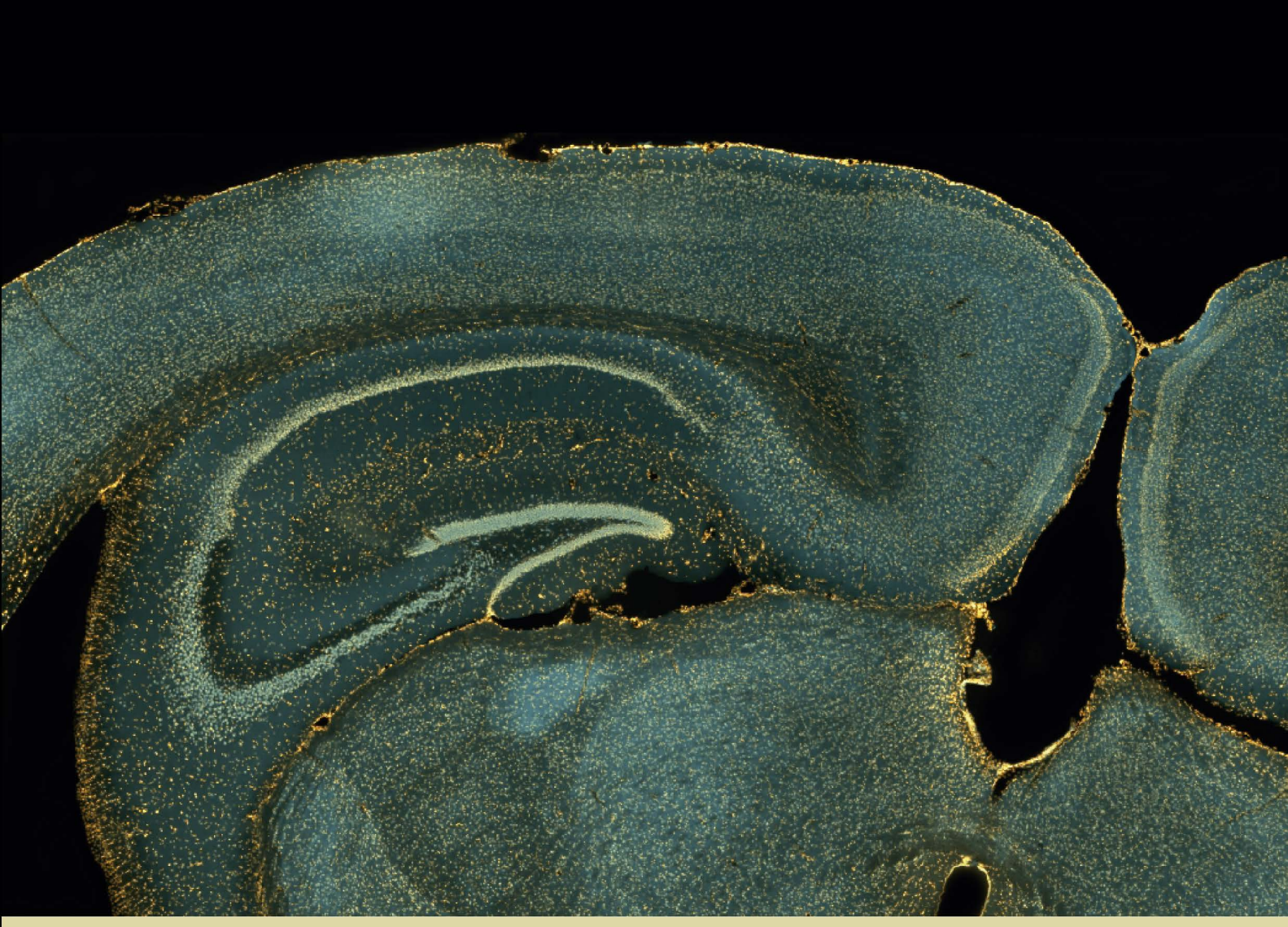

Dominique Moreira Fernandes

Role of Caspr1 and Caspr2 in the regulation of glutamatergic transmission and synaptic plasticity – implication for disease pathogenesis

Tese de doutoramento em Biologia Experimental e Biomedicina, ramo de Neurociências e Doença,
orientada pela Doutora Ana Luísa Carvalho e co-orientada pela Doutora Sandra Manuela Domingues dos Santos
e apresentada ao Instituto de Investigação Interdisciplinar da Universidade de Coimbra

Fevereiro 2018

· U C ·

Universidade de Coimbra

Dominique Moreira Fernandes

Role of Caspr1 and Caspr2 in the regulation of glutamatergic transmission and synaptic plasticity – implications for disease pathogenesis

PhD thesis presented to the Institute for Interdisciplinary Research from the University of Coimbra (IIIUC) for the fulfillment of the requirements for a Doctoral degree in Experimental Biology and Biomedicine, branch of Neuroscience and Disease, under the supervision of Doctor Ana Luísa Monteiro de Carvalho (Life Science Department, University of Coimbra) and co-supervision of Doctor Sandra Manuela Domingues dos Santos (CNC - Centre for Neuroscience and Cell Biology, University of Coimbra).

February 2018

· U C ·

UNIVERSIDADE DE COIMBRA

Dominique Moreira Fernandes

Papel da Caspr1 e Caspr2 na regulação da transmissão glutamatérgica e plasticidade sináptica - implicações para a patogénese de doenças neuropsiquiátricas

Tese de doutoramento apresentada ao Instituto para a Investigação Interdisciplinar da Universidade de Coimbra (IIIUC) para a prestação de provas de Doutoramento em Biologia Experimental e Biomedicina no ramo de Neurociências e Doença, sob a orientação da Doutora Ana Luísa Monteiro de Carvalho (Departamento de Ciências da Vida, Universidade de Coimbra) e co-orientação da Doutora Sandra Manuela Domingues dos Santos (CNC - Centro de Neurociências e Biologia Celular, Universidade de Coimbra).

Fevereiro 2018

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Cover image:

Coronal slice of the mouse brain stained for Caspr2

"*The brain is locked in total darkness, of course, children*, says the voice. *It floats in a clear liquid inside the skull, never in the light. And yet the world it constructs in the mind is full of light. It brims with color and movement. So how, children, does the brain, which lives without a spark of light, build for us a world full of light?*"

Anthony Doerr in "All the light we cannot see"

Ao Avô M. e ao Avô F.

Embora fazendo falta a cada dia, há
momentos em que a vossa presença
tornaria tudo mais completo.

Este é um desses momentos.

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Table of contents

Abbreviations

3'UTR	3'Untranslated region
λPP	Lambda protein phosphatase
aCSF	Artificial cerebral spinal fluid
ADAM	Disintegrin and metalloproteinase domain-containing protein
AMCA	Aminomethylcoumarin Acetate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	AMPA receptors
ANOVA	Analysis of variance
APV	D-(-)-2-Amino-5-phosphonopentanoic acid
Arc	Activity-regulated cytoskeleton-associated protein
ASD	Autism-spectrum disorder
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CAM	Cell-adhesion molecule
CaMKII	Calcium-calmodulin-dependent protein kinase II
Caspr1	Contactin-associated protein 1
Caspr1ΔPRO	Contactin-associated protein 1 lacking a proline domain
Caspr1ΔGNP	Contactin-associated protein 1 lacking a GNP motif
Caspr2	Contactin-associated protein 2
CDFE	Cortical dysplasia and focal epilepsy
cDNA	complimentary DNA
CNS	Central nervous system
CNTN	Contactin
CNTNAP	Contactin-associated protein
CSF	Cerebrospinal fluid
Ct	Cycle threshold
DE	Dark exposure
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
ECF	Enhanced chemifluorescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FERM	Four-point-one, Ezrin, Radixin, Moesin
FMRP	Fragile X mental retardation protein
FXS	Fragile X Syndrome
GAP-43	Growth associated protein 43
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein

GluRs	Glutamate receptors
GNP	Glycophorin C, Neurexin, Paranodin (GNP) motif
GPI	Glycosylphosphatidyl inositol
GRIP/ABP	Glutamate receptor interacting protein / AMPAR binding protein
HBSS	Hank's balanced salt solution
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
ID	Intellectual disability
IgG	Immunoglobulin G
IP	Immunoprecipitation
KD	Knock-down
kDa	kiloDalton
KH	hnRNP K homology domain
KO	Knock-out
LBD	Ligand binding domain
LGI1	Leucine-rich glioma-inactivated 1
LTD	Long-term depression
LTP	Long-term potentiation
MAGUK	Membrane associated guanylate kinase
MAP2	Microtubule associated protein 2
mEPSCs	Miniature excitatory postsynaptic currents
MRI	Magnetic ressonance imaging
mRNA	Messenger RNA
NBM	Neurobasal medium
NLG	Neuroligin
NMDA	*N*-methyl *D*-aspartate
NR	Normal reared
NRX	Neurexin
NTD	Amino terminal domain
PBS	Phosphate buffered saline
PDZ	Postsynaptic density-95 / Disc large / Zonula occludens-1 protein domain
PGY	Proline, glycine, tyrosine (PGY) repeats
PI3K	Phosphoinositide 3-kinase
PICK1	Protein interacting with C-kinase 1
pIgGs	Purified immonuglobulins
PKA	Protein kinase A
PKC	Protein kinase C
PLCγ	Phospholipase C gamma subunit
PMSF	Phenylmethylsulfonyl fluoride
PNS	Peripheral nervous system
PSD	Postsynaptic density
PSD95	Postsynaptic density protein 95
PVDF	Polyvinylidene difluoride

RA	Retinoic acid
RARα	Retinoic acid receptor-α
RBD	RNA-binding domain
RBP	RNA-binding protein
RIP	Ribonucleoprotein immunoprecipitation
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RRM	RNA recognition motif
RT	Room temperature
SAP97	Synapse-associated protein 97
SCZ	Schizophrenia
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylimide gel electrophoresis
SEM	Standard error of the mean
SH3	Src homology 3 domain
shRNA	Short hairpin ribonucleic acid
TARP	Transmembrane AMPAR-associated regulatory protein
TMD	Transmembrane domain
TNFα	Tumor necrosis factor-α
TTX	Tetrodotoxin
V1	Primary visual cortex
V1-layer2/3	Layer 2/3 of the primary visual cortex
VGKC	Voltage-gated potassium channels
vGluT1	Vesicular glutamate transporter 1
WB	Western blot
WT	Wild-type
ZBP1	Zipcode-binding protein 1

Keywords

CASPR1 & CASPR2

Glutamate AMPA receptors

Homeostatic synaptic plasticity

Neuropsychiatric disorders

Autoimmune encephalitis

Palavras-chave

CASPR1 & CASPR2

Receptores de glutamato do tipo AMPA

Plasticidade homeostática

Doenças neuropsiquiátricas

Encefalite autoimune

Abstract

The human brain is distinctively unique due to its remarkable ability for complex language, higher cognition, emotion regulation and executive control of behaviour. Yet, these are precisely the brain functions impaired in several neurological and neuropsychiatric disorders that afflict a large percentage of the population worldwide. Emerging evidence in the field suggests that one potential functional process thought to be compromised in pathological conditions is homeostatic synaptic plasticity. This form of plasticity is able to modulate the overall activity of neuronal networks so that it never goes off-balance, even in face of constant changes, such as occurring during development, sensory experience or even during learning-related adaptations. At the molecular level, bidirectional compensatory changes in the postsynaptic accumulation of glutamate receptors of the AMPA-type are thought to be one cellular substrate to achieve neuronal homeostasis. However, full comprehension of the AMPAR regulatory mechanisms that underlie this form of plasticity, and of how such mechanisms can fail in the context of disease, is still elusive.

Contactin-associated proteins 1 and 2 (Caspr1 and Caspr2) are integral transmembrane cell-adhesion molecules that have received considerable attention in the past few years due to emerging roles in the regulation of synaptic, cellular and functional processes in the brain. Previous evidence from our laboratory identified Caspr1 as an AMPAR-interacting protein necessary to regulate the trafficking of surface AMPARs into synapses. However, the molecular mechanisms underlying this role of Caspr1 are still uncharacterized, and no link has yet been established between its role in AMPAR regulation and synaptic plasticity mechanisms. Herein, we describe a posttranscriptional mechanism orchestrated by Caspr1 and the RNA-binding protein ZBP1 in the regulation of AMPAR. We found that ZBP1 binds to the mRNA of the GluA1 subunit of AMPARs in an activity-dependent manner, and regulates synaptic levels of cell surface GluA1-containing AMPARs. During periods of prolonged activity inhibition, when homeostatic responses are elicited to upscale surface AMPARs, endogenous expression of Caspr1 is upregulated, and the phosphorylation of ZBP1 increases, resulting in a significant decrease in GluA1 mRNA molecules bound to ZBP1, suggesting an activity-dependent release of GluA1 transcripts to undergo translation on demand. Importantly, when the expression of either Caspr1 or ZBP1 is lost, synaptic upscaling of AMPARs is compromised, indicating a specific requirement for both Caspr1 and ZBP1 in the regulation of postsynaptic AMPARs during homeostatic synaptic plasticity.

Mutations in the CASPR2-encoding gene *CNTNAP2* have been recurrently implicated in several neuropsychiatric disorders including autism, schizophrenia and intellectual disability. Recent findings have attributed an important function for CASPR2 in synapse regulation, but

the full-spectrum of mechanisms mediated by CASPR2 remains elusive. Importantly, it is still unclear how perturbations in CASPR2 function become pathogenic and drive the severe cognitive and psychiatric symptoms presented by patients. Herein, we find that Caspr2 is expressed in cortical excitatory synapses, and identify Caspr2 as a novel AMPAR-interacting protein capable of regulating the trafficking of AMPARs to synapses. Moreover, we demonstrate that loss of Caspr2 impairs AMPAR function and *in vivo* excitatory synaptic transmission in the cortex, and reveal a requirement for Caspr2 in the regulation of homeostatic mechanisms necessary for the expression of visually-driven experience-dependent plasticity.

Finally, autoantibodies targeting CASPR2 have been recently discovered in patients with autoimmune synaptic encephalitis that can manifest with severe memory deficits, cognitive impairments and psychosis. However, it is not clear to date if CASPR2 autoantibodies (CASPR2-Abs) perturb CASPR2 functions, and whether they can mediate a direct pathogenic effect that can drive the disease symptoms. Taking into consideration the role we describe for Caspr2 in the regulation of AMPARs, we hypothesized that CASPR2-Abs exert their pathogenic effect by disrupting this Caspr2 function. Using human immunoglobulin (IgGs) preparations from a patient with CASPR2 encephalitis, we found that patient IgGs significantly alter the synaptic distribution of Caspr2 and cell surface AMPARs. Moreover, patient IgGs hamper Caspr2 function *in vivo* and perturb basal glutamatergic synaptic transmission in the visual cortex of mice. Additionally, patient IgGs prevent the triggering of long-term potentiation, whilst sparing homeostatic synaptic scaling mechanisms. Finally, we reveal that patient IgGs bound to the neuronal surface can undergo time-dependent internalization, thus underpinning a likely mechanism of pathogenesis elicited by CASPR2-Abs.

Altogether, our findings identify Contactin-associated proteins as crucial regulators of glutamatergic synaptic transmission and synaptic plasticity mechanisms, and suggest that these processes are likely targets for the pathogenesis of several neurological and neuropsychiatric disorders, including those ensuing from genetic- or antibody-mediated disruptions of CASPR2.

Resumo

O cérebro humano é único, capaz de processos altamente complexos tal como cognição, formas de linguagem superior, regulação de emoções e controlo executivo do comportamento, que nos distinguem de outros mamíferos. No entanto, são precisamente estas as funções cerebrais afectadas em doenças neurológicas e do foro psiquiátrico, que atingem uma larga percentagem da população em todo o mundo. Evidências recentes sugerem que um dos processos cerebrais que possa estar comprometido em condições patológicas é a plasticidade homeostática. Esta forma de plasticidade é capaz de modular a actividade de redes neuronais de forma a mantê-la dentro de limites funcionais necessários para um adequado funcionamento do cérebro, mesmo face a constantes alterações potencialmente desestabilizadoras, como as que ocorrem durante o desenvolvimento neuronal, em resposta ao ambiente sensorial ou até durante processos de aprendizagem. Ao nível molecular, a homeostase neuronal é conseguida, por exemplo, através de alterações compensatórias e bidirecionais na acumulação pós-sináptica de receptores de glutamato do tipo AMPA (AMPAR). No entanto, os mecanismos regulatórios de AMPAR que estão na base deste tipo de plasticidade ainda não são completamente conhecidos, e não se compreende ainda de que forma estes mecanismos podem falhar num contexto de doença.

As proteínas 1 e 2 associadas à Contactina (Caspr1 e Caspr2) são moléculas de adesão celular que têm recebido considerável atenção nos últimos anos devido a novas funções que lhes foram atribuídas na regulação de processos sinápticos, celulares e funcionais do cérebro. Resultados anteriores obtidos no nosso laboratório identificaram a Caspr1 como uma nova interactora de receptores AMPA necessária para regular o seu tráfego para a sinapse. No entanto, os mecanismos moleculares que estão na base da função da Caspr1 ainda não foram caracterizados, e nenhuma relação foi ainda estabelecida entre a sua função na regulação de AMPAR e mecanismos de plasticidade sináptica. Neste estudo, descrevemos um novo mecanismo pós-transcripcional regulado pela Caspr1 e pela proteína de ligação a RNA, ZBP1. Descobrimos que a ZBP1 é capaz de se ligar ao RNA mensageiro da subunidade GluA1 dos AMPARs de uma forma dependente de actividade, e que regula os níveis sinápticos basais de AMPARs à superfície da célula. Durante períodos de bloqueio crónico de actividade, quando processos homeostáticos são activados para induzir o escalamento sináptico de AMPARs, a expressão endógena da Caspr1 é aumentada, bem como os níveis de fosforilação da ZBP1, o que reduz significativamente o número de moléculas de RNAm de GluA1 ligado à ZBP1, e sugere que a actividade neuronal poderá induzir uma libertação de transcriptos de GluA1 para que possam ser traduzidos quando necessário. Finalmente, inibição da expressão endógena da Caspr1 ou da ZBP1 compromete o escalamento sináptico de AMPARs, o que

indica que a Caspr1 e a ZBP1 são necessárias para a regulação de AMPARs durante mecanismos de plasticidade homeostática.

Mutações no gene codificante da Caspr2, *CNTNAP2*, foram já implicadas em várias doenças neuropsiquiátricas, incluindo autismo, esquizofrenia e défice intelectual. Evidências recentes sugerem que a Caspr2 tem um importante papel na sinapse, no entanto não se conhecem ainda os mecanismos através dos quais a Caspr2 regula a função sináptica. Além disso, é ainda incerto de que forma perturbações na função da CASPR2 se podem tornar patogénicas e causar o desenvolvimento de sintomas cognitivos e psiquiátricos nos pacientes. Neste estudo vimos que a Caspr2 é bastante expressa em sinapses excitatórias do cortéx, e identificámos a Caspr2 como uma nova proteína interactora de AMPARs capaz de regular o seu tráfego para a sinapse. Adicionalmente, demonstrámos que inibição da expressão endógena da Caspr2 perturba a função de AMPARs e a transmissão sináptica excitatória *in vivo*, e revelámos que a Caspr2 é necessária para mecanismos de escalamento sináptico que regulam a expressão de plasticidade sináptica induzida *in vivo* por experiência visual.

Autoanticorpos contra a CASPR2 foram recentemente identificados em pacientes com encefalite autoimune, que podem apresentar graves défices de memória, perturbações cognitivas e psicose. No entanto, não se sabe ainda se os autoanticorpos contra a CASPR2 perturbam a sua função, e se poderão mediar um efeito patogénico directo que possa ser causal para o desenvolvimento dos sintomas da doença. Tendo em consideração o papel que descrevemos para a Caspr2 na regulação de AMPARs, colocamos a hipótese de os autoanticorpos exercerem o seu efeito patogénico por disrupção desta função da Caspr2. Usando amostras de imunoglobulinas humanas purificadas a partir do plasma de um paciente com encefalite anti-CASPR2, descobrimos que estas alteram significativamente a distribuição sináptica da Caspr2 endógena e de AMPAR superficiais. Adicionalmente, vimos que as IgGs do paciente bloqueiam a função da Caspr2 *in vivo* e perturbam a transmissão glutamatérgica no córtex visual de ratinhos. Vimos também que as IgGs do paciente inibem a indução de mecanismos de potenciação de longo-termo, embora o escalamento sináptico se mantenha intacto. Finalmente, revelamos que IgGs do paciente que se ligam à superfície neuronal são internalizadas ao longo do tempo, sugerindo assim um potencial mecanismo patogénico induzido por estes anticorpos.

Em conclusão, os nossos resultados identificam as proteínas Casprs como reguladoras fundamentais da transmissão glutamatérgica e de mecanismos de plasticidade homeostática, e sugere que estes processos sejam facilmente perturbados no contexto de doença e contribuam assim para a patogénese de doenças neuropsiquiátricas, nomeadamente para doenças associadas à Caspr2.

Chapter I
Introduction

Part of the information revised in this chapter is included in the following published review:

Dominique Fernandes & Ana Luísa Carvalho (2016). Mechanisms of homeostatic plasticity in the excitatory synapse. *Journal of Neurochemistry*. **139**(6): 973-996.

The mammalian central nervous system (CNS) is, like no other system, remarkably complex. All brain processes, from the control of simple reflexes to higher-order brain functions, such as learning, memory and other processes that define human behaviour, are possible due to the intricate connectivity between neurons in the brain. The adult human brain has roughly 100 billion neurons, each potentially participating in thousands of connections. These connections, known as synapses, allow neurons to communicate with each other and establish functional neuronal circuits. To ensure a proper wiring of the neuronal circuitry, a highly complex sequence of events takes place. In broad terms, neurons, guided by specific extracellular cues, are able to send axonal branches to precise spatial locations in receptive dendrites, where synapse formation is triggered (O'Donnell *et al.*, 2009). Subsequent sensory experience refines circuitry through a combination of synapse maturation and pruning mechanisms, linking neurons that are active together in a functional context (Caroni *et al.*, 2012; Lu *et al.*, 2009a; Volk *et al.*, 2015). Finally, the continual change in neuronal communication occurs through an exquisite activity-dependent and long-lasting regulation of the mature synapses, a cellular mechanism best known as synaptic plasticity.

At individual synapses, synaptic transmission can be regulated either at the presynaptic side, by changing the efficacy of neurotransmitter release, or at the apposing postsynaptic side, by altering the type, density or properties of neurotransmitter receptors.

The Glutamatergic Synapse

Glutamatergic synapses convey most of the fast excitatory neurotransmission in the CNS, and intact glutamate signalling is thus critical for the majority of sensory processing and cognitive function. In a functionally mature glutamatergic synapse (Figure 1.1), presynaptic compartments are enriched with glutamate-filled synaptic vesicles and specialized active zones that support the release of glutamate to the synaptic cleft in response to depolarization of the presynaptic nerve terminal (Sudhof, 2013; Volk *et al.*, 2015). Postsynaptically, glutamate acts on two distinct categories of glutamate receptors (GluRs): metabotropic receptors (mGluRs) and ionotropic receptors (iGluRs), which differ in their molecular, biochemical, pharmacological and physiological properties (Hollmann & Heinemann, 1994; Kew & Kemp, 2005).

Metabotropic glutamate receptors are coupled to G proteins and their activation generates intracellular secondary messengers. As such, they mediate slow synaptic responses, occurring within seconds or even minutes. mGluRs can be located both

presynaptically or postsynaptically, depending on receptor subtype. However, on the postsynaptic side, metabotropic receptors are mainly located perisynaptically, outside of the active zone of the synapse (Ferraguti & Shigemoto, 2006; Okabe, 2007).

Ionotropic glutamate receptors usually mediate fast synaptic responses (in the order of milliseconds), due to their ligand-gated ion channel conformation. Upon binding by glutamate, the channel pore opens, triggering a rapid influx of cations that results in membrane depolarization of the postsynaptic neuron. Mammalian iGluRs [reviewed in (Smart & Paoletti, 2012)] exhibit great diversity and are encoded by a total of 18 genes that assemble into four major families, according to their electrophysiological properties and most selective agonists (Table 1.1): alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), kainate (KARs), N-methyl-D-aspartate (NMDARs) and delta (deltaRs) receptors (Dingledine *et al.*, 1999; Hollmann *et al.*, 1989; Nakanishi & Masu, 1994; Seeburg, 1993; Watkins, 1981).

Table 1.1 – The four families of iGluRs. Each subunit is encoded by a different gene, and there is no known mixing between subunits of different families. Functional receptors only form between subunits within the same family. [Adapted from (Smart & Paoletti, 2012)].

Ionotropic glutamate receptors (iGluRs)			
AMPARs	KainateRs	NMDARs	DeltaRs
GluA1	GluK1 GluK4	GluN1* GluN2A GluN3A*	GluD1
GluA2	GluK2 GluK5	GluN2B GluN3B*	GluD2*
GluA3	GluK3	GluN2C	
GluA4		GluN2D	

* These subunits bind glycine or D-serine.

At the synapse, iGluRs are highly accumulated in clusters at the postsynaptic density (PSD), a functional specialization of the postsynaptic membrane (Figure 1.1). This dense and rich protein network is comprised not only of the glutamate receptors, but also of a set of anchoring and scaffolding molecules that bind to the receptors, as well as to cell-adhesion molecules, signalling enzymes and cytoskeletal components, thus holding the PSD together [reviewed in (Harris & Weinberg, 2012; Sheng & Kim, 2011)]. AMPARs, NMDARs and KARs can also be expressed presynaptically (Corlew *et al.*, 2008; Jane *et al.*, 2009; Schenk & Matteoli, 2004), where they can act by altering neurotransmitter release.

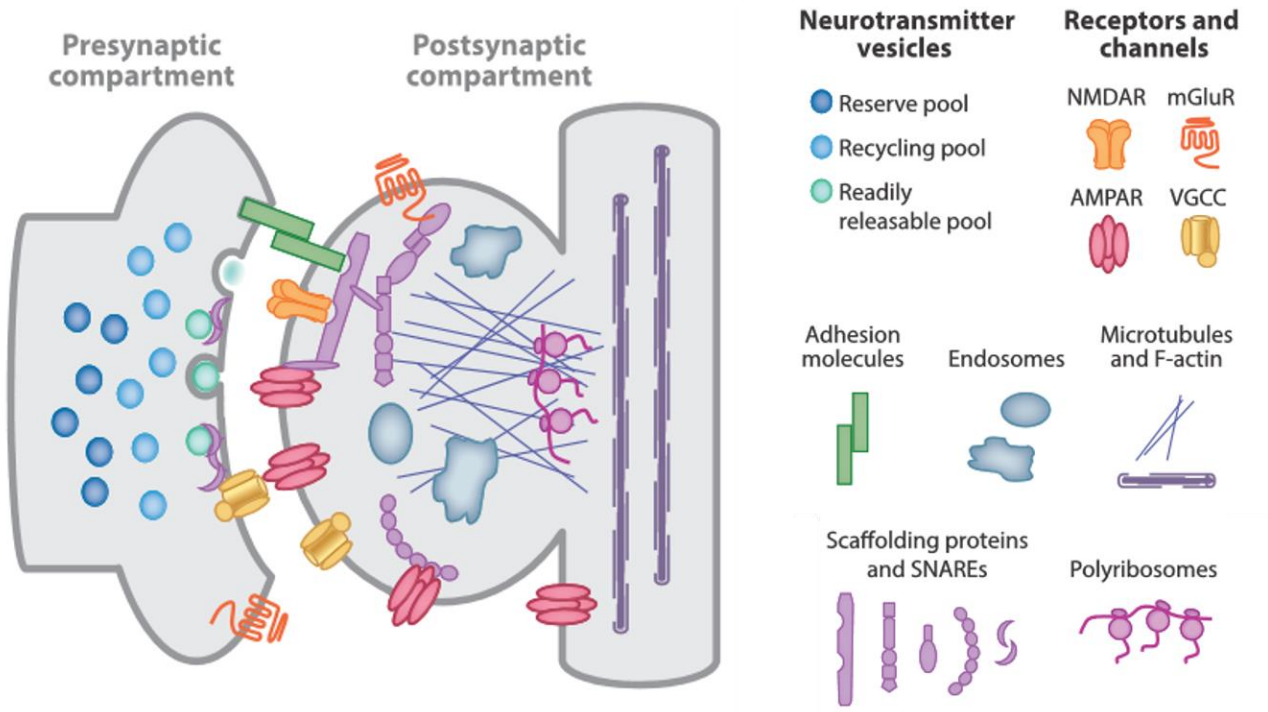


Figure 1.1 – Molecular organization of glutamatergic synapses. Simplified diagram of the main proteins that compose a glutamatergic synapse. A typical mature glutamatergic synapse is composed of a presynaptic compartment packed with membrane proteins (e.g., voltage-gated ion channels, mGluRs, and cell-adhesion molecules), neurotransmitter-filled synaptic vesicles, and SNARE protein complexes for vesicle fusion and glutamate release. This is opposed by a postsynaptic spine containing membrane proteins [e.g., iGluRs (AMPARs and NMDARs), mGluRs, voltage-gated ion channels, and cell-adhesion molecules], extensive scaffolding proteins, endosomes, and local protein translation machinery. [Adapted from (Volk *et al.*, 2015)].

AMPARs and KARs are voltage-independent ion channels, permeable to Na^+ and K^+, which allow a net depolarizing influx of cations upon activation with glutamate, making them responsible for most of the fast excitatory synaptic transmission. KARs, however, are thought to have more of a modulatory than a direct role at the synapse (Contractor *et al.*, 2011; Jane *et al.*, 2009; Lerma, 2006). Despite sharing high sequence homology with other iGluR families (Lomeli *et al.*, 1993; Yamazaki *et al.*, 1992), DeltaRs are the least understood iGluRs largely because, in contrast with the other families, their apparent incapacity to gate an ion channel following ligand binding makes them electrically "silent" (Kohda *et al.*, 2000; Schmid *et al.*, 2009). Unlike AMPARs, which have a great influence in the strength of the fast immediate synaptic response (Esteban, 2003; Ozawa *et al.*, 1998), NMDARs are ligand-gated ion channels that, at resting membrane potential, remain silent with a voltage-dependent magnesium blockade, even in the presence of glutamate and the coagonist glycine (Kauer *et al.*, 1988; Watkins, 1981). However, if the cell depolarizes, Mg^{2+} dissociates from its binding

site within the NMDAR channel pore, allowing a maximal influx of Ca^{2+}, as well as Na^{+}, into the dendritic spine, which activates intracellular signalling cascades ultimately responsible for altering synaptic efficacy. Hence, NMDARs function as molecular coincidence detectors, since they open only when both the pre- and postsynaptic neurons are active, and this is of particular importance for the induction of specific forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD) (Bear & Malenka, 1994; Luscher & Malenka, 2012; Malenka & Nicoll, 1999).

It is clear that iGluRs are very diverse and each receptor subtype displays unique functional properties. *In vivo*, the diversity of these receptors ultimately varies with gene expression in a given neuron, and even within the same neuron, iGluR subtypes can differ from one synapse to another in an input-specific manner. The synaptic content of iGluR subtypes, and the subunit composition of the receptors within the same family, is highly dynamic and varies according to developmental stages, synaptic activity and even disease (Bellone & Nicoll, 2007; Liu & Cull-Candy, 2000; Mameli *et al.*, 2007; Smart & Paoletti, 2012). Importantly, iGluRs, and in particular NMDARs and AMPARs, are essential for the induction and maintenance of synaptic plasticity mechanisms, which continually change synaptic efficacy and constitute the cellular substrate of learning and memory [reviewed in (Luscher & Malenka, 2012)]. Despite many forms of plasticity being dependent on the activation of NMDARs, AMPARs are the workhorse of glutamatergic transmission. Indeed, growing evidence show that changes in AMPARs, from phosphorylation/dephosphorylation events to their insertion or removal from the PSD, underlie the changes in synaptic strength associated with synaptic plasticity (Bredt & Nicoll, 2003; Carvalho *et al.*, 2000; Derkach *et al.*, 2007; Gomes *et al.*, 2003; Jiang *et al.*, 2006; Lee *et al.*, 2010; Santos *et al.*, 2009).

AMPA Receptors

The major mediators of the primary depolarization in glutamatergic neurotransmission are AMPARs, which drive large and rapid synaptic responses. Because they play key roles in synaptic plasticity, they are targets for dynamic regulation of multiple mechanisms that range from post-transcriptional and post-translational modifications of receptor subunits to alterations in traffic and cellular distribution of AMPARs. These regulated modifications in AMPARs ultimately underlie activity-dependent and long-lasting changes in synaptic strength and efficacy [reviewed in (Anggono & Huganir, 2012; Derkach *et al.*, 2007; Kessels & Malinow, 2009; Santos *et al.*, 2009; Shepherd & Huganir, 2007)].

Expression of AMPA receptors

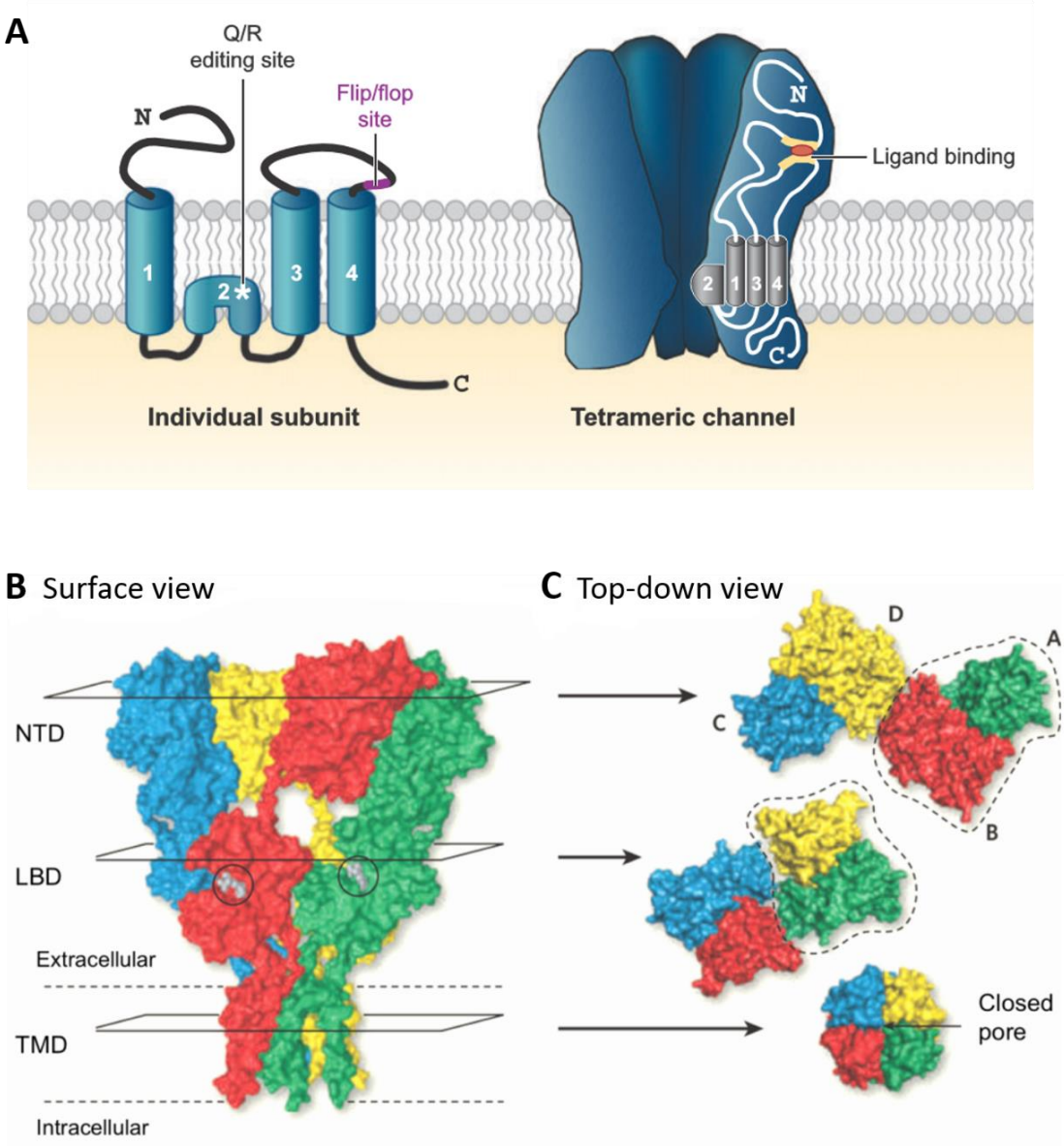
AMPARs are composed of four homologous subunits, GluA1 – GluA4 (Hollmann & Heinemann, 1994) encoded by four closely related genes, with about 70% sequence homology (Collingridge *et al.*, 2004). They comprise about 900 amino acids, have a molecular weight of about 105 kDa and assemble in tetramers (see Figure 1.2) to form AMPARs with different properties (Ozawa *et al.*, 1998; Rosenmund *et al.*, 1998; Santos *et al.*, 2009; Sobolevsky *et al.*, 2009). In conformity with their key role in excitatory neurotransmission, AMPARs are widely distributed throughout the brain, although the expression of the receptor subunits is brain region-specific and developmentally regulated (Santos *et al.*, 2009; Shepherd & Huganir, 2007). GluA1-GluA3 subunits seem to be ubiquitous, but particularly enriched in the cerebral cortex, hippocampus and amygdala (Keinanen *et al.*, 1990), whereas the GluA4 subunit is abundant in the cerebellum in the adult brain (Petralia & Wenthold, 1992). Curiously, despite being almost inexistent in the mature hippocampus, GluA4-containing AMPARs are primarily expressed in early postnatal development, in detriment of other subunits (Esteban, 2003; Zhu *et al.*, 2000).

AMPA receptor general structure and diversity

Subunit topology and tetrameric assembly

Functional AMPARs are formed by the association of two dimers in tetramers, composed of either homomers of the same AMPAR subunit, or heteromers of two differing subunits, typically GluA1/2 or GluA2/3 (Lu *et al.*, 2009b; Rosenmund *et al.*, 1998). Each individual subunit presents a characteristic modular organization that includes: i) an extracellular N-terminal domain (NTD) responsible for subunit-specific receptor assembly and modulation; ii) the ligand-binding domain (LBD); iii) the transmembrane domain (TMD), composed of three membrane-spanning segments (M1-M4) plus a short re-entrant membrane loop (M2) that forms the ion pore; and iv) the final intracellular C-terminal domain (Figure 1.2A). The individual subunits will then assemble to form the tetrameric channel (Greger & Esteban, 2007; Greger *et al.*, 2007; Madden, 2002). In 2009, Gouaux and colleagues were able to determine the first accurate atomic resolution of the subunit arrangement and tetrameric architecture of a full-length homomeric GluA2 AMPAR, through crystallographic studies (Figure 1.2B/C) (Sobolevsky *et al.*, 2009). This study confirmed some of the predictions for AMPAR assembly, but also uncovered some unanticipated features. The extracellular NTD and LBD domains are organized as dimers with an overall two-fold symmetry, whereas the pore region, formed by

the TMD, presents a typical four-fold symmetry (Figure 1.2C). Besides the symmetry mismatch, the GluA2 ultrastructure revealed the occurrence of domain swapping and crossover between subunits. The tetrameric complex presents two distinct pairs of subunits, referred to as A/C and B/D (Figure 1.2C). While at the level of the NTD the dimer pairs are A–B and C–D, at the level of the LBD, the dimer pairs interchange between subunits (A–D and B–C dimers) (Figure 1.2C). This pairwise arrangement is abolished in the TMD, in which four independent but equivalent subunits have four-fold symmetry [(Sobolevsky *et al.*, 2009), and reviewed in (Wollmuth & Traynelis, 2009)].



[Figure 1.2 - subtitle on the next page**]**

Figure 1.2 – Modular topology of AMPAR subunits and their tetrameric assembly. (A) Membrane topology of AMPAR subunits and schematic representation of the tetrameric channel. Each individual subunit is composed of an extracellular N-terminal, a ligand binding domain, four transmembrane domains and a final intracellular C-terminal. [Adapted from (Shepherd & Huganir, 2007)]. **(B, C)** Crystallographic structure of the AMPA GluA2 homotetrameric receptor. (Sobolevsky *et al.*, 2009). **(B)** Surface representation of the AMPAR with four subunits of identical amino-acid sequence (A–D) coloured green (A), red (B), blue (C) and yellow (D). The tetramer shows a typical layer organization with the extracellular NTD and LBD domains, and the TMD that forms the ion channel. The circles indicate competitive antagonists (grey) occupying the agonist-recognition sites. **(C)** Top-down view of the NTD, LBD and TMD, illustrating domain swapping and symmetry mismatch (between LBD and TMD). The dashed lines in ATD and LBD indicate a dimer containing the A subunit, which in the NTD associates with the B subunit, but in the LBD associates with the D subunit. The TMD shows four-fold symmetry. Because the LBD is bound by an antagonist, the permeation pore, located in the centre of the TMD, is closed. [Adapted from (Wollmuth & Traynelis, 2009)].

AMPA receptor diversity through alternative splicing, RNA editing and posttranslational modifications

The AMPAR ultrastructure determined by Gouaux (mentioned in the section above) focused on the extracellular and transmembrane regions of the receptors, which are highly homologous between different subunits. However, the intracellular C-tails are the most structurally and functionally divergent region among AMPAR subunits, playing crucial roles in controlling AMPAR function, including receptor trafficking and stabilization at synapses (Anggono & Huganir, 2012; Greger & Esteban, 2007; Santos *et al.*, 2009). The GluA1, GluA4, and an alternative splice form of GluA2 (GluA2L) have long cytoplasmic tails with high homology (Figure 1.3). In contrast, the predominant splice form of GluA2, GluA3, and an alternative splice form of GluA4 (Glu4c) have shorter, homologous cytoplasmic tails (Figure 1.3). Expression of these subunits undoubtedly changes the properties of AMPARs. For instance, receptors composed of subunits with short C-tails (e.g. GluA2/3) are able to cycle continuously in and out of the synapse (Passafaro *et al.*, 2001; Shi *et al.*, 2001), whereas AMPARs containing long C-tails (e.g. GluA1/2) are added to synapses in an activity-dependent manner (Hayashi *et al.*, 2000; Shi *et al.*, 2001). Alternative splicing of the C-terminal domains can further determine the binding of subunits to specific interacting proteins or dictate the type of posttranslational modification the subunits undergo (Figure 1.3) (Shepherd & Huganir, 2007). The long-tailed GluA1 subunit is able to bind to the actin-binding protein 4.1N (Shen *et al.*, 2000) and to SAP97 (Synapse-associated protein 97) (Leonard *et al.*, 1998), which regulate the trafficking and surface expression of AMPARs. On the other hand, short-tailed AMPAR subunits like GluA2 can interact with GRIP (Glutamate receptor-interacting protein) (Dong *et*

al., 1997), ABP (AMPA receptor-binding protein) (Srivastava *et al.*, 1998), and PICK1 (Protein interacting with C-kinase 1) (Dev *et al.*, 1999; Xia *et al.*, 1999) to regulate the stabilization and recycling of the subunit in and out of the synapse.

