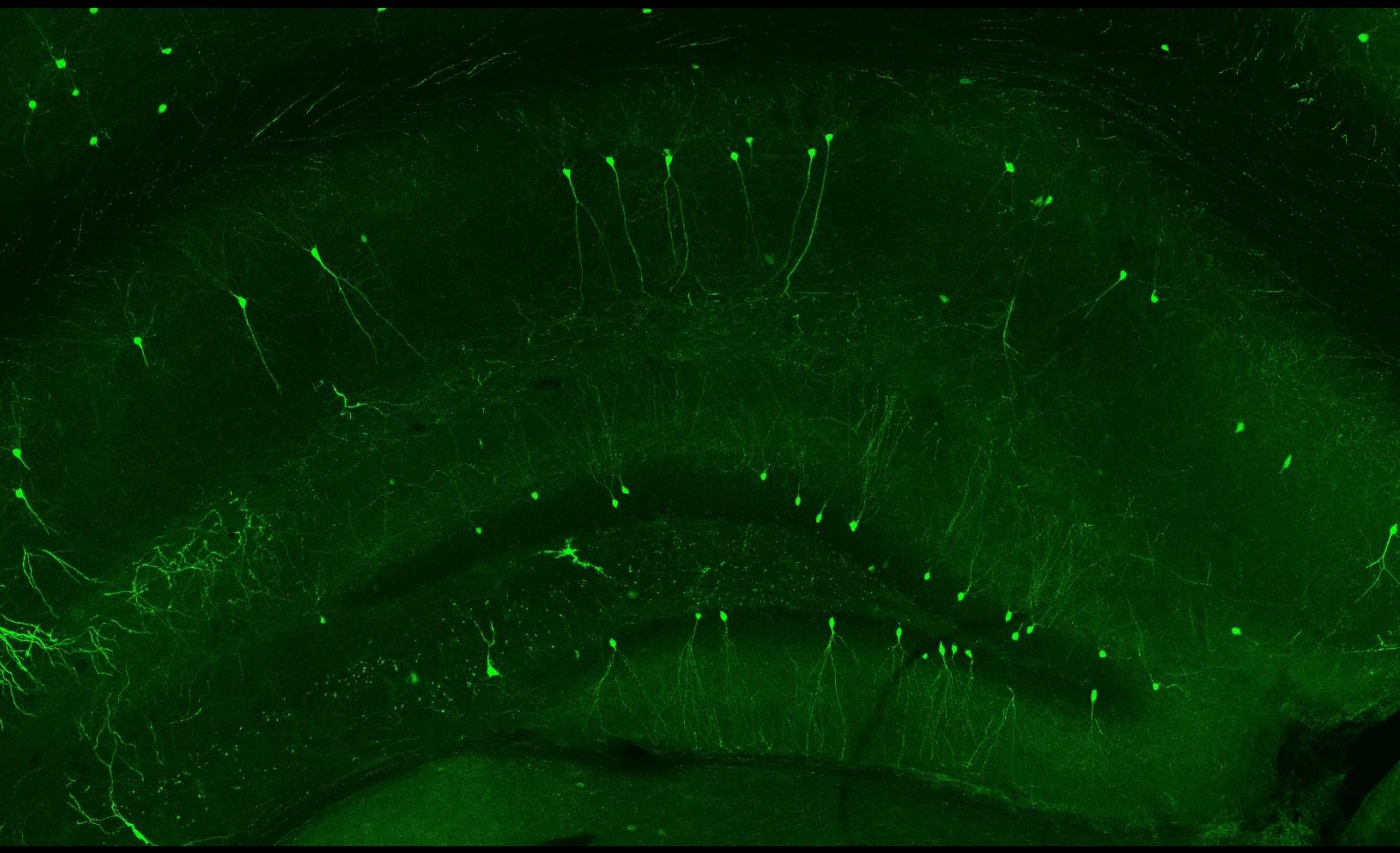

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Dissertação de mestrado em Biologia Celular e Molecular com especialização em Neurobiologia

Setembro/2017

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Universidade de Coimbra

**Cover image:** Hippocampal section of C57BL/6 mouse injected with AAV9 Syn-GFP
(Maximum Intensity Projection, Pln Apo 20x/0.8 DICII lens)

FCTUC FACULDADE DE CIÊNCIAS
E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

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# Characterization of *Gprasp2* knockout mice as a new model for autism spectrum disorders

Dissertação de Mestrado em Biologia Celular e Molecular, com especialização em Neurobiologia orientada pelo Doutor João Peça e coorientada pela Professora Ana Luísa Carvalho, apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Coimbra, Setembro 2017

UNIVERSIDADE DE COIMBRA

This work was conducted at the center for Neuroscience and Cell Biology (CNC) of University of Coimbra, under the scientific supervision of Doctor João Peça and Professor Ana Luisa Carvalho

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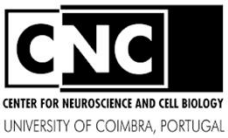











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# Table of Contents

# Abbreviation List

*5-HT7* - 5-hydroxytryptamine receptor 7
*Adcy5* - Adenylate Cyclase 5
*ADRB1* - Adrenoceptor beta 1
Agrp - Agouti-related protein
*Akt1* - AKT Serine/Threonine Kinase 1
AMPAR - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
*Ank3* - Ankyrin 3
ANOVA – Analysis of Variance
*ARC* - Activity Regulated Cytoskeleton Associated Protein
*ARMCX* - Armadillo Repeat Containing, X-Linked
AS – Asperger Syndrome
ASD – Autism Spectrum Disorders
*ATP6AP1* - ATPase H+ Transporting Accessory Protein 1
*B2M* – Beta-2-Microglobulin
*BARD1* - BRCA1 Associated RING Domain 1
*BHLHB9* – Basic Helix-Loop -Helix Family Member B9
BL – Brain Lysate
*BTRC* - Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase
CA1 - *Cornu Ammonis* 1
$Ca^{2+}$ - Calcium Cation
*CALCR* - Calcitonin Receptor
*CamkIIa* – Calcium/Calmodulin Dependent Protein Kinase II Alpha
*Cask* - Calcium/Calmodulin Dependent Protein Serine Protein Kinase
CDC – Center for Disease Control and Prevention
cDNA – Complementary DNA
*Cfl1* - Cofilin 1
*Chrm1* - Cholinergic Receptor Muscarinic 1
*Chrm2* - Cholinergic Receptor Muscarinic 2
CNS – Central Nervous System
*Cntnap2* - Contactin Associated Protein-Like 2
CNV – Copy Number Variations
*Creb1* – Cyclic adenosine monophosphate responsive element binding protein 1
CRH - Corticotropin-releasing hormone
*Crhr1* - Corticotropin Releasing Hormone Receptor 1
*Ctnnb1* - Catenin Beta 1
*Cttn* - Cortactin
*Cyfip* – Cytoplasmic FMR1 interacting protein 1
DAPI - 4',6-diamidino-2-phenylindole
DHPG - (*S*)-3,5-Dihydroxyphenylglycine
*DIMT1* - DIM1 Dimethyladenosine Transferase 1 Homolog
DISC1 - Disrupted in Schizophrenia 1
*Dlg1* - Discs Large MAGUK Scaffold Protein 1
Dlg2 - Discs Large MAGUK Scaffold Protein 2
*Dlgap2* - Discs Large Homolog Associated Protein 2
*Dlgap3* - Discs Large Homolog Associated Protein 2
*Dlgh4* - Discs Large Homolog 4
DNA - Deoxyribonucleic Acid
DOR - Delta opioid receptor

*Drd1/2* - Dopamine Receptor D1/2

DSM-V - Diagnostic and Statistical Manual of Mental Disorders, 5th Edition

E- Embryonic day

EIF4E - Eukaryotic Translation Initiation Factor 4E

EPSP - Excitatory postsynaptic potential

Foxg1 - Forkhead Box G1

*Frm1* - Fragile X Mental Retardation 1

FXS - Fragile X Syndrome

GABA - Gamma-AminoButyric Acid

*Gabbr* - Gamma-Aminobutyric Acid Type B Receptor Subunit

*Gabra* - Gamma-Aminobutyric Acid Type A Receptor Alpha Subunit

*Gabrb* - Gamma-Aminobutyric Acid Type A Receptor Beta Subunit

*Gabrg* - Gamma-Aminobutyric Acid Type A Receptor Gamma Subunit

*GABRQ* - Gamma-Aminobutyric Acid Type A Receptor Theta Subunit

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GASP - G protein-coupled associated sorting protein

*GDI1* - GDP Dissociation Inhibitor 1

GDP - Guanosine diphosphate

GEF - Guanine nucleotide exchange factor

GFP - Green fluorescent protein

*Ghrl* – Ghrelin

GPCR – G-protein coupled receptors

Gphn – Gephryn

*GPR50* - G Protein-Coupled Receptor 50

Gprasp – G-protein coupled receptor associated sorting protein

*Gria* - Glutamate Ionotropic Receptor AMPA Type Subunit 1

*Grin* - Glutamate Ionotropic Receptor NMDA Type Subunit

*GRIP1* - Glutamate Receptor Interacting Protein 1

Grm5 - Glutamate Metabotropic Receptor 5

Gsk3b - Glycogen Synthase Kinase 3 Beta

GTP - Guanosine diphosphate

Gα12/13 - G12/G13 alpha subunits

Gαi - $G_i$ alpha subunit

Gαq - Gq alpha subunit

Gαs - Gs alpha subunit

HAA – Hypothalamic Aggression Area

Hh - Hedgehog

Homer1 - Homer Scaffolding Protein 1

Homer3 - Homer Scaffolding Protein 3

*HRH2* – Histamine Receptor H2

Htt - Huntingtin

ID – Intellectual Disability

IL1RAPL1 - Interleukin 1 Receptor Accessory Protein Like 1

Ins1/2 – Insulin 1/2

IQ – Intelligence Quotient

KAR – Kainate Receptor

Kif3b - Kinesin Family Member 3B

KO - Knockout

Lep - Leptin

LH – Lateral Hypothalamus

LTD – Long-Term Depression

LTP – Long-Term Potentiation

Mapk1 - Mitogen-Activated Protein Kinase 1

MC4r - Melanocortin 4 Receptor

MeCP2 - Methyl-CpG Binding Protein 2

mEPSC – Miniature Excitatory postsynaptic potential
mGluR – Metabotropic Glutamate Receptors
mRNA – Messenger Ribonucleic acid
mTOR - Mechanistic target of rapamycin
Munc18 - Mammalian uncoordinated-18
NaCl – Sodium Chloride
NF1 - Neurofibromin 1
Nlgn – Neuroligin
NMDAR - *N*-methyl-D-aspartate receptor
NPY – Neuropeptide Y
NPYr – Neuropeptide Y receptors
*Nrxn1* – Neurexin 1
*Ophn1* - Oligophrenin 1
p53 - Tumor protein p53
PAS - Per-Arnt-Sim
patDp – Paternally inherited duplication
PCR - Polymerase Chain Reaction
PDD-NOS - Pervasive Developmental Disorder-Not Otherwise Specified
Period - Period Circadian Clock
*PI3kca* - Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
*PI3kcb* - Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Beta
*PI3kcd* - Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta
Pifo - Pitchfork
PIPS - Period1 interacting protein of the superchiasmatic nucleus
PKA - Protein Kinase A
PKC - Protein Kinase C
*Plcb1* - Phospholipase C Beta 1
*Plcb3* - Phospholipase C Beta 3
*Plcg1* - Phospholipase C Gamma 1
PLCβ - Phospholipase C Beta
PND – Post-natal Day
POMC - Proopiomelanocortin
PPP2R5E - Protein Phosphatase 2 Regulatory Subunit B'Epsilon
PSD – Post-Synaptic Density
*PTEN* - Phosphatase and Tensin Homolog
RNA - Ribonucleic acid
RT-qPCR - Reverse transcription polymerase chain reaction
SEM - Standard error of the mean
SHANK2 - SH3 And Multiple Ankyrin Repeat Domains 2
SHANK3 - SH3 And Multiple Ankyrin Repeat Domains 3
Shh – Sonic Hedgehog
shRNA - Short hairpin Ribonucleic acid
Smo - Smoothened
*SNX27* - Sorting nexin 27
*Sox10* - Sex Determining Region Y Box 10
SPM - Synaptosomal plasma membrane
*SynGAP* - Synaptic GTPase activating protein
*Syt17* - Synaptotagmin 17
*TCF25* - Transcription factor 25
TRH - Thyrotropin-releasing hormone
TSC – Tuberous Sclerosis
Tsc1/2 - Tuberous Sclerosis 1/2
Ube3A - Ubiquitin protein ligase E3A
WT – Wild-type

# Abstract

Autism Spectrum Disorders (ASD) and Intellectual Disability (ID) are neurodevelopmental disorders that share common aspects in genetic etiology and phenotypic presentation. Nevertheless, despite a significant number of studies the exact mechanisms triggering these neurodevelopmental disorders and the neuronal circuits disrupted are not completely understood. A recent hypothesis has implicated deficits in synaptic plasticity and spine structure on behavioral and cognitive alterations in patients.

G-protein coupled receptors (GPCRs) are known for their role in the regulation of synaptic signaling. Some of the members of this broad family, include the metabotropic glutamate receptor family (mGluR) which are known for their role in synaptic plasticity and in the mediation of long-term depression. Both these mechanisms have been shown to be deregulated in various neuropsychiatric and neurodevelopmental disorders. Additionally, the regulation and trafficking of mGluRs as well as their intracellular partners have been gaining increased attention. In line with this, the G-protein coupled receptor associated sorting protein (Gprasp) family is an interesting target since *Gprasp2* has been implicated in psychiatric disorders. *Gprasp2* is also a pleiotropic susceptibility candidate gene implicated in autism and is thought to be involved in the endocytic sorting of G-protein coupled receptors.

To understand the role of Gprasp2 *in vivo,* a *Gprasp2* KO model was recently developed. Here we explore the characterization of this animal model at a behavioral and neuronal morphology level. In our behavioral characterization, we found deficits in terms of memory and cognition in *Gprasp2* KO mice pointing towards a defect in terms of hippocampal function. Aligned with these results, decreased dendritic complexity and decreased number of mature spines was also found in the CA1 region of the hippocampus. Changes in mRNA levels were also found in the hippocampus that pointed towards a synaptic signaling deficiency. We also found an increase in body weight and changes in behaviors connected to social dominance and aggression in *Gprasp2* KO mice, suggesting a role for this gene in hypothalamic circuit function.

Our data suggest that *Gprasp2* mutations may contribute to the pathogenesis of neurodevelopmental disorders, affecting hippocampal dendritic and spine morphology

and hippocampal and hypothalamic-mediated behaviors. The *Gprasp2* KO mice display deficits consistent with ASD and ID-like behaviors.

**Keywords:** Autism Spectrum Disorders; Intellectual Disability; mGluR; Gprasp2; animal model

## Resumo

A perturbação do espectro do autismo (PEA) e o défice intelectual (DI) são doenças do desenvolvimento neurológico que partilham aspectos comuns no que toca à sua etiologia genética e apresentação fenotípica. No entanto, apesar de todos os estudos desenvolvidos no sentido de compreender estes distúrbios, os mecanismos exactos que os desencadeiam e os circuitos neuronais afectados ainda não foram precisamente identificados. Uma hipótese que tem vindo a crescer implica os défices encontrados a nível de plasticidade sináptica e a nível da estrutura sinática como causadores das alterações a nível comportamente e cognitivo observadas em pacientes que sofrem destes distúrbios.

Os receptores acoplados às proteínas G (GPCRs) são conhecidos pelo seu papel importante na regulação da sinalização sináptica. Um dos membros desta vasta família, um grupo, que inclui os receptores metabotrópicos de glutamato (mGluR), é conhecido pela sua função na plasticidade sináptica e na mediação da depressão sináptica a longo prazo. Alterações a nível destes mecanismos estão associados a várias doenças do foro neurológico. Com isto, mais atenção tem sido dada à regulação e tráfico dos mGluRs e dos seus parceiros intracelulares. Assim sendo, a família de proteínas Gprasp parece ser um alvo interessante tendo em conta que um dos seus membros, Gprasp2 já foi associado a distúrbios psiquiátricos. Gprasp2 é um forte candidato de susceptibilidade genética implicado em PEA cuja função parece estar relacionada com a regulação do tráfico de GPCRs.

De modo a entender o papel da Gprasp2 *in vivo*, um modelo de murganhos *knockout* (KO) para o gene *Gprasp2* foi desenvolvido. No trabalho aqui desenvolvido, é apresentada uma caracterização detalhada deste modelo animal a nível comportamental e da morfologia neuronal. A nível comportamental foram encontrados défices na cognitivos e memória nestes animais, apontando para um defeito relacionado com funções reguladas pelo hipocampo. De acordo com estes resultados, foi também encontrado na zona CA1 do hipocampo, uma redução da complexidade dendrítica e uma diminuição do número de espículas maduras. Alterações nos níves de mRNA sugestivas de alterações ao nível da sinalização sináptica foram também observadas em amostras de hipocampo. Aumentos no peso dos animais *Gprasp2* KO e alterações em comportamentos relativos a dominância social e agressão foram também encontrados neste modelo animal, sugerindo um papel desta proteína no circuito hipotalâmico.

Todos os dados recolhidos ao longo deste trabalho sugerem que mutações na proteína Gprasp2 poderão contribuir para a patogenecidade de doenças do foro neurológico, tendo um efeito na morfologia e densidade de espículas dos neurónios do hipocampo e afectando comportamentos mediados pelo hipocampo e hipótalamo. Os animais *Gprasp2* KO exibem assim défices consistentes com alterações encontradas em modelos de PEA e DI.

**Palavras-chave:** Perturbação do espectro do autism; Défice Intelectual; mGluR; Gprasp2; modelo animal

# CHAPTER I | INTRODUCTION

# Introduction

## 1. Autism

Autism is a neurodevelopmental disorder that belongs to a group of heterogeneous conditions termed Autism Spectrum Disorders (ASD). First diagnosed in the 1940s by Kanner and Asperger [1, 2], these disorders are diagnosed based on different criteria regarding key behavioral anomalies: deficits in language and communication, impaired or abnormal social interactions and restricted interests or repetitive behavior [3]. ASD also includes disorders such as Asperger's Syndrome (AS), Pervasive Developmental Disorder - Not Otherwise Specified (PDD-NOS) and Idiopathic Autism. AS was considered a mild form of autism, characterized by obsessive interests in patients showing normal to high intelligence [4]. PDD-NOS was diagnosed when an individual failed to meet specific criteria for autistic disorder but displayed difficulties in terms of social interactions and repetitive behavior [5]. Other disorders, known as syndromic ASD, include Rett Syndrome, Tuberous Sclerosis (TSC) and Fragile X Syndrome (FXS). These patients frequently display ASD phenotypes and the underlying genetic insult is known. For example, mutations in *MeCP2* (methyl CpG binding protein 2) are associated with Rett syndrome, *Tsc1/2* (tuberous sclerosis 1/2) with TSC and Frm1 (fragile X mental retardation 1) with FXS.

