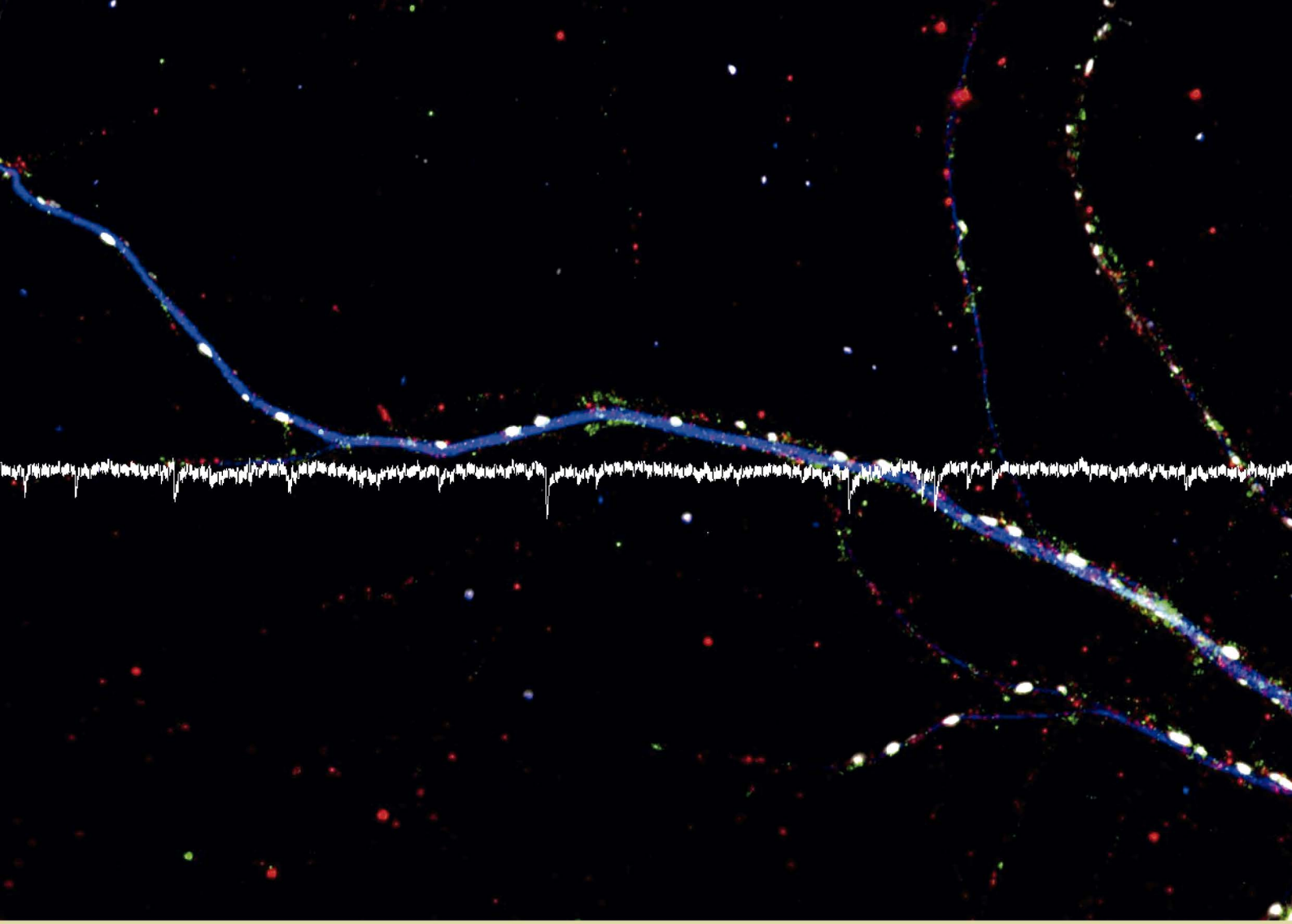

Inês Baião Santos

ARHGAP8 co-regulates excitatory and inhibitory synapse function

Dissertação de Mestrado em Biologia Celular e Molecular, especialização em Neurobiologia,
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UNIVERSIDADE DE COIMBRA

Cover illustration: Immunofluorescent stainings of MAP2, ARHGAP8, gephyrin and VGAT in dendrites of rat cortical neurons. Overlaid is an AMPAR-mediated mEPSC recording of a GFP-expressing cell.

The present work was performed in the Synapse Biology Laboratory of the CNC – Center for Neuroscience and Cell Biology (Universidade de Coimbra, Portugal), headed by Professor Ana Luísa Carvalho. The research work was supervised by Jeannette Schmidt and Ana Luísa Carvalho.

This work was supported by a NARSAD Independent Investigator Grant from the Brain and Behavior Research Foundation, by national funds through the Portuguese Science and Technology Foundation (FCT; PTDC/SAU-NMC/4888/2014 and UID/NEU/045S39/ 2013), by the European Union – European Fund for Economic and Regional Development funding through the Operational Competitiveness Program (COMPETE), and by Programa Mais Centro (CENTRO-07-ST24-FED ER-002002, 002006, 002008).

Agradecimentos

Na fase final de mais uma etapa, não poderia deixar de agradecer a todos os que contribuíram para o meu crescimento enquanto pessoa, enquanto estudante, enquanto cientista, e que me incentivaram ao longo deste último longo e desafiante ano. Sem estes contributos, esta investigação não teria sido possível.

Em primeiro lugar, deixo o meu profundo agradecimento à Professora Ana Luísa Carvalho, por me ter dado a oportunidade de integrar o seu grupo e realizar este projecto de Mestrado sob a sua orientação; pelos conhecimentos transmitidos e, especialmente, pela entusiástica forma de contornar as frustrações que a Ciência acarreta, pela disponibilidade, preocupação e motivação que sempre demonstrou.

To Jeannette Schmidt, I cannot be thankful enough. For your support over the past year, particularly during periods of insecurity and self-doubt, thank you for always reaching to me with helpful advice. I truly learned a lot from your supervision and kindness.

Quero agradecer ainda ao restante clã *ALC*, à Gladys, Dominique, Mariline, Tatiana, Sandra e às mais recentes adições, Beatriz, Débora e Marina, e ainda ao Tiago, pela recepção, por estarem sempre disponíveis para ajudar e pelas histórias e conhecimentos partilhados. Deixo ainda um agradecimento ao Paulo Pinheiro pela ajuda prestada e pelos conhecimentos transmitidos.

Agradeço também aos *CBD* e aos *Peças* por directa ou indirectamente terem contribuído para este percurso e, em especial aos colegas do Mestrado em Biologia Celular e Molecular, por todos os risos e frustrações compartilhadas e por animarem os encontros nocturnos aleatórios no CNC.

Aos meus amigos de sempre que, apesar de toda a ausência desde que comecei a jornada da minha formação em Coimbra, sempre me fizeram sentir a sua amizade, apoio e confiança incondicional. A todo o grupo, mas em especial ao Azra, à Nicole e à Raquel – porque sem vocês não seria a pessoa que sou hoje.

Às amizades que Coimbra me proporcionou; esta cidade moldou-me nas mais diversas formas porque tive a oportunidade de a viver convosco. Não podia deixar de salientar a Jú e a Bea por terem acompanhado todas as aventuras durante os anos da licenciatura,

a Beatriz pela companhia, paciência e motivação ao longo deste último e complicado ano e ainda, a Mariana pelas infindáveis conversas. Por fim, ao Ricardo e ao Patrick, não só pelos primeiros anos mas também pelo agradável reencontro no CNC, e ainda à Patrícia pela amizade ao longo dos últimos cinco anos, dentro e fora da faculdade.

Por último, deixo os meus agradecimentos mais importantes.

Às Mondeguinas – Tuna Feminina da Universidade de Coimbra, por todas as amizades, por todos os sorrisos, histórias, cantigas e desventuras incontáveis, por todas as terras que visitei, pessoas que conheci, pelo crescimento pessoal, pelos momentos indescritíveis… nunca vos poderei agradecer o suficiente. Coimbra será sempre sinónimo de capas negras e malmequeres.

Ao Pedro, por tudo o que vivemos ao longo do último ano e por todos os sonhos e aspirações que partilhamos. Sem o teu apoio constante e visitas inesperadas, esta caminhada teria sido muito mais difícil.

Ao meu irmão, que mesmo longe sempre fez sentir a sua segurança e confiança na minha capacidade em alcançar aquilo a que me proponho – não podia ter melhor exemplo.

Aos meus pais, que não o querendo, merecem o meu mais sentido agradecimento. Por todo o amor ao longo de uma vida, por desde pequena me incentivarem, pelo apoio e confiança incondicional e por toda a ajuda na superação dos obstáculos que ao longo deste percurso foram surgindo. O meu mais sincero, obrigada.

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Abbreviations

aa	Amino acids
AMPA	Alpha-amino-3-hydroxy-5-methyl-4 isoxazole proprionic acid
AMPAR	Alpha-amino-3-hydroxy-5-methyl-4 isoxazole proprionic acid receptor
AP-V	(2R)-amino-5-phosphonovaleric acid
ASD	Autism spectrum disorders
BAR	Bin/Amphiphysin/Rvs
BCH	BNIP-2 and Cdc42GAP homology/Sec14p-like
BPGAPs	BCH domain-containing, proline rich and Cdc42GAP-like proteins
BSA	Bovine serum albumin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CNS	Central nervous system
CTD	Cytoplasmic C-terminal domain
DIV	Days in vitro
E/I	Excitation/inhibition
EEN	Human homolog of rat endophilin II
EGF	Epidermal growth factor
FDU	5-Fluoro-2'-deoxyuridine
FXS	Fragile X syndrome
GABA	γ-aminobutyric acid
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDI	Guanine nucleotide dissociation inhibitors
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
HAP1	Huntingtin-associated protein 1
HBSS	Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution
HS	Horse serum
ID	Intellectual disability
KA	Kynurenic acid
LBD	Ligand-binding domain
LTD	Long-term depression
LTP	Long-term potentiation
MAP2	Microtubule associated protein 2
MECP2	Methyl-CpG-binding protein 2
MEM	Minimum essential medium Eagle
NBM	Neurobasal medium

mEPSC	Miniature excitatory post-synaptic currents
mGluRs	Metabotropic glutamate receptors
mIPSC	Miniature inhibitory post-synaptic currents
mTOR	Mammalian target of rapamycin
NKCC1	Chloride importer Na^+-K^+-$2Cl^-$ co-transporter 1
NKCC2	Chloride exporter K^+-Cl^- co-transporter 2
NMDAR	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NOS	Nitric oxide synthase
NTD	N-terminal domain
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PH	Pleckstrin homology
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PRR	Proline-rich region
PSD	Post-synaptic density
PTX	Picrotoxin
SH3	Src-homology 3
SH3-GK	Src homology 3-guanylate kinase-like
SmsGDS	Small G-protein GDP dissociation stimulator
SRGAP2	Slit-Robo Rho-GTPase Activating Protein 2
TARP	Transmembrane AMPAR regulatory proteins
TMD	Transmembrane domain
TTX	Tetrodotoxin
VGAT	Vesicular GABA transporter
VGluT	Vesicular glutamate transporter

Abstract

The activity-dependent modifications of synaptic strength are the molecular mechanism that underlies circuit plasticity, the molecular device for learning and memory. However, maintaining proper balance of excitation and inhibition (E/I balance) is critical for information processing and plasticity in the central nervous system (CNS). Correct excitatory glutamatergic transmission and inhibitory GABAergic signalling are essential for tight control of E/I balance and normal neural circuit function, and disruption of E/I often underlies the development of neuropsychiatric disorders. As key regulators of the actin cytoskeleton, Rho-family GTPases play a critical role in synapse development and plasticity. They shuttle between an active GTP-bound form and an inactive GDP-bound form. Their activation and inactivation cycle is under the regulation of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. Even though dozens of GEFs and GAPs have been detected in the brain (outnumbering Rho GTPases), the function of most of them has not been elucidated. Rho-regulatory proteins typically comprise multiple signalling domains, playing an important role as key signalling integrators and scaffolds. Given that Rho GTPases regulate a myriad of neurodevelopmental processes including neuronal migration, dendritic arborization and synaptogenesis, their precise regulation is important for circuitry development and normal cognitive function. In fact, aberrant Rho GTPase signalling can cause synaptic defects that can ultimately lead to cognitive impairments. Furthermore, mutations in genes encoding regulators and effectors of the Rho GTPase family have already been associated with intellectual disability (ID) and other neurodevelopmental disorders.

Here, we focus on the characterization of a novel Rho-GTPase activating protein, ARHGAP8, in the brain. Preliminary data from our group showed that ARHGAP8 is present at the post-synaptic densities of excitatory synapses in an NMDA receptor-dependent way, and that this Rho-GAP might be involved in the regulation of excitatory synapses. Considering this hypothesis, further studies were conducted testing the functional effects of overexpressing ARHGAP8 in AMPA receptor-mediated transmission. Our data show that overexpression of ARHGAP8 decreases the amplitude and frequency of miniature excitatory post-synaptic currents, indicating that ARHGAP8 downregulates AMPA receptor-mediated excitatory synaptic transmission. Furthermore, we characterized the presence of this protein in inhibitory synapses to further extend our knowledge of its

role in neurons. Our results indicate that ARHGAP8 is present in inhibitory synapses, and regulates the synaptic accumulation of inhibitory synapse markers. Collectively, these observations suggest that ARHGAP8 coordinates the development of excitatory and inhibitory synapses. Further investigation should be done in order to unravel the mechanisms through which ARHGAP8 modulates AMPAR-mediated synaptic transmission and inhibitory synapse composition, and to evaluate if endogenous ARHGAP8 is involved in the regulation of both excitatory and inhibitory synapses.

Keywords: E/I balance, Rho-family GTPases, Excitatory synapse, Inhibitory synapse, ARHGAP8.

Resumo

A remodelação das estruturas sinápticas, dependente do tipo de estímulos que recebem, é o mecanismo molecular responsável pela plasticidade dos circuitos neuronais – um processo que se julga estar na base da aprendizagem e da memória. O processamento de informação e a plasticidade dos circuitos no sistema nervoso central dependem do equilíbrio entre a função excitatória e a função inibitória. O estabelecimento de uma correta transmissão glutamatérgica (excitatória) e GABAérgica (inibitória) é essencial para o controlo do equilíbrio entre excitação e inibição e para o funcionamento normal dos circuitos neuronais; a perda deste equilíbrio está geralmente associada ao desenvolvimento de neuropsiquiátricos. As GTPases são uma família de proteínas associadas com a regulação do citoesqueleto de actina, e que têm um papel relevante no desenvolvimento e plasticidade da sinapse. As GTPases apresentam um ciclo de activação (quando ligadas a GTP) e inactivação (quando ligadas a GDP) que é regulado pelos factores de troca de nucleótidos de guanina (GEFs) e pelas proteínas activadoras de GTPase (GAPs), respectivamente. Apesar de existirem dezenas de GEFs e GAPs no cérebro (um número superior ao de GTPases), a função da maioria destas proteínas ainda não foi descrita. Tipicamente, as proteínas reguladoras de GTPases possuem vários domínios proteicos, que lhes conferem um papel importante como integradores de sinalização intracelular. Uma vez que as GTPases estão envolvidas em vários processos do desenvolvimento neuronal, como por exemplo, a migração neuronal, a formação da árvore dendrítica e o desenvolvimento sináptico – quer excitatório quer inibitório, a sua regulação é de extrema importância para o normal desenvolvimento dos circuitos neuronais e normal função cognitiva. De facto, distúrbios na sinalização pelas GTPases podem causar defeitos sinápticos que originam défices cognitivos. Para além disso, mutações em genes que codificam proteínas reguladoras e sinalizadores das GTPases já foram extensamente associadas a défices cognitivos e outros distúrbios comportamentais.

Neste estudo, focamo-nos na caracterização da função neuronal da proteína ARHGAP8, uma nova proteína potenciadora da actividade de GTPases de Rho-GTPases. Resultados preliminares do nosso grupo indicam que a proteína ARHGAP8 está presente nas densidades pós-sinápticas das sinapses excitatórias e que esta GAP pode estar envolvida na regulação deste tipo de sinapses. Tendo em consideração esta hipótese,

foram realizadas experiências com o objectivo de testar os efeitos da sobre expressão de ARHGAP8 na transmissão sináptica mediada por receptores AMPA. Os nossos resultados demonstraram que a sobre expressão de ARHGAP8 causa uma diminuição na frequência e amplitude de correntes excitatórias pós sinápticas miniatura, o que indica que a proteína ARHGAP8 regula negativamente a transmissão sináptica excitatória mediada pelo receptor AMPA. Para além disto, caracterizámos a presença desta proteína nas sinapses inibitórias. Os nossos resultados indicam que a proteína ARHGAP8 está presente nas sinapses inibitórias e que regula a acumulação de marcadores sinápticos inibitórios. Estes resultados sugerem que a proteína ARHGAP8 coordena o desenvolvimento de ambos os tipos de sinapses (excitatórias e inibitórias). Mais experiencias são necessárias de forma a desvendar os mecanismos através dos quais a proteína ARHGAP8 regula a transmissão sináptica medida por receptores AMPA e a composição da sinapse inibitória, bem como, para avaliar se a proteína endógena está envolvida na regulação de sinapses excitatórias e inibitórias.

Palavras-chaves: Equilíbrio excitação/inibição, Rho-GTPases, Sinapse excitatória, Sinapse inibitória, ARHGAP8.

Chapter 1 - Introduction

1 Introduction

1.1 Connectivity of neuronal networks

The brain is probably the most complex and fascinating organ in the human body. It contains around 86 billion neurons each interconnected by thousands of synapses – specialized structures that process and transmit information in the form of chemical or electric signals (Azevedo, F. et al., 2009; Tolias, K. et al., 2011). These intricate structures are believed to be under constant modification during development and by experiences throughout life, i.e. some neuronal pathways within a circuit are selectively stabilized while others are weakened, depending on their synaptic input – a concept first introduced by Donald O. Hebb in 1949 which is now known as synaptic plasticity. Synaptic plasticity is viewed as the molecular translation of the human capability for learning from experience and adapting to new situations (Denève, S. et al., 2017). The tight regulation of synaptic rearrangement during critical periods of development and the fact that this process slows with age are consistent with the notion that the control of synapse dynamics by activity plays a central role in shaping the organization of local synaptic networks during development (Bernardinelli, Y. et al., 2014). Neural circuits sit in a balance between synaptic excitation (E) and inhibition (I), typically consisting of fast glutamate and slower γ-aminobutyric acid (GABA) inputs, respectively (Xue et al., 2014).