AMPARs can further be diversified through posttranslational modifications such as glycosylation, palmitoylation, sumoylation, ubiquitination and phosphorylation and these changes can be subunit-specific [reviewed in (Jiang *et al.*, 2006; Lu & Roche, 2012)]. Phosphorylation, for instance, has been described to modify and regulate all four AMPAR subunits, but through distinct signalling mediators and in different target regions (Figure 1.3) (Jiang *et al.*, 2006; Lee, 2006). Four distinct phosphorylation sites have already been reported in the intracellular C-tail of the GluA1 subunit alone. The first identified site was a serine residue 831 (S831) prone to be phosphorylated by protein kinase C (PKC) (Roche *et al.*, 1996) and calcium-calmodulin-dependent protein kinase II (CaMKII) (Mammen *et al.*, 1997). Serine 845 (S845) was identified as a target site for protein kinase A (PKA) and cGMP-dependent protein kinase II (cGKII) phosphorylation (Roche *et al.*, 1996; Serulle *et al.*, 2007). Serine 818 (S818) and Threonine 840 (T840) were only later discovered to be major regulatory phosphorylation sites of PKC in GluA1 (Boehm *et al.*, 2006; Lee *et al.*, 2007). Phosphorylation of these residues is thought to be important for regulating AMPAR trafficking and synaptic insertion, as well as the induction of synaptic plasticity events (Carvalho *et al.*, 2000; Jiang *et al.*, 2006; Lu & Roche, 2012). Phosphorylation of S831 is increased with LTP (Lee *et al.*, 1998), however, it does not seem required for synaptic insertion of receptors (Hayashi *et al.*, 2000) and S831 phosphodead knockin mice have intact hippocampal LTP (Lee *et al.*, 2010). Similarly, knockin mice lacking phosphorylation at S845 express normal LTP (Lee *et al.*, 2010), although mutations in the residue prevent the delivery of GluA1 to synapses (Esteban *et al.*, 2003). Interestingly, LTP is impaired in double knockin mice lacking phosphorylation in both S831 and S845, indicating that, while none of the residues is absolutely required, they can substitute one another and be sufficient for LTP induction (Lee *et al.*, 2003). Phosphorylation of S818 by PKC, on the other hand, is critical for the synaptic insertion and stabilization of GluA1-AMPARs and for LTP induction (Boehm *et al.*, 2006). By contrast, GluA1 phosphorylation on T840 seems to have an important role in hippocampal LTD, and not LTP (Delgado *et al.*, 2007; Lee *et al.*, 2007).

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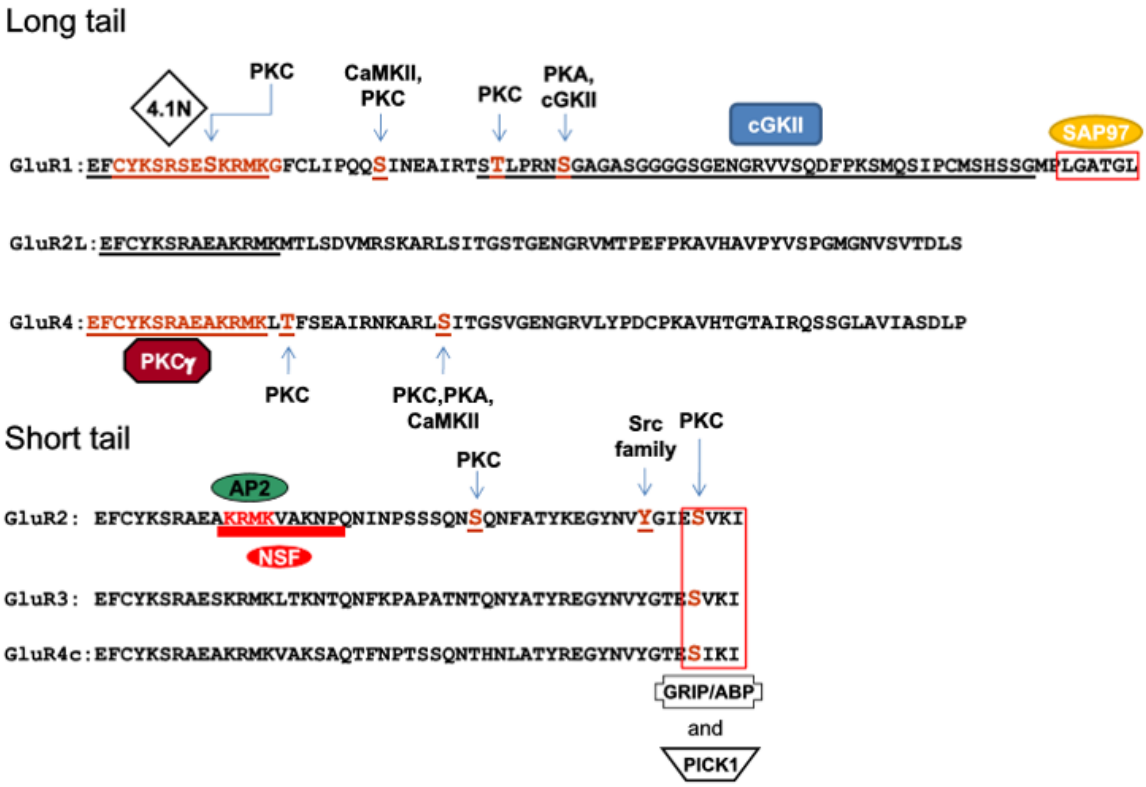


Figure 1.3 – Schematic representation of the intracellular C-terminal region of AMPAR subunits. Sequence alignment of the intracellular C-terminal region of the long-tailed (GluA1, GluA2L and GluA4) and short-tailed (GluA2, GluA3 and GluA4c) AMPAR subunits. Phosphorylation sites for each subunit and binding sites for interacting proteins are underlined and highlighted in red or with boxes, respectively. [Adapted from (Santos *et al.*, 2009)].

AMPAR function is further diversified by RNA processing events, including alternative splicing and RNA editing. In the adult brain, particularly at mature hippocampal synapses, AMPARs consist predominantly of GluA1/2 or GluA2/3 subunits. The presence of the GluA2 subunit in the receptors has a profound effect on the channel properties of AMPARs. Following transcription, the GluA2 subunit mRNA undergoes RNA editing in the channel pore-encoding region (Q/R site), whereby the RNA coding for a glutamine (Q) residue is exchanged for the RNA coding for arginine (R), which has a positive charge, thus preventing divalent ions from entering the cell (Figure 1.2A). As such, GluA2-lacking receptors have a high Ca^{2+} permeability, as opposed to GluA2-containing receptors that are highly impermeable to Ca^{2+}. Besides calcium permeability, this Q/R editing also regulates channel conductance properties, endoplasmic reticulum (ER) export kinetics, and subunit assembly into functional receptors (Geiger *et al.*, 1995; Greger *et al.*, 2003; Greger *et al.*, 2002; Mansour *et al.*, 2001; Sommer *et al.*, 1991; Swanson *et al.*, 1997).

Regulation of AMPA receptors by protein interactions

Unquestionably, the presence of AMPARs at synapses is of major importance. AMPARs not only mediate basic synaptic functions, but are fundamental for plasticity events that allow synaptic strengthening. As such, mechanisms that allow trafficking and stabilization of AMPARs at synapses are carefully regulated. These regulatory mechanisms are only possible through a myriad of proteins that interact with AMPARs and regulate their function. Indeed, accessory and scaffolding proteins interact with AMPARs at numerous subcellular domains. As previously mentioned, because the intracellular C-tail of AMPARs contains several regulatory domains, this region is particularly targeted by distinct binding partners, which can be subunit-specific. However, several proteins, from transmembrane proteins to cell-adhesion complexes, were found interacting with extracellular domains of AMPARs, thus contributing to the idea that AMPARs are subject to complex regulation.

The large collection, certainly still incomplete, of AMPAR partners identified and characterized until today has given an insight not only on the roles of individual interactors regulating AMPARs, but on how these partners influence one another to function within an intricate network in the synapse. Importantly, the discovery of AMPAR partners has begun to explain the details of how AMPARs reach the synapse and how they are regulated upon activity within the PSD to modulate synaptic function (Anggono & Huganir, 2012; Bredt & Nicoll, 2003; Cheng *et al.*, 2012; Henley, 2003; Jackson & Nicoll, 2011; Jacobi & von Engelhardt, 2017; Sheng & Kim, 2011).

AMPA receptor subunit-specific intracellular interactions

AMPA receptor insertion in the synaptic membrane involves tightly regulated events that depend on the subunit composition of the receptor and on specific signals contained within their C-termini. As previously mentioned, the C-tails are highly divergent between AMPAR subunits and each can be targeted by specific interacting proteins.

SAP97 was the first protein reported to directly interact with the GluA1 subunit (Figure 1.3), through a PDZ domain (Leonard *et al.*, 1998), and it allows the transport of GluA1-containing AMPARs from the ER and Golgi apparatus to the plasma membrane (Sans *et al.*, 2001). Also, SAP97 highly accumulates at GluA1-containing synapses and recruits PKA to GluA1, suggesting that it could further act to facilitate GluA1 phosphorylation and its trafficking to the synapse (Colledge *et al.*, 2000; Valtschanoff *et al.*, 2000). Protein 4.1N is an actin-binding protein highly expressed in excitatory synapses, where it interacts and binds to the

intracellular C-tail of GluA1 to regulate its surface expression (Figure 1.3) (Shen *et al.*, 2000). Disrupting 4.1N binding to GluA1 decreases the surface expression of the receptor (Coleman *et al.*, 2003), whereas enhancing the interaction by phosphorylation of GluA1 in S816 and S818, promotes exocytosis of AMPARs in a PKC-dependent manner (Lin *et al.*, 2009). Importantly, loss of 4.1N expression decreases the insertion of GluA1 on extrasynaptic sites and impairs the maintenance of LTP (Lin *et al.*, 2009).

The GluA2 subunit is also target to specific interacting proteins, previously mentioned, that regulate its trafficking in and out of the synapse (Figure 1.3). GRIP and ABP are both localized in synapses where they bind to a PDZ domain of GluA2 and promote its anchoring in the synapse. Blocking the interaction of GRIP/ABP with GluA2 by specifically mutating the GluA2 PDZ domain accelerates the endocytosis of the receptor (Osten *et al.*, 2000). Despite also binding to the PDZ domain of GluA2 (Chung *et al.*, 2000), PICK1 acts to promote the receptor endocytosis. PICK1 is able to chaperone AMPARs with activated PKC, which, in turn, phosphorylates GluA2 and promotes its binding to PICK1, resulting in the endocytosis of GluA2 (Lin & Huganir, 2007; Terashima *et al.*, 2004).

AMPA receptor transmembrane interactions

Transmembrane AMPAR-associated proteins (TARPs) bind to AMPARs within a stable complex at the cell surface, and are able to regulate AMPAR functions, including targeting and mediating surface trafficking of the receptors to the synapse [reviewed in (Jackson & Nicoll, 2011; Nicoll *et al.*, 2006; Opazo *et al.*, 2012; Straub & Tomita, 2012; Ziff, 2007). Stargazin (also known as TARP γ2) and its closely related TARPs γ3, γ4 and γ8 proteins interact with AMPARs early in the secretory pathway to control their assembly, maturation and trafficking (Tomita *et al.*, 2003). The C-terminal domain of Stargazin is a substrate for phosphorylation by CaMKII, and this regulation promotes the interaction of Stargazin with PSD95 (Schnell *et al.*, 2002; Sumioka *et al.*, 2010), which, in turn, allows the trapping of AMPAR complexes in the postsynaptic density (Bats *et al.*, 2007; Opazo *et al.*, 2010). Importantly, Stargazin phosphorylation is crucial for synaptic plasticity events. Tomita and colleagues showed that phosphorylation/dephosphorylation of Stargazin can bidirectionally regulate Hebbian plasticity, being required for the induction of either LTP or LTD, respectively (Tomita *et al.*, 2005). Moreover, a recent study from our lab demonstrated that Stargazin, and its phosphorylation, is further required for synaptic scaling of AMPARs, playing an important role in the induction of homeostatic and experience-dependent plasticity (Louros *et al.*, 2014).

Several other transmembrane proteins were more recently identified as auxiliary subunits to AMPARs and important for their regulation. Cornichons, for example, can be found associated with native AMPARs together with TARPs, possibly controlling the stoichiometry of TARPs associated with receptors. Cornichons further act to modulate channel properties of the receptor and regulate AMPAR surface expression (Schwenk *et al.*, 2009). Genetic deletion of cornichon family members CNIH-2 and -3 together causes a profound and selective loss of synaptic and surface GluA1-containing AMPARs (Herring *et al.*, 2013). SynDIG1 (Synapse Differentiation Induced Gene 1) was very recently identified as a new transmembrane regulator of AMPAR content at developing hippocampal synapses. SynDIG1 seems to be important to cluster GluA2-containing AMPARs in extrasynaptic sites; however, overexpression of SynDIG1 induces a selective increase in the synaptic content of GluA1 (Kalashnikova *et al.*, 2010).

AMPA receptor interaction with cell-adhesion molecules

Synaptic cell-adhesion molecules (CAMs) are usually localized in very close proximity to the synaptic cleft. These molecules interact in a homo- or heterophilic fashion across the synaptic cleft and are able to bridge pre- and postsynaptic specializations (trans-synaptic complexes) that are fundamental for synaptic formation, differentiation and maturation [Figure 1.4, extensively reviewed in (Bukalo & Dityatev, 2012; Dalva *et al.*, 2007; McMahon & Diaz, 2011; Murase & Schuman, 1999)]. However, recent studies show that synaptic CAMs are not merely static structural components of the synapse, but are often dynamic modulators of synaptic function, able to regulate AMPARs and synaptic plasticity events. Integrins are transmembrane heterodimers of α- and β-subunits shown to be highly accumulated in synapses (Chavis & Westbrook, 2001; Einheber *et al.*, 1996; Mortillo *et al.*, 2012). Several studies using inhibitors or mutant/knockout mice for β1 integrins revealed an impairment in basal excitatory synaptic transmission through AMPARs as well as in the induction of hippocampal LTP (Chan *et al.*, 2003; Chan *et al.*, 2006; Huang *et al.*, 2006b; McGeachie *et al.*, 2011). More recent studies have also shown that postsynaptic β3 integrins interact with the C-terminal of GluA2, and disruption of this interaction promotes GluA2 endocytosis, reducing AMPAR-mediated currents. On the other hand, overexpression of β3 integrins increases synaptic currents mediated by GluA2-containing AMPARs. Importantly, β3 integrin expression is bidirectionally regulated by manipulation of neuronal activity and its interaction with GluA2 is fundamental for the expression of homeostatic synaptic scaling mechanisms (Cingolani & Goda, 2008; Cingolani *et al.*, 2008; Pozo *et al.*, 2012; Thalhammer & Cingolani, 2014).

Cadherins also play a role in AMPAR regulation. N-cadherin is the most prominent cadherin in the brain and it mediates homophilic adhesions in a Ca^{2+}-dependent manner (Figure 1.4). Its intracellular C-tail binds to the cognate β-catenin, a PDZ-containing protein, which provides a link to the cytoskeleton and allows regulation of dendritic spine morphology (Fannon & Colman, 1996; Hirano & Takeichi, 2012; Uchida *et al.*, 1996). Recently, it has been shown that postsynaptic N-cadherin and β-catenin bind to and form a complex with the GluA2 subunit of AMPARs to regulate AMPAR surface expression (Nuriya & Huganir, 2006; Saglietti *et al.*, 2007). Furthermore, it is well established that preventing N-cadherin adhesion blocks the remodelling of dendritic spine morphology and the induction of LTP (Bozdagi *et al.*, 2000; Bozdagi *et al.*, 2010). More recent studies also provide evidence that β-catenin, and to a lesser extent N-cadherin, plays a key role in the regulation of AMPAR synaptic content in response to chronic deprivation of activity (Okuda *et al.*, 2007; Vitureira *et al.*, 2012).

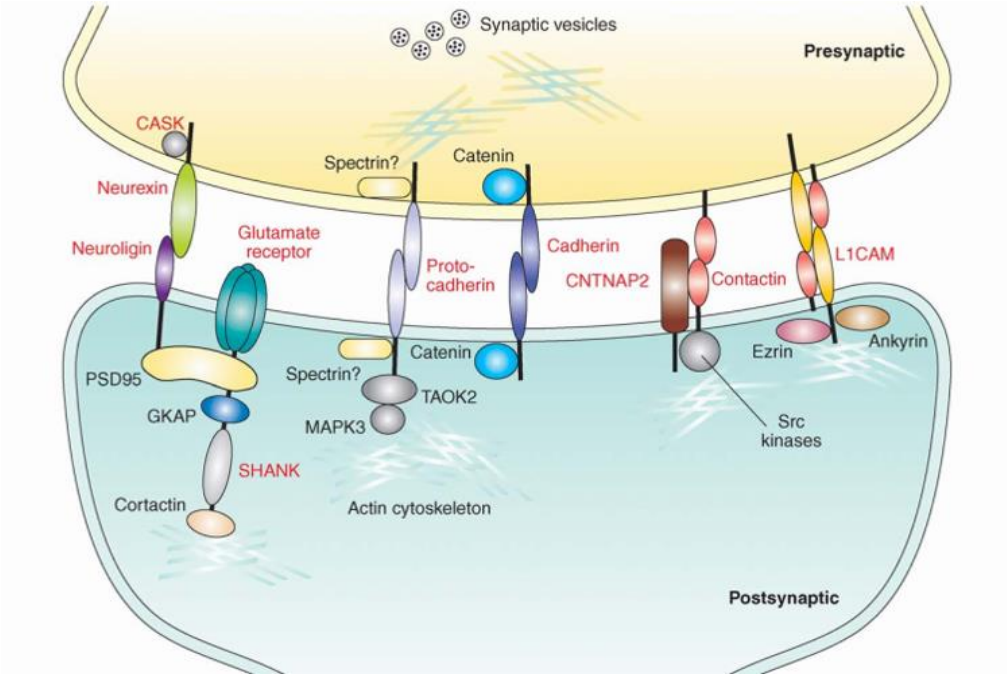


Figure 1.4 – Molecular distribution of trans-synaptic cell-adhesion molecules known to regulate synaptic function. Cell-adhesion molecules connect pre- and postsynaptic sites through homophilic (e.g. cadherins, protocadherins) and heterophilic (e.g. neuroligins-neurexins, Caspr2 and contactins) interactions and have roles in synapse development, function and plasticity. These CAMs also interact with several cytoplasmic proteins, namely synaptic scaffolding proteins, cytoskeletal proteins and signalling molecules, all of which are involved in synapse function and plasticity. The expression and localization of CAMs can be modulated by neuronal activity, contributing to the regulation of synaptic strength and sculpting of the molecular components of the synapse. Proteins labelled in red have already been implicated in Autism spectrum disorder (ASD) or intellectual disability (ID) [Adapted from (Betancur *et al.*, 2009)].

Neuroligins (NLGs) constitute a family of cell-adhesion proteins that bind trans-synaptically to presynaptic neurexins (NRXs) and are crucial for the formation and specification of synaptic properties (Figure 1.4) (Bukalo & Dityatev, 2012; Krueger *et al.*, 2012; McMahon & Diaz, 2011; Sudhof, 2008). Despite extensive knowledge on their role as synaptic organizers, NLGs and NRXs are now thought to also play roles in the regulation of AMPARs. At the postsynaptic membrane, the interaction between NLGs and NRXs results in an increased clustering of PSD95, with increased subunit-specific accumulation of GluA2-AMPARs (Heine *et al.*, 2008b). Interestingly, alternative splicing of a member of the neurexin family, NRX3, is able to trans-synaptically control the postsynaptic trafficking of AMPARs: inclusion of a splicing sequence in NRX3 that blocks its binding to NLGs enhances AMPAR endocytosis and abrogates AMPAR recruitment during the induction of LTP (Aoto *et al.*, 2013). Furthermore, recent studies by Südhof and collaborators demonstrated that an autism-associated point mutation in the cytoplasmic tail of NLGs selectively impairs GluA1-containing AMPAR trafficking to synapses and significantly decreases AMPAR-mediated synaptic transmission (Chanda *et al.*, 2015; Etherton *et al.*, 2011). Other postsynaptic neurexin-interacting proteins similar to NLGs, the LRRTMs (leucine-rich repeat transmembrane neuronal), also bind trans-synaptically to NRXs to promote formation of excitatory synapses (de Wit *et al.*, 2009; Ko *et al.*, 2009; Linhoff *et al.*, 2009). Moreover, specific knockdown of LRRTM2 results in a significant reduction in GluA1 synaptic content (de Wit *et al.*, 2009), and in vivo loss of LRRTM2 further impairs AMPAR-mediated currents and maintenance of LTP (Soler-Llavina *et al.*, 2013; Soler-Llavina *et al.*, 2011).

AMPA receptor trafficking

Long-lasting changes in synaptic strength in response to neuronal activity occur through exquisitely regulated changes in the number of AMPARs present in synapses. Interestingly, these receptors are not static components of the synaptic membrane. Instead, they are highly dynamic and are continuously being delivered and removed in and out of the synapse (Figure 1.6). This cycling is dependent on relative rates of exocytosis and endocytosis of the receptors at the postsynaptic membrane and these processes are at the basis of synaptic plasticity events (Anggono & Huganir, 2012; Huganir & Nicoll, 2013; Jacobi & von Engelhardt, 2017; Kessels & Malinow, 2009; Shepherd & Huganir, 2007). Because neurons are highly polarized and arborized, they pose many unique problems for the trafficking of membrane proteins, when compared to other cell types. As such, new AMPARs synthesized in the cell body have to travel long distances and undergo a series of trafficking steps before being delivered to distal

synapses. After synthesis and processing in the ER and Golgi apparatus, respectively, AMPARs can be transported to synapses through several possible pathways (Anggono & Huganir, 2012; Derkach *et al.*, 2007; Groc & Choquet, 2006; Jacobi & von Engelhardt, 2017; Santos *et al.*, 2009; Shepherd & Huganir, 2007). One possibility is that newly synthesized receptors can be directly inserted to the cell membrane and then move along dendrites by lateral diffusion until they reach the synapse and get anchored in the PSD (Borgdorff & Choquet, 2002). Another hypothesis is the intracellular dynein/kinesin-dependent transport of AMPAR-containing vesicles (endosomes) along dendrites to the vicinity of synaptic sites, and only then followed by exocytosis to the cell membrane (Passafaro *et al.*, 2001). One other alternative requires the transport of AMPAR mRNAs and their local dendritic synthesis close to synaptic sites, followed by synaptic insertion (Steward & Schuman, 2001; Steward & Worley, 2001). Despite high controversy among studies about the actual mechanism that conducts AMPARs to synapses, there is consensus in the fact that the trafficking of AMPARs is an exquisitely complex mechanism crucial for activity-dependent changes in synaptic strength.

Local dendritic synthesis of AMPA receptors

Nowadays, it is well established that synaptic potentiation requires gene transcription and is highly dependent on new protein synthesis [reviewed in (Holt & Schuman, 2013; Swanger & Bassell, 2013)]. Due to their polarized and highly compartmentalized structure, neurons developed extremely dynamic translational processes that have evolved into highly elaborate and crucial regulatory mechanisms of neuronal function (Holt & Schuman, 2013; Shepherd & Huganir, 2007; Sutton & Schuman, 2006; Tom Dieck *et al.*, 2014). In particular, neurons have evolved specific pathways to transport mRNAs out into dendrites, which contain localized outposts with virtually all the requisite machinery for translation, from ribosomes to initiation and elongation factors (Asaki *et al.*, 2003; Cajigas *et al.*, 2012; Steward & Levy, 1982), and where subsequent translation can occur (Figure 1.5) [reviewed in (Bramham & Wells, 2007; Buxbaum *et al.*, 2015a; Buxbaum *et al.*, 2015b; Czaplinski, 2014; Doyle & Kiebler, 2011; Swanger & Bassell, 2013)]. Specifically, actively transported mRNAs contain *cis*-acting sequences or targeting elements, typically located within the 3' untranslated region (UTR) of the mRNA (Andreassi & Riccio, 2009; Chatterjee & Pal, 2009). These elements interact with RNA-binding proteins (RBPs) and other regulatory molecules to form ribonucleoprotein (RNP) complexes that are transported into dendrites along microtubules by motor proteins (Figure 1.5) (Andreassi & Riccio, 2009; Xing & Bassell, 2013). Moreover, localized transcripts travel within RNPs to their final destination in a translationally dormant state, until a specific synaptic stimulus is able to activate translation. Once an individual synapse is activated, RNPs partially

disassemble and translational repression is relieved locally (Figure 1.5) (Buxbaum *et al.*, 2014). This translational repression is often accomplished by the *trans*-acting RBPs already regulating the transport of the mRNA (Tolino *et al.*, 2012; Xing & Bassell, 2013).

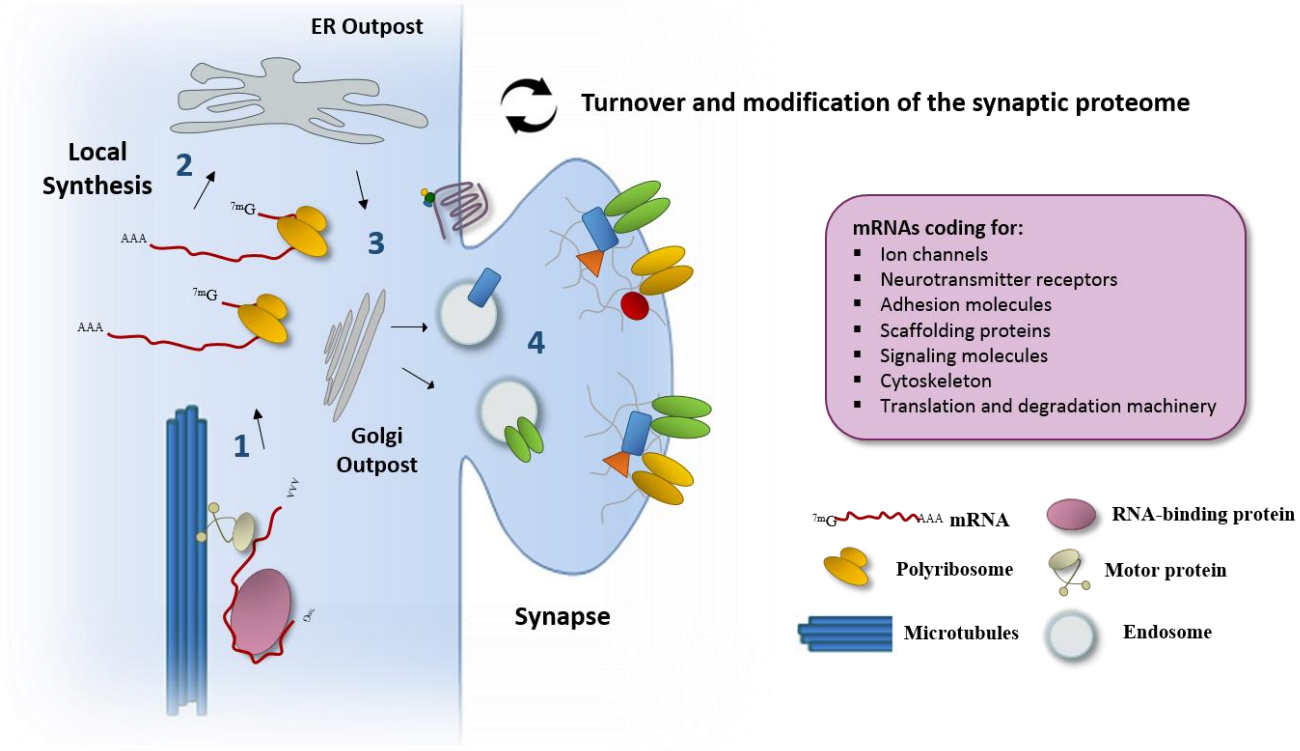


Figure 1.5 – Transport and local translation of neuronal transcripts fundamental for synaptic function. RNA-binding proteins (RBPs) can bind to specific mRNAs and promote their translational silencing and incorporation into ribonucleoprotein (RNP) complexes that travel to dendrites, via motor proteins, along the microtubular network **(1)**. Following synaptic activation, the translational repression of the mRNA is abrogated through disassembly of the RNP granule, resulting in the release of the transcript to undergo translation **(2)**. After synthesis in the ER and processing in the Golgi apparatus (which contain dendritic outposts) **(3)**, newly synthesized proteins can then be inserted in the synapse to exert their functions **(4)**. The number of mRNAs found locally in dendrites is ever increasing and it includes transcripts belonging to the categories mentioned in the pink box. Importantly, their local dendritic synthesis allows the creation of a nearby toolbox for the turnover of the synaptic proteome and this is crucial for activity-induced changes in synaptic strength.

One striking example comes from the well-orchestrated regulation of β-actin mRNA transport and local translation by zipcode-binding protein 1 (ZBP1) (Ming, 2006; Paquin & Chartrand, 2005; Wu *et al.*, 2015). ZBP1 binding to a 54-nucleotide zipcode in the 3'UTR of β-actin mRNA (Ross *et al.*, 1997) induces both translational silencing of the transcript and its incorporation into RNPs for transport. Local β-actin synthesis is possible through phosphorylation of ZBP1. Upon specific stimulus, the Src kinase phosphorylates ZBP1 at a

key tyrosine residue required for its binding to RNA, resulting in the release of β-actin mRNA from the RNP and the activation of β-actin synthesis at sites of high actin dynamics (Huttelmaier *et al.*, 2005; Sasaki *et al.*, 2010; Wu *et al.*, 2015). In developing neurons, this ZBP1-dependent temporal and spatial control over β-actin translation is fundamental for mechanisms of axonal guidance and neurite outgrowth (Huttelmaier *et al.*, 2005; Sasaki *et al.*, 2010; Welshhans & Bassell, 2011). Later in development, ZBP1 further contributes to proper dendritic branching, and ZBP1 deficiency or impairment of its phosphorylation alters dendritic morphology (Perycz *et al.*, 2011). Moreover, ZBP1-β-actin mRNA complexes selectively traffic into dendritic spines upon synaptic stimulation to regulate dendritic structure and density (Eom *et al.*, 2003; Tiruchinapalli *et al.*, 2003). Other RBPs have also been identified to regulate the transport and local translation of transcripts fundamental for proper synaptic function. For example, cytoplasmic polyadenylation element-binding protein (CPEB) binds to and regulates experience-dependent translation of CaMKII mRNA at synapses (Wu *et al.*, 1998), whilst fragile X mental retardation protein (FMRP), impaired in individuals with Fragile X syndrome (FXS), regulates the translation of synaptic transcripts such as PSD95, CaMKII and the immediate early gene Arc/Arg3.1 (Kao *et al.*, 2010; Niere *et al.*, 2012; Zalfa *et al.*, 2007; Zalfa *et al.*, 2003).

The targeting and localization of specific transcripts to synapses offers neurons a flexible "at hand" toolbox for the maintenance, turnover and modification of the synaptic proteome, which might provide an important means to fine-tune synaptic strength with great temporal and spatial resolution. Early *in vitro* and *in vivo* studies suggested that dendritic mRNA location was specific only to a handful of transcripts. However, recent advances in genome-wide deep sequencing and single-molecule RNA imaging in live cells and whole organisms have dramatically expanded the local transcriptome in dendrites, suggesting that local translation is the rule rather than the exception [(Cajigas *et al.*, 2012; Lionnet *et al.*, 2011; Park *et al.*, 2014; Poon *et al.*, 2006) and reviewed in (Buxbaum *et al.*, 2015a; Buxbaum *et al.*, 2015b; Holt & Schuman, 2013; Tom Dieck *et al.*, 2014)]. One of the most surprising findings from these studies is the vast number of mRNAs present in dendrites, which surpasses 2500 transcripts spanning many different functional classes of synaptic molecules, from scaffolding and cytoskeletal proteins, cell-adhesion and signalling molecules to ion channels and neurotransmitter receptors, including AMPARs (Figure 1.5) (Cajigas *et al.*, 2012). Indeed, it has been known for a while that a substantial fraction of synaptic sites contain mRNA molecules for the GluA1 and GluA2 AMPAR subunits, which can be locally synthesized upon manipulations of neuronal activity (Grooms *et al.*, 2006; Ju *et al.*, 2004; Kacharmina *et al.*, 2000). Using transected dendrites from hippocampal neurons, a couple of studies showed that exogenously expressed GluA1 and GluA2 subunits could still be locally synthesized,

independently of the cell soma (Ju *et al.*, 2004; Kacharmina *et al.*, 2000). Moreover, they demonstrated that activation of mGluRs led to increased dendritic synthesis and membrane insertion of both AMPARs subunits, whereas a chronic blockade of synaptic activity specifically increased the synthesis of GluA1 alone (Ju *et al.*, 2004; Kacharmina *et al.*, 2000). One other study also found endogenous mRNA molecules for AMPARs localized to distal and proximal dendrites in hippocampal neurons, whose synthesis is decreased upon activation of NMDARs (Grooms *et al.*, 2006).

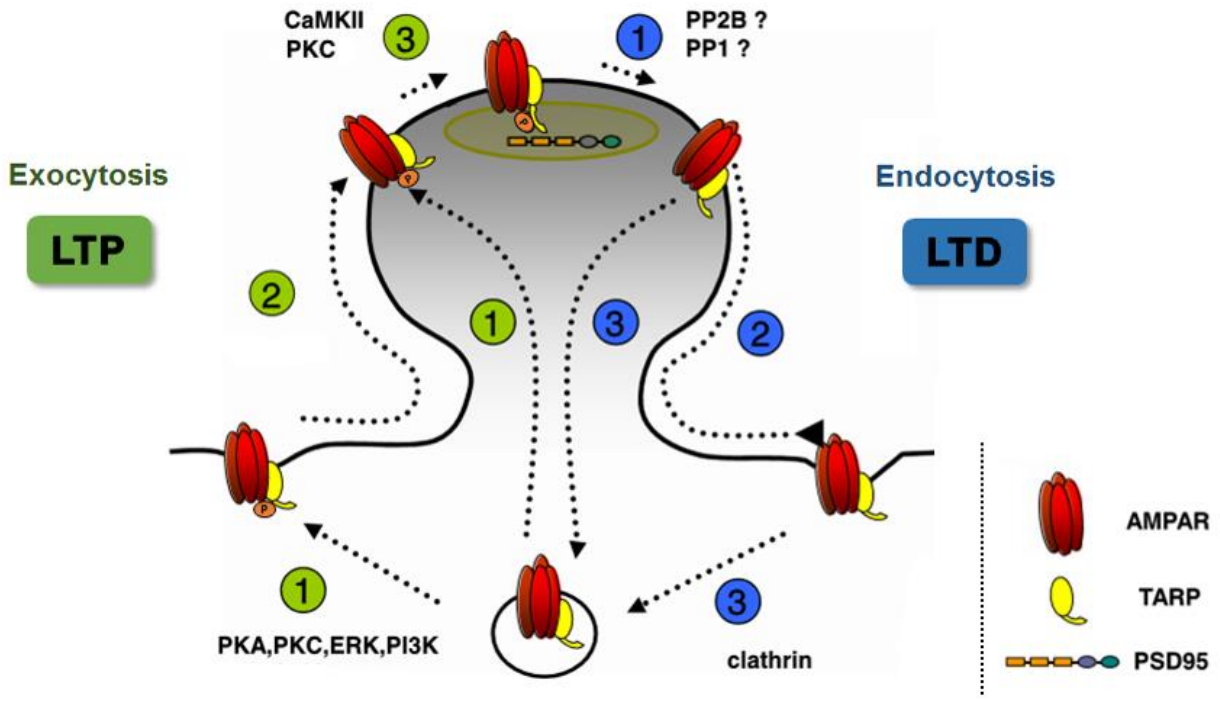
Despite still poorly understood, some recent studies are starting to shed some light on the mechanisms regulating the targeting and local translation of AMPARs in dendrites. For instance, CPEB3, a CPEB-like RNA-binding protein, seems to act as a specific translational repressor of GluA2 mRNA. CPEB3 binds to and represses the translation of the GluA2 mRNA, which is reverted upon activation of NMDARs. Moreover, loss of expression of CPEB3 stimulates the local translation of GluA2 mRNA (Huang *et al.*, 2006a). Also, FMRP and its autosomal paralogs FXR1P and FXR2P, whose loss of function leads to intellectual disability traits in individuals with Fragile X Syndrome, have recently been shown to be located near synaptic sites and regulate AMPAR mRNA with distinct functions. FXRP1, for instance, was shown to repress the local translation and synaptic incorporation of GluA2 and impair hippocampal LTP (Cook *et al.*, 2014), whereas FXR2P and FMRP promote the stabilization and translation of GluA1 mRNA and its subsequent membrane insertion, respectively (Guo *et al.*, 2015). GluA1 mRNA was just recently shown to be further regulated by the Fused in Sarcoma (FUS) RNA-binding protein, which binds to and stabilizes the GluA1 transcript. Moreover, *in vitro* and *in vivo* loss of FUS leads to a decrease in surface levels of GluA1 and reduction of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) amplitude (Udagawa *et al.*, 2015). AMPAR local translation has further been shown to be regulated through other mechanisms such as retinoic acid and its receptor or small non-coding microRNAs (miRNA), and these seem to be crucial for the homeostatic regulation of AMPARs (please see section “Homeostatic regulation of AMPA receptors” in Chapter I). Altogether, these evidence converge to point out that the local regulation of AMPAR translation, in close proximity to synapses, empowers neurons to rapidly respond to activity-dependent changes in synaptic AMPARs and subsequent modulation of synaptic strength.

Surface dynamics of AMPA receptors

Changes in the number of synaptic AMPARs underlie long-lasting modifications in synaptic strength, and contribute to mechanisms of synaptic plasticity. In the absence of neuronal activity, AMPARs undergo constitutive recycling between synapses and local pools in the cytosol, where they are sorted for either degradation or reinsertion at synapses (Ehlers, 2000; Passafaro et al., 2001). Upon synaptic activity, the cycling of AMPARs rapidly shifts to adapt to changes in activity and increases either the insertion or removal of AMPARs from the synapse (Figure 1.6). Whilst an increased synaptic insertion of AMPARs correlates with synaptic potentiation, increased removal of the receptors from the PSD is associated with synaptic weakening (Figure 1.6). Despite under intense study, there is still high controversy on the mechanisms regulating the trafficking and insertion of AMPARs into synapses. Although it is likely that a combination of several pathways mediates the synaptic insertion of AMPARs, the consensual hypothesis is that SNARE protein-mediated exocytosis (Kennedy *et al.*, 2010; Lu *et al.*, 2001; Suh *et al.*, 2010) of nearby local pools of AMPARs occurs firstly on extra/perisynaptic regions of the membrane, with subsequent lateral diffusion of the receptors to the synapse (Figure 1.6) (Adesnik *et al.*, 2005; Borgdorff & Choquet, 2002; Makino & Malinow, 2009; Passafaro *et al.*, 2001; Patterson *et al.*, 2010; Yang *et al.*, 2008). This extrasynaptic insertion of AMPARs seems to be largely regulated by AMPAR interaction partners and phosphorylation of AMPAR subunits [reviewed in (Anggono & Huganir, 2012; Henley *et al.*, 2011)]. For instance, overexpression of Stargazin (Schnell *et al.*, 2002) and PKC-dependent phosphorylation of GluA1 S816 and S818 residues, through binding of the 4.1N protein (Lin *et al.*, 2009), enhance the surface insertion of GluA1-containing AMPARs to extrasynaptic pools (Figure 1.6). Also, PKA-dependent phosphorylation of GluA1 at S845 specifically targets and clusters receptors to extrasynaptic sites but not synapses (Man *et al.*, 2007; Oh *et al.*, 2006).

Studies with advanced imaging approaches to directly visualize the mobility of individual AMPARs, like single particle tracking (SPT) or fluorescence recovery after photobleaching (FRAP), show that, once at the cell surface, extrasynaptic AMPARs are highly mobile and able to laterally diffuse into synapses, where they can be retained (Figure 1.6) (Bats *et al.*, 2007; Borgdorff & Choquet, 2002; Groc *et al.*, 2004; Heine *et al.*, 2008a). Interestingly, not all extrasynaptic receptors are necessarily retained, since, under basal conditions, extrasynaptic AMPARs can easily enter and scan the synaptic surface, and eventually exit it (Borgdorff & Choquet, 2002; Tardin *et al.*, 2003). In agreement with the lack of retention of the receptors, neither Stargazin overexpression nor PKA activation correlate with increased synaptic accumulation of AMPARs and subsequent synaptic potentiation, despite increased number of

surface AMPARs. Instead, Stargazin- or PKA-mediated synaptic accumulation of AMPARs occurs only after activation of NMDARs (Oh *et al.*, 2006). This evidence suggests that both Stargazin and PKA prime AMPARs for synaptic plasticity by inducing their extrasynaptic accumulation, and point out the necessity for an additional activity-dependent trapping step to retain AMPARs in the synapse. This diffusional trapping of AMPARs from extra/perisynaptic sites into the synapse is mediated by an activity-dependent interplay between Stargazin, PSD95 and CaMKII that allows the immobilization of passing AMPARs in the postsynaptic membrane (Figure 1.6) [reviewed in (Opazo & Choquet, 2011; Opazo *et al.*, 2012). Several studies performed by Choquet and collaborators show that activation of NMDARs triggers the immobilization of diffusing surface AMPARs at synapses, through a Ca^{2+}-dependent signalling cascade (Borgdorff & Choquet, 2002; Heine *et al.*, 2008a; Petrini *et al.*, 2009). Activation of NMDARs promotes the rapid translocation of CaMKII into active synapses, which in turn mediates the phosphorylation of GluA1 at S831 and of Stargazin. Phosphorylation of Stargazin will facilitate its binding to PSD95 in the postsynaptic membrane, thus allowing the trapping of AMPARs in the synapse (Bats *et al.*, 2007; Opazo *et al.*, 2010). Taken together, these findings suggest a three-step mechanism for the trafficking of AMPARs (Figure 1.6), in which AMPARs are inserted to extra/perisynaptic sites and intrinsically diffuse within the neuronal surface to synapses, where they can finally be trapped in an activity-dependent manner [extensively reviewed in (Chater & Goda, 2014; Lisman *et al.*, 2012; Opazo & Choquet, 2011)].