Some of the symptomatology attributed to ASD is described in the DSM-V [3] to include: responding inappropriately in conversations, misreading nonverbal interactions and having difficulty building friendships appropriate for their age. Individuals with ASD may also be overly dependent on routines, highly sensitive to changes in their environment or intensely focused on specific objects. Besides these core symptoms, patients also suffer from secondary symptoms such as self-injurious or self-mutilatory behaviors, hyperactivity, aggression and comorbidity with neuropsychiatric conditions such as seizures, major depression and anxiety [6]. In particular, intellectual disability (ID) is highly prevalent in ASD patients and is one of the most common cognitive deficits, affecting up to 69% of patients [7].

In terms of incidence, a study from the CDC's Autism and Developmental Disabilities Monitoring Network estimated the prevalence of children identified with ASD at about 1 in 68 [8]. This high prevalence comprehends all the disorders in the spectrum and reflects the variety of phenotypes and symptoms observed. All of these symptoms vary

in terms of severity between patients and are in some cases dependent on the age of the individual [9].

In most cases, the exact cause behind ASD cannot be determined but it is believed that the onset and development is influenced by both genetic and environmental factors [10]. In terms of environmental factors, it has been shown that the maternal lifestyle and diet (use of drugs of abuse, vitamin D, fatty acids) can alter brain development and have an effect in neurological processes such as cell differentiation, synaptogenesis and axon myelination, for instance [11]. Although the high number of studies establishing an association between environmental factors and ASD [11, 12], it is the combination of several factors and not the effect of a single one that might have the ability to significantly impact the predisposition for ASD [13]. Nevertheless, ASD is a highly heritable disease with reports of more than 80% concordance in monozygotic twins [14]. ASD shows an increased prevalence in males (a 4:1 male to female ratio) [15]. Additionally, there is also an increased prevalence of autism in individuals with sex chromosome aneuploidy [16], suggesting an influence of X-linked genetics factors in these disorders. In fact, X-linked genes have been involved in brain development, cognition and emotional regulation [16] and genes like *GPR50*, *ATP6AP1* and *GABRQ* have been strongly implicated in ASD [17]. This suggests a potentially important role for the X-chromosome in the development of this disorder. However, genomic analysis of autistic patients shows that autism is a complex and heterogeneous disorder with many of the genetic variables and a high degree of pleiotropy (one gene affects more than one phenotype, and can be related to different disorders) [18].

To date, there are up to 200 genes implicated in autism [18] associated with allelic variants such as rare mutations (5%), chromosomal abnormalities (5%), rare copy number variations (CNV) (5-10%), de novo and transmitted point mutations and single nucleotide polymorphisms. Large-scale genome wide association studies have associated the presence of CNVs (duplications, microdeletions) in genes such as *Nlgn4* [19], *SHANK3* [20] and *Nrxn1* [21], which are related to the regulation of synaptogenesis. These defects in proteins associated with synaptic function and structure and neuronal circuits has steered to the recent awareness of autism as a synaptopathy [22]. This led to an increase in studies trying to understand the role of synaptic dysfunction in this disorder. Also, as previously mentioned, syndromic ASD disorders such as Rett Syndrome and FXS are caused by genetic mutations that lead to alterations in protein synthesis that ultimately have an impact in terms of synaptic plasticity.

Another one of the theories that try to unravel the origins of ASDs includes the theory of impeded plasticity, which correlates the decrease in long-distance connectivity in the brains of ASD individuals [23] and the morphological abnormalities such as early overgrowth of several brain structures including the frontal cortex, the amygdala and the cerebellum [24] with the deficits in behavior observed in these patients. The excitation and inhibition (E/I) unbalance theory tries to explain the intellectual disabilities, epileptic seizures often observed in these patients, [25] and the alteration in GABAergic levels detected in several autistic patients [26]. Altered excitation/inhibition (E/I) can lead to the disruption of the normal function of sensory and cognitive brain networks. Another recent theory tries to explains the cognitive and social impairments based on dysfunctions in the mirror neuron systems [27] which consist in a group of neurons involved in the regulation of social, emotional, and cognitive tasks and recognition of motor acts being performed by others. The mirror neurons fire not only when an individual performs a specific action but also when the individual sees that action being performed, and as such are thought to be important for social learning. However, the theories mentioned above can only, by themselves, account for certain phenotypes and symptoms of ASD.

The interaction between genetic alterations and the environment has also been explored. It has been shown that some environmental factors (such as maternal malnutrition, stress and exposure to toxins or drugs) can have a direct action in some susceptibility genes, leading to epigenetic changes in gene expression (such as DNA methylation, acetylation) that can increase the risk for ASD [28].

As the genetics behind autism are better understood new hypothesis have started to surface. The development of genetics, alongside with animal models and new methods of analysis allow for the identification of various etiologies and common molecular and cellular pathways responsible for the disorder. Unraveling these mechanisms can advance our ability to classify, diagnose and treat patients in the future.

## 2. Intellectual Disability

Intellectual Disability is characterized by significant limitations in terms of intellectual functioning and adaptive behavior. In humans, deficits in intellectual functioning are measured by Intelligence Quotient (IQ) tests and include limitations in terms of conceptual skills such as reasoning, problem solving, learning and abstract thinking. Significant cognitive deficits are diagnosed when the IQ values are two standard deviations below the average, typically 70 or below. Impairments in adaptive functioning include deficits in skills required to live in an independent and responsible manner such as communication, social and practical skills [3].

In developed countries, ID presents a high prevalence of 1-3% of the population and, like ASD, there is a higher occurrence in males rather than females mainly due to an X-linked genetic mutation. The etiology of ID is also not fully understood and much like ASD, the known causes of this disorder have been shown to be heterogeneous, including environmental influences (neurotoxicity, maternal malnutrition, prenatal infections and premature birth), chromosomal abnormalities and gene specific point mutations [29].

Intellectual Disability is a developmental disorder with an early onset. The appearance of the disability is noticed normally during childhood or adolescence usually before the age of 18 years. ID can be subdivided into two categories: non-syndromic in which deficits in cognition are the only manifestation of the disorder and syndromic ID in which patients present one or more clinical features or co-morbidities in addition to ID. Many syndromic genetic forms of ID have been widely studied and are often linked to ASD which is the case of FXS, Rett Syndrome, TSC and Angelman Syndrome that show alterations in genes that code for proteins involved in neuronal and synaptic function, signaling and differentiation [29].

The genetic background behind non-syndromic forms of this disorder are not as well understood but screening studies have been able to unravel candidate genes that seem to be dysregulated in patients with some non-syndromic forms of ID. Among several targets are: *SHANK2*, *SHANK3* (scaffolding proteins involved in glutamatergic synapses), *Nlgn4* and *Nrxn1* (synaptic maintenance) [30]. Similarly, to what was observed in ASDs (and given the overlap between the two disorders), synaptic signaling seems to play an important role in this pathology as well, as most of the genes deregulated, as the ones previously mentioned, are involved in neuronal and synaptic structure and function, as depicted in Figure 1.

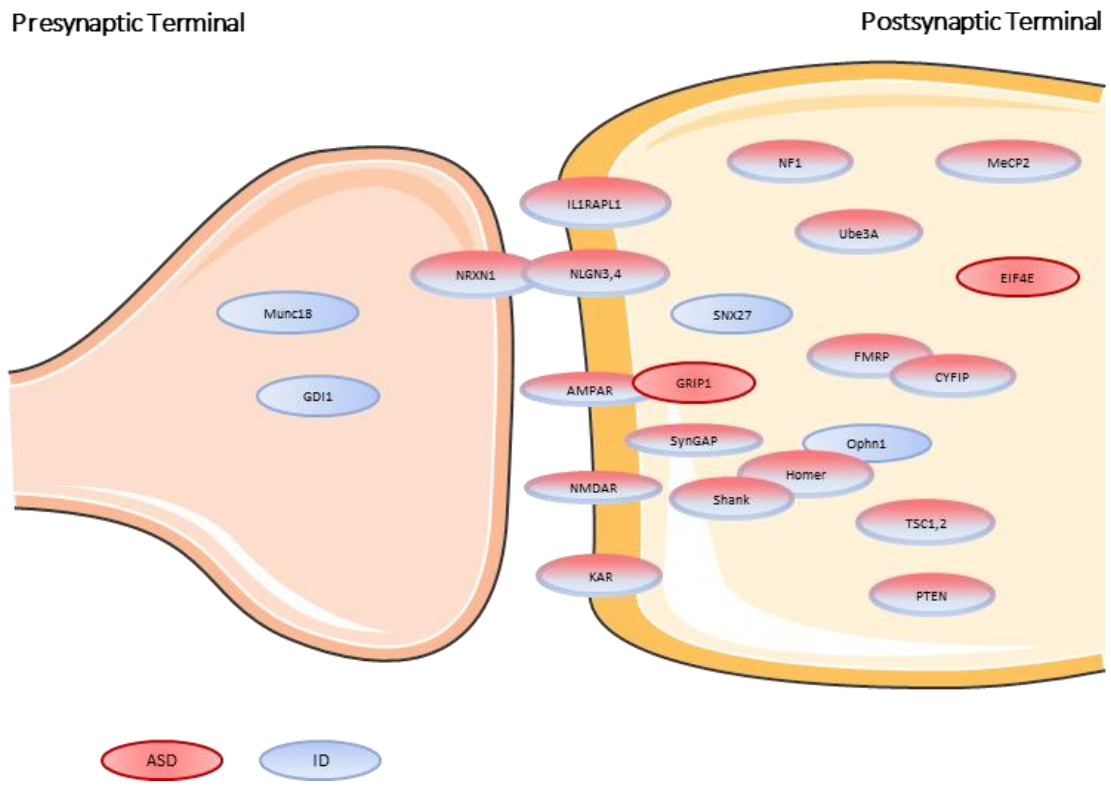


**Figure 1. Overview of human genes implicated in ASD and ID.** Proteins are color-coded based on their genetic association with ID (blue) or ASD (red), with protein interactions indicated by overlapping symbols. Abbreviations: *AMPAR*, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; *Cyfip*, cytoplasmic FMRP-interacting protein; *EIF4E*, eukaryotic translation initiation factor 4E; *Frm1*, fragile X mental retardation protein; *GDI1*, guanosine diphosphate–dissociation inhibitor; *GRIP1*, glutamate receptor-interacting protein 1; *IL1RAPL1*, interleukin-1 receptor accessory protein-like 1; *KAR*, kainate receptor; *MeCP2*, methyl-CpGbinding protein 2; *Munc18*, mammalian uncoordinated 18; *NF1*, neurofibromin 1; *Nlgn3,4*, neuroligin 3 and 4; *NMDAR*, N-methyl-D-aspartate receptor; *Nrxn1*, neurexin 1; *Ophn1*, oligophrenin1; *PTEN*, phosphatase and tensin homolog; *SNX27*, sorting nexin 27; *SynGAP*, synaptic GTPase activating protein; *Tsc1,2*, tuberous sclerosis complex 1 and 2; *Ube3A*, ubiquitin protein ligase E3A. Adapted from [30].

## 3. Spine pathology in ASD and ID

Autism Spectrum Disorders and ID are both neurodevelopmental disorders that have been shown to have synaptic dysfunctions as a common background, either by dysregulations in synaptic proteins or proteins involved in synaptic signaling [31, 32]. Alterations in terms of synaptic signaling, especially in long-term potentiation (LTP) and long-term depression (LTD) lead to changes in synaptic strength that end in changes to the number and morphology of spines [33]. LTD, for example, has been shown to cause a decrease in the number of spines through the removal of weak synapses [34]. Variations in spine morphology and spine dynamics, including spinogenesis and spine turnover, are

crucial for brain functions involving memory, cognition and regulation of behaviors, mediating connectivity within neuronal circuits [33]. In line with this, several animal models of ASD and ID, such as the *Fmr1* KO, *15q11-13 patDp*/+ or *SynGAP*(+/-), for example, have been shown to manifest impairments in LTD across different brain regions which are accompanied by spine rearrangements and altered behavior, supporting the link between LTD and changes in spines that lead to deficits in behavior across different disease conditions [35-37].

These alterations in terms of spine density have also been observed in patients suffering from neuropathologies such as ASD and ID. Studies performed in postmortem brain tissue in ASD patients showed an increase in cortical spine density when compared to control cases, being this increase inversely correlated to the patient's cognitive ability [38]. In ID patients, however, spine dysgenesis and a reduction in spine density has been reported in cortical neurons [39]. In hippocampal sections collected from patients with Rett Syndrome, a disorder known to have a high comorbidity with both ASD and ID, a decrease in spine density has been reported [40]. This variability (Figure 2) shows, once again the phenotypical diversity, etiology and the different impacts this spectrum of disorders can have. It also reinforces the importance of well characterized animal models, which have a clear genetic dysregulation, so the mechanisms behind these cellular deficits that lead to alterations in behavior can be unraveled.

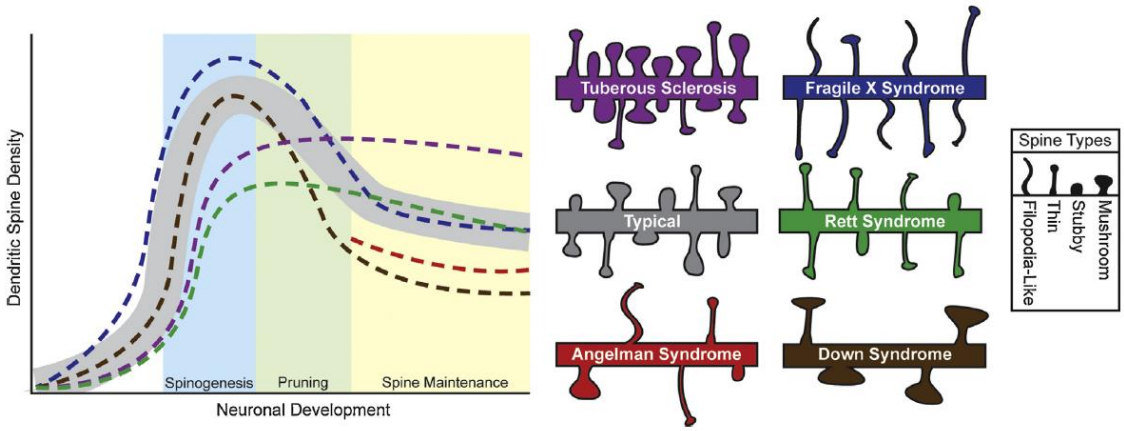


**Figure 2. Characterization of dendritic spines in autism related disorders.** Dendritic spine density during neurodevelopment stages and changes in mature spine morphology in different autism related disorders. [41]

## 4. Hypothalamus

The hypothalamus is a brain region known for the coordination between intrinsic needs of the individual with dynamic changes in its environment, synchronizing key physiological and behavioral responses towards homeostatic balance [42]. To do so, the hypothalamus possesses various nuclei with different functions. One of the central roles of this region is in neuroendocrine regulation, by mediating the release of various pituitary hormones through the production and release of hypothalamic hormones. These have an important role in growth (growth-hormone releasing hormone, somatostatin), reproduction (gonadotropin-releasing hormone), lactation (thyrotropin-releasing hormone (TRH), dopamine and oxytocin), metabolism and also the response to stress through the mediation of the hypothalamic-pituitary axis (corticotropin-releasing hormone (CRH)). The release of these hormones and subsequent effect on the pituitary leads to a set of endocrine feedback mechanisms that ultimately influences metabolic activity.

In addition to the previously mentioned functions, the hypothalamus also plays an important role in the control of food intake (further explored in section 4.1), aggression (further explored in section 4.2), thermic regulation, circadian rhythm and sleep-wake cycles. Within the hypothalamus, there are nuclei responsible for the control of sleep states, such as the ventrolateral preoptic and median preoptic nuclei, and others that promote wakefulness, e.g. lateral hypothalamus (LH). To control sleep-wake cycles, a coordination between neural networks (similar to a switch) has been reported to be present in this region. Neurons from the ventrolateral preoptic nucleus and the median preoptic nucleus are two centers know to promote sleep and repress wakefulness while the ascending arousal system, that include the LH and tuberomammillary nucleus, are active during wakefulness. Through the release of different neurotransmitters (histamine, orexin, GABA and NPY) these systems are able to ensure rapid transitions from one state to the other [43].

Dysregulations in this region, given its broad spectrum of function, have been known to underlie a variety of disorders such as obesity, Kallmann Syndrome, Prader-Willi Syndrome, depression and hypogonadism.

Taking all this into account it is clear that the hypothalamus may be implicated in different pathologies and be responsible for several endophenotypes seen in

neuropsychiatric and neurodevelopmental disorders. In the next few sections, the role of this region in feeding and aggressive behaviors will be explored in more detail.

### 4.1 Hypothalamic role in: Feeding

The hypothalamus was studied as a possible control center for feeding and metabolism in the 1940s, when reports of lesions performed in the arcuate and ventromedial nucleus of rats led to massive hyperphagia (i.e. insatiable appetite) and obesity. Conversely aphagia and weight loss were observed after ablation of the LH [44, 45]. Since then, other nuclei have been also linked to satiety and hunger and the neuronal circuits in which the hypothalamic nuclei crosstalk with other brain regions has been further dissected.

Within the hypothalamic nuclei, a complex and diverse network of neuronal populations, neuropeptides and hormones have been reported to have a role in the regulation of hunger. The two main neuronal populations involved are the Agrp (agouti related protein) and the POMC (pro-opiomelanocortin) neurons both being present within the arcuate nucleus of the hypothalamus. Agrp neurons co-express NPY, GABA and Agrp (an inverse agonist of melanocortin signaling) and stimulate feeding when they are activated [46]. Optogenetic activation of this neuronal population was shown to be able to rapidly induce feeding in well-fed mice, being this behavioral response proportional to the level of activity in this neurons, proving the impact of this neurons in feeding behavior [47]. POMC neurons, on the other hand, express POMC, a precursor of α-melanocyte stimulating hormone which binds to melanocortin-4 receptors ($MC_4R$) leading to the inhibition of food intake.

The activity of these neuronal populations is regulated in response to humoral signals that circulate in concentrations proportional to the animal's body fat content. Leptin is one of the hormones that regulates this activity. This “satiety messenger” is produced in white adipose tissue when the fat content is adequate, leading to a decrease in food intake. The impact of this hormone was demonstrated in the *ob*/*ob* mice, a mouse model that lacks leptin. These animals presented hyperphagia which led to obesity, type II diabetes and elevated levels of NPY in the hypothalamus [48]. Other cue that provides inputs to the regulation of food intake is ghrelin, a peptide produced in the stomach before meal onset [49]. Ghrelin increases or leptin deficiency lead to activation of Agrp neurons and inhibition of the POMC neuronal population [50]. The changes and balance between

this two neuronal populations aim to reach a physiological homeostasis in terms of hunger and energy expenditure levels.

Besides the arcuate nucleus, other nuclei from the hypothalamus have been shown to be involved in the feeding circuitry with the production and release of different neuropeptides. The LH, which when ablated leds to aphagia, has been shown to produce orexigenic compounds (appetite stimulants) such as orexin and melanin concentrating hormone, and promote food intake. Neurons from the paraventricular nucleus were shown to produce anorexigenic peptides such as oxytocin, CRH and TRH that have a role in the metabolic processes that regulate food intake [51].

