, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice in the EPM are shown in Figure 3.3 A. Neither the time spent in the open arms (Figure 3.3 B,D) nor the latency to enter the open arms for the first time (Figure 3.3 C, E) showed significant differences among genotypes, supporting the inexistence of differences in the anxiety levels between WT and StgV143L$^{+/+}$ animals. Taken together, these data show that mice expressing the ID-associated stargazin mutation in homozygosity present hyperactivity without displaying anxiety-like phenotypes.

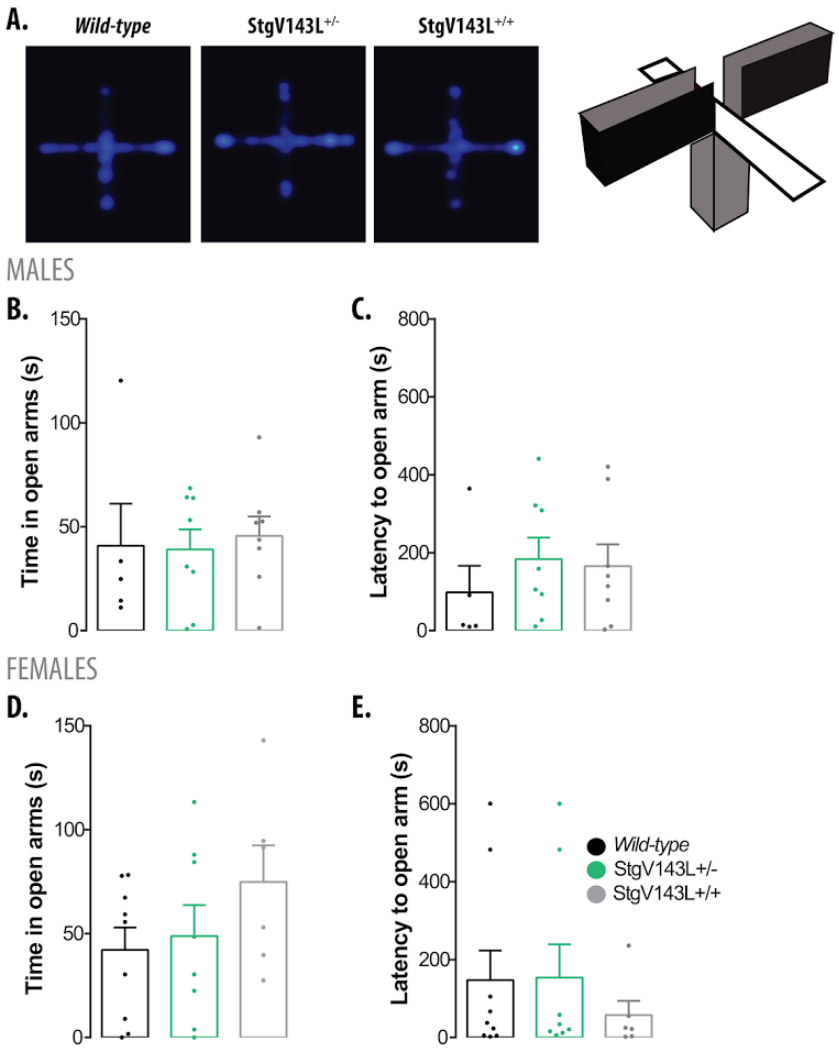


Figure 3.3. Stg^{ID}*knock-in* mice do not show anxiety-like behaviors. Anxiety-like behavior was evaluated using the elevated plus maze, a plus-shaped platform containing two arms enclosed by walls and two open arms that were exposed to 100 lux. Animals explored the maze for 10 minutes and the preference for the open and closed arms was assessed (**A**). The latency to enter open arms for the first time (**C,E**) and the time spent in the open arms (**B, D**) did not vary significantly between genotypes either for males (**B,C**) or females (**D,E**), suggesting that animals carrying the ID-associated stargazin mutation do not present increased anxiety comparing to controls. N=5,8,8 for WT, StgV143L$^{+/-}$, StgV143L$^{+/+}$ males, respectively, and N=9,8,6 for WT, StgV143L$^{+/-}$, StgV143L$^{+/+}$ females, respectively.

3.3.3. The ID-associated stargazin mutation affects motor learning in the rotarod

Stargazin is highly expressed in the hippocampus, cortex and cerebellum; however, whereas in the hippocampus and cortex other TARPs are also expressed (γ8 and γ3, respectively), in the cerebellum stargazin is by large the most abundant TARP (Tomita *et al.*, 2003). In agreement with an important function for stargazin in the cerebellum, *stargazer* mice, which lack stargazin, present cerebellar ataxia (Chen *et al.*, 2000).

Thus, aiming at assessing the effect of the ID-associated stargazin mutation in cerebellum function, and to assess if motor abnormalities could interfere with the performance of StgV143L mice in other behavior tests, mice were submitted to a cerebellum-dependent task, the rotarod assay. This test consists on the evaluation of the time that a mouse withstands in the top of a rotating beam with increasing velocity, a behavior that directly correlates with motor balance. The time spent in the beam (in seconds) was measured for two consecutive days, averaged from three trials each day.

On average, no significant differences among genotypes were observed in the time the animals endure on the apparatus, in the first or second day (Figure 3.4 A, E). Although StgV143L$^{+/-}$ and StgV143L$^{+/+}$ females present a slight increase in the mean time spent on the rotating beam, comparing to WT females, which is in agreement with the increased locomotor activity presented in the open field arena, these differences are not statistically significant (Figure 3.4 E). These observations suggest that the ID-associated stargazin mutation does not interfere with the motor ability of these animals in this particular cerebellum-dependent task. In the rotarod assay, the performance improvement on the second day, compared to the first day, correlates with the ability to learn and adapt to the task. Analysis of the performances on the two consecutive days shows that on average animals from the three genotypes are able to learn the task and improve in the second day; however, paired analysis showed that although WT animals always endure significantly more time in the second day (Figure 3.4 B,F), StgV143L$^{+/-}$ females and StgV143L$^{+/+}$ males and females fail to do so (Figure 3.4 G, D, H), suggesting an effect of the ID-associated mutation in motor learning in this task.

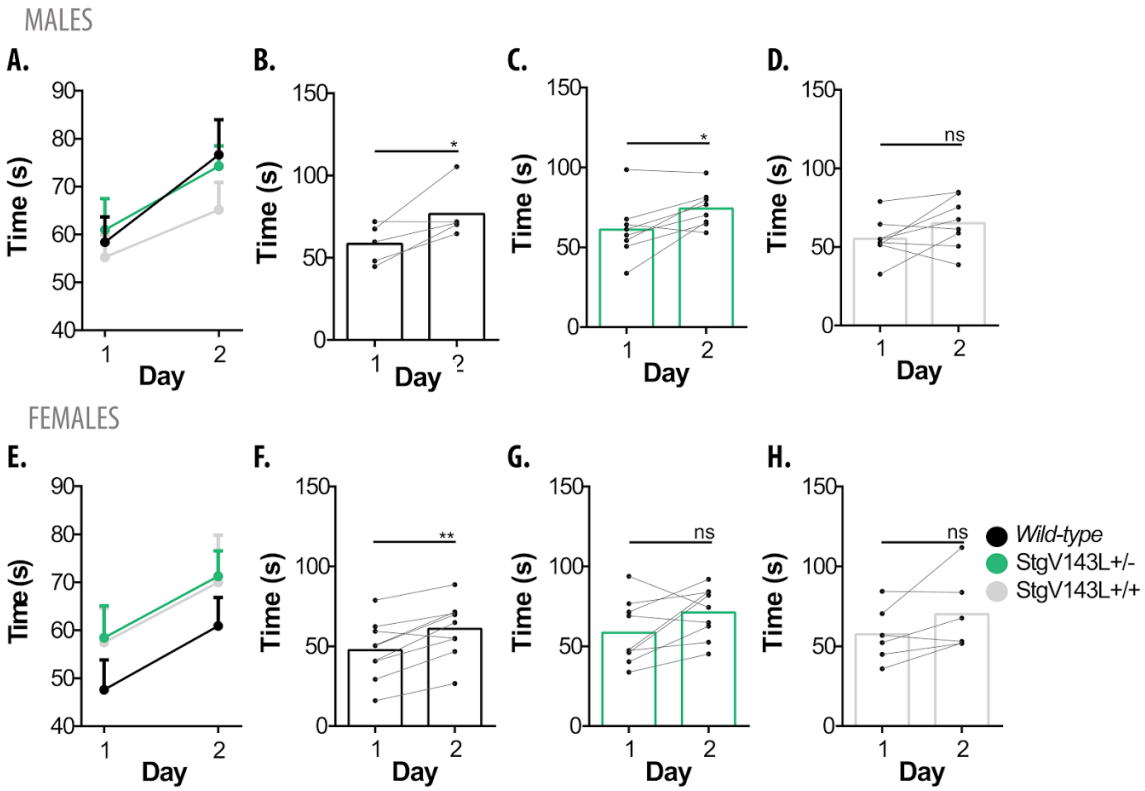


Figure 3.4.Stg^{ID} *knock-in* mice motor learning in the rotarod assay is impaired. Evaluation of motor function was performed using the rotarod test. In this test, the time withstanded on a rotating beam with increased velocity (4 to 40 mps in 5 minutes) was evaluated in two consecutive days. The average time spent on the rotarod did not significantly vary between genotypes in either of the two days (**A**, **E**). Analysis of the improvement in the second day, for each animal, showed that although WT (**B**,**F**) and StgV143L$^{+/-}$ male animals (**C**) perform significantly better in the second day, StgV143L$^{+/-}$ females (**G**) and StgV143L$^{+/+}$ mice of both genders (**D**,**H**) do not improve significantly, suggesting that the ID-associated mutation affects learning in this task.*p<0.05, **p<0.01, Paired t-test. N=5,8,8 for WT, StgV143L$^{+/-}$, StgV143L$^{+/+}$ males, respectively, and N=9,9,6 for WT, StgV143L$^{+/-}$, StgV143L$^{+/+}$ females, respectively.

3.3.4. Stg^{ID}*knock-in* mice present impaired spontaneous alternation phenotype

Stargazin is required for synaptic plasticity mechanisms such as LTP and LTD (Tomita *et al.*, 2005b), which are thought to underlie learning and memory. Furthermore, it is expressed in the cortex and hippocampus, two of the most important brain regions for those processes. Working memory is associated with the ability to process and retain information regarding the surrounding environment. This type of memory has been widely associated with the prefrontal cortex both in rodents (Sanchez-Santed *et al.*, 1997) and in humans and primates (Yang *et al.*, 2014), but other regions, such as the hippocampus and the thalamus, have also been implicated in this type of memory [reviewed in (Lalonde, 2002; Yoon *et al.*, 2008).

The T-maze is used for different tests that enable the evaluation of several features of spatial working memory and that take advantage of the fact that rodents are naturally motivated to explore their environment to look for food, water or shelter. One of those tests evaluates the spontaneous alternation. Animals are placed at the base of the T and are allowed to choose one of the arms. If a second trial is performed shortly after the first, the rodent usually chooses the arm not visited before (alternation) in order to fully explore the environment, demonstrating memory of the first arm (Deacon and Rawlins, 2006; Dudchenko, 2004).