1.2 Excitatory and Inhibitory Balance

Neuronal networks need to sustain tightly correlated levels of activity of both excitatory and inhibitory neurons (Bourdoukan & Denève, 2015). Balance between excitation and inhibition (E/I) is a fundamental feature of network activity. This dynamic balance was first theorized (Shadlen & Newsome, 1994; van Vreeswijk & Sompolinsky, 1996) and later shown experimentally in vitro (Shu et al., 2003) and in vivo (Haider et al., 2006). Remarkably, even though the density of synapses is significantly higher in human cortical neurons, the E/I ratio is conserved between rodents and humans, suggesting that the developmental mechanisms that set the equilibrium between the number of excitatory and inhibitory synapses might be conserved (Defelipe, 2011). Break down of E/I could be an important factor in defining pathological states. An altered proportion between excitation and inhibition presupposes a disruption of homeostatic plasticity resulting from either

insufficient or excessive compensatory mechanisms in response to a change in network activity (Fritschy, 2008). Indeed, improper spine morphogenesis is associated with neurodevelopmental (Dehghani, N. et al., 2016), neuropsychiatric and neurodegenerative disorders, as a consequence of impaired information processing in the brain (Lai, K., 2013). The fact that alterations in the tight E/I balance are generally consistent with disruptions in neurological and psychiatric disorders highlights the emerging role of E/I balance and synaptic homeostasis as crucial for neural information processing and learning (Eichler & Meier, 2008; Yizhar et al., 2011, Ziburkus et al., 2013).

1.2.1 E/I Imbalance in neurodevelopmental disorders

Neurodevelopmental defects of multiple origins lead to structural and functional abnormalities in neurons that often amount to an E/I imbalance. Most commonly, this altered stasis generates a hyper-excitability, whether it is a consequence of the GABAergic transmission (Frye et al., 2016; Frye & Rossignol, 2016), or an effect caused by an upsurge in glutamate receptor signalling (Oberman, 2012). Physiologically, the balance between excitation and inhibition is essential for neuronal homeostasis (Turrigiano & Nelson, 2004); when disturbed, it can result in profound consequences, particularly if it occurs during key developmental periods where it can lead to neurological and psychiatric disorders (González-Ramirez et al., 2015) as well as cognitive delay, intellectual disability and behavioural impairments (Yizhar et al., 2011). A role for interneurons and GABAergic inhibition in intellectual disability (ID) (Penzes et al., 2013; Marin, 2012; Hernandez-Gonzalez et al., 2015), in models of schizophrenia (Lewis et al., 2005; Cho et al., 2015), Down syndrome (Hernandez-Gonzalez et al., 2015), Rett syndrome (Calfa et al., 2015; Chao et al., 2010) and Autism Spectrum Disorders (ASD) (Blatt et al., 2001; Fatemi et al., 2010; Blackmon, 2015; Schür et al., 2016; Bozzi et al., 2017) has already been documented.

1.2.2 Altered Rho GTPase signalling in neurodevelopmental disorders

Several authors have successfully established a correlation between altered Rho GTPase signalling, spine abnormalities and mental retardation, highlighting the critical role of rigorous Rho GTPase signalling for proper circuit development and normal cognitive function (reviewed in Ramarkers, 2002; Newey et al., 2005). Interestingly, Rho GTPases may co-regulate the development of excitatory and inhibitory synapses (e.g. Fossati et al.,

2016; Zamboni et al., 2016), thus constituting a hub for coordinating the E/I balance in the brain.

1.2.2.1 Rho-GTPases

Given the actin-rich nature of dendritic spines, the Rho GTPase family, known for their ability to control actin dynamics and organization, have emerged as key regulators of spine morphogenesis (reviewed in Ba et al., 2013; Tolias et al., 2011). Rho-family GTPases are a subfamily of the Ras superfamily of small (±21 kDa) GTPases. Rho proteins are guanine nucleotide-binding proteins, which act as binary switches cycling between an active GTP-bound form and an inactive GDP-bound form (Figure 1) (reviewed by Ba et al., 2013). The three best described GTPases in the Rho-family are Cdc42 (which plays an important role in the formation of filopodia), RhoA and Rac1 (which when activated result in the formation of stress fibers and lamellipodia, respectively) (reviewed by Hall, 1998). Rho GTPases regulate a myriad of neurodevelopmental processes, such as neuronal migration, dendritic arborization and synaptogenesis (Govek et al., 2005). The level of specificity necessary for the generation of such distinct cellular outcomes suggests a tight regulation by Rho regulatory proteins.

1.2.2.2 Rho-regulatory proteins

Precise spatio-temporal control of Rho GTPase signalling is orchestrated by positive and negative regulators. Guanine nucleotide exchange factors (GEFs), activate Rho GTPases by catalyzing GDP/GTP exchange, whereas GTPase activating proteins (GAP) inhibit Rho-GTPases by enhancing their intrinsic GTPase activity causing GTP to be hydrolyzed to GDP. Additionally, guanine nucleotide dissociation inhibitors (GDI), prevent the GDP/GTP exchange and sequester inactive Rho GTPases in the cytoplasm (Figure 1). These regulatory proteins possess multiple signalling domains that are involved in receiving upstream inputs and recruiting downstream components of Rho GTPase signalling pathways, acting both as signal integrators and scaffolds (reviewed by Tolias et al., 2011). Furthermore, GEFs and GAPs outnumber Rho GTPases with more than one regulatory protein existing for each GTPase. All of these distinct features suggest a level of specificity necessary to the activity of Rho-GTPases in various neurodevelopmental processes (Heasman and Ridley, 2008; Govek et al., 2011). All Rho-GAP proteins contain a conserved GAP domain of ~140 amino acids (aa) that targets GTP-bound Rho

GTPases through a common mechanism of action by utilizing an “arginine-finger” motif in trans to stabilize the transition state of GTP hydrolysis (Gambli and Smerdon, 1998; Peck et al., 2002).

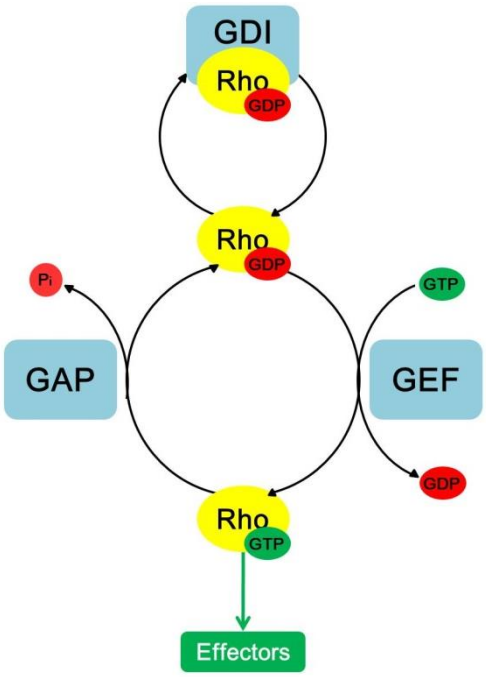

Figure 1: Regulatory cycle for the activation and inactivation of Rho GTPases. Rho GTPases act as molecular switches, cycling between an active, GTP-bound state, and an inactive, GDP-bound state. The state of activation of Rho proteins is regulated by GEF, which mediates exchange of GDP by GTP. Inactivation occurs through the intrinsic GTPase activity of the protein (hydrolysis of the bound GTP) and is stimulated by GAPs. GDP-bound Rho GTPases can be sequestered in the cytoplasm by GDIs which prolongs its inactivation. Among the effector proteins we can highlight kinases and scaffold proteins involved in the regulation of actin dynamics, and kinases involved in gene expression regulation (based on Ramarkers *et al.*, 2002).

1.3 Excitatory synapses

The majority of excitatory synapses localize to dendritic spines, small actin-rich protrusions on the surface of dendrites. These spines contain the post-synaptic density (PSD) – an architecturally and functionally highly specialized structure that opposes the pre-synaptic active zone (Gray, 1959; Siekevitz, 1985).

1.3.1 Components of the PSD

Proteomic analysis of rat PSD fractions by mass spectrometry revealed ~400 PSD proteins, of which actin-cytoskeletal, kinase signaling and GTPase signaling pathways were highly represented (Peng et al., 2004). Besides anchoring the glutamate receptors, the PSD serves as a host for associated signaling and structural molecules that include adhesion molecules, cytoskeletal components and cytoplasmic signaling enzymes (Figure 2).

1.3.1.1 Glutamate receptors

Glutamate receptors are the primary mediators of excitatory synaptic transmission in the brain and can be divided into metabotropic glutamate receptors (mGluRs) and ionotropic receptors (alpha-amino-3-hydroxy-5-methyl-4 isoxazole proprionic acid receptor (AMPAR), N-methyl-D-aspartate receptor (NMDAR) and kainate receptors) (Dingledine et al., 1999; Traynelis et al., 2010). Ionotropic glutamate receptors are cation permeable (to Na^+ and K^+) receptor tetramers that drive the post-synaptic neuron to depolarize after activation by pre-synaptically released L-glutamate (Lüscher & Malenka, 2012). At the PSD, NMDAR are stably incorporated, whereas AMPAR are more dynamic, showing rapid lateral diffusion in and out of the post-synaptic membrane. This dynamic exchange underlies the strengthening and weakening of synaptic transmission (Cognet et al., 2006; Shepher & Huganir, 2007). Given their role in synaptic transmission, AMPAR are further described in a paragraph below.

1.3.1.2 Scaffold proteins of the PSD

The PSD contains a large number of scaffolding proteins families including PSD-95, GKAP, Shank and Homer. Synaptic scaffolding proteins usually contain multiple domains for protein-protein interaction, the PDZ domain being one of the most common – a ~90 aa sequence that interacts with a peptide motif located at the carboxyl (C-) terminus of several binding partners (Funke et al., 2004; Kim & Sheng, 2004; Feng & Zhang, 2009).

1.3.1.3 PSD-95

PSD-95 is the most widely studied PSD scaffold and is known to bind to and tether various membrane proteins, adhesion and signaling molecules in the PSD of excitatory synapses (Kim & Sheng, 2004). It does so via its three PDZ domains and one Src homology 3-guanylate kinase-like (SH3-GK) module. By binding two of these PDZ domains, NMDARs can stabilize at the cell surface (Kornau et al., 1995; Prybylowski et al., 2005) allowing for the assembly of NMDA receptor-associated protein complex by interconnecting proteins such as nitric oxide synthase (NOS) (Aarts et al., 2002), GEFs (Penzes et al., 2008) and GAPs (Kim et al., 1998), facilitating the functional coupling of the receptor with its downstream signaling molecules. PSD-95 also binds the C-terminus of transmembrane AMPAR regulatory proteins (TARP) thus recruiting AMPARs to synapses. The N-terminus of PSD-95 is palmitoylated and thus located at the membrane,

which is necessary for its synaptic localization, clustering of receptors and stability at the PSD (Won et al., 2016).

1.3.1.4 GKAP, Shank, Homer and other PSD scaffolds

GKAP connects with both the C-terminus of PSD-95 (Kim et al., 1997) and with the PDZ domain of Shank, which in turn binds to Homer (Sheng & Kim, 2000). These three protein families can form an axis, tethering several proteins at the synapse. Additionally, GRIP and PICK1 are scaffolds for AMPARs present at the synapse but have other relevant roles in AMPAR trafficking (Shepherd & Huganir, 2007).

1.3.1.5 Signaling proteins of the PSD

A huge diversity of signaling molecules are located at the PSD, some of the most relevant being kinases, phosphatases, small GTPases and several GEF and GAP that regulate them. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) binds NMDAR at the PSD, locking CaMKII in an active conformation, which in turn stimulates synaptic delivery of AMPAR (Shen & Meyer, 1999; Hayashi et al., 2000). Synaptic small GTPases and their tuning by GEF and GAP regulate both synaptic structure and function (Penzes et al., 2008).

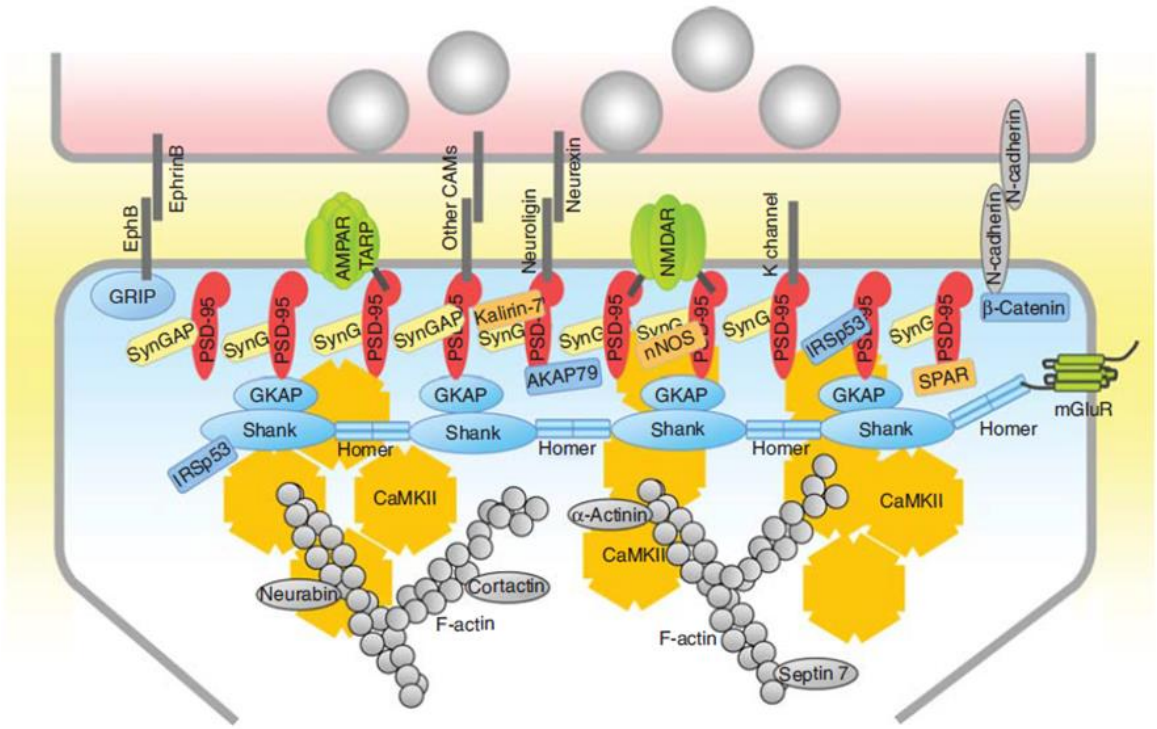

Figure 2. Schematic diagram of the molecular organization of the post-synaptic density of excitatory synapses. Protein interactions are indicated by direct contacts or overlaps between the shapes. (Sheng and Kim, 2011).

1.4 AMPAR

AMPA receptors are tetrameric ionotropic channels that mediate fast synaptic excitatory transmission in the CNS. Four different genes encode AMPA receptors subunits (GluA1-4) which form channels as homo- or heterotetramers built from combination of the four subunits. (Wenthold et al., 1996). The extraordinarily fast kinetics of the AMPARs (sub-millisecond timescale) ensures fast depolarization of the post-synaptic membrane, allowing high fidelity propagations of impulses between neurons. AMPARs also acts as gate-keepers of NMDARs by relieving their voltage-dependent channel block by Mg^{2+} (Mayer et al., 1984) allowing the post-synaptic Ca^{2+} entry (Kessels & Malinow, 2009). It is generally accepted that there is a common mechanism controlling the postsynaptic expression of long-term potentiation (LTP) and long-term depression (LTD), the two most widely studied forms of synaptic plasticity: the addition and removal, respectively, of synaptic AMPAR (Malinow & Malenka, 2002; Kessels & Malinow, 2009). According to Kessels and Malinow, AMPA receptors trafficking is hypothesized as following: (1) synaptic strengthening involves activity-dependent addition of long-tailed (typically, GluA1-containing) AMPA receptors to synapses; (2) synaptic weakening occurs through activity-

dependent endocytosis of AMPA receptors from synapses; (3) short-tailed (typically, GluA1-lacking) AMPA receptors constitutively traffic in and out of synapses, independent of activity and without change in synaptic strength. The passive replacement of long-tailed synaptic AMPAR for GluA1-lacking AMPAR may be important to stabilize synaptic strength in the absence of activity and may represent a molecular mechanism for the consolidation of encoded memories (Cingolani et al., 2008).

1.4.1 Architecture of AMPAR

The architecture of AMPARs is quite distinctive; each subunit consists of four domain layers: an extracellular N-terminal domain (NTD), a ligand-binding domain (LBD), a transmembrane domain (TMD) and a cytoplasmic C-terminal domain (CTD) (Sobolevsky et al., 2009). The TMD which constitutes the ion channels is made up by four helical elements: M1-M4. Interestingly, the M2 segment presents a Q/R editing site in GluA2 which induces the switch of glutamine (Q) to arginine (R) that renders GluA2-containing AMPAR far less permeable to Ca^{2+} (Higuchi et al., 2000). The NTD is a bulky domain that drives receptor assembly (Herguedas et al., 2013) and plays a role in AMPAR anchoring at synapses (Watson et al., 2017).

1.4.2 Auxiliary subunits of AMPAR

Unlike NMDA- and kainate receptors, AMPARs assemble with a wide variety of auxiliary subunits that play a role in the receptors trafficking and their expression at the synapses. TARPs associate with AMPARs and enhance synaptic transmission by slowing receptor desensitization and increasing the size of synaptic current (Jackson & Nicoll, 2011). The interaction of the C-terminal PDZ binding motif of the AMPAR-associated TARPs with scaffolding proteins in the PSD (PSD-95/93 MAGUKs) is required for AMPAR accumulation at post-synaptic sites (Jackson & Nicoll, 2011). The first AMPAR auxiliary subunit identified was stargazin, a protein that has been shown to anchor AMPARs at post-synaptic sites (Chen et al., 2000). It is thought that AMPAR desensitization induces a partial-loss of the AMPAR-stargazin interaction, enhancing AMPAR mobility and allowing for their fast lateral diffusion and replacement for naïve receptors (Constals et al., 2015).