One of the most prominent pathologies that exemplifies the impact of abnormal hypothalamic function in normal feeding behavior and also cognition is the Prader-Willi Syndrome. This disorder is mainly characterized by hyperphagia which ultimately leads to obesity, diabetes, hypogonadism and developmental delays. Patients suffering from this disorder also show aggressive behaviors, sleep disturbances, repetitive and compulsive behaviors and increased plasma levels of ghrelin [52]. A high comorbidity of this disorders with intellectual disability and ASD has also been observed. This pathology presents a genetic background being caused by a lack of paternally derived imprinted material on chromosome 15q11-q13 that targets genes responsible for hypothalamic function and regulation [53].

### 4.2 Hypothalamic role in: Aggression

Since the 1930s studies have been suggesting the hypothalamus as a possible anatomical center for emotional behavior. Electrical stimulation of the lateral part of the hypothalamus was first performed in cats and was shown to induce responses suggestive of rage, such as increased respiration, biting, salivation and sweating [54]. In human patients, stimulation and disturbance of this region was also reported to lead to acceleration of heart rate and respiration, elevation of blood pressure, expressions of terror and rage and general hyperactivity [55, 56]. More detailed studies performed in cats, were able to demonstrate that lesions performed in the immediate region of the ventromedial hypothalamic nuclei of cats were able to elicit savage and aggressive behavior, further dissecting the source of this aggressive behaviors [57]. In rats, a “hypothalamic attack area” (HAA) has been identified and characterized as the response area in the brain for stimulation-induced attack and comprises the region that includes the lateroanterior

hypothalamic and anterior hypothalamic nuclei and the region from the dorsolateral to the ventromedial nucleus [58]. In mice, a specific locus for aggression was identified through optogenetic stimulation [59]. Activation of neurons in the ventrolateral subdivision of the ventromedial hypothalamus was shown to elicit males to attack castrated males, females or inanimate objects [59] and also had a role in the control of aggression seeking behavior when no threat was present [60].

In terms of neural substrates, the serotonin system seems to play a negative role in the control of hypothalamic aggressive behavior in rodents [61, 62]. The balance between excitatory glutamate and inhibitory GABA inputs also seems to play a role in mice, with studies showing that hypothalamic induced aggressive responses can be elicited by the overriding of local inhibition, through local infusion of GABA receptor antagonists [63] and a combination of GABA receptor antagonists and glutamate receptor agonists [64]. However, these responses seem to vary slightly between nuclei and appear to differ if different subunits of the receptors are modulated [65]]. Nevertheless, the precise nuclei involved in these behaviors and the molecular mechanisms that underlie the control of aggressive responses are still not well understood.

## 5. G-protein coupled receptors and social behavior

G-protein coupled receptors (GPCRs) are one of the most abundant family of proteins encoded in the human genome and account for around 40% of the targets of all drugs available in the market [66]. GPCRs are characterized by a common structural feature: the presence of seven hydrophobic transmembrane segments. The ligands to which these receptors respond vary and include glycoprotein hormones, peptides, lipid-like substances, aminoacids and $Ca^{2+}$ ions. After the binding of the different ligands, conformational changes in the intracellular and transmembrane domains of GPCRs occur, allowing for interactions with heterotrimeric G proteins and the initiation of ligand-dependent cellular signaling. When GPCRs are activated, they act as guanine nucleotide exchange factors (GEFs), catalysing the release of GDP and the binding of GTP to a α subunits of heterotrimeric G proteins leading to their activation. The different subtypes of α-GTP subunits include Gαs (stimulator of adenylyl cyclase), Gαi (inhibitor of of adenylyl cyclase), $G\alpha_{12/13}$ (activator of RhoGEF) and Gαq (activator of phospholipase C) and can modulate downstream effectors directly or generate second messengers that modulate further downstream effectors, such as PKA and PKC. Following their release from the

heterotrimeric G protein complex, the βγ subunits can also bind and regulate certain downstream effectors, such as ion channels and PLCβ [66]. Through these interactions, GPCR activation can regulate various signaling pathways. In the following paragraphs, a group of GPCRs with the highest relevance for ASD and ID will be further explored.

### 5.1 mGluR receptors

Metabotropic glutamate receptors (mGluR) are members of the GPCR family that bind the excitatory neurotransmitter glutamate. They are broadly present in the central nervous system (CNS) both in pre- and post-synaptic terminals but have higher levels of expression in the hippocampus, cerebellum and cortex [67]. These receptors are subdivided in eight types which are inserted into three groups based on their structure, localization and physiological activity. Recently, deficits in signaling mediated by both members of Group I, mGluR1 and mGluR5 have been linked to some disorders of the autism spectrum, mainly FXS and TSC [68, 69].

Long-term depression is thought as a neuronal correlate for learning, and it is now well established that the adaptation induced by LTD can be induced by the activation of this group of mGluRs. Specifically, activation of mGluR1/5 mediates the internalization of AMPA receptors via a mechanism that requires protein translation in the postsynaptic compartment [70]. This form of synaptic plasticity inevitably leads to alterations in synaptic strength and was shown to be deregulated in several neurodevelopmental disorders [68, 69]. Additionally, *mGluR5* KO mice showed deficits in spatial working memory, hyperlocomotion and lack of novelty seeking behavior which correlate with some of the deficits observed in patients suffering from ASD [71]. The increase in mGluR5 activity has been seen as a possible therapeutic target for this disorder, since modulating the levels of mGluR5 activity ameliorates the functional and behavioral defects in animal models of ASD, such as the *SHANK3* KO mice [72]. This family of receptors has then been gaining increased attention for their possible role in these psychiatric disorders.

## 6. Grasp family: as a novel target to modulate mGluRs

### 6.1 G-protein coupled receptor associated sorting proteins

G-protein coupled receptor activity is tightly regulated by a great variety of proteins that modulate their membrane targeting, intracellular trafficking and signaling

properties. One family of proteins that have been associated with the intracellular regulation of GPCRs is the G-protein coupled receptor associated sorting proteins (Gprasp) family. This family is composed by 10 members, from Gpraspı to Gpraspı0, being sometimes subdivided into two subfamilies.

All members of this family were discovered in different screenings and began by having different official nomenclatures, more related to the specific interactions through which these proteins were found [73]. The first member to be identified was Gpraspı through its interaction with the PAS domain of Periodı from rat superchiasmatic nucleus, which gave the protein its first official nomenclature: PIPS (Periodı interacting protein of the superchiasmatic nucleus) [74]. Afterwards, the same protein was identified in a different screening by its interaction with the carboxyl-terminal tail (C-tail) of the delta opioid receptor (a member of subfamily A of GPCRs). Since it was also found that it had a role in the post-endocytic sorting of this receptors, it was renamed G protein-coupled associated sorting protein (GASP) [75]. Gprasp2, the second member of the family, was first identified as an interacting partner of huntingtin which gave rise to the alias: HIP15 (huntingtin interacting partner 15) [76]. Due to the preexistence of a Drosophila protein called Gasp, the official nomenclature for this family of proteins was changed to Gprasp, being this the present official nomenclature.

Gpraspı to Gpraspı0 share significant resemblance in terms of amino acid sequence [77]. All of these proteins display a conserved C-terminal domain of 250 amino acids, represented in Figure 3, showing also the percentage of identical amino acids shared with Gpraspı. Also based on the amino acid sequences, the Gprasp family was divided in two subfamilies: subfamily 1 (Gpraspı to 5), which shows a higher affinity for GPCRs and contains a conserved and repeated motif of fifteen amino acids present outside the conserved C-terminal domain (represented in Figure 3 by the bold black lines) and subfamily 2 (Gprasp6 to 10) which does not [78]. For subfamily 1, the Gprasp motif (a small repeated motif of 15 AA in the central domain of Gprasps which is present 22 times in Gpraspı and twice in Gprasp2 to 5) was shown to be the protein-protein interaction motif mediating the interaction between GPCRs and Gprasps [78]. Almost all members of the subfamily 2 (except Gpraspı0 and Gprasp5) contain armadillo repeats within the conserved C-terminal region (represented in Figure 3 by the parallel rectangles with a small capital A).

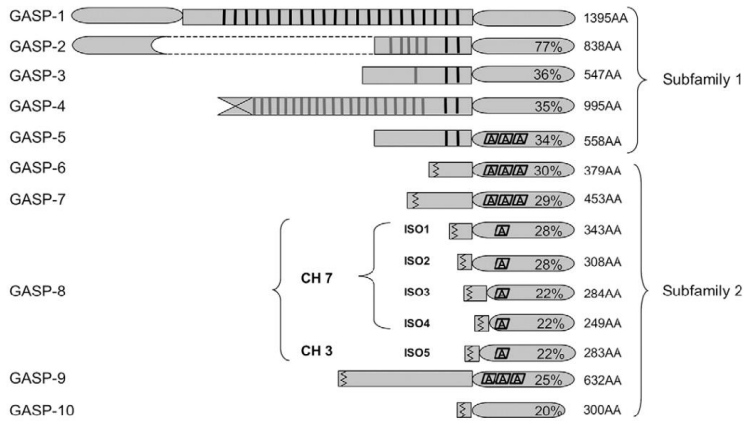


**Figure 3. Sequence comparisons between full-length human sequences of all the members of the Gprasp family** [77]

Another characteristic is that with the exception of Gprasp8, all other Gprasps are located in the chromosome loci Xq22.1-q22.2 (a region specific to mammals). All these genes contain a single protein coding exon that includes the entirety of the 3' non-coding sequence. Gprasp8, on the other hand, has two copies, one in chromosome 3 (with a single coding exon, alike the other Gprasps) and another in chromosome 7 (which contains seven coding exons) [77].

In terms of tissue distribution, mRNA expression of this family of proteins appears to be enriched in the CNS, with some exceptions. Gprasp4 is homogeneously distributed in all tissues and Gprasp9 has an enriched expression in the kidney and ovaries. Besides the predominant expression in the CNS, Gprasp6 mRNA is also expressed in the immune cells, lungs and liver and Gprasp8 has some expression in pancreas, immune cells and liver [73]. In terms of subcellular distribution, most members seem to have a cytoplasmic localization. Some members of the subfamily 1, mainly Gprasp1 and 3, appear to be able to be translocated between the nucleus and the cytoplasm under special conditions, having a nucleocytoplasmic localization [74, 79]. Most members of subfamily 2 have been co-localized with specific organelles such as mitochondria (Gprasp6 [80]), endoplasmic reticulum (Gprasp8 [81] and Gprasp9 [82]) and Golgi apparatus (Gprasp9 [82]) The main function of each of the members of this family is not yet fully understood but, as previously mentioned, an array of screenings has been performed that have been able to provide some information regarding possible functions through protein-protein interactions and different selectivity profiles. The most studied and understood member of this family is Gprasp1. This particular member has been shown to interact *in vitro* with the following

GPCR C-tail receptors (Figure 4): opioid (δ, κ, μ) dopamine D2 and D4, oxytocin, beta-1 and beta-2 adrenergic, muscarinic M1 and M2, bradykinin B1, cannabinoid CB1, thromboxane A2, calcitonin and mGluR1a [73, 83]. The same studies suggested that a small conserved region with the C-terminal domain of opiod receptors which contained a putative α-helix 8 is crucial for binding to Gpraspı [73]. Studies exploring the *in vitro* interactions of Gprasp2 have also been carried out showing a strong interaction with calcitonin, muscarinic M1 and dopamine D2 receptors. It also seems to interact, with lower strength, to 5-HT7, beta-1 adrenergic, muscarinic M2 and Histamine H2 receptors showing no preference in binding to opioid receptors [73, 83]. Gprasp3, 5 and 7 have been also shown to interact *in vitro* with GPCRs [84]. The existence and prevalence throughout the different members of the interaction between Gprasps and GPCRs led to studies that tried to understand the exact function of this interaction and the physiological repercussions it might have. Through the disruption of the DOR-Gprasp interaction (through either receptor mutation or overexpression of a dominant negative fragment of Gpraspı) Whistler *et al.,* suggested that this family may have a role in the modulation of lysosomal sorting and functional down-regulation of a variety of GPCRS, since recycling was promoted and receptor trafficking of lysosomes was inhibited. Other members of the GPCR family, such as β2-adrenergic, dopamine D2 and cannabinoid CB1 receptors, were also examined and Gpraspı seemed to function through the lysosomal pathway also [75, 85, 86]. It has also been shown that under sustained receptor stimulation Gpraspı stimulates recycling rather than receptor degradation [84]. These studies have not been performed for every member of the family, so the exact function and sorting pathway used is not yet fully understood but since the endocytosis and modulation of the activity of these receptors is important for conditions such as drug tolerance this topic is worth exploring.

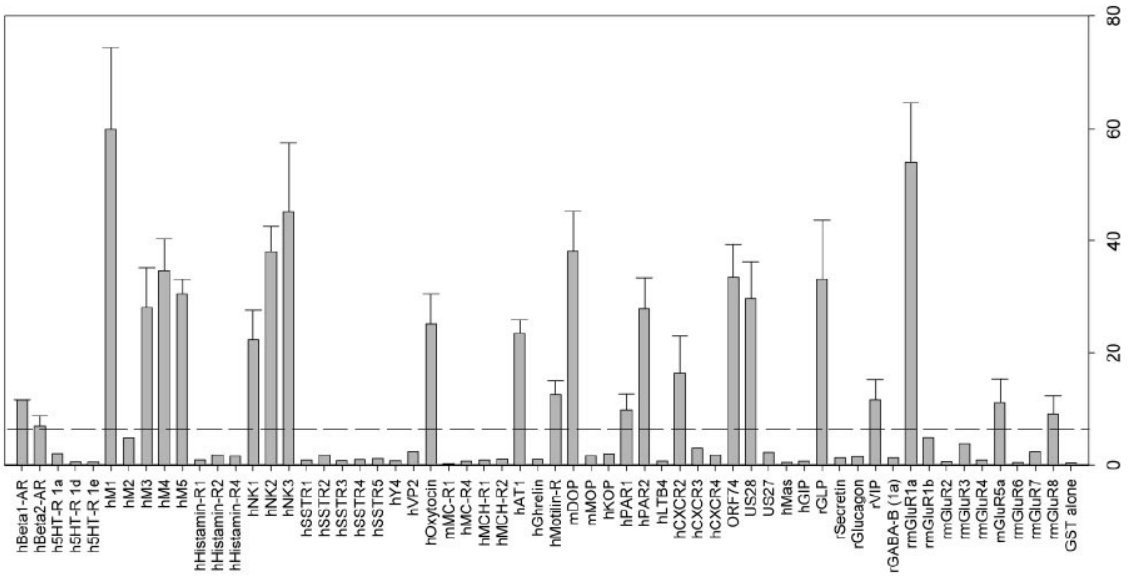


**Figure 4. 7TM receptor tail interaction with the C-terminal fraction of Gprasp.** [87]

As previously mentioned Gprasps have other interacting partners besides GPCRs that have given rise to other possibilities in terms of functions for this family of proteins. Gprasp1 is thought to be involved in the modulation of the activity of single transmembrane receptors and have an impact in circadian rhythm based on its interaction with Growth factor receptor-bound protein 2-associated-binding protein 1, Nerve growth factor [88] and Period-1 [74], respectively. Gprasp2 has been shown to interact with huntingtin (htt) and four other partners, htt interacting partner 5 , Breast cancer 1-associated ring domain protein 1 (BARD1), BARD1 associated interacting protein 3 and pleiotrophin [89]. Gprasp3 has been associated to some degree with the negative control of cell growth and division and in the nuclear import of proteins due to its interaction with PPP2R5E (a protein phosphatase 2A) and importin 5, respectively [79]. Gprasp6 and Gprasp8 have been also implicated with a role in modulation of transcription due to their interaction with Sox10 [80] and p53 [90], respectively, although the precise physiological role is not known. Some members have also been implicated in apoptosis and cell survival such as Gprasp1 and Gprasp8 [88, 90]. Inhibition of isoform 2 of Gprasp8, for example, was shown to induce apoptosis while its overexpression accelerated cell growth [90].

In terms of pathological effects, Gprasps have been associated with some diseases and disorders, most of them cancer related. The exceptions have been Gprasp3 whose mRNA levels have been shown to be decreased in Alzheimer's patients [79], Gprasp4 has been characterized as one of the dysregulated priority in Parkinson patients brains [91] and Gprasp2, which will be further explored, has been associated with

neurodevelopmental disorders such as schizophrenia and autism [17]. Gprasps 1 , 2, 3, 7, 8 and 9 have been to some degree implicated in cancer, either by having an impact in transcription, apoptosis or cellular transformation [77], which coincides with the previously mentioned suggested functions for this family of proteins.

## 6.2 Gprasp2 in human disorders

Gprasp2, as mentioned previously, is a member of the subfamily 1 of the Gprasp family. This 130 kDa protein, coded by a gene present in the X chromosome, has been associated either through known interactions or by sequencing analysis to many disorders. This protein has been shown to interact with many GPCRs (as shown in Figure 5) and many other proteins such as htt [89], a protein usually associated with microtubules, involved in cytoskeletal dynamics and vesicle trafficking which further supports the role of this protein in receptor trafficking. Gprasp2 has been also shown to make connections with DISC1, which is linked to psychiatric disorders such as schizophrenia and with the known interaction with huntingtin, the protein mutated in Huntington's disease, an overlap of dysregulated pathways between both disorders has been suggested [92]. A study performed with different selection methods has also identified Gprasp2 as new potential biomarker for Alzheimer's disease [93].

Gprasp2 has also been associated with the human Xq22.1 deletion syndrome. Deletion of a 0.35 Mb subregion of chromosome Xq22.1 in mice, which contains *Gprasp2*, has been shown to be sufficient to cause the Xq22.1 deletion syndrome which is characterized by respiratory failure, cleft palate and epilepsy [94].

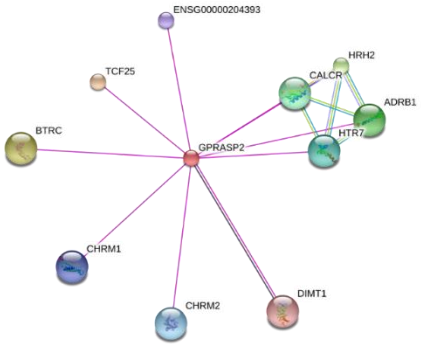


**Figure 5. Protein-protein interaction network for Gprasp2.** *Chrm1*: cholinergic receptor, muscarinic 1; Chrm2: cholinergic receptor, muscarinic 2; *HTR7* (*5-HT7*): 5-hydroxytryptamine receptor 7; *CALCR*: calcitonin receptor; *HRH2*: histamine receptor H2; *ADRB1*: adrenoceptor beta 1; *DIMT1*: dimethyladenosine transferase 1; *TCF25*: transcription factor 25; *BTRC*: beta-transducin repeat containing E3 ubiquitin protein ligase. [95]

A recent study by *Jung et. al* showed Gprasp2 expression in the nervous system and at early embrionic stages in neural crest cells and in the neural tube. Also, expression of this protein was found in the liver and in the pancreas (in the β-cells of the islets of Langerhans) of adult mice [96]. This study suggested a role for Gprasp2 in the Hedgehog signaling pathway, due to its expression in regions of high pitchfork (Pifo) and sonic hedgehog (Shh) activity, being this way involved in early development in Shh-dependent specification of the ventral neural tube and Shh target gene regulation. They also suggest a role for Gprasp2 in intracellular transport machinery. More specifically, they discovered a protein-protein interaction between Gprasp2 and both smoothened (Smo) and Pitchfork which in turn interacts with Kif3b, a motor protein, part of the kinesin II complex, allowing this complex the anterograde transport of Smo [96]. Since the abnormal activation of the Hh signaling pathway has been implicated in tumorogenesis [97], the authors also propose the Pifo-Gprasp2-Smo as a good therapeutic target through prevention of the pathways activation [96].