Taking into account the importance of stargazin for synaptic plasticity mechanisms underlying memory and learning, and also its high expression in the cortex and hippocampus, we evaluated the effect of the ID-associated stargazin mutation in spatial working memory, by testing Stg^{ID} *knock-in* mice for spontaneous alternation in the T-maze. Spontaneous alternation was evaluated in five trials divided in two rounds. In the first round, immediately after mice chose an arm, their movement was restricted to that arm for 30 seconds. After this time, animals were removed from the maze and placed back in the start arm. If the previously unexplored arm was now chosen, we considered there was spontaneous alternation.

Although WT animals presented a percentage of alternations significantly higher than 50%, *i. e.* than what would be expected by chance, both $StgV143L^{+/-}$ and $StgV143L^{+/+}$ mice failed to do so, suggesting an impairment in spontaneous alternation, compatible with impaired working memory (Figure 3.5 A). As observed before for locomotor activity and motor function/learning, when evaluated separately in the T-maze, males and females show a distinct phenotype; whereas $StgV143L^{+/-}$ and $StgV143L^{+/+}$ males show a tendency for decreased spontaneous alternation (Figure 3.5 B), $StgV143L^{+/+}$ females were able to alternate as efficiently as WT females (Figure 3.5 C), suggesting that protective or compensatory mechanisms might take place differently between genders.

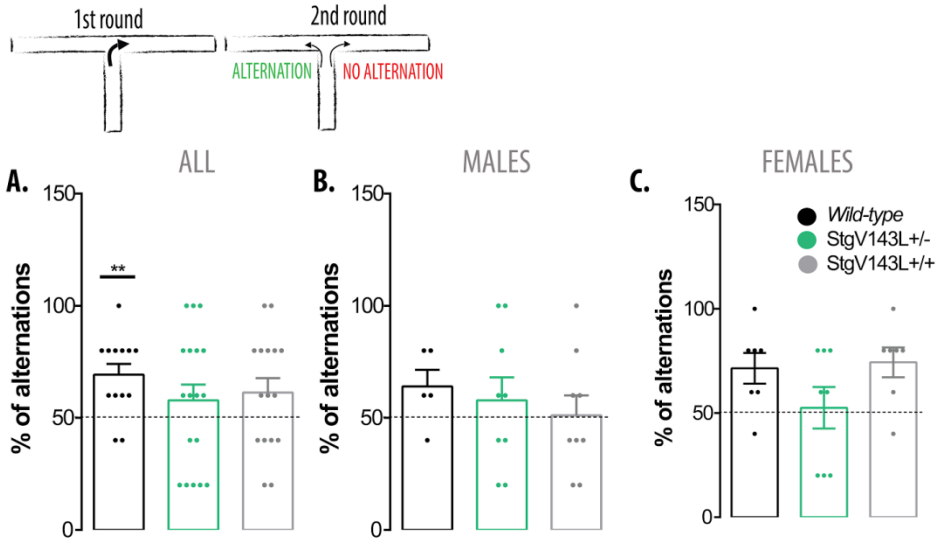


Figure 3.5. Spontaneous alternation is impaired in StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice. Spontaneous alternation was evaluated in five trials using a T-maze. In this task, divided in two rounds, the animals are allowed to choose one of the two arms of the maze and are kept in that arm for 30 seconds. After another 30 seconds they are re-placed in the start arm and, again, allowed to choose one of the arms. Alternation is considered when the animal does not visit the same arm twice. WT animals alternated significantly more than 50% of the times whereas StgV143L$^{+/-}$ and StgV143L$^{+/+}$ animals failed to do so (**A**). A similar trend was observed for males (**B**) when evaluated separately, whereas StgV143L$^{+/+}$ females alternated as efficiently as the WT females (**C**). **p<0.01 One sample t-test against a value of 50. N=5,9,9 for WT, StgV143L$^{+/-}$, StgV143L$^{+/+}$ males, respectively, and N=7,8,7 for WT, StgV143L$^{+/-}$, StgV143L$^{+/+}$ for females, respectively.

3.3.5. Nesting behavior is affected by the ID-associated mutation

When provided with suitable material, mice build nests, which in the wild allow them to hide, conserve their temperature and protect them during reproduction periods. The ability to build nests is impaired by different stimuli and genetic manipulations. In fact, several models for ASD, SCZ and other syndromes that include intellectual disability as major symptom showed deficits in nesting behavior (Blundell *et al.*, 2010; Etherton *et al.*, 2009; Miyakawa *et al.*, 2003; Moretti *et al.*, 2005).

To investigate whether the nesting behavior was intact in Stg^{ID}*knock-in* animals, animals were isolated and a cotton disk was included in the cage. After 12 hours, the nest built with the cotton disk was evaluated by seven observers blinded to animal genotype, who graded the nest from 1 to 5 according to the rating scale described in (Deacon, 2006). We observed that most of the WT animals gathered the completely shredded cotton to form a high, round shaped nest, whereas nests coming from StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice often presented intact pieces of cotton with random shapes; furthermore, cotton was sometimes found spread throughout the cage (Figure 3.6 A). Accordingly, nests from both StgV143L$^{+/-}$ and StgV143L$^{+/+}$, either male or female, were scored

significantly worse than nests built by WT animals, suggesting that the ID-associated stargazin mutation interferes with the animal's ability to build a nest (Figure 3.6 B, C). Considering that no motor problems were found in these mice, the decrease in the nest score is not likely caused by the inability to shred the cotton disk, but possibly by lack of interest or carelessness. Furthermore, for WT animals, nests made by males were scored substantially higher than the ones made by females.

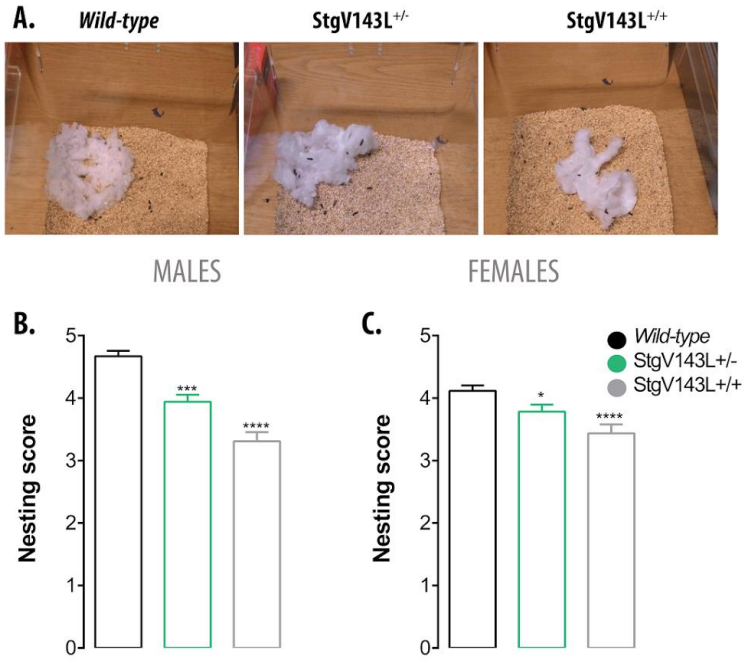


Figure 3.6. Nest building is impaired in StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice. Nesting behavior was evaluated by isolation of the animals and inclusion of a cotton disk in the cage for 12 hours. Nests were scored, by observers blinded to the animal genotype, from 1 to 5, where 5 corresponds to a well-built nest. Representative nests for WT, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice show that although nests from WT animals include totally shredded cotton pieces, put together to form a round and high structure, StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice nests included intact pieces of cotton, displaying random shapes (**A**). Nests from StgV143L$^{+/-}$ and StgV143L$^{+/+}$ males (**B**) and females (**C**) were scored significantly lower than those from WT animals, suggesting that *knock-in* mice have impaired nest building behavior. *$p<0.05$, ***$p<0.001$, ****$p<0.0001$, Unpaired t-test.

3.4. Discussion

In the previous chapter, we characterized two mutations in the *CACNG2* gene, identified in patients with two different psychiatric disorders, and that result in shared and distinct cellular phenotypes when expressed in neurons. Although both mutations affect stargazin surface diffusion properties and impair the trafficking of AMPAR to the surface, only the ID-associated mutation, V143L, affects homeostatic plasticity.

To further evaluate the consequences of this mutation in stargazin function and animal behavior, we generated *knock-in* mice that express the ID-associated form of stargazin. Using a gene targeting approach we induced the homologous recombination-mediated modification of one nucleotide in the third exon of the *CACNG2* gene, leading to the production of the stargazin V143L variant.

Preliminary data from our lab show that stargazin is expressed in the brain of both StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice, but its levels are decreased in postsynaptic densities (PSDs) isolated from StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice whole brain lysates (data not shown), in comparison to PSDs isolated from WT littermates, suggesting impaired synaptic accumulation of mutant stargazin.

In this chapter, we describe the performance of Stg^{ID} *knock-in* animals in a set of behavior tests that enable the evaluation of some features that depend on the function of brain regions where stargazin is highly expressed, such as the cortex, cerebellum and hippocampus and that correlate with phenotypes previously observed in models of ID and SCZ. We assessed locomotor activity, the presence of anxiety-like behaviors, nesting behavior, motor learning and cognitive performance.

Female Stg^{ID}*knock-in* mice present hyperactivity

In order to assess locomotor activity, we evaluated the behavior of Stg^{ID} *knock-in* animals in the open field arena. StgV143L$^{+/+}$ females showed an increase in the total distance traveled as well as in the velocity displayed, suggesting increased locomotor activity. Hyperactivity has been observed in several mouse models bearing mutations or lacking proteins implicated in psychiatric disorders, namely the model that mimics high expression levels of Neuregulin1 (Yin *et al.*, 2013), and *knock-out* animals for GluA1 (Wiedholz *et al.*, 2008), calcineurin (Miyakawa *et al.*, 2003) and Fmr1 (The Dutch-Belgian Fragile X Consortium, 1994).

Accordingly, several reports indicate a higher incidence of attention deficit and hyperactivity disorder (ADHD) in intellectual disability patients than in the general population [reviewed in (Seager and O'Brien, 2003)]. Furthermore, syndromes in which intellectual disability is a common

feature, such as tuberous sclerosis and fragile X, also include attention deficit hyperactivity disorder (ADHD) as a major symptom [reviewed in (de Vries *et al.*, 2005; McLennan *et al.*, 2011)].
Although StgV143L$^{+/+}$female mice show increased locomotor activity in the open field, no changes were observed in the time spent in the center or in the latency to enter the center for the first time, either in StgV143L$^{+/-}$ or StgV143L$^{+/+}$ mice, suggesting that these animals do not present anxiety-like behaviors. In agreement, no differences were observed in the elevated plus maze comparing Stg^{ID} mice to WT animals, either in the time spent in the open arms or in the time spent to access open arms for the first time.