1.5 Rho-GAP signalling in excitatory synaptogenesis

Given the role or Rho GTPases in regulating excitatory synapse formation, maintenance and dynamics, it is not surprising that their negative regulators, Rho-GAPs, are major players in these processes. For a few Rho-GAPs, there is detailed understanding of their function in excitatory synapses.

1.5.1 SRGAP2

The *srGAP2* gene codes for the Slit-Robo Rho-GTPase Activating Protein 2 (SRGAP2), a protein highly conserved in mammals that regulates several aspects of cortical development, including the migration and differentiation of pyramidal neurons as well as the maturation and density of dendritic spines (Guerrier et al., 2009; Charrier et al., 2012, Dennis et al., 2012). SRGAP2C is the human-specific gene that is co-expressed with the ancestral SRGAP2A in the adult human brain (Charrier et al., 2012; Dennis et al., 2012). SRGAP2A contains three functional domains: an N-terminal F-BAR domain, a central Rho-GAP domain specific for Rac1, and a C-terminal Src-homology 3 (SH3) domain (Guerrier et al., 2009). SRGAP2C differs from SRGAP2A in the corresponding truncated F-BAR domain lacking its last 49 aa (Charrier et al., 2012, Dennis et al., 2012) and it is able to physically interact with SRGAP2A and inhibit its function (Charrier et al., 2012). Fossati et al. identified a conserved proline-rich motif corresponding to a class II EVH1 binding site in the F-BAR domain of SRGAP2 which is a canonical binding site for Homer family proteins (Tu et al., 1998). Through co-immunoprecipitation, the authors were able to show direct interaction between SRGAP2A and Homer1 in brain lysates of P15 mouse brains. Point mutations introduced to the class II EVH1 binding motif were sufficient to abolish SRGAP2A-Homer interaction, validating its specificity. By knocking down the endogenous SRGAP2A and replacing it with mutant forms, the authors were able to pin point the contribution of each domain to synaptogenesis. While the Rac1-GAP domain contributes to the setting of spine density by helping the formation of the Homer-based postsynaptic scaffold, the EVH1 interferes with spine head size determining the degree of spine maturation.

1.5.2 ARHGAP15

The *ArhGAP15* gene codes for a Rac1-specific GAP protein. It comprises a Rho-GAP domain that binds the C-terminus of Rac1 and promotes the GDP-bound state, with a consequent inactivation of the downstream pathway, and a pleckstrin homology (PH) domain which mediates the activation of ARHGAP15 via binding to the PI3K product phosphatidylinositol 3,4,5-triphosphate (PIP3) (Costa et al., 2010). Loss of ARHGAP15 has been documented in a rare variant of the Mowat-Wilson disease, characterized by serious neurological deficits, severe ID, speech impairment and autism (Smigiel et al., 2010). Zamboni et al. described an increase in excitatory synapse density in the CA1-CA2 region and a decrease in the CA3 region of the hippocampus of ARHGAP15$^{-/-}$ P30 mouse brains, when compared to the controls. The authors also reported abnormalities in neuritogenesis and the morphology of ARHGAP15$^{-/-}$ hippocampal neurons which presented a decline in the ramifications of their dendritic arborization. The spontaneous electrical activity of primary cultures of ARHGAP15$^{-/-}$ hippocampal neurons seems to be raised (increase in the overall burst number and firing frequency) but randomly distributed, when compared to wild-type neurons. The over-excitation and reduced synchronicity of ARHGAP15$^{-/-}$ neurons might be a result of the decreased complexity, ultimately showing in hippocampus-dependent cognitive performance tests (determined by hippocampal-dependent behavioural tests performed on adult ArhGAP15$^{-/-}$ mice) (Zamboni et al., 2016).

1.5.3 Oligophrenin-1

OPHN1, firstly identified as an X-linked mental retardation gene (Billuart et al., 1998), encodes for Oligophrenin-1, a RhoA-specific GAP that regulates synapse development (Govek et al., 2009; Kasri et al., 2009). Oligophrenin-1 is abundantly expressed in the CNS during development and localizes both at pre- and post-synaptic sites. This multi-domain protein possesses in its structure a Bin/Amphiphysin/Rvs (BAR) domain, a PH domain, a Rho-GAP domain and three PRR (Billuart et al., 1998). Oligophrenin-1 normally maintains spine length by suppressing the RhoA/Rho-kinase (ROCK) pathway. In Oligophrenin-1 mutant mice, RhoA inhibition is relieved. ROCK activation induces actin remodelling thus promoting spine shrinkage. These mutant animal models display spine abnormalities and altered pre-synaptic function that culminates in behavioural, cognitive and social impairments (Khelfaoui et al., 2007). Overexpression of Oligophrenin-1 enhances AMPAR-mediated currents and increases spine size in hippocampal neurons.

Contrarily, its downregulation reduces spine length and density and depresses AMPAR-mediated currents due to increased RhoA activity (Kasri et al., 2009). This RhoA-specific GAP regulates synaptic activity at both a structural and a functional level, and its role in regulating basal synaptic function depends on the interaction with the scaffold protein, Homer1.

1.5.4 ARHGAP12

ARHGAP12, a Rac1-specific Rho-GAP recently characterized in the brain, is specifically expressed in the CA1 region of the hippocampus (Ba et al., 2016). Through manipulation of its protein levels, the authors characterized this Rho-GAP as a "structure-function coordinator of excitatory synapses". Overexpression of ARHGAP12 depressed both AMPAR and NMDAR-mediated currents. Contrarily, downregulation results in potentiated AMPAR-mediated currents (enhanced frequency and amplitude of mEPSC) but not NMDAR-mediated currents. Additionally, ARHGAP12 has been described as a "developmental break" due to its inhibitory effects in unsilencing synapses during early development (Ba et al., 2016).

1.6 Inhibitory synapses

Although many studies of synaptic transmission focus on excitatory synapses, inhibitory transmission by GABA plays a fundamental role in controlling neuronal excitability and network synchronization in the CNS (Xu, 2007). Inhibitory synapses are mainly formed on the shaft of dendrites, or on cell bodies and axon initial segments. Even though the inhibitory post-synaptic specialization presents a lower degree of complexity (Figure 3) when compared to the PSD of excitatory synapses, the main organization principles seem to be conserved (Gray, 1959). The primary mediators of inhibitory synaptic transmission in the CNS are the $GABA_A$ and glycine receptors (Jacob et al., 2008).

1.6.1 Scaffold proteins at inhibitory synapses

Gephyrin – a 93-kDa polypetide (Pfeiffer et al., 1982) – is a well-known post-synaptic scaffold in inhibitory synapses that is able to self-multimerize and directly interacts with both $GABA_A$ and glycine receptors, anchoring them to synapses (Figure 3) (Levi et al.,

2004; Tyagarajan et al. 2011). Around a dozen binding partners of gephyrin have been identified (Fritschy et al., 2008). Neuroligin-2, a synaptic transmembrane adhesion molecule, is specifically located at inhibitory post-synaptic sites. On the extracellular side, it associates with pre-synaptic neurexins on GABAergic neurons, while it intracellularly recruits gephyrin-bound collybistin (a GEF activating Cdc-42) (Graf et al., 2004; Sudhof, 2008). When unbound by neuroligins, neurexin has been found to interact with $GABA_A$ receptors and suppress GABAergic transmission (Zhang et al., 2010). Mammalian target of rapamycin (mTOR) binds with gephyrin and is thought to be important to the regulation of local protein synthesis (Sabatini et al., 1999). Gephyrin also adheres to actin through the actin-associated proteins profilin and Mena/VASP (Bausen et al., 2006). In this fashion, gephyrin is able to oligomerize and form a mesh-like anchor system that interacts with the cytoskeleton, receptors and signalling molecules, mimicking what happens with PSD-95 in the excitatory synapses.

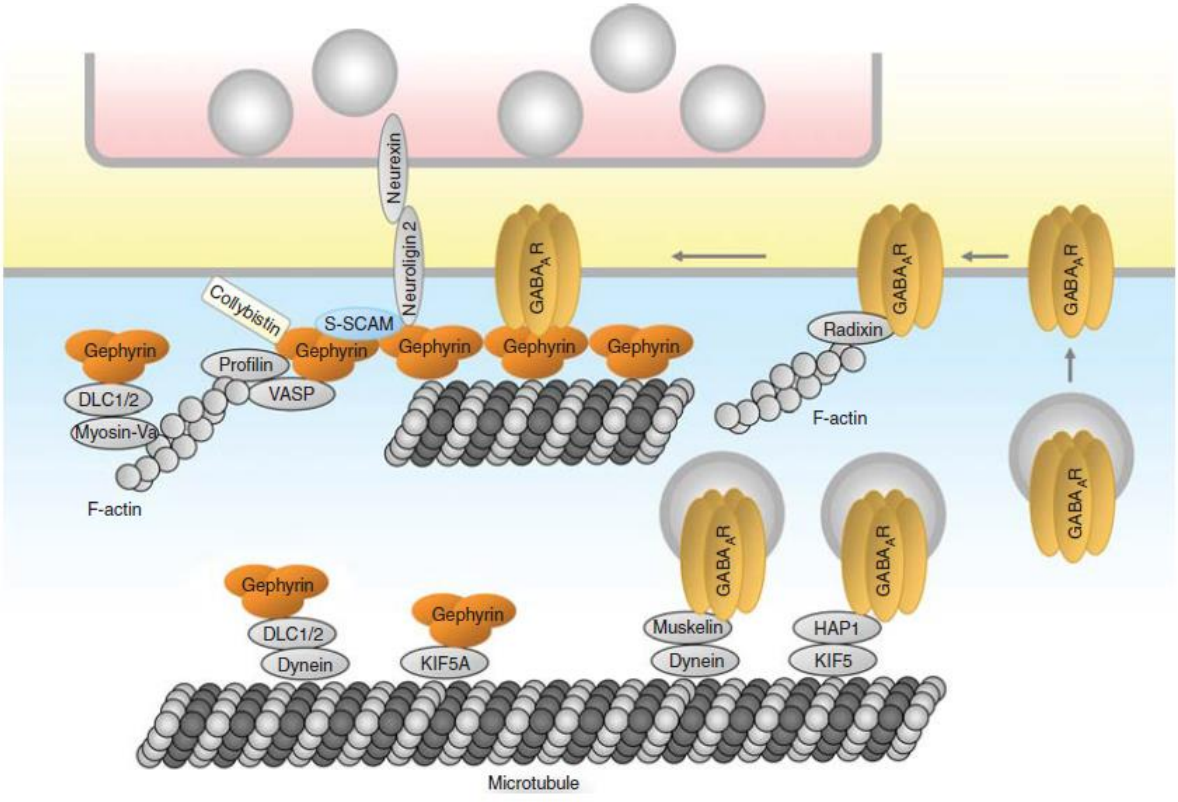

Figure 3. Schematic diagram of the molecular organization of the post-synaptic structure of inhibitory GABAergic synapses. Protein interactions are indicated by direct contacts or overlaps between the shapes. (Sheng and Kim, 2011).

1.6.2 $GABA_A$ receptors

GABA – the most abundant inhibitory neurotransmitter in the CNS – activates three major classes of receptors, termed $GABA_{A-C}$ (Xu, 2007). $GABA_A$ receptors are ionotropic ligand-gated chloride channels that mediate most of the fast synaptic transmission in the CNS (Fritschy, 2008). $GABA_A$ receptors are composed of five subunits arranged around a central pore, selected from 19 known receptor subunits: α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π, ρ_{1-3} (typically, comprising two α's, two β's and one γ subunit – α2β2γ1) (Mody & Pearce, 2004). $GABA_A$ receptors that contain an α_{1-3} subunit are mainly synaptic, whereas α_{4-6} and δ-containing receptors are primarily peri- or extrasynaptically located (Glykys and Mody, 2007). Synaptic receptors mediate fast phasic inhibition while extra-synaptic receptors produce persistent tonic inhibition (Ben-Ari, 2002). The existence of multiple $GABA_A$ receptor subtypes differing in subunit composition, localization and functional properties underlies their role for fine tuning of neuronal circuits (Fritschy, 2008).

1.6.2.1 Depolarizing-to-hyperpolarizing GABA shift

In contrast to the inhibitory action in the adult brain, $GABA_A$ receptors are depolarizing and often excitatory during early development. The chloride importer Na^+-K^+-$2Cl^-$ co-transporter 1 (NKCC1) maintains high intracellular chloride concentrations in immature neurons; therefore, activation of $GABA_A$ receptors in these neurons results in chloride efflux and membrane depolarization. During development, an upregulation of the chloride exporter K^+-Cl^- co-transporter 2 (NKCC2) is accompanied by a downregulation of NKCC1. This shift in ratio between chloride importers and exporters results in a progressive decrease in intracellular chloride concentration, which induces the excitation-inhibition reversal in $GABA_A$ receptor-mediated signalling (Ben-Ari, 2002). In the adult brain, activation of $GABA_A$ receptors causes influx of the chloride ion, hyperpolarization of the post-synaptic membrane and thus, inhibition of neuronal excitability (Xu, T. 2007). Timing of the depolarizing-to-hyperpolarizing GABA shift is controlled via the oxytocin receptor that upregulates NKCC2 activity by phosphorylation (Leonzino et al., 2016).

1.6.2.2 $GABA_A$ receptor trafficking

Newly synthesized $GABA_A$ receptors are initially inserted into the extra-synaptic plasma membrane through the secretory pathway, and laterally diffused into synaptic sites where they are stabilized by the interaction with the scaffold protein, gephyrin (Bogdanov et al.,

2006). The number of synaptic $GABA_A$ receptor is determined by the lateral diffusion of the receptors from and into the synaptic region and by the rate of exo- and endocytosis of the receptor within the synaptical membrane (Bogdanov et al., 2006; Thomas et al., 2005). When in the extra-synaptic compartment, $GABA_A$ receptors, if dephosphorylated, bind to clathrin adaptor complex AP2 and undergo endocytosis in a clathrin- and dynamin-dependent manner (Kittler et al., 2005). Internalized $GABA_A$ receptors can be recycled back to the plasma membrane or trafficked to the lysosomal pathway for degradation, depending on their interaction with the huntingtin-associated protein 1 (HAP1) which acts as an adaptor protein for the KIF5 kinesin motor for microtubule-dependent and anterograde trafficking of $GABA_A$ receptors to the plasma membrane (Twelvetrees et al., 2010). Muskelin promotes retrograde trafficking of these receptors by linking them to the actin-dependent motor dynein for receptor endocytosis and degradation (Heisler et al., 2011).

1.6.3 GABAergic dysfunctions in neurodevelopmental disorders

Proper inhibitory GABAergic signalling is essential for normal neural circuit function and its influence on cell firing and network oscillations is constrained spatially and temporally (Fritschy, 2008). Accordingly, evidence is accumulating that the GABAergic system is affected in several neurodevelopmental disorders. GABAergic signalling is a key pathway that is commonly disturbed in the pathophysiology of Fragile X syndrome, Dravet syndrome, Rett syndrome, Tourette syndrome, Down syndrome, Autism Spectrum Disorders and schizophrenia, among others (Braat and Kooy, 2015).

1.6.3.1 Fragile X Syndrome

Fragile X syndrome (FXS) is a frequent form of inherited intellectual disability (ID) in most of the cases associated with ASD (Kaufmann et al., 2004) and epileptic seizures (Hagerman, 2002). The disorder is caused by an inactivating mutation in the *FMR1* gene that encodes the FMRP protein (Verkerk et al., 1991), which interacts with hundreds of neuronal mRNAs (Ascano et al., 2012). In its absence, several molecular pathways are affected (Darnell and Klan, 2013). Differential expression of several $GABA_A$ receptor subunits, enzymes involved in $GABA_A$ synthesis, $GABA_A$ transporters and degradation and even the clustering protein gephyrin, have been described (reviewed by Cea-Del Rio and Huntsman, 2014; Paluszkiewicz et al., 2011). Decreased GABA concentrations and a

delayed excitation-inhibition switch of $GABA_A$ receptors have also been detected in animal models (He et al., 2014). All of this evidence supports the hypothesis of an impaired inhibitory GABAergic system in FXS.

1.6.3.2 Autism Spectrum Disorders

Several neuropathological findings hint to the hypothesis that inhibitory circuits are disrupted in the autistic brain. Alterations in GABAergic neurotransmission, decreased levels of $GABA_A$ receptor subunits as well as reduced binding to $GABA_A$ receptors, have been documented (Blatt et al., 2001; Fatemi et al., 2010). Histopathological studies revealed malformations during cortical development in ASD brain tissues and brain imaging studies have identified abnormal gray/white matter volumes (Blackmon, 2015). In 2016, Schür et al. provided evidence for the hypothesis of disrupted inhibitory signalling in the autistic brain, as the authors presented a systematic literature review and meta-analysis of proton magnetic resonance spectroscopy studies that confirmed that GABA levels are indeed reduced in ASD brains (Schür et al., 2016). Taken together, the data indicates that the GABAergic transmission is compromised in ASD, shifting the E/I balance to a dysfunctional state (Bozzi et al., 2017).

1.6.3.3 Rett syndrome

The clinical spectrum of patients with Rett syndrome includes autistic-like features alongside with ID and epileptic seizures. Rett syndrome is caused by a loss-of-function mutation of the *methyl-CpG-binding protein 2 (MECP2)* gene (Amir et al., 1999) which leads to a dysregulation of the GABAergic system, with several components differentially expressed due to MECP2 deficiency (El-Khoury et al., 2014). The decreased NKCC2 protein expression and NKCC2/NKCC1 ratio is evocative of immature $GABA_A$ receptor function (Duarte et al., 2013).

1.6.4 Rho-GAP signalling in inhibitory synaptogenesis

Similarly to excitatory synapses, inhibitory synaptogenesis and synapse maintenance are regulated by Rho-GTPases and their regulators, e.g. Rho-GAPs. A few examples of Rho-GAPs with a function in inhibitory synapses are highlighted below.