[Figure 1.6 - subtitle on the next page**]**

Figure 1.6 – Three-step model for the surface dynamics of AMPARs during synaptic plasticity. During LTP **(in green)**, AMPARs are inserted to the cell membrane in extra/perisynaptic sites via phosphorylation events **(1)**. Following exocytosis, AMPARs rapidly diffuse to synaptic sites **(2)**, where they can be trapped by phosphorylation events triggered by CaMKII that target Stargazin and increase its affinity to PSD95 **(3)**. During LTD **(in blue)**, the reverse order of events is likely to take place. Dephosphorylation of AMPARs and Stargazin mediated by PP1 and PP2B protein phosphatases might destabilize AMPARs from the PSD **(1)**, and allow their lateral diffusion out of the synapse **(2)**, where they can undergo clathrin-mediated endocytosis **(3)**. [Adapted from (Opazo & Choquet, 2011)].

In addition to their regulated insertion into synapses, the number of AMPARs expressed in the synaptic membrane is also dependent on their regulated internalization (Figure 1.6). This process is thought to occur through dynamin-dependent endocytosis via clathrin-coated vesicles that form in specializations stably positioned adjacent to the PSD (endocytic zone - EZ) (Carroll *et al.*, 1999; Ehlers, 2000; Man *et al.*, 2000). These endocytic zones and complementary components of the endocytic machinery seem to lie in lateral domains of the spine (Blanpied *et al.*, 2002; Lu *et al.*, 2007; Petrini *et al.*, 2009; Racz *et al.*, 2004), suggesting that AMPAR removal from the synapse, similarly to exocytosis, occurs in extra- or perisynaptic sites (Figure 1.6). This possibility implies that, as they get stabilized in the PSD for synaptic accumulation, AMPARs should be conversely destabilized from the postsynaptic membrane and laterally diffuse out of the synapse to undergo endocytosis. Consistent with this idea, synaptic proteins mediating AMPARs stabilization, like Stargazin, are targeted to dephosphorylation by the protein phosphatases PP1 and calcineurin (PP2B) following NMDAR activation (Mulkey *et al.*, 1994; Mulkey & Malenka, 1992). Accordingly, dephosphorylation of Stargazin seems to be required for the induction of LTD (Tomita *et al.*, 2005), and this event may be the trigger necessary to promote the destabilization and escape of AMPARs to extrasynaptic endocytic domains of the cell membrane (Figure 1.6).

Following internalization, AMPARs can be differentially sorted between recycling and degradative pathways. AMPARs sorted to late endosomes are usually fused with lysosomes and subsequently degraded, and this mechanism may underlie LTD phenomena. On the other hand, receptors targeted to recycling endosomes can then be reinserted to the cell membrane, allowing the maintenance of a stable pool of AMPARs nearby synaptic sites, which, in turn, underlies LTP events. Moreover, these sorting cycles between recycling/ degradation pathways can also underlie scaling mechanisms associated with homeostatic plasticity (Hanley, 2010).

Synaptic Plasticity

Learning and memory as well as other processes involved in all human behaviour are possible due to the ability of the mammalian brain to undergo experience-based adaptations. Such plasticity occurs at the level of synapses, which become stronger or weaker in response to specific patterns of activity. These changes in synaptic strength are mostly mediated by mechanisms regulating the number, distribution and function of postsynaptic AMPARs, and later on, through new protein synthesis-dependent structural changes that modulate the shape of the synapse. Changes in synaptic strength as a mechanism underlying learning and memory had already been proposed by Cajal at the beginning of the last century and formulated into a concrete synaptic model by Hebb in 1949 (Hebb, 1949). However, experimental evidence backing up the synaptic hypothesis was only obtained when Bliss and Lomo first discovered long-term potentiation (LTP), by showing that repetitive activation of excitatory synapses in the hippocampus triggered a persistent and long-lasting increase in synaptic transmission (Bliss & Lomo, 1973). LTP and long-term depression (LTD), the weakening counterpart of synaptic strength, are the two most studied and prevailing cellular models of synaptic plasticity [reviewed in (Huganir & Nicoll, 2013; Luscher & Malenka, 2012; Malenka & Bear, 2004; Nicoll & Roche, 2013)]. In addition to these input-specific forms of Hebbian plasticity, neurons have also developed mechanisms of homeostatic synaptic plasticity to respond to chronic changes in the overall level of network activity [reviewed in (Chater & Goda, 2014; Fernandes & Carvalho, 2016; Turrigiano, 1999; Turrigiano, 2008; Vitureira & Goda, 2013)].

Hebbian synaptic plasticity

Hebbian synaptic plasticity is the most widely studied form of long-lasting activity-dependent changes in synaptic strength and includes both long-term potentiation (LTP) and long-term depression (LTD). These Hebbian mechanisms are thought to be the cellular basis of learning and memory because they typically function in an input-specific manner, are rapidly induced and long-lasting, and are associative in requiring correlated firing of the pre- and postsynaptic neurons, thus reinforcing precise synaptic connections [reviewed in (Huganir & Nicoll, 2013; Luscher & Malenka, 2012; Malenka & Bear, 2004)].

Although resulting in opposing outcomes in terms of synaptic strength, both LTP and LTD can be elicited by activation of NMDARs. In brief, following presynaptic stimulation and partial postsynaptic depolarization, NMDARs are activated by glutamate binding, increasing Ca^{2+} intracellular concentration. The rise in intracellular Ca^{2+} is what typically distinguishes the

trigger for either LTP or LTD: a brief but strong increase in postsynaptic Ca^{2+} promotes the induction of LTP, whereas a sustained low-level Ca^{2+} elevation induces LTD. Different intracellular Ca^{2+} levels will then trigger distinct downstream signalling cascades, known to regulate either AMPARs or other players involved in their trafficking (Luscher & Malenka, 2012; Malenka & Bear, 2004; Malenka & Nicoll, 1999). These Ca^{2+}-triggered mechanisms regulating AMPARs, from posttranslational modifications of the receptors to their subcellular redistribution, largely underlie the changes in synaptic strength associated with LTP or LTD: phosphorylation and insertion of AMPARs in the PSD potentiates synapses, whereas dephosphorylation and endocytosis of AMPARs leads to synaptic weakening (Figure 1.6).

Typically induced following a short period of high-frequency tetanic stimulation, LTP is characterized by a persistent increase in AMPAR-mediated EPSCs, as a direct consequence of a higher number of synaptic AMPARs (Figure 1.6). Although the adult hippocampus contains both GluA1/2 and GluA2/3 heteromers, only GluA2/3-containing AMPARs are constitutively trafficked to the synapse (Malinow *et al.*, 2000). GluA1-containing AMPARs, on the other hand, are only recruited to the synapse upon LTP induction (Hayashi *et al.*, 2000; Shi *et al.*, 2001; Shi *et al.*, 1999). GluA1 plays a central role in the induction of LTP, since GluA1-knockout mice show impaired hippocampal LTP, which is recovered by genetically expressing GluA1 (Mack *et al.*, 2001; Zamanillo *et al.*, 1999). Curiously, LTP seems to be normal in GluA2/3 double KO mice (Meng *et al.*, 2003), emphasizing the requirement for GluA1 in LTP induction and the distinct activity-dependent trafficking behaviour of this subunit. The activity-dependent regulation of GluA1 trafficking into synapses requires NMDAR activation and the subsequent Ca^{2+}-dependent activation of several kinases such as PKA, PKC and CaMKII (Figure 1.6). Firstly, phosphorylation of GluA1 at S845 and S818/831 by PKA and PKC, respectively, mediates the insertion of AMPARs to extrasynaptic sites and their lateral diffusion to the synapse. CaMKII-dependent phosphorylation of GluA1 at S831 is then further required for the diffusional trapping of GluA1 in the PSD (for more detail, please see sections “AMPA receptor diversity through alternative splicing, RNA editing and posttranslational modifications” and “Surface dynamics of AMPARs”). Importantly, phosphorylation of GluA1 by these kinases, as well as of GluA1-interacting proteins such as Stargazin, has been extensively implicated and shown to be required for the induction and expression of LTP [extensively reviewed in (Carvalho *et al.*, 2000; Jiang *et al.*, 2006; Lu & Roche, 2012; Luscher & Malenka, 2012; Santos *et al.*, 2009)].

Prolonged low levels of synaptic stimulation can trigger NMDAR activation and cause a persistent weakening of synaptic strength that is characterized by the loss of synaptic AMPARs by endocytosis (Figure 1.6) (Beattie *et al.*, 2000; Carroll *et al.*, 1999; Man *et al.*, 2000). Similarly

to LTP, the phosphorylation status of AMPARs plays a central role in the induction and expression of LTD. NMDAR-dependent LTD causes a moderate postsynaptic increase in calcium influx that results in the activation of protein phosphatases such as calcineurin and PP1 (Figure 1.6). These phosphatases catalyse the dephosphorylation of GluA1 at S831 and S845, as well as of AMPAR-interacting proteins, and trigger the destabilization of GluA1-containing AMPARs from the synapse, with subsequent endocytosis and lysosomal degradation (for details check section “Surface dynamics of AMPARs”) (Ehlers, 2000). Importantly, mice containing mutations in GluA1 S831 and S845 exhibit major deficits in LTD, with impaired AMPAR internalization (Lee *et al.*, 2003; Lee *et al.*, 2010). The phosphorylation of the GluA2 subunit has also been implicated in LTD. Evidence shows that GluA2 phosphorylation at S880 disrupts its interaction with GRIP/ABP and shifts GluA2-containing AMPARs to PICK1, which results in the removal of synaptic AMPARs by increased endocytosis (Chung *et al.*, 2003; Chung *et al.*, 2000; Matsuda *et al.*, 1999; Perez *et al.*, 2001).

Hebbian plasticity mechanisms are widely considered to be the cellular correlates of learning and memory and are fundamental for information storage in the brain. Nevertheless, Hebbian plasticity is a positive-feedback process (Figure 1.7); for example, once LTP is induced, potentiated synapses can be excited to undergo further LTP with greater ease; similarly, depressed synapses easily undergo further LTD. These forms of unconstrained synaptic modulation could potentially result in synapses prone to either functional hyperexcitability or silence, driving neuronal activity to a runaway state [Figure 1.7; extensively reviewed in (Fernandes & Carvalho, 2016; Turrigiano, 1999; Turrigiano, 2008; Vitureira & Goda, 2013; Watt & Desai, 2010)]. In order to prevent such instability, neurons are able to sense their own excitability and trigger negative-feedback homeostatic mechanisms to counteract perturbations in activity and restrain it within a physiological range (Figure 1.7).

Homeostatic synaptic plasticity

Many of the plastic changes that underlie developmental or learning-related adaptations require input-specific modifications in synaptic strength, which can, nevertheless, exert destabilizing influence on network function, through unconstrained positive-feedback cycles of activity. To overcome this limitation, neurons have developed multiple mechanisms of homeostatic plasticity to adapt to overall changes in synaptic activity, which are critical for proper brain functions. Several distinct modes of homeostatic regulation of neural responses have been identified so far, involving possible changes in either the pre- or the postsynaptic

side of a neuron that range from adjustment of excitatory and inhibitory synaptic gain, changes in intrinsic excitability, and alterations in the threshold for LTP or LTD induction [reviewed in (Lee, 2012; Pozo & Goda, 2010; Turrigiano, 1999)]. Nevertheless, the best-studied form of homeostatic plasticity at central excitatory synapses occurs postsynaptically through mechanisms of synaptic scaling.

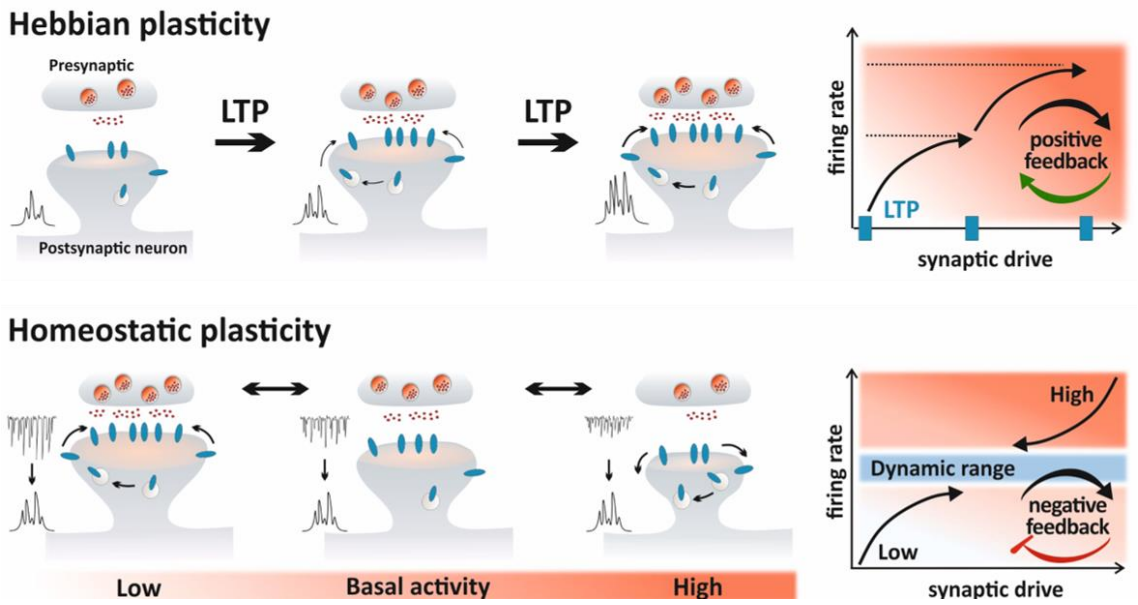


Figure 1.7 – Schematic representation of the circuitry in the primary visual cortex, which integrates visual experience through glutamatergic feed-forward inputs. Hebbian forms of synaptic plasticity, such as long-term potentiation (LTP), induce positive-feedback long-lasting changes in synaptic strength, which can, nonetheless, be destabilizing and drive activity to saturation, resulting in synapses prone to hyperexcitability. In a converse mechanism, unconstrained LTD can lead to synapse elimination. On the other hand, homeostatic plasticity bidirectionally regulates pre- and postsynaptic mechanisms through negative-feedback responses, in order to compensate for prolonged activity changes, thus stabilizing neuronal firing within a dynamic physiological range for adequate brain function.

Homeostatic synaptic scaling

The most extensively studied form of homeostatic plasticity at excitatory synapses occurs through postsynaptic mechanisms that maintain the overall firing of neurons within dynamic but functional boundaries, by fundamentally regulating their synaptic strength (Figure 1.7). This homeostatic regulation of synaptic strength, otherwise known as synaptic scaling, is typically expressed as a compensatory and bidirectional change in the postsynaptic accumulation of AMPARs (Figure 1.7; 1.9). Experimental evidence supporting a synaptic

scaling homeostatic mechanism was initially obtained *in vitro* from cortical, spinal cord and hippocampal neuronal cultures, via chronic pharmacological manipulations of neuronal activity that elicited compensatory and bidirectional changes in AMPAR-mediated synaptic transmission (Burrone *et al.*, 2002; O'Brien *et al.*, 1998; Turrigiano *et al.*, 1998). When neuronal firing was blocked over many hours with tetrodotoxin (TTX), a specific blocker of voltage-gated sodium channels, the amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) increased significantly, whereas increasing firing rates over time, by blocking GABA-mediated inhibition, resulted in the opposite effect.

Since this initial proposal of a homeostatic scaling mechanism by which neurons are able to adapt to chronic changes in their activity, numerous studies have followed up to demonstrate that such mechanism also occurs *in vivo* [reviewed in (Lee, 2012; Turrigiano, 2008)]. Physiologically, *in vivo* homeostatic plasticity mechanisms are thought to be of particular importance during development, when a massive adjustment of synapses and neural circuits occurs, and during periods of heightened plasticity, triggered by prolonged changes in the sensory environment or from neurological conditions [reviewed in (Turrigiano, 2011; Turrigiano & Nelson, 2004; Whitt *et al.*, 2014)]. Homeostatic scaling processes were also shown to occur during cross-modal plasticity of sensory modalities [(Goel *et al.*, 2006; Petrus *et al.*, 2014), and reviewed in (Lee & Whitt, 2015)], a process elicited to compensate for the loss of function of an affected modality in either deaf or blind people, for instance. Furthermore, a relevant role for homeostatic plasticity has just been recently established in the regulation of sleep/wake physiology (de Vivo *et al.*, 2017; Diering *et al.*, 2017; Hengen *et al.*, 2016). Homeostatic downscaling mechanisms were shown to occur during the sleep phase, promoting a “reset” of the overall network activity for the next vigilant state. These mechanisms were considered to be fundamental for memory consolidation and learning [reviewed in (Cirelli, 2017; Tononi & Cirelli, 2014)].

The most frequently used system to study *in vivo* homeostatic plasticity is the visual cortex (Figure 1.8), since it is amenable to manipulations of sensory experience and, thus, an excellence model to investigate experience-driven homeostatic changes. In this model system, several paradigms to manipulate visual experience have been shown to scale up or down excitatory synapses in the visual cortex. In particular, intraocular injections with TTX (to block action potentials) or chronic visual deprivation (such as dark rearing or dark exposure) are able to homeostatically scale up the amplitude of AMPAR-mediated mEPSCs in the layer 2/3 pyramidal neurons of the primary visual cortex (V1), while re-exposing visually deprived animals to light scales down mEPSCs back to basal levels [reviewed in (Lee, 2012; Whitt *et al.*, 2014)].

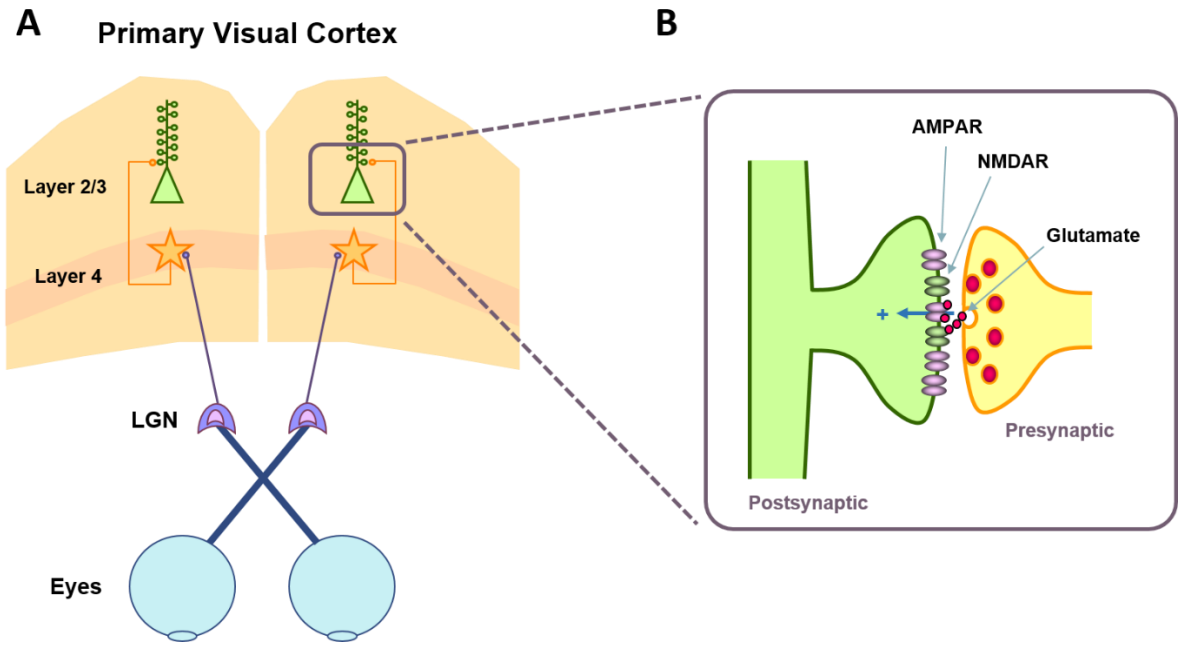


Figure 1.8 – Schematic representation of the circuitry in the primary visual cortex, which integrates visual experience through glutamatergic feed-forward inputs. **(A)** Homeostatic plasticity mechanisms occur *in vivo* through integration of sensory experience in the primary sensory cortices, including in the primary visual cortex circuitry (V1). Visual experience is integrated through sensory inputs from the eye onto the Lateral Geniculate Nucleus (LGN) of the thalamus, which outputs onto layer 4 of the V1. Pyramidal neurons on layer 2/3 of the V1, in turn, receive feed-forward inputs from layer 4. **(B)** Feed-forward inputs onto layer 2/3 pyramidal neurons of the V1 are known to be mainly glutamatergic and are known to be homeostatically regulated following prolonged periods of visual deprivation.

Both *in vitro* and *in vivo* studies of homeostatic synaptic scaling highlight an obvious dependence on the regulation of AMPARs. Interestingly, the functional changes in AMPAR-mediated currents are often accompanied by changes in the composition and abundance of synaptic AMPARs, both *in vitro* and *in vivo* (Figure 1.9). Hours to days of neuronal inactivity *in vitro* or decreased sensory input to neurons *in vivo* results in a synaptic accumulation of AMPAR subunits that correlates with increases in the amplitude of mEPSCs (Figure 1.9) (Goel *et al.*, 2006; Goel *et al.*, 2011; Ju *et al.*, 2004; O'Brien *et al.*, 1998; Thiagarajan *et al.*, 2005; Wierenga *et al.*, 2005). However, while most studies agree that synaptic scaling mechanisms depend on the accumulation of AMPARs at synapses, there is less agreement on the subunit composition of these receptors. Several reports show that both *in vitro* pharmacological blockade of neuronal activity (Aoto *et al.*, 2008; Ju *et al.*, 2004; Sutton *et al.*, 2006; Thiagarajan *et al.*, 2005), and *in vivo* chronic visual deprivation (Goel *et al.*, 2006; Goel *et al.*, 2011), trigger synaptic scaling mechanisms by selectively increasing the accumulation of Ca^{2+}-permeable (CP) GluA1-containing AMPARs at synapses, without much change in GluA2 levels. Other

studies, however, report a proportional and concurrent increase in the synaptic content of both GluA1 and GluA2 subunits following synaptic scaling triggered by pharmacological (Anggono *et al.*, 2011; O'Brien *et al.*, 1998; Wierenga *et al.*, 2005) or sensory manipulations of activity (Gainey *et al.*, 2009).

The discrepancy in results regarding the subunit regulation of AMPARs during synaptic scaling still remains to be clarified, but one possible explanation is that it may depend on the paradigm to manipulate neuronal activity. Homeostatic synaptic scaling has traditionally been considered a global phenomenon that uniformly changes synaptic strength across the entire synapse population of a given neuron. This is important as it conserves the relative differences in synaptic strength among synapses, which is crucial for information storage. However, it is now clear that synaptic scaling can operate locally at single dendritic branches or specific synapses to individually tune their own activity (Barnes *et al.*, 2017; Beique *et al.*, 2011; Branco *et al.*, 2008; Hou *et al.*, 2008; Ju *et al.*, 2004), without disrupting input-specific storage and processing [extensively reviewed in (Fernandes & Carvalho, 2016; Rabinowitch & Segev, 2008; Turrigiano, 2012; Turrigiano, 2008; Vitureira & Goda, 2013; Yu & Goda, 2009)]. While the details are not fully understood yet, there is some evidence that the mechanisms underlying the induction of global versus local synaptic scaling may differ. For instance, blocking action potentials with TTX may trigger a global synaptic scaling response that regulates both GluA1 and GluA2 subunits, in a manner that depends in decreased somatic Ca^{2+} signals and CaMKIV-mediated regulation of transcription (Ibata *et al.*, 2008). Local synaptic scaling, on the other hand, seems to require local NMDAR inhibition in conjunction with action potential blockade, resulting in a selective increase in the dendritic protein synthesis of GluA1-containing AMPARs (Aoto *et al.*, 2008; Ju *et al.*, 2004; Sutton *et al.*, 2006). *In vivo* sensory experience-induced synaptic scaling can also be global or restricted to a subset of synapses depending on the age of the animal or the particular neuronal type studied in an intact circuit (Gao *et al.*, 2010; Goel *et al.*, 2006; Goel & Lee, 2007; Petrus *et al.*, 2011). Altogether, these observations suggest that, similarly to Hebbian plasticity events, the postsynaptic expression of homeostatic plasticity relies on mechanisms that regulate not only the trafficking and accumulation of AMPARs at synapses, but also the dendritic availability of AMPAR transcripts and their local translation.

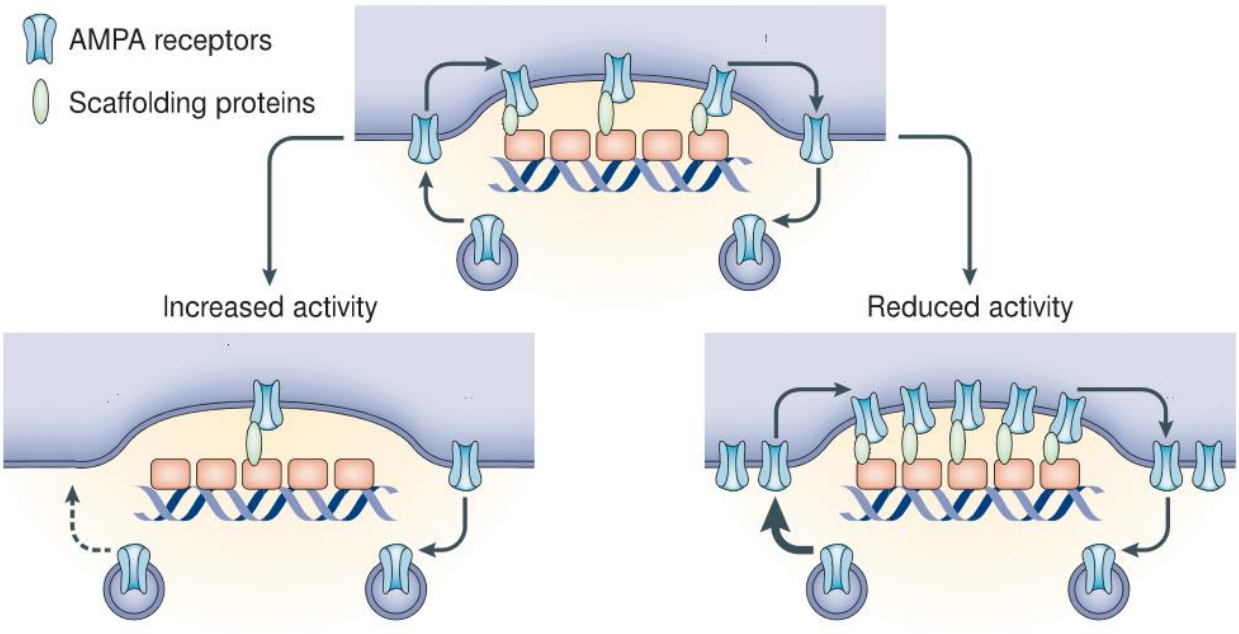


Figure 1.9 – Homeostatic synaptic scaling is accompanied by changes in the synaptic accumulation of AMPARs. In response to chronic changes in activity, neurons trigger compensatory mechanisms of synaptic scaling to adapt and maintain their overall firing within a dynamic range. These mechanisms are accompanied by changes in the synaptic accumulation of AMPARs, as well as turnover of scaffolding proteins that tether AMPARs to the synapse. Synaptic scaling triggered by pharmacological blockade of activity or by sensory deprivation results in an increased trafficking of AMPARs and respective scaffolding proteins to synapses **(bottom right)**. Conversely, increased neuronal activity leads to a decrease in the synaptic content of AMPARs **(bottom left)**. [Adapted from (Turrigiano & Nelson, 2004)].

Molecular mechanisms of homeostatic synaptic scaling

Since the first descriptions of homeostatic synaptic plasticity, the knowledge on the cellular properties of this type of plasticity has increased significantly. However, the molecular mechanisms underlying synaptic scaling are still poorly understood. Nevertheless, several novel players have been identified to interfere with the regulation of AMPARs and expression of homeostatic plasticity (Figure 1.10), contributing to emphasize the complexity of possible signalling pathways that neurons use to maintain their homeostasis [extensively reviewed in (Chater & Goda, 2014; Fernandes & Carvalho, 2016; Pozo & Goda, 2010; Turrigiano, 2008; Wang *et al.*, 2012)]. For a more comprehensive view of the mechanisms underlying synaptic scaling, please refer to Table 1.2 and (Fernandes & Carvalho, 2016).

The synaptic trafficking of AMPARs during Hebbian plasticity depends critically on the activation of CaMKII, but there is now evidence for an involvement of CaMKII function in synaptic scaling (Thiagarajan *et al.*, 2002). Distinct isoforms of CaMKII have been suggested to respond inversely to neuronal inactivity, such that CaMKIIα is down-regulated, while CaMKIIβ increases. Furthermore, knocking down CaMKIIβ expression prevents the inactivity-induced increase in GluA1 synaptic accumulation and AMPAR-mediated mEPSC amplitude (Groth *et al.*, 2011). In line with these evidence, mice lacking the GluA1-S831 site for CaMKII-mediated phosphorylation display abnormal synaptic scaling of visual cortex synapses, such that visual deprivation scales down mEPSCs, which is opposite from what is observed in wild-type mice (Goel *et al.*, 2011). These results provide evidence for a requirement of CaMKII signalling in the expression of synaptic scaling. As previously mentioned, CaMKII-dependent phosphorylation of Stargazin is fundamental for the synaptic trapping of AMPARs (please see section “Surface dynamics of AMPARs”). Interestingly, a recent study from our lab has implicated Stargazin in homeostatic synaptic scaling (Figure 1.10 and Table 1.2). Synaptic Stargazin is increased following neuronal inactivity both *in vitro* and *in vivo*, and loss of its expression blocks the GluA1 synaptic increase induced by chronic blockade of activity. Synaptic scaling is also prevented with the expression of Stargazin phosphodead mutants, further indicating a requirement for Stargazin phosphorylation in the expression of homeostatic synaptic plasticity (Louros *et al.*, 2014).

Other players recently involved in mechanisms regulating the expression of synaptic scaling are cell-adhesion molecules (Figure 1.10) [extensively reviewed in (McGeachie *et al.*, 2011; Thalhammer & Cingolani, 2014)], which had previously been implicated in the regulation of AMPAR surface expression and Hebbian plasticity (please see section “AMPAR interaction with cell-adhesion molecules”). One such example is the postsynaptic N-cadherin/β-catenin complex that regulates the GluA2 subunit. Recent studies have shown that bidirectional synaptic scaling is impaired in the absence of β-catenin. Moreover, expression of β-catenin deletion mutants, lacking either its PDZ domain or the N-cadherin binding region, cause a similar effect (Okuda *et al.*, 2007). Accordingly, the postsynaptic expression of dominant-negative constructs of N-cadherin impairs synaptic upscaling as effectively as ablating β-catenin (Vitureira *et al.*, 2012). Similarly to the N-cadherin/β-catenin complex, postsynaptic β3 integrins have recently been implicated in the homeostatic regulation of GluA2-containing AMPARs. β3 integrin expression is bidirectionally regulated by manipulations of neuronal activity. Furthermore, chronic suppression of activity is completely ineffective in scaling up synapses with loss of function or genetic ablation of β3 integrins (Cingolani & Goda, 2008; Cingolani *et al.*, 2008; Pozo *et al.*, 2012). These evidence suggesting a fundamental role of cell adhesion molecules in the homeostatic regulation of AMPARs are of particular interest to

the present study, where we show that Contactin-associated proteins 1 and 2 have a similar effect.

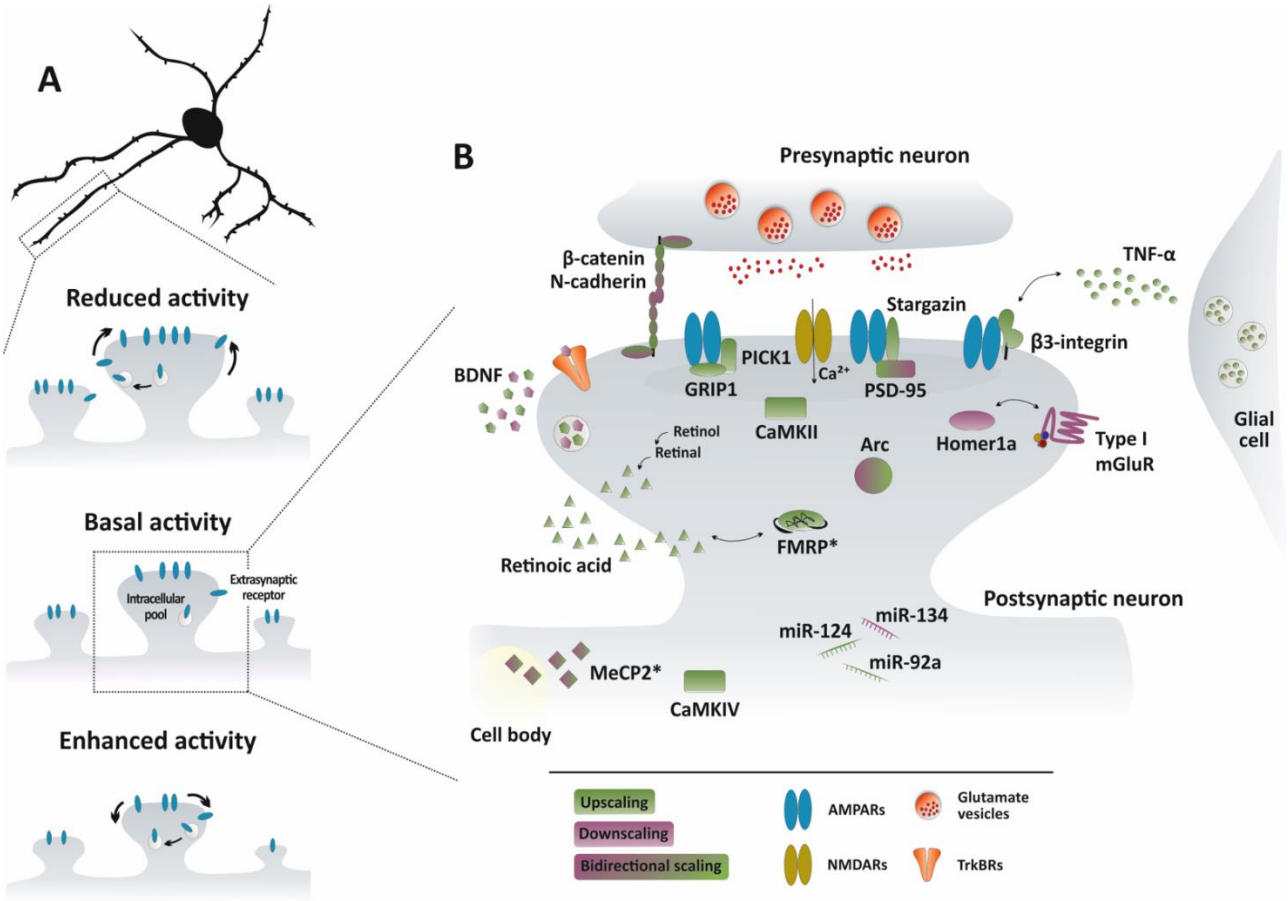


Figure 1.10 - Homeostatic synaptic scaling of excitatory synapses maintains neuronal activity within a dynamic range. (A) Synaptic scaling occurs through compensatory and bidirectional changes in the postsynaptic accumulation of AMPARs. Prolonged inhibition of neuronal activity will enhance AMPAR-mediated excitatory synaptic transmission, whilst there is a decrease of AMPAR-mediated transmission when neuronal activity is chronically enhanced. **(B)** Major molecular players and pathways implicated in synaptic scaling. Molecules involved in synaptic upscaling are indicated in green, whereas those involved in synaptic downscaling are drawn in magenta. Dual colour players are implicated in both forms of homeostatic scaling. Disease-related molecules are indicated (*).

Table 1.2 – Summary of the molecular mechanisms underlying the postsynaptic expression of homeostatic synaptic scaling.

Pathway	Role	Scaling up or down	References
Secreted molecules			
BDNF	Released in activity-dependent manner; acutely regulates AMPAR trafficking.	Reduced BDNF scales up synapses, and prevents scaling down.	(Reimers *et al.*, 2014; Rutherford *et al.*, 1998)
TNFα	Released by glia when activity falls.	Increased TNFα scales up synapses; TNFα KO or scavenging abolishes scaling up. No effect in scaling down.	(Kaneko *et al.*, 2008; Steinmetz & Turrigiano, 2010; Stellwagen & Malenka, 2006)
Trans-synaptic complexes and Cell adhesion molecules			
MHC-1	Synaptic localization and expression regulated by neuronal activity.	Reduced expression of MHC-1 prevents scaling up of synapses. Not tested in scaling down.	(Goddard *et al.*, 2007)
Presinilin 1	Integral member of γ-secretase enzyme; affects PI3/Akt signalling.	Loss of PSEN1 or Alzheimer's-linked mutation impairs synaptic upscaling. Scaling down not tested.	(Pratt *et al.*, 2011)
Intracellular pathways, proteins and mediators			
PKA signalling	GluA1-S845 phosphorylation. AMPAR synaptic targeting.	Scaling up increases PKA and GluA1-S845 phosphorylation; impaired visual deprivation-induced scaling up in mice lacking GluA1-S845 site.	(Diering *et al.*, 2014; Goel *et al.*, 2006; Goel *et al.*, 2011)
PICK1	Interacts with GluA2 to regulate AMPAR endocytosis	PICK1 loss of function occludes scaling up. No effect in scaling down.	(Anggono *et al.*, 2011)
GRIP1	Interacts with GluA1 to regulate AMPAR synaptic insertion.	GRIP1 synaptic expression bidirectionally regulated by synaptic scaling. GRIP1 loss of function blocks scaling up. Not tested in scaling down.	(Gainey *et al.*, 2015; Tan *et al.*, 2015)
PSD95/PSD93	Scaffold proteins essential for synaptic organization.	Altered synaptic expression with bidirectional scaling. Double KO prevents scaling up, but only PSD95 is required for scaling down.	(Sun & Turrigiano, 2011)
Arc	Immediate-early gene regulated by activity. Protein regulates AMPAR endocytosis.	Altered expression with bidirectional scaling. Increased Arc blocks upscaling Arc KO occludes both scaling up and down. Required for in vivo experience-dependent synaptic scaling.	(Gao *et al.*, 2010; Rial Verde *et al.*, 2006; Shepherd *et al.*, 2006)
MeCP2	Transcriptional factor regulating GluA2 transcription.	Loss of MeCP2 blocks both scaling up and down. MeCP2 required for in vivo experience-dependent scaling up.	(Blackman *et al.*, 2012; Qiu *et al.*, 2012)
miRNAs	Translational repressors. miR-485 and -134 regulate GluA2. miR-92a regulates GluA1.	miRs-485 and -134 required for scaling down. Not tested in scaling up. miR-92a blocks scaling up of synapses; not tested in scaling down.	(Cohen *et al.*, 2011; Fiore *et al.*, 2014; Letellier *et al.*, 2014)

Several studies have also proposed a role for some secreted molecules in shaping homeostatic adaptations of synaptic strength, such as TNFα, BDNF and Retinoic acid (for more details on TNFα and BDNF, please check Table 1.2). A series of recent studies by Chen and collaborators showed that scaling up of synapses following chronic neuronal inactivity is accompanied by an increased synthesis of Retinoic acid (RA). Moreover, exogenous application of RA increases the synaptic accumulation of AMPARs and occludes the same effect mediated by chronic inactivity, while suppression of RA synthesis prevents synaptic scaling (Aoto *et al.*, 2008). Importantly, RA-induced scaling of AMPARs occurs through a specific increase in the local dendritic synthesis of the GluA1 subunit, through signalling via the RA receptor RARα, which binds to and represses GluA1 mRNA translation in basal conditions (Maghsoodi *et al.*, 2008; Poon & Chen, 2008). Another study by the same group further uncovers a role of the RNA-binding protein FMRP in synaptic scaling and RA-induced GluA1 local translation (Soden & Chen, 2010). In Fmr1 KO mice, both inactivity-induced and RA-mediated upregulation of AMPARs is impaired, while expression of WT FMRP is able to restore the homeostatic upscaling of AMPAR-mediated mEPSCs. This evidence suggests that regulation of homeostatic plasticity, via FMRP, may be implicated in the neurodysfunction that characterizes the fragile X syndrome.

Undoubtedly, homeostatic synaptic scaling plays a crucial role in fundamentally stabilizing neuronal networks in face of exceedingly perturbing circumstances, such as massive circuit remodelling during development, prolonged changes in the sensory environment, or heightened plasticity caused by injury, for instance. These situations, if not counterbalanced by proper homeostatic mechanisms, eventually lead to neurological malfunctions, ranging from synaptic dysfunctions caused by aberrant protein translation or trafficking of synaptic proteins, to imbalances in excitation/inhibition. Ultimately, these abnormalities can lead to the onset of neurological disorders. Nevertheless, compelling evidence actually linking brain diseases to defects in homeostatic synaptic signalling is just starting to emerge. Indeed, many of the genes and molecules required for homeostatic synaptic plasticity are now being implicated in a variety of seemingly disparate neurological and neuropsychiatric conditions, including autism spectrum disorders, intellectual disability, schizophrenia, and Fragile X syndrome. Nonetheless, most of the molecular mechanisms through which defective homeostatic signalling may lead to disease pathogenesis still remain elusive.

Contactin-associated proteins

Contactin-associated proteins (CASPRs) are integral single-pass transmembrane proteins that typically form trans-synaptic cell-adhesion complexes with members of the Contactin (CNTN) family, and play fundamental roles in neuron-glia cell-adhesion and intercellular communication, crucial for the proper development and functioning of both the peripheral and central nervous system [(Peles *et al.*, 1997; Poliak *et al.*, 1999; Spiegel *et al.*, 2002; Traut *et al.*, 2006), and reviewed in (Poliak & Peles, 2003; Simons & Trajkovic, 2006)].