Motor learning is impaired in Stg^{ID} *knock-in* mice

Assessing motor functions in genetically modified animals is one of the most important requirements, since the vast majority of tests rely on the movement of the animals. If motor functions are impaired, complex tasks that demand movement towards food, inanimate objects or other animals will be confounded. Different alterations may lead to the outcome of abnormal motor function, as changes in the formation of dendrites and/or axons, alterations in neurotransmitter receptor-mediated signaling, and changes in the development of bones, tendons or muscles can potentially interfere with motor function.

Furthermore, stargazin is highly expressed in the cerebellum and, considering the importance of this brain region for motor balance and learning, we evaluated the performance of these mice in the rotarod, a widely used task to study cerebellum function.
Our results show that the average time the animals from different genotypes can withstand in the rotating beam does not vary significantly, suggesting that animals expressing the ID-associated stargazin variant do not present gross motor abnormalities.
When repeatedly evaluated in this task, WT animals are able to adapt to the task and significantly improve their performance; this observation corresponds to motor learning, which requires coordination and balance and is described to be correlated to the cerebellum function. The latency to fall was increased in WT animals across training days. On the other hand, for both StgV143L$^{+/-}$ or StgV143L$^{+/+}$ the performance in the first and second days did not significantly differ, suggesting that they were not able to substantially improve in the task, which is compatible with an impairment in motor learning mediated by changes in the function of stargazin. The role for stargazin in the cerebellum function has already been addressed by several studies given the striking ataxia phenotype of the *stargazer* mouse. The absence of stargazin expression in these mice leads to impairment in the trafficking of AMPAR to the surface of cerebellum granule cells, with striking

consequences to neuronal transmission. This effect is particularly deleterious in the cerebellum since the synapses of cerebellar granule cells are virtually devoid of functional AMPAR (Chen *et al.*, 2000; Hashimoto *et al.*, 1999). Accordingly, besides presenting ataxia, these mice also display impaired acquisition of the eyeblink conditioning, a paradigm of motor learning dependent on cerebellar function and plasticity (Hashimoto *et al.*, 1999).

Impairment of motor learning has been previously described in genetically modified mice lacking or expressing variants of proteins implicated in psychiatric disorders. In fact, mice lacking the expression of Dysbindin1, a protein implicated in SCZ (Takao *et al.*, 2008), and mice expressing ASD- and SCZ-associated variants of Shank3 present impaired motor learning in the rotarod (Zhou *et al.*, 2016a). Furthermore, *knock-out* mice for Erbin, a protein that interacts with stargazin, present both increased locomotor activity and decreased motor learning in the rotarod (Tao *et al.*, 2013).

Further studies are necessary to assess if cerebellum-related tasks are affected by the disease-associated mutation, namely other tests that evaluate motor learning and motor balance/coordination should be performed, such as conditional eyeblinking, the balance beam and footprint pattern evaluation.

Besides the behavioral analysis, evaluation of total and synaptic levels of stargazin and AMPAR in cerebellar cells would be informative of the impact of this mutation on stargazin function and regulation.

The V143L stargazin mutation affects spontaneous alternation phenotype

Despite being widely studied, the mechanisms underlying memory and learning are not yet fully characterized. It is accepted that changes in synaptic strength might be at the basis of information storage, and that mechanisms such as LTP and LTD play a pivotal role in those processes. Numerous glutamatergic synapse proteins are indispensable for the induction and maintenance of Hebbian and homeostatic plasticity mechanisms, and changes in the expression of several of these proteins were correlated with impairments in domains of learning and memory in mouse models.

Spatial working memory correlates with the ability to obtain and retain information regarding the surrounding environment for a short period of time and different regions of the prefrontal cortex are thought to correlate with its acquisition and recall. In fact, the dorsolateral prefrontal cortex, a region that is affected in SCZ, was recently shown to be important in keeping spatial information for short periods (van Asselen *et al.*, 2006) and, accordingly, spatial working memory impairment has been described in SCZ patients (Glahn *et al.*, 2003; Park and Holzman, 1992). Different spatial mazes are used to assess spatial working memory. In most cases, the animals are allowed to choose between arms to receive reinforcements, in the form of food, water or shelter, which drive the

animals to recall the places where those rewards were found. Although the T-maze is often used for tests following the same rationale, it also enables the evaluation of spontaneous alternation. When placed in a T-shaped maze, rodents display a tendency for alternation between the arms as a measure of their exploratory behavior.

We observed that contrarily to WT animals which alternate significantly more than chance level, both StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice alternate around 50% of the times, suggesting that the choice is random, compatible with an impairment in recalling the memory of the previously visited arm. These observations are in accordance with the previously described pivotal role of stargazin not only for LTP and LTD but also for homeostatic scaling of AMPAR and experience-dependent plasticity (Louros *et al.*, 2014; Tomita *et al.*, 2005b). The role of stargazin in these processes makes it a critical regulator of synaptic strength, and, most likely, an important player in memory and learning.

Importantly, stargazin interacts with several proteins whose importance in learning and memory, and consequently in the development of cognitive disorders, has been described. In fact, altered spatial working memory was described in GluA1 *knock-out* mice, which perform poorly on tests of non-matching to place on T and Y-mazes (Reisel *et al.*, 2002; Sanderson *et al.*, 2009). Also, stargazin was recently shown to interact with ARC, an immediate early gene required for memory and learning that has been implicated in cognitive disorders such as fragile X and Angelman syndromes, characterized by intellectual disability (Zhang *et al.*, 2015).

Furthermore, as previously described in Chapter 2, we identified several new interaction partners for stargazin that are associated with aspects of memory and learning, such as Arvcf (Suzuki *et al.*, 2009), mGluR5 [reviewed in (Dolen and Bear, 2008)] and IQSEC2 (Shoubridge *et al.*, 2010). Thus, evaluation of the expression of these proteins in the brains of StgV143L$^{+/-}$ and StgV143L$^{+/+}$ mice, as well as evaluation of the strength of interaction with the mutated stargazin would give important insight regarding the role of these interactions in the observed phenotypes.

Stg^{ID} *knock-in* mice present altered nesting behavior

In nature, rodents build nests in order to provide shelter and protection from predators. They usually share nests with parents and offspring and nest building is therefore considered a phenotype correlated with social behavior. Several mouse models for psychiatric and neurodegenerative disorders present impairments in nest building behavior. In fact, nesting behavior is impaired in a Rett syndrome mouse model (Moretti *et al.*, 2005), in conditional *knock-out* animals for calcineurin (Miyakawa *et al.*, 2003) and for the Phospholipase C β1, a protein implicated in SCZ (Koh *et al.*, 2008),

and in the triple transgenic mouse model for Alzheimer's disease (Torres-Lista and Gimenez-Llort, 2013).

Impairments in nest building are often correlated with motor abnormalities that would affect the ability for shredding and combining the pieces of material. In our observations, both StgV143L$^{+/-}$ and StgV143L$^{+/+}$showed poorly constructed nests, and since no motor abnormalities were observed in these mice, either in the open field arena or in the rotarod assay, it is unlikely that the inability in nest building can be accounted for by motor impairment; it is more likely related to a lack of interest in the nesting material, or in making a nest. Interestingly, ongoing analyses on the marble burying behavior test indicate that StgV143L$^{+/-}$ and StgV143L$^{+/+}$mice show an expressive decrease in the number of marbles buried over 20 min, in comparison to WT animals (personal communication, Tiago Rondão), corroborating the hypothesis of decreased interest on the interaction with the provided materials.

Male and female mice perform differently in behavior tests

Gender differences in psychiatric disorders constitute one of the most intriguing features in psychiatry (Riecher-Rossler, 2017b). In many psychiatric disorders, age of onset, symptoms and drug response are influenced by gender (Kokras and Dalla, 2014). During adolescence women present a higher prevalence of depression and mood disorders. As adults, this tendency is maintained as women present a higher prevalence of affective disorders, whereas men more often present social deficits and substance abuse. Nevertheless, women present a later onset for SCZ psychosis (Afifi, 2007). Many studies have shown that estradiol has a protective effect on schizophrenic psychosis and concordantly, gonadal dysfunction and estrogen deficiency in women suffering from SCZ have been reported (Riecher-Rossler, 2017a). An hypothesis linking stress, sex hormones and dopamine proposes that the increase of prolactin release following stress events culminates in a decrease in estrogen levels and a concomitant increase in dopamine levels, which in turn favors the development of psychosis in vulnerable people (Riecher-Rossler, 2017a). On the other hand, women are more vulnerable to mood and anxiety disorders, which has been hypothesized to be influenced by sex hormones.

It has been proposed that males have increased susceptibility to changes in genes involved in synaptic plasticity due to a lower threshold for the development of changes triggered by a genetic or environment factor, which may explain the higher prevalence of autism in males (Mottron *et al.*, 2015). Many studies report a higher prevalence of intellectual disability among males than females, in both adult and children populations (Maulik *et al.*, 2011). A meta-analysis study reports that among adults with ID, the female-to-male ratio varies between 0.7 and 0.9, while in children it varies

between 0.4 and 1.0 (Maulik *et al.*, 2011). The reasons for this discrepancy are not clear, but may include X-linked intellectual disability (XLID). XLID is a common cause of monogenic ID, overrepresented in males due to their single X chromosome, which lacks compensation. In fact, the prevalence of XLID in males is about 1:4000, accounting for 2% of the male ID cases. In females, only 0.3% of the ID cases are X-linked. Importantly, mutations in XLID genes in sporadic males is much lower, suggesting that the inheritance pattern is important to distinguish X-linked from autosomal mutations (Carlo and Verpelli, 2016).

Gender differences were also observed in the Stg^{ID} *knock-in* mice for specific behaviors, including locomotor activity, spontaneous alternation and, to a smaller extent, motor learning.

In fact, $StgV143L^{+/+}$ female mice present hyperactivity while no changes are observed among genotypes in males. Interestingly, there is evidence that locomotor activity is affected by estrogen, since females but not males, showed increased activity in the open field when an estradiol benzoate pellet was implanted following gonadectomy (Ogawa *et al.*, 2003). Accordingly, several behaviors mediated by dopamine sensitive sites are affected by estradiol (Joyce *et al.*, 1984), and enhanced dopaminergic activity in rodents leads to enhanced motor activity (van den Buuse, 2010). The same trend for increased locomotor activity specifically in females was observed in a transgenic mouse model for S100β, a calcium-binding protein that affects brain development and LTP, whose coding gene is located in the chromosome 21, and which is therefore overexpressed in Down syndrome (Gerlai and Roder, 1993).

Motor learning was more severely impaired in Stg^{ID} females than males, which is in accordance with the observation in humans that males show a better performance and adaptation to motor tasks than females (Dorfberger *et al.*, 2009; Moreno-Briseno *et al.*, 2010).