1.6.4.1 SRGAP2

Gephyrin was recently identified as a binding partner of SRGAP2A via a SH3-based photo-trapping assay (Okada et al., 2011). Through co-immunoprecipitation introduction of point mutation, Fossati and her team confirmed the direct interaction between the PGLP motif of gephyrin and SH3 domain of SRGAP2A. Direct interaction between SRGAP2 and gephyrin is necessary for inhibitory synapse maturation but it is the Rac1-GAP activity that regulates the density and subcellular distribution of inhibitory synapses. Fossati et al. unraveled the molecular mechanisms by which SRGAP2A links both excitatory (homer) and inhibitory (gephyrin) scaffolding molecules promoting maturation of both excitatory and inhibitory synapses (Fossati et al., 2016). The Rac1-GAP domain activity determines the density of both types of synapses, showing that co-regulation of excitatory and inhibitory synaptic development by SRGAP2A maintains the equilibrium between excitatory and synaptic transmission. The unique property of SRGAP2A to bind both Homer and gephyrin during synaptogenesis, suggest that SRGAP2C may universally modify synaptic development in human cortical pyramidal neurons (Fossati et al., 2016).

1.6.4.2 ARHGAP15

Zamboni et al. described that disruption of ARHGAP15 alters neuritogenesis and the balance between excitatory and inhibitory synapses. Interneurons appear to be the most affected, with altered directional migration and reduced number of specific sub-populations (CR+ PV+ and SST+) in ARHGAP15$^{-/-}$ mice. The altered interneuron migration might be a result of the hyperactive Rac1/3 in the ARHGAP15$^{-/-}$ brain, since Rac1 is required for the formation of the leading edge which directs neuron migration (Konno et al., 2005; Kawachi et al., 2003). In the absence of ARHGAP15, the authors observed fewer inhibitory synapses in CA1-CA3 neurons (determined by the number of VGAT-positive puncta), suggesting a globally reduced inhibition that prevails over the reduced excitation. The authors conclude that in ARHGAP15$^{-/-}$ hippocampi, the excitatory and inhibitory synapses are unbalanced in favour of excitation.

1.7 ARHGAP8 – a novel Rho-GAP

As a result of bioinformatic searches through human genome public databases, Low et al. identified sequences encoding for putative GAP proteins, and which contained additional signalling protein domains. One of them, the human *ARHGAP8* locus on chromosome 22q13.31 codes for a specific subclass of GAPs with a unique domain organization: BPGAPs (for BCH domain-containing, proline-rich and Cdc42GAP-like proteins) (Shang et al., 2003). Interestingly, the chromosomal 22q13.3 region where human ARHGAP8 gene is positioned has been connected to a number of disorders involving intellectual and cognitive deficits collectively described as 22q13.3 Deletion Syndrome or Phelan-McDermid Syndrome (Phelan and McDermid, 2012). Additionally, ARHGAP8 is located between the gene loci encoding for stargazin and shank3, two proteins indispensable to proper synaptic function and that are implicated in ID (Hamdan et al., 2011) and ASD (Peça et al., 2011), respectively. Human-mouse homology maps show a large region of conserved synteny between human chromosome 22 and mouse chromosome 15 (Shan et al., 2002). In the same article, Shan et al. reported the identification in chromosome 15 of the murine ortholog of ARHGAP8 – Arhgap8 which is expressed widely in adult mouse tissues.

1.7.1 BPGAP1 encoded from the *ARHGAP8* locus

The group of Prof. Boon Chuan Low was the first to describe the subclass of proteins from the ARHGAP8 locus and proposed to name them after their unique domain organization – BCH (BNIP-2 and Cdc42GAP homology/Sec14p-like) domain-containing, proline-rich and Cdc42GAP-like proteins (BPGAPs) (Shang et al., 2003). There are four putative isoforms and as the first researchers to study these proteins they have succeeded in cloning the first isoform, BPGAP1 (Figure 4). The structure of the protein is consistent with the suggested subclass architecture – an N-terminal BCH domain, a central proline-rich (PRR) domain and at the C-terminal the Rho-GAP domain containing an invariant "arginine finger" motif that is critical to its GAP function (Shang et al., 2003).

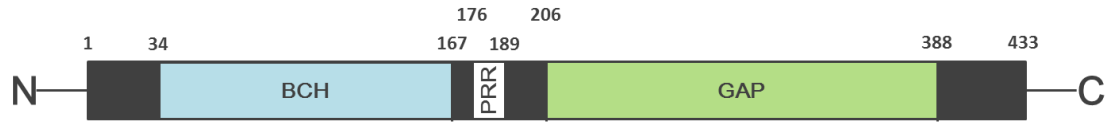

Figure 4: BPAGP1 structure and domain organization. BPGAP1 arbors near the N-terminal, a BCH domain, near the C-terminal, the obligatory GAP domain and in between these two, a proline-rich moiety. BPGAP1 is also known as ARHGAP8.

1.7.2 GTPase activity of BPGAP1

GTPase activity assays revealed that BPGAP1 is able to differentially modulate RhoA, Cdc42 and Rac1 activity in vitro, but only exerts its GAP activity on RhoA in vivo. Modulation of these GTPases is dependent on the BCH and GAP domains of BPGAP1 (Shang et al., 2013).

1.7.3 BPGAP1/cortactin-induced cell migration

Because the PRR domain of BPGAP1 contains multiple potential binding sites for SH3 or WW domains (Zarrimpar et al., 2003), it is no wonder that protein interaction assays have revealed binding partners to BPGAP1 (Figure 5) in a PRR-dependent manner, making BPGAP1 a potential partner for regulation of proteins containing these domains. In vitro and in vivo protein interaction assays using full-length BPGAP1 and domain-containing fragment reconstructs, showed that cortactin is able to interact with BPGAP1 in a manner that requires its PRR domain to connect to the SH3 domain present in cortactin (Figure 5) (Lua and Low, 2004). Cortactin, a multi-domain protein, interacts with multiple signaling partners and has the potential role of acting as an adaptor/scaffold since it interacts with Shank to organize the clustering of receptor complexes, and binds to dynamin-2 to regulate receptor-mediated endocytosis (Weed et al., 2000). In HeLa cells, BPGAP1 mediates translocation of cortactin from the cytosol to the membrane periphery, and overexpression of BPGAP1 together with cortactin promotes cell migration (Lua and Low 2004). This was the first evidence that a Rho-GAP functionally interacts with cortactin and could potentially provide a link between Rho GTPases and cortactin in regulating spatial and temporal cell dynamics (Lua and Low, 2004).

1.7.4 BPGAP1/EEN-enhanced ERK activation

The PRR domain of BPGAP1 was also found to be involved in the interaction with the human homolog of rat endophilin II (EEN) (Lua and Low, 2005), an SH3-containing GRB2-like protein I which belongs to a family of proteins known to be involved in the endocytic pathway and function in connection with several endocytic proteins (So et al., 2000). Protein binding assays revealed that EEN directly attaches to BPGAP1 in a manner that requires its SH3 domain binding to the PRR domain of BPGAP1 (Figure 5) (Lua and Low, 2005). Overexpression of BPGAP1 in HeLa cells, alone or in conjunction with EEN, enhances the epidermal growth factor (EGF)-triggered internalization of the EGF receptor, in a manner dependent on the activity of the BPGAP1 GAP domain, and promotes ERK activation (Lua and Low, 2005).

1.7.5 BPGAP1/Pin1-supressed Erk activation

Pin1 is another protein that was identified by Low and his colleagues as a binding partner for BPGAP1. Pin1 is a peptidylprolyl isomerase and a regulator of protein conformation that uses its WW domain to target specific phosphorylated Ser/Thr-Pro motifs (Lu et al., 2002). It regulates diverse cell fates, including cell proliferation and neuronal survival, while its deregulation is linked to neurological disorders (reviewed by Lu and Zhou, 2007). Pin1 also regulates various signaling events linked to cell motility, including the feedback loop of Raf-Mek-Erk pathway (Dougherty et al., 2005). Pin1 uses its WW and PPI domains to interact with specific motifs within the PRR and GAP domains of BPGAP1 (Figure 5) and suppresses BPGAP1-induced ERK activation (Low et al., 2010). These results indicate the unique interplay by different domains in BPGAP1 in exerting cell dynamics (Shang et al., 2013; Lua and Low, 2004; Low et al., 2010).

Interestingly, Pin1 is expressed in postsynaptic structures and interacts with PSD-95 through Ser/Thr-Pro consensus motifs in the linker region connecting PDZ domains in PSD-95 (Antonelli et al., 2016). Pin1 binding to PSD-95 negatively affects PSD-95 interaction with NMDARs, in agreement with larger NMDAR-mediated synaptic currents in the hippocampus of Pin1 knock-out mice (Antonelli et al., 2016).

1.7.6 BPGAP1/SmgGDS-supressed K-Ras activation

Besides ERK activation downstream of EGF receptor endocytosis, BPGAP1 activates ERK independently of its interaction with EEN (Lua and Low, 2005). In fact, the unique BCH domain of BPGAP1 induces robust ERK activation and leads to PC12 cell differentiation under EGF stimulation (Ravichandran and Low, 2013). This domain was also found to bind to the small GTPase K-Ras, promoting its activation (Figure 5), and this interplay is regulated by the small G-protein GDP dissociation stimulator (SmgGDS), as demonstrated by super induction of K-Ras activation and PC12 differentiation mediated by the BCH domain upon SmgGDS knockdown (Ravichandran and Low, 2013).

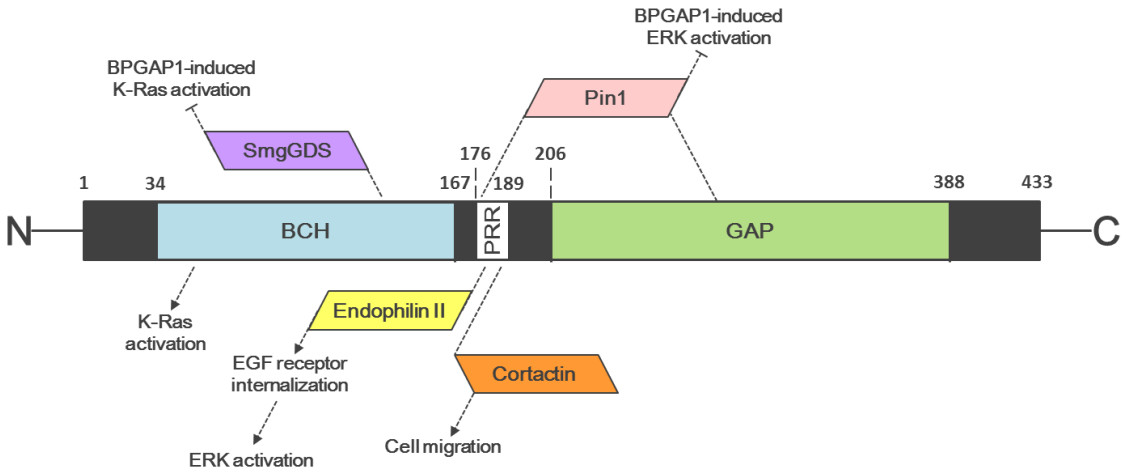

Figure 5: Domain organization of BPGAP1 (a.k.a. ARHGAP8) and known interactions. The BCH domain of BPGAP1 induces K-Ras (a small GTPase) activation; it is also able to interact with SmgGDS, inhibiting BPGAP1-induced K-Ras activation. The PRR domain of BPGAP1 interacts with both the SH3 domain of cortactin (inducing cell migration) and the SH3 domain of EEN (promoting EGR receptor internalization and ERK activation). The GAP and PRR domains of BPGAP1 interact with the WW and PPI motifs in Pin1 inhibiting BPGAP1-induced ERK activation.

Overall, the myriad of BPGAP1 (henceforth termed ARHGAP8) interactors and cellular effects (Figure 5) highlight its unique role, particularly as a dual regulator of Ras and Rho signaling. However, the neuronal role of ARHGAP8 has never been explored.

1.7.7 Preliminary supporting data

Our group is interested in understanding synaptic function at the cellular and molecular levels. Given the central role of NMDA receptors in neurotransmission and synaptic plasticity induction, we subjected PSDs isolated from wildtype mice and mice lacking the developmentally regulated NMDA-type glutamate receptor subunit GluN2B to quantitative mass spectrometry as part of a study that looked at the synaptic role of GluN2B-NMDARs (Ferreira et al., 2015). We identified ARHGAP8 as a new constituent of the PSD and, more importantly, our data showed its complete loss from PSDs isolated from cortical mouse neurons lacking the GluN2B subunit (Table 1, work performed by Joana Ferreira, *unpublished data*).

Protein significantly changed in the PSDs isolated from GluN2B (-/-) cortical neurons

(compared to PSDs isolated from GluN2B (+/+) & GluN2B (+/-) cortical neurons)

Category	Protein name	Gene symbol	Acession number	% coverage	Number of peptides (CI ≥ 95%)	GluN2B (+/+) and (+/-) : 113 ± SEM		GluN2B (-/-) : 113 ± SEM	
Actin cytoskeleton	Actin-related protein 3	Actr3	IPI:IPI00115627.4	37.59	9	0.879	0.089	0.464	0.021
	Actin filament-associated protein 1	Afap1	IPI:IPI00467327.4	22.33	2	1.057	0.064	0.697	0.049
	Profilin-2	Pfn2	IPI:IPI00845675.1	35.12	4	1.092	0.064	1.399	0.044
GTPases	Rho GTPase-activating protein 8 [1]	Arhgap8	IPI:IPI00318371.1	24.23	1	0.956	0.018	-	-
	RAB5C. member RAS oncogene family	Rab5c	IPI:IPI00404579.1	49.5	3	1.156	0.063	1.542	0.143
	Ras-related protein Rab-10	Rab10	IPI:IPI00130118.1	78.13	6	0.960	0.110	1.752	0.220
	Guanine nucleotide-binding protein subunit beta-5	Gnb5	IPI:IPI00127930.1	54.93	7	0.825	0.049	0.408	0.045

Table 1. Actin cytoskeleton-related protein expression alterations in the PSDs isolated from GluN2B$^{-/-}$ mice compared to wild-type PSDs. PSDs isolated from wildtype mice and mice lacking the developmentally regulated NMDA-type glutamate receptor subunit GluN2B were subjected to quantitative mass spectrometry. The table (cropped section) presents some of the proteins which values showed statistical significance between genotypes. ARHGAP8 expression is completely abolished in PSDs isolated from cortical mouse neurons lacking the GluN2B subunit.

Further preliminary work performed in our group confirmed these findings in GluN2B$^{-/-}$ hippocampal neurons. The dendritic and synaptic levels of ARHGAP8 are significantly decreased when compared to wild-type amounts (Figure 6, work performed by Jeannette Schmidt, *unpublished data*). These data prompted us to study the synaptic role of ARHGAP8.

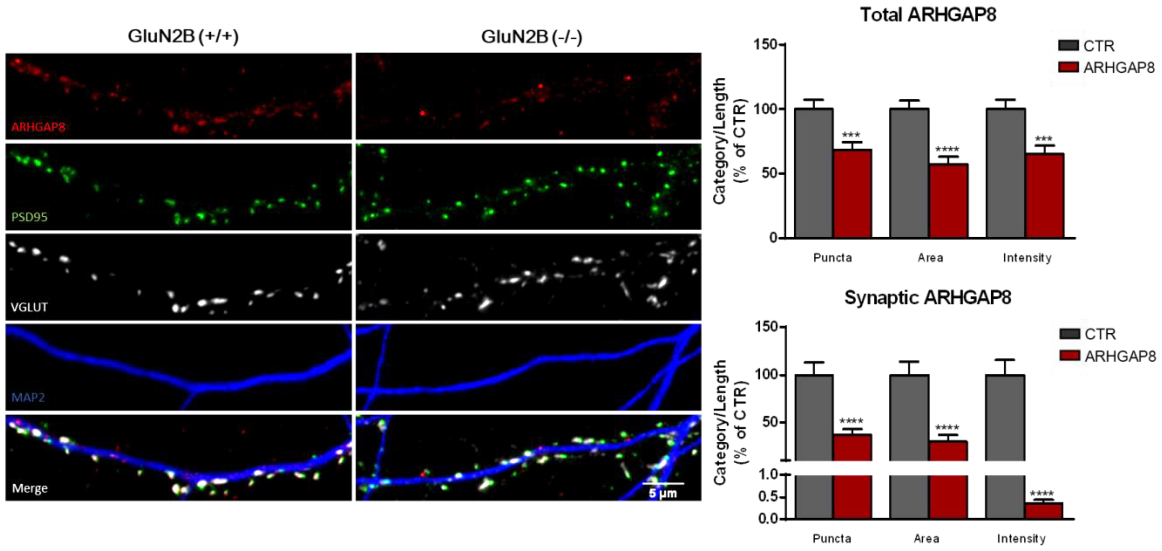

Figure 6: The dendritic and synaptic levels of ARHGAP8 are significantly decreased in GluN2B$^{-/-}$ hippocampal neurons. Hippocampal neurons of GluN2B$^{-/-}$ and WT mice were stained for MAP2, ARHGAP8, PSD-95 and VGLUT. Neurons were analyzed for total ARHGAP8 puncta (top right) or synaptic ARHGAP8 puncta (bottom right) number, area and intensity. Synaptic ARHGAP8 puncta was determined as ARHGAP8 signal that overlaps with both PSD-95 and VGLUT signals. Results are presented as percentage of GluN2B$^{+/+}$ neurons. Statistical analysis was determined by unpaired two tailed t test. Significance: *** P-value < 0.001, **** P-value < 0.0001.

Western blot analysis of brain tissue samples taken from adult *Wistar* rats (more than 10 weeks old) reveal that ARHGAP8 is widely expressed in the rat adult brain (Figure 7 top left panel, work performed by Jeannette Schmidt, *unpublished data*). Moreover, looking at the developmental expression pattern we are able to identify ARHGAP8 as early as E17 with an apparent raise throughout development and a peak at post-embryonic day 21 after which its expression stabilizes (Figure 7 right and bottom left panels, work performed by Jeannette Schmidt, *unpublished data*).