Expression and structure of Caspr1 and Caspr2

Contactin-associated protein 1 and 2 (Caspr1 and Caspr2, respectively; Figure 1.11) were the first family members of the CASPR family of proteins to be identified nearly 20 years ago as the mammalian orthologs of the *Drosophila* Neurexin IV (Peles *et al.*, 1997; Poliak *et al.*, 1999). In humans, they are encoded by the *CNTNAP1* and *CNTNAP2* genes as 190 and 150 kDa proteins, respectively. Alternative splicing of *CNTNAP2* has been reported to additionally generate either non-coding transcripts or developmentally-expressed shorter isoforms of Caspr2 (Poliak *et al.*, 1999; Poot, 2015). The expression of both Caspr1 and Caspr2 starts early on during embryonic stages, but increases significantly as development progresses into adulthood, when peak expression is achieved. Both Caspr1 and Caspr2 are highly expressed throughout the brain, presenting similar expression patterns in the hippocampus, cortex and thalamus, although some region-specificity has been reported in the cerebellum and amygdala, where Caspr1 or Caspr2, respectively, are preferentially expressed instead of the other (Peles *et al.*, 1997; Poliak *et al.*, 1999). A recent study also reports an enrichment of Caspr2 in primary sensory organs and related sensory cortical areas (Gordon *et al.*, 2016).

At the molecular level, Caspr1 and Caspr2 are composed of a mosaic of domains typically involved in protein-protein interactions, encompassing a long extracellular region and a short cytoplasmic tail (Figure 1.11). The overall extracellular architecture of both proteins is very similar, and shares high homology to that of neurexins, a family of highly polymorphic cell surface molecules (Peles *et al.*, 1997; Poliak *et al.*, 1999). Their extracellular tails contain a discoidin I homology domain right next to the N-terminal, several laminin G neurexin-like motifs, two epidermal growth factor-like repeats and a region related to fibrinogen β/γ. Caspr1 contains additional unique PGY (proline – glycine – tyrosine) repeats, whereas Caspr2 contains a signal peptide domain at the N-terminal (Figure 1.11).

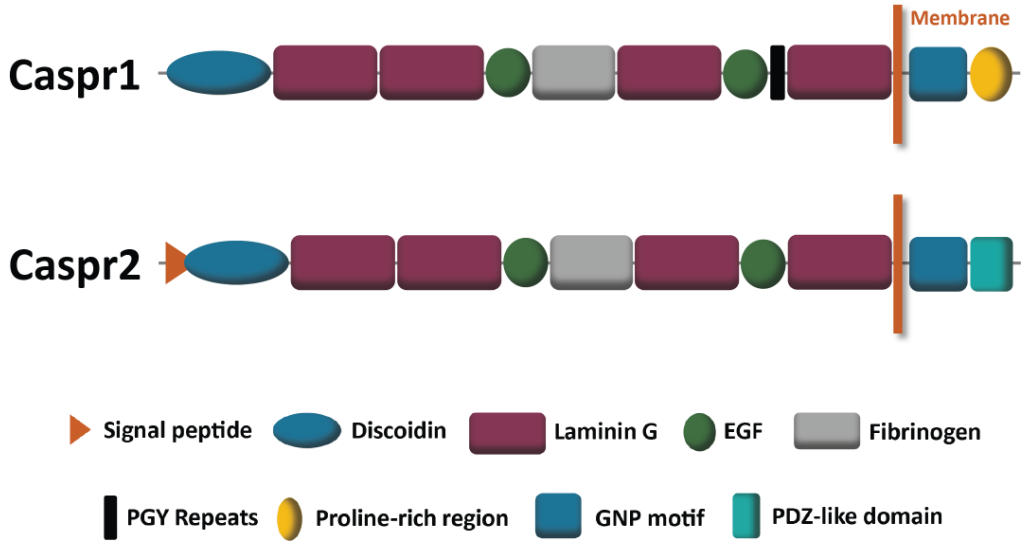


Figure 1.11 – Schematic representation of the protein domains present in Caspr1 and Caspr2. Caspr1 and Caspr2 are transmembrane neuronal proteins belonging to the neurexin family, which contain a mosaic of domains implicated in protein-protein interactions. Their extracellular terminal is very similar, containing a discoidin I homology domain, a region related to fibrinogen β/γ, epidermal growth factor- like repeats and several laminin G neurexin-like motifs. Caspr1 contains additional unique PGY repeats, whereas Caspr2 contains a signal peptide domain. The major difference between both proteins lies on their intracellular C-terminus. Caspr1 contains a juxtamembrane conserved GNP motif for the binding of the cytoskeleton adaptor 4.1 protein as well as a proline-rich region capable of binding SH3 domains. Caspr2, despite also having the GNP motif, contains an additional PDZ-like domain.

The intracellular C-tail of Caspr1 and Caspr2, despite being a short region, possesses particular interest, since it contains domains of a potential regulatory nature (Figure 1.11). Both Caspr1 and Caspr2 contain a juxtamembrane GNP (Glycophorin C, Neurexin IV, Paranodin) motif (Figure 1.11) for the binding of molecules containing FERM (Four-point-one, Ezrin, Radixin, Moesin) domains, such as β1 integrins, schwannomin and the cytoskeleton adaptor protein 4.1 (Denisenko-Nehrbass *et al.*, 2003a; Denisenko-Nehrbass *et al.*, 2003b). Caspr1 contains an additional unique proline-rich (PRO-rich) region (Figure 1.11) capable of binding canonical Src homology 3 (SH3) domains, which suggests the likelihood of Caspr1 interacting with molecules typically involved in signalling pathways (Peles *et al.*, 1997). Caspr2, on the other hand, presents a shorter cytoplasmic region that contains a type II PDZ-binding domain (Figure 1.11), which is thought to mediate the interaction of Caspr2 with *Shaker*-type rectifier K^+ channels (Poliak *et al.*, 1999).

Extracellularly, Caspr1 and Caspr2 are known to bind to Contactin-1 and Contactin-2 (Peles *et al.*, 1997; Poliak *et al.*, 2003; Rubio-Marrero *et al.*, 2016; Traka *et al.*, 2003), cell surface proteins that anchor to the cell membrane through a glycosylphosphatidyl inositol (GPI)

moiety, to form heterophilic membrane complexes that participate in neuron-glia interactions and play fundamental roles in cell adhesion and axonal myelination [reviewed in (Poliak & Peles, 2003; Simons & Trajkovic, 2006)]. Through their juxtamembrane GNP motif, both Caspr1 and Caspr2 have been shown to interact with the cytoskeleton adaptor protein 4.1B/N (Denisenko-Nehrbass *et al.*, 2003b), which is necessary for the distribution and clustering of Caspr1 and Caspr2 at subcellular specialized axonal microdomains (Cifuentes-Diaz *et al.*, 2011; Horresh *et al.*, 2010), indicating that it most likely serves as a local anchor of these membrane proteins to the actin-based cytoskeleton. Caspr1, through its other C-tail proline-rich motif, has been shown to selectively interact with the SH3 domains of the Src tyrosine kinase, Fyn, p85 and PLCγ (Peles *et al.*, 1997), indicating that it probably mediates the activation of downstream signalling cascades. On the other hand, recent studies have analysed the intracellular proteome of Caspr2 and found that it interacts not only with the Kv1 family of K^+-channels (Horresh *et al.*, 2008) but also with G protein-coupled receptor 37 [(GPR37), (Tanabe *et al.*, 2015)], members of the ADAM (Disintegrin and metalloproteinase domain-containing protein) family of proteins, in particular ADAM22 in complex with the secreted LGI1 (Leucine-rich glioma-inactivated 1) protein (Chen *et al.*, 2015), as well as with PSD93 and PSD95, SAP97, CASK and other PSD-MAGUKs (Chen *et al.*, 2015; Horresh *et al.*, 2008; Poliak *et al.*, 1999).

It is remarkable that, despite the high similarity between Caspr1 and Caspr2, a single-domain difference in their cytoplasmic regions opens up a series of potentially different biological processes in which they can participate, thus likely entailing a significant functional distinction between Caspr1 and Caspr2.

Biological functions of Caspr1 and Caspr2

Caspr1 and Caspr2 were initially identified in specialized axonal microdomains of peripheral and central myelinated neurons, where they have been thoroughly studied and their roles in mediating axonal organization and myelination, and nerve conduction well characterized. Interestingly, recent investigations have shed light on additional roles played by these proteins in the brain, including at the level of synapse regulation, cellular development and neuronal network activity.

Role in axonal organization, myelination and nerve conduction

Several studies have thoroughly characterized the role of Caspr1 and Caspr2 in peripheral and central myelinated axons, where they are known to play fundamental functions in the organization and maintenance of axonal microdomains at the nodes of Ranvier, which are critical for myelin ensheathing, correct saltatory propagation of action potentials and overall axonal excitability [Figure 1.12, reviewed in (Faivre-Sarrailh & Devaux, 2013; Poliak & Peles, 2003; Simons & Trajkovic, 2006)].

Caspr1 was one the first identified constituents of the paranodal junctions (Einheber *et al.*, 1997; Menegoz *et al.*, 1997), which act as a physical and electrical barrier to restrict the localization of voltage-gated Na^+-channels to the nodes of Ranvier, hence promoting action potential propagation (Figure 1.12). With a diffuse axonal expression during development, Caspr1 rapidly increases at the onset of myelination and localizes into clustered hotspots near the nodes of Ranvier to promote the initial steps of myelin ensheathing (Menegoz *et al.*, 1997). Here, by binding intracellularly to the cytoskeleton adapter 4.1 protein and extracellularly in *cis* with Contactin-1, which, in turn, binds in *trans* to glial cell surface-expressed Neurofascin155, Caspr1 initiates the formation of the paranodal junction and promotes the tethering of the myelin sheath to axons [Figure 1.12; reviewed in (Faivre-Sarrailh & Devaux, 2013; Poliak & Peles, 2003; Simons & Trajkovic, 2006)]. Importantly, mice lacking Caspr1 exhibit tremor, ataxia and motor paresis ensuing from severe perturbations in the organization of the paranodes, with altered distribution of the paranodal components Contactin-1 and Neurofascin155 and diffusion of voltage-gated Na^+-channel from the nodes of Ranvier, resulting in a marked decrease in nerve conduction velocity (Bhat *et al.*, 2001).

Caspr2, on the other hand, localizes with Contactin-2 to the juxtaparanodal region, a nodal specialization adjacent to the paranodes (Figure 1.12), highly enriched in *Shaker*-like rectifier K^+-channels that are crucial for the regulation of axonal excitability. In the juxtaparanodes, Caspr2 serves as a membrane scaffold to cluster K^+-channels (Poliak *et al.*, 2003; Traka *et al.*, 2003), in a mechanism that requires cytoskeleton anchoring of Caspr2 via 4.1-binding, and that also involves the scaffolding PSD93 and PSD95 proteins (Horresh *et al.*, 2010; Horresh *et al.*, 2008). Conversely, it seems that Caspr2 is not required for PSD93/95-mediated clustering of K^+-channels at the axon initial segment (Ogawa *et al.*, 2008; Pinatel *et al.*, 2017). Absence of Caspr2 in mice results in a complete disorganization of the juxtaparanodes, with K^+-channels diffusing along the internodal region. Interestingly, however, no gross alterations were reported in axonal myelination or conduction velocity of peripheral nerves in *Cntnap2* KO mice, despite K^+-channel mislocalization (Poliak *et al.*, 2003; Traka *et al.*, 2003). Recent reports in the CNS are confounding, with one study reporting defects in

nodal formation and myelin ensheathing, as well as abnormal action potential propagation in *Cntnap2* KO mice (Scott *et al.*, 2017), whilst one other study reports reduced local and long-range prefrontal functional connectivity, albeit observing no changes in myelination (Liska *et al.*, 2017).

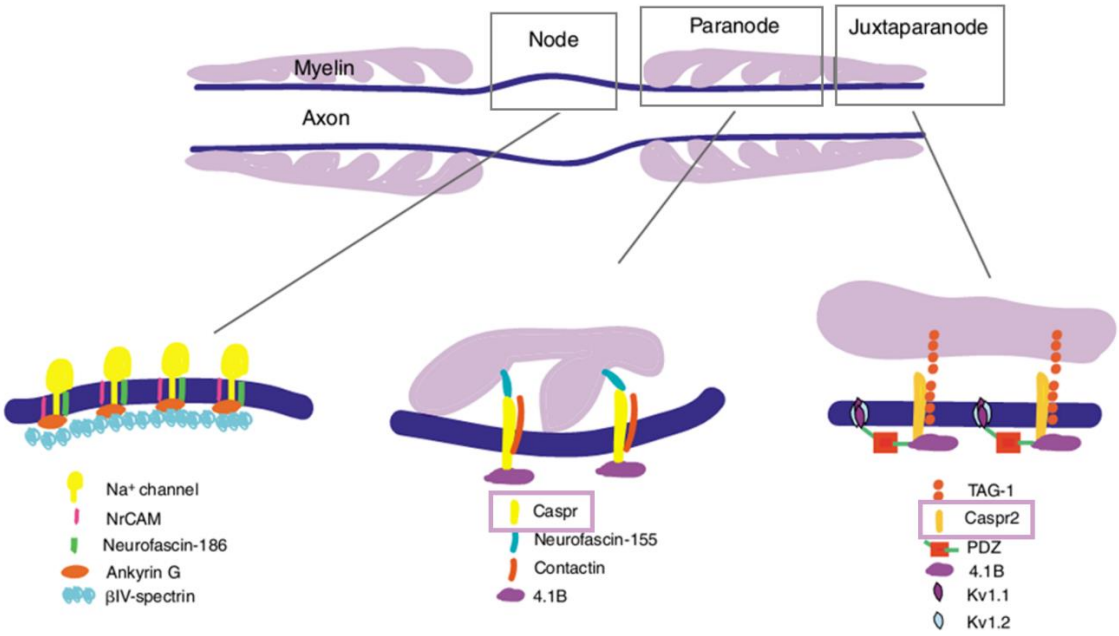


Figure 1.12 – Caspr1 and Caspr2 play critical roles during axonal myelination. Voltage-gated Na^+ channels in the nodes of Ranvier are anchored in clusters in the axonal membrane through interaction with the CAMs neurofascin-186 and NrCAM, and several cytoskeleton/scaffolding proteins. The myelin sheath is initially formed in the paranodes, which surround the nodes of Ranvier. Formation of the paranodes depends on the interaction between a Contactin1/Caspr1 complex in the axonal membrane with the glial loop, with further stabilization by the 4.1 cytoskeleton-binding protein. The assembly of the myelin sheath next progresses to the juxtaparanodes, which are enriched in *Shaker*-type K^+ channels. These are tethered in the axonal membrane by a complex containing the 4.1 protein, Caspr2 and TAG-1, or Contactin2; and linked to the myelin sheath through a *trans*-interaction with TAG-1, also present in the glial membrane. [Adapted from (Simons & Trajkovic, 2006)].

Role in synaptic regulation, cell development and network activity

Multiple central roles have been attributed to Caspr2 in recent years, including in the regulation of synapses, cell development and migration, and neuronal network activity [reviewed in (Penagarikano & Geschwind, 2012; Poot, 2015; Poot, 2017; Rodenas-Cuadrado *et al.*, 2014)]. One seminal milestone in understanding the role of Caspr2 in brain function came from the characterization of severe histopathological brain defects and behavioural abnormalities in the *Cntnap2* KO mouse that recapitulated the pathology of neuropsychiatric patients carrying *CNTNAP2* mutations [(Penagarikano *et al.*, 2011), and reviewed in (Penagarikano & Geschwind, 2012; Poot, 2015; Poot, 2017; Rodenas-Cuadrado *et al.*, 2014)].

One overarching phenotype reported in *CNTNAP2* KO animal models is a loss of GABAergic interneurons and asynchronous neuronal firing (Hoffman *et al.*, 2016; Penagarikano *et al.*, 2011; Vogt *et al.*, 2017), that results in impaired hippocampal and cortical GABAergic inhibitory transmission in adult mice (Bridi *et al.*, 2017; Jurgensen & Castillo, 2015). Animals also present abnormal migration of excitatory projection neurons and altered cortical layer patterning (Penagarikano *et al.*, 2011), likely contributing to a reduction in local and long-range prefrontal functional connectivity assessed by fMRI (Liska *et al.*, 2017). These observations hint at severe circuitry and neuronal network activity defects occurring in the absence of Caspr2, thus indicating that its role goes well beyond the regulation of juxtaparanodal K^+-channel clustering.

Further arguing in favour of an important role of Caspr2 in the establishment of neuronal networks and circuits is the evidence that transient loss of Caspr2, induced by shRNA-mediated knockdown, significantly alters the dendritic complexity of cortical neurons and decreases the overall density of excitatory spines, resulting in impaired excitatory synaptic transmission (Anderson *et al.*, 2012). This study also reports a decrease in basal inhibitory transmission, which, together with previous evidence, pinpoint a critical role for Caspr2 in the regulation of excitatory/inhibitory (E/I) balance in the brain. Recent studies have provided additional compelling evidence to establish a fundamental role for Caspr2 in synapse regulation and proper development of synaptic circuitry. Resorting to super-resolution imaging methods, one study further demonstrated that Caspr2 is enriched in dendritic spines (Varea *et al.*, 2015), in accord to previous biochemical evidence reporting its presence in synaptic membranes (Bakkaloglu *et al.*, 2008). Additionally, *Cntnap2* KO cortical neurons displayed reduced spine density and altered spine morphology (Varea *et al.*, 2015), thus proposing a role for Caspr2 in spine dynamics. Confirming this hypothesis, an *in vivo* imaging study reported that, whilst formation of new spines remains intact in *Cntnap2* KO mice, there is an impairment in the stabilization of newly formed spines (Gdalyahu *et al.*, 2015).

A synaptic function for Caspr1 has also been recently established. Several studies have already uncovered an extensive localization of Caspr1 in dendrites and synaptic sites (Cajigas *et al.*, 2012; Collins *et al.*, 2006; Murai *et al.*, 2002; Santos *et al.*, 2012), and a recent study performed in our laboratory established Caspr1 as novel regulator of AMPAR trafficking to synapses (Santos *et al.*, 2012). Santos and colleagues identified Caspr1 as a novel interactor of the GluA1 AMPAR subunit capable of regulating the synaptic trafficking of cell surface GluA1-containing AMPARs: overexpression of Caspr1 in hippocampal neurons resulted in a significant increase in the synaptic content of cell surface AMPARs, and co-expression of Caspr1 with GluA1 in a heterologous system increased the amplitude of AMPAR-currents evoked by glutamate. Conversely, shRNA-mediated loss of Caspr1 expression led to a significant reduction in synaptic cell surface AMPARs, indicating that Caspr1 is required for the

regulation of their trafficking. Interestingly, the C-tail domain of Caspr1 (through which Caspr1 binds to GluA1) alone is sufficient to upregulate surface AMPARs at the synapse, or to rescue AMPARs to the surface following loss of endogenous Caspr1 (Santos *et al.*, 2012). Interestingly, a potential role of Caspr2 in the regulation of AMPARs has recently been suggested since *Cntnap2* KO cortical neurons present cytoplasmic aggregates of AMPARs in parallel to synaptic abnormalities (Varea *et al.*, 2015).

Implication of Caspr1 and Caspr2 in the pathogenesis of neurological and neuropsychiatric disorders

Past investigation on the biological functions of Caspr1 and Caspr2 emphasize their likely relevance for mediating important physiological processes required for proper brain function. Confirming this hypothesis is surmounting clinical and experimental evidence implicating both Caspr1 and Caspr2 in the pathogenesis of several neurological and neuropsychiatric disorders.

Although evidence linking Caspr1 to pathological conditions is rather recent, mutations in the CASPR1-encoding gene *CNTNAP1* have been found in patients with arthrogryposis multiplex congenita and other congenital forms of hypomielynating disorders, and peripheral neuropathy (Hengel *et al.*, 2017; Lakhani *et al.*, 2017; Laquerriere *et al.*, 2014; Mehta *et al.*, 2017; Nizon *et al.*, 2016; Vallat *et al.*, 2016). Additionally, autoantibodies targeting CASPR1 have recently been found in patients with inflammatory neuropathy (Doppler *et al.*, 2016). These disorders, in particular arthrogryposis multiplex congenita (most affected children die within months or few years from birth), are severely debilitating, presenting with severe motor and sensory nerve dysfunctions, including cramps, fasciculations, muscle fibrosis and juncture contractions. Although the primary aetiology of some of these disorders can be orthopedic, in most cases patients' symptoms are secondary to neurological dysfunction. Interestingly, *Cntnap1* KO mice exhibit tremors, ataxia and motor paresis secondary to abnormal paranodal junctions and aberrant myelination in peripheral and central axons (Bhat *et al.*, 2001), and similar phenotypes have been observed in the *shambling* mouse, caused by a frameshift mutation in *Cntnap1* (Sun *et al.*, 2009). Interestingly, a similar perturbation in the paranodes and reduced myelination have been reported by electron microscopy imaging of the sciatic nerves of *CNTNAP1* patients (Laquerriere *et al.*, 2014).

Interestingly, a recent meta-analysis of transcriptomic profiling studies across several neuropsychiatric disorders, reveals that gene expression of *CNTNAP1* is significantly downregulated in psychiatric disorders such as ASD, SCZ, bipolar disorder and depression

(Gandal *et al.*, 2018), giving a first prospect of the potential relevance of CASPR1 in brain function. Curiously, some of the patients with congenital hypomyelination carrying *CNTNAP1* mutations have been diagnosed with severe intellectual disability (Hengel *et al.*, 2017), which could correlate with a potential disruption of synaptic functions played by CASPR1.

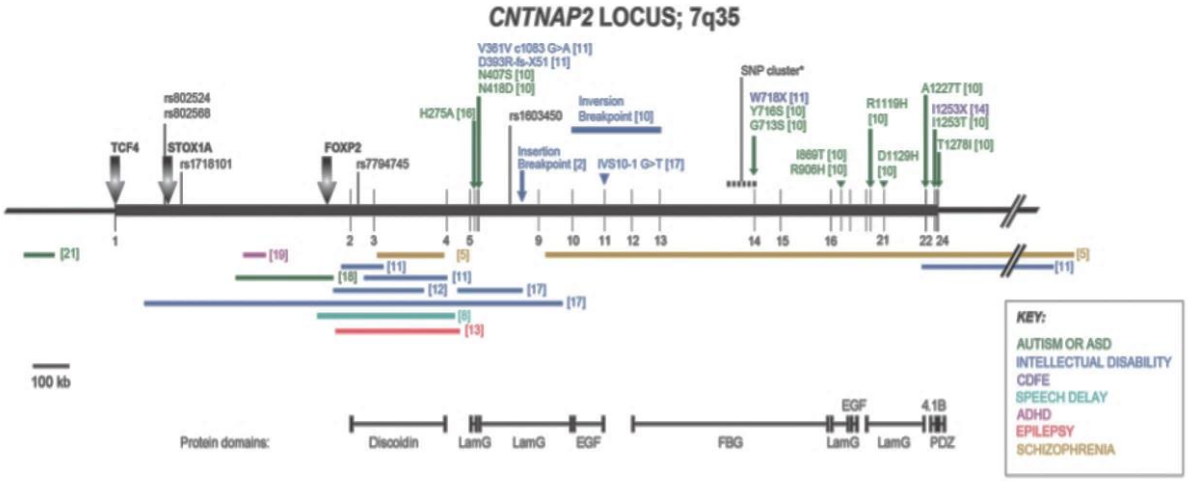


Figure 1.13 – The human *CNTNAP2* locus at chromosomal region 7q35 harbouring mutations implicated in several neuropsychiatric disorders. Schematic representation of the 24 exons (black bar) of the *CNTNAP2* gene. Large grey arrows indicate binding sites for regulation by the transcription factors: TCF4, STOX1A and FOXP2. Mutation and deletions identified in patients are colour-coded for the primary patient diagnosis indicated in the key box. *The SNP cluster contains the following SNPs: rs851715, rs10246256, rs2710102, rs759178, rs1922892, rs2538991, rs17236239, rs2538976, rs2710117 and rs4431523. The protein domains of Caspr2 are represented below in black, with vertical lines corresponding to each encoding exon [Adapted from (Rodenas-Cuadrado *et al.*, 2014)].

On the other hand, several linkage, association, gene expression analysis and imaging data studies conducted in the past decade found countless mutations in the *CNTNAP2* gene (Figure 1.13) that have been implicated, with more or less robustness, in the pathogenesis of several neuropsychiatric disorders including ASD (Alarcon *et al.*, 2008; Arking *et al.*, 2008; Bakkaloglu *et al.*, 2008; O'Roak *et al.*, 2011; Poot *et al.*, 2010), SCZ and bipolar disorder (Friedman *et al.*, 2008; O'Dushlaine *et al.*, 2011; Wang *et al.*, 2010b), ID and language impairment (Mikhail *et al.*, 2011; Petrin *et al.*, 2010; Sehested *et al.*, 2010; Smogavec *et al.*, 2016; Vernes *et al.*, 2008; Zweier *et al.*, 2009), epilepsy and cortical dysplasia focal epilepsy (CFDE) syndrome (Friedman *et al.*, 2008; Mefford *et al.*, 2010; Smogavec *et al.*, 2016; Strauss *et al.*, 2006), Tourette syndrome (Belloso *et al.*, 2007; Verkerk *et al.*, 2003), among other disorders (Figure 1.13). Patients harbouring *CNTNAP2* mutations typically present with a very complex phenotypic variability, but their overarching features can include seizures, language

impairments, intellectual disability and varying autistic-core behaviours [reviewed in (Penagarikano & Geschwind, 2012; Poot, 2017; Rodenas-Cuadrado *et al.*, 2014)]. Histopathological analysis of temporal-lobe specimens obtained during resective surgeries done to patients with CFDE showed evidence of abnormalities in neuronal migration, altered cortical layering and widespread astrogliosis (Strauss *et al.*, 2006). Additionally, human MRI studies revealed dramatic losses in frontal-lobe grey matter and alterations in functional connectivity in patients carrying *CNTNAP2* variations (Scott-Van Zeeland *et al.*, 2010; Tan *et al.*, 2010). Importantly, *Cntnap2* KO animal models display histopathological defects (Liska *et al.*, 2017; Penagarikano *et al.*, 2011) and develop behavioural abnormalities (Hoffman *et al.*, 2016; Penagarikano *et al.*, 2011) that recapitulate the core features of patients with *CNTNAP2* mutations.

Caspr2 has also been recently associated to autoimmune synaptic encephalitis (Irani *et al.*, 2010; Lancaster *et al.*, 2011a), a rare neurological / neuropsychiatric disorder recently characterized in which autoantibodies are aberrantly produced against neuronal receptors and synaptic cell surface proteins, leading to the development of severe neurological and psychiatric symptoms. Some of the identified synaptic antigen targets identified to date include NMDA- (Dalmau *et al.*, 2008; Dalmau *et al.*, 2007) and AMPA-type (Lai *et al.*, 2009), GABAergic (Lancaster *et al.*, 2010; Petit-Pedrol *et al.*, 2014), glycinergic (Hutchinson *et al.*, 2008) and metabotropic receptors (Lancaster *et al.*, 2011b; Marignier *et al.*, 2010), as well as proteins other proteins of the voltage-gated K^+-channel (VGKC) complex (Vincent *et al.*, 2004), including the leucine-rich glioma-inactivated 1 protein [LGI1; (Irani *et al.*, 2010; Lai *et al.*, 2010)], among others [extensively reviewed in (Chefdeville *et al.*, 2016; Coutinho *et al.*, 2014; Crisp *et al.*, 2016; Dalmau, 2016)]. Autoantibodies targeting CASPR2 have been identified less than a decade ago in the serum and cerebrospinal fluid of patients presenting with severe neurological syndromes that can include neuromyotonia, Morvan's syndrome and limbic encephalitis [reviewed in (Bastiaansen *et al.*, 2017; Binks *et al.*, 2017; Pruss & Lennox, 2016; van Sonderen *et al.*, 2017)]. Overarching features of these patients reveal a complex dysfunction of both the PNS and CNS, with patients often manifesting with peripheral nerve hyperexcitability and neuropathic pain, sleep alterations, seizures, memory impairment, cognitive deficits and psychosis (Bien *et al.*, 2017; Irani *et al.*, 2010; Irani *et al.*, 2012; Joubert *et al.*, 2016; Klein *et al.*, 2013; Lancaster *et al.*, 2011a; Somers *et al.*, 2011; Sunwoo *et al.*, 2015; van Sonderen *et al.*, 2016).

These disorders are treatable with timely immunotherapy, and most patients present a good clinical outcome. Nevertheless, the risk for relapse is particularly high and most patients require continuous immunosuppression throughout life, with some retaining permanent cognitive sequelae [reviewed in (Bastiaansen *et al.*, 2017; Binks *et al.*, 2017; Pruss & Lennox,

2016; van Sonderen *et al.*, 2017)]. Importantly, a good clinical outcome following immunotherapy can be correlated with decreasing circulating titres of CASPR2 autoantibodies in patients' sera [reviewed in (Bastiaansen *et al.*, 2017; Pruss & Lennox, 2016; van Sonderen *et al.*, 2017)], which is suggestive of a potential direct pathogenic effect of CASPR2 autoantibodies. Unfortunately, potential pathogenic mechanisms elicited by CASPR2 antibodies still remain uncharacterized and compelling evidence for a direct antibody-mediated pathogenesis is only now starting to surface. A couple of studies have recently developed maternal-to-foetus antibody transfer models by exposing mice *in utero* to CASPR2 autoantibodies (Brimberg *et al.*, 2016; Coutinho *et al.*, 2017b). Curiously, they found that exposed offspring mice developed behavioural abnormalities comparable to those of the *Cntnap2* KO mouse model, and that recapitulate the psychiatric phenotypes of patients with anti-CASPR2 encephalitis, thus suggesting a direct pathogenic effect elicited by CASPR2 autoantibodies. Interestingly, circulating autoantibodies against CASPR2 were recently detected during pregnancy in mothers of children with autism-spectrum disorders (Brimberg *et al.*, 2016) or intellectual disability (Coutinho *et al.*, 2017a), which suggest that gestational transfer of CASPR2 autoantibodies might contribute to the development of neuropsychiatric disorders in the progeny.

Objectives of the present study

During development, sensory experience and throughout learning-related adaptations, the mammalian brain undergoes constant changes that can compromise its function. To prevent this, neurons can elicit homeostatic responses that maintain neuronal activity within dynamic, yet stable boundaries that keep neuronal networks in-balance, even in face of constant changes of activity. Hence, homeostatic synaptic plasticity has stood out as a fundamental process to conserve brain function, and ultimately behaviour and cognition. The dynamic regulation of synaptic glutamate receptors of the AMPA-type has been pinpointed as a major cellular correlate to achieve neuronal homeostasis. Emerging evidence implicating a failure in glutamatergic transmission and neuronal synaptic homeostasis in the pathogenesis of distinct neuropsychiatric disorders warrants a full comprehension of the mechanisms underlying these processes, and how they can be compromised in the context of disease.

Contactin-associated proteins 1 and 2 are cell-adhesion molecules with emerging roles in the regulation of fundamental cellular and synaptic processes, including in glutamatergic synaptic transmission. Taking into consideration the established association of Caspr1 and Caspr2 with several neurological and psychiatric disorders, herein we aimed at exploring potential roles played by these proteins in the regulation of glutamatergic synaptic transmission and synaptic plasticity phenomena.

1) Previous studies carried out in our laboratory have identified Caspr1 as an AMPAR-interacting protein required for the regulation of cell surface AMPARs at the synapse. Here, we aimed at exploring the molecular mechanisms underlying the function of Caspr1 in AMPAR regulation, and investigated a link between its function and the expression of homeostatic synaptic plasticity. (Chapter III)

2) Mutations in the CASPR2-encoding gene *CNTNAP2* have been found in association with cognitive and psychiatric disorders; however, the deleterious effects of these mutations in CASPR2 function remain elusive. Given the shared homology with Caspr1, we hypothesized Caspr2 to play a similar role in AMPAR regulation. We evaluated the presence of Caspr2 at glutamatergic synapses of cortical neurons and evaluated the synaptic content of cell surface AMPARs, as well as the expression of homeostatic synaptic scaling mechanisms upon loss of expression of endogenous Caspr2. We also aimed at determining the physiological relevance of Caspr2 in the

regulation of glutamatergic function *in vivo*, and in the expression of experience-dependent mechanisms of plasticity driven by visual experience. (Chapter IV)

3) Autoantibodies against CASPR2 have been recently found in patients with autoimmune synaptic encephalitis that manifest with severe cognitive and psychiatric symptoms. However, the mechanisms elicited by CASPR2 autoantibodies are still uncharacterized, and a causal link establishing their direct pathogenic effect is still missing. Here, we hypothesized that CASPR2 autoantibodies exert their pathogenesis by disrupting the role of CASPR2 in the regulation of AMPARs. We used human immunoglobulins purified from a patient with CASPR2 encephalitis and evaluated how they impinged on the regulation of AMPAR trafficking, basal glutamatergic transmission and synaptic plasticity mechanisms. We further aimed at identifying the mechanisms elicited by CASPR2 autoantibodies to elicit pathogenesis. (Chapter V)

This study provides compelling evidence of a fundamental role played by both Contactin-associated proteins 1 and 2 in the regulation of AMPAR trafficking and function and in underlying important mechanisms to achieve neuronal synaptic homeostasis. Importantly, our findings provide insights into the pathogenesis of neurological and neuropsychiatric disorders.

Chapter II
Materials & Methods

Materials

Horse serum, gentamycin, penicillin/streptomycin, Neurobasal Medium (NBM), Fetal Bovine Serum (FBS), Lipofectamine LTX with Plus reagent, Opti-MEM, RNAse-free water and trypsin were purchased from GIBCO, as part of Thermo Fisher Scientific (MA, USA). Cytosine arabinoside, kynurenic acid, glutamine, Minimum Essencial Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM) and poly-D-lysine were purchased from Sigma-Aldrich, as part of Merck (MA, USA). SM1 supplement was acquired from STEMCELL Technologies (Vancouver, Canada). TRIzol reagent was purchased from Invitrogen, as part of Thermo Fisher Scientific (MA, USA). All other reagents were purchased from Sigma-Aldrich (Sintra, Portugal or St. Louis, MO, USA), Fisher Scientific (Loures, Portugal or Pittsburgh, PA, USA), Merck (Oeiras, Portugal), Gibco, as part of Life Technologies (Alfagene, Carcavelos, Portugal), and NZYTech (Lisboa, Portugal), unless stated otherwise.

The drugs used in this study were: Tetrodotoxin (TTX), picrotoxin and D-(-)-2-Amino-5-phosphonopentanoic acid (APV) for *in vitro* assays were purchased from Tocris Bioscience (Bristol, UK). Strychnine was purchased from Sigma-Aldrich (Sintra, Portugal). For electrophysiology experiments, TTX was purchased from Abcam (Cambridge, MA, USA), APV from Sigma-Aldrich (St. Louis, MO, USA) and Bicuculline (Bicc) was acquired from Enzo Life Sciences (Farmingdale, NY, USA).

Antibodies

Table 2.1 – Primary antibodies used in this study.

Primary antibodies	Dilution (application)	Source
β-Tubulin	1:5000 (WB)	Abcam (Cambridge, UK)
Caspr1	1:50 (ICC) / 1:250 (WB)	Abcam (Cambridge, UK)
Caspr2	1:200 (ICC) / 1:250 (WB/IP)	Abcam (Cambridge, UK)
GAPDH	1:5000 (WB)	Abcam (Cambridge, UK)
GFP	1:500 (ICC) / 1:1000 (WB)	MBL International (MA, USA)
GluA1 C-terminal	1:1000 (WB)	Millipore (Oeiras, Portugal)
GluA1 N-terminal	1:200 (ICC)	kind gift from Dr. Andrew Irving (U. of Dundee, Scotland)
GluA1 N-terminal	1:100 (ICC)	Millipore (Oeiras, Portugal)
GluA2 N-terminal	1:100 (ICC)	Millipore (Oeiras, Portugal)
Human IgG1 subtype	1:50 (ICC)	Sigma-Aldrich (UK)

Human IgG2 subtype	1:50 (ICC)	Sigma-Aldrich (UK)
Human IgG3 subtype	1:50 (ICC)	Sigma-Aldrich (UK)
Human IgG4 subtype	1:50 (ICC)	Sigma-Aldrich (UK)
Human Transferrin receptor	1:500 (WB)	Invitrogen (UK)
MAP2	1:5000 (ICC)	Abcam (Cambridge, UK)
Phospho-ZBP1 (Tyr396)	1:250 (ICC) / 1:500 (WB)	kind gift from Dr. Gary Bassell (Emory U., Atlanta, USA)
Phospho-Src (Tyr416)	1:500 (WB)	Cell Signalling (MA, USA)
PSD95	1:200 (ICC)	Thermo Fisher Scientific (Loures, Portugal)
PSD95	1:750 (ICC)	Cell Signalling (MA, USA)
Synaptophysin	1:20000 (WB)	Abcam (Cambridge, UK)
vGluT1	1:5000 (ICC)	Millipore (Oeiras, Portugal)
ZBP1	1:500 (WB / IP)	SantaCruz Biotechnologies (Frilabo, Maia, Portugal)

Abbreviations: ***ICC*** *– Immunocytochemistry,* ***IP*** *– Immunoprecipitation,* ***WB*** *– Western Blot*

Table 2.2 – Secondary antibodies used in this study.

Secondary antibodies	Dilution (application)	Source
Alexa 488-conjugated anti-human	1:500 (ICC)	Molecular Probes (Leiden, Netherlands)
Alexa 488-conjugated anti-rabbit	1:500 (ICC)	Molecular Probes (Leiden, Netherlands)
Alexa 488-conjugated anti-sheep	1:500 (ICC)	Molecular Probes (Leiden, Netherlands)
Alexa 568-conjugated anti-human	1:500 (ICC)	Molecular Probes (Leiden, Netherlands)
Alexa 568-conjugated anti-human	1:750 (ICC)	Invitrogen (UK)
Alexa 568-conjugated anti-mouse	1:500 (ICC)	Molecular Probes (Leiden, Netherlands)
Alexa 568-conjugated anti-mouse	1:750 (ICC)	Invitrogen (UK)
Alexa 568-conjugated anti-sheep	1:500 (ICC)	Molecular Probes (Leiden, Netherlands)
Alexa 594-conjugated anti-rabbit	1:500 (ICC)	Molecular Probes (Leiden, Netherlands)
Alexa 647-conjugated anti-guinea pig	1:500 (ICC)	Molecular Probes (Leiden, Netherlands)
Alexa 647-conjugated anti-mouse	1:500 (ICC)	Molecular Probes (Leiden, Netherlands)
Alexa 647-conjugated anti-rabbit	1:500 (ICC)	Molecular Probes (Leiden, Netherlands)
Alkaline phosphatase-conjugated anti-mouse	1:20000 (WB)	GE Healthcare (Carnaxide, Portugal)

Alkaline phosphatase-conjugated anti-rabbit	1:20000 (WB)	GE Healthcare (Carnaxide, Portugal)
Alkaline phosphatase-conjugated anti-rabbit light chain specific	1:10000 (WB)	Jackson ImmunoResearch (PA, USA)
AMCA-conjugated anti-chicken	1:200 (ICC)	Jackson ImmunoResearch (PA, USA)

Abbreviations: ***ICC*** *– Immunocytochemistry,* ***WB*** *– Western Blot*

Constructs and primers

The GluA1 construct was a kind gift from Dr. Juan Lerma (Instituto de Neurociencias de Alicante, Spain). The deletion construct GluA1Δ3'UTR was prepared with the QuickChange II XL-site-directed mutagenesis kit (Agilent Technologies, CA, USA). The GluA1 construct was used as template and the primers (sense primer: 5' GAG CCA CAG GAT TGT AAG TCG ACC T 3'; antisense primer: 5' AGG TCG ACT TAC AAT CCT GTG GCT C 3') were designed to match all regions upstream of the 3'UTR, to specifically exclude this region. Caspr1 was kindly provided by Dr. Catherine Faivre-Sarrailh (CNRS, Marseille, France) and cloned as described by Bonnon and colleagues (Bonnon *et al.*, 2003). The deletion constructs Caspr1ΔPro, deleted for the proline-rich region of Caspr1 C-terminal, and Caspr1ΔGNP, deleted for the FERM domain, were prepared with the QuickChange II XL-site-directed mutagenesis kit (Agilent). The Caspr1 construct was used as template and primers were designed to match the upstream and downstream regions of each domain, in order to specifically delete only that region. Primers were the following: Caspr1ΔPro - sense primer: 5' GGC CAC CCA TGA TTC CCA CAG GGA CCA GAA CC 3', antisense primer: 5' GGT TCT GGT CCC TGT GGG AAT CAT GGG TGG CC 3'; Caspr1 ΔGNP - sense primer: 5' TGC TGT TCT ATT CCC ACC CTG GCG GC 3', antisense primer: 5' GCC GCC AGG GTG GGA ATA GAA CAG CA 3'. Dr. Thomas Südhof (Stanford University, USA) generously provided the Caspr2 construct, together with the shRNA plasmids for knocking down Caspr1 and Caspr2. The ZBP1 constructs (ZBP1*, ZBP1-Y396F) and the shRNA plasmid against ZBP1 were a kind offer from Dr. Jacek Jaworski (International Institute of Molecular and Cell Biology, Warsaw, Poland). The constructs pEGFP-N1 (Clontech, Saint-Germain-en-Laye, France) and pBK-CMV (Stratagene, Cambridge, UK) were used as controls for transfection. The constructs pMDLg/pRRE, pRSV-Rev and pCMV-VSV-G were used for the production of lentivirus and were kindly provided by Dr. Ann Marie Craig (Djavad Mowafaghian Centre for Brain Health and Department of Psychiatry, University of British Columbia, Vancouver, Canada). All DNA constructs were verified by DNA sequencing.

Methods

Animals

For electrophysiological studies, C57BL/6J mice (Jackson Laboratories) were raised under a normal 12 hour (h) light/dark cycle until the beginning of experiments. For primary cultures of hippocampal and cortical neurons, either pregnant Wistar rats, Wistar pups at postnatal day 0 (P0) or pregnant *Cntnap2* knockout (KO) mice [B6.129(Cg)*Cntnap2*^{tm1Pele}/J; Jackson Laboratory] were used. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Johns Hopkins University, Baltimore, USA, or by the Portuguese National Authority for Animal Health (DGAV).