Although not statistically significant, $StgV143L^{+/-}$ and $StgV143L^{+/+}$ males showed a tendency to alternate less than genotype-matched females, suggesting that the impairment in this task was more pronounced in males than in females. It is plausible that differences in the locomotor activity and in the latency to choose the second arm could account for the differences between sexes; however, neither the velocity and total distance traveled nor the time the animals took to choose the arms were evaluated in this test. Sex differences in spontaneous alternation have been observed previously. In a study involving hamsters, males spontaneously alternated significantly less in a T-maze than females (Hughes, 1989). A superior spatial memory was observed for female mice, comparing to age-matched males, following repetitive concussive brain injuries, which led to the suggestion of a role for female sex hormones in neuroprotection, as previously described in the literature (Velosky *et al.*, 2017). As reviewed in (Hill, 2016), in some developmental models of SCZ, males present a higher vulnerability for early developmental insults in the prefrontal cortex, and in several genetic models cognitive performance is more disrupted in males.

The account for gender differences in animal behavior represents an underrated subject of study as the vast majority of reports involving animal models of disease disregard gender for data analysis or do not include animals from both genders in experimental designs. Given the important observations concerning the effect of sex hormones in pivotal processes such as memory and learning or the predisposition to specific conditions, we believe that the inclusion of the gender variable to animal behavior studies, especially if in the context of psychiatric disorders, is of utmost importance.

Concluding remarks and future perspective

Taken together, these results suggest that mice carrying the ID-associated stargazin mutation present defects in locomotor activity, motor learning, working memory and social behavior, which might be correlated to the changes in synaptic composition and plasticity evidenced in the previous chapter. The elucidation of the behavioral phenotypes triggered by the ID-associated mutations in stargazin helps not only on the elucidation of the mechanisms in which stargazin takes part but also on the correlation between specific mechanism or circuits and disease-relevant phenotypes.

Many other behavior tests could be performed in order to determine the effect of the ID-associated mutation in cognition, namely the novel object recognition (NOR) test, a paradigm associated to both cortex and hippocampus function, which enables the evaluation of short-, middle- and long-term memory (Antunes and Biala, 2012). The Morris water maze and the Barnes maze allow the evaluation of spatial memory in rodents, since both tests evaluate the latency to recall the localization of a previously visited shelter-comparable platform and hole, respectively. Although the Morris water maze is more widely used than the Barnes maze, the latter is considered less anxiogenic since it does not involve swimming (Harrison *et al.*, 2006).

Sensory gating corresponds to a filtering mechanism that prevents the flooding of information. Sensory gating deficits are often observed in psychiatric disorders patients, as deficits in the ability to inhibit a response to a stimulus are commonly observed in these patients. As previously mentioned, the PPI test is used to evaluate sensorimotor gating in both humans and animals (Brody *et al.*, 2004). In fact, as described in the Table 1.4, several models of psychiatric disorders present deficits in PPI; thus, it would be interesting to evaluate if this disease-associated mutation in stargazin results in alterations in PPI. Furthermore, given the importance of stargazin in the visual-dependent refinement of the retino-thalamic connection (Louros *et al.*, 2014), the evaluation of the visual perception is particularly interesting in this case, and can be performed using the visual cliff test (Fox, 1965) for evaluating visual depth perception, and the optomotor system (Abdeljalil *et al.*, 2005) to study spatial vision.

Social behavior deficits are one of the hallmarks of psychiatric disorders, and are often comorbid with intellectual disability (Charman *et al.*, 2011). Several behavior tests have been developed to enable the evaluation of social behavior in rodents, as are the cases of the tube test, which allows the evaluation of social dominance, or the three-chamber test, which tests the preference for the exploration of an animal over the exploration of an inanimate object, and the discrimination between a known and a new animal. The observed defects in the nest building behavior of the Stg^{ID} *knock-in* mice thrust further evaluation of the behavior of these mice in social paradigms to unveil the role of stargazin in social behavior.

Interestingly, most of the behavior defects observed for Stg^{ID} *knock-in* mice have been described in animals modelling intellectual disability, ASD or SCZ, highlighting the overlap between different psychiatric disorders. As a future perspective, we aim at further studying the molecular and functional alterations occurring in these animals, namely at evaluating the effect of this mutation on the interaction between stargazin and other synaptic proteins with roles on excitation and inhibition and consequently in circuit formation and regulation.

The observation of an effect of the disease-associated mutations on stargazin-mediated AMPAR trafficking and synaptic scaling (Chapter 2) prompts the evaluation of their impact in synaptic transmission *in vivo.* Electrophysiological analyses of these animals would shed light on the impact of Stg^{ID} on synapse function and plasticity, and allow the assessment of functional alterations in specific circuits. Given the expression of stargazin in the cortex and hippocampus, electrophysiology studies in these brain regions are of relevance. Thus, the determination of the frequency, amplitude and kinetics of mEPSC as well as evaluation of paired pulse facilitation would largely contribute to evaluate both postsynaptic and presynaptic function. Furthermore, the importance of stargazin in plasticity and the identification of mGluR5 as a stargazin interaction partner (Chaper 2) instigate the evaluation of LTP and LTD in these mice.

Given the shared and distinct effects mediated by the SCZ and ID-associated mutations in stargazin on the function of stargazin and related cellular and molecular phenotypes (Chapter 2), it would be interesting to compare the effects of the two mutations in the behavior and biochemical features of both lines. More, provided that the differences between the two mutations include the distinct response to the drug tianeptine, which reverts cellular effects mediated by the SCZ-associated mutation but not by the ID-associated variant, it is relevant to assess the effect of this drug on the reversion of disease-associated phenotypes.

The generation and thorough characterization of *knock-in* mice for the SCZ-associated stargazin mutation would strengthen our knowledge about the common pathways among psychiatric disorders and help on enlightening how mutations in the same gene can culminate in different psychiatric disorders.

3.5. Material and Methods

We generated *knock-in* mice bearing the ID-associated stargazin mutation, V143L, using a gene targeting technique, which enables the introduction of directed modifications in the genome through homologous recombination (Peca *et al.*, 2011).

Unlike other engineering methods such as transgenic technology and gene trap, gene targeting does not involve the random insertion of genetic material or the interruption of the expression of other genes. Gene targeting makes use of mouse embryonic stem cells (ES cells), which are pluripotent cells from the inner cell mass of the blastocyst and can contribute to the formation of both somatic and germline tissues. These cells can be cultured, manipulated and implanted into blastocysts, allowing, with a high degree of precision, the generation of genetically engineered mice for the study of gene function and disease modeling (Starkey and Elaswarapu, 2010). The procedures regarding the vector design and animal generation were performed as described in [Heyer, Feliciano, Peça & Feng, "Elucidating gene function through use of genetically engineered mice", in Genomic: Essential methods, edited by (Starkey and Elaswarapu, 2010)].

3.5.1. Vector design

Targeted modifications can be introduced into ES cells by homologous recombination of a targeting vector; since the rate of homologous recombination in mammals is extremely low, positive and negative selection mechanisms are needed to increase the success of the method. Thus, a neomycin resistance gene under the control of a strong promoter is present in the targeting vector pLFN-DTA (kindly provided by Guoping Feng, MIT), allowing the selection of cells that incorporated the vector into the genome and were, therefore, resistant to G418/geneticin. Also, a diphtheria toxin A (DTA) cassette is present in the targeting vector for further negative selection. Most of the cells that incorporate the vector and are therefore resistant to G148/geneticin, do not undergo homologous recombination. In these cells, the DTA will be produced, killing the cells. In the ones where homologous recombination took place, the DTA cassette, because it sits outside the homologous regions is excised preventing cell death. However, if the DTA cassette was randomly inserted in a transcriptionally inactive region of the genome, it will not be produced, resulting in false positive colonies. In order to overcome this issue, a DNA extraction protocol was performed, followed by PCR with one primer that binds on the newly introduced Neo cassette and another that binds

outside of the short homology arm (SA); thus, amplification only occurred if homologous insertion took place.

The recombination rate increases with homology between a vector and its target and this is maximized by using two homology arms, a long arm (LA) and a short arm (SA). For our vector we designed a ~8 kb LA and a ~800 bp SA. In addition, a ~400bp middle arm (MA), containing the point mutation to be introduced in the genome, was designed.

3.5.1.1. SA and MA

All the homology arms were obtained using bacterial artificial chromosomes (BACs) DNA as template. Commercial bacteria containing the BAC (Source BioScience) were grown with the appropriate antibiotic at 37ºC, overnight, and 5 ml of cell suspension were collected by centrifugation at 16,100 x *g* for 1 min. The pellet was ressuspended in 250 µl of P1, 250µl of P2 and 350µl of P3 buffers from the NZYMiniprep kit (NZYTech). Tubes were centrifuged at 16,100 x*g* for 4 min. The supernatant was centrifuged again with the same speed and after discarding the pellet, the supernatant was incubated for 10 min at RT with 750µl of isopropanol to precipitate de DNA. The DNA was collected by spinning the tubes at 16,100 x *g* for 10 min. The resultant pellet was washed with 70% ethanol, air dried and resuspended in TE. The SA and MA were obtained by standard PCR and amplification primers were designed to introduce restriction sites to allow cloning into the final vector (Figure 3.7 A) and an additional site to allow the combined cloning of both SA and MA into the pLFN-DTA in a single step (SA was flanked by KpnI and ApaI restriction sites and MA was flanked by ApaI and AscI sites). MA forward primer also included a random sequence, non-homologous to the mouse genome, to allow the genotyping of the animals (Table 3.1).

Both the SA and the MA were subcloned into a pBluescript vector by blunt cloning with EcoRI to facilitate the site-directed mutagenesis of the MA and subsequent cloning into the final vector. Site-directed mutagenesis was performed by PCR (Primers shown in Table 3.1) and confirmed by Sanger sequencing (Figure 3.7 B).

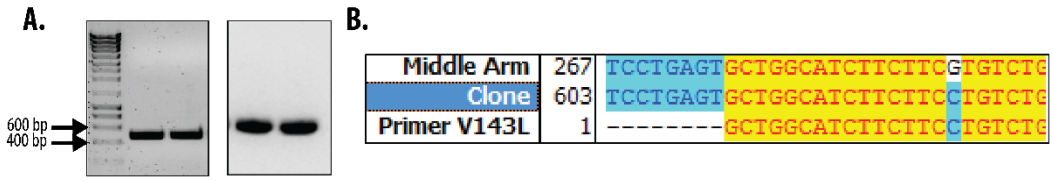


Figure 3.7 SA and MA amplification. SA and MA were obtained by standard PCR (**A**). A single point mutation was introduced into the MA by site-directed mutagenesis and confirmed by Sanger sequencing analysis (**B**).

3.5.1.2. LA retrieval

Due to its size, the LA could not be directly amplified by PCR and had to be obtained by "recombineering" *i.e.* recombination-mediated genetic engineering. In this strategy, large sequences of DNA are retrieved from BAC DNA by using a modified *E.coli* strain that allow recombination, the SW102 strain.

3.5.1.2.1. Amplification of the subcloning vector

Retrieval of the LA required the amplification of a subcloning vector, the pNAPP. Therefore, standard PCR primers were designed, complementary to regions of the pNAPP and overlapping the multiple cloning site containing rare cutting restriction enzyme sites (Table 3.1). The primers had an additional 50 bp sequence at their 3' end that was homologous to the ends of the LA sequence to be retrieved, as shown in Figure 3.8. The confirmation of pNAPP amplification is shown in Figure 3.8 B.