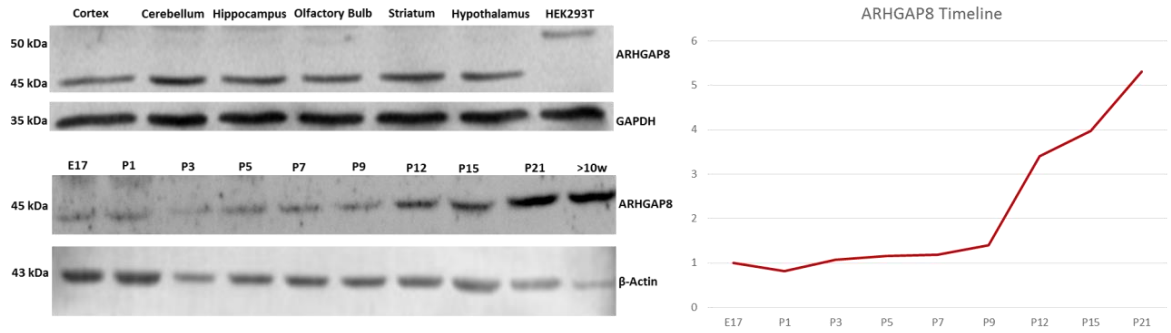

Figure 7: ARHGAP8 is widely expressed in the brain and its expression increases throughout development. Western blot analysis of brain tissue samples taken from adult *Wistar* rats (more than 10 weeks old, top left pannel) were stained for ARHGAP8 and GAPDH (housekeeping gene, internal control). Western blot analysis of cortical tissue samples taken from mouse (C57BL6) (bottom left pannel) were stained for ARHGAP8 and β-actin (internal control). Results are plotted as the amount of ARHGAP8 normalized with its internal control in a timeline graph (right pannel).

Sholl analysis performed in excitatory neurons of primary cultures of mouse hippocampal neurons transfected with a GFP-tagged full length ARHGAP8 or the GFP control did not reveal any alterations in the dendritic arborization triggered by ARHGAP8 overexpression (Figure 8, work performed by Jeannette Schmidt, *unpublished data*).

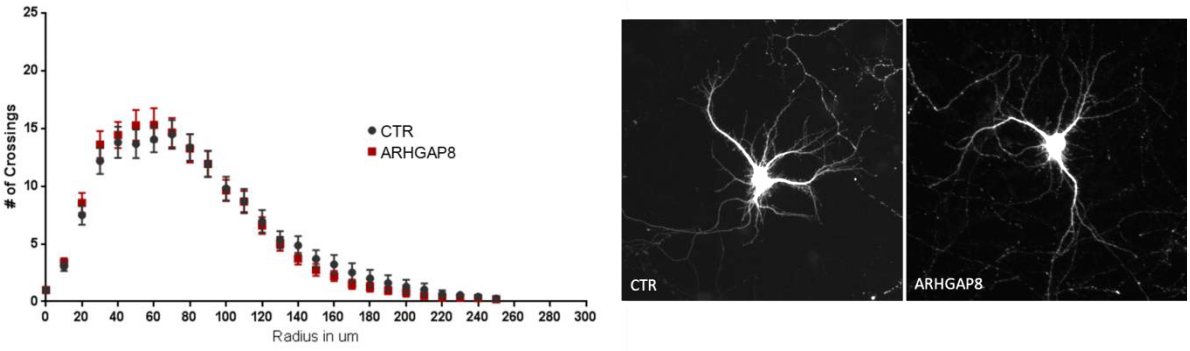

Figure 8: ARHGAP8 overexpression does not affect the dendritic morphology of excitatory neurons. Sholl analysis for DIV 10 primary cultures of mouse hippocampal neurons shows no significant difference in the extend of dendritic arborization between GFP controls (n = 37 cells) and ARHGAP8 overexpression (n = 36 cells). Concentric circles are spaced 10 µm apart. Statistical analysis was determined by unpaired two tailed t test.

Trying to understand ARHGAP8 function in synapses, and given the altered levels of synaptic AMPAR in GluN2B$^{-/-}$ neurons (Ferreira et al., 2015), primary cultures of rat hippocampal neurons transfected with a GFP-tagged full length ARHGAP8 construct or a GFP-encoding control vector were immunostained for the surface GluA1 subunit of the AMPAR and synaptic GluA1 staining was determined by co-localization with PSD-95 (Figure 9, work performed by Jeannette Schmidt, *unpublished data*). Synaptic levels of surface GluA1 are greatly decreased when ARHGAP8 is overexpressed, with no effect on the overall total GluA1 staining puncta or area but with an acute loss in intensity of the puncta.

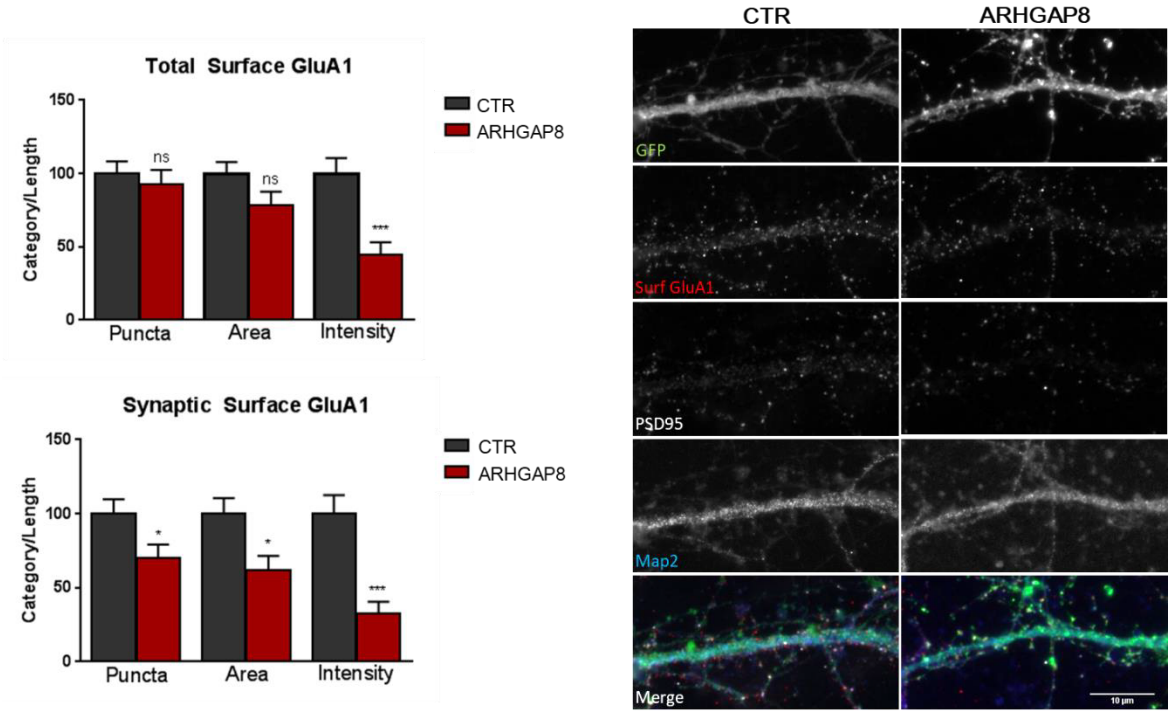

Figure 9: Synaptic levels of surface GluA1 are decreased upon ARHGAP8 overexpression. Hippocampal neurons were stained for MAP2, surface GluA1 and PSD-95. Neurons were analyzed for total surface GluA1 puncta (top left panel) or synaptic surface GluA1 puncta (bottom left panel) number, area and intensity. Synaptic surface GluA1 puncta was determined as GluA1 signal that overlaps with PSD-95 signal. Results are presented as percentage of control neurons, and are averaged from 13-14 cells. Statistical analysis was determined by unpaired two tailed t test. Significance: * P-value < 0.05, *** P-value < 0.001.

Combining our preliminary data and all the information so far available, we are prompted to believe that ARHGAP8 may have a regulatory role in excitatory synaptogenesis that may not depend on its pure function as a GAP. Yet, still no analysis has been made as for the role of ARHGAP8 in inhibitory synapses. This study first aims to investigate whether ARHGAP8 is indeed able to influence excitatory synaptical function and secondly to look

at the protein in the context of inhibitory synapses to further characterize the role of this Rho-GAP in neurons.

1.8 Objectives

Previous results from our group led to the identification of ARHGAP8 as a novel constituent of the post-synaptic density, and its localization to the PSD seems to be NMDAR-GluN2B dependent. Indeed, in GluN2B$^{-/-}$ mice, synaptic ARHGAP8 is significantly decreased. Furthermore, upon ARHGAP8 overexpression, the surface levels of synaptic GluA1 are significantly decreased.

Therefore, we firstly aimed to assess if excitatory synaptic transmission is affected by ARHGAP8. Secondly, we aimed to characterize ARHGAP8 in the context of the inhibitory synapse. To conduct this characterization, we pursued the following objectives:

1. Evaluate whether ARHGAP8 affects excitatory synaptic transmission in cortical neurons. Whole-cell patch clamp methods are applied to evaluate the frequency and amplitude of AMPAR-mediated mEPSC in ARHGAP8 overexpressing neurons.

2. Determine the linear density of ARHGAP8 and inhibitory synapse markers in cultured cortical neurons and determine if ARHGAP8 overexpression affects their dendritic expression levels. Quantitative immunocytochemistry in cortical neuronal cultures is used to determine co-localization of these proteins, and their levels of expression.

3. Assess whether overexpression of ARHGAP8 induces changes in the morphology of the dendritic arbor of inhibitory cortical neurons.

Overall, these three sets of experiments will clarify the role of ARHGAP8 in the excitatory synapse and determine if ARHGAP8 also presents a role in the inhibitory synapse.

Chapter 2 – Materials and Methods

2 Materials and Methods

2.1 Cortical neuron cultures

Cultured neurons were prepared based on (Banker & Goslin, 1998). Cerebral hemispheres of E17-18 *Wistar* rat embryos were dissected, washed three times with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS: 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red) and chemically dissociated using trypsin 0.06% (m/v) in HBSS for 15 minutes at 37ºC. To stop trypsin activity, cortices were washed six times with HBSS. Cortical tissue was mechanically dissociated, single-cell suspension was isolated using a cell strainer and the obtained cortical cells were plated in neuronal plating medium (minimum essential medium Eagle (MEM) supplemented with 10% horse serum (HS), 0.6% glucose and 1 mM pyruvic acid) onto poly-D-lysine-coated glass coverslips.

Low density cultures. Cells were plated onto coverslips at a final density of $3x10^5$ cells/60 mm culture dish. After 2 hours of incubation at 37ºC in a humidified incubator of 5% CO_2/95% air, coverslips were flipped onto an astroglial feeder layer in neurobasal medium (NBM) supplemented with SM1 neuronal supplement (1:50 dilution, StemCell technologies), 0.5 mM glutamine and 0.12 mg/mL gentamycin.

High density cultures. Cells were plated onto coverslips at a final density of $1,8x10^5$ cells/cm^2 in 12-well plates. After 2 hours of incubation at 37ºC in a humidified incubator of 5% CO_2/95% air the plating medium was replaced for supplemented NBM.

To further prevent glia overgrowth, high and low density neuronal cultures were treated with 5-Fluoro-2'-deoxyuridine (FDU) (10µM final concentration) after 2 days in vitro (DIV) (Meyers et al., 2005). Cultures were maintained at 37ºC in a humidified incubator of 5% CO_2/95% air, up to 18 days. The medium of all cultures was replenished every 3-4 days, starting at DIV 7, with fresh NBM.

2.2 Transfection of primary neuronal cultures by calcium phosphate co-precipitation

Constructs were recombinantly expressed using a calcium phosphate transfection protocol adapted from Jiang and collaborators (Jiang, Deng & Chen, 2004). DNA precipitates were prepared by diluting plasmid DNAs in Tris-EDTA transfection buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.3) followed by the addition of $CaCl_2$ solution (2.5 M in 10 mM HEPES) to the diluted DNA, to the final concentration of 250 mM $CaCl_2$. The DNA-$CaCl_2$ mix was then added drop-wise to an equivalent volume of HEPES-buffered transfection solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 15 mM glucose and 42 mM HEPES, pH 7.2) and thoroughly mixed. The precipitates were left to develop at room temperature for 25-30 minutes and vortexed every 5 minutes. Prior to DNA precipitate addition, neurons were pre-treated with 2 mM kynurenic acid (KA) in conditioned NBM, for at least 15 minutes. DNA precipitates were added drop-wise to each coverslip and left incubating for 2 hours in order to allow the DNA precipitates internalization via endocytosis. Afterwards, the remaining precipitates were dissolved by replacing the medium with new pre-warmed supplemented NBM with 2 mM KA, slightly acidified with HCl (~3.125 mM final concentration) for 15 minutes at 37ºC. Finally, each coverslip was transferred to the original dish/plate containing the conditioned medium and returned to a 37ºC, 5% CO_2/95% air incubator to allow expression of the transfected constructs.

Plasmids. The pXJ40 plasmids containing the GFP control or the GFP-tagged full length ARHGAP8 were a kind gift from Professor Boon Chuan Low (Cell Signaling and Developmental Biology Laboratory, Department of Biological Science, National University of Singapore). The pLentiLox 3.7 plasmid containing mCherry was kindly provided by Professor Ann Marie Craig (University of British Columbia, Vancouver).

2.3 Immunocytochemistry

Low density cortical neurons were fixed in 4% paraformaldehyde (PFA)/4% sucrose/phosphate buffered saline (PBS) for 15 minutes at room temperature and washed 3 times in PBS before being permeabilized with 0.25% Triton X-100/PBS for 5 minutes at 4ºC. Afterwards, neurons were again washed in PBS and wax dots removed from coverslips before being place into 10% (w/v) bovine serum albumin (BSA)/PBS for 30 minutes at 37ºC to block non-specific staining. After blocking, coverslips were incubated overnight with the primary antibody mix diluted in 3% BSA/PBS, at 4ºC (Table 2). Posteriorly, coverslips were washed 6 times with PBS and incubated overnight with the appropriate secondary antibodies diluted in 3% BSA/PBS, at 4ºC (Table 3). Coverslips were then washed 6 times in PBS and mounted using fluorescent mounting medium from DAKO. Preparations were preserved overnight, protected from light and sealed with nailpolish until microscope analysis.

Imaging. Fluorescence imaging was performed on a Zeiss Observer Z.1 inverted microscope, with an AxioCamHRm camera and Zen imaging software. Images were acquired with a Plan-Apochromat 63x/1.4 oil DIC objective. Secondary dendrites of at least 7 cells per condition were randomly chosen based on the health and similarity of their morphology, using the MAP2 channel.

Protein puncta quantification and co-localization. Gephyrin, VGAT and ARHGAP8 signals or $GABA_A$-receptor-α1 and ARHGAP8 signals were analyzed after thresholds were set, such that clusters and/or puncta were included in the analysis and the background intensity of each image was subtracted. Synaptic gephyrin puncta were selected by co-localization with VGAT. For each selected cell, integrated density, area and number of total puncta per dendritic length were determined with Image J 1.51n analysis software. Images were quantified blind to experimental condition.

2.4 ARHGAP8 overexpression

Changes in synaptic number and protein localization were determined by comparing immunofluorescence staining of non-transfected neurons with the ones of transfected neurons overexpressing the GFP-tagged full length ARHGAP8 or the GFP control. Neurons were transfected at DIV 11 (1 µg of DNA per coverslip) and DNA expression was allowed until DIV 14.

2.5 Sholl analysis

Transfection. Low density cultures were transfected as described above. Neurons were simultaneously transfected with plasmids enconding mCherry and either the GFP or the GFP-tagged full length ARHGAP8 (2 µg of total DNA per coverslip, at a ratio of 1:1). Neurons were transfected at DIV 7 or DIV 11 and DNA expression was allowed until DIV 10 or DIV 14, respectively. Subsequently, cells were fixed and immunostained, as previously outlined, for MAP2 and GABA for identification of the dendritic tree of inhibitory neurons.

Imaging. Fluorescence imaging was performed on a Zeiss Imager.Z2 upright microscope, with an AxioCamHRm camera and Zen imaging software. Images were acquired with an EC Plan-Neofluar 10x/0.3 DIC objective. At least 15 cells per condition were imaged based on immunofluorescence against GFP (marker for transfected neurons), mCherry (neuronal filling marker) and GABA (inhibitory neurons marker).

Neurite tracing. Sholl analysis was carried out using using the Simple Neurite Tracer Plugin in Image J/FIJI 1.51n analysis software (Longair et al. 2011; Ferreira et al. 2014). Quantification of the number of dendrite intersections with concentric circles of gradually increasing radii (annulus of 10 µm) centered at the centroid of the cell body was carried out in mCherry images.

2.6 Electrophysiology

Transfection. High density cultures were transfected at DIV 14 with the pXJ40 plasmids expressing GFP or the GFP-tagged full length ARHGAP8 (1 µg of DNA per coverslip). DNA expression was allowed up to DIV 18.

Miniature excitatory post-synaptic currents (mEPSC) recordings. Whole-cell voltage clamp recordings were carried out at room temperature (~23ºC). The recording chamber was mounted on a fixed-stage inverted microscope (Zeiss Observer.A1) and perfused at a constant rate in extracellular solution (140 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose, 4 mM $CaCl_2$, 4 mM $MgCl_2$, pH was adjusted to 7.3 and osmolarity to 300-310 mOsm, supplemented with 100 µM picrotoxin (PTX) (Tocris Bioscience), 500 nM tetrodotoxin (TTX) (Tocris Bioscience), 50 µM (2R)-amino-5-phosphonovaleric acid (AP-V) (Tocris Bioscience)). Fluorescent illumination was used to identify transfected neurons and transmission illumination was used to visualize and patch the selected neurons. Patch electrodes (3-5 mΩ) were made from borosilicate glass (Science Products, Germany) and filled with a solution composed of 115 mM $CsMeSO_3$, 20 mM CsCl, 2.5 mM $MgCl_2.6H_2O$, 10 mM HEPES, 0.6 mM EGTA, 10 mM Na-phosphocreatine, 4 mM Na_2-ATP, 0.4 mM Na-GTP, adjusted to pH 7.3 with CsOH and osmolarity 298-300 mOsm. Cells were voltage-clamped at -70 mV and access resistance was not compensated. mEPSC were acquired using Patchmaster software (HEKA Elektronik). mEPSC events were recorded over a period of 5 minutes in a gap-free acquisition mode, the signals filtered at 2.9 kHz and acquired with a sampling rate of 25 kHz. After conversion of the files to .abf format, the pClamp software was used to detect the events and quantify amplitude, frequency and decay tau of mEPSC recordings. Only events larger than 2x the recording noise and decay tau below 80 ms were considered. Recordings were quantified blind to experimental condition. For each cell, the median value was obtained and averaged across all recorded cells.