Cell line culture – maintenance and transfection

COS7, immortalized kidney cells of the African green monkey, and Human embryonic kidney (HEK) 293T cell lines were maintained at 37°C in a humidified incubator with 5% CO_2 / 95% air, incubated in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 44 mM $NaHCO_3$ at pH 7.2, to a subconfluence of 60-80% and diluted 1:5 every three days.

COS7 cells in 6-well plates and cultured to 70 - 80% confluency were transiently transfected with Lipofectamine reagent (Invitrogen, Barcelona, Spain) as follows: Lipofectamine was diluted in OptiMEM reduced serum medium, to which a total of 12 µg of plasmid DNA, previously diluted in OptiMEM to an equivalent volume, was added. The DNA-Lipofectamine mix was then gently vortexed for 2-3 seconds (sec) and incubated at room temperature (RT) for 20 minutes (min) to allow the formation of complexes. Precipitates were then added to the cells and incubated for 5 hours (h) at 37°C. HEK293T cells in 6-well plates and cultured to 70 - 80% confluency were transfected using the calcium phosphate method. Briefly, to allow formation of DNA precipitates, a solution of 2 M $CaCl_2$ was added dropwise to a total of 12 µg of DNA previously diluted in water. This mixture was then added to HEPES-buffered transfection solution (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.05) and incubated for 20 min, at RT, in the dark. The precipitates were added to the cells and incubated at 37°C for 5 h. For both transfection methods, after the period of incubation with the precipitates, the medium was completely changed to fresh culture medium in order to remove the remaining precipitates and prevent toxicity. Cells were returned to the incubator and allowed to express the transfected constructs for 48 h.

For live cell-based assays, HEK293T cells were grown in DMEM supplemented with 10% Fetal Calf Serum (FCS) and 1% penicillin G / streptomycin /amphotericin (PSA) and maintained

in flasks at 37°C in a humidified incubator with 5% CO_2 / 95% air. For screening purposes, cells grown in flasks were transferred to 6-well plates with 13 mm poly-L-lysine-coated glass coverslips, by gentle detachment with 1% trypsin in Phosphate buffer saline (PBS) and re-seeded in DMEM/10% FCS/1% PSA at a concentration of $2.5x10^5$ cells/mL. The following day, cells were transfected using 1.5 µL polyethylenimine (PEI) with 3 µg of the cDNA plasmid of EGFP-tagged CASPR2 per well. After 14-16 h, medium was replaced with fresh DMEM/10% FCS/1% PSA.

Primary culture of cortical and hippocampal neurons (high-density and Banker low-density cultures)

High- and low-density primary cultures of rat cortical and hippocampal neurons were prepared from the cortices and hippocampi of E18 Wistar rat embryos, as previously described (Santos *et al.*, 2012). Briefly, after dissection, tissue was treated for 15 min at 37°C with 0.06% trypsin, in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS: 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). Cells were then washed 6 times in HBSS and mechanically dissociated. Cells were plated in neuronal plating medium [Minimal Essential Medium (MEM) supplemented with 10% horse serum, 0.6% glucose and 1 mM pyruvic acid)] at a density of 8.9×10^4 cells/cm^2 in 6-well plates, coated with poly-D-lysine (0.1 mg/mL). After 2-4 h, the medium was replaced with fresh Neurobasal medium [NBM - supplemented with SM1 neuronal supplement (StemCell Technologies, Grenoble, France), 0.12 mg/mL gentamycin, 0.5 mM glutamine and 25 µM glutamate (to prevent excitotoxicity, glutamate was not added to cortical cultures)]. For imaging purposes, low-density Banker cultures were prepared as previously described by Banker and colleagues (Banker & Goslin, 1998). Low-density cells were plated onto poly-D-lysine-coated coverslips in 60 mm culture dishes, at a final density of 3×10^5 cells/dish. After 2-4 h, coverslips were flipped over an astroglial feeder layer in NBM. Wax dots on the neuronal side of the coverslips allowed the physical separation of neurons from the glia, despite neurons growing face down over the feeder layer. To further prevent glia overgrowth, neuron cultures were treated with 5 µM cytosine arabinoside after 3 days *in vitro* (DIV). Cultures were maintained at 37°C in a humidified incubator of 5% CO_2 / 95% air, for up to 15 DIV.

For screening of human samples, primary hippocampal neurons were prepared either from Wistar rat pups at postnatal day 0 (P0), or from E18.5 *Cntnap2* knockout mice embryos [B6.129(Cg)*Cntnap2*^{tm1Pele}/J; Jackson Laboratory]. Briefly, hippocampi were dissected, washed with HBSS and dissociated in 1% trypsin-HBSS solution for 20 min at 37˚C. Trypsin

was aspirated and replaced with complete MEM, supplemented with 10% FCS and 1% PSA, and cells gently triturated until no visible clumps of tissue remained. The cell suspension was gently centrifuged at 1,000 rpm for 5 min, the supernatant aspirated and the cell pellet resuspended in complete MEM medium. Cells were plated onto poly-lysine-coated glass coverslips in a 6-well plate at a final density of $2x10^5$ cells/well. After 2 h plating medium was replaced with NBM (supplemented with 1% B-27 serum free supplement, 200 mM L-glutamine and 1% PSA). Cultures were maintained at 37°C in a humidified incubator of 5% CO_2 / 95% air, until 14-18 DIV.

Neuronal transfection

DNA plasmids were recombinantly expressed in both high- and low-density primary cultures of hippocampal or cortical neurons using an adapted calcium phosphate transfection protocol (Jiang *et al.*, 2004). Briefly, plasmid DNAs were diluted in Tris-EDTA transfection buffer (10 mM Tris-HCl, 2.5 mM EDTA, pH 7.3). A $CaCl_2$ solution (2.5 M in 10 mM HEPES) was added, drop-wise, to the diluted DNA to a final concentration of 250 mM $CaCl_2$, and added to an equivalent volume of HEPES-buffered solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose and 42 mM HEPES, pH 7.2). The final transfection solution was then gently vortexed and incubated at room temperature for 30 min to allow the formation of precipitates. Precipitated DNA was then added, drop-wise, to DIV 7 neurons and cultures were incubated at 37°C for 1-3 h in the presence of kynurenic acid (2 mM in Neurobasal medium). To dissolve the precipitates, cells were incubated at 37°C for 20 min with slightly acidified culture medium containing kynurenic acid. Finally, reserved, conditioned medium was added to the cells (or coverslips were transferred back to the original astroglial plate), which returned to the incubator to allow expression of the transfected constructs, until DIV 13 (cortical) or DIV 15 (hippocampal).

Anti-CASPR2 encephalitis patient – clinical history

Plasma samples (obtained during periods of disease exacerbation) of a 72 year-old male patient with anti-CASPR2 autoimmune encephalitis were used in this study. The patient, presenting with Morvan's syndrome secondary to high levels of CASPR2 autoantibodies, has developed progressive neuropathic pain, episodes of lack of perception with goosebumps, and memory complaints (short- and long-term memory, including word-finding difficulties), with no indication of paraneoplastic causes. The patient was subjected to plasma exchange and immunosuppressive therapy, with good clinical outcome. Some of the symptoms persisted, and the patient retained permanent cognitive sequelae, despite continuous

immunosuppression. Serum from an age- and sex-matched healthy subject was also kindly donated. Written informed consent was obtained from both patient and healthy control.

Preparation of purified human IgG

Purification of human immunoglobulins (IgGs) from patient samples was done using the ammonium sulphate precipitation method, as previously described (Coutinho *et al.*, 2017b). Briefly, saturated ammonium sulphate solution was added to the human plasma to produce a 45% final saturation and left to stir at 4°C overnight. The following day, the solution was centrifuged at 12000 rpm for 2 h. The supernatant was discarded and the precipitate was re-suspended in 10% of the original volume in Hartmann's solution. After homogenization, the solution was dialysed against a minimum of three changes of Hartmann's buffer (2L per change). IgG concentration was determined using a commercially available human IgG radial immunodiffusion kit (The Binding Site, Birmingham, UK): healthy pIgG - 21.2 mg/mL; patient pIgG - 15.1 mg/mL.

In vitro paradigms

To study the pathogenic effects of CASPR2 autoantibodies, 200 ηg/mL or 50 µg/mL of purified human IgGs from the plasma of the CASPR2-encephalitis patient or the healthy control were incubated in DIV 14-15 hippocampal neurons or DIV 11-13 cortical neurons for periods of 1, 2, 7 and 21 h, at 37°C, in conditioned medium.

To elicit paradigms of homeostatic synaptic scaling, neuronal activity in hippocampal or cortical neurons was chronically blocked with 1 µM tetrodotoxin (TTX), or TTX together with 100 µM D-(-)-2-Amino-5-phosphonopentanoic acid (APV). Hippocampal neurons at DIV 13/14 or cortical neurons at DIV 11 (from primary high density or Banker cultures) were incubated for 24 or 48 h, at 37°C, in conditioned medium.

To induce a chemical protocol of NMDAR-dependent long-term potentiation (cLTP), 13 DIV cortical neurons were preincubated for 15 min at 37°C in chemLTP buffer [125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 33 mM glucose, 25 mM HEPES (pH 7.4), supplemented with 20 µM Bicuculline to block $GABA_A$ receptors, 500 ηM TTX to block voltage-gated Na^+-channels and 1 µM strychnine to block Glycine receptors], followed by 200 µM glycine treatment for 5 min at room temperature for activation of NMDARs (in chemLTP buffer without Mg^{2+}). Neurons were allowed to recover (in original chemLTP buffer without glycine) for 20 min at 37°C. This protocol was previously described in (Diering *et al.*, 2016; Hussain *et al.*, 2014).

Following *in vitro* paradigms, cells were either lysed for Western blot analysis (high density cultures) or fixed for immunocytochemistry experiments (Banker cultures).

Immunocytochemistry

Live cell-based assays for human IgG titration and subtyping

To determine antibody levels in patient serum, a live cell-based assay (CBA) expressing EGFP-tagged human CASPR2 was used, as previously reported (Coutinho *et al.*, 2017b). Patient serum samples were diluted at a 1:100 dilution ratio in DMEM supplemented with 1% BSA (v/v) and 200 mM HEPES and added to HEK293T cells expressing EGFP-CASPR2. Patient samples and healthy controls were added in each assay. Diluted sera were incubated with the live cells on coverslips at room temperature for 1 h, after which the supernatant was aspirated and washed 3 times in DMEM/HEPES. Cells were fixed in 4% formaldehyde in PBS and incubated for 10 min at RT and then washed 3 times in DMEM or PBS. Afterwards, cultures were incubated for 45 min, at room temperature, with an anti-human IgG fluorophore-conjugated secondary antibody in DMEM+HEPES+BSA. The coverslips were washed 4 times in PBS and mounted using fluorescent mounting media (DakoCytomation, Cambridge, UK), containing 4',6'-diamidino-2-phenylindole (DAPI; 1:1000). After drying, cells were viewed with a Leica DM 2500 immunofluorescent microscope. Human IgG binding was scored in a scale from 0-4 according to immunofluorescence intensity (0= no binding, 1= weak binding, 2= moderate binding, 3=strong binding, 4=very strong binding). Any sample scoring 1 or above was repeated and double scored by a second observer. Positive samples (score≥1) were then titrated and end-point titration determined. Patient antibody titre was 1:6400, as determined by the last titration at which a positive signal was still observed.

To analyse IgG subclasses, diluted serum at 1:100 was incubated for 1 h with EGFP-CASPR2-expressing HEK293T cells as mentioned above. After fixation, cultures were incubated for 45 min, at room temperature, with a mouse IgG-specific anti-human IgG primary antibody (IgG1 - IgG4), followed by 3 washes in PBS, and then incubated for 45 min with an anti-mouse fluorophore-conjugated secondary antibody. The coverslips were washed and mounted as described above. Binding of specific IgG subclasses was scored, and their specific titres determined as previously mentioned.

Detection of antibody binding to the surface of live primary neurons and pre-adsorption assays to test binding specificity

To recognize human IgGs bound to the neuronal surface, patient serum diluted at 1:100 in NBM supplemented with 1% BSA were incubated live with primary WT or *Cntnap2* KO hippocampal neurons for 1 h, at room temperature. Coverslips were washed three times in NBM, fixed for 15 min in 3% formaldehyde in PBS and then re-washed before incubating with an anti-human IgG fluorophore-conjugated secondary antibody for 45 minutes. Cells were then permeabilised with 0.25% Triton-X-100 in PBS for 15 min. Coverslips were incubated with an anti-MAP2 antibody to identify the neuronal cells in the preparation. After 1 h coverslips were washed and incubated with the appropriate secondary antibody for 45 minutes, washed 3 times in NBM/BSA, twice in PBS and mounted as described previously.

To test specificity of binding of patient antibodies, a pre-adsorption assay was performed. HEK293T cells either un-transfected or transfected with EGFP-CASPR2 were trypsinised and re-suspended in DMEM-1% HEPES. Patient serum, diluted 1:10, was incubated against live cells in solution for 1 h at room temperature on a rotator. This procedure was repeated 3 times. The adsorbed samples were collected and re-tested on the cell-based assay and on the neuronal primary cultures (at a 1:100 dilution) to confirm complete adsorption.

Immunocytochemistry

Low-density cortical or hippocampal neurons in coverslips were fixed for 15 min in 4% sucrose / 4% paraformaldehyde in PBS, at room temperature, and permeabilized with 0.25% Triton X-100 in PBS for 5 min, at 4°C. Neurons were then incubated in 10% (w/v) BSA in PBS for 30 min, at 37°C, to block nonspecific staining, and incubated with the indicated primary antibodies diluted in 3% BSA in PBS (2 h, 37°C or overnight, 4°C). Following several washes with PBS to remove primary antibodies, cells were incubated with the appropriate fluorophore-conjugated secondary antibodies diluted in 3% BSA in PBS, for 1 h at 37°C. Coverslips were then washed with PBS and mounted using fluorescent mounting medium from DAKO (Glostrup, Germany). Preparations were cured overnight at 4°C, protected from light, sealed with nailpolish and kept at 4°C until microscopy analysis.

Labelling of cell surface AMPARs

To label surface GluA1-containing AMPA receptors, live cortical or hippocampal neurons in coverslips were incubated for 10 min at room temperature with a primary antibody against an extracellular epitope in the GluA1 N-terminus, diluted in conditioned neuronal culture medium. Each coverslip was then briefly rinsed in PBS and fixed as described above.

Coverslips were then incubated with an anti-sheep fluorophore-conjugated secondary antibody diluted in 3% BSA in PBS, overnight at 4°C. Coverslips were subsequently permeabilized and probed as described above. Alternatively, surface GluA1 or GluA2-containing AMPARs were immunolabelled in fixed, but un-permeabilized neurons. Briefly, cells were fixed as mentioned above and incubated in 10% (w/v) horse serum for 30 min, at 37°C, to block nonspecific staining. Primary antibodies against extracellular epitopes in the GluA1 and GluA2 N-terminals were diluted in 5% horse serum and incubated overnight, at 4°C. Cells were then washed 5 times, permeabilized with 0.25% Triton X-100 in PBS for 5 min, at 4°C, and re-blocked. Subsequent steps were performed as described above.

Human IgG internalization assays

To test if CASPR2 autoantibodies bound to cell surface of primary neurons are internalized, cortical neurons incubated for 1 or 7 h with human IgGs purified from the plasma of either the healthy control or the encephalitis patient were fixed as before. Coverslips were incubated in 10% (w/v) BSA for 30 min, at 37°C, to block nonspecific staining, and then incubated overnight, at 4°C, with an excess concentration (1:50) of a first anti-human IgG fluorophore-conjugated secondary antibody in order to label all human IgGs bound to the neuronal surface. Coverslips were thoroughly washed to remove the excess of unbound antibody, permeabilized as described above and then re-blocked. Internalized human IgGs were then labelled with a normal concentration (1:500) of a second anti-human IgG fluorophore-conjugated secondary antibody for 1 h, at 37°C. Coverslips were incubated with an anti-MAP2 primary antibody (2 h, 37°C) to identify dendritic processes, and with an appropriate secondary antibody and probed as before.

Culture imaging and quantitative fluorescence analysis

Imaging was performed on a Zeiss Axiovert 200M microscope using a 63 X 1.4 numerical aperture oil objective. Images were quantified using the image analysis software FIJI (FIJI Is Just ImageJ). For quantification, sets of cells were cultured and stained simultaneously, and imaged using identical settings. The region of interest was randomly selected avoiding primary dendrites, and dendritic length was measured using MAP2 staining. In experiments where cells had been previously transfected, fields for imaging were chosen by the GFP or mCherry channels, for the presence of transfected, GFP/mCherry-positive, neurons. Surface GluA1 or GluA2, phospho-ZBP1, Caspr2 and human IgG digital images were thresholded such that recognizable clusters were included in the analysis, and measured for cluster intensity, number, and area for the selected region. Synaptic clusters of GluA1, GluA2, phospho-ZBP1

and Caspr2 were selected by their overlap with thresholded and dilated PSD95 or vGluT1 (or both) signal. The number of glutamatergic synapses containing Caspr2 per dendritic length was determined by identifying PSD-95- and vGluT1- positive clusters that were also labelled for Caspr2. The same criteria was used to identify glutamatergic synapses that contained both GluA1 and Caspr2 signal. Measurements were performed in a minimum of 3 independent experiments, and at least 10 cells per condition were analysed for each preparation.

Cell culture lysates

Total protein extracts of COS7 cells and high-density neuronal cultures were prepared for Western Blot (WB) analysis. Total extracts of COS7 cells were prepared at 48 h after transfection. Protein extracts from cortical or hippocampal neurons were prepared at DIV 13 or 15, respectively. Cells were washed once with cold phosphate buffered saline (PBS – 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and protein extracts were prepared in ice-cold lysis buffer RIPA (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5, with phosphatase inhibitors [2 mM sodium orthovanadate (Na_3VO_4), 50 mM sodium fluoride (NaF) and 1 µM okadaic acid] supplemented immediately before use with a cocktail of protease inhibitors [200 µM PMSF and 1 µg/ml each of chymostatin, leupeptin, antipain and pepstatin – CLAP]). The lysates were sonicated with an ultrasonic probe, on ice, for 30 sec (6 pulses of 5 sec each). After centrifugation at 16100 x *g* for 10 min at 4°C, cell debris and insoluble material were discarded and the supernatant submitted to protein quantification by the Bicinchoninic acid (BCA) assay kit (Pierce, Thermo Fisher Scientific). The protein was aliquoted and frozen at -20°C, until needed.

For the dephosphorylation assays using the Lambda protein phosphatase (λPP – New England Biolabs through Izasa, Lisbon, Portugal), total cell extracts of COS7 cells were washed in ice-cold PBS and extracted with the lysis buffer RIPA without phosphatase inhibitors (sodium orthovanadate and sodium fluoride). Lysates were then solubilized and protein was quantified, as described above. Approximately 150 µg of protein of each sample to be dephosphorylated were incubated with 2 µl of λPP in 1xNEBuffer for protein metallophosphatases (PMP - 50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, pH 7.5) supplemented with 1 mM $MnCl_2$, for 2 h at 30°C. Following this period, samples were denatured at 95°C for 5 min, aliquoted and stored at -20°C, until needed.

Tissue lysates

Rat cerebellum and mouse cortex were collected and lysed as previously described (Santos *et al.*, 2012). Briefly, tissue blocks were homogenized in a potter with 10 volumes (10 x weight) of ice-cold 10 mM Tris-HCl buffer, pH 7.4, containing 320 mM sucrose. The homogenate was centrifuged at 700 x *g* for 10 min, at 4°C. The pellet was homogenized again and centrifuged at 700 x *g* for 10 min, at 4°C. Both supernatants were pooled and supplemented with protease inhibitors (0.2 mM PMSF, 100 mM DTT, 1 µg/ml each of CLAP). All samples were submitted to protein quantification by the BCA method.

For biochemical experiments using the primary visual cortex, the brain of C57BL/6J mice was removed and immersed in ice-cold dissection buffer (212.7 mM sucrose, 2.6 mM KCl, 1.23 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 10 mM glucose, supplemented with 3 mM $MgCl_2$ and 1 mM $CaCl_2$) saturated with 5% CO_2 / 95% air mixture. Blocks of primary visual cortices were rapidly dissected and snap-frozen at -80°C. Samples were homogenized in ice-cold 1% Triton X-100 lysis buffer (20 mM Na_3PO_4, 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, 10 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4, pH 7.4 freshly supplemented with 1 µM okadaic acid and 10 U/mL aprotinin) by sonication (15 pulses). To ensure lysis, homogenates were incubated with 0.2% SDS for 30 min with rotation, at 4°C. Samples were then centrifuged at 1000 x *g* for 10 min, at 4°C, to remove unhomogenized tissue and protein concentration was quantified with the BCA method. Samples were denatured with 3x sample buffer [2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol in 0.125 M Tris, pH 7.4] and boiled for 5 min.

Immunoprecipitation assays

The interaction between Caspr2 and AMPAR subunits was evaluated through immunoprecipitation assays done from rat whole cerebellum or mouse cortical lysates, as previously described (Santos *et al.*, 2012). Briefly, 4 mg of protein lysates were solubilized in TEEN buffer [25 mM Tris (pH=7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1% TritonX-100, supplemented with protease inhibitors], and sonicated with an ultrasonic probe, on ice, for 30 sec. The insoluble material was removed by centrifuging the sample at 13000 x *g* for 10 min, at 4°C. At this point the supernatant was collected and 50 µL were removed for analysis by Western Blot (input). The supernatant was transferred to a tube containing 30 µL of a 50% slurry of protein A sepharose beads (GE Healthcare, Carnaxide, Portugal) suspended in TEEN and rotated at 4°C for 1 h for pre-clearing. After a 5 min centrifugation step, the sedimented sepharose beads were discarded. The supernatant was split and incubated either with 3 µg of anti-Caspr2 antibody (IP) or with the same amount of non-immune rabbit IgGs (negative IP).

This incubation step was performed at 4°C with orbital rotation, for 3 h or overnight. Samples were then incubated with 80 µL of a 50% slurry of protein A sepharose beads and rotated for 2 h at 4°C. To further avoid non-specific binding, samples were washed in subsequent steps: 4x TEEN + 0.1% TritonX-100, 3x TEEN + 1% TritonX-100 + 500 mM NaCl and 2x TEEN + 1% TritonX-100. Proteins were eluted by boiling the beads in 40 µl of sample buffer 2x [125 mM Tris-HCl (pH 6.8), 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM Na_3VO_4, and 0.01% bromophenol blue] for 5 min and processed for Western blot analysis.

Purification of postsynaptic density fractions

Postsynaptic densities (PSDs) were purified from the whole brain of P60 C57BL/6 mice. Briefly, brains were collected and homogeneized in HEPES-buffered sucrose solution [4 mM HEPES (pH=7.4), 0.32 M sucrose, containing protease and phosphatase inhibitors] in a motor driven homogenizer at 900 rpm (30-40 strokes). The homogenate was collected and centrifuged at 700 × *g* for 15 min to obtain the non-nuclear fraction (S1), a portion of which was recovered and stored as the brain lysate (Bl) for Western blot (WB) analysis. The remaining S1 was further centrifuged for 15 min at 18000 × *g* to yield the crude synaptosomal pellet (P2). P2 was resuspended in HEPES-buffered sucrose solution and homogenized, and then centrifuged again at 25000 × *g* for 20 min to yield the lysed synaptosomal membrane fraction (P3). P3 was resuspended in a HEPES/EDTA-buffered solution [50 mM HEPES (pH=7.4), 2 mM EDTA, with protease and phosphatase inhibitors] and solubilized in 0.5% TritonX-100. A fraction of the synaptic plasma membrane (Pm) lysate was recovered and stored for WB analysis. The remaining P3 fraction was centrifuged at 32000 × *g* for 20 min and the resulting pellet (PSD-1P) was again resuspended in HEPES/EDTA-buffered solution with 0.5% TritonX-100, incubated with orbital rotation for 15 min at 4°C, and centrifuged at 200000 × *g* for 20 min. The remaining pellet, corresponding to the PSDs, was resuspended in HEPES/EDTA solution and stored for WB analysis.

Gel electrophoresis and Western Blot

All protein samples were denatured with sample buffer concentrated 2x or 5x [5x: 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.01% bromophenol blue)], and boiled for 5 min. Protein extracts were resolved by SDS-PAGE in Tris-glycine-SDS (TGS) buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) in 7.5-8% polyacrylamide gels 1.5 mm thick at 60-80 V. For Western blot analysis, proteins were transferred onto a PVDF membrane (Millipore, Oeiras, Portugal) by electroblotting overnight at 40 V and 4°C. Membranes were blocked for 1

h at room temperature with 5% (w/v) non-fat dry milk or BSA (NZYTech, Lisboa, Portugal) in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBS-T). Membranes were probed during 1 h, at room temperature, or overnight, at 4°C, with the primary antibodies diluted in 5% BSA in TBS-T. Following several washes with TBS-T, the membranes were incubated for 1 h, at room temperature, with the appropriate alkaline phosphatase-conjugated IgG secondary antibodies. Immunostaining was resolved with the enhanced chemifluorescence (ECF) substrate (GE Healthcare, Carnaxide, Portugal) for a maximum of 5 min or until protein bands were visible. Membranes were scanned on a VersaDoc Imaging System (Model3000 – BioRad, Amadora, Portugal) or on Storm 860 scanner (GE Healthcare, Carnaxide, Portugal). Bands corresponding to the proteins of interest were quantified using the FIJI software and normalized with the loading controls (GAPDH, Human transferrin receptor or β-tubulin) indicated in figure captions. When necessary, the membranes were stripped (0.2 M NaOH for 5 min) and re-probed.

Ribonucleoprotein Immunoprecipitation Protocol (RIP)

To evaluate mRNA molecules bound to the RNA-binding protein ZBP1, a ribonucleoprotein immunoprecipitation protocol (RIP) was performed. Briefly, total protein extracts were obtained by lysing cells with ice-cold RIPA buffer, as previously described, but further supplemented with NZY RNase inhibitor (NZYTech, Lisbon, Portugal) to prevent RNA degradation and preserve protein-RNA interactions. After processing and quantification of protein concentration, samples were stored at -80°C, until necessary. To immunoprecipitate ZBP1, 6 µg of anti-ZBP1 antibody were firstly mixed with 100 µL of protein G agarose beads (SantaCruz Biotechnology, Frilabo, Maia, Portugal) in 100 µL of RNase-free $NT_2$2x buffer [100 mM Tris-HCl (pH 7.4), 300 mM NaCl, 2 mM $MgCl_2$, 0.1% IGEPAL and supplemented with phosphatase and protease inhibitors (previously mentioned) and NZY RNase inhibitor] and incubated with rotation overnight, at 4°C. Non-immune goat IgGs were used as negative control for the IP. For each experiment, 100 µg of the initial protein sample were saved as input, while 2 mg of the remaining sample were diluted to 700 µg/mL in $NT_2$2x buffer and used for the immunoprecipitation. To avoid non-specific binding, protein samples were initially pre-cleared with 50 µL of protein G agarose beads for 30 min, at 4°C. The mix was centrifuged at 2000 x *g* for 2 min, at 4°C, and the protein supernatant collected. The 2 mg of pre-cleared protein were then split in two for each of the tubes containing the protein G agarose beads together with either the anti-ZBP1 antibody or the control IgGs, and incubated with rotation for 1 h, at 4°C. To remove any non-specific binding, samples were centrifuged at 2000 x *g* for 2 min and washed with 500 µL of $NT_2$1X buffer four times. Following the IP, total RNA from the

input sample or RNA bound to the immunoprecipitate was extracted and analysed by real-time qPCR as described below.

Semi-quantitative Real-time PCR

Total RNA isolation, RNA quality and RNA concentration

Total RNA extraction from immunoprecipitation samples, as well as from COS7 cells and cultured hippocampal neurons transfected with the constructs of interest was performed with TRIzol reagent (Invitrogen, Barcelona, Spain). Briefly, 1 mL of TRIzol was added to each well of a 6-well plate (or vial in case of IPs) and the content of each experimental condition was collected. Chloroform was then added for phase separation and the RNA was precipitated by isopropanol addition. The precipitated RNA was washed with 75% ethanol, centrifuged, air-dried and resuspended in 20 µL of RNase-free water. RNA quality and integrity was evaluated using the Experion automated gel-electrophoresis system (Bio-Rad, Amadora, Portugal). A virtual gel was created for each sample, allowing the detection of degradation of the reference markers RNA 18S and 28S. Samples showing RNA degradation or contamination by DNA were discarded. RNA concentration was determined using a NanoDrop 2000c/2000 UV-Vis Spectrophotomer (Thermo Fisher Scientific, Rockford, USA). The samples were stored at -80°C, until further use.

Reverse Transcription

First strand cDNA was synthesized from 500 ng – 1 µg of total RNA using the NZY First-Strand cDNA Synthesis kit (NZYTech, Lisboa, Portugal) following the manufacturer's specifications. Briefly, total RNA was mixed with a combination of random hexamers and oligo$(dT)_{18}$ primers (included in a NZYRT Mastermix, also containing dNTPs, $MgCl_2$ and an optimized buffer), and a NZYRT Enzyme mix containing a NZY Reverse Transcriptase and a ribonuclease inhibitor. The mix was incubated at 25°C for 10 min for primer annealing to the template, followed by a 30 min incubation at 50°C for cDNA synthesis. The reaction was inactivated by heating at 85°C for 5 min and then chilled on ice. To specifically degrade the RNA template in cDNA:RNA hybrids after the cDNA synthesis, samples were further incubated with a NZY RNase H (*E.coli*) for 20 min at 37°C. Samples were stored at -80°C, until further use.

Primer Design

Primers for real-time PCR were designed using the Beacon Designer 7 software (Premier Biosoft International, Palo Alto, CA, USA), with the following considerations taken into account: (1) GC content about 50%; (2) annealing temperature (Ta) between 55 ± 3°C; (3) secondary structures and primer-dimers were avoided; (4) primer length between 18-24 bp; (5) final product length between 100-200 bp. The primers used for Real-time PCR experiments were the following: 5' ACT ACA TCC TCG CCA ATC TG 3' (GluA1 *forward*); 5' AGT CAC TTG TCC TCC ATT GC 3' (GluA1 *reverse*); 5' TCC AGA TAG ACT TAA TGA AGA AG 3' (Caspr1 *forward*); 5' TGA CCC AAT CCC AAG AAT 3' (Caspr1 *reverse*); 5' GAT GAC CAT CCT TTT CCT TAC 3' (BDNF *forward*); 5' ATT CAC GCT CTC CAG AGT CC 3' (BDNF *reverse*); 5' ACT ACA TCC TCG CCA ATC TG 3' (β-actin *forward*); 5' AGT CAC TTG TCC TCC ATT GC 3' (β-actin *reverse*); 5' TCC CAT TCT TCC ACC TTT 3' (GAPDH *forward*); 5' TAG CCA TAT TCA TTG TCA TAC C 3' (GAPDH *reverse*); 5' CAT CCT CAC CAC CCA CAC 3' (Tubulin *forward*); 5' GGA AGC AGT GAT GGA AGA C (Tubulin *reverse*); 5' GCA CCA TCT TTC AAG G 3' (GFP *forward*); 5' TTG TGG CTG TTA GTT G 3' (GFP *reverse*).

Real-time PCR

Gene expression analysis was performed using SsoFast SuperMix (Bio-Rad). Briefly, 2 µL of 1:100 diluted cDNA were added to 10 µL of 2x EvaGreen and to specific primers, with a final concentration of 250 nM each in 20 µL total volume. The thermocycling reaction was initiated with 1) the activation of the Sso7d fusion DNA polymerase at 95°C for 30 sec, followed by 2) 45 cycles of a 10 sec denaturation step at 95°C, 3) a 30 sec annealing step at the optimal annealing temperature for each set of primers, and 4) a final elongation step of 30 sec at 72°C. The fluorescence was measured after the extension step, using the iQ5 Multicolor Real-Time PCR Detection System (BioRad). At the end of the thermocycling reaction a melting step was performed with slow heating (starting at 55°C, with a rate of 0.5°C per 10 sec, up to 95°C), and continuous measurement of fluorescence for detection of possible non-specific products. The assay included a non-template control and a standard curve of cDNA (in 10-fold steps) to assess the efficiency of each set of primers. The reactions were run in duplicate to reduce confounding variance.

Data processing and analysis

Data were processed and analysed based on the value of the threshold cycle (Ct). The Ct represents the detectable fluorescence signal above background resulting from the accumulation of amplified product, and it is a proportional measure of the starting concentration of the target sequence. Ct base line was always set at the beginning of the exponential phase,

and therefore was not affected by possible limiting components in the reaction. Data analysis was performed using the GenEx software (MultiD Analyses, Sweden) for Real-Time PCR expression profiling. Relative changes in mRNA levels for the GluA1 AMPAR subunit, Caspr1, β-actin or BDNF were assessed. The constitutively expressed housekeeping genes encoding tubulin and GAPDH, or the co-transfected GFP, were used as control.

Generation of Lentivirus

Generation of the lentiviral vectors used in this study was previously described by Anderson and colleagues (Anderson *et al.*, 2012). Lentivirus were produced for the expression of shRNA sequences against Caspr1 and Caspr2, or corresponding empty-vector. Briefly, HEK293T cells were transfected using the calcium phosphate protocol with the lentiviral expression vectors of interest together with three lentiviral packaging vectors: pMDLg/pRRE, pRSV-Rev and pCMV-VSV-G, for the expression of gag/pol genes, rev gene and vesicular stomatitis virus G (VSVG) envelope glycoprotein gene, respectively. Supernatant containing viral particles was collected 48 h after transfection, filtered and centrifuged at 95 x *g* for 10 min to remove cell debris. Viral particles were concentrated by centrifugation at 87300 x *g* for 2 h at 20°C, and the pellet was resuspended in 10 mL of PBS with 1% BSA (Calbiochem through Merck Millipore, Oeiras, Portugal), gently vortexed for 10 min and left resuspending for 30 min. The pellets were collected and ultracentrifuged again for 1 h 30 min at 83000 x *g* at 20°C. The pellets were then finally resuspended in 60 µL of PBS with 1% BSA for 1 h, with rotation, aliquoted and stored at -80°C.

Stereotactic surgeries

Stereotactic surgeries were performed as previously described (Petrus *et al.*, 2015). Briefly, P21-P28 C57BL/6J mice were anesthetized with 1-3% isoflurane mixed with O_2 in an induction chamber and properly placed in a stereotactic apparatus with constant flow of isoflurane. The skin and periosteum were carefully cut and the wound properly disinfected. Stereotactic coordinates for the primary visual cortex were calculated after identification of the Bregma and intersections with the Sagittal and Lamdoid sutures of the skull. A craniotomy was done uni- or bilaterally using a hand-held drill under a surgical microscope at the location of -3.6 mm from the Bregma and 2.5 mm lateral for the primary visual cortex (V1). To specifically target layer 2/3 of the V1, injections were done by lowering a glass micropipette into the brain 0.36 mm from the pia. Glass micropipettes were filled with either: 1 µL of recombinant L309mCherry lentivirus expressing either the empty vector or specific shRNAs against Caspr1

or Caspr2 for the knock-down experiments; 200 ηg (0.4 µL at 0.5 mg/mL) of either healthy or patient purified IgGs for the CASPR2-Abs experiments. Injections were done at a rate of 0.10 µL/min using a digital pump. After injection, the wound was cleaned with a 0.9% saline solution and the skin sutured and treated with an antibiotic gel. Animals recovered from the surgery in a heating pad and returned to the colony. Mice injected with human IgGs were recovered for 7 h before sacrifice. Lentiviral-infected mice were returned to the animal colony for 4-5 weeks post-infection to allow sufficient lentiviral expression before experimental paradigms were initiated. Wounds and behaviour were checked daily. All the procedures mentioned above were reviewed and approved by the IACUC at Johns Hopkins University, Baltimore, USA.

Paradigms for manipulation of visual experience

Both male and female C57BL/6J mice (Jackson Laboratories, USA) were raised in a normally lighted environment (12 h light/dark cycle). Visual deprivation was induced in the form of dark exposure (DE), as described previously (Goel & Lee, 2007). Briefly, mice within the critical period for visual plasticity (Desai *et al.*, 2002; Whitt *et al.*, 2014), were kept in a dark room for 2 days, and cared for using infrared vision goggles under dim infrared light. Age-matched control [normally reared (NR)] animals were continuously raised in normal light conditions for the same duration. Experimental dark-reared animals were transferred to the lab for protein extraction or slice preparation in an opaque box to minimize light exposure prior to sacrifice. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Johns Hopkins University, Baltimore, USA.

Primary visual cortex acute slice preparation

Mice were deeply anesthetized in a chamber (the chamber was light-tight for DE experimental groups) with isoflurane vapors and killed by decapitation. The brain was rapidly removed and immersed in ice-cold dissection buffer (212.7 mM sucrose, 2.6 mM KCl, 1.23 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 10 mM dextrose, freshly supplemented with 3 mM $MgCl_2$ and 1 mM $CaCl_2$) saturated with 5% CO_2 / 95% air mixture. Blocks of tissue containing the primary visual cortex were rapidly dissected and coronally sectioned into 300 µm-thick slices using the PELCO easiSlicer™ vibratome (Ted Pella, CA, USA). The slices were then gently transferred to a submersion holding chamber with artificial cerebral spinal fluid (aCSF – 124 mM NaCl, 5 mM KCl, 1.23 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 10 mM dextrose, freshly supplemented with 1.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$) saturated with 5% CO_2 / 95% air. The slices were allowed to recover for 1 h at room temperature, before recording.

Electrophysiology

Visual cortical slices (prepared as described above) were transferred to a submersion-type chamber mounted on a fixed stage of an upright microscope (Eclipse FN-1, Nikon, Japan) equipped with infrared oblique illumination. AMPA receptor (AMPAR)-mediated miniature excitatory postsynaptic currents (mEPSCs) were pharmacologically isolated by adding 1 µM TTX, 20 µM Bicc and 100 µM APV to aCSF at 30°C and saturated with 5% CO_2 / 95% air, which was continuously perfused at 2 mL/min. Pyramidal neurons on layer 2/3 were identified by their pyramidal-shaped soma and apical dendrite pointing to the pia, and patched using a whole cell patch pipette (tip resistance 3–5 MΩ), which was filled with internal solution (130 mM Cs-gluconate, 8 mM KCl, 1 mM EGTA, 10 mM HEPES, 4 mM ATP, 0.5 mM GTP, 10 mM phosphocreatine and 5 mM Lidocaine; pH 7.3, 280–290 mOsm). Recordings were initiated 2–3 min after break-in, and each cell was recorded for 8–10 min to collect enough mEPSCs for analysis. The Axon patch-clamp amplifier 700B (Molecular Devices, CA, USA) was used for voltage-clamp recordings. Cells were held at -80 mV, and the recorded mEPSC data were digitized at 10 kHz with a data acquisition board (National Instruments, TX, USA) and acquired using the Igor Pro software (WaveMetrics). The MiniAnalysis program (Synaptosoft) was used to analyse the acquired mEPSCs, and the threshold for detecting mEPSCs was set at three times the root mean square (RMS) noise. Recordings were excluded from analysis if the RMS noise was > 2, the series resistance was > 25ΩM, and input resistance was < 150ΩM. To minimize the impact of dendritic filtering, we adopted the standard approach of excluding mEPSCs with rise time > 3 msec. At least two hundred consecutive mEPSCs that met the rise time criteria were analyzed from each cell.

Statistical analysis

Graphs and statistical analysis were performed using Graph Pad Prism 6 software. Results are presented as normalized mean ± S.E.M. Biochemical and immunocytochemistry data are presented from at least three different experiments, performed in independent preparations, unless stated otherwise. Statistical differences were calculated according to non-parametric tests for most part of the cases. Mann-Whitney test was used to compare statistical differences between any two groups. Comparisons between multiple groups were performed with the Kruskal-Wallis analysis of variance followed by Dunn's Multiple Comparison test, with the parametric one-way ANOVA analysis followed by Fisher's least significant difference (PLSD) test, or with a two-way ANOVA analysis followed by Tukey's multiple comparison test. Significance of cumulative distributions was calculated with the Kolmogorov-Smirnov test. For all tests, $p<0.05$ was considered statistically significant.

Chapter III
Results & Discussion

Homeostatic regulation of synaptic AMPA receptors by Caspr1 and ZBP1

The results in this chapter are partially presented in the following publication in preparation:

Dominique Fernandes, Sandra Santos, Luís Ribeiro, Anna Ceccarelli, Ana Luísa Carvalho. Homeostatic regulation of synaptic AMPA receptors by Caspr1 and ZBP1. *In preparation.*

Summary

During development and throughout learning-related adaptations, the mammalian brain undergoes constant changes that can compromise its function. Nonetheless, the integrity and function of neuronal circuits is preserved. At the cellular level, such stability is accomplished by homeostatic synaptic plasticity, which allows experience-based adaptations to occur while maintaining the activity of neuronal networks in-balance. Amongst other processes, an ever-growing body of evidence pinpoints the dynamic regulation of synaptic glutamate receptors of the AMPA-type by distinct molecular players as a fundamental mechanism to achieve neuronal homeostasis.

Herein, we describe a post-transcriptional mechanism orchestrated by the cell-adhesion molecule Caspr1 and the RNA-binding protein ZBP1 that regulates the synaptic content of surface AMPARs in an activity-dependent manner. Caspr1 induces Src activation, and the phosphorylation of ZBP1 in neurons. During prolonged periods of neuronal inactivity, when mechanisms of homeostatic plasticity are triggered to upscale synaptic AMPARs, Caspr1 expression is upregulated, and the phosphorylation of Src and ZBP1 increases. ZBP1 binds to GluA1 mRNA in an activity-dependent manner, and regulates synaptic levels of GluA1-containing AMPARs. Accordingly, when the expression of either Caspr1 or ZBP1 is lost, synaptic upscaling of AMPARs is compromised. Overall, this study uncovers a specific requirement for both Caspr1 and ZBP1 in the regulation of postsynaptic AMPARs during homeostatic synaptic plasticity, which places Caspr1 and ZBP1 in the map of potential regulators of neuronal homeostasis.