Through whole exome sequencing, the use of association rules and various screenings performed in ASD and schizophrenia patients, Gprasp2 has also been identified as one of the candidate and susceptibility genes for both disorders, with rare missense variants observed [17, 98-100].

### 6.3 Gprasp2 and neuropsychiatric disorders: previous results

Recent work developed by Mohamed Edfawy under the supervision of Dr. João Peça at the Neuronal Circuits and Behavior Group in the Center for Neuroscience and Cell Biology, has been studying the dysfunction in mGluR signaling implicated in the pathophysiology of ASD by dissecting their regulation and trafficking. To do so the exact role of Gprasp2 in the normal nervous system function and the consequences of its dysregulation are being determined, given the link of this protein to autism and other neurodevelopmental disorders and its interaction with mGluRs.

Given the fact that not a lot is known regarding the function and expression of Gprasp2, initial work was performed to try and understand the expression pattern of the protein through time and in different brain regions. It was found that the expression of this protein was different throughout development, being detected from early stages (E18) increasing until postnatal day 15 (PND15, an age known for proliferation and migration of neuronal cells and robust synaptogenesis [101]), where it has a peak of expression which

then gradually decreases through adult stages (Fig. 6A). The protein was also found to be present in the postsynaptic density (Fig. 6B). Gprasp2 was also found to be highly expressed in specific brain regions in adult mice particularly hippocampus, hypothalamus, cortex and thalamus (Fig. 6C).

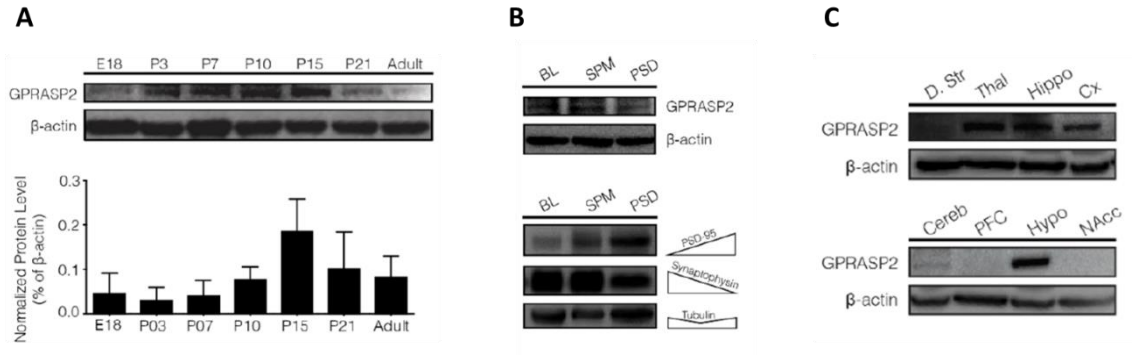


**Figure 6. Expression profile of Gprasp2 in the mouse brain.** (A) Expression patterns of Gprasp2 in brain lysates of C57BL/6 mice from embryonic stage (E18) to adult ages analyzed by western blot. (B) Western blot analysis of Gprasp2 in brain lysate (BL), synaptosomal plasma membrane (SPM) and postsynaptic density (PSD) fraction purified from whole mouse brain. (C) Immunoblot analysis of adult mouse brain subregions using anti-Gprasp2 antibody. (*Edfawy* et. al, *unpublished data*)

To assess a possible role in synaptic function, neuronal and spine morphology Gprasp2 was manipulated in primary neuronal cultures. The knockdown of this protein led to a decrease in dendritic arborization and complexity (Fig. 7A-D) and also to deficits in terms of spine density and morphology (Fig. 7E-I). Given the known interactions of Gprasp2 with different GPCRs [87], the impact of this protein in mGluR trafficking was also studied. Overexpression of Gprasp2 led to a decrease in surface levels of mGluR5 being observed the opposite when Gprasp2 was knocked-down, suggesting that indeed Gprasp2 has a role in the regulation of this receptors trafficking (Edfawy *et al.*, *unpublished data*).

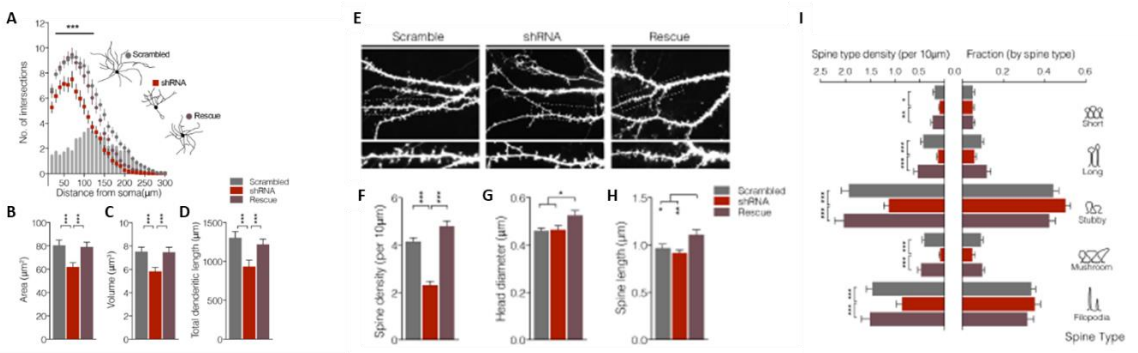


**Figure 7. Effect of *Gprasp2* knockdown in cultured hippocampal neurons.** Transfected with scramble shRNA (Scramble), shRNA *Gprasp2* (shRNA) or GFP- *Gprasp2*+shRNA (Rescue) at DIV10 and fixed at DIV15. Quantification of (A) sholl analysis, (B) total dendritic area, (C) length and (D) volume. Analysis of (E, F) spine

density per 10 µm, (G) spine head diameter and (H) spine length in scramble, shRNA and rescue neurons. (I) Dendritic spine classification and quantification. (Edfawy *et al., unpublished data*).

To complement and validate the results mentioned above with *in vivo* data a *Gprasp2* knockout animal model was generated by deleting exon 7 using the Cre/lox system (Figure 8). Anatomically, these animals did not show any gross abnormalities but after behavioral characterization, some alterations were observed.

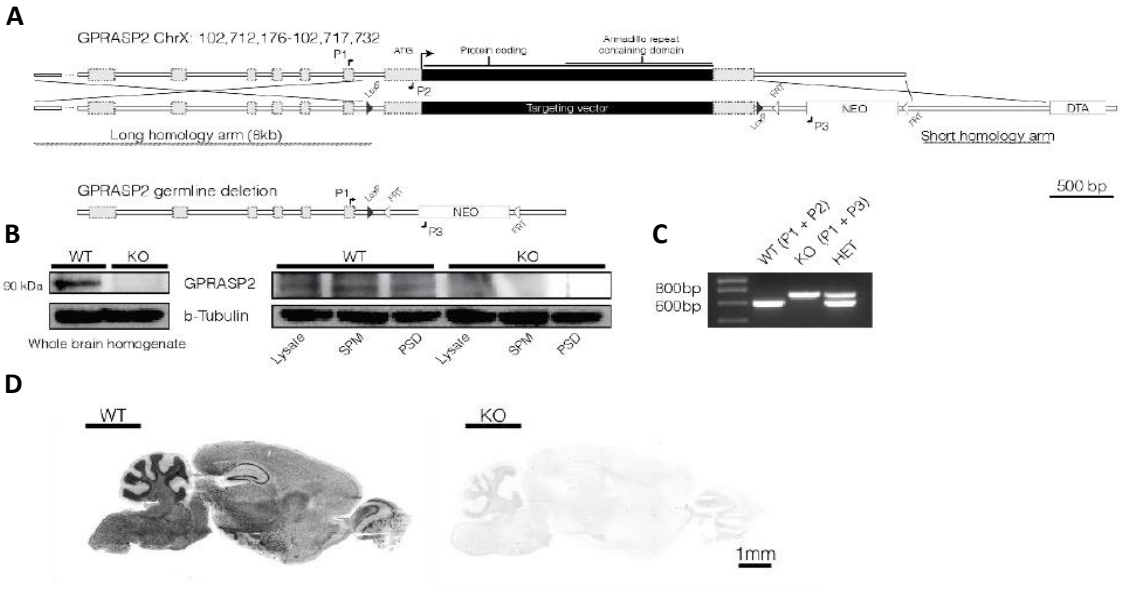


**Figure 8. Development and validation of *Gprasp2* knockout mice.** (A) Schematic drawing of *Gprasp2* gene structure, functional domain and strategy to create *Gprasp2*-knockout mouse. (B) (left) Gprasp2 antibody staining in whole brain homogenate in WT and *Gprasp2* KO mice. (right) Western blot showing GPRASP2 antibody staining in brain lysate (lysate), SPM and PSD fractions in WT and *Gprasp2* KO mice. (C) PCR genotyping confirms deletion of exon 7 from genome of male knockout mice. (D) Validation of the *Gprasp2* KO mouse model through *in situ* hybridization probes targeting exon 7. (Edfawy *et al., unpublished data*).

*Gprasp2* KO mice showed reduced anxiety-like behavior in the open field and in the elevated plus maze. Impairments in social interaction were assessed through the three-chamber test and social dyadic paradigm and deficits in social recognition and reciprocal interactions were also observed. When compared to WT (wild-type) littermates the *Gprasp2* KO mice also showed memory impairments in the novel object recognition test (Figure 9), hinting to a possible dysfunction in hippocampal function and circuitry.

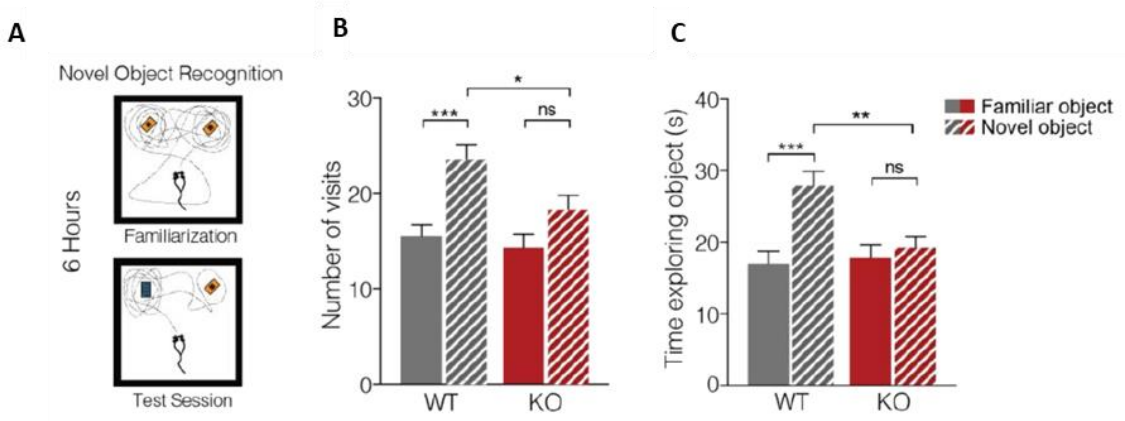


**Figure 9. *Gprasp2* KO mice show memory impairment in novel object recognition test.** (A) Schematic diagram of the novel object recognition task. Quantification of time spent in exploration (B) and frequency of exploration of the novel and the familiar objects (C) was performed during the test session for WT and *Gprasp2* KO animals. (Edfawy *et al.*, *unpublished data*).

In terms of functional alteration to neuronal circuits, DHPG-induced LTD was found to be significantly increased in the hippocampus of *Gprasp2* KO mice when compared to WT (Figure 10), suggesting an impairment in hippocampal synaptic plasticity.

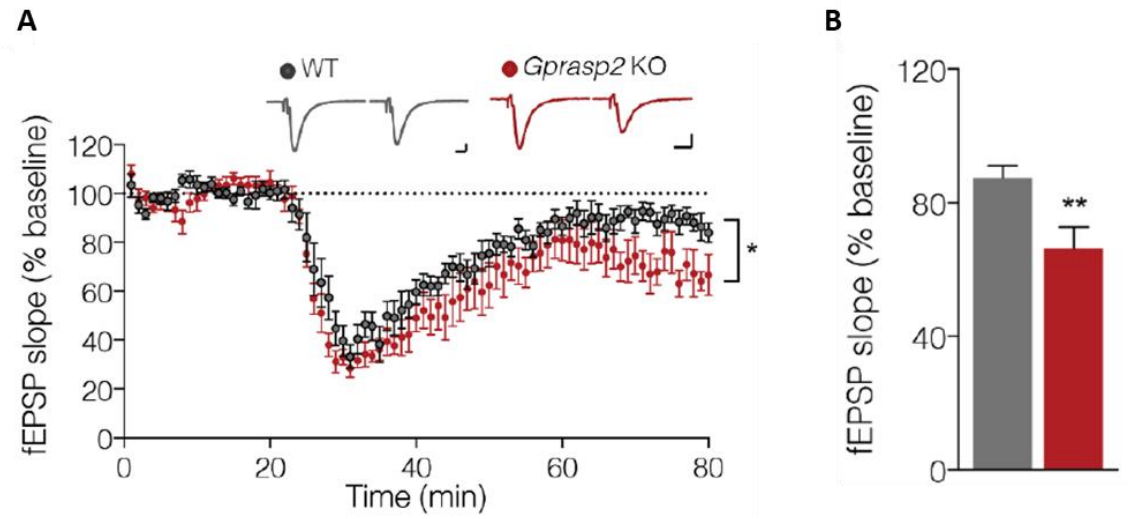


**Figure 10. Enhanced mGluR-dependent LTD in *Gprasp2* KO mice.** (A) Time-course of fEPSP slope; normalized to baseline from WT (gray) and KO mice (red). (B) Quantification of change in fEPSP slope following DHPG protocol, showing the average responses to the last 5 minutes in control and *Gprasp2* KO mice. (Edfawy *et al.*, *unpublished data*).

The memory deficits, other behavioral deficits and the dysfunctions in hippocampal mGluR-dependent plasticity encountered in the *Gprasp2* KO mice, resemble previously reported features of ASD and ID, giving some support to the possible impact of Gprasp2 in these disorders and for this *Gprasp2* KO mice as a useful animal model to dissect the circuit mechanisms behind ASD and ID.

However, several questions remain regarding the *in vivo* role of Gprasp2. Can the *in vitro* alterations pertaining to neuronal morphology be observed in the *in vivo* model? Do the behavioral alterations go beyond the ones observed so far? Does the lack of Gprasp2 have an impact in other brain regions besides the hippocampus? These are some of the crucial questions that still need to be answered for a further understanding of the role and reach of Gprasp2 in ASD and ID.

## Objectives

Autism Spectrum Disorders (ASD) and Intellectual Disability (ID) research has pointed towards an implication of mGluRs in their pathophysiology. To target and unravel the mechanisms behind this dysregulation we will study the interaction between Gprasp2 (a regulator of GPCR signaling) and these well-known receptors to see if the disruption in these interactions can lead to disease-like phenotypes *in vivo*. To assess this, we will take advantage of a recently generated *Gprasp2* KO model and perform a detailed characterization at the following levels:

- Behavioral characterization;
- Neuronal morphology and spine density characterization through confocal imaging;
- changes to mRNA levels using qRT-PCR analysis.

# CHAPTER II |MATERIALS AND METHODS

# Materials and Methods

## Animals

Male *Gprasp2* knockout mice (hemizygous *Gpras2*$^{-/y}$ generated through deletion of exon 7 using the Cre/lox deletion) and WT littermates were used in all the experiments. Mice were maintained at constant temperature (22 °C) and relative humidity (60%) under a cycle of 12 hours / light (lights on from 7 a.m. to 7 p.m.) with individual cage ventilation system. Animals were allowed access to water and food ad libitum. The maintenance and treatment of the animals were performed according to the Animals Use and Care Guidelines issued by FELASA. All experiments were carried out according to the protocols approved by the ORBEA (Body responsible for Animal Welfare of the University of Coimbra / CNC) and the DGAV (Direcção Geral de Alimentação e Veterinária).

## Behavior

Mice were transferred from the animal house one day before the experiment to acclimatize to the behavioral room; tests were conducted from 09:00 AM until 17:00 PM after which the animals were returned to the vivarium. Animal identification was performed by subcutaneous injection of green and/or black dyes in the paws one week before the experiment start date. The investigator was blind to genotype during the behavioral tests.

### Tube Test

The tube test was performed in a transparent plexiglass tube, 33 cm long with an inner diameter of 3 cm. Acrylic ramps allowed the animals to easily access and retreat back from the tube. Testing started by introducing two different age-matched subjects to the edges of the tube. Testing ended as soon as one of the subjects had all paws outside of the tube for at least 4 seconds. All animals were weighed before each round and weight-matched as closely as possible. A subject was declared a "winner"/dominant when its opponent was pushed or backed out of the tube.

Barnes Maze

The Barnes Maze test was performed to assess spatial memory and learning. The apparatus consisted in a white circular platform (122 cm diameter) with 20 equally spaced holes with a diameter of 4.4 cm positioned 2.5 cm from the edge of the maze. The circular platform was mounted on top of a table, 92 cm above the ground. In the test the animal is placed in the center of the maze and is given a negative reinforcement, in this case bright light (350 lux). This will motivate the animal to seek a dark, safe place which is provided by a goal box placed beneath one of the 20 holes. During the training sessions, the 19 holes that do not possess the goal box are closed off making it hard to distinguish the location from the surface of the maze. The animal has to orient itself and locate the box based only on spatial cues that surround the maze. The test includes: one day of habituation (to reduce anxiety), 3 training days and a probe day. In the habituation phase, the animal is placed in the center of the maze underneath a transparent 1,5mL glass beaker for 30 seconds before being slowly guided (10-15 s) towards the target hole by moving the glass beaker. The mice are given 3 minutes in order to independently enter through the target hole into the escape cage. If the animal does not enter during that time, it is nudged with the beaker to enter. The animal is allowed to stay in the escape cage for 1 min before being placed in the home cage. The spatial memory was consolidated through 3 training sessions. The first session consisted in three 2-minute trials/day and the last 2 consisted in only 2 trials/day. Each trial was similar to the habituation phase but in this case when the animal was placed in the center of the maze the beaker was covered making it impossible for the mice to assess its location based on the spatial cues presented. After 15s the beaker was removed and the animal was allowed to move freely for 2 minutes. Mice that failed to find the goal box within the 2 minutes of the trial were gently guided to the location.

On day 5 the animals were submitted to a probe trial to assess short-term memory. In this trial, the goal box was removed and the hole was covered. The animal was allowed to freely explore the maze for 2 minutes.

After 2 days of rest, the animals were submitted to the Reversal phase of the test to assess learning and memory flexibility. To do so, the placement of the target box is changed and the animals go through the 5 days of trials similar to the first week, being this way possible to evaluate the ability to extinguish a previously acquired location and to learn a new one. Thirty minutes before the first trial of each day the animals were isolated to different cages that are kept during the 2 weeks of the test (including reversal).