2.7 Statistical analysis

Graphs and statistical analysis were performed using GraphPad Prism 6 software. Results are plotted as normalized means ± SEM and p-value inferior to 0.05 was chosen for statistical significance. Two samples comparisons were evaluated using non-paired two-tailed t-test. Outliers were removed using ROUT for immunocytochemistry data or Grubbs' test for electrophysiology data.

Primary antibody	Source	Cat #	Dilution
anti-**ARHGAP8** raised in rabbit	Abcam	Ab133851	1:250
anti-**gephyrin** raised in mouse	Synaptic Systems	147 011	1:1000
anti-**VGAT** raised in guinea-pig	Synaptic Systems	131 004	1:300
anti-**GABA-A-R-α1** raised in mouse	UC Davis/NIH NeuroMab Fac.	N95/35	1:200
anti-**GABA** raised in rabbit	Sigma-Aldrich	A2052	1:750
anti-**MAP2** raised in rabbit	Abcam	Ab5392	1:5000

Table 2. Primary antibodies used in the immunocytochemistry experiments.

Secondary antibody	Source	Cat #	Dilution
Alexa Fluor 488 goat anti-mouse	Thermo Fisher Scientific	A11001	1:500
Alexa Fluor 568 goat anti-rabbit	Thermo Fisher Scientific	A11036	1:500
Alexa Fluor 568 goat anti-mouse	Thermo Fisher Scientific	A11004	1:500
Alexa Fluor 647 goat anti-guinea	Thermo Fisher Scientific	A21450	1:500
Alexa Fluor 647 goat anti-rabbit	Thermo Fisher Scientific	A21244	1:500
AMCA AffiniPure goat anti-chicken	Jackson ImmunoResearch	103-155-155	1:200

Table 3. Secondary antibodies used in the immunocytochemistry experiments.

Chapter 3 - Results

3 Results

3.1 ARHGAP8 overexpression decreases the frequency and amplitude of AMPAR-mediated mEPSC

Over the last couple of years, more and more Rho-GAPs are being studied for their function in the context of neurons and many of them have now been shown to be crucial to the proper functioning of synapses. Probably one of the most prominent examples is Oligophrenin-1 (OPHN1). Interest in this protein first arose when it was found to be mutated in cases of mental disability (Bienvenu, et al., 1997; Billuart, et al., 1998). Since then evidence has been provided for its role in the morphology of dendritic spines of the hippocampal CA1 region (Govek et al, 2004; Khelfaoui et al. 2007) as well as its regulatory role in excitatory synaptic function. Downregulation or defects of OPHN1 in rat hippocampal slices caused not only reduced spinal length and density but also a destabilization of synaptic AMPARs due to increased RhoA activity (Govek et al., 2004; Kasri et al, 2009). A more recent example saw ARHGAP12, a Rac1-GAP that is almost exclusively expressed in the CA1 region and the dentate gyrus of the hippocampus, investigated for its respective function in the postsynaptic context. By manipulating the protein levels, the authors were able to provide evidence for ARHGAP12 being a developmental coordinator for synapse structure and function in the hippocampus. Upregulation of this specific GAP caused significant synaptic depression whereas knockdown led to an augmentation of AMPAR-mediated EPSCs and enhanced frequencies and amplitudes of mEPSCs. Additionally, ARHGAP12 has been described as a “developmental break” due to its inhibitory effects in unsilencing synapses during early development (Ba et al., 2016).

As part of a previous project that aimed to examine the effects of the NMDAR subunit GluN2B on AMPAR trafficking our group has identified ARHGAP8 as a novel component of the synapse. The PSD protein content of cortical neurons lacking the GluN2B subunit were compared to the wildtype control by quantitative mass spectrometry analysis (Ferreira et al., 2015) and, more strikingly, in addition to proving the presence of ARHGAP8 in this very specialized spinal substructure we have also found a complete depletion of the protein from PSDs of the GluN2B$^{-/-}$ condition. One of the main findings of the study was that GluN2B$^{-/-}$ hippocampal neurons presented with increased levels of surface GluA1 due to an impairment in GluA1-AMPAR endocytosis that we could link to

deficient synaptic proteasome activity (Ferreira et al, 2015). However, rescue experiments involving the pharmacological enhancement of the proteasomal activity only partially recovered GluA1 amounts to GluN2B wildtype levels, leading us to believe that there are additional pathways that need to be considered.

Looking at the emerging evidence for the role of GTPases and their regulatory proteins in excitatory synapses we have begun to take a closer look at ARHGAP8s putative role in synaptic connections. Preliminary immunocytochemistry stainings for superficial GluA1 in rat hippocampal neurons that overexpressed ARHGAP8 showed a pronounced loss in synaptical GluA1 levels compared to control conditions (unpublished preliminary data, Figure 9). These results are in accordance with our earlier findings showing a total loss of ARHGAP8 combined with an increase in surface GluA1-AMPARs in GluN2B$^{-/-}$ hippocampal neurons.

We therefore reasoned that ARHGAP8 may play a regulatory role in excitatory synapses, possibly through interaction with glutamatergic receptors or the protein complexes that anchor them. We aimed to assess the role of ARHGAP8 in modulating excitatory synaptic function. Therefore, we investigated the effects of its overexpression on AMPAR-dependent synaptic transmission.

DIV 14 rat cortical neurons were transfected in order to overexpress GFP-tagged ARHGAP8 or GFP, in the control condition, and effects on synaptic transmission were assessed by whole-cell recordings of AMPAR-mediated miniature EPSC (mEPSC). DIV 16-18 (at least 2 days of DNA expression) cortical dense cultures were used for whole-cell recording over a period of 5 minutes in the presence of 100 µM picrotoxin (to block inhibitory transmission through $GABA_A$ receptors), 500 nm TTX (to block Na^+ channels and depolarization of neurons) and 50 µM of AP-V (to block NMDAR-mediated transmission). Spontaneous AMPAR-mediated currents were measured and detected as inward currents, in transfected neurons identified by the expression of GFP.

Overexpression of ARHGAP8 significantly decreased both the amplitude (Figure 10A, 10E) and frequency (Figure 10B) of AMPAR-mediated synaptic transmission, showing no effects on the kinetics of the receptor (no differences on the rise tau or decay tau were detected; Figure 10C-D). Ectopically expressed ARHGAP8 is sufficient to depress AMPAR-mediated transmission. These changes could be a consequence of a change of synaptic AMPAR at individual synapses, a change in the number of functional synapses, or both. Typically, a change in frequency reflects a change in the number of active synapses or in the presynaptic release probability. Given that in this experiments

recording were performed from neurons overexpressing ARHGAP8 in sparsely transfected cultures, we do not expect pre-synaptic effects of the overexpressed protein, and favour the possibility that the decrease in mEPSC frequency is due to a decrease in the number of AMPAR-containing synapses. The decrease in amplitude and frequency of AMPAR-mediated mEPSC supports our previous findings showing that synaptic levels of surface GluA1 are decreased when ARHGAP8 is overexpressed (Figure 9).

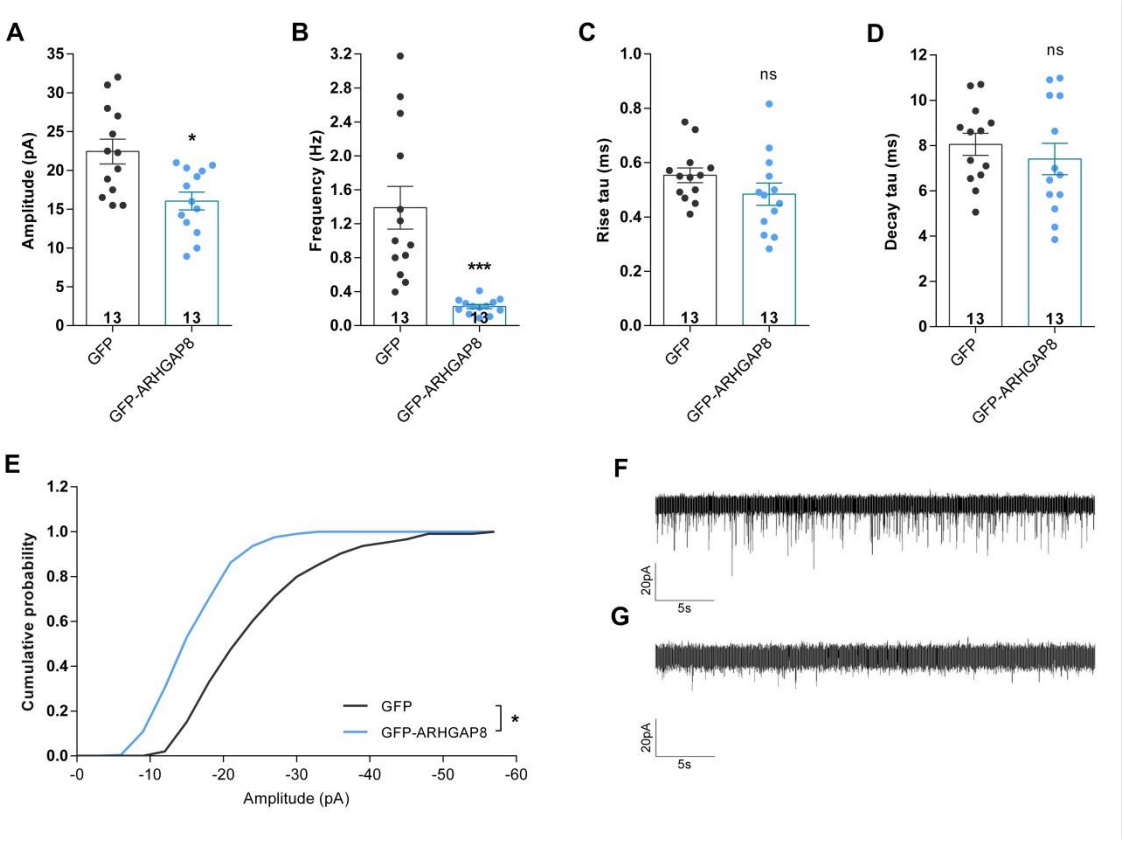

Figure 10. Overexpression of ARHGAP8 decreases the frequency and amplitude of AMPAR-mediated mEPSC without affecting AMPAR current kinetics. A-D. Quantification of excitatory miniature events recorded from control neurons and neurons overexpressing ARHGAP8 at DIV 16-18. **A-B.** Overexpression of ARHGAP8 results in a significantly reduced amplitude **(A)** of AMPAR-mediated mEPSC and in a marked decrease in the frequency **(B)** of these events. **C-D.** When overexpressing ARHGAP8, changes in the rise tau **(C)** and decay tau **(D)** of mEPSC were not detected. **E.** Cumulative probability distribution of mEPSC amplitudes for control neurons (416 events, n = 13 cells) and ARHGAP8 overexpressing neurons (416 events, n = 13 cells). Overexpression of ARHGAP8 decreased mEPSC amplitude, as indicated by a significate leftward shift to the cumulative probability distribution. **F-G.** Representative traces of whole-cell recording of mEPSC mediated by AMPAR, performed in cortical rat cells expressing GFP **(F)** or a GFP-ARHGAP8 **(G)**. Scale bars, 5 s and 20 pA. Data are presented as means ± SEM **(A-D)** for n = 13 cells from three independent experiments. Statistical analysis was determined by unpaired two tailed t test. Significance: * P-value < 0.05, *** P-value < 0.001..

3.2 ARHGAP8 co-localizes with gephyrin, VGAT and $GABA_A$ receptor subunit α1

Given that the role of ARHGAP8 in neuronal cells is largely unknown, basic characterization of ARHGAP8 overall neuronal distribution and its presence in synapses are important in order to further understand the function of this novel Rho-GAP. Besides addressing the role of ARHGAP8 in regulating excitatory synapses, here we focused on evaluating the presence of ARHGAP8 in inhibitory synapses. For this purpose, we quantified the linear density of ARHGAP8 and inhibitory synapse markers along secondary dendrites (Figure 11A). We labelled ARHGAP8, the postsynaptic scaffold protein gephyrin, and the presynaptic vesicular GABA transporter VGAT in cultured cortical neurons, and detected ~4.72 ARHGAP8 clusters per 10 µm dendrite, a linear density higher than those measured for gephyrin (~3.02 clusters per 10 µm dendrite) and VGAT (~1.90 clusters per 10 µm dendrite) (Figure 11B). We also evaluated the co-localization of ARHGAP8 with gephyrin and/or VGAT, as well as measured puncta of gephyrin that co-localize with VGAT (~1.09 clusters per 10 µm dendrite), to assess the density of inhibitory synapses (Figure 11C) in secondary dendrites of cortical neuron cultures. Even though ARHGAP8 clusters that co-localize with both gephyrin and VGAT are a small percentage of the total ARHGAP8 clusters, about half of the total gephyrin clusters that co-localizes with VGAT (accounting for the synaptic gephyrin clusters) contain ARHGAP8 (~0.6 clusters per 10 µm dendrite), indicating that ARHGAP8 may be present in around 50% of inhibitory synapses.

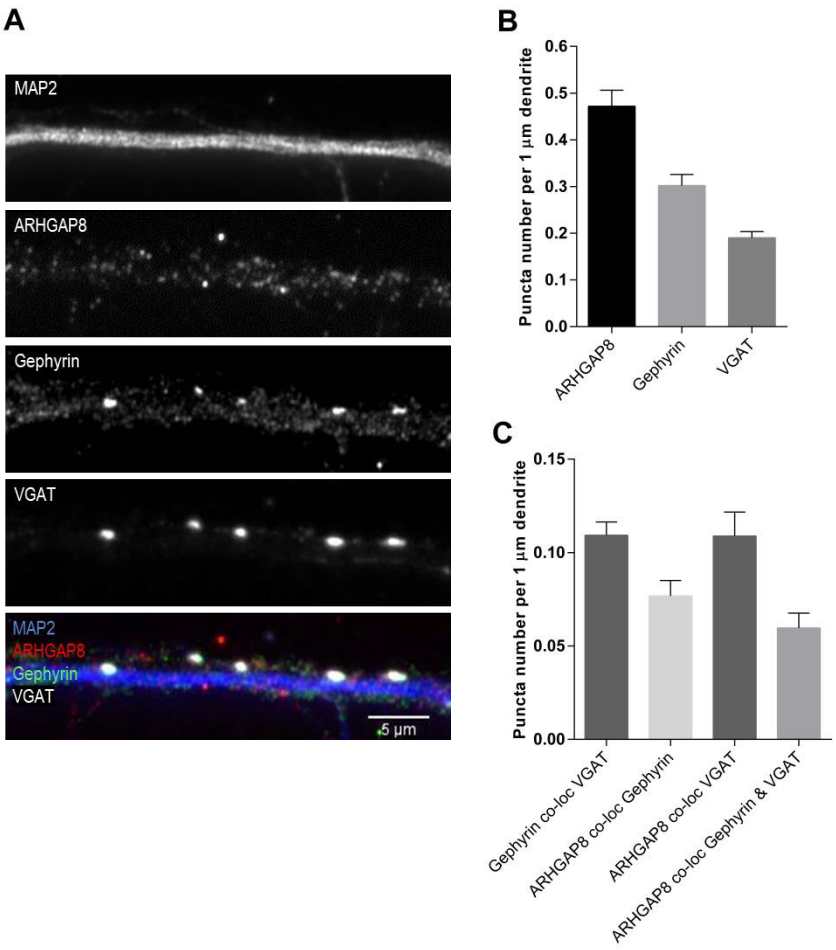

Figure 11. Distribution of ARHGAP8, gephyrin and VGAT along secondary dendrites. Cortical neurons (DIV 14) were stained for MAP2 (to identify dendrites), ARHGAP8, gephyrin and VGAT. Neurons were analyzed for total number of puncta per dendritic length **(B-C)**. **(C)** Gephyrin puncta that co-localize with VGAT, as well as ARHGAP8 puncta that co-localize with gephyrin, VGAT or with both gephyrin and VGAT were quantified. Results are averaged from three independent experiments (n = 39 cells). Data are presented as means ± SEM.

We also assessed the distribution of the α1 $GABA_A$ receptor subunit along secondary dendrites, and ARHGAP8 co-localization with this receptor subunit (Figure 12). As for the co-localization of ARHGAP8 with $GABA_A$ receptor subunit α1, only a small percentage co-localizes with this subunit that is typically expressed in $GABA_A$ receptors that are located at synapses. However, it is important to take in consideration that we are evaluating total $GABA_A$ receptor α1 subunit expression; it is expected that only a fraction of the clusters that we detect are synaptic. Also, these measurements were performed in non-simulated neurons; it would be interesting to test whether neuronal activity alters the expression pattern of ARHGAP8 and its co-localization with inhibitory synapse proteins.

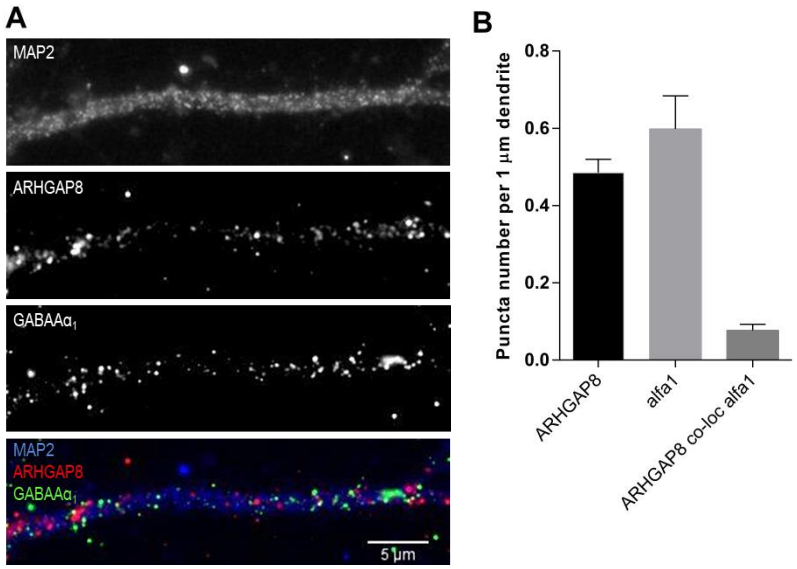

Figure 12. Linear density of $GABA_A$ receptor subunit α1 staining along cortical neuronal secondary dendrites, and ARHGAP8 co-localization with the $GABA_A$ receptor subunit α1. Cortical DIV 14 neurons were stained for MAP2 (to identify dendrites), ARHGAP8 and $GABA_A$ receptor subunit α1. **B.** Neurons were analyzed for total number of puncta per dendritic length, and for ARHGAP8 puncta that co-localize with the $GABA_A$ receptor subunit α1. Results are averaged from three independent experiments (n = 24 cells). Data are presented as means ± SEM.