3.5.1.2.2. Electroporation of BAC into the recombinogenic bacteria

SW102 bacteria were grown overnight at 32ºC, to minimize undesired recombination, and were collected by centrifugation at 2000 × *g* for 5 min at 4ºC. The pellet was resuspended in ice-cold water, spinned (15-20s) at maximum speed, the supernatant was discarded and the pellet was resuspended in 50µl of ice-cold water. After repeating this washing step 3 to 5 times, 1 µl of BAC DNA, extracted as described above, was added to the tube and the cells were transferred to a pre-cooled electroporation cuvette. Cells were electroporated using the following conditions: 1.75kV and 25µF, and plated in the antibiotic selection for the BAC. A PCR with primers to amplify the MA was performed to confirm BAC DNA incorporation by the SW102 cells (Figure 3.8 C).

3.5.1.2.3. Electroporation of the pNAPP DNA into the recombinogenic bacteria containing BAC DNA

SW102 cells containing the BAC were grown overnight at 32 ºC and then incubated at 42ºC, with shaking, for 15 min, to activate their recombinogenic capacity. In order to decrease the temperature as quickly as possible, the flasks were incubated on ice for 5 min. Cells were collected again by centrifuging at 2000 × *g* for 5 min, at 4ºC, washed with ice-cold water and electroporated with the PCR product obtained as described in 3.5.1.2.1. The electroporation was performed as described above. Cells were plated with antibiotic selection for the pNAPP vector (kindly provided by Guoping Feng, MIT). Retrieval of the LA sequence from the BAC DNA was confirmed by restriction analysis (Figure 3.8 D) and Sanger sequencing.

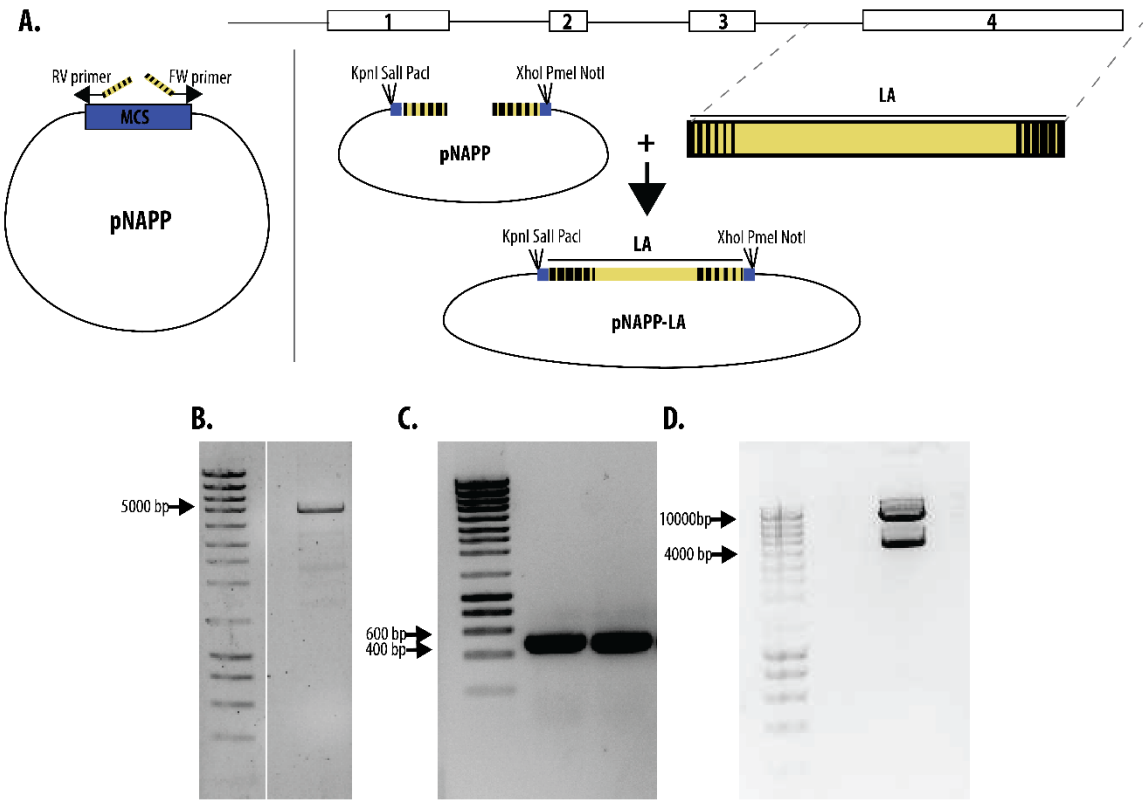


Figure 3.8. pNAPP vector amplification. The pNAPP vector was amplified with a forward and a reverse primer containing a 50 bp-long tail, homologous to the end and to the beginning of the long homology arm, respectively. The linearized vector and a BAC containing the *CACNG2* gene sequence were both electroporated into recombinogenic bacteria. Once activated, the recombinogenic bacteria allowed the retrieval of the LA sequence from the BAC DNA into the pNAPP vector through homologous recombination (**A**). pNAPP was amplified by standard PCR (**B**). Both the amplified pNAPP and BAC DNA were electroporated into recombinogenic bacteria and the latter was confirmed by PCR with the MA amplification primers (**C**). Retrieval of the LA sequence into the pNAPP vector was confirmed by restriction analysis (**D**).

3.5.1.3. Cloning of the homology arms into the pLFN-DTA

The LA was cloned into the PacI and NotI sites of the pLFN-DTA. The cloning was confirmed by restriction analysis (Figure 3.9A) SA and MA were then cloned into the KpnI and AscI sites of the pLFN-DTA vector already containing the LA. The cloning was confirmed by restriction digestion (Figure 3.9 B). Both cloning steps were confirmed by Sanger sequencing (data not shown). The final vector map is displayed in Figure 3.9 C.

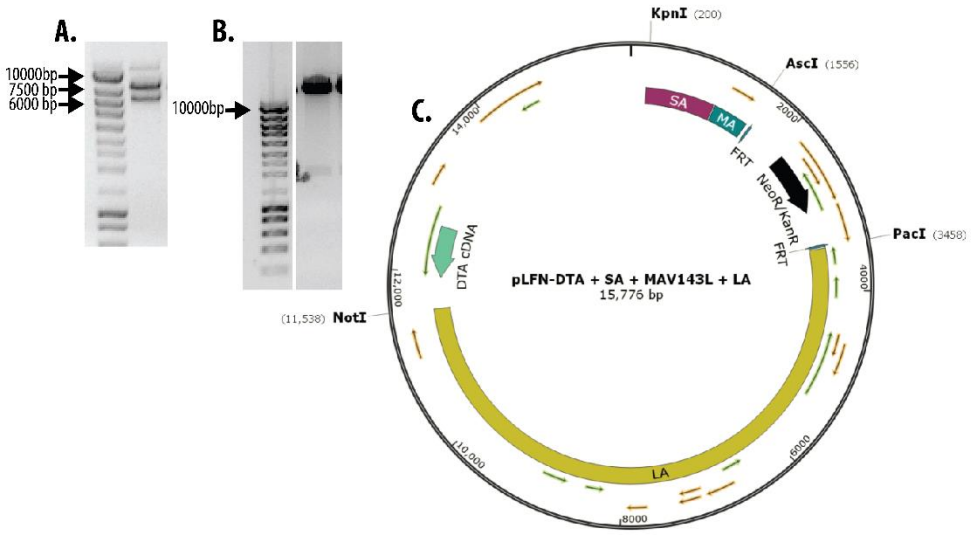


Figure 3.9. Cloning of the homology arms into the final vector. LA was cloned into the pLNF-DTA vector between the PacI and NotI restriction sites (**A**). SA and MA were then cloned simultaneously into the pLFN-DTA already containing the LA (**B**). The final construct (**C**) was electroporated into the ES cells.

3.5.2. Embryonic Stem Cells and Mouse Embryonic Fibroblasts cell culture

As mentioned before, targeted modifications can be introduced into ES cells by homologous recombination of a targeting vector. After the targeting vector was designed, it was incorporated into ES cells through electroporation, and rounds of selection were performed in order to pick ES cell colonies where homologous recombination took place, and thus suitable for injection into a blastocyst.

For this work, we used the R1 line of ES cells, a well-established line with good rates of germline transmission, derived from the crossing of 129/Sv and 129/SvJ substrains. These cells were kindly provided by Andras Nagy, Mount Sinai Hospital, Toronto.

One critical aspect of this strategy is that ES cells had to be maintained in an undifferentiated status throughout the whole process. In the case of the R1 ES cells this is accomplished by plating these cells on top of a mouse embryonic fibroblast (MEF) cell feeder-layer, which provides the ES cells with differentiation inhibitory signals, and by adding the leukemia inhibitory factor (LIF) to the culture medium.

3.5.2.1. Mouse embryo fibroblasts (MEFs)

As mentioned before, the pLFN-DTA contains a Neomycin resistance cassette that allows the selection of ES cells that incorporated the plasmid. Thus, the MEF cells were derived from a transgenic mouse line expressing the Neomycin resistance cassette allowing them to be cultured in the same selection medium as the ES cells containing the targeting vector. EmbryoMAX primary mouse embryo fibroblasts, Neo resistant, FVB strain were purchased from Millipore.

3.5.2.1.1. Expansion, harvesting and cryopreservation of MEF cells

MEF cells were plated in 0.1% gelatin-coated 100mm Petri dishes, in MEF medium: DMEM (high glucose, high bicarbonate, without pyruvate, with L-glutamine, Invitrogen), 1x penicillin/gentamicin (1000x stock – 0.59% penicillin and 8% gentamicin, Sigma) and 10% FBS (FBS cell screened, heat inactivated, Thermo Scientific Hyclone). When confluent, cells were trypsinized with 0.25% trypsin/EDTA (Gibco) and dissociated by pipetting up and down with glass pipettes. Cells were spinned for 3 min at 600 × *g* and the pellet was resuspended in MEF medium. One third of the cell suspension was plated in 150 cm^2 cell culture flask (Corning) and incubated at 37ºC, 5%CO_2. Medium was changed every two days until confluency (Figure 3.10).

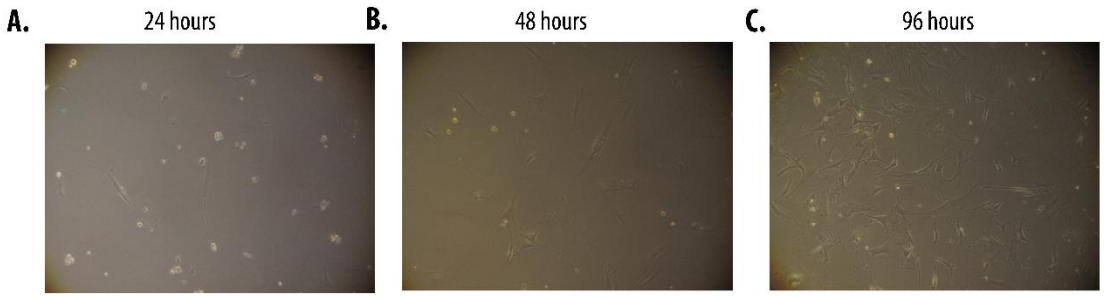


Figure 3.10. MEF cells culture at different time points after plating. MEF cells at 24 (**A**), 48 (**B**) or 96 (**C**) hours after plating.