Introduction

Throughout the years, many efforts have been made to uncover the cellular processes governing distinct higher-order brain functions, from behavioural mechanisms to learning and memory events, and cognition. These phenomena rely on the ability of the mammalian brain to undergo experience-based adaptations that occur at the level of glutamatergic synapses, which become stronger or weaker in response to specific patterns of activity (Shepherd & Huganir, 2007). Glutamate receptors of the AMPA-type mediate most of the fast excitatory synaptic transmission in the brain, and altering postsynaptic AMPAR number or function results in long-term changes in synaptic strength and efficacy. Understandably, the dynamic regulation of synaptic AMPARs has thus emerged as a critical mechanism underlying several forms of synaptic plasticity in different areas of the brain.

In Hebbian forms of plasticity, such as LTP and LTD, the trafficking and accumulation of AMPARs in synapses is modulated in a manner that positively correlates with synaptic potentiation or weakening: while increased AMPAR synaptic insertion triggers LTP, AMPAR removal from the synapse leads to LTD. These forms of plasticity, however, enable already potentiated or depressed synapses to undergo further LTP or LTD, respectively, and potentially lead to runaway excitation or synapse elimination. To counterbalance the destabilizing nature of Hebbian plasticity, neurons have developed homeostatic mechanisms to sense and adapt to overall changes in synaptic activity and restrain it within a physiological range (Fernandes & Carvalho, 2016; Turrigiano, 2008; Vitureira & Goda, 2013). In face of prolonged perturbations in activity, homeostatic mechanisms, such as synaptic scaling, modulate the postsynaptic accumulation of AMPARs in order to globally scale up or down the strength of synapses (Aoto *et al.*, 2008; Ju *et al.*, 2004; O'Brien *et al.*, 1998; Thiagarajan *et al.*, 2005; Wierenga *et al.*, 2005). Interestingly, the mechanisms underlying AMPAR trafficking and insertion at synapses seem to be partially shared between distinct forms of plasticity (Fernandes & Carvalho, 2016; Vitureira & Goda, 2013).

AMPAR trafficking and localization to synapses are dynamically modulated by multiple mechanisms ranging from post-transcriptional and post-translational modifications of AMPAR subunits, to specific interacting partners (Anggono & Huganir, 2012; Jiang *et al.*, 2006; Santos *et al.*, 2009). In particular, AMPAR subunits bind to a variety of accessory and scaffolding proteins that not only control the targeting and insertion (or removal) of AMPARs to extrasynaptic sites, but also regulate their anchoring to the synaptic membrane. Importantly, several of these binding partners regulate AMPARs in an activity-dependent manner, playing crucial roles in synaptic plasticity mechanisms, either Hebbian or homeostatic, or, in some

cases, even both. Although a fairly vast collection of studies already exists, a more comprehensive knowledge of AMPAR-interacting proteins would further contribute to fully uncover the mechanisms regulating AMPAR trafficking and function, and how they underlie the molecular basis for synaptic plasticity.

Taking this into account, a recent proteomic screening carried out in our laboratory to explore the AMPAR interactome allowed the identification of the transmembrane protein Contactin-associated protein 1 (Caspr1) as a novel binding partner of AMPARs (Santos *et al.*, 2012). Caspr1 is a neurexin-like cell-adhesion molecule that has been thoroughly studied in axons, where it plays a fundamental role in the initial steps of axonal myelination (Einheber *et al.*, 1997; Peles *et al.*, 1997). Despite this canonical function, the possibility of Caspr1 having a role in the regulation of AMPARs is not that implausible. Firstly, cell-adhesion molecules are no longer considered just mere structural components of the synapse, solely regulating synapse formation and differentiation; instead, they are now increasingly recognized as dynamic modulators of synaptic function (Bukalo & Dityatev, 2012; Yamagata *et al.*, 2003). Interestingly, Caspr1 typically forms a membrane complex with the cell-adhesion molecule Contactin 1 [also found in the screening (Santos *et al.*, 2012)], which has been implicated in synaptic plasticity and memory (Murai *et al.*, 2002; Puzzo *et al.*, 2015; Puzzo *et al.*, 2013). Secondly, several studies have already uncovered an extensive localization of Caspr1 in dendrites and synaptic sites (Cajigas *et al.*, 2012; Collins *et al.*, 2006; Murai *et al.*, 2002). This evidence is in accordance with data obtained in our lab showing that Caspr1 is present in almost 50% of all hippocampal glutamatergic synapses, and also enriched in biochemically purified postsynaptic densities from cortical neurons (Santos *et al.*, 2012).

Santos and colleagues demonstrated that Caspr1 interacts with AMPAR subunits, and that overexpression of Caspr1 in hippocampal neurons leads to a significant increase in the synaptic content of superficial GluA1-containing AMPARs. Co-expression of Caspr1 with GluA1 in HEK293 cells increases the average amplitude of currents evoked by glutamate. On the other hand, loss of endogenous Caspr1 results in decreased surface levels of synaptic GluA1, indicating that Caspr1 is indeed required for the synaptic localization of AMPARs (Santos *et al.*, 2012). The presence of Caspr1 in glutamatergic synapses and its regulation of AMPAR response to glutamate and AMPAR synaptic content point to a novel postsynaptic function for Caspr1 in regulating AMPARs.

Despite these exciting findings on a novel player in the regulation of synaptic AMPARs, the mechanism underlying the effect of Caspr1 in synaptic GluA1-containing AMPARs remains elusive, and evidence linking Caspr1 to synaptic plasticity is still missing. Here, we found that besides increasing GluA1 surface levels, Caspr1 also increases total protein levels of GluA1,

possibly through the post-transcriptional regulation of GluA1 mRNA. We further determined that this level of AMPAR regulation by Caspr1 occurs through a mechanism dependent on a specific intracellular C-tail proline-rich domain of Caspr1, which induces the activation of the tyrosine kinase Src. This signalling pathway, in turn, modulates the function of the RNA-binding protein Zipcode-binding protein 1 (ZBP1), which we found to bind to and regulate GluA1 mRNA, and to regulate the synaptic content of GluA1-AMPARs. Additionally, our results indicate that chronic manipulations of neuronal activity, which homeostatically scale up synaptic AMPARs, also increase the endogenous levels of Caspr1 and modulate the activation of the Src signalling pathway. Importantly, we determined that loss of either Caspr1 or ZBP1 in hippocampal neurons blocks synaptic scaling of GluA1-AMPARs induced by chronic neuronal inactivity. Lastly, we demonstrate that the modulation of ZBP1 function by Src-dependent phosphorylation is a specific requirement for the role ZBP1 plays in the activity-dependent regulation of synaptic AMPARs. Altogether, our results demonstrate that Caspr1, through activation of the Src signalling pathway, regulates GluA1-containing AMPARs in an activity-dependent manner, and further uncover a specific requirement for both Caspr1 and ZBP1 in the expression of mechanisms underlying homeostatic synaptic scaling.

Results

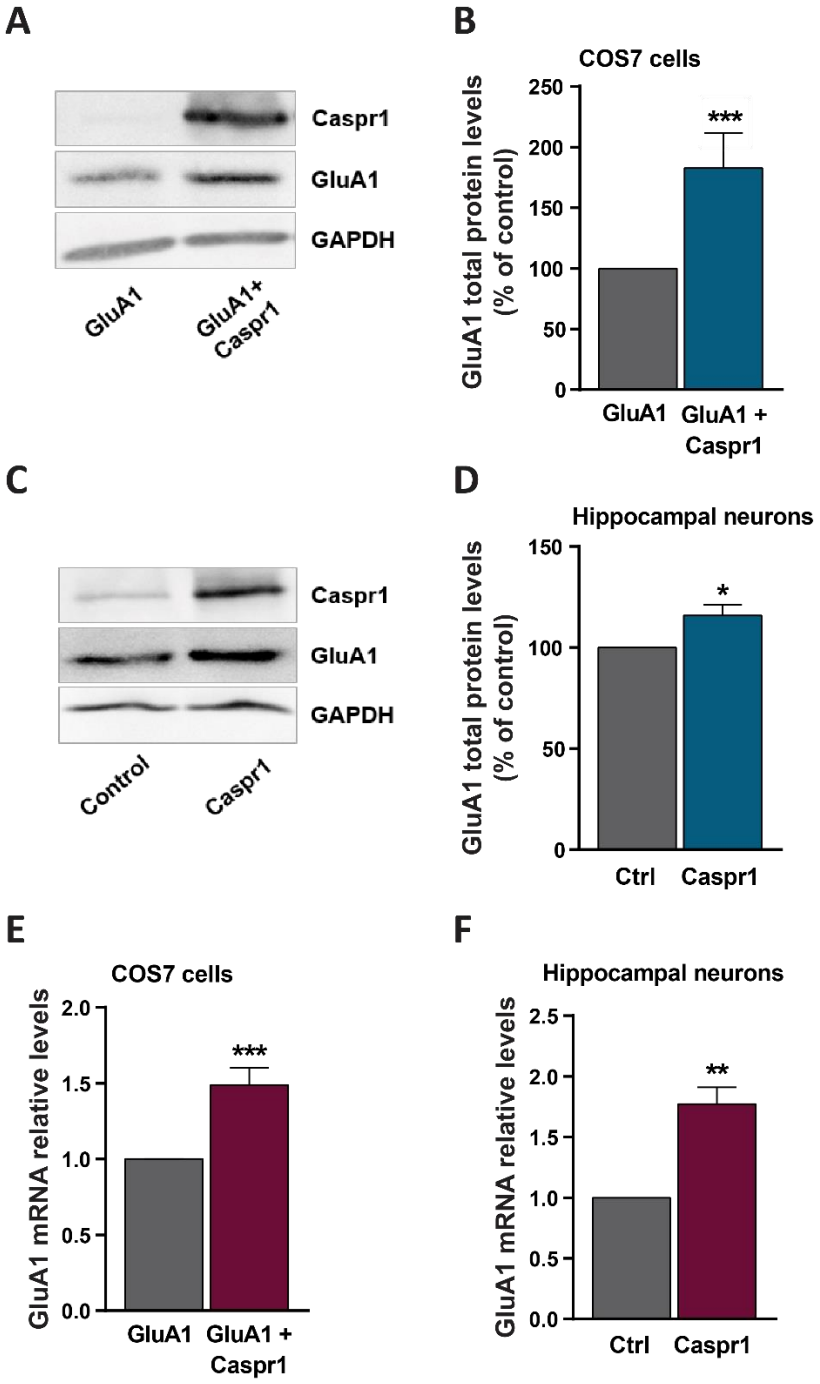
Caspr1 regulates total protein and mRNA levels of GluA1-containing AMPARs through an intracellular proline-rich domain

The regulation of AMPARs at the levels of transcription and translation plays fundamental roles for plasticity events (Sutton & Schuman, 2006), being a means to control available pools of new AMPARs to be inserted at the synapse. Nevertheless, the mechanisms underlying this type of regulation of AMPARs are poorly characterized. We used a heterologous system to co-express GluA1 and Caspr1, and evaluated total levels of GluA1 by western blot (WB) analysis (Figure 3.1A). We observed that co-expression of Caspr1 and GluA1 in COS7 cells resulted in a significant 82.8 ± 29.0% increase on total protein levels of the GluA1 subunit, when compared to GluA1-transfected cells (Figure 3.1B). To confirm if a similar effect can be observed in a neuronal system, Caspr1 was overexpressed in high-density cultured hippocampal neurons, and total protein levels of GluA1 were analysed by WB (Figure 3.1C). Similarly to what was observed in the heterologous system, overexpression of Caspr1 in hippocampal neurons resulted in a significant increase on GluA1 total protein levels (115.8 ± 5.3% of control; Figure 3.1D). This evidence indicates that Caspr1, besides increasing superficial and synaptic levels of GluA1 (Santos *et al.*, 2012), also regulates GluA1 protein expression, suggesting that Caspr1 plays an unexpected wide-ranging role in the regulation of AMPARs.

The increase on GluA1 total levels following expression of Caspr1 could be explained by an effect in protein stabilization. However, co-expression of Caspr1 and GluA1 in COS7 cells did not affect GluA1 half-life, despite increasing GluA1 protein expression, indicating that Caspr1 does not alter GluA1 protein stability (Santos, 2009). Taking this into account, we hypothesized that the increase in GluA1 protein could be due to an upregulation of GluA1 mRNA levels in the presence of Caspr1. The evaluation of GluA1 mRNA relative levels using a semi-quantitative real-time PCR approach, in COS7 cells transfected with GluA1, alone or together with Caspr1, showed that Caspr1 significantly upregulates GluA1 mRNA in COS7 cells (1.48 ± 0.11-fold increase when compared to control; Figure 3.1E). To understand if Caspr1 also regulates endogenous GluA1 mRNA in neurons, we overexpressed Caspr1 in high-density cultured hippocampal neurons and evaluated endogenous GluA1 mRNA levels by real-time PCR (Figure 3.1F). Similarly to what occurs in the heterologous system, Caspr1 overexpression resulted in a significant increase on the endogenous levels of neuronal GluA1 mRNA (1.77 ± 0.14-fold increase when compared to control; Figure 3.1F), further suggesting that the observed increase in GluA1 protein levels following Caspr1 expression occurs through

a regulation of GluA1 mRNA. Since GluA1 in COS7 cells is expressed from the transfected plasmid, under the control of the CMV promoter, it is unlikely that the increase on GluA1 mRNA in the presence of Caspr1 is a transcriptional effect. In fact, in the same system, Caspr1 expression fails to upregulate mRNA levels of another transcript under the same promoter as GluA1 (Santos, 2009). Altogether, these pieces of evidence hint at a novel post-transcriptional role for Caspr1 in regulating the GluA1 subunit of AMPARs.

These results, together with earlier evidence found in our lab (Santos, 2009; Santos *et al.*, 2012), suggest that Caspr1 mediates a multi-level regulation of AMPARs, controlling the total amount of GluA1 protein and mRNA, as well as the surface expression and synaptic content of GluA1-containing AMPARs. Interestingly, this multi-function profile of Caspr1 might be consistent with its diverse structure, composed of a mosaic of distinctive domains. The extracellular architecture of Caspr1 is very similar to that of neurexins, containing domains usually associated with cell adhesion functions. The intracellular region of Caspr1, on the other hand, is composed of domains typically involved in protein-protein interactions (Figure 3.2A). Its juxtamembrane portion comprises a short peptide with a conserved GNP (glycophorin C, neurexin IV and paranodin) motif (Figure 3.2A), capable of interacting with proteins encompassing FERM domains, such as the cytoskeleton adaptor 4.1 protein (Denisenko-Nehrbass *et al.*, 2003b). Besides the GNP motif, the intracellular C-tail portion of Caspr1 contains an additional sequence rich in proline residues (Figure 3.2A) that is capable of selectively interacting with the SH3 domain of several signalling molecules, particularly with the tyrosine kinases Src and Fyn, PLCγ and the p85 subunit of PI3K (Peles *et al.*, 1997). Given the overall architecture of Caspr1, comprising domains with such diverse functions, it is likely that one, or more, of these domains is responsible for the effects of Caspr1 in regulating GluA1-containing AMPARs. In accordance, Santos and collaborators highlighted the importance of the intracellular C-terminal portion of Caspr1 in the regulation of cell surface GluA1: on one hand, overexpression of this region alone in hippocampal neurons is sufficient to promote the surface insertion of GluA1-containing AMPARs; on the other hand, expression of the C-tail of Caspr1 is able to rescue the decrease in the synaptic content of GluA1-AMPARs induced by loss of endogenous Caspr1 (Santos *et al.*, 2012).



[Figure 3.1 - subtitle on the next page**]**

Figure 3.1 – Caspr1 overexpression increases total protein and mRNA levels of the GluA1 subunit of AMPARs in COS7 cells and in cultured hippocampal neurons. **(A-D)** Caspr1 increases total protein levels of GluA1 in COS7 cells **(A, B)** and hippocampal neurons **(C, D)**. **(A)** GluA1 was expressed in COS7 cells, in the presence or absence of Caspr1, as indicated. 48 h after transfection, total protein levels of GluA1 and Caspr1 were assessed by Western blot. **(C)** Cultured rat hippocampal neurons at DIV7 were transfected with either Caspr1 or GFP (Control), as indicated. Following total protein extraction at DIV15, GluA1 and Caspr1 protein levels were evaluated by Western blot. **(B, D)** Results are presented as mean ± S.E.M., when compared to control and normalized to the loading control GAPDH. Statistical significance was determined by the non-parametric test Mann-Whitney, ***p<0.001 (N=8) **(B)**, and *p<0.05 (N=10) **(D)**. **(E, F)** Caspr1 upregulates relative levels of GluA1 mRNA in COS7 cells **(E)** and hippocampal neurons **(F)**. **(E)** COS7 cells were transfected with GluA1, alone or together with Caspr1, and RNA isolation was performed 48 h after transfection. **(F)** Caspr1 was overexpressed in cultured hippocampal neurons at DIV7, and allowed to express until DIV15, when total RNA was isolated. Relative levels of GluA1 mRNA were evaluated by real-time PCR. Results are presented as mean ± S.E.M., when compared to control and normalized to the reference gene GFP (control of transfection). Statistical significance was determined by the non-parametric test Mann-Whitney, ***p<0.001 (N=11) **(E)**, and **p<0.01 (N=4) **(F)**.

Taking into account the architecture and properties of the domains that compose Caspr1, and the evidence implicating the intracellular portion of Caspr1 in the regulation of AMPARs (Santos *et al.*, 2012), we dissected the molecular determinants of Caspr1 underlying its effect on GluA1 total levels. To achieve this, we generated two constructs expressing deletion mutants of Caspr1, lacking either the GNP motif (Caspr1ΔGNP) or the proline-rich domain (Caspr1ΔPRO) present in the intracellular C-tail of wild-type (WT) Caspr1 (Figure 3.2A). We expressed GluA1 in COS7 cells, alone or together with either the full-length WT Caspr1 or the deletion mutants Caspr1ΔPRO or Caspr1ΔGNP, and evaluated relative levels of GluA1 mRNA by qPCR (Figure 3.2B). We observed that, whilst WT Caspr1 or its mutant form lacking the GNP motif (Caspr1ΔGNP) significantly upregulate GluA1 mRNA levels (1.69 ± 0.25- and 1.65 ± 0.44-fold increase respectively, when compared to control; Figure 3.2B), the Caspr1 deletion mutant lacking its proline-rich sequence (Caspr1ΔPRO) failed to increase GluA1 mRNA levels, which were comparable to mRNA levels in the absence of Caspr1 (Figure 3.2B). These results indicate that the proline-rich sequence located in the intracellular C-tail of Caspr1 is the one responsible for mediating the upregulation of GluA1 transcripts. To test if the same domain in Caspr1 is required for the upregulation of GluA1 protein levels by Caspr1, we expressed GluA1 alone, or together with either WT Caspr1 or the Caspr1ΔPRO deletion mutant, and evaluated GluA1 protein levels by WB analysis (Figure 3.2C). As expected, expression of full-length WT Caspr1 in COS7 cells resulted in a marked increase of 65.7 ± 21.2% on GluA1 total protein levels, when compared to control values. However, when cells were transfected with the

deletion mutant Caspr1ΔPRO, the increase on GluA1 total protein was lost, with GluA1 levels similar to control (96.4 ± 19.0%, Figure 3.2D).

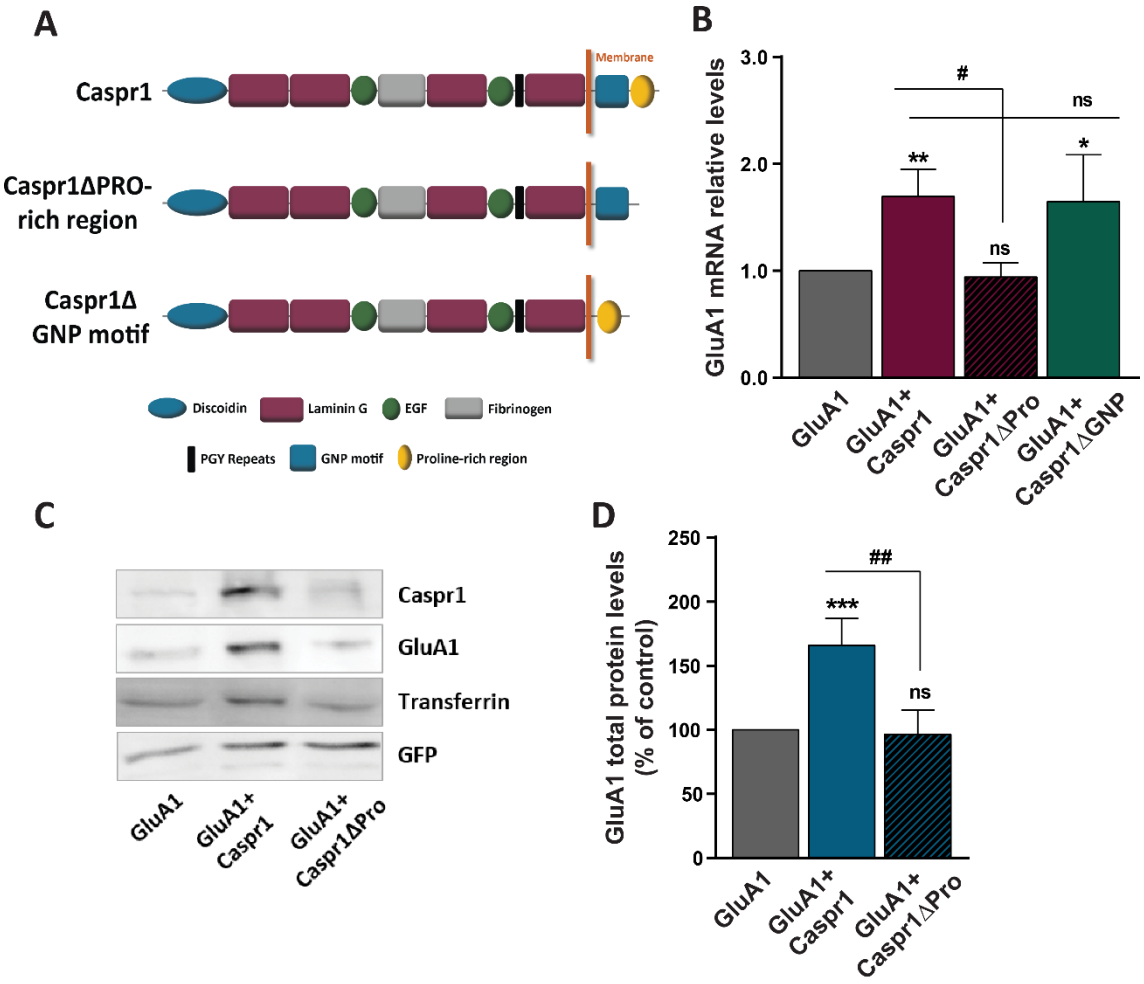


Figure 3.2 – Caspr1 regulates total protein and mRNA levels of the GluA1 subunit of AMPARs through its intracellular proline-rich domain. **(A)** Diagram of the protein domains present in Caspr1 and in the deletion mutants of Caspr1 used in this study: Caspr1ΔPRO and Caspr1ΔGNP. **(B-D)** The intracellular proline-rich domain of Caspr1 is required for the regulation of GluA1 mRNA **(B)** and total protein **(C, D)** levels. GluA1 was expressed in COS7 cells, alone or together with the full-length WT Caspr1 or with either one of the Caspr1 deletion mutants, as indicated. 48 h after transfection total RNA **(B)** and total protein **(C, D)** extracts were obtained. **(B)** Relative levels of GluA1 mRNA were evaluated by real-time PCR. Results are presented as mean ± S.E.M., when compared to control and normalized to the reference gene GFP (control of transfection). Statistical significance was determined by the non-parametric Kruskal-Wallis Test, followed by the Dunn's Multiple Comparison Test, **p<0.01 and *p<0.05 when compared to control (GluA1), and #p<0.05 when compared to GluA1+Caspr1 (N≥4). **(C)** Protein levels of GluA1 and Caspr1 were assessed by Western blot. **(D)** Results are presented as mean ± S.E.M., when compared to control and normalized to the loading control Transferrin. Statistical significance was determined by the non-parametric Kruskal-Wallis Test, followed by the Dunn's Multiple Comparison Test, ***p<0.001 when compared to control (GluA1), and ##p<0.01 when compared to GluA1+Caspr1 (N≥4).

Altogether, this first set of results indicates that Caspr1 regulates total amounts of the GluA1 subunit of AMPARs, and confirms that this occurs as a consequence of GluA1 mRNA regulation. Moreover, the evidence pinpoints a specific requirement for the proline-rich sequence of Caspr1 in mediating its effect on GluA1-containing AMPARs.

Caspr1 modulates the phosphorylation status of the RNA-binding protein ZBP1 through activation of the Src kinase signalling cascade

We demonstrated that total levels of the GluA1 subunit of AMPARs are regulated by the cell-adhesion molecule Caspr1, which increases levels of GluA1 protein as a consequence of a post-transcriptional regulation of GluA1 mRNA. Furthermore, we showed that the proline-rich sequence located in the intracellular C-tail region of Caspr1 is responsible for the observed effects on GluA1 levels. This evidence is rather interesting, as it starts hinting at the possible mechanisms underlying the role of Caspr1 in regulating AMPARs. The proline-rich domain of Caspr1 contains at least one canonical SH3 domain-binding site and is thought to interact with proteins involved in signalling pathways. Indeed, in previous studies Caspr1 was selectively pulled-down bound to the SH3 domain of several signalling molecules, namely PLCγ, the p85 subunit of PI3K, and members of the Src family of protein tyrosine kinases, namely Src and Fyn (Peles *et al.*, 1997). It is possible that Caspr1, through its proline-rich region, interacts with these signalling molecules to trigger the activation of biochemical responses that lead to a specific regulation of AMPARs. For instance, the interaction between Caspr1 and the tyrosine kinase Src has already been thoroughly confirmed by co-immunoprecipitation (Peles *et al.*, 1997). One curious detail about Src is that its activation can be mediated through interaction of a binding partner with its SH3 domain, which results in conformational changes in the structure of Src, such that a key tyrosine residue (Y416) is exposed to auto-phosphorylation, ultimately rendering Src active (Dikic *et al.*, 1996). Interestingly, Src has already been shown to be enriched in the PSD (Huang *et al.*, 2001), and several studies implicate Src on multi-level regulation of both glutamate receptors of the NMDA-type and synaptic plasticity [reviewed in (Salter & Kalia, 2004)].

Taking into account the fact that 1) Caspr1 regulates GluA1-containing AMPARs through its intracellular proline-rich sequence, 2) Caspr1, through the same domain, is able to interact with the tyrosine kinase Src, and 3) Src is known to play established roles in synaptic plasticity through regulation of NMDARs, we hypothesized that Caspr1 is able to regulate GluA1 levels through interaction with Src and activation of its downstream signalling cascade. To confirm this, we exogenously expressed Caspr1 in COS7 cells, together with the GluA1 subunit, and

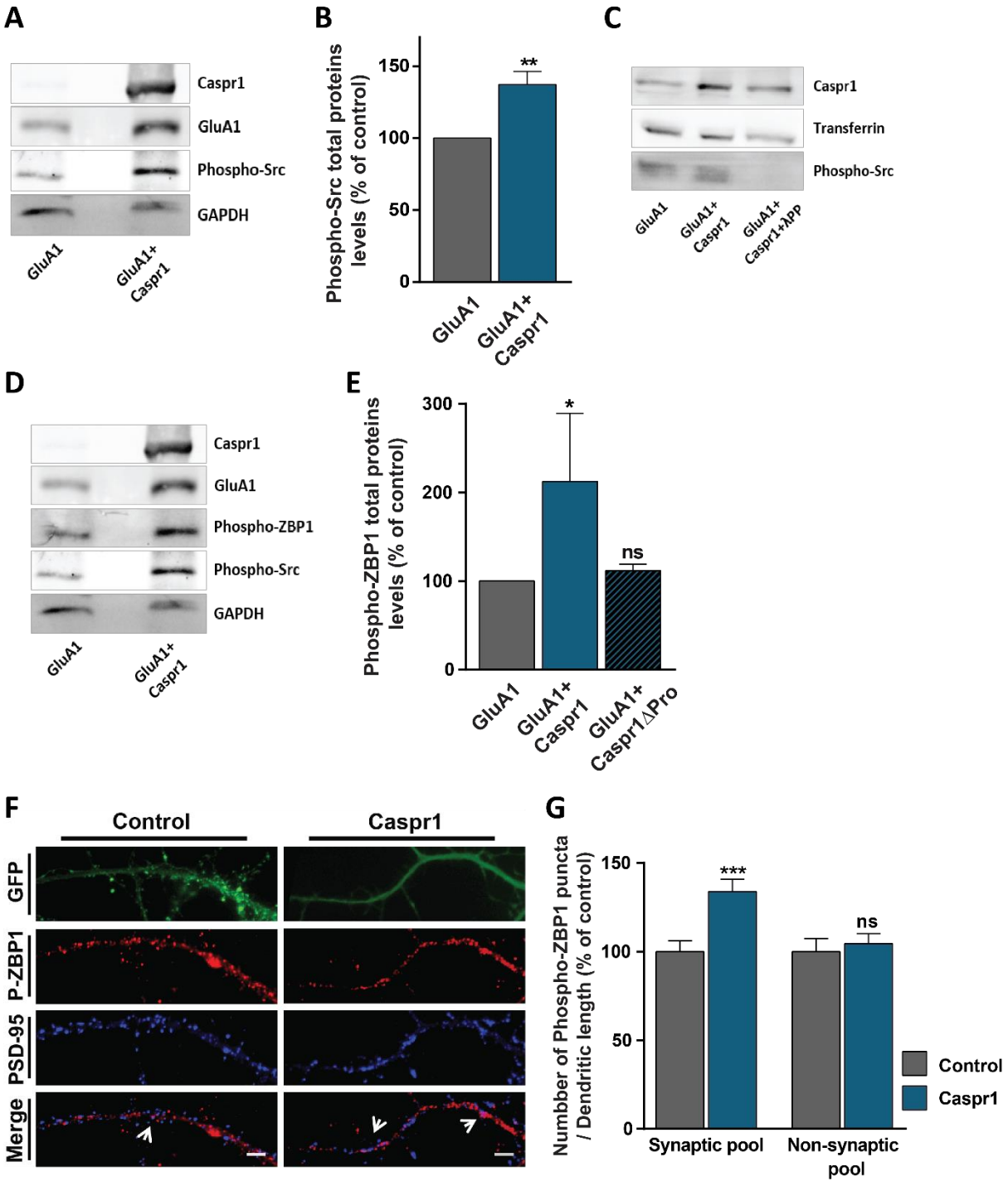
evaluated levels of the tyrosine kinase Src phosphorylated at Y416, by western blot analysis (Figure 3.3A). Indeed, expression of Caspr1 in COS7 cells resulted in a significant increase of 37.2 ± 9.1% on total levels of phosphorylated Src, when compared to control (Figure 3.3B). To confirm that the increase that we measured was a specific result of phosphorylation, we performed a dephosphorylation assay. We incubated lysates obtained from Caspr1-transfected COS7 cells with the protein phosphatase Lambda (λPP), and evaluated levels of phosphorylated Src by WB (Figure 3.3C). WB analysis of COS7 cells expressing Caspr1 revealed an increase in the labelling of a band of 70 kDa, detected by the anti-phospho Src antibody, which disappeared when samples were subjected to dephosphorylation with λPP, confirming that we are measuring a specific product of increased phosphorylation. As previously mentioned, interactions with the SH3 domain of Src result in its auto-phosphorylation at Y416, and consequent activation. As such, our results confirm that increased expression of Caspr1 (which interacts with the SH3 domain of Src) in COS7 cells renders the tyrosine kinase Src active, likely triggering a downstream signalling cascade.

Despite rather promising, the results we have obtained so far keep raising fundamental questions. We have shown that Caspr1 is capable of inducing the activation of a major signalling mediator, known to trigger various downstream biochemical responses that control a wide-range of physiological mechanisms. Indeed, Src has already been identified as an ubiquitous regulator of several cellular processes that include cell growth and survival, proliferation, cell communication, shape, differentiation and migration [extensively reviewed in (Huveneers & Danen, 2009; Ingley, 2008; Parsons & Parsons, 2004)]. In the CNS, Src has also been thoroughly implicated in neuronal differentiation and neurite outgrowth, as well as in the regulation of ion channel activity and synaptic transmission (Salter & Kalia, 2004).

One interesting downstream target of the signalling cascade triggered by Src is the oncofetal protein Zipcode-binding protein 1 (ZBP1). ZBP1 is a RNA-binding protein best known for its crucial role in the regulation of β-actin mRNA transport and local translation. ZBP1 is able to induce both translational silencing of β-actin mRNA and its incorporation into ribonucleoproteins for transport, through binding to a conserved zipcode present in the 3'UTR of β-actin transcripts (Oleynikov & Singer, 2003; Ross *et al.*, 1997; Wu *et al.*, 2015). Upon specific stimuli, Src phosphorylates ZBP1 at a key tyrosine residue (Y396) required for its binding to RNA, resulting in the release of β-actin mRNA from ZBP1 and activation of its translation at sites of high actin dynamics (Huttelmaier *et al.*, 2005; Sasaki *et al.*, 2010; Wu *et al.*, 2015). This temporal and spatial control over β-actin synthesis by ZBP1 plays critical roles in several mechanisms that range from the regulation of axonal guidance and neurite outgrowth (Huttelmaier *et al.*, 2005; Sasaki *et al.*, 2010; Welshhans & Bassell, 2011), dendritic

morphology and branching (Perycz *et al.*, 2011; Urbanska *et al.*, 2017), to dendritic spine structure and density (Eom *et al.*, 2003; Tiruchinapalli *et al.*, 2003). Furthermore, ZBP1 has also been implicated in the regulation of several other target mRNAs, including the mRNAs for cofilin and β-catenin (Gu *et al.*, 2008; Jonson *et al.*, 2007; Piper *et al.*, 2006). Altogether, it is evident that ZBP1 plays a key role in the control of transcripts with major importance in neuronal development and function, which endows ZBP1 with the potential to functionally regulate other neuronal transcripts.

The evidence reported so far in this chapter indicates that Caspr1 regulates the levels of GluA1 in a manner that depends on its intracellular proline-rich domain. In turn, through the same domain, Caspr1 promotes the activation of the tyrosine kinase Src. Taking into account that ZBP1 is a downstream target of Src, capable of regulating several transcripts, it would be extremely interesting to test if Caspr1 regulates ZBP1 as a downstream effect of the activation of Src. To achieve this, Caspr1 or its deletion mutant for the proline-rich sequence, Caspr1ΔPRO, were exogenously expressed in COS7 cells, together with the GluA1 subunit, and total protein levels of ZBP1 phosphorylated at Y396 (target for Src-dependent phosphorylation) were assessed by WB analysis (Figure 3.3D). We observed that expression of Caspr1 in COS7 cells resulted in a significant 112.5 ± 77.0% increase on total levels of phosphorylated ZBP1, when compared to control (Figure 3.3E). On the other hand, the Caspr1ΔPRO deletion mutant of Caspr1 completely failed to increase the phosphorylation of ZBP1, whose levels were comparable to the control (Figure 3.3E). Given the requirement for the proline-rich domain of Caspr1, these results suggest that Caspr1 regulates the phosphorylation status of ZBP1 downstream of the activation of the Src signalling pathway. Overall, these results confirm that Caspr1 controls the RNA-binding protein ZBP1 downstream of the activation of Src kinase, and suggest a possible role of ZBP1 in regulating GluA1 mRNA.



[Figure 3.3 - subtitle on the next page**]**

Figure 3.3 – Caspr1 regulates the phosphorylation status of the RNA-binding protein ZBP1, by activating the signalling cascade downstream of the tyrosine kinase Src. **(A-E)** Caspr1 expression in COS7 cells increases levels of phosphorylated tyrosine kinase Src **(A-C)** and phosphorylated ZBP1 **(D, E)**. COS7 cells were transfected with GluA1, alone or together with either WT Caspr1 or the deletion mutant Caspr1ΔPRO, as indicated, and allowed to express for 48 h, before total protein extraction. **(A)** Levels of phosphorylated Src, Caspr1 and GluA1 were assessed by WB. **(B)** Total levels of phosphorylated Src were determined. Results are presented as mean ± S.E.M., when compared to control and normalized to the loading control GAPDH (N=5). Statistical significance was determined by the non-parametric Mann-Whitney Test, **p<0.01. **(C)** To confirm the specificity of the phospho-Src signal, protein extracts from Caspr1-transfected cells were subjected to a dephosphorylation assay with the lambda protein phosphatase (GluA1 + Caspr1 + λPP) for 2 h, at 30°C. Levels of phosphorylated Src were then assessed by WB. **(D)** Levels of phosphorylated ZBP1 were assessed by WB in parallel with GluA1 and Caspr1. **(E)** Total levels of phosphorylated ZBP1 were determined. Results are presented as mean ± S.E.M., when compared to control and normalized to the loading control GAPDH (N=4). Statistical significance was determined by the non-parametric Kruskal-Wallis Test, followed by the Dunn's Multiple Comparison Test, *p<0.05. **(F, G)** Overexpression of Caspr1 in hippocampal neurons increases the phosphorylation of ZBP1 in synapses. Cultured hippocampal neurons were transfected at DIV7, as indicated. At DIV15, cells were fixed and immunolabeled against phospho-ZBP1 and PSD95. **(F)** Representative immunofluorescence images of transfected, GFP-positive neurons stained for phospho-ZBP1 and PSD95. Scale bars represent 5 µm. **(G)** Phospho-ZBP1 clustering was quantified and co-localized with PSD95 to assess the number of synaptic phospho-ZBP1 puncta. Puncta in the non-synaptic pool were determined by subtracting the synaptic fraction of phospho-ZBP1 from the number of total puncta. Results are presented as mean ± S.E.M., when compared to control (N≥3, n≥ 30cells). Statistical significance was determined by the non-parametric Mann-Whitney Test, ***p<0.001.

To test whether Caspr1 can regulate ZBP1 in a neuronal system, we transfected low-density hippocampal neurons with either GFP alone or together with Caspr1. Cells were then fixed and immunolabeled with antibodies against phosphorylated ZBP1, and the postsynaptic marker PSD95 to visualize excitatory synapses. To specifically evaluate the effect of Caspr1, imaging fields were chosen by the GFP channel for the presence of transfected, GFP-positive neurons (Figure 3.3F). The clustering of phosphorylated ZBP1 was quantified and co-localized with PSD95 to determine the number of phospho-ZBP1 puncta at synapses. We observed that overexpression of Caspr1 in hippocampal neurons resulted in a significant 33.9 ± 6.9% increase in the number of synaptic puncta of phosphorylated ZBP1, when compared to control (Figure 3.3G). Interestingly, Caspr1 regulates ZBP1 phosphorylation selectively at synapses, since we observed that Caspr1 failed to increase the phosphorylation of the non-synaptic pool of ZBP1 puncta (Figure 3.3G). Indeed, there is evidence showing large ZBP1 granules, carrying β-actin mRNA, localized in dendrites that rapidly translocate to spines in response to synaptic activity (Eom *et al.*, 2003; Tiruchinapalli *et al.*, 2003). Other studies have reported that dendritic granules containing β-actin mRNA, and most likely ZBP1, specifically disassemble

following induction of chemical LTP or KCl-induced neuronal depolarization, to subsequently allow the local translation of β-actin (Buxbaum *et al.*, 2014; Park *et al.*, 2014; Whalley, 2014). Because the translational competence of ZBP1 depends on its phosphorylation status, these studies suggest that ZBP1 must undergo phosphorylation nearby synapses in order to restrict the translation of β-actin with such spatial resolution following synaptic activity. It is conceivable that the expression of Caspr1, through activation of the Src signalling pathway, regulates the phosphorylation status of ZBP1 in a similar manner, restricting it specifically to synaptic granules.

Altogether, this set of results starts to uncover the mechanisms underlying the effect of Caspr1 in the regulation of AMPARs, through activation of an important signalling cascade that involves the control over the function of a RNA-binding protein.

ZBP1 binds to GluA1 mRNA and further regulates the surface expression and synaptic content of GluA1-containing AMPARs

The results presented so far show that Caspr1 regulates total levels of GluA1 by modulating GluA1 mRNA. Additionally, we identified a promising signalling pathway, activated by Caspr1, which ultimately controls the phosphorylation status of the RNA-binding protein ZBP1. Correlating these pieces of evidence, we hypothesize that the post-transcriptional effect of Caspr1 on GluA1 mRNA is potentially mediated by ZBP1.

ZBP1 is a RNA-binding protein known to play important roles in the stability, localization and translational control of target mRNAs (Yisraeli, 2005). The protein contains a characteristic arrangement of six canonical RNA-binding domains (RBD), consisting of two RNA recognition motifs (RRMs) followed by four hnRNP K-homology (KH) domains (Ross *et al.*, 1997). Two of these KH domains, KH3 and KH4, recognize and preferentially bind to a 54-nucleotide-long zipcode sequence typically located in the 3'UTR of target mRNAs, and initially identified in the mRNA coding for β-actin (Chao *et al.*, 2010; Kislauskis *et al.*, 1994; Ross *et al.*, 1997). The interaction of ZBP1 with β-actin mRNA is one of the best described and understood examples of mRNA control by RNA-binding proteins. By binding to the zipcode present in the transcript, ZBP1 is able to translationally repress β-actin mRNA and mediate its transport in a dormant state to sites where β-actin synthesis is most required, thus providing an efficient and energy-saving mechanism to control the translation of β-actin both temporally and spatially (Huttelmaier *et al.*, 2005; Oleynikov & Singer, 2003; Sasaki *et al.*, 2010; Welshhans & Bassell, 2011). Despite several studies and evolution of methodologies, the identification of mRNA

ligands that are targeted by ZBP1 has proven hard to achieve, although some, besides β-actin, have already been determined, including Igf2, tau, c-myc, cofilin, spinophilin and β-catenin [(Atlas *et al.*, 2004; Gu *et al.*, 2008; Jonson *et al.*, 2007; Patel *et al.*, 2012; Piper *et al.*, 2006) and reviewed in (Yisraeli, 2005)]. The important role that ZBP1 seems to play in the control of mRNA localization and translation, particularly of mRNAs that are crucial for neuronal function, raises the possibility that many more important neuronal transcripts might be regulated by ZBP1.