3.3 Overexpression of ARHGAP8 changes the expression of inhibitory synapses markers

Previous findings in our group show that upon ARHGAP8 overexpression, the surface expression of synaptic GluA1 is altered (Figure 9), suggesting that ARHGAP8 is able to modulate excitatory synaptic function. These observations are supported by our results regarding the effect of ARHGAP8 overexpression on AMPAR-mediated mEPSC (Figure 10). Some Rho-GAPs are able to modulate the function of both excitatory and inhibitory synapses. One such Rho-GAP is SRGAP2A, a Rac1-specific GAP, which is able to interact with the scaffold protein Homer 1 (in excitatory synapses) and gephyrin (in inhibitory synapses), linking excitatory and inhibitory modulation and promoting maturation of both types of synapses (Fossati et al., 2016). Given the significant fraction of inhibitory synapses that contain ARHGAP8 (Figure 11) we were interested in investigating whether ARHGAP8 overexpression could affect inhibitory synapse density and the dendritic expression of $GABA_A$ receptors. To assess this, we quantified the linear density of synaptic VGAT-co-localized gephyrin clusters (Figure 13B-D) and $GABA_A$ receptor subunit α1 puncta (Figure 14B-D) in neurons overexpressing ARHGAP8 compared to the GFP-transfected control neurons and non-transfected neurons. Upon ARHGAP8

overexpression, the total number of synaptic gephyrin clusters (determined by the overlapping of gephyrin and VGAT signalling) shows a strong tendency to decrease (Figure 13B), while there is a significant increase in the area of these clusters (Figure 13C) and no significant change to their fluorescence intensity (Figure 13D). These results may indicate that when ARHGAP8 is overexpressed, inhibitory synapses are decreased in number but increased in size.

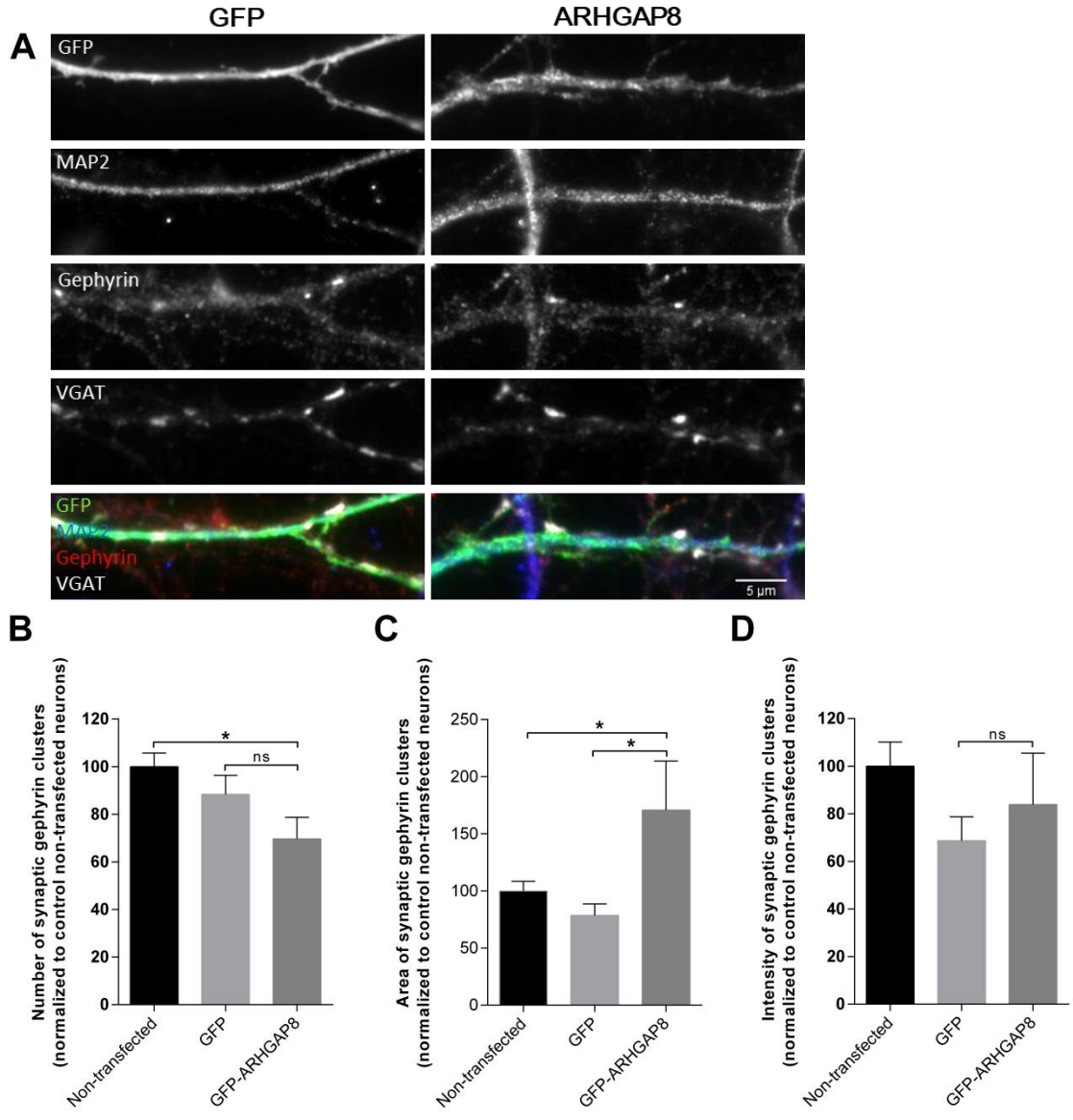

Figure 13. Overexpression of ARHGAP8 leads to an increase in the area of synaptic gephyrin clusters. Cortical DIV 14 neurons were transfected with a GFP-encoding vector or with a plasmid expressing GFP-ARHGAP8, and stained for MAP2, gephyrin and VGAT. Neurons were analyzed for synaptic gephyrin cluster **(B)** number, **(C)** area or **(D)** fluorescence intensity, per dendritic length. Synaptic gephyrin is defined as gephyrin signal that overlaps with VGAT. Results are presented as percentage of non-transfected (control)

cells, and are averaged from three independent experiments (n = 21-39 cells). Data are presented as means ± SEM **(B-D)**. Statistical analysis was determined by unpaired two tailed t test. Significance: * P-value < 0.05.

As for the $GABA_A$ receptor subunit α1, no significant change was detected between the GFP control and ARHGAP8 overexpression conditions, but there seems to be a strong tendency for an increase in both puncta number (Figure 14B) and area (Figure 14C), with no changes in the fluorescence intensity (Figure 14D). These results may indicate that ARHGAP8 modulates the distribution of $GABA_A$ receptor subunits (in particular the α1 subunit) along dendrites. However, more experiments should be performed to confirm these data and to determine if ARHGAP8 affects synaptic, extra-synaptic or even the total population of $GABA_A$ receptors containing the α1 subunit.

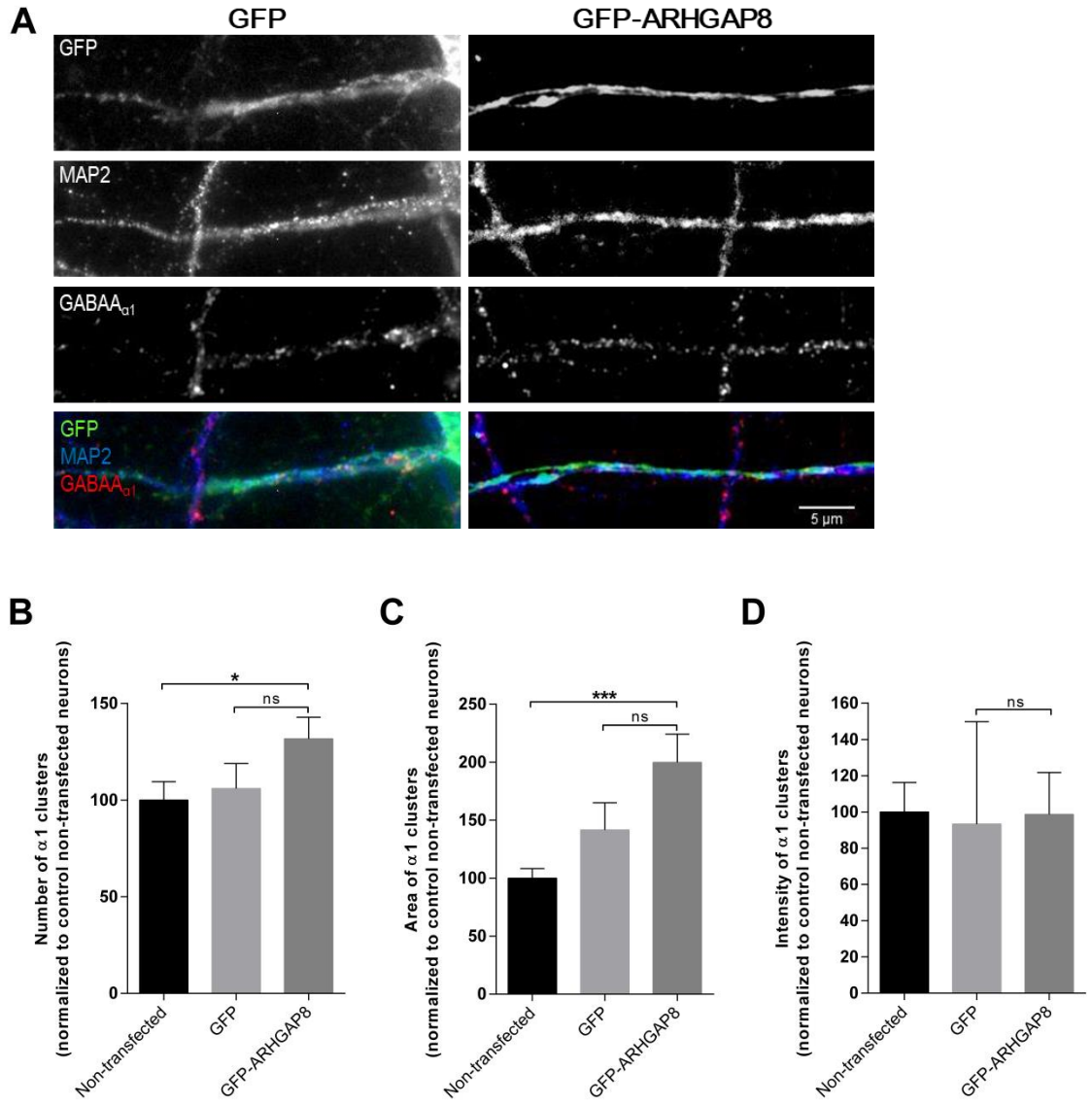

Figure 14. Effect of the overexpression of ARHGAP8 on the dendritic expression of α1-containing $GABA_A$ receptors. Cortical DIV 14 neurons were transfected with a GFP-encoding vector or with a plasmid expressing GFP-ARHGAP8, and stained for MAP2 and the $GABA_A$ receptor subunit α1. Neurons were analyzed for total $GABA_A$ receptor subunit α1 **(B)** number, **(C)** area or **(D)** fluorescence intensity, per dendritic length. Results are presented as percentage of non-transfected (control) cells, and are averaged from three independent experiments (n = 21-39 cells). Data are presented as means ± SEM **(B-D)**. Statistical analysis was determined by unpaired two tailed t test. Significance: * P-value < 0.05, *** P-value < 0.001.

3.4 Overexpression of ARHGAP8 had no effect in the dendritic arborization of inhibitory cortical neurons

Patients with mental retardation and ID show a particularly common feature in their neurons called spine dysgenesis – high degree of spine loss and morphological abnormalities – and/or abnormal dendritic structure (reviewed by Newey, S. et al., 2004). As an example, neurons from patients with Down's syndrome show a decreased length and branching of theirs dendritic arborization (Takashima et al., 1994) – a feature that is replicated in the mouse model Ts65Dn, which shows cognitive, behavioral and anatomic deficits consistent with the pathology. Rho signaling has been suggested to be relevant for the development of normal cognition due to its well defined role in determining dendritic development and morphology (reviewed by Newey, S. et al., 2004). Furthermore, mutations in some genes that result in mental retardation are directly linked to aberrant GTPase signaling (as reviewed by Govek et al., 2005). Through manipulation of the three most common GTPases: RhoA, Rac1 and Cdc42, it has become clear that interplay between GTPases determines the complexity of the dendritic arbor. In general, RhoA activation negatively impacts dendritic outgrowth, and its effects are mediated by the RhoA effector, Rho-kinase (ROCK) (reviewed by Newey, S. et al., 2004). As regulators of GTPases, Rho-GAPs have been described to regulate dendritic complexity of cortical neurons. For example, loss of NOMA-GAP, a negative regulator of Cdc42, reduces the dendritic complexity of layer II/III pyramidal neurons with no effects in layer V cortical neurons (Rosário et al., 2012).

We have already analyzed the effects of an upregulation of ARHGAP8 on dendritic arborization, and did not find any significant differences between control neurons and cells overexpressing the protein (Figure 8). However, in this analysis we evaluated excitatory pyramidal neurons, the most abundant in hippocampal neuronal cultures. As we have demonstrated opposite outcomes for the overexpression of ARHGAP8 in excitatory versus inhibitory synapses before (Figure 9 vs. Figures 13,14), here we went ahead and tested the effect of ARHGAP8 overexpression specifically on inhibitory neuron dendritic ramifications. Additionally, we have so far only looked at the consequences of upregulating ARHGAP8 in hippocampal cells of a relatively mature age (DIV 10) (Figure 8) but it is possible that ARHGAP exerts effects at earlier developmental stages. We therefore decided to assess the effect of increasing the ARHGAP8 levels on inhibitory dendritic morphology at two different time points.

DIV 7 and DIV 11 rat cortical neurons were transfected to overexpress ARHGAP8 or the GFP control, and co-express mCherry (for neuronal filling) and effects on morphological structure of inhibitory neurons were assessed by Sholl analysis at DIV 10 and DIV 14, respectively (DNA expression was allowed for three days). Immunostaining against GABA allowed detection of inhibitory neurons in the cortical dense cultures. The mCherry signal was used to analyze neuronal morphology.

Similarly to our data from excitatory neurons, our results show that overexpression of ARHGAP8 does not affect the overall dendritic morphology of inhibitory rat cortical neurons (Figure 15), at either of the two examined ages. Together with the previous data from our group relative to the morphology of excitatory neurons, our findings indicate that ARHGAP8 is not essential to the development of the dendritic arborization of neuronal cells. Even though ARHGAP8 doesn't seem to present a structural role in the modulation of neuronal morphology, at least at the development stages that we tested, our data do not exclude the hypothesis that ARHGAP8 presents a more functional role in modulating aspects of neuronal development or communication.

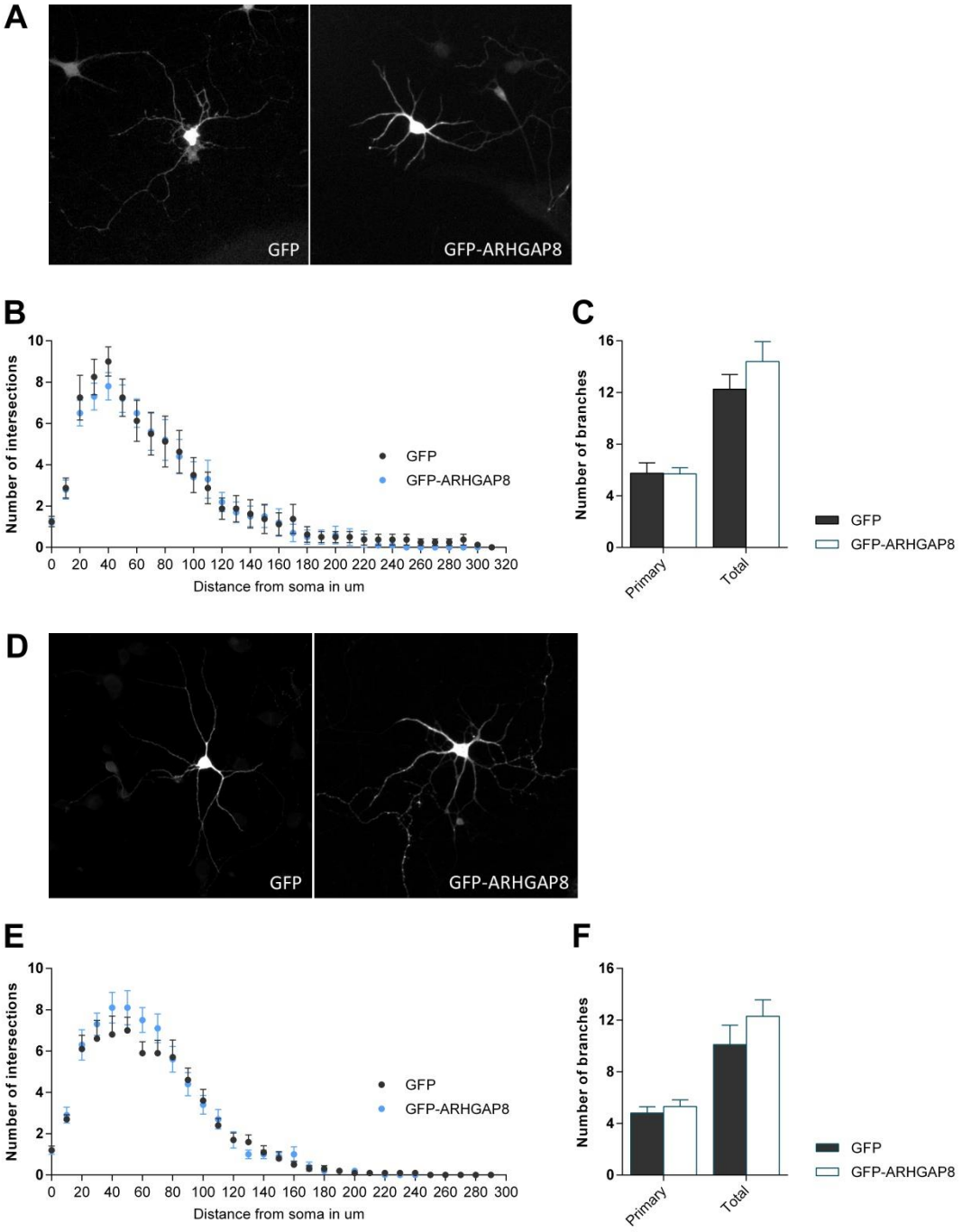

Figure 15. ARHGAP8 overexpression does not affect the dendritic morphology of DIV 7-14 inhibitory neurons. **B.** Sholl analysis for DIV 10 inhibitory neurons shows no significant difference in the complexity of the dendritic arbor between GFP-expressing (n = 13 cells) and neurons overexpressing GFP-ARHGAP8 (n = 15 cells). **C.** Histogram shows comparisons of the number of primary dendritic branches and total dendritic branch points **E.** Sholl profile for DIV 14 inhibitory neurons. Again, there is no significant difference in the morphology of the dendritic trees between control (n = 15 cells) and ARHGAP8 overexpressing (n = 16 cells) neurons. **F.** Histogram shows comparisons of the number of primary dendritic branches and total dendritic branch points. Statistical analysis was determined by unpaired two tailed t test. No significant difference was found in these parameters. Concentric circles are spaced 10 μm apart.