Prior to the addition of ES cells to the MEF feeder layer, MEF cells must be mitotically inactivated. In order to do so, we treated P4 MEF cells with 10µg/ml of Mitomycin C (Sigma) for 3 hours. The cells were washed 3 times, dissociated with trypsin/EDTA and cryopreserved with 10% DMSO in liquid nitrogen.

3.5.2.2. Embryonic stem cells (ES cells)

3.5.2.2.1. ES cells plating, passaging and electroporation

At least 48h prior to ES cells plating, MEF cells were plated in a 0.1% gelatin coated 100 mm dish. After being rapidly thawed at 37ºC, cells were centrifuged for 3 min at 600 × *g* and cultured in ES cells medium: DMEM (high glucose, high bicarbonate, no pyruvate, with L-glutamine, Invitrogen), 20% FBS (FBS cell screened, heat inactivated, Thermo Scientific Hyclone), 1x penicillin/gentamicin (1000x stock – 0.59% penicillin and 8% gentamicin, Sigma), 1:10000 dilution of LIF (10^7 units/ml, Chemicon) and1xβ-mercaptoethanol (from the Embryomax 100x stock for ES Cells, Millipore). The medium was changed daily until cells reached about 70% confluency. At this stage cells were trypsinized and either cryopreserved as described previously for MEF cells, or electroporated with the targeting vector.

3.5.2.2.2. ES cells electroporation

The targeting vector was amplified in non-recombinogenic bacteria and purified by the endotoxin free Nucleobond Xtra Midi Plus kit (Macherey-Nagel). About 80µg of the purified vector was linearized with the NotI enzyme and purified with the NucleoSpin Gel and PCR Clean-up (Macherey-Nagel). 60-70% confluent ES cells were incubated with 0.25% trypsin/EDTA at 37ºC, 5% CO_2 for 5 min, after which ES medium was added to stop the action of trypsin. Cells were completely dissociated by pipetting up and down with glass pipettes and centrifuged at 600 × *g* for 3 min. After resuspension in DPBS (without calcium and magnesium, Gibco), cells were diluted 5 times in trypan blue (Invitrogen) and counted. For each electroporation, 20 x10^6 cells were centrifuged at 600 × *g* for 3 min and resuspended in DPBS with 25 µg of the linearized targeting vector DNA in a total volume of 800 µl. The suspension was added to a 4mm cuvette (Bio-Rad) and incubated for 5 min at RT. Cells were electroporated with a 225 V, 500µF pulse and after 10 min on ice, they were transferred to 10 ml of medium. ES cells were then plated on 5-6 dishes containing a feeder layer of MEF cells in ES medium. After 24 h the medium was changed by ES medium + 100 mg/ml G418/Geneticin (Sigma) in order to select the cells that incorporated the vector. The medium was changed by ES medium with 50 mg/ml G418/Geneticin (Selection ES medium), 24 hours later.

3.5.2.2.3. Picking colonies

Nearly 48 h before colony picking, MEF cells were plated on 0.1% gelatin-coated 24-well plates and 1 h before the picking, the medium was changed to Selection ES medium. A plate containing ES cell colonies was placed in a clean microscope stage, and isolated and undifferentiated colonies (Figure

3.11) were picked with a 20 µl pipette set for 8 µl. Each colony was added to a well of a 96-well plate containing 100 µl of trypsin/EDTA and pipetted up and down a few times to break the colony. After 10 min at RT, the colonies were transferred to a 24-well plate (containing MEF cells in Selection ES medium). The medium was replaced 48 h after picking and daily after then until the harvesting.

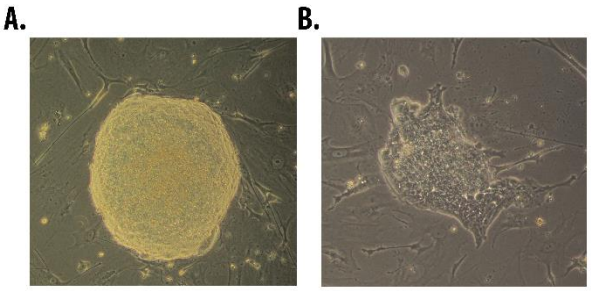


Figure 3.11. ES cells colonies. Only undifferentiated colonies were picked to be screened. Undifferentiated colonies presented well defined margins and rounded shapes (**A**) whereas differentiated colonies were diffuse and often lacked distinguishable margins (**B**).

3.5.2.2.4. Harvesting and Cryopreservation

Wells containing 20 to 100 medium to large colonies were selected to be harvested. The medium was replaced by 350 µl 0.25%/EDTA trypsin and incubated at 37 ºC for 5 min. After 5 min, 400 µl of warm FBS was added to each well and pipetted up and down to dissociate the cells. 400 µl of cell suspension were immediately frozen at -80ºC with 10% DMSO and the remaining was centrifuged at maximum speed for 2 min. The pellets were stored at -20ºC until used for PCR screening.

3.5.2.2.5. ES cells screening by PCR

ES cells colony pellets, previously stored at -20 ºC, were resuspended in 50 µl of digestion buffer (50 mM Tris pH 8.0, 1 mM $CaCl_2$, 1% Tween 20 in H_2O) supplemented with 2µg/ul proteinase K (GRISP). The cells were digested for 3h at 55ºC and then immersed in boiling water for 10 min to denature proteinase K. After cooled to room temperature, 2 µl of the digested cell suspension were used for PCR reaction. Reaction primers were designed in order to amplify a fragment if homologous insertion occurred. The forward primer hybridized a region outside the vector and the reverse primer hybridized a region flanking the Neo cassette which is not present in the mouse genome.

3.5.2.2.6. Expansion of positive colonies

Tubes containing the positive colonies were quickly thawed in a 37 ºC water bath and the content was transferred to a falcon tube containing Selection ES medium. The tubes were centrifuged for 3

min at 600x*g* and the pelleted cells were resuspended in 1ml Selection ES medium and then plated in dishes containing the MEF feeder layer. The medium was changed daily until the colonies were clearly visible. Cells were then trypsinized and half of the suspension was plated in a dish containing MEF cells. The other half of the suspension was freezed in 10% DMSO and regular ES medium. When the colonies became dense, they were trypsinized again and 1/5 of the content was plated in a non-coated 10 cm dish for genomic DNA extraction, whereas the remaining volume was frozen in 5 vials, with 10% DMSO, in regular ES medium.

3.5.2.2.7. Blastocyst microinjection

This part of the strategy was performed at vivarium in the Champalimaud Foundation for the Unknown. Shortly, positive ES cells were plated in dishes containing MEF cells in ES cell medium and allowed to grow until large colonies were apparent. The cells were then trypsinized as described before, centrifuged for 3 min at 600 x *g* and resuspended in 2 ml of injection buffer (8.3 g/L of DMEM without phenol red and sodium bicarbonate, Sigma D5030, 4.5 g/L D-glucose, 25 mM HEPES and 584 mg/L L-glutamine). Visually identified ES cells were microinjected into the fluid-filled blastocoele cavity of 3.5-day-old embryos at the blastocyst stage, obtained from a C57BL/6 mouse. The injected embryos were then implanted in the uterus of pseudopregnant females.

3.5.2.3. Chimera mating and backcrossings

The targeted R1 ES cells were from the 129 genetic background, which presents agouti coat color. The blastocysts where the ES cells were injected were obtained from the C57BL/6 mouse strain which presents black coat color. Thus, the resulting offspring included chimeric animals presenting black and agouti coat (Figure 3.12). Chimeric males were mated with C57BL/6 females and the presence of agouti coat in the F1 generation confirmed germline transmission. Tail DNA from the brown pups was used for PCR screening of the germline transmission of the targeted gene. Heterozygous animals were backcrossed to the C57BL/6 strain for 4 generations to decrease the contribution of genetic background to the observed phenotypes. Heterozygous animals were then intercrossed to generate homozygous and WT littermates.

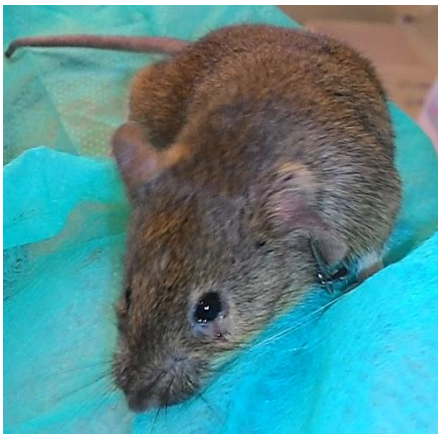


Figure 3.12. Male chimera. Chimeras presented a mixed color coat due to the mixed background of C57BL/6 and 129 strains

3.5.2.4. Genotyping

Animals were genotyped by standard PCR with a reverse primer for the end of the MA and a forward primer for the boundary between the SA and MA that differs in the WT and in the KI form due to the insertion of a random sequence in the initial segment of the MA of the latter (Table 3.1). The homologous insertion of the engineered sequence was also confirmed by the amplification of a ~1.5 kb fragment by standard PCR using a primer against a region flanking, but not included in the SA, and a primer against a sequence flanking the Neo cassette that is not present in the mouse genome (Figure 3.1 A,B).

3.5.2.5. Neo cassette deletion

The targeting vector included a Neo cassette containing the Neo resistance gene and its promoter flanked by two FRTR sites to allow the selection of ES cells that incorporated the plasmid and were therefore resistant to geneticin/G148. In order to prevent protein expression issues, the Neo cassette was eliminated by crossing the *knock-in* mice with Rosa FLP transgenic mice which express the FLP recombinase, an enzyme that recognizes the two FRTR2 sites and excises the sequence flanked by them. Confirmation of the presence of the FLP gene and of deletion of the Neo cassette was obtained by standard PCR (Primers shown in Table 3.1). In animals in which the Neo cassette was deleted, the fragment amplified by PCR was substantially smaller since it did not include the sequence between the two FRTR2 sites.

3.5.3. Behavioral characterization of stargazin V143L *knock-in* mice

In order to study how the mutation in stargazin could interfere with its function and consequently be contributing to disease development, we characterized selected behavioral features of these mice. Considering brain regions that are affected in intellectual disability and/or SCZ and also the expression patterns of stargazin in the brain, we performed a set of behavioral tests that are dependent on those regions. The behavior analysis was divided in three main categories: locomotor activity, anxiety-like tests and cognitive performance tests.