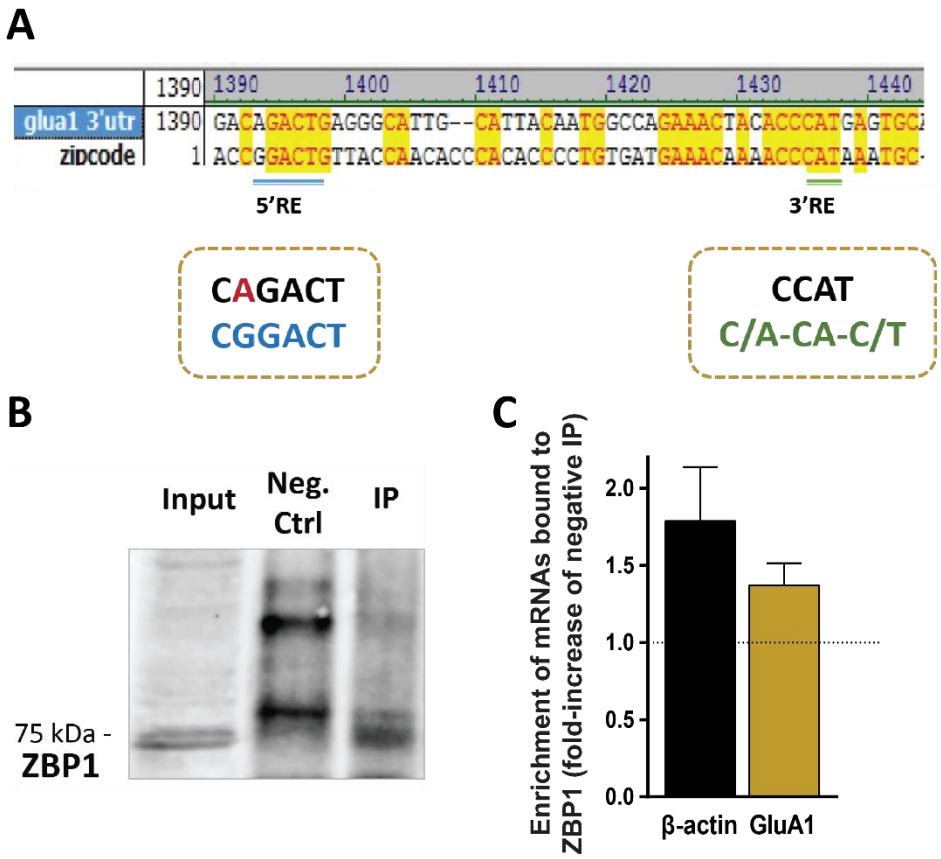


Figure 3.4 – ZBP1 binds to and regulates mRNA levels of the GluA1 subunit of AMPARs in an activity-dependent manner. **(A)** Sequence alignment between the 3'UTR of GluA1 mRNA and the 54-nucleotide zipcode in β-actin targeted by ZBP1. Homologous nucleotides in both sequences are highlighted in yellow. The bipartite 5' and 3' response elements targeted by ZBP1 KH4 and KH3 domains are underlined in blue and green, respectively, and highlighted in the boxes. **(B)** ZBP1 was immunoprecipitated from 15DIV cultured hippocampal neurons using a specific antibody against the protein. IP was confirmed by WB analysis of ZBP1 levels when compared to the initial protein sample (input) and a negative IP using non-immune IgGs. **(C)** ZBP1 binds to GluA1 mRNA in hippocampal neurons. Following RIP of ZBP1 in RNAse-free conditions, total RNAs bound to ZBP1 were isolated and levels of β-actin and GluA1 mRNA were assessed by RT-qPCR. Results are presented as mean ± S.E.M., when mRNA levels obtained from the ZBP1 RIP are compared to the negative RIP with non-immune IgGs (N=4).

Taking into consideration the canonical function of ZBP1, together with our evidence implicating Caspr1 in the control of ZBP1 phosphorylation, as well as in the regulation of GluA1 mRNA, we evaluated if ZBP1 can bind to and regulate GluA1 mRNA. Sequence alignment of the 3'UTR of the GluA1 mRNA and the 54-nucleotide β-actin zipcode revealed 54% identity between them in a particular region of the GluA1 3'UTR (Figure 3.4A), which is quite significant considering that there is a lot of variability amongst the zipcodes present in distinct mRNAs targeted by ZBP1 (Kislauskis *et al.*, 1994). Previous work, however, has shown that the KH4 and KH3 domains of ZBP1 recognize a bipartite RNA sequence, with KH4 binding to a highly conserved element (CGGACT) near the 5' end of the zipcode, and KH3 binding to the 3' element (C/A-CA-C/U) (Patel *et al.*, 2012). These consensus sites are present in the 3'UTR of GluA1, despite one substitution in the 5' response element (Figure 3.4A). Taking this into consideration, we evaluated if ZBP1 indeed binds to GluA1 mRNA. We performed a ribonucleoprotein immunoprecipitation (RIP) protocol with a specific antibody to pull-down ZBP1 from cultured hippocampal neurons, and isolated RNAs bound to the precipitate (Figure 3.4B, C). To confirm the specificity of the IP, a fraction of the precipitate was used for WB analysis, where we identified a specific pulled-down 75 kDa-band corresponding to total ZBP1 (Figure 3.4B). RT-qPCR analysis of the mRNAs isolated from the ZBP1 RIP revealed an enrichment in GluA1 mRNA when compared to the control RIP performed using non-immune IgGs, in levels that did not differ significantly from the typical ZBP1 target, β-actin (Figure 3.4C).

Overall, this result confirms that ZBP1 binds to GluA1 transcripts and pinpoints ZBP1 as a potential novel regulator of GluA1 mRNA. Moreover, this evidence further substantiates our hypothesis of ZBP1 being responsible for the post-transcriptional effect of Caspr1 over GluA1 mRNA. Considering the canonical function attributed to ZBP1, future experiments will be required to fully understand how ZBP1 regulates GluA1 mRNA, and, in particular, ascertain if ZBP1 exerts a translational control over GluA1 that is required for its local synthesis in dendrites and near synaptic sites.

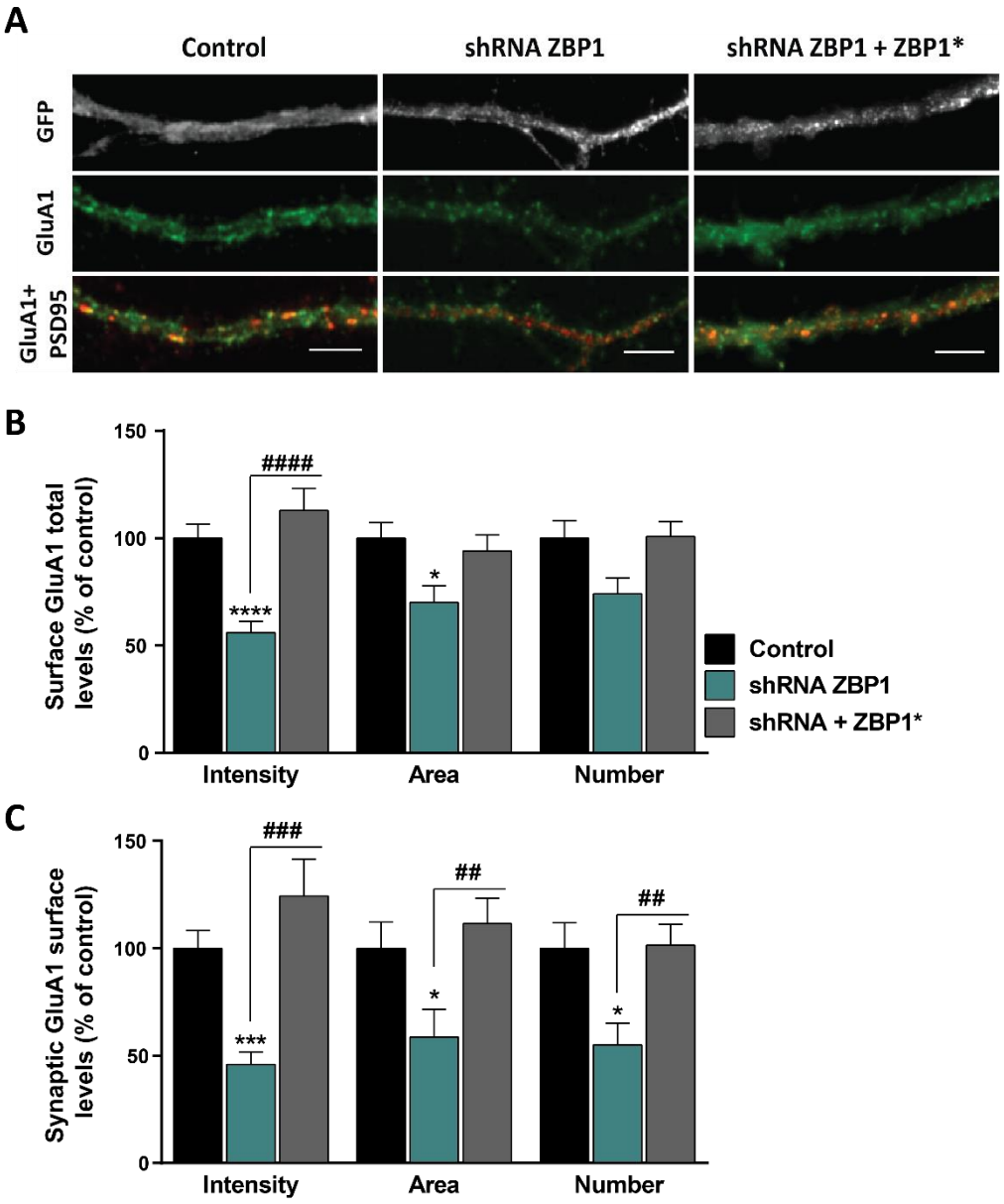


Figure 3.5 – ZBP1 is required to regulate the surface expression and synaptic content of GluA1-containing AMPARs. (A) Representative immunofluorescence images of GFP-positive low-density cultured hippocampal neurons transfected at DIV7 either with a scrambled shRNA construct (control - left), a plasmid encoding GFP and ZBP1-shRNA (middle), or with the ZBP1-shRNA plasmid and a construct encoding the ZBP1 rescue mutant (ZBP1* - right). At DIV15, cells were live-stained with an antibody against an extracellular epitope in the GluA1 N-terminus, fixed, and then stained for the synaptic marker PSD95. Scale bars represent 5µm. **(B)** The intensity, area and number of surface GluA1 total clusters were quantified, and **(C)** synaptic GluA1 clusters were determined by colocalization with the postsynaptic marker PSD95. Total number of analysed cells was obtained from at least three independent experiments (N≥3; n≥30 cells, and results are presented as mean ± S.E.M. Statistical significance was determined by the non-parametric Kruskal-Wallis Test, followed by the Dunn's Multiple Comparison Test, *p<0.05, ***p<0.001 and ****p<0.0001 when compared to control, and #p<0.05, ##p<0.01 and ####p<0.0001 when relative to the ZBP1 shRNA condition.

Taking into account the previous results, together with the role that Caspr1 plays in regulating the synaptic content of GluA1-containing AMPARs, and the fact that Caspr1 modulates the function of ZBP1, particularly in synapses, we evaluated if ZBP1 is required to regulate the surface expression of AMPARs. Low-density cultured hippocampal neurons were transfected with a plasmid encoding GFP and expressing a shRNA sequence against ZBP1 (Perycz *et al.*, 2011), to knock-down its endogenous expression, or with a control scrambled shRNA construct. We then performed quantitative immunofluorescence analysis of the surface expression of GluA1 by live-staining neurons with an antibody against an extracellular epitope in the GluA1 N-terminus. After fixation, neurons were stained for PSD95 as a postsynaptic marker to visualize excitatory synapses. Imaging fields were chosen by the presence of transfected, GFP-positive neurons (Figure 3.5A). We observed that loss of ZBP1 in hippocampal neurons resulted in a significant decrease of approximately 50% in the intensity of surface GluA1 total clusters (56.0 ± 5.3%; Figure 3.5B). The area and number of GluA1 clusters were also markedly reduced following knock-down (KD) of ZBP1. Furthermore, loss of ZBP1 induced a significant decrease in the synaptic content of GluA1 clusters, as determined by colocalization with the postsynaptic marker PSD95 (Figure 3.5C). The intensity, area and number of surface GluA1 synaptic clusters were significantly decreased by over 50% when compared to GluA1 labelling in neurons transfected with a control scrambled shRNA construct.

To exclude the contribution of off-target effects of the ZBP1 shRNA, we performed a rescue experiment by transfecting hippocampal neurons with the ZBP1 shRNA, in parallel with a mutant construct of ZBP1 resistant to the knock-down (ZBP1*, Figure 3.5A - C). Expression of the mutant ZBP1 refractory to the shRNA rescued the KD-mediated decrease of total (Figure 3.5B) and synaptic (Figure 3.5C) GluA1 cluster intensities. The area and number of GluA1 puncta were also rescued to values comparable to the control. These results indicate that the defects in the surface expression of GluA1 clusters observed with the ZBP1 shRNA were specifically caused by the loss of endogenous ZBP1.

We have previously shown that ZBP1, as a RNA-binding protein, binds to GluA1 mRNA. Interestingly, however, the latter set of results suggests that ZBP1 regulation over GluA1 goes beyond a mere control of the transcript, and impacts the surface expression and synaptic content of GluA1-containing AMPARs.

Caspr1 expression and ZBP1 function are both regulated during mechanisms of homeostatic synaptic plasticity

The present study led to the identification of novel players in the regulation of AMPARs. We uncovered a role for Caspr1 in regulating total protein levels of the GluA1 subunit of AMPARs, through a possible post-transcriptional effect on GluA1 mRNA. Moreover, we demonstrated that, through its intracellular proline-rich domain, Caspr1 activates a signalling cascade downstream of the tyrosine kinase Src, and regulates the phosphorylation status of the RNA-binding protein ZBP1. Given the physiological importance of AMPARs, one central question that arises is whether Caspr1, and the downstream mechanism triggered by Caspr1, are of relevance in the activity-dependent regulation of AMPARs. Caspr1, as well as both Src and ZBP1, are localized at dendritic spines and at the actual postsynaptic density (Cajigas *et al.*, 2012; Collins *et al.*, 2006; Eom *et al.*, 2003; Huang *et al.*, 2001; Murai *et al.*, 2002; Santos *et al.*, 2012; Tiruchinapalli *et al.*, 2003). Nevertheless, there are very few studies regarding the synaptic function of Caspr1 and ZBP1, whereas Src was already implicated in mechanisms of synaptic plasticity, since LTP induction is impaired upon application of specific Src inhibitors or in animals lacking Src [reviewed in (Salter & Kalia, 2004)].

Taking these pieces of evidence into account, and since Caspr1 is the most upstream effector of the mechanism we have proposed thus far, we asked whether Caspr1 is regulated by neuronal activity. Synaptic scaling is a form of homeostatic plasticity by which neurons elicit changes in their overall synaptic strength to compensate for prolonged alterations in network activity (Turrigiano, 2008). This homeostatic scaling of neuronal activity is typically translated as a change in the accumulation of AMPARs, particularly in the subunit content at excitatory synapses, with many studies reporting a selective regulation of the GluA1 subunit (Aoto *et al.*, 2008; Goel *et al.*, 2006; Goel *et al.*, 2011; Ju *et al.*, 2004; Sutton *et al.*, 2006; Thiagarajan *et al.*, 2005). The molecular mechanisms underlying the scaling of AMPARs in face of chronic changes in activity are still far from understood, but several players have already been implicated, including cell-adhesion molecules, for instance [extensively reviewed in (Chater & Goda, 2014; Fernandes & Carvalho, 2016; Pozo & Goda, 2010; Thalhammer & Cingolani, 2014; Wang *et al.*, 2012)]. Interestingly, the expression or function of many of these players is itself changed by synaptic scaling mechanisms (Schanzenbacher *et al.*, 2016). We evaluated whether endogenous levels of Caspr1 are regulated during homeostatic synaptic scaling. Neuronal activity in cultured hippocampal neurons was blocked for 24 h with 1 µM tetrodotoxin (TTX), a specific blocker of voltage gated Na^+-channels that inhibits action potential generation, and total protein levels of both GluA1 and Caspr1 were evaluated by WB (Figure 3.6A). As previously described (Ju *et al.*, 2004; Wierenga *et al.*, 2005), chronic blockade of neuronal

activity resulted in a significant increase of 82.3 ± 25.6% on total levels of the GluA1 subunit of AMPARs (Figure 3.6B). This result indicates that blocking neuronal activity in hippocampal neurons for 24 h with 1µM TTX induces homeostatic mechanisms that scale up GluA1-containing AMPARs. Interestingly, this increase in GluA1 total levels following chronic neuronal activity blockade with TTX was paralleled by a significant 38.7 ± 10.6% increase on total protein levels of endogenous Caspr1, when compared to control (Figure 3.6B).

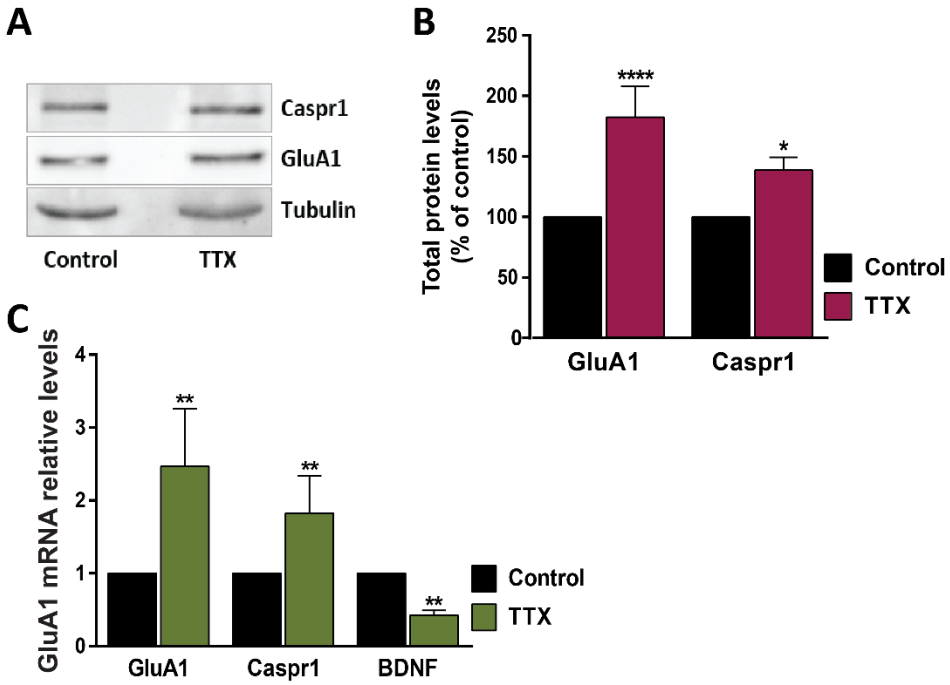


Figure 3.6 – Homeostatic mechanisms of synaptic scaling regulate the expression of endogenous Caspr1. Cultured rat hippocampal neurons at 14DIV were treated for 24 h with 1µM TTX. **(A)** Protein extracts were obtained and total protein levels of GluA1 and Caspr1 were assessed by WB. **(B)** Results are presented as mean ± S.E.M., when compared to control and normalized to the loading control Tubulin (N=10). Statistical significance was determined by the non-parametric Mann-Whitney Test, ****p<0.0001 and *p<0.05. **(C)** Total RNA was extracted from cell lysates and relative levels of mRNA for the GluA1, Caspr1 and BDNF genes were evaluated by RT-qPCR. Results are presented as mean ± S.E.M., when compared to control (N≥5). Statistical significance was determined by the non-parametric Mann-Whitney Test, **p<0.01.

These results show that endogenous Caspr1 protein levels can be regulated by paradigms that elicit homeostatic plasticity; however, they fall short on explaining how this is happening. One possibility to answer this question comes from evidence pointing to transcriptional mechanisms as the trigger for global synaptic scaling responses following blockade of action potentials with TTX (Ibata *et al.*, 2008). Taking this into consideration, we evaluated endogenous levels of GluA1, Caspr1 and BDNF mRNAs following a chronic period of TTX-induced neuronal inactivity (Figure 3.6C). We observed that chronically blocking action

potentials in hippocampal neurons results in a marked downregulation in mRNA levels of BDNF, whose release is known to be reduced during homeostatic scaling up (Rutherford *et al.*, 1998). On the contrary, prolonged neuronal inactivity induced by TTX application significantly increased not only GluA1 (2.47 ± 0.79-fold increase compared to control) but also Caspr1 mRNA levels (1.82 ± 0.51-fold increase, when compared to control). Overall, these results indicate that the expression of Caspr1, possibly through a transcriptional response, is regulated by mechanisms of homeostatic synaptic scaling, raising the possibility of Caspr1 being required for this type of plasticity.

Taking into account the evidence showing that 1) increased expression of Caspr1 in COS7 cells induces the activation of the signalling pathway downstream of Src, and regulates the phosphorylation status of the RNA-binding protein ZBP1, and that 2) the endogenous expression of Caspr1 is itself upregulated following chronic neuronal inactivity, we hypothesized that the paradigms inducing homeostatic scaling of GluA1 and Caspr1 would also result in activation of the Src signalling pathway and regulation of ZBP1. To confirm this, we evaluated total protein levels of phosphorylated Src and phosphorylated ZBP1 in neurons incubated with 1 µM TTX for 24 h (Figure 3.7A). Indeed, we observed that a prolonged period of inactivity induced by application of TTX in hippocampal neurons resulted in a significant increase of 65.9 ± 17.3% on total levels of phosphorylated Src, when compared to control (Figure 3.7B). Moreover, this blockade of neuronal activity caused a parallel significant increase on the phosphorylation levels of ZBP1 (199.7 ± 36.6%; Figure 3.7B). Furthermore, in agreement to our hypothesis, we observed that the increase in phosphorylation of both Src and ZBP1 following neuronal inactivity parallels the increase on total levels of GluA1 and Caspr1 (Figure 3.7A). These results provide evidence that the signalling pathway downstream of the tyrosine kinase Src is activated endogenously following prolonged inactivity in hippocampal neurons, with increased phosphorylation of the downstream RNA-binding protein ZBP1. Most likely, chronic neuronal inactivity increases the phosphorylation of Src and ZBP1 as a direct consequence of an upregulated expression of endogenous Caspr1 in these conditions. Overall, these results show that paradigms that induce mechanisms of homeostatic synaptic scaling of AMPARs regulate endogenous Caspr1, and further control post-translational modifications on both Src and ZBP1.

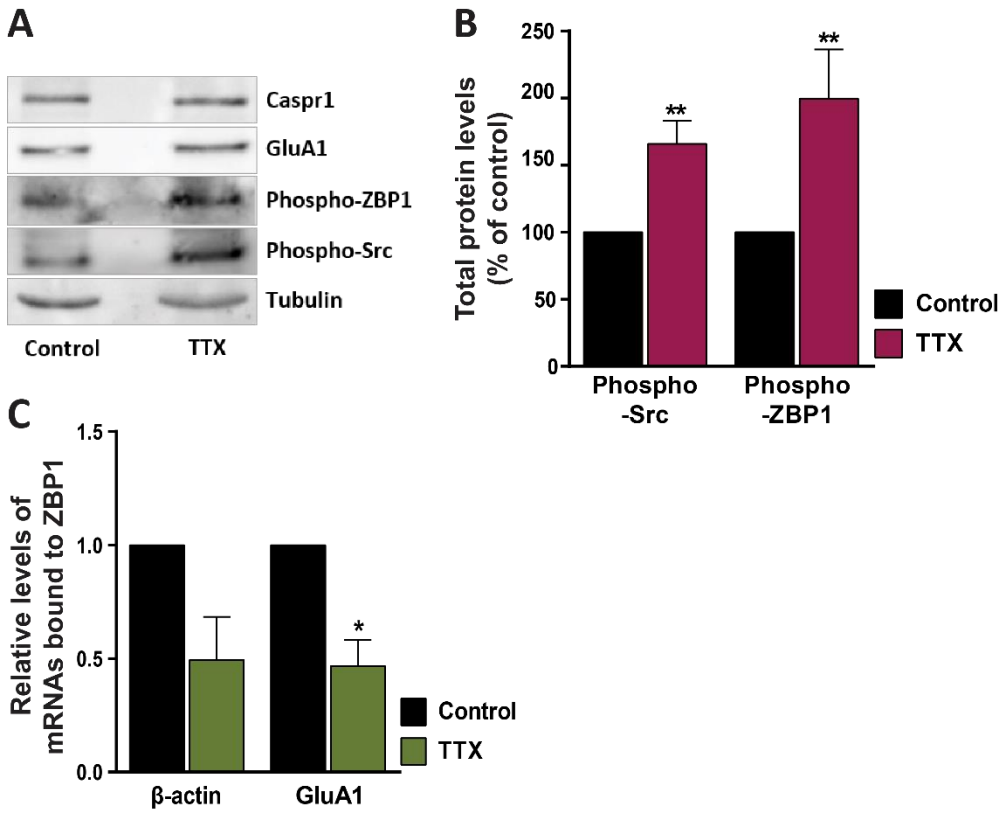


Figure 3.7 – Homeostatic mechanisms of synaptic scaling induce the activation of the signalling cascade downstream of Src, which increases the phosphorylation levels of ZBP1, and consequently reduces its binding to GluA1 mRNA. (A-B) Chronic blockade of activity in hippocampal neurons induces the activation of the signalling pathway downstream of Src and controls phosphorylation levels of ZBP1. Cultured rat hippocampal neurons at 14DIV were treated for 24 h with 1 µM TTX, after which protein extracts were obtained. **(A)** Total protein levels of phosphorylated Src and phosphorylated ZBP1 were evaluated by WB analysis. **(B)** Results are presented as mean ± S.E.M., when compared to control and normalized to the loading control Tubulin (N≥5). Statistical significance was determined by the non-parametric Mann-Whitney Test, **p<0.01. **(C)** Chronic inactivity in hippocampal neurons induces the release of GluA1 mRNA from ZBP1. ZBP1 was immunoprecipitated from 15DIV cultured hippocampal neurons following a TTX-induced 24 h-blockade of neuronal activity. Total RNAs bound to ZBP1 were isolated from the immunoprecipitate and mRNA levels of β-actin and GluA1 were assessed by RT-qPCR. Results are presented as mean ± S.E.M., when compared to control and normalized to the negative RIP with non-immune IgGs (N=4). Statistical significance was determined by the non-parametric Mann-Whitney Test, *p<0.05.

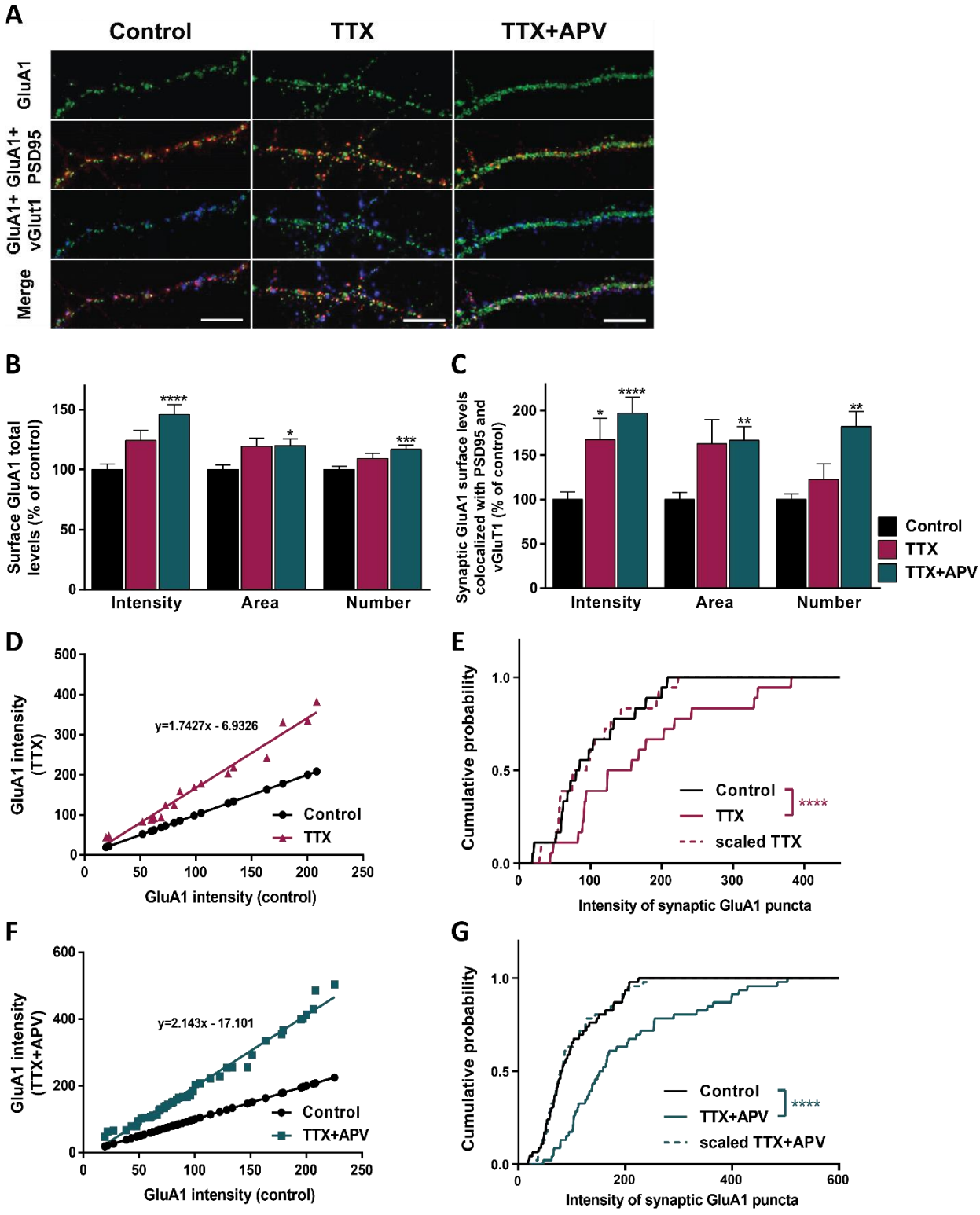
These pieces of evidence are rather interesting as they raise a tantalizing role for ZBP1 in the regulation of homeostatic mechanisms. ZBP1 is modulated by phosphorylation; following Src-dependent phosphorylation in a specific tyrosine residue, ZBP1 undergoes conformational changes that induce the release of bound mRNAs from its grip (Huttelmaier *et al.*, 2005; Sasaki *et al.*, 2010). Previous results in this study revealed that both increased expression of Caspr1 and chronic neuronal inactivity induce an increase in the phosphorylation of ZBP1, possibly

contributing to the release of ZBP1-bound transcripts. Given that ZBP1 binds to GluA1 mRNA in basal conditions, we evaluated their interaction following chronic blockade of neuronal activity (Figure 3.7C). We found that, when activity is chronically blocked in hippocampal neurons, the relative levels of GluA1 mRNA bound to ZBP1 significantly decrease when compared to basal conditions (0.47 ± 0.11-fold decrease compared to control), similarly to an expected decrease in the levels of β-actin mRNA bound to ZBP1.

Firstly, these results are consistent with the previously described phosphorylation-mediated loss of function of ZBP1 (Huttelmaier *et al.*, 2005; Sasaki *et al.*, 2010). Secondly, these results indicate that ZBP1 is able to bind to GluA1 mRNA in basal conditions, possibly repressing its translation. Finally, these pieces of evidence further suggest that homeostatic mechanisms of synaptic plasticity induce an activity-dependent release of GluA1 transcripts from ZBP1, possibly creating an available pool of GluA1 mRNA that can readily undergo translation and contribute to mechanisms underlying the homeostatic scaling of AMPARs. Future experiments should be undertaken in order to determine if ZBP1 exerts a translational control over GluA1 and whether that regulation is required for homeostatic plasticity events. Further along in this chapter we will assess the requirement for ZBP1 in the induction of homeostatic synaptic scaling mechanisms.

Caspr1 is required for the homeostatic synaptic scaling of GluA1-containing AMPARs in hippocampal neurons

So far in the present study we reported that Caspr1 increases total protein levels of the GluA1 subunit of AMPARs in hippocampal neurons, possibly through post-transcriptional regulation of GluA1 mRNA. Also, a previous report from our lab determined that Caspr1 interacts with and regulates the synaptic content of AMPARs (Santos *et al.*, 2012). In particular, while overexpression of Caspr1 leads to a synaptic accumulation of GluA1, loss of endogenous Caspr1 significantly decreases the surface synaptic expression of GluA1-containing AMPARs. Furthermore, in the present study, we show that homeostatic mechanisms of synaptic scaling increase endogenous Caspr1, in parallel to GluA1. Thus, it would be of particular importance to understand if Caspr1 is required for homeostatic mechanisms of synaptic plasticity that scale AMPARs.



[Figure 3.8 - subtitle on the next page**]**

Figure 3.8 – Chronic inactivity in cultured hippocampal neurons induced by 48 h blockade of action potentials, alone or in conjunction with NMDAR inhibition, results in a significant multiplicative scaling of synaptic GluA1-AMPARs. Chronic neuronal inactivity increases the superficial expression of synaptic GluA1-containing AMPARs. Neuronal activity in 13DIV Banker cultured hippocampal neurons was inhibited for 48 h either with 1 µM TTX alone, or together with 100 µM APV. Cells were then live-stained with a specific antibody against an extracellular epitope in GluA1 N-terminus, fixed, and then stained for the synaptic markers PSD95 and vGluT1. **(A)** Representative immunofluorescence images of cultured hippocampal neurons treated for 48 h with TTX or TTX + APV, and stained for superficial GluA1, PSD95 and vGluT1. Scale bars represent 5 µm. **(B)** The intensity, area and number of surface GluA1 total clusters were quantified. Results are presented as mean ± S.E.M. Statistical significance was determined by the nonparametric Kruskal-Wallis Test, followed by the Dunn's Multiple Comparison Test, *p<0.05; ***p<0.001, ****p<0.0001 when compared to the control. **(C)** The intensity, area and number of surface GluA1 clusters that co-localize with synaptic markers (PSD95 + VGluT1) was determined. Total number of analysed cells was obtained from at least two independent experiments (TTX: N=2, n=20 cells; TTX+APV: N≥3, n≥30 cells), and results are presented as mean ± S.E.M. Statistical significance was determined by the nonparametric Kruskal-Wallis Test, followed by the Dunn's Multiple Comparison Test, *p<0.05, **p<0.01, ****p<0.0001 when compared to the control. **(D-G)** Chronic inactivity scales up the intensity of surface GluA1 synaptic puncta by a multiplicative factor. **(D, F)** Ranked intensities of synaptic GluA1 clusters from the control condition were plotted against either ranked TTX **(D)** or TTX + APV **(F)** intensities and the best-fit linear function was determined. **(E, G)** Cumulative distributions of the control and TTX **(E)** or TTX + APV **(G)** surface GluA1 intensities were plotted. The original TTX **(E)** or TTX + APV **(G)** distributions were transformed by their respective best-fit equations and scaled against the control plot (dashed lines). Statistical significance was determined by the Kolmogorov-Smirnov Test, ****p<0.0001

Synaptic scaling is a form of homeostatic plasticity in which neurons adjust their overall synaptic strength, through compensatory changes in the synaptic content of AMPARs, in order to keep network activity within a dynamic physiological range (Fernandes & Carvalho, 2016; Turrigiano, 2008). While many studies resort to electrophysiological measurements of AMPAR-mediated currents to evaluate the scaling in the synaptic content of AMPARs, we used immunocytochemical methods to evaluate the surface expression of synaptic AMPARs following chronic neuronal inactivity. We treated low-density cultured hippocampal neurons for 48 h with 1 µM TTX to block action potential generation, and performed quantitative immunofluorescence analysis of the surface expression of AMPARs by live-staining neurons with an antibody against an extracellular epitope in the GluA1 N-terminus (Figure 3.8A). After fixation, neurons were stained for MAP2 to visualize the dendritic structure (not shown), and for PSD95 (postsynaptic marker) and vGluT1 (presynaptic marker) to visualize excitatory synapses (Figure 3.8A). We observed that chronic inactivity induced by TTX treatment in hippocampal neurons significantly increased the intensity of GluA1 clusters at the synapse, as defined by GluA1 puncta that co-localize with the synaptic markers PSD95 and vGluT1 (167.3

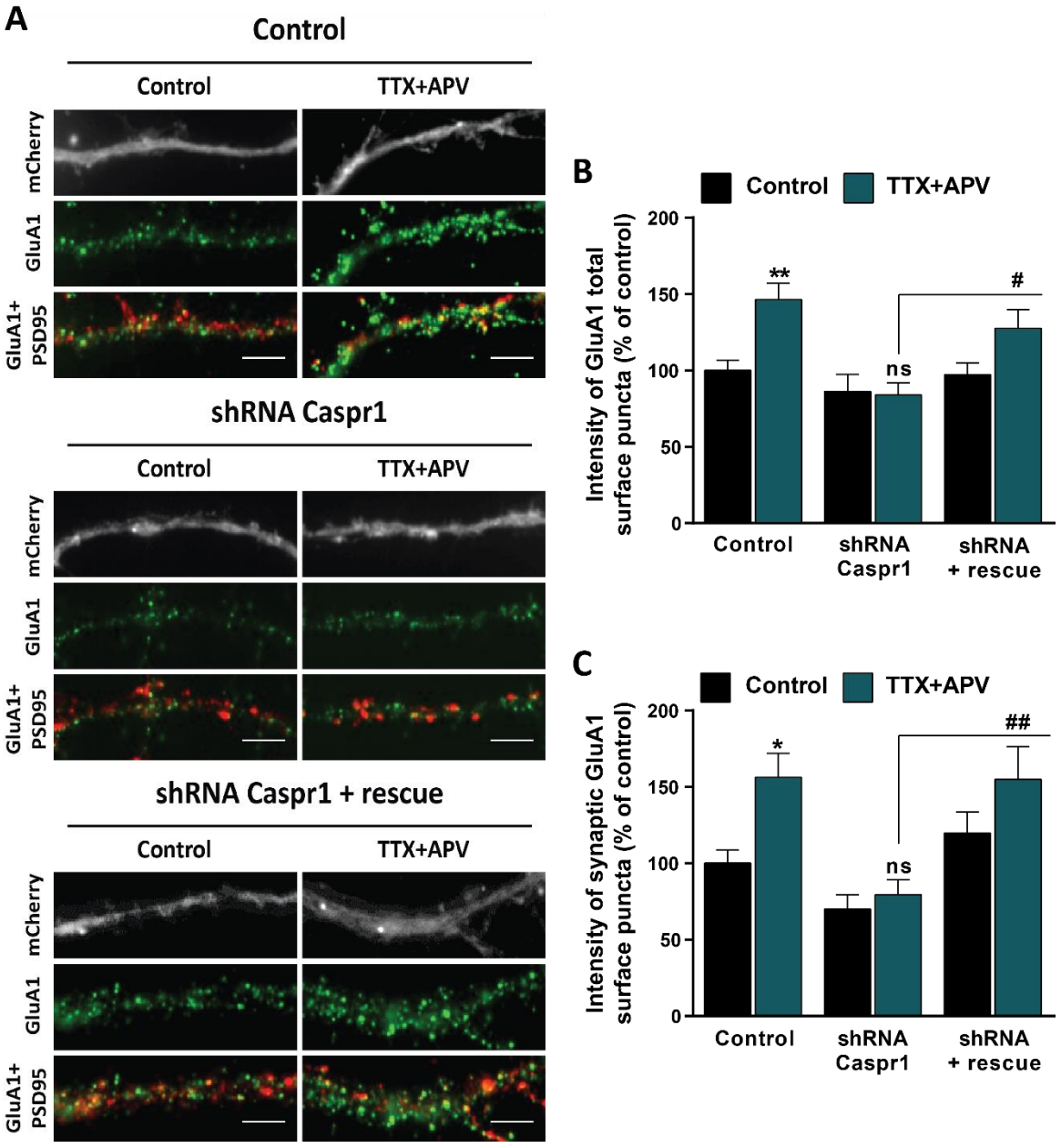
± 23.8%; Figure 3.8C). These results are in agreement with previous studies reporting an increase in the synaptic expression of GluA1 following action potential blockade, both in cortical and hippocampal neurons (Jakawich *et al.*, 2010; Wierenga *et al.*, 2005).

Previous results in this chapter report a homeostatic-dependent regulation of GluA1 mRNA by the RNA-binding protein ZBP1. Considering the overall role of ZBP1 in RNA regulation, it is possible that ZBP1 exerts a temporal and spatial control over the synthesis of GluA1, allowing the translation of its mRNA to occur locally and only when required. Interestingly, recent studies have proposed that synaptic scaling mechanisms can operate locally at single dendritic branches or synapses, in a manner that requires local inhibition of NMDARs in conjunction with action potential blockade, resulting in a selective increase in the dendritic protein synthesis of GluA1 (Aoto *et al.*, 2008; Ju *et al.*, 2004; Sutton *et al.*, 2006). Taking this into account, we decided to evaluate the synaptic content of GluA1-containing AMPARs following chronic neuronal inactivity induced by both action potential blockade together with NMDAR inhibition. For that, we treated cultured hippocampal neurons for 48 h with 1 µM TTX together with 100 µM of a specific NMDAR antagonist, D-(-)-2-Amino-5-phosphonopentanoic acid (APV), and performed quantitative immunofluorescence analysis of the surface expression of GluA1, as previously described (Figure 3.8A). Chronic neuronal inactivity induced by TTX + APV treatment in hippocampal neurons resulted in a significant increase in the intensity of total (145.8 ± 8.1%; Figure 3.8B) and synaptic (197.2 ± 18.0%; Figure 3.8C) surface GluA1 clusters, when compared to control. The area and number of surface GluA1 puncta also increased significantly. Interestingly, the scaling of GluA1 puncta following chronic inactivity induced by TTX + APV treatment was higher than that induced by blocking action potentials alone (Figure 3.8C).