All trials were digitally recorded and analyzed using the automated software Ethovision XT (Noldus). Information regarding latencies to reach the target hole, target quadrant, time spent and distance were extracted automatically.

### T-maze

To assess working memory, spontaneous alternation in the T-maze test was performed. The apparatus consisted in a T shaped maze with a stem (45.5cm*5cm) and 2 arms (45.5cm*5.5cm) made of white plastic with bedding placed on the floor. The mice were placed in the initial part of the stem and allowed to explore the maze. After the animal entered one the arms a sliding door was placed in the initial part of the arm chosen, trapping the mouse allowing exploration of a single arm. After 30s, the animal was gently removed and returned to the homecage. Next, the animal was returned to the start arm and a second run was initiated. Directions of choice were recorded for each mouse and the percentage of alternation was obtained. 5 trials (2 runs each) were conducted in the space of 2 consecutive days (3 on the first day and the rest on the second). The floor in the maze was illuminated at 15-20 lux.

## Tissue Collection

### Dendritic and spine morphology analysis

Animals (3 per genotype) were anesthetized with isofluorane (Abbott Laboratories) and perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Whole brain was dissected and post-fixed in 4% paraformaldehyde for 24h, followed by 30% sucrose in 0,1M phosphate buffer. Serial coronal section of 100 µm were collected from both WT and *Gprasp2* knockout animals using a vibratome (Leica) and mounted in gelatinized slides using Vectashield with DAPI (Vector Laboratories) as the mounting medium. Slides were store at 4 ºC protected from light.

### RNA analysis

Naïve animals (WT/KO) were sacrificed at two different ages (PND20 and PND90) by decapitation after deep anesthesia with isoflurane (Abbott Laboratories). The brain was dissected and regions of interest, including the hippocampus, hypothalamus, cortex,

striatum and cerebellum were collected. Immediately after, tissue was preserved at - 80 ºC for further processing.

## qRT-PCR analysis

Total RNA was extracted from the different tissues previously isolated (3 animals/genotype/age, 5 brain regions) with the use of the NucleoSpin RNA kit and according to the instructions of the supplier (Macherey-Nagel). Complementary DNA (cDNA) was synthesized from 1ong of total extracted RNA by using the Fluidigm Reverse Transcription MasterMix kit, following the instructions of the supplier (Fluidigm). The resulting cDNA was subjected to quantitative PCR analysis using a 96.96 Dynamic Array Integrated Fluidic Circuit (Fluidigm) and the Fast Gene Expression Analysis using EvaGreen on the Biomark HD System protocol from Fluidigm. PCR primer sequences used were as follows in Table 1. Beta-2-Microglobulin (*B2M*) was used as an internal control for all samples collected at PND20 and Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*) was used for PND90 samples, as they were shown to display the least variability between genotypes from age-matched animals at each particular age. Analysis was performed using the Fluidigm Real-Time PCR Analysis Software.

**Table 1. RT-qPCR primer sequences**

| Gene | Name | Primer FW | Primer RV |
|---|---|---|---|
| | Gprasp Family | | |
| *GPRASP1* | G protein-coupled receptor associated sorting protein 1 | CCCAGGCAAAGGCTGAAAATA | GATTTGTGTCCTAACCTTGGGTC |
| *BHLHB9* | G protein-coupled receptor associated sorting protein 3 | AGGGTCTAAGGGAAAGGTAGTTG | CGTGTGGATCTAGCAAACTTGT |
| *ARMCX4* | G protein-coupled receptor associated sorting protein 4 | ACTGGAGTGGACACGAAGTC | AGCACCAGCCATATCATCATTTT |
| *ARMCX5* | G protein-coupled receptor associated sorting protein 5 | GGAGAAGAGCCTAGTGTAGGG | AGTCGAATGTATCTGGGGGTT |
| *ARMCX3* | G protein-coupled receptor associated sorting protein 6 | AAGGGCTTCTCCTAATTCAGACG | GCAGCATTATTACCCAGAGCAA |
| *ARMCX1* | G protein-coupled receptor associated sorting protein 7 | CTGGTGCCTGCTACTGTGTAT | CCCCTACCCCAACATTAGTCT |
| *ARMCX6* | G protein-coupled receptor associated sorting protein 10 | TGGGAAGAAGTGAGGGGAAC | GTCGAGCCATTGCTGTGAAAT |
| | mGluR signaling | | |
| *Adcy5* | Adenylate Cyclase 5 | AACGCCAAGCAGGAGGATATG | CCCCGAGGATCTTAATCCGTAA |
| *Akt1* | AKT Serine/Threonine Kinase 1 | ATGAACGACGTAGCCATTGTG | TTGTAGCCAATAAAGGTGCCAT |
| *ARC* | Activity Regulated Cytoskeleton Associated Protein | AAGTGCCGAGCTGAGATGC | CGACCTGTGCAACCCTTTC |
| *CamkIIa* | Calcium/Calmodulin Dependent Protein Kinase II Alpha | TATCCGCATCACTCAGTACCTG | GAAGTGGACGATCTGCCATTT |
| *Creb1* | CAMP Responsive Element Binding Protein 1 | CAGGGGTGCCAAGGATTGAAG | ACTGCTAGTTTGGTAAATGGGG |
| *Fmr1* | Fragile X Mental Retardation 1 | AGGTGCCAGAAGATTTACGACA | CTCGCTTTGAGGTGACTTCATT |
| *Foxg1* | Forkhead Box G1 | CACTTTGAGTTACAACGGGACC | CGAGTTTTGAGTCAACACGGA |
| *Gsk3b* | Glycogen Synthase Kinase 3 Beta | AAGCGATTTAAGAACCGAGAGC | AGAAATACCGCAGTCGGACTAT |
| *Mapk1* | Mitogen-Activated Protein Kinase 1 | TTGCTTTCTCTCCCGCACAAA | AGAGCCTGTTCAACTTCAATCC |
| *MeCP2* | methyl-CpG binding protein 2 | AAACCACCTAAGAAGCCCAAATC | TTGACAACAAGTTTCCCAGGG |

| | | | |
|---|---|---|---|
| ***mTOR*** | Mechanistic Target Of Rapamycin | CACCAGAATTGGCAGATTTGC | CTTGGACGCCATTTCCATGAC |
| ***Pi3kca*** | Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha | CACTCGTCACCATCAAACATGA | AGGGTTGAAAAAGCCGAAGGT |
| ***Pi3kcb*** | Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Beta | CTATGGCAGACAACCTTGACAT | CTTCCCGAGGTACTTCCAACT |
| ***Pi3kcd*** | Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta | GTAAACGACTTCCGCACTAAGA | GCTGACACGCAATAAGCCG |
| ***Plcb1*** | Phospholipase C Beta 1 | ACCTGAGCGGAGAAGAAAATG | TGTTGTGCGAGGAATTGATGAA |
| ***Plcb3*** | Phospholipase C Beta 3 | CTGCCGCTCTATCTTTGGGG | GCCGATGTCGCTTCTTATTCTTC |
| ***Plcg1*** | Phospholipase C Gama 1 | GAGACGCGCCAGATCACAT | AAAGTCCCGAGAAGTCTTCCC |
| ***PTEN*** | Phosphatase And Tensin Homolog | TGGATTCGACTTAGACTTGACCT | GCGGTGTCATAATGTCTCTCAG |
| ***Tsc1*** | Tuberous Sclerosis 1 | CAAGGAGTCCCTCAATTCTGAAC | GATGTGCAATACCGGCTGAGA |
| ***Tsc2*** | Tuberous Sclerosis 2 | AGTTCTCACCTTATTGAAGGCCA | CATTGGAGGGGTAGTCCTTGA |
| | Synaptic Structure | | |
| ***Ank3*** | Ankyrin 3 | CTGACGTTCACGAGGGAGTTT | GGGCTTGAGACCAGGTTCA |
| ***Cask*** | Calcium/Calmodulin Dependent Serine Protein Kinase | AAAACTCGGCACCTGTTAAACT | AGGCTTTCCGTAAGGCTCTCT |
| ***Cfl1*** | Cofilin 1 | ATGACATGAAGGTTCGCAAGT | GACAAAAGTGGTGTAGGGGTC |
| ***Ctnnb1*** | Catenin Beta 1 | TCCCATCCACGCAGTTTGAC | TCCTCATCGTTTAGCAGTTTTGT |
| ***Cttn*** | Cortactin | ATGGGGTGCTAAAACCGTG | CTCCTTGAGCGTCTGGTGTTC |
| ***Dlg1*** | SAP 97 | CAGGATGGAAGATTGCGGGTA | ACTGCTTTGCTGTGGGTTACA |
| ***Dlg2*** | PSD 93 | AAACGCTCCCTGTATGTCAGA | CCCCATCTAGTGTGACCCTTC |
| ***Dlgap2*** | SAPAP2 | ACAAGGGATGATTGCGCCAT | TGGACTCTCATTCCGCTGTTC |
| ***Dlgap3*** | SAPAP3 | GAGAGTCCTAGCCGCATCC | GGCCCGTTATAGTCTCGCTT |
| ***Dlgh4*** | PSD95 | ACCAGAAGAGTATAGCCGATTCG | GGTCTTGTCGTAGTCAAACAGG |
| ***Gphn*** | Gephyrin | TGGTCCAGGGGATCGTTTCAT | TTGTAACCCGCATCACTTGTC |
| ***Homer 1*** | Homer Scaffolding Protein 1 | GCCAATGGGCTGATAGCCG | AGCTCCATCTTCTCCTGCGAC |
| ***Homer 3*** | Homer Scaffolding Protein 3 | ATT CCA GAT CGA CCC CAC TAC | CACATTTCGGGTTGCATCATAGA |
| ***Nlgn1*** | Neuroligin 1 | GGTACTTGGCTTCTTGAGCAC | AAACACAGTGATTCGCAAGGG |
| ***Nlgn2*** | Neuroligin 2 | ATTCCTCAACTACGACATGCTCA | GTTGGAGACGGTGAAGTCAAA |
| ***Nlgn3*** | Neuroligin 3 | AGTTACGGCAACGTCATCGTC | GCGAAGGGCCTGGATTTGAT |
| ***SHANK 3*** | SH3 And Multiple Ankyrin Repeat Domains 3 | GAGATCAGCTCATTGTTTGA | ACAGATTTGGTCCGTGGAAT |
| ***Syt17*** | Synaptotagmin 17 | GTCAGAGGTGCTATGAGTCCA | GGGGTCAAAGGAACATCGCT |
| | Receptors | | |
| ***Chrm1*** | Cholinergic Receptor Muscarinic 1 | AGTCCCAACATCACCGTCTTG | CAGGTTGCCTGTCACTGTAGC |
| ***Chrm2*** | Cholinergic Receptor Muscarinic 2 | TGCCATTGCGGCTTTCTATCT | CTTGCACTAGACTCGGAGACA |
| ***Crhr1*** | Corticotropin Releasing Hormone Receptor 1 | GGAACCTCATCTCGGCTTTCA | GTTACGTGGAAGTAGTTGTAGGC |
| ***Drd1*** | Dopamine Receptor D1 | ATGGCTCCTAACACTTCTACCA | GGGTATTCCCTAAGAGAGTGGAC |
| ***Drd2*** | Dopamine Receptor D2 | ACCTGTCCTGGTACGATGATG | GCATGGCATAGTAGTTGTAGTGG |
| ***Gabra1/2*** | Gamma-Aminobutyric Acid Type A Receptor a1/2 Subunit | AACAGTGTCAGCAAAATCGACA | CTCCCTGTTTAAATAGGTAGC |
| ***Gabra 3*** | Gamma-Aminobutyric Acid Type A Receptor Alpha 3 Subunit | ATGTGGCACTTTTATGTGACCA | CCCCAGGTTCTTGTCGTCTTG |
| ***Gabra4*** | Gamma-Aminobutyric Acid Type A Receptor Alpha 4 Subunit | AGGTGCCAAAGGAGTCTTCTA | TAGCCCATCTTCCGTCTGAGG |
| ***Gabra5*** | Gamma-Aminobutyric Acid Type A Receptor Alpha 5 Subunit | GCAGGTGCGAACAGACATCTA | CCTTAAACCGCAGCCTTTCATC |
| ***Gabrb3*** | Gamma-Aminobutyric Acid Type A Receptor Beta3 Subunit | AAGCTGTTGAAAGGCTACGAC | ACTCGATTGTCAAGCGTGAGG |
| ***Gabbr1*** | Gamma-Aminobutyric Acid Type B Receptor Subunit 1 | ACGTCACCTCGGAAGGTTG | CACAGGCAGGAAATTGATGGC |
| ***Gabbr2*** | Gamma-Aminobutyric Acid Type B Receptor Subunit 2 | AAGACCCCATAGAGGACATCAA | GGGTGGTACGTGTCTGTGG |
| ***Gabrg2*** | Gamma-Aminobutyric Acid Type A Receptor Gamma 2 Subunit | GCTCTACCCAGGCTTCACAAG | CCAGCAGGTTGTTTAAGATGACA |
| ***Gria1*** | Glutamate Ionotropic Receptor AMPA Type Subunit 1 | CAAGTTTTCCCGTTGACACATC | CGGCTGTATCCAAGACTCTCTG |
| ***Gria2*** | Glutamate Ionotropic Receptor AMPA Type Subunit 2 | TTCTCCTGTTTTATGGGGACTGA | CTACCCGAAATGCACTGTATTCT |
| ***Grin1*** | Glutamate Ionotropic Receptor NMDA Type Subunit 1 | CTGCGACCCCAAGATTGTCAA | TATTGGCCTGGTTTACTGCCT |
| ***Grin2a*** | Glutamate Ionotropic Receptor NMDA Type Subunit 2a | ACGTGACAGAACGCGAACTT | TCAGTGCGGTTCATCAATAACG |
| ***Grin2b*** | Glutamate Ionotropic Receptor NMDA Type Subunit 2b | CAGCAAAGCTCGTTCCCAAAA | GTCAGTCTCGTTCATGGCTAC |

| | | | |
|---|---|---|---|
| ***Grm5*** | Glutamate Metabotropic Receptor 5 | ACCAACCAACTGTGGACAAAG | CAAGAGTGTGGGATCTGAATTGA |
| | Hypothalamic Function | | |
| ***Ghrl*** | Grehlin | TGTCCTCACCACCAAGACCA | CTGGGCTTTCTGGTGCTCTG |
| ***Ins1/2*** | Insulin 1/2 | GCTTCTTCTACACACCCATGTC | AGCACTGATCTACAATGCCAC |
| ***Lep*** | Leptin | GAGACCCCTGTGTCGGTTC | CTGCGTGTGTGAAATGTCATTG |
| ***NPY*** | Neuropeptide Y | ATGCTAGGTAACAAGCGAATGG | TGTCGCAGAGCGGAGTAGTAT |
| ***NPY1r*** | Neuropeptide Y Receptor Y1 | ACCTTGGCTCTCGCTTATGG | CACGATCAGAATGTTGGTGACA |
| ***NPY2r*** | Neuropeptide Y Receptor Y2 | TCTTTCTCCTACACCCGTATCTG | CGCTGATGGTAATGGTCACTT |
| ***NPY5r*** | Neuropeptide Y Receptor Y5 | ATGAAAAAGCGCAATCAGAAGAC | GAGCAAAACAGGACAACCAAAAT |
| ***Per1*** | Period Circadian Clock 1 | ACCAGCCATTCCGCCTAAC | CGGGGAGCTTCATAACCAGA |
| ***Per2*** | Period Circadian Clock 2 | GAAAGCTGTCACCACCATAGAA | AACTCGCACTTCCTTTTCAGG |
| ***Mc4r*** | Melanocortin 4 Receptor | TGCTCGCATCCATTTGCAG | ATGATCCCGACCCGCCTAA |

## Viral Injection

4-week-old animals were injected via the tail vein with 5µL of AAV9 Syn-GFP diluted in 0.9% NaCl in a final volume of 100 µL. Six weeks after the injection the animals were sacrificed, the brains collected and processed for neuronal imaging.

## Neuron and Spine acquisition and Analysis

Images of pyramidal neurons from the CA1 region of the hippocampus and neurons of the hypothalamus from fixed brain sections were acquired with a LSM 710 Confocal microscope from Zeiss with a Pln Apo 20x/0.8 DICII lens. Each image consisted of a stack of images taken through the z-plane of the section. Confocal microscope settings were kept the same for all scans in each experiment. Neurons expressing GFP were chosen randomly for quantification from at least 4 different sections containing the region of interest and at least 8 neurons were acquired per animal. Spines on primary and secondary dendrites of hypothalamic and hippocampal neurons respectively, were acquired with a Pln Apo 63x/1.4 Oil DICII lens and analyzed. Spine categorization, density and size was performed using Neurolucida software. Each spine was included in one of two categories: immature spines which included filopodia (spines without a defined head) and mature spines which included stubby spines (spines without a defined neck); thin (spines with a neck and head diameter smaller than double the width of the neck) and mushroom spines (spines with a neck and head diameter bigger than double the width of the neck). Sholl analysis was performed using the Neurolucida software.

The investigator was blind to the genotypes during both image acquisition and image analysis.

## Statistical analysis

All graphs represent average values ± SEM. Statistical differences were performed using unpaired student t-test with Welch's correction, Mann-Whitney test, one sample t-test or two-way ANOVA analysis followed by Sidak post test: ***p<0.001, **p<0.01, *p<0.05.

# CHAPTER III | RESULTS

# Results

## *Gprasp2* KO animals show decreased performance in memory and cognition tasks and increased social dominance

Autism spectrum disorder is characterized by deficits in social behaviors, restricted interests and communication problems. Additionally, there is a very strong overlap between ASD and intellectual disability (ID). Several animal models have been developed to understand these disorders by targeting proteins with a role in synaptic signaling. These knockout mice often show deficits in cognition, social behaviors and anxiety-like behavior among other dysfunctions. As previously mentioned, *Gprasp2* KO animals showed memory impairments in a novel object recognition test (Figure 9), which suggests the possibility for this protein to have an impact in memory and cognition.

As memory and cognition include a broad variety of conceptual processes, we tried to dissect and validate the role of Gprasp2 in learning and memory by performing two additional behavioral tests. We performed the Barnes Maze test in order to tackle spatial learning and memory. In the first week of the test, we observed that the KO animals had significantly higher latency to reach the goal box (Figure 11B). In the probe trial (last day of each test, where the goal box is not present) the *Gprasp2* KO animals took longer periods of time to reach the target (Figure 11C), had a displayed tendency to spend less time on the target (Figure 11E, 11F, 11M), had longer latencies to reach the quadrant in which the target was placed (Figure 11N) and "visited" the target approximately the same number of time as the WT animals (Figure 11O). We did not observe a significant difference in terms of strategy in the probe trial between the two genotypes although the WT animals showed slightly lower distances from the target throughout the trial, meaning a higher proximity to the goal hole (Figure 11D).

In the reversal part of the Barnes Maze test (second week) the KO animals showed some improvement when compared to the first week. However, the animals still displayed higher latencies to reach the goal box during the training phase (Figure 11H), higher latency to reach the target hole on the probe day (Figure 11I) when compared to WT animals. They showed a slightly lower time spent in the target box (Figure 11K, 11L, 11M) and lower frequency when it came to target visits (Figure 11O). Regarding the travel patterns on the probe day, despite no significant changes between both genotypes, the KO

animals showed a decreased distance from the target hole during most of the trial (Figure 11J).