Chapter 4 - Discussion

4 Discussion

As the first investigators to study *ARHGAP8*, Prof. Boon Chuan Low and his group cloned the first of four isoforms encoded by it, which they named BPGAP1 after its protein domains. A lot of research has been done and published by this group in identifying and characterizing the functional domains present on this novel Rho GTPase activating protein. The authors have described how this Rho-GAP is able to interact with cortactin in regulating spatial cell dynamics, with endophilin II, a protein known to be involved in the endocytic pathway, and with Pin1, a peptidylprolyl isomerase also known to be expressed in postsynaptic structures and interact with PSD-95, negatively affecting its interaction with NMDAR. Besides the previously mentioned functions for ARHGAP8, it is able to activate other signalling pathways through interaction of its unique domains, emerging as a potential regulator of cell dynamics. Even though a lot of characterization work has been put into investigation of ARHGAP8, its domains and interactions, up until now the neuronal role of this Rho-GAP has yet to be explored.

Our group first took an interest at this protein when a quantitative mass spectrometry study that looked at the synaptic role of GluN2B-NMDARs revealed that in mice lacking the developmentally regulated NMDA-type glutamate receptor subunit GluN2B, ARHGAP8 was abolished from the PSDs, while in wild-type mice there was detection of this Rho-GAP in these excitatory post-synaptic structures. Besides identifying ARHGAP8 as a new component of the PSDs, the loss of ARHGAP8 in GluN2B$^{-/-}$ motivated us to understand why ARHGAP8 is located at the PSD and what kind of synaptic function it might exert.

Given that ARHGAP8 existence at the synapse was apparently dependent on the presence of GluN2B-NMDAR and having in mind that other GAPs have been attributed roles in the formation and dynamics of excitatory synapses, it is reasonable to think that ARHGAP8 function might be related to the regulation of glutamatergic receptors or of the synaptic structure. Thus, one of the aims of this study is to investigate whether ARHGAP8 presence translates into a functional role in excitatory synapses. Even though the first piece of evidence that lead our group to study this Rho-GAP indicated a possible role in excitatory synapses, being that the neuronal role of ARHGAP8 has never been explored and given that other GAPs have been shown to have relevant effects in both excitatory

and inhibitory synapses, we also wanted to look at ARHGAP8 in the context of inhibitory synapses to further characterize the function of this Rho-GAP in neurons.

4.1 ARHGAP8 regulates AMPAR-mediated synaptic transmission

Preliminary data from our group showed that in GluN2B$^{-/-}$ neurons, the presence of ARHGAP8 in synapses is cut down, as compared to GluN2B$^{+/+}$ neurons (Figure 6). Moreover, upon ARHGAP8 overexpression, the density of AMPARs was affected, with an acute decrease of the surface level of expression of the GluA1 subunit specifically at the synapse (Figure 9). Considering these results, the hypothesis that ARHGAP8 can play a role in regulating excitatory synaptic function has arisen. To tackle this question, we took advantage of whole-cell patch clamp recordings to measure AMPAR-mediated miniature excitatory post-synaptic currents in ARHGAP8 overexpressing cortical neurons. Both the frequency and amplitude of AMPAR-mediated mEPSC were decreased in ARHGAP8 overexpressing cortical neurons, compared to neurons transfected with a control vector (Figure 10). Typically, there is a direct correlation between mEPSC frequency and either synapse number or pre-synaptic function. On the other hand, decreased mEPSC amplitude is a result of any (or a combination) of the following factors: less neurotransmitter content per quanta (per presynaptic vesicle), fewer post-synaptic receptors or a change in the type of receptors at the post-synaptic sites. Given that in this experiment recordings were performed from neurons overexpressing ARHGAP8 in sparsely transfected cultures, we do not expect pre-synaptic effects of the overexpressed protein. Since no significant alterations were detected in the rise tau or decay tau, indicating that the kinetics of the receptor doesn't seem to be affected by ARHGAP8 overexpression, we also do not expect a change in the type of receptor subunits. Therefore, we favour the possibility that the decrease in mEPSC frequency and amplitude is due to a decline in the number of AMPAR-containing synapses; the change in amplitude may also be due to a decreased AMPAR content per synapse. Indeed, our results support our previous data (Figure 9) and the hypothesis that ARHGAP8 affects synaptic AMPAR function.

Interestingly, the effects provoked by ARHGAP8 overexpression in AMPAR-function and surface expression resemble the ones described for a Rac1-specific GAP (Ba et al., 2016), ARHGAP12, as opposed to the ones described for a RhoA-specific GAP (Kasri et al., 2009), Oligophrenin-1. ARHGAP12, a Rac1-specificic GAP specifically expressed in

the CA1 region of the hippocampus, was shown to depress AMPAR-mediated signalling when overexpressed. This down-regulation of AMPAR function was accompanied by a significant decrease in spine density and volume, and an increase in the percentage of immature spines (Ba et al., 2016). Paradoxically, overexpression of Oligophrenin-1, a RhoA-specific GAP coded by the first identified Rho-linked ID gene *(OPHN1)*, enhances AMPAR-mediated currents and increases spine-density in hippocampal neurons (Kasri et al., 2009). One could expect that GAPs with the same specific GTPase target would act similarly to each other. This is not the case when comparing ARHGAP8 and Oligophrenin-1. The articulation of different signalling domains provides a unique mode of action that result in the interaction with different binding partners and differential modulation of synaptic transmission. However, it remains to be unveiled which are the activated pathways and what domain(s) contribute to the function of ARHGAP8, specifically, in the post-synaptic AMPAR regulation. Since we found that overexpression of this Rho-GAP induces a decrease in the number of AMPAR present at synapses resulting in a functional depression of AMPAR-mediated currents, our future work aims to unravel the specific ARHGAP8 domains that are contributing to this effect. A study of the AMPAR-mediated transmission upon overexpression of different ARHGAP8 constructs lacking specific domains, could help us clarify which is the domain (or the interaction between domains) that is responsible for the effects that we observe when recording miniature EPSC. Prof. Boon Chuan Low kindly provided us with five different deletion constructs of ARHGAP8 that could be used in understanding the contribution of each of the domains in ARHGAP8 function. By overexpressing each of these constructs and comparing their effects with the results that we obtained for full-length ARHGAP8 overexpression in recorded AMPAR-mediated mEPSC, we could progress in our understanding of synaptic ARHGAP8 activity.

It is important to highlight that the effects that we observe for synaptic AMPAR receptors number and function are both achieved with the addition of ectopic ARHGAP8. If we want to further determine what the effect of the endogenous protein is, we need to downregulate the endogenous levels of ARHGAP8 and check if the effects that we obtain are consistent with what we observed previously. To do so, we propose to use small hairpin RNA (shRNA) construct vector encoding interference RNA against endogenous ARHGAP8, to downregulate the levels of this protein in cultured neurons and study the effect of this manipulation in AMPAR-mediated synaptic transmission, assessed by mEPSC recordings. We are currently validating different shRNA sequences against ARHGAP8, in order to conduct these experiments.

Given the dynamic exchange of AMPAR that underlies the strengthening and weakening of synaptic strength (Kessels & Malinow, 2009) and having in mind that our results indicated a decrease in the number of synaptic AMPARs that translates into a functional depression of AMPAR-mediated transmission, the question arises whether ARHGAP8 is implicated in synaptic plasticity. It would be interesting to either overexpress or silence ARHGAP8 in neuronal cultures, and to test a chemical LTP (c-LTP) protocol, in order to check whether the cLTP-induced increase in synaptic AMPAR content is affected by ARHGAP8. Alternatively, testing whether ARHGAP8 affects LTP in the hippocampus, e.g. in the Schaffer collaterals-CA1 synapse, is a possibility. Organotypical hippocampal slices biolistically-transfected with the construct encoding full-length ARHGAP8 or the ARHGAP8-shRNA construct can be used for this effect; a high-frequency stimulation protocol can be used to induce LTP, and recordings from transfected CA1 neurons will inform on whether ARHGAP8 is implicated in synaptic plasticity mechanisms in the hippocampus.

4.2 ARHGAP8 overexpression changes the expression of synaptic gephyrin clusters

Given that there was no preliminary data regarding the presence of ARHGAP8 in inhibitory synapses, the first step that we took towards the characterization of this Rho-GAP in this type of synapses was to check for its co-localization with inhibitory synapse markers. Interestingly, our immunocytochemistry images revealed that around half of the detected synaptic gephyrin clusters co-localize with ARHGAP8 (Figure 11C). This is a significant fraction, especially when comparing to that of the excitatory synapse co-localization with ARHGAP8 (~20% of excitatory synapses contain ARHGAP8, *data not shown*). Since inhibitory synapses are formed at the dendritic shaft, and lack morphologically specialized post-synaptic structure, such as the one existing for excitatory synapses (spines), it is possible that some of the co-localization occurs by chance and not through direct interaction between ARHGAP8 and the inhibitory synapse structure. Even if this true, we detected that when overexpressing ARHGAP8, the area of synaptic gephyrin clustering significantly increases with an apparent decrease of the number of clusters (Figure 13), which might indicate that ARHGAP8 can also be involved in the regulation of inhibitory synapses. We assessed the distribution of the α1 $GABA_A$ receptor subunit along dendrites, and ARHGAP8 co-localization with this receptor subunit (Figure 12). Even though only a small percentage of ARHGAP8 appears to co-localize with this receptor

subunit, it is important to take in consideration that we are evaluating total $GABA_A$ receptor α1 subunit expression; it is expected that only a fraction of the clusters that we detect are synaptic. Even so, ARHGAP8 overexpression reveals an apparent increase in both number and area of total α1 $GABA_A$ receptor subunit along dendrites (Figure 14). Prior to any new approach, the number of experiments described here should be increased to confirm this tendency. Detection of synaptic (instead of total) α1 $GABA_A$ receptor subunit could also bring important insights to the characterization of the role of ARHGAP8 in inhibitory synapses.

Since we detected an increased area and an apparent (but not significant) increase in number of synaptic gephyrin clusters upon ARHGAP8 overexpression, and given that other Rho-GAPs (such as SRGAP2A) have been shown to interact with the inhibitory post-synaptic scaffold gephyrin, we propose to check for interaction of ARHGAP8 with gephyrin, through a co-immunoprecipitation assay. This would give us further insights on the mechanistical characterization of the function of ARHGAP8 in inhibitory synapses.

Given that there are Rho-GAPs known for their interaction and regulation of both excitatory and inhibitory synapses, future work should be done in further exploring ARHGAP8 capability of such regulation and its functional role. We propose to do so, by assessing the role of ARHGAP8 in modulating inhibitory synaptic function through recordings of GABAR-mediated miniature inhibitory post-synaptic currents (mIPSC) in ARHGAP8 overexpressing neuronal cultures.

4.3 ARHGAP8 overexpression shows no effect in the maintenance of dendritic arbors

Given the link between aberrant GTPase signaling, mental retardation and abnormal dendritic development and morphology (reviewed by Newey et al., 2004), and having in mind that the chromosomal region where the human ARHGAP8 gene is positioned has been extensively associated to disorders involving intellectual and cognitive deficits, we hypothesized that the dendritic branching/arborization of neurons could be affected by overexpressing ARHGAP8. Previous data, showed no significant effects of an upregulation of ARHGAP8 on the dendritic arborization of excitatory pyramidal neurons, the most abundant neurons in hippocampal neuronal cultures (Figure 8). Given that we had opposite outcomes for the overexpression of ARHGAP8 in excitatory vs. inhibitory synapses, it seemed relevant to also examine for effects of upregulating ARHGAP8 in the

dendritic arbor of inhibitory cortical neurons. To assess the morphological structure of inhibitory neurons, we performed Sholl analysis at two different time points (DIV 10 and DIV 14) in transfected neurons immunostained for GABA (to identify inhibitory neurons). Similarly to the previous results in excitatory neurons, Sholl analysis did not reveal any significant changes to the morphology and the dendritic arbor of ARHGAP8 overexpressing inhibitory neurons. Even though one could think that ARHGAP8 does not represent a relevant function for determination of the dendritic outgrowth of neurons, it might be that we are introducing the overexpression plasmid too late in development to produce any morphological changes. We should bear in mind that we are only affecting the maintenance stage of neurons morphology. Knowing that ARHGAP8 is able to interact with cortactin and promote cell migration, it could be expected that it might exert some influence on the development of the dendritic arborization. It would be interesting to introduce ARHGAP8 during the beginning of neuronal development, i.e., DIV 0, to determine whether this Rho-GAP could exert an effect in the formation of the dendritic ramifications. We could achieve this early transfection by resorting to a nucleofection protocol – an electroporation-based transfection method – instead of the calcium phosphate transfection protocol used up until now. Still, even if a structural role doesn't seem evident, we cannot exclude the possibility that ARHGAP8 presents a functional role in modulating aspects of neuronal development or communication.

Chapter 5 - Conclusion

5 Conclusion

The work in this thesis provides the first insights on the regulation of ARHGAP8, a novel Rho-GTPase activating protein, in neuronal cells. Given the preliminary data that we had available (from experiments performed previously in our lab), we hypothesised that ARHGAP8 could be playing a functional role in the regulation of AMPA receptor transmission. We proposed to assess this by recording miniature excitatory post-synaptic currents from cortical neuronal cultures overexpressing ARHGAP8, as hypothesized, we detected a strong depression in AMPAR-mediated currents (both in frequency and amplitude) that correlates with the preliminary immunostaining data that indicated a decreased number of surface GluA1 upon ARHGAP8 overexpression. To further develop our knowledge on the signalling domains responsible for ARHGAP8 regulatory role in AMPAR-mediated synaptic transmission, we will record AMPAR-mediated mEPSCs in cultures overexpressing ARHGAP8 deletion constructs. The outcome of these experiments should elucidate the protein domain(s) responsible for the change in AMPAR-mediated currents, thus providing us with insights to which signalling pathways might be affected. Given that these results are achieved with the addition of ectopic ARHGAP8, future work should be done to evaluate whether down-regulation of the endogenous protein results in effects that are consistent to those here described.

Even though the first piece of evidence that lead our group to study this novel Rho-GAP indicated its possible role in excitatory synapses, there is evidence in the literature of other GAPs involved in both excitatory and inhibitory synapse regulation. Being this the first characterization study of ARHGAP8 in neuronal cells, the second aim of this study was to characterize ARHGAP8 in the context of inhibitory synapses. We found that in non-stimulated neuronal cortical cultures, ARHGAP8 partially co-localizes with the inhibitory synapses markers gephyrin, VGAT and the α1 $GABA_A$ receptor subunit. Strikingly, ARHGAP8 is present in approximately half of the synaptic gephyrin clusters. Given the significant percentage presence of ARHGAP8 in inhibitory synapses, we overexpressed ARHGAP8 and assessed the number, area and fluorescence intensity of synaptic gephyrin clusters. We detected a significant increase in area of these clusters with a tendency to a diminished number of clusters. Interestingly, the total number and area of the $GABA_A$ receptor subunit α1 also show an augmentation tendency (even though data does not reach significance). This second set of results provides confirmation that ARHGAP8 does co-localize with inhibitory synapse markers in cortical neurons and its overexpression affects the expression of these markers. Still, in the work performed for

this Master thesis, we did not approach the existence of a direct interaction between ARHGAP8 and inhibitory synapse markers, or if there is a functional effect in this type of synapse. Future work needs to be done regarding these two objectives to further characterize ARHGAP8 role in regulating inhibitory synapse function. Carrying out electrophysiology experiments regarding GABAR-mediated inhibitory post-synaptic currents, and performing co-immunoprecipitation assays to test the interaction of ARHGAP8 with inhibitory synapse scaffold proteins, would give us insights on the functional, and structural role of ARHGAP8 in inhibitory synapses, respectively.

Overall, the group of experiments performed in the framework of this thesis, gave us important knowledge about the regulatory role of ARHGAP8 in excitatory AMPAR-mediated synaptic transmission, and uncovered the possibility of a regulatory role of this protein in the inhibitory synapse context. Additionally, the suggested group of experiments should further elucidate the role of this novel Rho-GAP in the regulation of synaptic transmission in neurons.

Chapter 6 - References

6 References

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