3.5.3.1. Locomotor activity tests

Stargazin is highly expressed in the cerebellum, a brain region that is extremely important for balance and coordination. In fact, *stargazer* mice, lacking stargazin expression, present ataxic gait. To determine whether the ID-associated stargazin mutation, V143L, affected locomotor activity and motor function/learning, Stg^{ID} *knock-in* mice were tested in the rotarod apparatus and in the open field arena.

3.5.3.1.1. Rotarod

Motor coordination and learning were evaluated with a rotarod apparatus (Med Associates) that accelerates from 4 to 40 rpm in 5 minutes. Each animal was tested for three trials in two different days. Latency to fall (in seconds) was determined for each trial and averaged for each day. The improvement in performance in the second day was considered motor learning.

3.5.3.1.2. Open-field

Spontaneous locomotor activity was assessed in a 40x40 cm open-field arena subjected to 100 lux of illumination. The total distance traveled (m), speed, latency to enter the center and the time spent in the center were automatically determined Ethovision (Noldus, Netherlands). Activity was quantified over a period of 60 minutes.

3.5.3.2. Anxiety-like behavior tests

3.5.3.2.1. Elevated plus maze

The elevated plus maze consists of a cross-shaped platform, containing four arms, that is elevated from the ground; two of the arms are enclosed by walls whereas the other two are open and thus subjected to bright illumination (100 lux). The animals initiated the test in the center of the maze and their preference for the open or closed arms, as well as the latency to enter each of them, was evaluated for 10 minutes.

3.5.3.3. Cognitive behavior test

Stargazin plays an important role in synaptic plasticity and it is highly expressed in regions associated to memory and learning. Furthermore, the *knock-in* mice generated in this work carry a mutation that is associated with intellectual disability. Therefore, one of the main goals of this work was to assess the effect of this mutation in cognition and further study the importance of stargazin for these processes. To do so, we evaluated the performance of these mice in the T-maze, in a spontaneous alternation task correlated to spatial working memory.

3.5.3.3.1. T-maze

The T-maze is a T-shaped apparatus used for several types of cognition tests. One of the most common tests performed in this maze is the spontaneous alternation task. It is based on the observation that, when placed in the base of the arm (base of the T), the animal rapidly chooses one arm, left or right. If subjected to a second round, most of the animals tend to choose the previously unexplored arm in order to look for food or shelter. This is called spontaneous alternation. On the other hand, animals displaying spatial working memory deficits fail to alternate in a second round due to the inability to recall which arm was visited in the first place.

Thus, in this test, the animals were allowed to choose an arm, where they were restricted for 30 seconds. After additional 30 seconds, the animals were re-placed in the base of the T, and repeated the task. If two different arms were visited, spontaneous alternation was considered; on the contrary, if the same arm was visited twice, no alternation occurred. After five trials, the percentage of alternation was compared with a percentage of 50%, *i. e.*, to randomness.

3.5.3.4. Social behavior test

3.5.3.4.1. Nesting behavior

To assess the nesting behavior, the animals were transferred to individual cages about one hour before the dark phase. A cotton disk was added to the cage and in the next morning the nests built with the provided material were evaluated from 1 to 5 using the following rating scale: 1 – Untouched nest; 2 – Nesting partially torn (50-90% intact); 3 – Less than half of the material remain intact, shredded material dispersed in the cage (50-90% shredded); 4 – Flat nest with walls (for less than 50% of its circumference), more than 90% of the material is torn and the material is gathered in one place; 5 – More than 90% of the material is shredded, nest is a crater with high walls (for more than 50% of its circumference) (Deacon, 2006). The evaluation of the nests was performed by seven observers.

Table 3.1 Primers

Use	Section	Sequence
SA amplification primer (FW)	3.5.1.1.	5′GGTACCGCCTCCGTTGACTGGCATTC3′
SA amplification primer (RV)	3.5.1.1.	5′GGCGCGCCGGGCCCCCCTTAAAGACTCTCCATGGCC3′
MA amplification primer (FW)	3.5.1.1.	5′GGTACCGGGCCCGGTGCAATACACGCCCTCTCCTTACACCGCTAAACC3′
MA amplification primer (RV)	3.5.1.1.	5′GGCGCGCCCCCTTTCCTTAGAGACAGGAGG3′
Mutagenesis primers for the V143L mutation in the MA (FW)	3.5.1.1.	5′GCTGGCATCTTCTTCCTGTCTGCAGGTAAGGC3′
Mutagenesis primers for the V143L mutation in the MA (RV)	3.5.1.1.	5′CCTGCCTTACCTGCAGACAGGAAGAAGATGCC3′
pNAPP amplification primer with LA-homologous overhangs (FW)	3.5.1.2.1.	5′GCGGCCGCGCCACTTGGCCCTGCCTACTGCTGGCAGTCAC AGATGCAAATCCAGTTCCCTAAGCGGCCGCCACCGCG3′
pNAPP amplification primer with LA-homologous overhangs (RV)	3.5.1.2.1.	5′TTAATTAAGAGGGCAGAGGGCAAGTGCTGTGGGTG GGCCTGGTGTCCTGAAAATTACAGCCCTTAATTAACCGGTCG3′
Genotyping primer (Common SA sequence) (FW)	3.5.2.4	5′GCTACGGTCAGGTTCCAGCC3′
Genotyping primer specific for WT form of *CACNG2* (RV)	3.5.2.4	5′GAGAGGACGGAGGGTCCCTT3′
Genotyping primer specific for KI form of *CACNG2* (RV)	3.5.2.4	5′GCGTGTATTGCACCGGGCC3′
Homologous insertion confirmation primer (outside the targeted sequence) (FW)	3.5.2.4	5′GGCAGCTGGGTCATGACGCC3′
Homologous insertion confirmation primer (LRFN0 – flanking Neo cassette) (RV)	3.5.2.4	5′AGG AAC TTC GCG CTA TAA CTT CG3′
Genotyping primer for FLP (FW)	3.5.2.5.	5′CACTGATATTGTAAGTAGTTTGC3′
Genotyping primer for FLP (RV)	3.5.2.5	5′CTAGTG CGAAGTAGTGATCAGG3′
Neo cassette deletion confirmation primer (FW)	3.5.2.5	5′GAGTGCTGGCATCTTCTTCCTG3′
Neo cassette deletion confirmation primer (RV)	3.5.2.5	5′GAGGGCAGAGGGCAAGTGCTGT3′

CHAPTER 4

Closing remarks and Future Perspective

Psychiatric disorders present a significant genetic overlap as many common circuits and gene alterations have been identified in different disorders. Recently, glutamatergic synapses have been on the spotlight in the context of these disorders due to the identification of a substantial number of mutations in genes encoding synaptic networks components (Fromer *et al.*, 2014; Hamdan *et al.*, 2011; Kirov *et al.*, 2012; Krumm *et al.*, 2014; Network and Pathway Analysis Subgroup of Psychiatric Genomics, 2015; Purcell *et al.*, 2014; Schizophrenia Working Group of the Psychiatric Genomics, 2014). The association of SNPs and CNVs in the *CACNG2* gene with psychiatric disorders has suggested a role for stargazin in these disorders (Ament et al., 2015; Drummond et al., 2013; Liu et al., 2008; Nissen et al., 2012; Wilson et al., 2006; Yang et al., 2013). Interestingly, a recent study reported four ID cases caused by microdeletions in chromosome 17q24.2, where the genes *CACNG1, 4* and *5*, which code for other TARPs, are located. Furthermore, all of these patients presented seizures and half of those experienced hallucinations and mood swings (Vergult et al., 2012). Recently, a missense mutation was identified in an 8 year-old non-syndromic intellectual disability patient, V143L, and was shown to affect the interaction with AMPAR and to reduce both total and surface levels of GluA1(Hamdan et al., 2011).

In order to study the role of stargazin in psychiatric disorders we searched for mutations in the exons of the *CACNG2* gene from psychiatric disorder patients from the Portuguese Island Collection. We identified a missense mutation in the third exon of the *CACNG2* gene in a SCZ patient. Analysis of DNA sequences from other family members showed this mutation was inherited from his mother who suffered from major depression. The mutation was not found in any of the other, non-affected, family members. Herein we evaluated the effect of these mutations on stargazin function, by expressing the disease-associated stargazin variants in dissociated cortical neurons where endogenous stargazin was depleted by interference RNA. As predicted by several computational tools, both the mutations affect stargazin function. However, expression of disease-associated stargazin variants results in shared and distinct cellular phenotypes

Disease-associated mutations produce shared and distinct effects on stargazin function

Stargazin-AMPAR interaction was shown to be important not only for the traffic of AMPAR to the surface (Chen et al., 2000; Vandenberghe et al., 2005a) but also for their stabilization at the synapse (Chen et al., 2000; Opazo et al., 2010) and for synaptic plasticity (Constals et al., 2015; Louros et al., 2014; Tomita et al., 2005b). In fact, LTP requires the activation of CaMKII which phosphorylates stargazin, enhancing its binding to PSD95 and consequently the synaptic stabilization of AMPAR.

On the other hand, once bound to glutamate, AMPAR become desensitized and more mobile due to the dissociation from stargazin, allowing the exchange of desensitized receptors by naïve ones,

and rapid recovery from short-term synaptic depression (Constals et al., 2015). These observations highlight the central role of stargazin in excitatory transmission and synaptic plasticity, and hint at the importance of fine-tuned expression, function and interactions of stargazin with other synaptic components for the maintenance of plasticity mechanisms.

Disease-associated variants of stargazin present altered surface diffusion properties, displaying a more mobile behavior than the WT form of stargazin. Providing the importance of stargazin for the stabilization of AMPAR at the synapses, changes in stargazin mobility likely interfere with synaptic AMPAR function and synaptic plasticity. Indeed, besides impacting the stargazin-mediated trafficking of AMPAR the disease-associated mutations affect the stabilization of AMPAR at synapses. Given that no significant changes in the interaction between stargazin variants and AMPAR were found, we hypothesize that these changes in the mobility of stargazin and in the stabilization of AMPAR are most likely due to changes in the interaction with other synaptic proteins. Stargazin interacts with PSD95 in a phosphorylation-dependent manner, and this interaction was shown to be essential for the synaptic immobilization of stargazin and, consequently, for the clustering of AMPAR (Chen et al., 2000). Hereupon, the evaluation of the interaction of these variants with PSD95 and other scaffold proteins would be important to further understand how stargazin variants impact AMPAR synaptic expression.

The chronic blockade of neuronal activity results in a stargazin-dependent homeostatic increase of GluA1-containing AMPAR at the cell surface (Louros et al., 2014); however, the ID-associated stargazin mutation prevents synaptic scaling of AMPAR. Oppositely, although the SCZ-associated mutation affects the basal traffic of AMPAR it does not prevent synaptic scaling. Interestingly, contrarily to what was observed in neurons expressing the SCZ-associated mutation, the effects mediated by the ID-associated mutation in the trafficking and stabilization of AMPAR are not reverted by tianeptine, the anti-depressant drug that increases stargazin phosphorylation and consequently its interaction with PSD95. These observations suggest that the mechanisms underlying the effects of the two stargazin mutations in AMPAR function may differ, and that the SCZ-associated variant is more responsive to stimuli that enhance stargazin function.