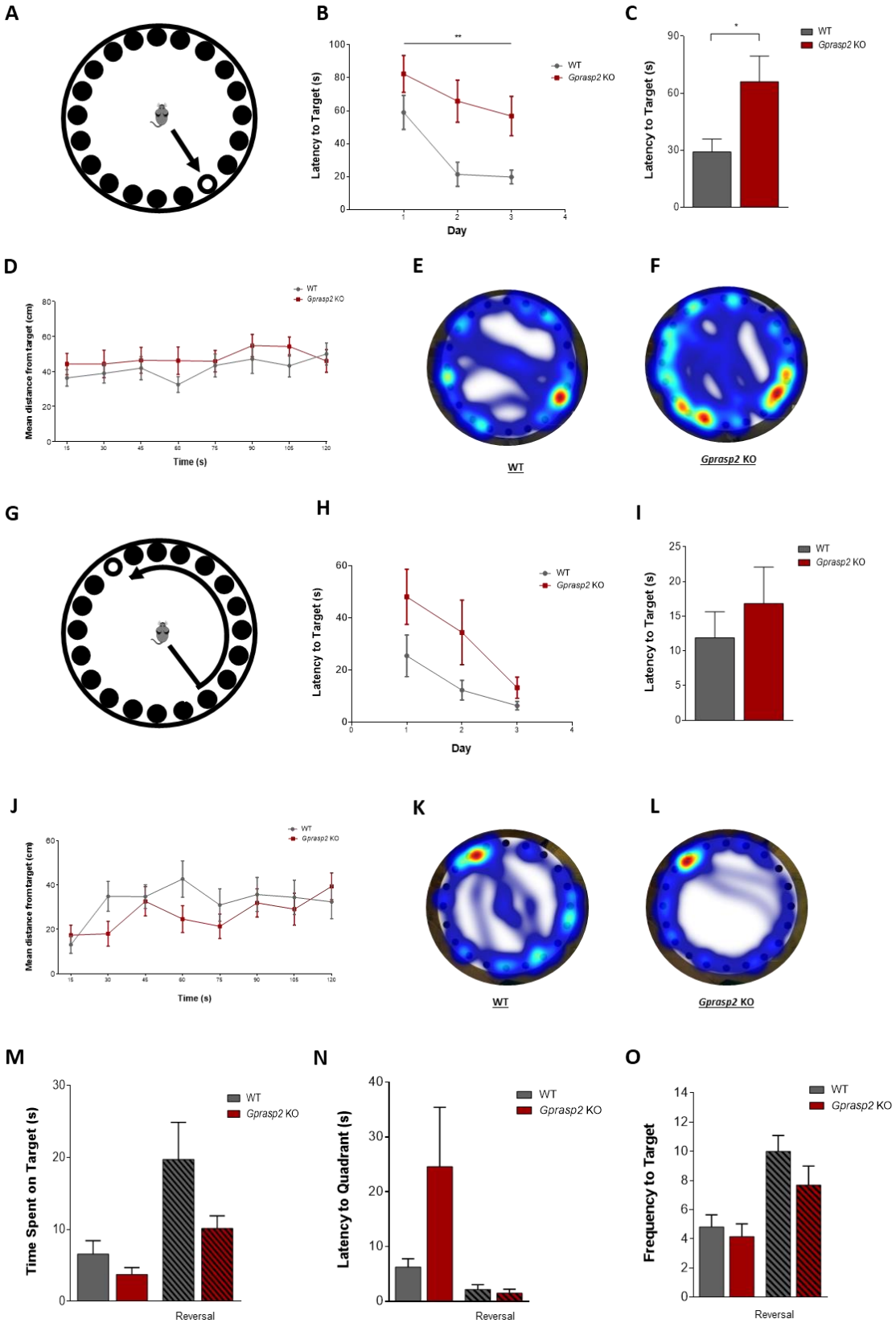

**Figure 11. *Gprasp2* KO mice show spatial and working memory impairments in the Barnes Maze test.** (A-F) Results obtained in the first phase of the test, acquisition. (G-L) Results obtained in the reversal phase of the test. (A, G) Schematic drawing of the Barnes maze platform and the target hole location. (B, H) Latency to reach the target hole during the training trials. (C, I) Latency to reach the target hole during the probe trial. (D, J) Mean distance between the animal and the target hole throughout the probe trial. (E-F, K-L) Representative heat map images from the probe trial from WT and *Gprasp2* KO mice. Data obtained in the probe trial in both acquisition and reversal phase for the following parameters: (M) time spent on the target hole; (N) latency to reach the target quadrant; (O) number of target hole visits (frequency). (WT n=11; *Gprasp2* KO n=13). (C, I, M-O) All values represented as mean ± SEM, statistical comparison was performed using unpaired t-test with Welch's correction *p<0.05. (B, D, H, J) All values represented as mean ± SEM, statistical comparison was performed using Two-way repeated measures ANOVA followed by Sidak test **p<0.01.

The other behavioral test performed to understand the impact of Gprasp2 in memory and cognition was the T-maze test for spontaneous alternation, to assess spatial and working memory. This test is based on the willingness of rodents to forage and explore a new environment, leading to a preference in the exploration of a new arm rather than a familiar one in two consecutive trials. In this test, the *Gprasp2* KO animals showed deficits in spontaneous alternation, with a statistically significant difference in the ability to alternate between the two arms of the maze in two consecutive trials (Figure 12B).

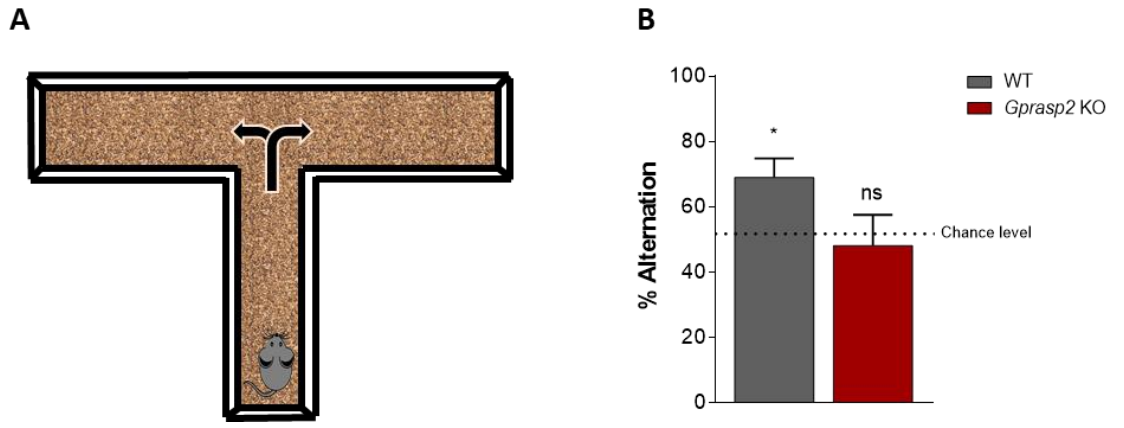


**Figure 12. *Gprasp2* KO mice show spatial memory impairments in the T-maze.** (A) Schematic drawing of the T-maze apparatus. (B) Alternation rates in the T-maze for WT and *Gprasp2* KO animals. WT n=9; *Gprasp2* KO n=10. All values represented as mean ± SEM, statistical comparison was performed using a one-sample t-test against the hypothetical value of 50% alternation at chance level, *p<0.05.

Considering that many animal models of autistic disorders have been shown to have changes in social dominance and aggression and since *Gprasp2* is highly enriched in the hypothalamus (Figure 6C), a region linked to the control of aggressive behavior, we decided to assess if these animals displayed alterations in social dominance. To do so, the tube test paradigm was performed in naive animals. Interestingly, we observed that the *Gprasp2* KO animals displayed a more dominant behavior with a significantly higher percentage of win trials when compared to the WTs in the tube test (Figure 13B).

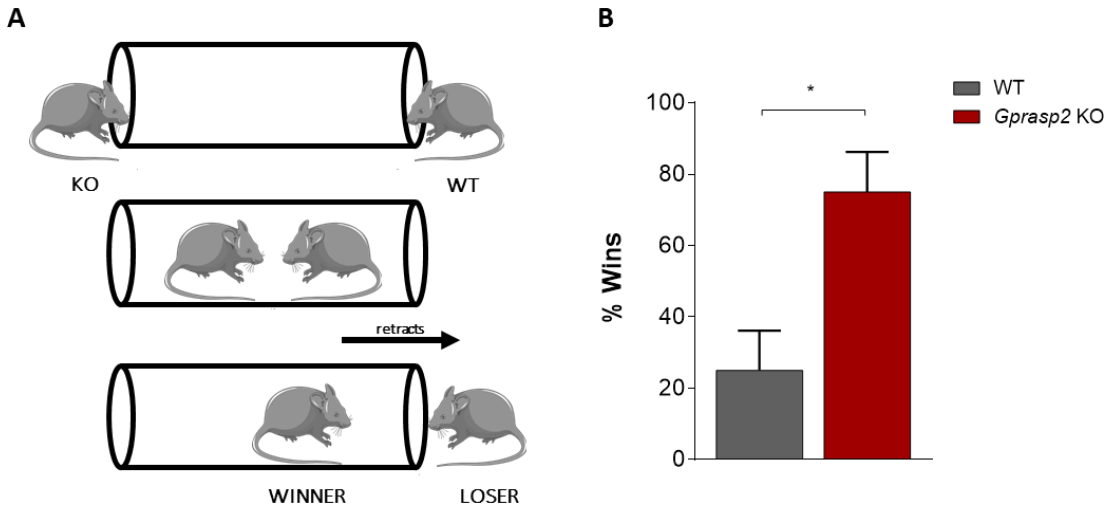


**Figure 13. *Gprasp2* KO mice show increased social dominance behavior.** (A) Schematic drawing of the tube test paradigm. The animals are placed in both ends of the tube (top) during the test the animals interact (middle). The test finishes when one of the animals is pushed or backs out of the tube being considered the loser (bottom). (B) Percentage of wins for WT and *Gprasp2* KO animals. (WT n=16; *Gprasp2* KO n=16). All values represented as mean ± SEM, statistical comparison was performed using a one-sample t-test against the hypothetical value of 50% chance of winning rate, *p<0.05.

## *Gprasp2* KO animals showed increased body weight

Through the handling of the various cohorts of animals we were able to observe that there was a difference in terms of weight between the wild-type animals and KOs when the animals grew older. In order to more accurately assess this variation, naïve cohorts were kept exclusively for weight measurements (i.e. did not run any behavior test) (Figure 14). We observed that at 5 months of age, *Gprasp2* KO mice showed statistically significant higher body weight when compared to age-matched WT animals.

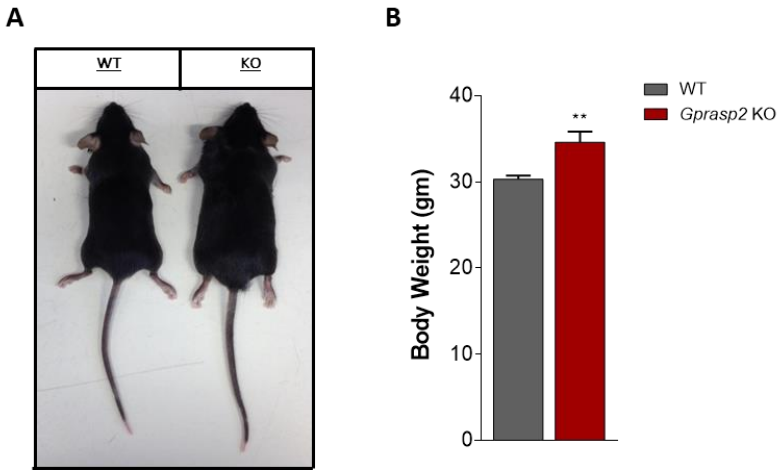


**Figure 14. *Gprasp2* KO mice show increased body weight.** (A) 5 months old male *Gprasp2* KO mice compared with WT (B) Quantification of body weight in *Gprasp2* KO compared to WT mice. (WT n = 13; *Gprasp2* KO n = 13). All values represented as mean ± SEM, statistical comparison was performed using unpaired t-test with Welch's correction **p<0.01.

## *Gprasp2* KO animals present changes in neuronal complexity and spine density in the hippocampus and hypothalamus

With previous in vitro work performed in our lab showing alterations in neuronal complexity and spine density in hippocampal cultures when *Gprasp2* was knockeddown *in vitro* (Figure 7), the knowledge that this protein is highly enriched in the postsynaptic density and that it belongs to a family known to have a synaptic function, the next step was to understand if the loss of this protein *in vivo* would lead to alterations in neuronal morphology. This analysis was performed in two brain regions: the hippocampus and the hypothalamus. The hippocampus was one of the chosen regions in order to more easily correlate this results with the in vitro work and because of the deficits we found in memory and cognition that are strongly related to hippocampal function. The hypothalamus was also selected because of the alterations observed in social dominance and body weight and due to the high expression of Gprasp2 in this region.

In the hippocampus, we were able to observe changes in terms of dendritic complexity between the two genotypes. Neurons from the CA1 region in Gprasp2 KO were less complex (lower number of interactions) specially towards the more medial and distal parts of the neurons (Figure.15C-F). When we look at total dendritic length levels, no significant changes can be observed in terms of total dendritic length between the two genotypes (Figure.15G). However, if we analyze in particular the regions previously

defined, we can see a clear decrease in dendritic length in medial and distal regions of *Gprasp2* KO hippocampal neurons in comparison to WT controls (15H).

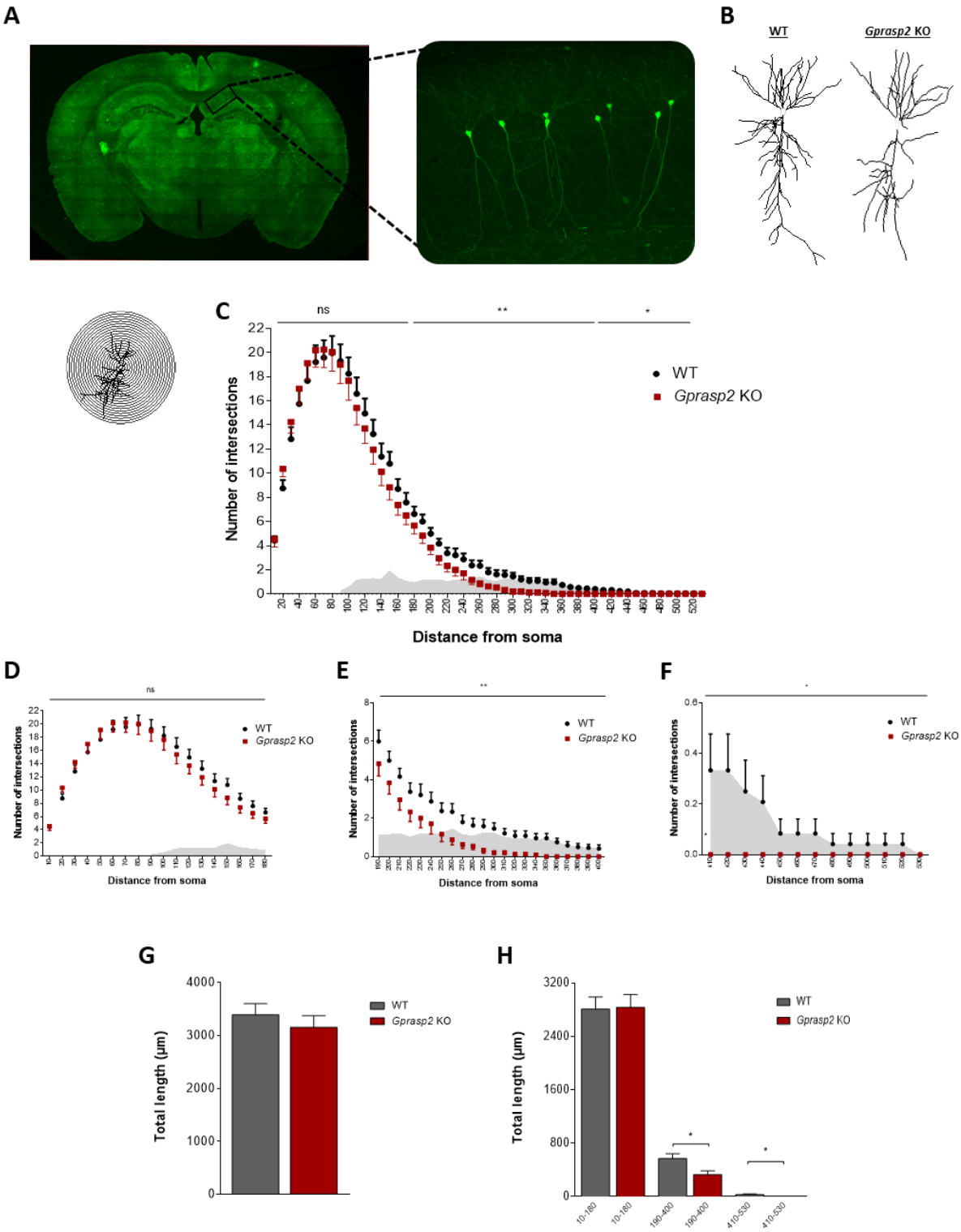


**Figure 15. *Gprasp2* KO mice show decreased dendritic complexity in hippocampal neurons.** (A) Pyramidal neurons were acquired from the CA1 region of the hippocampus. (B) Representative images of the neuronal morphology encountered in WT and *Gprasp2* KO neurons. (C) Sholl analysis quantification measured as number of intersections across the soma with 10 µm radius in Gprasp2 KO neurons compared to

WT control. Sholl analysis compartmentalized in proximal from 10-180µm (C), medial from 190-400µm (D) and distal from 410-530µm (E) sections related to the distance from the soma. (G) Effect of Gprasp2 deletion in total dendritic length and in the different sections (H). (WT n=24 neurons; *Gprasp2* KO n=24 neurons, 8 neurons from 3 different animals/genotype). (C-F) All values represented as mean ± SEM, statistical comparison was performed using Two-way repeated measures ANOVA followed by Sidak test, *p<0.05: **p<0.01. (G, H) All values represented as mean ± SEM, the statistical comparison was performed using the Mann-Whitney test, *p<0.05.

In terms of spine density, the characterization was performed distinguishing apical from basal dendrites. In apical secondary dendrites, no significant changes were observed in terms of total spine density between the two genotypes although a tendency to a decrease in the KO animals was noted (Figure 16B). After due characterization and division of the spines in their two major classes, mature and immature, we can more clearly observe differences in terms of mature spine density, with a statistically significant decrease in KO animals and concomitant tendency for an increase in immature spines (Figure 16B). As for secondary basal dendrites, no significant changes were observed although a tendency for a decrease in both total and mature and immature spine density was observed (Figure 16D).

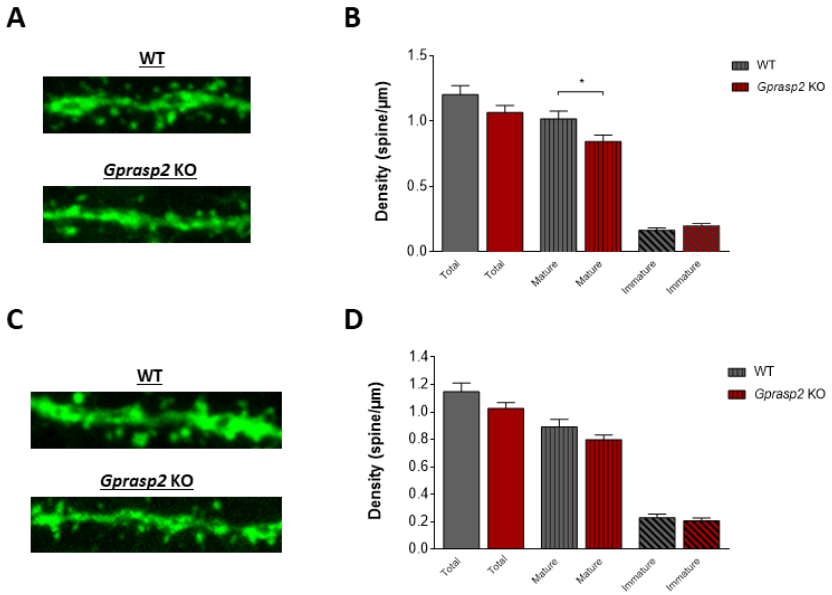


**Figure 16. *Gprasp2* KO mice show alterations in mature spine density.** (A) Representative images of secondary apical dendrites of hippocampal neurons from WT and *Gprasp2* KO mice. (B) Analysis of spine density per 1µm in *Gprasp2* KO apical dendrites and comparison with WT control. (C) Representative images of secondary basal dendrites of hippocampal neurons from WT and *Gprasp2* KO mice. (D) Analysis of spine density per 1µm in *Gprasp2* KO basal dendrites and comparison with WT control (B,D) Dendritic spines were classified into mature or immature spines. (WT n=24 apical/basal dendrites; *Gprasp2* KO n=24 apical/basal dendrites, 8 dendrites from 3 different animals/genotype). All values represented as mean ± SEM, the statistical comparison was performed using the Mann-Whitney test, *p<0.05.

After Sholl analysis of hypothalamic neurons, no significant differences were observed in terms of dendritic complexity or total dendritic length between both genotypes (Figure 17C-D). Also, total, mature and immature spine density did not differ significantly (Figure 17F).

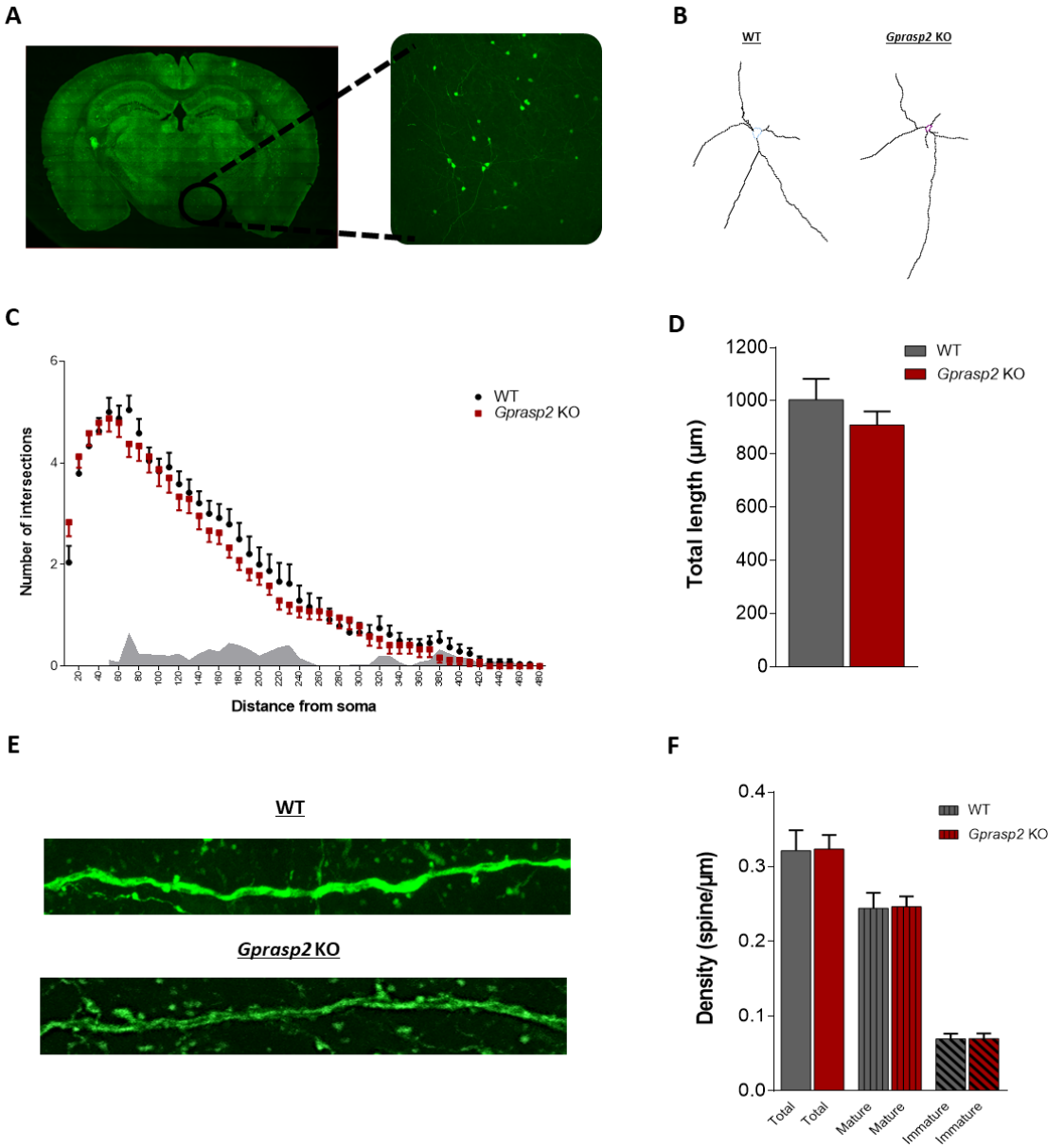


**Figure 17. *Gprasp2* KO mice do not seem to have an effect in hypothalamic neuronal complexity and spine density.** (A) Hypothalamic neurons were acquired and analyzed. (B) Representative images of the neuronal morphology encountered in WT and *Gprasp2* KO hypothalamic neurons. (C) Sholl analysis quantification measured as number of intersections across the soma with 10 µm radius in Gprasp2 KO neurons compared to WT control (D) Effect of Gprasp2 deletion in total dendritic length (E) Representative images of dendrites from hypothalamic neurons from WT and *Gprasp2* KO mice. (F) Analysis of spine density per 1µm in *Gprasp2* KO dendrites and comparison with WT control. Dendritic spines were classified into mature or immature spines. (WT n=24 apical/basal dendrites; *Gprasp2* KO n=24 apical/basal dendrites, 8 dendrites from

3 different animals/genotype). (C) All values represented as mean ± SEM, statistical comparison was performed using Two-way repeated measures ANOVA followed by Sidak test. (D,F) All values represented as mean ± SEM, the statistical comparison was performed using the Mann-Whitney test.

## mRNA analysis

Given that deficits and alterations were observed in this animal model not only at a behavior level, with the memory and cognition deficits, but also at a cellular level, with differences in terms of dendritic complexity and spine density, the next step was to try and understand more broadly what could be the basis of this changes in terms of signaling and synaptic structure.

To do so, we took advantage of microfluidic chambers that allowed us to performed RT-qPCR reactions for a wide number of samples with the ultimate goal of detecting quantitative changes in a wide range of genes that: i) coded for other members of the Gprasp family, ii) proteins that have an effect in mGluR signaling, iii) proteins present at the synapse, iv) various receptor subunits and vi) regulators of hypothalamic function. To have a better understanding of what would be the effect of Gprasp2 deletion in a more general capacity we combined the results obtained from the 5 different brain regions from into a single pool referred to as "whole brain" herein. The results were divided depending on the function of the protein that each gene codes for: Gprasp family, mGluR signaling, synapse structure, receptors and hypothalamic function, as mentioned above.

At PND20, other members of the Gprasp family, showed a slight tendency for an increase in expression of mRNA in the whole brain KO samples (Figure 18A) however, when we analyzed the expression specifically in the hippocampus, we can find a tendency for an overall downregulation (Figure 18B). As for members of the mGluR signaling pathways, at a whole brain level most genes seem to be unaltered with exceptions in *CamkIIa*, *Akt*, *Mapk1*, *Tsc1* and *Tsc2* that seem to be slightly upregulated (Fig. 18C). At a hippocampal level, there seems to be a statistically significant downregulation for *Adcy5* and *PTEN* genes (Figure 18D). At a synaptic structure level, we observed an overall upregulation at a whole brain level, especially in *Homer 3* and *Nlgn1* (Figure 18E) but not in the hippocampus (Figure 18F). In this specific region, we can observe varying degrees of downregulations in *Dlg1, Gphn*, *Homer 1* and *Syt17* mRNA levels (Figure 18F). When the mRNA levels of specific receptors are analyzed, we observed alterations in *GABAAR* and *Drd1* in the whole brain level (Figure 18G). Trends for upregulated *GABABR*, *AMPAR* and *NMDAR* are also of note (Figure 18G), although we lacked analytical power to confirm

these alterations. In the hippocampus, we also noted an overall downregulation in GABAA, AMPA receptor subunits (*Gria 1*, *Gria 2*) and metabotropic glutamate receptor *Grm5* (Figure18H). Regarding hypothalamic function genes in whole brain samples, we can observe that most do not shown any alterations with the exception of *NPY* and *NPYr* which appeared to be slightly upregulated (Figure 19A).

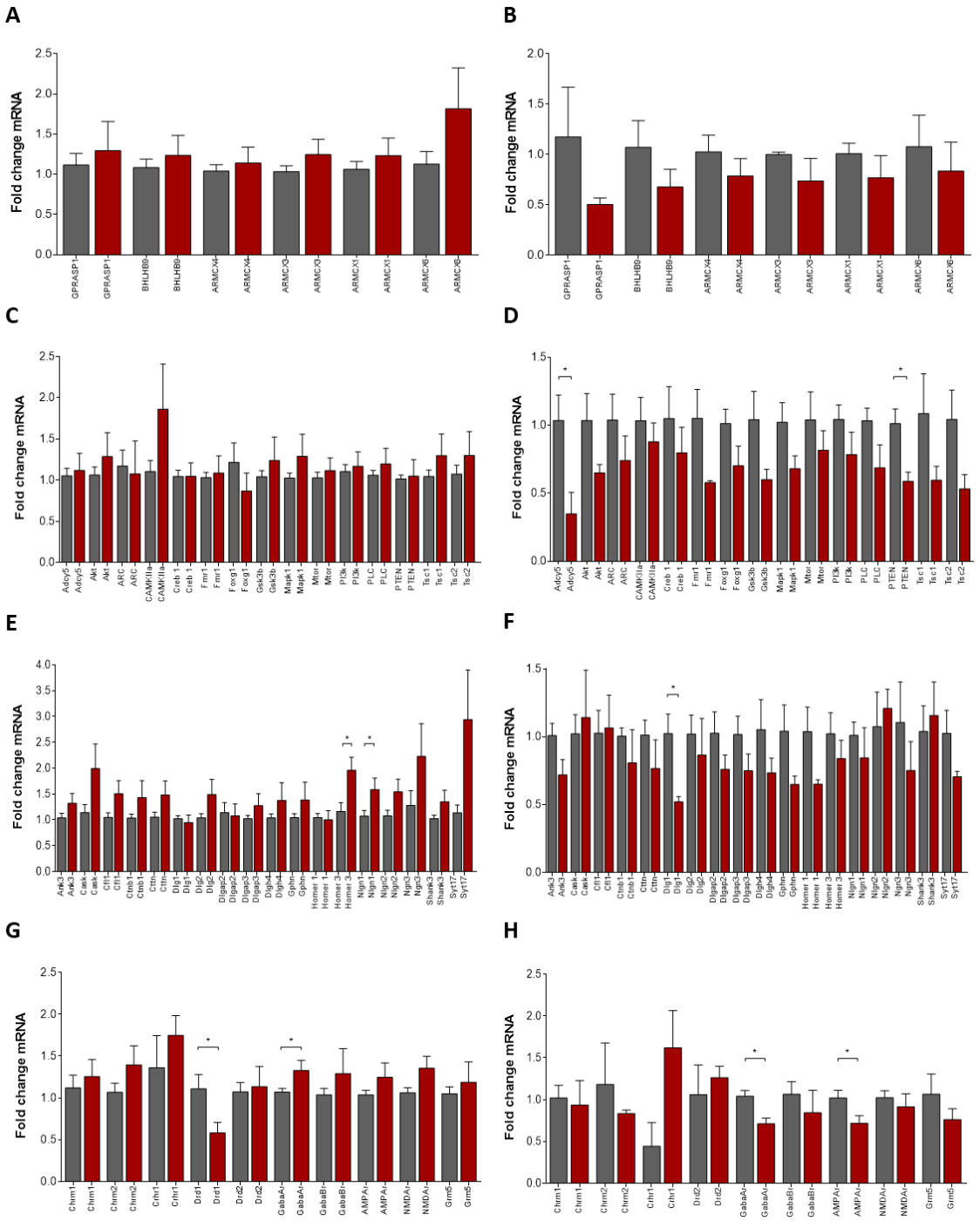


**Figure 18. Quantitative RT-PCR analysis of the various selected genes from samples obtained at PND20 from WT and *Gprasp2* KO animals.** Data are shown as fold changes relative to age-matched WT controls. (A, C, E, G) Results obtained from "whole brain" samples (combination of the results attained in the

hippocampus, hypothalamus, cortex, striatum and cerebellum). (B,D,F,H) Results obtained from hippocampal samples. The results were subdivided into groups pertaining to the gene function: (A-B) Members of the Gprasp2 family; (C-D) Genes selected by their involvement in mGluR signaling; (E-F) Genes selected due to their function in synaptic structure; (G-H) Receptor subunits. ("Whole brain" n=15/genotype, 3 samples/region; Hippocampus n=3 animals/genotype). All values represented as mean ± SEM, the statistical comparison was performed using the Mann-Whitney test, *p<0.05.

At PND 90, regarding other members of the Gprasp family, no significant alterations can be observed at a whole brain or hippocampal level (Figure 20 A and B, respectively). As for genes involved in mGluR signaling pathways, a slight tendency for an increase in expression of mRNA in the KO samples was observed at a whole brain level, especially in *PLC* subunits (Figure 20C). In the hippocampus, *mTor* appeared slightly upregulated and *ARC*, *PI3k* and *Tsc1* slightly downregulated (Figure 20D). As for transcripts involved in synaptic structure, at a whole brain level there seemed to be an overall modest upregulation of mRNA expression (Figure. 20E), however, this was not observed in the hippocampus, were most genes seem to be unaltered (with the exception of *Nlgn3* that seems to be slightly downregulated) (Figure 20F). When the mRNA levels of specific receptor subunits were analyzed, we noted a slight overall upregulation at a whole brain level especially in GABAA receptors (Figure 20G). In the hippocampus, these alterations were not identified (Figure 20H). Regarding genes involved in "hypothalamic function" in the whole brain sample, we noted a slight increase throughout, with a significant upregulation of *NPYr* (Figure 19B).

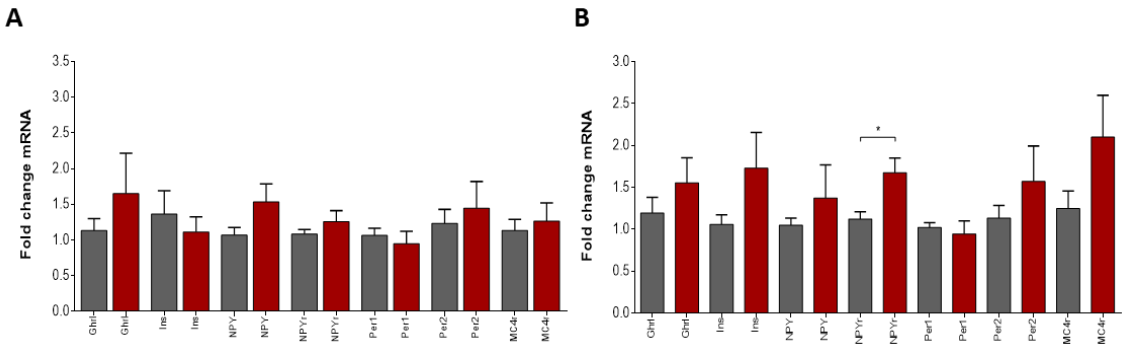


**Figure 19. Quantitative RT-PCR analysis of the various selected genes from samples obtained WT and *Gprasp2* KO animals.** Data are shown as fold changes relative to age-matched WT controls. (A, B) Results obtained from "whole brain" samples (combination of the results attained in the hippocampus, hypothalamus, cortex, striatum and cerebellum) for reactions targeting genes involved in hypothalamic function. (A) Samples obtained from PND20 animals. (B) Samples obtained from PND90 animals. ("Whole brain" n=15/genotype, 3 samples/region). All values represented as mean ± SEM, the statistical comparison was performed using the Mann-Whitney test, *p<0.05.