Additionally, the SCZ-linked mutation (but not the ID-associated one) affects both dendritic arborization and the number of inhibitory synapses onto neurons expressing the Stg^{SCZ} mutation. These alterations potentially contribute to changes in the neuronal circuitry and to unbalanced excitation/inhibition ratio, respectively. In agreement with these observations are the reports of a decrease in the volume of cortical gray matter of SCZ patients, attributed mainly to the regression of synapses and changes in the dendritic arborization (Bennett, 2011b).

Also, an increase in the ratio of excitation/inhibition in the mouse prefrontal cortex was described to induce an impairment in social function and cognition without motor abnormalities or increased anxiety (Yizhar et al., 2011).

The observation of distinct effects of these mutations, both located in the same transmembrane domain, on the function of stargazin is noteworthy. These observations corroborate the reports on the overlap of susceptibility genes and symptoms among different disorders (Cross-Disorder Group of the Psychiatric Genomics, 2013; Fromer et al., 2014; Network and Pathway Analysis Subgroup of Psychiatric Genomics, 2015) and highlight the importance of identifying disease-associated variants and establishing genotype/phenotype correlations. The identification of novel stargazin interactors that are associated to psychiatric disorders creates new opportunities to further explore these effects of stargazin mutations on stargazin function.

The Stg^{ID}*knock-in* mice present behavior phenotypes associated to psychiatric disorders symptomatology

The generation of the *knock-in* mouse for the ID-associated mutation aimed at further studying the effect of this mutation on stargazin function and at correlating these effects with behavioral phenotypes that are relevant for psychiatric disorders. Stg^{ID} *knock-in* mice were generated by gene targeting, a technique that allowed the directed targeting of a single nucleotide in the third exon of the *CACNG2* gene. In cultured cortical neurons the surface expression of both variants was decreased, which is in agreement with the observed decrease of stargazin levels in the post-synaptic densities of StgV143L$^{+/-}$ and StgV143L$^{+/+}$mice (personal communication, Tiago Rondão).

Considering the expression pattern of stargazin and the behavioral features more often associated to psychiatric disorder models, we evaluated the behavior of the Stg^{ID}*knock-in* mice in tasks that assess locomotor activity, anxiety-like behaviors, motor balance, nesting behavior and cognition.

StgV143L$^{+/+}$ female mice presented increased locomotor activity, characterized by increased speed and total distance traveled in an open field arena. Hyperactivity is a common feature in genetic mouse models harboring mutations or lacking the expression of ID- and SCZ-associated proteins, including *knock-out* mice for GluA1 (Wiedholz et al., 2008) and *knock-out* mice for the dopamine transporter (DAT) which reuptakes dopamine into presynaptic terminals. The hyperactivity in DAT *knock-out* model was further increased by blockade of NMDAR with MK801, corroborating a functional interaction between dopaminergic and glutamatergic systems in the regulation of this phenotype (Gainetdinov et al., 2001). Accordingly, another study showed that the increase in locomotor activity mediated by PCP and amphetamine could be reversed by antipsychotic treatment (Freed et al., 1984; Powell and Miyakawa, 2006)

Interestingly, a subset of schizophrenic patients have been described to display psychomotor agitation, which includes stereotypic movements and hyperactivity (Powell and Miyakawa, 2006) and several reports indicate a higher incidence of attention deficit and hyperactivity disorder (ADHD) in intellectual disability patients than in the general population [reviewed in (Seager and O'Brien, 2003)], and syndromes in which intellectual disability is a common feature, such as tuberous sclerosis and fragile X, also include attention deficit hyperactivity disorder (ADHD) as a major symptom [reviewed in (de Vries *et al.*, 2005; McLennan *et al.*, 2011)].

Stargazin is highly expressed in the cerebellum, a brain region implicated in motor function and learning. Thus, we evaluated the performance of Stg^{ID} *knock-in* models in the rotarod. Although no differences were observed in the motor balance between genotypes, both $StgV143L^{+/-}$ and $StgV143L^{+/+}$ present motor learning deficits, since contrarily to WT animals they do not significantly improve their performance in the second day. Other cerebellum-dependent tasks could be performed to study the effect of this mutation in cerebellum functioning, including the balance beam, footprint pattern or the acquisition of eyeblink conditioning, a task that is impaired in the *stargazer* mice, which lack surface expression of AMPAR in cerebellar granule cells (Hashimoto et al., 1999). The striking effect of the ID-associated mutation in the trafficking of AMPAR in cultured cortical neurons prompts the evaluation of the AMPAR expression in the post-synaptic densities of different brain regions of the Stg^{ID} *knock-in* mice. Analogously to what was observed in the *stargazer* mice, a decrease in the synaptic levels of AMPAR in cerebellar neurons is a plausible explanation for the motor learning deficits presented by the Stg^{ID} mice.

The role of stargazin in several forms of plasticity anticipates its importance in memory and learning processes. Working spatial memory was evaluated using the spontaneous alternation task in the T-maze. In this test, whereas WT animals alternate significantly more than 50%, $StgV143^{+/-}$ and $StgV143L^{+/+}$ mice alternate randomly, reflecting the inability to recall which arm was previously visited. Electrophysiological analysis of LTP and LTD induction in cortical and/or hippocampal slices from these mice would be important to elucidate functional changes at the basis of cognitive defects. Interestingly, as described in the Chapter 2, stargazin interacts with several proteins implicated in cognitive disorders, including ARC, IQSEC2 and mGluR5, with a role in synaptic plasticity. Analysis of the interactome of Stg^{ID} using brain samples from these mice or heterologous systems (to differentiate between direct and indirect interactions) would contribute to unravel the mechanisms and/or brain regions underlying this phenotype.

Several other behavior tests have been developed to evaluate cognitive function, namely the novel object recognition, the Barnes maze and the Morris water maze. A further behavior characterization is necessary to evaluate which domains of memory and learning are affected by the StgV143L mutation.

Providing that many of the mouse models for psychiatric disorders present social deficits, we evaluated social behavior of the Stg^{ID} knock-in mice. Besides the nesting behavior, that is impaired in $StgV143L^{+/-}$ and $StgV143L^{+/+,}$ other tests that evaluate social behavior are currently being performed at our laboratory, namely the three-chamber social test and the dyadic social interaction test.

Social interaction and social discrimination are important features to be evaluated in any model of psychiatric disorders since social dysfunction is a hallmark for most of them.

Interestingly, in the case of SCZ, social dysfunction is often noticeable years before the diagnosis and is considered a robust predictor of the development of SCZ. A correlation between social function and the frontal cortex has been made early in the history of SCZ, when Kraepelin recognized that patients with frontal lesions showed emotional flattening and social withdrawal similarly to SCZ patients (Chemerinski et al., 2002). Accordingly, imaging studies showed that the morphology of the ventral frontal cortex has a significant association to social dysfunction in a group of SCZ patients (Chemerinski et al., 2002).

Future perspectives

Taking into consideration the pattern of effects elicited by the disease-associated variants on stargazin function and providing that StgID *knock-in* mice display behavior phenotypes correlated to symptoms that are transversal to ID and SCZ, we believe that the comparison of cellular and behavior phenotypes displayed by mice expressing the distinct variants would largely contribute to the elucidation of the mechanisms implicated in both disorders. Furthermore, it would allow the correspondence between behavior phenotypes and the cellular changes that are specific for one variant versus the other. Thus, the generation of the Stg^{SCZ} *knock-in* mice would constitute a valuable tool for further studying the role of stargazin in the pathogenesis of psychiatric disorders.

Besides the tests used to evaluate behavior of the Stg^{ID} *knock-in* mice in this brief characterization, many others should be considered for the clear and robust characterization of both mouse lines, namely tests focusing on the cognitive function and on sensorimotor gating. Furthermore, given the important role of stargazin on the experience-dependent plasticity of the retinogeniculate synapse, it would be particularly interesting to test the visual perception on these animals.

The numerous reports on changes in the morphology of several brain regions in SCZ (Bennett, 2011b), the described effect of stargazin in activity-dependent dendritogenesis of immature neocortical pyramidal neurons (Hamad et al., 2014), and the observation of impaired dendritic arborization in cortical neurons where stargazin has been depleted or expressing the SCZ-linked

stargazin variant, highlight the need for evaluating dendritic arborization and spine morphology in mice harboring disease-associated stargazin variants.

The effect of the disease-associated variants on AMPAR trafficking and homeostatic plasticity likely result on functional alterations in synaptic transmission *in vivo*. These observations instigate the electrophysiological analysis of functional alterations in circuits in brain regions where stargazin is expressed, namely hippocampus, cortex and thalamus. The recording of frequency, amplitudes and kinetics of mEPSCs and mIPSCs and the AMPAR/NMDAR ratio in cortex and hippocampus would help unveiling defects on basal synapse function and allow determining whether the excitation/inhibition balance is compromised in these mice.

Considering the role of stargazin for the experience-dependent development of the retinogeniculate synapse (Louros et al., 2014), analyzing activity-dependent remodeling of this synapse in Stg^{ID} *knock-in* mice would be relevant.

Despite the shared effect of stargazin mutations on AMPAR trafficking and synapse stabilization, only the SCZ-linked mutation effects are reverted by tianeptine. This antidepressant drug, which increases stargazin phosphorylation and thus the levels of surface AMPAR, has also been proposed as a cognitive enhancer since it was shown to have a beneficial effect on learning and working memory (Jaffard et al., 1991). Furthermore, tianeptine also showed beneficial effects on stress-induced defects on working memory and on hippocampal neuronal remodeling. Interestingly, stargazin has been shown to play a role in stress coping. In fact, a study involving monkeys showed that learning to cope with stress induced by social isolation protects these animals from subsequent stress-induced deficits in emotionality tests. These observations were accompanied by an increase in the stargazin mRNA levels in the anterior cingulate cortex (Lee et al., 2016).

In collaboration with Dr. Célia Carvalhos's group (University of Azores), a psychological assessment of the susceptibility to positive, negative and neutral stressors (Carvalho et al., 2015) was performed for the SCZ patient where the missense mutation on the *CACNG2* gene was identified, and for his mother who suffers from major depression and also carries the mutation. Interestingly, although neither of the two individuals showed alterations in the response to positive or negative stressors, they presented an exacerbated vulnerability to neutral stressors in comparison with healthy individuals (Data not shown).

Hereupon, we suggest that the potential of tianeptine in the reversion of the defects associated to the disease-linked mutations should be addressed. To do so, we propose the evaluation of the effect of tianeptine on the biochemical, functional and behavioral features of the mice harboring the disease-associated mutations.

Taken together, our data provide evidence for a role of stargazin in cellular and behavioral phenotypes that correlate to psychiatric disorders, which prompts the research and development of therapies that target the pathways in which stargazin takes part.

The observation of common and distinct effects mediated by the two mutations in the *CACNG2* gene corroborates evidence suggesting genetic overlap between different disorders, and highlights that the correlation of risk gene variants to specific disease endophenotypes would stem the development of more personalized and efficient therapeutic strategies.

CHAPTER 5

References

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