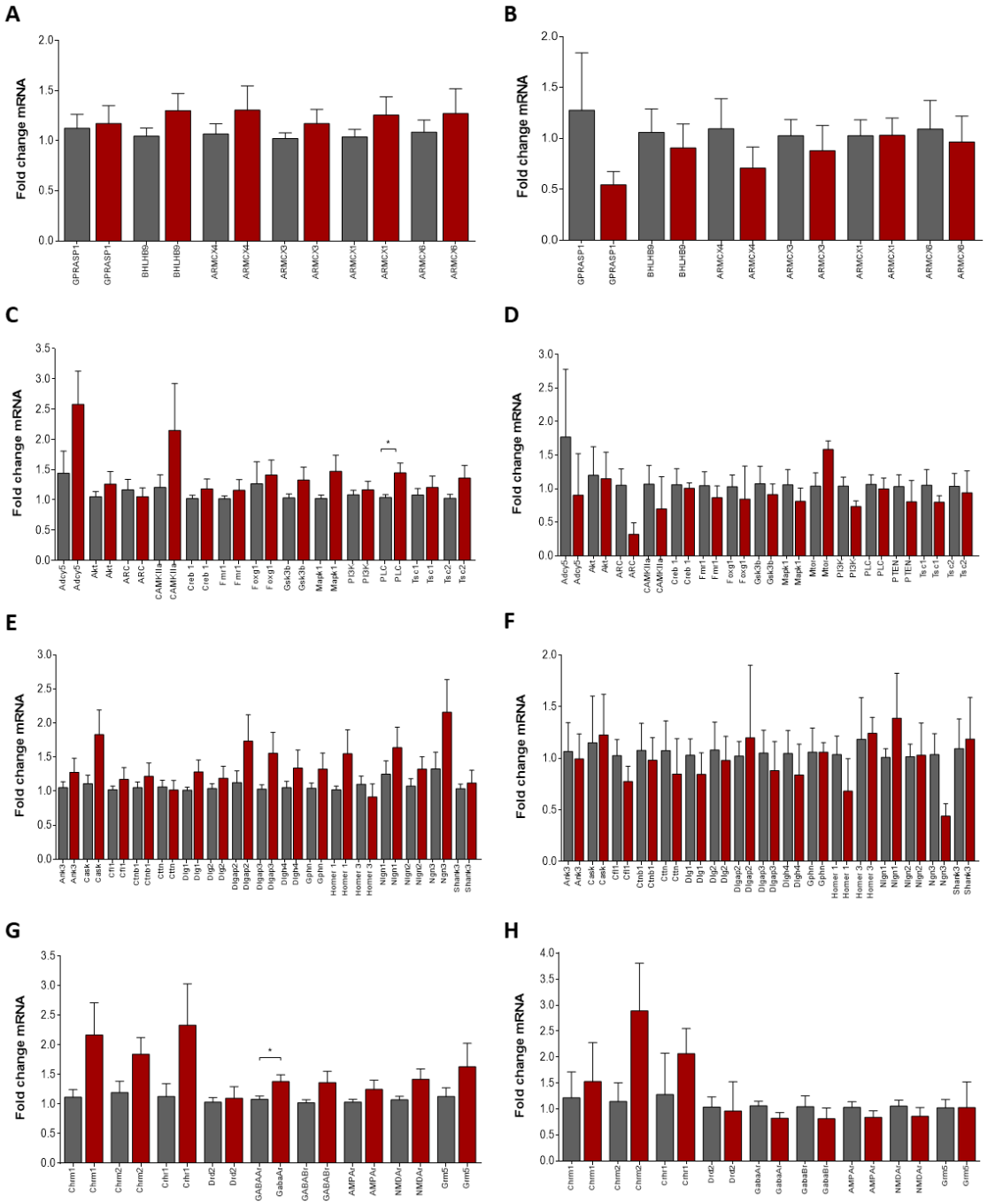


**Figure 20. Quantitative RT-PCR analysis of the various selected genes from samples obtained at PND90 from WT and *Gprasp2* KO animals.** Data are shown as fold changes relative to age-matched WT controls. (A, C, E, G) Results obtained from "whole brain" samples (combination of the results attained in the hippocampus, hypothalamus, cortex, striatum and cerebellum). (B,D,F,H) Results obtained from hippocampal samples. The results were subdivided into groups pertaining to the gene function: (A-B) Members of the Gprasp2 family; (C-D) Genes selected by their involvement in mGluR signaling; (E-F) Genes selected due to their function in synaptic structure; (G-H) Receptor subunits. ("Whole brain" n=15/genotype, 3 samples/region; Hippocampus n=3 animals/genotype). All values represented as mean ± SEM, the statistical comparison was performed using the Mann-Whitney test, *p<0.05.

For a more summarized view of the results obtained by this analysis, Table 2 shows all the significant alterations observed in *Gprasp2* KO samples when compared to WT controls. This work was performed as a pilot experiment to guide future experiments and as such, advanced statistical analysis, power analysis or false discovery rates were not performed.

**Table 2. Significant changes observed after qRT-PCR analysis in *Gprasp2* KO samples when compared to WT controls at different ages.** (↑ - upregulation; ↓ - downregulation)

| | "WHOLE BRAIN" | HIPPOCAMPUS |
|---|---|---|
| PND20 | • ↑ *Homer3*<br>• ↑ *Nlgn1*<br>• ↑ *GabaAr*<br>• ↓ *Drd1* | • ↓ *Adcy5*<br>• ↓ *PTEN*<br>• ↓ *Dlg1*<br>• ↓ *GabaAr*<br>• ↓ *AMPAr* |
| PND90 | • ↑ *PLC*<br>• ↑ *GabaAr*<br>• ↑ *NPYr* | |

# CHAPTER IV | DISCUSSION & FUTURE PERSPECTIVES

# Discussion and Future Perspectives

Autism spectrum disorders have been gaining increasing levels of attention due to their high prevalence and wide range of phenotypes that do not seem to possess a common genetic or molecular background. In recent years, the mGluR signaling pathway has started to gain recognition as a possible core dysfunction found in many models of ASD [22]. The *Gprasp2* KO animal model may shed new insight into possible dysregulations in these neuropsychiatric disorders given that Gprasp2 not only regulates mGluRs it has also been implicated in human neurodevelopmental disorders [98-100].

As mentioned before, previous work has been developed towards the characterization of the *Gprasp2* KO model and the role of Gprasp2 in normal neuronal function. The results and conclusions obtained from the work here reported aim at further characterizing and validating these mice complementing the results previously obtained with behavioral and neuronal morphology characterization and preliminary mRNA analysis.

Previous work has shown hippocampal deficiencies in the *Gprasp2* KO animal model. The hippocampus is a region well known for its impact in memory formation and changes in synaptic strength (including alterations in mGluR-dependent LTD) and synaptic structure in this region have for long been linked with alterations in cognitive function [102, 103]. A physiological impact of this alterations is observed in the *Fmr1* KO mouse model which has been shown to present increased mGluR-induced LTD in the hippocampus [32, 35].

At a behavior level, deficits in the novel object recognition test, a test that targets memory, were observed in the *Gprasp2* KO mice. Additionally, electrophysiological experiments provided evidence that the *Gprasp2* KO mice exhibited increased mGluR-dependent LTD following DHPG stimulation in the CA1 region of the hippocampus; similarly, to what is found in *Fmr1* KO mice. With the work done in this study we showed that the *Gprasp2* KO animals possess deficiencies in spatial learning and working memory in the Barnes Maze. We also performed a reversal task in the Barnes Maze to evaluate behavior flexibility and even though a difference can be noted between the two genotypes, it is important to note that the KO animals improved from the first week to the reversal (decreased latencies throughout the reversal) which might be an indication that these animals do not have complete impairment in learning but that this learning process might

be to some degree delayed. This deficit is consistent with impaired cognitive performance as was also detected in the spontaneous alternation in the T-maze. In this task the *Gprasp2* mutant mice showed a decreased ability in alternating between the two different arms of the maze in two consecutive trials, interpreted here as an inability to distinguish between a familiar (previously explored) and a novel arm.

These behavioral deficiencies are also observed in other well-studied animal models like the *Cntnap2*$^{-/-}$ *and the Fmr1* KO mice model per example, one of the most used models for FXS (one of the most commonly inherited forms of intellectual disability and monogenic causes of ASD). These models have been shown to have deficits in terms of spatial learning and memory when submitted to these tests or other that have a similar goal [104, 105], establishing a link between the *Gprasp2* KO model and other well-known models of ASD, both at a behavioral and physiological level.

In order to understand if a possible physiological alteration could be behind the deficits observed in the behavioral characterization of the *Gprasp2* KO mice we explored the dendritic and spine morphology of neurons in the hippocampus. Dendritic branches and spines have been shown to be major regulators of neuronal function and neuronal circuitry plasticity and alterations in the morphology, function and density of this neuronal components have been shown to be present in neuropsychiatric disorders such as ASDs and ID being these changes linked to the deficits encountered in behavior in animal models like *Tsc2*$^{+/-}$ and *Fmr1* KO [32, 33, 106]. Here, we showed that *Gprasp2* KO mice present a decrease in dendritic complexity in pyramidal neurons of the CA1 region of the hippocampus when compared to WT animals. This difference was shown to be more prominent in medial and distal regions of branches. We also determined spine density and found a slight decrease in the number of spines per µm in secondary apical dendrites, these changes were found to be mostly due to a decrease in mature spine density. In basal secondary dendrites from pyramidal neurons of the CA1 region of the hippocampus, no significant changes were observed between the two genotypes. These results converge towards what was observed in the *in vitro* work previously performed in the lab, in which the knockdown of Gprasp2 through shRNA in hippocampal cultures resulted in a decrease in dendritic complexity and spine density, further validating the idea that indeed Gprasp2 could possibly have a role in neuronal complexity and spine maturation.

Given the decrease in mature spine density observed in apical dendrites in the hippocampus of *Gprasp2* KO mice it might be plausible to say that Gprasp2 could have a

role in the maintenance of mature spines. The increase observed in mGluR-dependent LTD is correlated with this alteration since, as mentioned, LTD has already been linked to a decrease in spine number through elimination of synapses [34], which in this case, can lead to the presence of less mature synapses. Spine and PSD analysis through electron microscopy could be a helpful technique to dissect *in vivo* morphological changes in both excitatory and inhibitory synapses of *Gprasp2* KO mice to better understand the role of Gprasp2 in spine maturation.

Alterations and deficits in terms of dendritic arborization and spine morphology are, as previously mentioned, two common features of animal models of neuropsychiatric disorders. Similar disruptions to the ones here observed in the *Gprasp2* KO mice model have been reported in other models that mimic ASDs and mental retardation, namely the *Cyfip1*$^{+/-}$ model and the *Mecp2* KO model that show decreased dendritic complexity and spine density in the hippocampus, respectively [107, 108].

Taken together, through behavioral tests and morphology analysis, we were able to provide evidence for the impact of Gprasp2 in hippocampal-dependent learning, neuronal complexity and spine maturation that are similar to previous observations done in mouse models of syndromic autism and ID, as mentioned [32]. The high expression levels of Gprasp2 in the hippocampus (observed through both western blot analysis and in situ hybridization), the changes observed in neuronal complexity and spine maturation through knockdown of Gprasp2 in hippocampal neuronal cultures and the alterations observed in synaptic plasticity previously reported further solidify the results here obtained and strengthen the role of Gprasp2 in hippocampal function.

As an attempt to understand and dissect the impact of Gprasp2 at a deeper level, we performed an exploratory study to try and detect small alterations at the mRNA level in these animals in different brain regions and at different ages. We were able to target different neuronal functions and signaling pathways that could in some way be related to the regulation performed by this family of proteins, more specifically Gprasp2.

From the analysis and combination of the results obtained we were able to conclude that at PND20 (age at which Gprasp2 seems to be highly expressed in wild-type animals) in the hippocampus an overall downregulation of mRNA levels of substrates of signaling pathways, synaptic structure proteins and receptors seems to be present. At PND90, this overall downregulation is to some degree present, not as evident in terms of

synaptic structure where the mRNA levels seem to return to "normal" WT levels, but still noticeable in terms of signaling substrates and receptor subunits.

Two possible distinct lines of thought can to some degree explain the variations observed. Based on the fact that Gprasp2 is known to be present at the post synaptic density, that its loss was shown here to lead to spine alterations and the fact that other members of the family were previously reported as having an impact in transcription [80, 90] it is plausible to link the loss of this protein to high levels of synaptic dysregulation that ultimately lead to this overall decrease in mRNA levels. Steering into this last option are the results obtained regarding AMPA receptor subunits mRNA expression. mEPSC recordings performed in Gprasp2 KO hippocampal slices, suggested a decrease in the surface levels of AMPA receptors in these animals in basal conditions (Joana Guedes, personal communication). These results added to the downregulation observed in the mRNA levels of these receptors hint towards a possible synaptic dysregulation and overall decrease in AMPA receptors. Another line of thought is based on the changes in synaptic plasticity observed in *Gprasp2* KO neurons. The increased LTD and mGluR signaling could possibly trigger a compensatory mechanism in the cell to try and downregulate certain genes in order to reach homeostatic levels. The changes observed between PND20 and PND90 might be due to the expression levels of Gprasp2 in "normal conditions" since it was shown that this protein's expression reaches a peak around PND15 and starts decreasing after that which would explain to some extent the higher impact and the biggest differences being observed at PND20.

Of course, these are alterations observed at a mRNA level and we do not have sufficient information to understand if these translate to the protein level or if they are distinct in any way. Therefore, additional analysis needs to be performed so these results can be further validated and a stronger hypothesis formed.

Also, these results are preliminary, as the number of samples is relatively low which leads to a high degree of variability and deviation that may mask potential alterations, as such, we provide only a broad picture of what might be happening.

However, the combination of the results obtained for mRNA expression, the behavioral deficits and the anomalies found in dendritic complexity and spine density in the *Gprasp2* KO are sufficient to confidently say that this protein plays an important function in the hippocampus and that the lack of Gprasp2 leads to hippocampal and hippocampal-dependent deficiencies.

To have a broader understanding of what were the effects of the *Gprasp2* deletion in terms of mRNA levels, we combined the results obtained from the different brain regions. When doing this analysis, an overall dysregulation of genes involved in synapse structure, mGluR signaling and receptor subunits can be observed in both ages. This variation in results, when compared to hippocampus alone, further validates the need and importance for a more specific analysis. It also means that Gprasp2 might have a differential effect depending on the region being analyzed. Nevertheless, there are still some alterations worth mentioning:

Significant downregulation of *Drd1* was observed at PND20. Dopamine D1 receptors are highly abundant G-protein coupled receptors in the central nervous system having already been established as susceptibility genes for ASD given their role in social cognition and previous genetic link to families where ASD are present [109, 110]. With this in mind, exploring a possible dopaminergic deficit could be interesting to understand the social deficits (in the 3-chamber test and social dyadic paradigm) observed previously in the *Gprasp2* KO mice.

One alteration that seemed to be present throughout the different ages and regions, although with some disparity, was the deregulation observed in GABA receptor subunits. One of the major theories for the disruptions in normal function of sensory and cognitive brain networks observed in ASD and ID patients is based on the excitation and inhibition unbalance seen. Alterations in GABAergic signaling and glutamatergic levels are often observed in patients, being this thought to be behind the behavioral and cognitive dysfunctions reported [25, 26]. The alterations observed at an mRNA level could then be an indication that the GABAergic signaling is to some degree defective in the *Gprasp2* KO mice and that this might be one of the causes behind the cognitive deficits encountered.

Again, although the deletion of Gprasp2 in vivo resulted in significant changes in mRNA levels, analysis of the protein levels by Western-Blot could help understand whether Gprasp2 is important for the maintenance of the structures and macromolecular complexes here targeted.

The hypothalamus was one of the other regions were Gprasp2 was shown to be highly expressed in wild type animals. With this in mind the idea of a possible hypothalamic dysfunction was tackled. Dysregulations in hypothalamic function, given the broad spectrum of hypothalamic roles can have an effect in many physiologic functions

and behaviors. We started by understanding if the *Gprasp2* mutant mice displayed alterations in terms of aggressive behavior. Particularly, since alterations in terms of aggression are common in patients that suffer from autistic disorders and ID. However, various types of aggressive behavior have been found in the animal models [111-113].

We started by performing the tube test paradigm, usually used to assess social dominance through the measurement of non-violent aggression [114]. Here, the *Gprasp2* KO mice showed increased social dominance with a higher percentage of wins. In a great part of the trials the animals won by pushing the opponent out of the tube, showing signs of aggression, hinting as a possible hypothalamic dysfunction. More behavior tests that target aggression circuits, such as the resident-intruder paradigm, should be performed to further validate the dysregulation observed in these animals.

Changes in terms of body weight were also observed in this animal model, with the *Gprasp2* KO mice showing significant increases in weight at later stages of development when compared with WT animals. There has been increasing evidence supporting the concurrence of neuropsychiatric disorders and metabolic disturbances such as diabetes and obesity both in animal models and human studies, which suggests the possibility of shared pathophysiological mechanisms [115]. In ASDs, the prevalence of obese children does not differ significantly from the general population of children. However, sleep deprivation, the food selectivity and the impairments in motor development make these children more vulnerable to other risk factors and make it harder to control and treat the disorder [116]. The hypothalamus is, as mentioned, a "feeding control center" of the brain, and problems in hypothalamic function are often noticed through changes in body weight and obesity [117], further solidifying a possible link between hypothalamic function and Gprasp2.

With this two clues pointing towards a possible hypothalamic dysfunction, we looked into possible changes in terms of neuronal morphology, complexity and dendritic spine density in this brain region. No significant alterations were observed in terms of dendritic complexity and spine density, however, our study may be lacking in statistical power, so no strong conclusion may be drawn.

The hypothalamus is known to regulate a wide variety of physiological functions and behaviors and does so through different nuclei and different neuronal populations within those nuclei. This wide range of neuronal populations, that have different morphologies and contents, might explain the dispersion of results obtained in the sholl

analysis as no specific region or neuronal morphology was targeted while performing the imaging acquisition. To overcome this issue, specific staining for each neuronal population would have to be performed in order to be able to have a targeted analysis and correlate the possible alterations observed with specific hypothalamic functions and neuronal populations.

"Whole brain" mRNA analysis showed, at both ages studied (PND20 and PND90), a slight upregulation of the mRNA levels of various NPY receptors, associated with a slight increase of NPY mRNA levels at PND20. Stimulation of NPY-expressing neurons is known to be linked to the stimulation of feeding [46]. NPY receptors are G-protein coupled receptors and although a direct connection between this family of receptors and Gprasp2 is not yet known, it seems plausible that a lack of regulation of this receptors by Gprasp2 could lead to an increase in their production which would ultimately led to an increase in NPY/feeding signaling causing the increase in body weight observed in these animals. Again, these are only results from an exploratory study so more work needs to be done in order to comprehend in detail what might be the causes being this physiological disturbance.

The Prader-Willi syndrome is one example that mimics to some degree the dysregulations observed in this animal model being there a high comorbidity between this disorder and ASDs and ID. Patients with this disorder suffer from obesity, hyperphagia, increased aggression and cognitive deficits all of which has been linked to an hypothalamic dysregulation [53], providing some insight into the reach that this type of dysregulations can achieve.

All the results obtained in this thesis project, point towards a possible function of Gprasp2 both in hippocampal and hypothalamic function. Of course, the exact mechanism through which Gprasp2 functions is still not understood, and this is a crucial step to unravel how the changes here observed can be linked at a mechanistical level. However, this work generates lines of thought and possibilities to be further explored. How does Gprasp2 impact hypothalamic function? Is it done through alterations in a specific neuronal population, mainly Agrp neurons? Regarding its hippocampal impact: Is the lack of Gprasp2 causing alterations at synaptic structural level or is the effect only seen through signaling dysregulations? Is the pharmacological blockade of mGluR activity able to rescue the memory deficits encountered? Is Gprasp2 having an impact in just these two brain regions or is its effect more global?

In terms of future directions, it would be interesting to see if the impact this protein has shown in terms of cognition may be reversed by pharmacological manipulation of mGluR activity.

Hypothalamic function also seems to be affected by the loss of Gprasp2. Alterations in terms of body weight, changes in terms of social dominance and aggression and upregulation of receptors known to mediated feeding were observed in these animals. This region-specific impact needs to be further analyzed with more behavioral tests and a more detailed and specific characterization of the different neuronal populations present in this region to better comprehend the mechanisms and signaling pathways affected by this loss.

Better comprehending the interaction between Gprasp2 and mGluRs and their impact in terms of behavior can help reveal new therapeutic targets to ameliorate the deficits in behavior and mGluR signaling observed in ASD and ID patients.

Additionally, in order to comprehend the region and circuit-specific alterations caused by the loss of Gprasp2, our conditional model could be crossed with different Cre lines or the administration of viral vectors containing specific Cre recombinases for different neuronal populations could be performed.

Taken together, this work has identified learning and memory defects in *Gprasp2* KO mice, highlighted a role for *Gprasp2* in the regulation of neuronal structure and spine morphology in the hippocampus, and provided first evidence for the role of Gprasp2 *in* hypothalamic relevant behaviors.

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