One of the most defining characteristics of synaptic scaling mechanisms is that they uniformly change synaptic strengths across the entire synapse population of a given neuron through the same multiplicative factor (Turrigiano *et al.*, 1998). This uniform regulation allows the conservation of relative differences in strength among synapses, which is crucial for information storage. To confirm that the previously observed scaling of surface GluA1 clusters following chronic neuronal inactivity, induced by either TTX alone or together with APV, occurred in a multiplicative manner, we plotted the ranked values of intensity of synaptic GluA1 clusters from the control condition against ranked TTX (Figure 3.8D) or TTX + APV (Figure 3.8F) GluA1 intensities and found that the data are well fit by a linear function, with a slope of 1.7 (Figure 3.8D) or 2.1 (Figure 3.8F), respectively. Cumulative histograms of the acquired data showed that the entire distribution of GluA1 cluster intensities shifts uniformly and significantly towards larger values in hippocampal neurons treated either with TTX alone

(Figure 3.8E) or with TTX and APV (Figure 3.8G), when compared to basal conditions. Importantly, when the original TTX (Figure 3.8E) or TTX + APV (Figure 3.8G) data were transformed by the respective multiplicative factors, their scaled distributions of GluA1 cluster intensities were almost completely superimposable over the distribution of data from control neurons. This distribution profile is similar to previous reports of TTX-induced multiplicative upscaling of synaptic AMPAR function in neocortical and hippocampal neurons (Jakawich *et al.*, 2010; Turrigiano *et al.*, 1998; Wierenga *et al.*, 2005). This analysis demonstrates that we can evaluate AMPAR synaptic scaling following chronic neuronal inactivity by quantifying total and synaptic clusters of surface GluA1 by immunofluorescence assays.

Taking these pieces of evidence into account and considering the important role of Caspr1 in the regulation of AMPARs, together with the fact that Caspr1 is itself regulated by homeostatic mechanisms, we evaluated if Caspr1 is required for the homeostatic upscaling of synaptic GluA1-containing AMPARs following chronic neuronal inactivity. We knocked-down the endogenous expression of Caspr1 in low-density cultured hippocampal neurons by transfection with a bicistronic plasmid encoding mCherry and a specific shRNA sequence against Caspr1 (Anderson *et al.*, 2012). We then treated neurons for 48 h with 1 µM TTX together with 100 µM APV, and performed quantitative immunofluorescence analysis of the surface expression of GluA1, as previously described (Figure 3.9A). After fixation, neurons were stained for PSD95 as a postsynaptic marker of excitatory synapses. Imaging fields were chosen by the presence of transfected, mCherry-positive hippocampal neurons (Figure 3.9A). As shown earlier, chronic inactivity induced by blockade of action potentials and NMDAR inhibition resulted in a significant increase on the intensity of total (146.4 ± 10.8%; Figure 3.9B) or PSD95-colocalized synaptic (156.2 ± 15.7%; Figure 3.9C) surface GluA1 clusters. On the other hand, knocking-down (KD) the endogenous expression of Caspr1 with a specific shRNA prevented the scaling of surface and synaptic GluA1 puncta induced by chronic inactivity (Figure 3.9B, C). To exclude the contribution of off-target effects of the Caspr1 shRNA, we rescued Caspr1 levels by transfecting hippocampal neurons with the Caspr1 shRNA and in parallel with a mutant construct of Caspr1 resistant to the shRNA (Figure 3.9A - C). Indeed, expression of the Caspr1 construct refractory to shRNA significantly rescued the KD-mediated decrease of GluA1 levels, and further led to upscaling of total (127.6 ± 12.3%; Figure 3.9B) and synaptic (154.9 ± 21.6%; Figure 3.9C) GluA1 cluster intensities following chronic blockade of activity. These results indicate that the defects in the surface expression of GluA1 clusters observed with the shRNA were specifically caused by the loss of endogenous Caspr1. Altogether, this set of results indicates that Caspr1 is required for the homeostatic regulation of synaptic GluA1-containing AMPARs.


A
Control
Control
TTX+APV
mCherry
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Intensity of GluA1 total surface puncta (% of control)
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Intensity of synaptic GluA1 surface puncta (% of control)
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Control
shRNA Caspr1
shRNA + rescue

Figure 3.9 – Loss of endogenous Caspr1 prevents homeostatic upscaling of synaptic GluA1-containing AMPARs upon chronic inactivity in hippocampal neurons. (A) Representative immunofluorescence images of mCherry-positive low-density cultured hippocampal neurons transfected at DIV7 either with an empty vector construct (control - top), a Caspr1-shRNA plasmid (middle), or with the Caspr1-shRNA plasmid together with a plasmid encoding the Caspr1 rescue mutant (bottom). At DIV13, neurons were treated for 48 h with 1 µM TTX and 100 µM APV to chronically block neuronal activity. Cells were then live-stained with a specific antibody against an extracellular epitope in GluA1 N-terminus, fixed, and then stained for the synaptic marker PSD95. Scale bars represent 5 µm. **(B)** The intensity of surface GluA1 total clusters was quantified, and **(C)** synaptic GluA1 clusters were determined by colocalization with the postsynaptic marker PSD95. Total number of analysed cells was obtained from three independent experiments (N=3; n≥30 cells), and results are presented as mean ± S.E.M. Statistical significance was determined by the parametric two-way analysis of variance (ANOVA) Test, followed by the Tukey's Multiple Comparison Test, *p<0.05, **p<0.01 when compared to control, #p<0.05, ##p<0.01 when relative to the shRNA Caspr1 TTX+APV condition, and ns - non significant when the shRNA Caspr1 TTX+APV condition is compared to the control Caspr1 KD.

Caspr1 plays a fundamental role in the regulation of the GluA1 subunit of AMPARs, in basal conditions and during homeostatic scaling mechanisms triggered by prolonged changes in neuronal activity. Since the GluA2 subunit is also fundamental for adequate plasticity mechanisms, and several studies report a regulation of GluA2 during homeostatic synaptic scaling events (Anggono *et al.*, 2011; O'Brien *et al.*, 1998; Wierenga *et al.*, 2005), we evaluated if Caspr1 is required to control the synaptic content of GluA2. To determine this, we knocked-down the expression of Caspr1 in low-density hippocampal neurons as described above, and performed quantitative immunofluorescence analysis of the surface expression of GluA2. Neurons were stained for GluA2 with an antibody against an extracellular epitope in the GluA2 N-terminus, and for PSD95 as a postsynaptic marker to visualize excitatory synapses. Imaging fields were chosen by the presence of transfected, mCherry-positive neurons (Figure 3.10A). We observed that, unlike what happens with GluA1, loss of Caspr1 expression induced by specific shRNA-mediated KD had no effect in regulating surface GluA2 total (Figure 3.10B) or PSD95-colocalized synaptic (Figure 3.10C) cluster intensities. Interestingly, expression of a mutant construct of Caspr1 refractory to its shRNA resulted in a significant increase on the intensity of total (136.5 ± 10.6%; Figure 3.10B) and synaptic (135.0 ± 11.0%; Figure 3.10C) surface GluA2 clusters. These results suggest that, besides controlling GluA1, Caspr1 may also play a role in the regulation of the GluA2 subunit of AMPARs, albeit not being required for maintaining basal levels of GluA2.

Altogether, the previous sets of results unravel a critical role for Caspr1 in underlying homeostatic scaling mechanisms that regulate the synaptic content of GluA1-containing AMPARs. Furthermore, evidence indicates that, although Caspr1 might also be involved in the regulation of the GluA2 subunit of AMPARs, there is a functional requirement for Caspr1 to selectively control GluA1 levels.

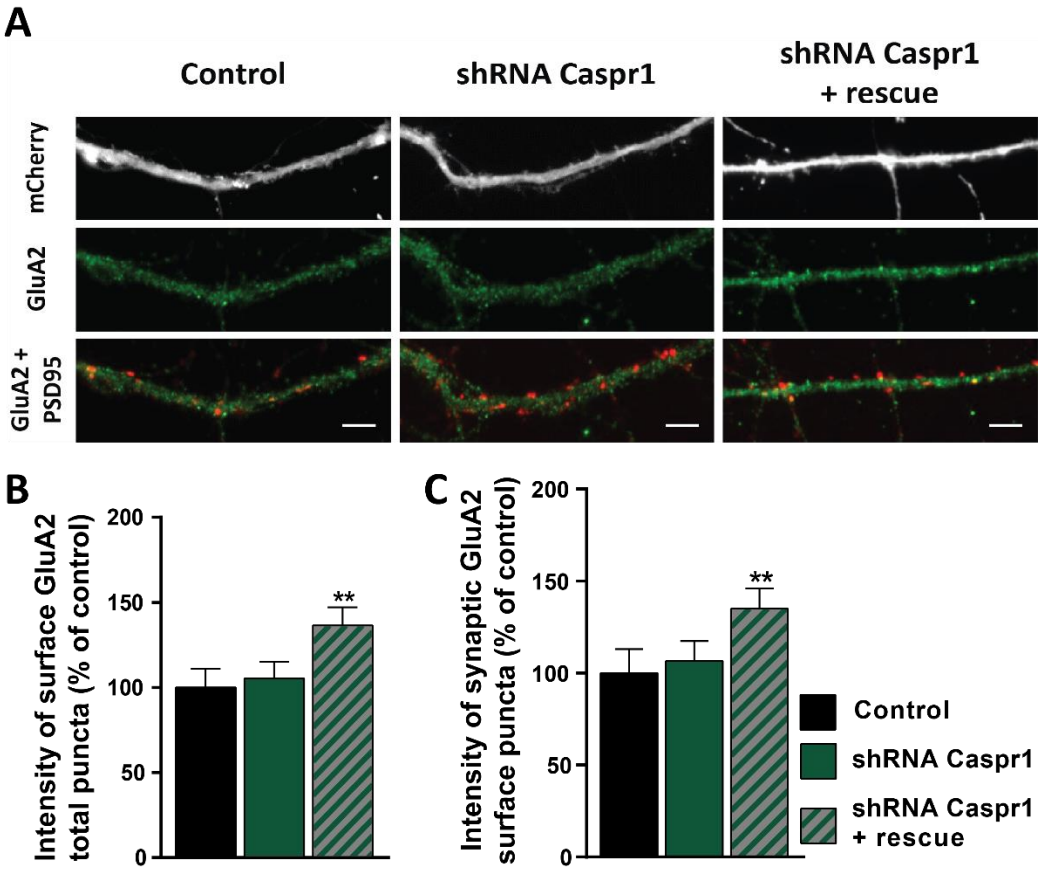


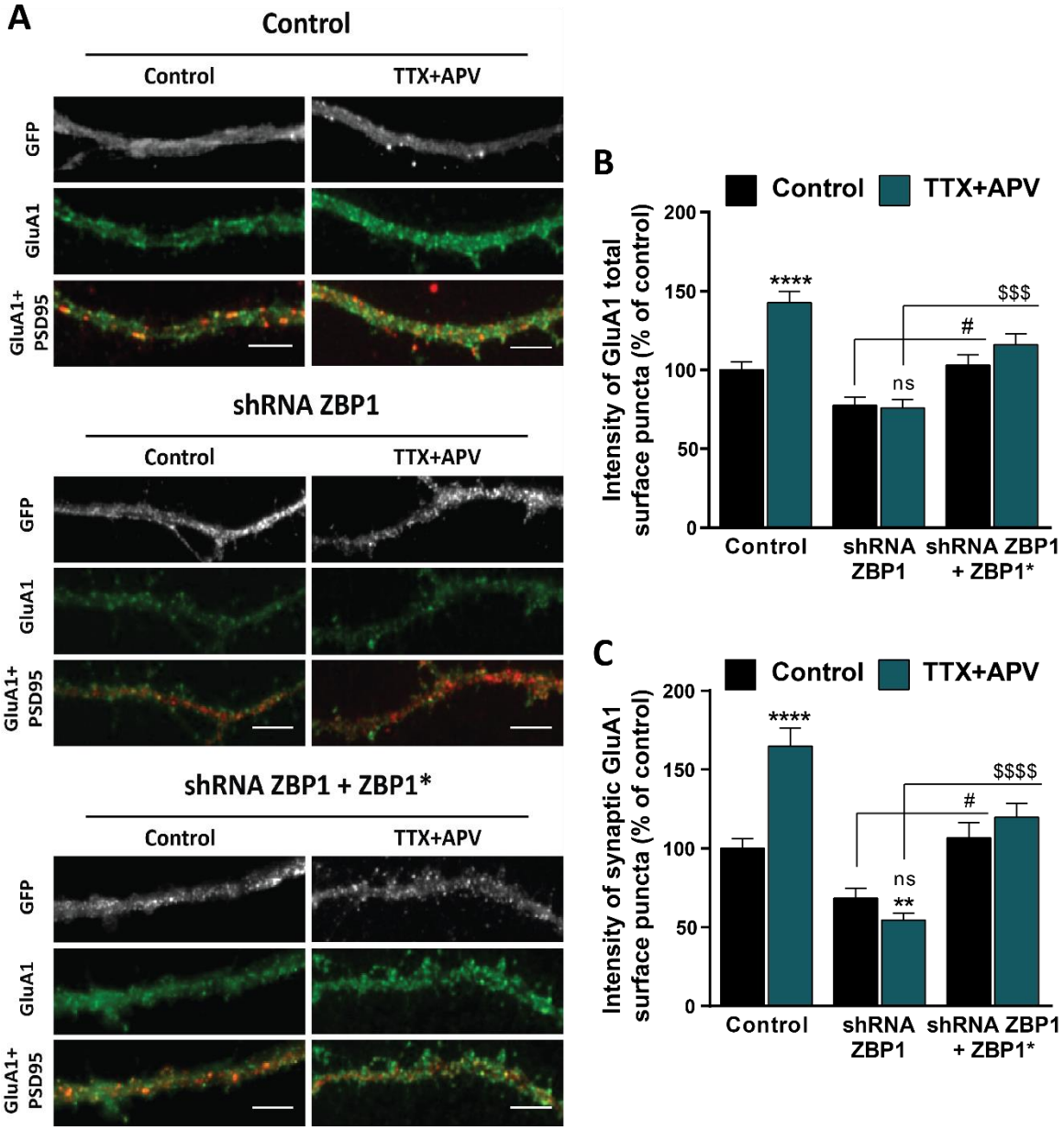
Figure 3.10 – Caspr1 is not required for maintaining basal levels of surface GluA2-containing AMPARs. (A) Representative immunofluorescence images of mCherry-positive low-density cultured hippocampal neurons transfected at DIV7 either with an empty vector construct (control - left), a plasmid expressing Caspr1-shRNA (middle), or with the Caspr1-shRNA plasmid and a Caspr1 rescue mutant (right). At DIV15, cells were stained for GluA2 with an antibody against an extracellular epitope in GluA2 N-terminus, and for the synaptic marker PSD95. Scale bars represent 5 µm. **(B)** The intensity of surface GluA2 total clusters was quantified, and **(C)** synaptic GluA2 clusters were determined by colocalization with the postsynaptic marker PSD95. Total number of analysed cells was obtained from three independent experiments (N=3, n=30 cells), and results are presented as mean ± S.E.M. Statistical significance was determined by the non-parametric Kruskal-Wallis Test, followed by the Dunn's Multiple Comparison Test, **p<0.01 when compared to control.

ZBP1 is required for homeostatic synaptic scaling of GluA1-containing AMPARs in hippocampal neurons

In the previous section, we demonstrated that in the absence of Caspr1, neurons fail to upscale synaptic AMPARs in face of prolonged neuronal inactivity, thus pinpointing a requirement for Caspr1 in the regulation of homeostatic synaptic plasticity. Moreover, earlier in this study we identified the RNA-binding protein ZBP1 as a potential player involved in the molecular mechanism underlying the effect of Caspr1 on GluA1-containing AMPARs. Specifically, we showed that ZBP1 not only binds to GluA1 mRNA, but also regulates the synaptic content of surface GluA1 clusters. Taking these pieces of evidence into consideration, we evaluated if ZBP1, similarly to Caspr1, is required for homeostatic regulation of synaptic GluA1-containing AMPARs. Low-density cultured hippocampal neurons were transfected with a plasmid encoding a specific shRNA against ZBP1 to knockdown its expression, or with a control scrambled shRNA construct, and then treated for 48 h with 1 µM TTX and 100 µM APV to chronically inhibit neuronal activity. We performed quantitative immunofluorescence analysis for superficial GluA1, as described previously (Figure 3.11A). In control neurons expressing the scrambled shRNA sequence a significant increase on the intensity of total (142.7 ± 7.0%; Figure 3.11B) or PSD95-colocalized synaptic (164.9 ± 11.4%; Figure 3.11C) surface GluA1 clusters was observed following chronic inactivity, induced by both blockade of action potentials and NMDAR inhibition. However, shRNA-induced loss of expression of ZBP1 resulted not only in a decrease on the surface expression of GluA1 in basal conditions, but prevented scaling of surface GluA1 cluster intensities induced by chronic inactivity (Figure 3.11B, C). Expression of a mutant rescue form of ZBP1 refractory to its shRNA recovered surface GluA1 clusters to control levels, although it did not fully recover the scaling effect of chronic inactivity on surface GluA1 puncta (Figure 3.11B, C).

Overall, this result reveals that ZBP1 regulates the basal synaptic content of surface GluA1-containing AMPARs, and is required for homeostatic mechanisms that mediate synaptic scaling of AMPARs in face of prolonged changes in neuronal activity. This evidence is in accordance with previous results obtained in this study showing a modulation of ZBP1 function upon chronic activity blockade. In these conditions, the phosphorylation of ZBP1 is increased and its binding to GluA1 mRNA is decreased, suggesting an activity-dependent release of GluA1 transcripts. Taking these results into consideration, it is conceivable that the control ZBP1 exerts over GluA1 mRNA influences the local availability of novel GluA1-containing AMPARs to be inserted into the synapse following neuronal inactivity. Future experiments should be performed to determine if ZBP1 is able to regulate the translation of GluA1 locally

and ascertain if such a mechanism underlies the role of ZBP1 in mediating the homeostatic scaling of synaptic AMPARs.



[Figure 3.11 - subtitle on the next page**]**

Figure 3.11 – Loss of endogenous ZBP1 prevents the homeostatic upscaling of surface GluA1-containing AMPARs following chronic inactivity in hippocampal neurons. (A) Representative immunofluorescence images of GFP-positive low-density cultured hippocampal neurons transfected at DIV7 either with a scrambled shRNA construct (control - top), a specific shRNA against ZBP1 (middle), or co-transfected with the ZBP1 shRNA sequence and a ZBP1 rescue mutant (bottom). At DIV13, neurons were treated for 48 h with 1 µM TTX and 100 µM APV to chronically block neuronal activity. Cells were then live-stained with a specific antibody against an extracellular epitope in GluA1 N-terminus, fixed and stained for the synaptic marker PSD95. Scale bars represent 5 µm. **(B)** The intensity of surface GluA1 total clusters was quantified, and **(C)** synaptic GluA1 clusters were determined by colocalization with the postsynaptic marker PSD95. Total number of analysed cells was obtained from at least three independent experiments (N≥3, n≥30 cells), and results are presented as mean ± S.E.M. Statistical significance was determined by the parametric two-way ANOVA Test, followed by the Tukey's Multiple Comparison Test, **p<0.01 and ****p<0.0001 when compared to control, #p<0.05 and ns - non significant when relative to the control ZBP1 shRNA, and $$$p<0.01 and $$$$p<0.0001 when compared to the TTX+APV ZBP1 shRNA condition.

The translational competence of ZBP1, modulated by Src-dependent phosphorylation, is required for homeostatic scaling of synaptic GluA1-containing AMPARs

Throughout this study we aimed at deconstructing the mechanisms behind the previously described Caspr1-mediated regulation of the GluA1 subunit of AMPARs (Santos *et al.*, 2012), and we discovered an unexpected role for the RNA-binding protein ZBP1. Firstly, we showed that ZBP1 binds to GluA1 mRNA in basal conditions, while following chronic neuronal inactivity the levels of GluA1 transcripts bound to ZBP1 significantly decrease. This evidence is in accordance with the canonical function attributed to ZBP1 in recognizing and binding to specific zipcode sequences located in the 3'UTR of target mRNAs (Chao *et al.*, 2010; Kislauskis *et al.*, 1994; Ross *et al.*, 1997), allowing the stabilization and transport of the mRNA cargo to distinct locations, while halting its translation. Although we still have to test if ZBP1 exerts a similar post-transcriptional control over GluA1 mRNA, this level of regulation could, in principle, provide an efficient and energy-saving manner to fine-tune GluA1 availability, particularly during periods of high temporal and/or spatial demand, such as during chronic periods of neuronal inactivity. Several studies have also thoroughly described the step of de-repression of translation of ZBP1-bound transcripts, occurring upon Src-dependent phosphorylation at ZBP1 tyrosine 396, which, in turn, conformationally triggers the release of the mRNA cargo from ZBP1 and subsequent translation de-repression (Huttelmaier *et al.*, 2005; Sasaki *et al.*,

2010; Welshhans & Bassell, 2011). Interestingly, we observed an increase in the phosphorylation of ZBP1 at tyrosine 396 upon chronic blockade of activity, which is consistent with the decrease in levels of ZBP1-bound GluA1 transcripts in these conditions, suggesting an activity-dependent release of GluA1 mRNA from ZBP1 and possible activation of translation following chronic neuronal inactivity. Moreover, we further uncovered a requirement for ZBP1 to regulate the surface expression of GluA1 synaptic clusters, particularly under homeostatic scaling events.

Taken together, our results highlight a fundamental synaptic role for ZBP1, since its potential post-transcriptional control over GluA1 mRNA ultimately influences and is required to regulate the surface expression of GluA1-containing AMPARs in synapses. To further investigate the mechanisms by which ZBP1 regulates GluA1, we evaluated whether the translational competence of ZBP1 affects the surface expression of the receptor subunit. We evaluated surface GluA1 in hippocampal neurons where the expression of endogenous ZBP1 was downregulated as previously described, while overexpressing in parallel either the shRNA-resistant WT ZBP1 or a phosphodead ZBP1.Y396F mutant, which cannot undergo Src-dependent phosphorylation at Y396 and, hence, cannot de-repress bound transcripts (Figure 3.12A).

As described in the previous section, loss of endogenous ZBP1 in hippocampal neurons resulted in a significant decrease in the intensity of total (76.4 ± 5.1%; Figure 3.12B) and PSD95-colocalized synaptic (68.5 ± 6.1%; Figure 3.12C) GluA1 surface clusters, when compared to the expression of a control scrambled shRNA construct. The area and number of synaptic GluA1 clusters were also significantly reduced following KD of ZBP1. Furthermore, expression of a WT ZBP1 construct refractory to the shRNA was able to significantly rescue all parameters of the KD-mediated decrease of total (ZBP1*, Figure 3.12B) and synaptic (ZBP1*, Figure 3.12C) GluA1 clusters to values comparable to the control. Nevertheless, and unlike WT ZBP1, expression of the shRNA-resistant phosphodead ZBP1.Y396F mutant did not differ significantly from the ZBP1 KD-induced phenotype and failed to rescue the intensity of synaptic GluA1 surface clusters back to control levels (71.9 ± 10.3%; ZBP1.Y396F, Figure 3.12C). This result indicates that the Src-mediated phosphorylation of ZBP1 at Y396 and, consequently, the translational competence of ZBP1 are required for the regulation of surface GluA1-containing AMPARs.

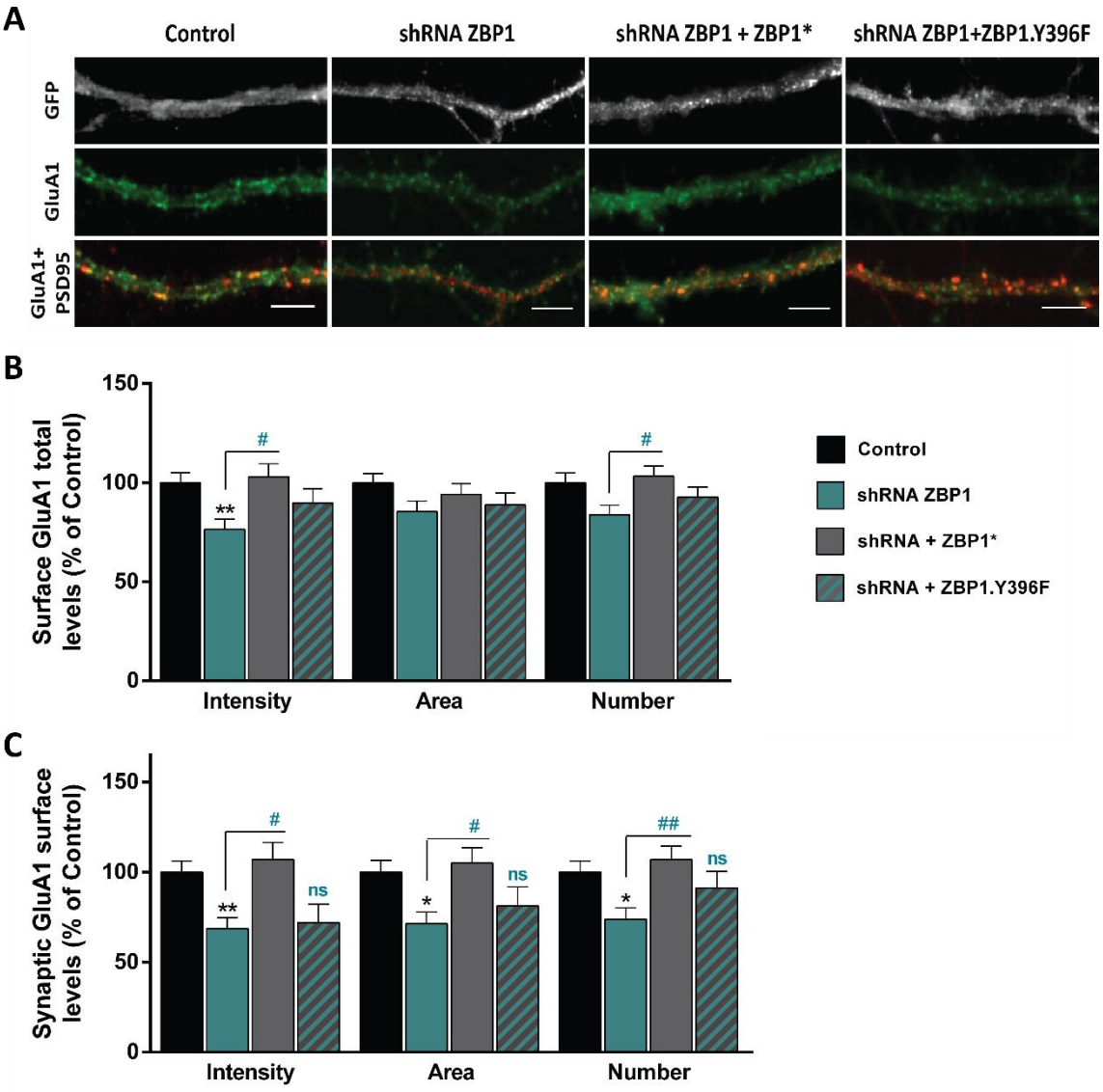


Figure 3.12 – Src-dependent phosphorylation of ZBP1 is required to regulate the surface expression and synaptic content of GluA1-containing AMPARs in hippocampal neurons. **(A)** Representative immunofluorescence images of GFP-positive low-density cultured hippocampal neurons transfected at DIV7 with a scrambled shRNA construct (control), a specific shRNA against ZBP1, or co-transfected with the ZBP1 shRNA sequence and with either a WT ZBP1 rescue mutant (ZBP1*) or a phosphodead ZBP1 rescue mutant unable to undergo Src-dependent phosphorylation (ZBP1.Y396F). At DIV15, cells were live-stained with a specific antibody against an extracellular epitope in the GluA1 N-terminus, fixed, and then stained for the synaptic marker PSD95. Scale bars represent 5 µm. **(B)** The intensity, area and number of surface GluA1 total clusters were quantified, and **(C)** synaptic GluA1 clusters were determined by colocalization with the postsynaptic marker PSD95. Total number of analysed cells was obtained from at least three independent experiments (N≥3, n≥ 30 cells, and results are presented as mean ± S.E.M. Statistical significance was determined by the non-parametric Kruskal-Wallis test, followed by the Dunn's Multiple Comparison Test, *p<0.05 and **p<0.01 when compared to control, and #p<0.05, ##p<0.01 and ns - non significant when relative to the ZBP1 shRNA condition.

Prolonged manipulations of neuronal activity homeostatically modulate the function of ZBP1, by increasing its phosphorylation and disrupting its binding to GluA1 mRNA. Moreover, ZBP1 is required for the homeostatic synaptic scaling of GluA1-containing AMPARs. Taking these results into consideration, together with the interesting preceding evidence indicating that the translational competence of ZBP1 is required for the role of ZBP1 in regulating the basal surface expression of GluA1, we tested whether the canonical function of ZBP1 as a translational competent RNA-binding protein is important for its role in the homeostatic regulation of GluA1 synaptic scaling. Low-density cultured hippocampal neurons were transfected as described above, then treated for 48 h with 1 µM TTX and 100 µM APV to chronically block action potentials and NMDARs, respectively, and surface GluA1 was analysed (Figure 3.13A).

While control hippocampal neurons expressing a scrambled shRNA suffered a significant 50 to 60% increase on the intensity of their total (Control, Figure 3.13B) and synaptic (Control, Figure 3.13C) GluA1 surface clusters following a prolonged period of neuronal inactivity, KD-induced loss of endogenous ZBP1 significantly decreased the basal surface expression of GluA1, and further prevented the homeostatic scaling of synaptic GluA1 surface levels (shRNA ZBP1, Figure 3.13B, C). Moreover, when we co-expressed the ZBP1-shRNA plasmid with the shRNA-resistant WT ZBP1, the intensity of GluA1 clusters was rescued back to control levels, although the homeostatic scaling of surface GluA1 puncta did not recover fully (ZBP1*, Figure 3.13B, C). On the contrary, overexpression of the loss-of-function ZBP1.Y396F mutant, which cannot be phosphorylated by Src at Y396, not only failed to rescue the ZBP1 KD-induced phenotype back to control levels, but it was also not sufficient to restore homeostatic synaptic upscaling of surface GluA1 clusters upon chronic blockade of activity (ZBP1.Y396F, Figure 3.13B, C).

This last set of results unveils part of the mechanism underlying the effect of ZBP1 in the regulation of GluA1-containing AMPARs. By taking advantage of a loss-of-function mutant of ZBP1, the evidence obtained underscores a fundamental requirement for the translational competence of ZBP1, modulated by Src-dependent phosphorylation, to regulate the surface expression of GluA1 puncta, and further contribute to homeostatic mechanisms underlying synaptic scaling of AMPARs.

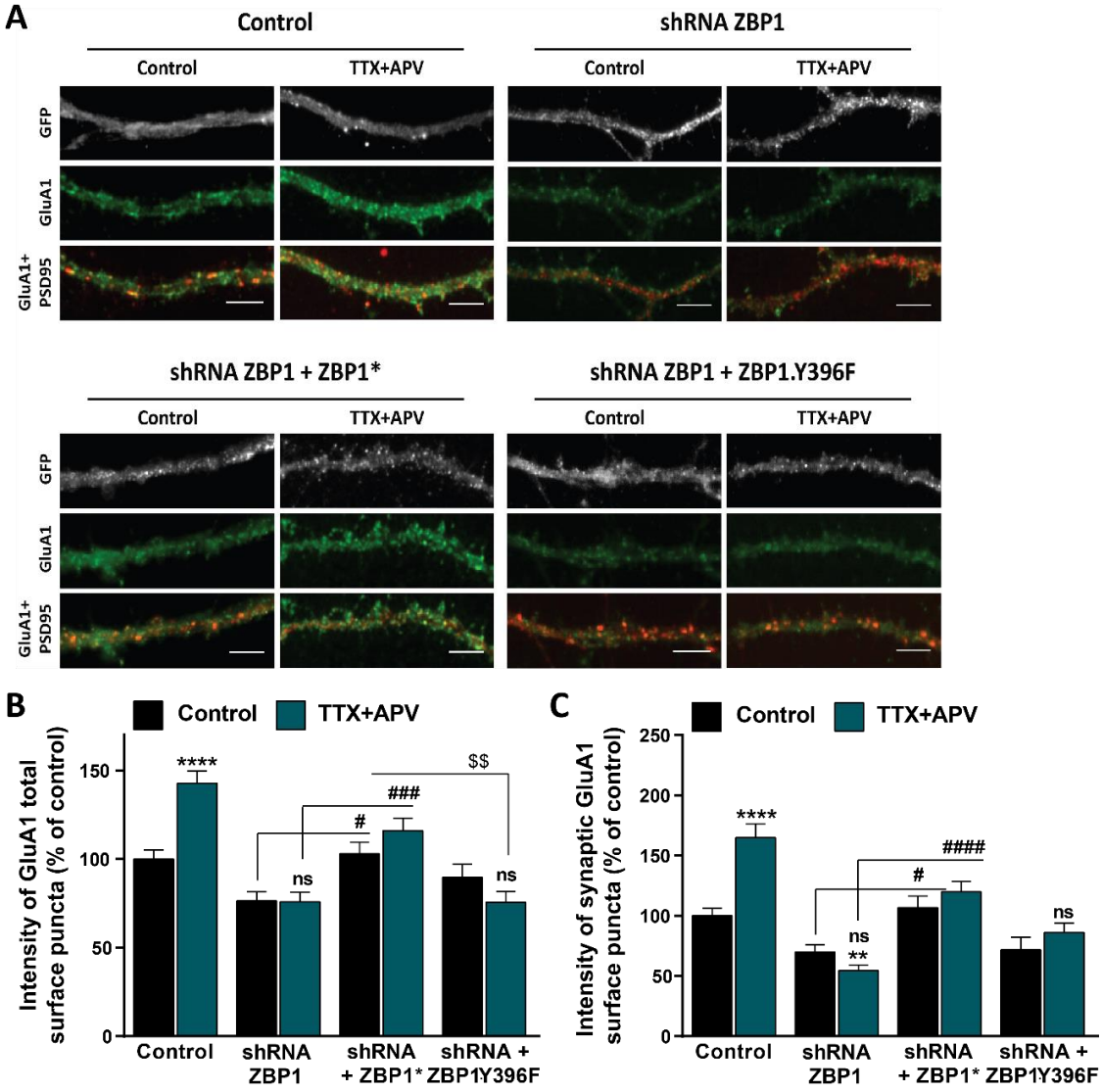


Figure 3.13 – The phosphodead mutant form of ZBP1 cannot mediate homeostatic upscaling of surface GluA1-containing AMPARs following chronic inactivity. **(A)** Representative immunofluorescence images of GFP-positive low-density cultured hippocampal neurons transfected at DIV7 with a scrambled shRNA construct (control – top left), the ZBP1-shRNA construct (top right), or co-transfected with the ZBP1-shRNA construct and either a WT ZBP1 rescue mutant (ZBP1* - bottom left) or a phosphodead ZBP1 rescue mutant unable to undergo Src-dependent phosphorylation (ZBP1.Y396F – bottom left). At DIV13, neurons were treated for 48 h with 1 µM TTX and 100 µM APV to chronically block neuronal activity. Cells were then live-stained with a specific antibody against an extracellular epitope in GluA1 N-terminus, fixed, and then stained for the synaptic marker PSD95. Scale bars represent 5µm. **(B)** The intensity of surface GluA1 total clusters was quantified, and **(C)** synaptic GluA1 clusters were determined by colocalization with the postsynaptic marker PSD95. Total number of analysed cells was obtained from at least three independent experiments (N≥3, n≥30 cells), and results are presented as mean ± S.E.M. Statistical significance was determined by the parametric two-way ANOVA Test, followed by the Tukey's Multiple Comparison Test, **p<0.01 and ****p<0.0001 when compared to control; #p<0.05 and ns - non significant when relative to the control ZBP1 shRNA; ###p<0.001 and ####p<0.0001 when relative to the TTX+APV shRNA ZBP1 condition; $$p<0.01 when compared to the TTX+APV shRNA ZBP1+ZBP1* condition; and ns - non significant when the shRNA ZBP1 + ZBP1.Y396F TTX+APV condition was compared to its control.

Discussion

Synaptic plasticity mechanisms, of either Hebbian or homeostatic type, highly depend on changes in the synaptic content of AMPARs to modulate activity-dependent alterations in synaptic strength. The trafficking and localization of AMPARs into synapses, in turn, is dynamically regulated by multiple mechanisms ranging from post-transcriptional and post-translational modifications of AMPAR subunits, to specific interacting partners (Anggono & Huganir, 2012; Jiang *et al.*, 2006; Santos *et al.*, 2009). These mechanisms control the trafficking and insertion/removal of receptors to synapses, and the stabilization and anchoring of AMPARs to the synaptic membrane.

A recent study from our laboratory identified the cell-adhesion molecule Caspr1 as a novel regulator of AMPAR subunits, capable of regulating their synaptic content (Santos *et al.*, 2012). Overexpression of Caspr1 in hippocampal neurons increases the trafficking of GluA1 to synapses, whereas loss of this protein leads to a significant decrease in the synaptic accumulation of GluA1-containing AMPARs. However, the mechanisms underlying the effect of Caspr1 and whether the Caspr1-mediated regulation of AMPARs might be linked to synaptic plasticity events was not known.

Regulation of GluA1-containing AMPARs by Caspr1

In the present chapter, we uncover yet additional roles for Caspr1 in the regulation of AMPARs, and identify possible mechanisms underlying them. We demonstrate that overexpression of Caspr1 in either heterologous cells or hippocampal neurons leads to an increase on the total protein amount of the GluA1 subunit (Figure 3.1A - D), without affecting its protein stability. We show that Caspr1 increases GluA1 protein as a consequence of a transcription-independent upregulation of GluA1 mRNA (Figure 3.1E, F), suggesting a novel post-transcriptional mechanism underlying the role of Caspr1. This is rather interesting considering that some synaptic plasticity events also depend on post-transcriptional and translational regulatory mechanisms to control AMPAR protein expression, particularly when localized to dendrites or close to synaptic sites (Sutton & Schuman, 2006). Most of the post-transcriptional mechanisms controlling a particular mRNA, at the level of either its stability, turnover, localization or translation, are typically mediated by regulatory elements present in the 3'UTR of mRNAs (Chatterjee & Pal, 2009). Indeed, Caspr1 fails to increase GluA1 mRNA in heterologous cells, if expressed together with a mutant form of GluA1 lacking its 3'UTR (data not shown). This evidence suggests that Caspr1 requires the presence of the 3'UTR of GluA1

mRNA to exert its effect, raising the possibility of Caspr1 *trans*-acting on some regulatory *cis*-element located in that region of the GluA1 transcript.

In order to understand the exact molecular mechanisms underlying the effect of Caspr1, we took into consideration the diverse structure of Caspr1, which might account for its multi-function regulatory profile on AMPARs. The intracellular region of Caspr1 accounts for its effects on GluA1 surface levels, since this region alone is sufficient to rescue synaptic levels of GluA1 following knock-down of Caspr1 (Santos *et al.*, 2012). The intracellular region of Caspr1 is composed of domains typically involved in protein-protein interactions (Figure 3.2A). It contains a juxtamembrane short peptide with a conserved GNP motif which interacts with proteins encompassing FERM domains, such as the cytoskeleton adaptor 4.1 protein (Denisenko-Nehrbass *et al.*, 2003b). Additionally, it contains a C-tail portion rich in proline residues with multiple binding sites that selectively interact with SH3 domains of several signalling molecules, including members of the Src family of tyrosine kinases, such as Src and Fyn, as well as PLCγ and the p85 subunit of the PI3K pathway (Peles *et al.*, 1997). To understand the mechanisms downstream of Caspr1 and discern the importance of these two intracellular domains, we generated two deletion mutant constructs of Caspr1 lacking either the GNP motif or the proline sequence (Figure 3.2A). We observed that, while deletion of the GNP motif does not affect the role of Caspr1 (Figure 3.2B), the proline-rich sequence domain of Caspr1 is required for the upregulation of both GluA1 mRNA (Figure 3.2B) and protein (Figure 3.2C, D).

Taking into account the structure of the proline-rich domain together with its specific requirement for the role of Caspr1, we reasoned that the effect of Caspr1 on AMPARs could occur through the triggering of an intracellular signalling cascade. Indeed, we found that Caspr1 increases the phosphorylation of the tyrosine kinase Src, rendering it active (Figure 3.3A - C). Moreover, through its proline-rich domain, Caspr1 increased the phosphorylation of ZBP1, a target downstream of the Src kinase, both in a cell line (Figure 3.3D, E) and in synaptic sites of hippocampal neurons (Figure 3.3F, G). ZBP1 is a RNA-binding protein best known for binding to specific zipcodes in the 3'UTR of target mRNAs to regulate their stability, localization and translation (Huttelmaier *et al.*, 2005; Kislauskis *et al.*, 1994; Ross *et al.*, 1997), and its function is regulated by Src-dependent phosphorylation. This evidence pointing to a role for Caspr1 in triggering a signalling cascade that ultimately controls the function of an important RNA-binding protein such as ZBP1, fits adequately with the upregulatory effect of Caspr1 on GluA1 mRNA and its dependence on the 3'UTR of GluA1, and highlights a possible regulation of GluA1 transcripts by ZBP1. Accordingly, we identified the presence of a conserved zipcode for the binding of ZBP1 in the 3'UTR sequence of rat GluA1 (Figure 3.4A), and demonstrated,

through an immunoprecipitation protocol of ribonucleoproteins, that ZBP1 interacts with GluA1 mRNA (Figure 3.4B, C). Considering the role of ZBP1 in regulating other transcripts, for instance the mRNA for β-actin, we propose that ZBP1 binds to GluA1 mRNA to repress its translation and possibly contribute to localize GluA1 transcripts to sites where GluA1 synthesis is most required. This level of regulation would be of crucial importance, particularly in the vicinity of synaptic sites, where local translation of GluA1 mRNA has already been shown to occur, with fundamental outcomes for synaptic plasticity events (Cajigas *et al.*, 2012; Grooms *et al.*, 2006; Ju *et al.*, 2004; Kacharmina *et al.*, 2000; Maghsoodi *et al.*, 2008). Future experiments should be performed to further understand if ZBP1 plays a role in the regulation of dendritically / synaptically localized GluA1 transcripts, and their translation.

Altogether, this novel post-transcriptional regulation of the total amount of GluA1, mediated by Caspr1 and ZBP1, does not disagree with previous results obtained in our lab. On the contrary, this evidence raises the interesting possibility of Caspr1 playing distinctive multiple roles that complement each other, and that underlie its effect in the synaptic content of GluA1-containing AMPARs, as shown in (Santos *et al.*, 2012). On one hand, we have pinpointed a crucial and specific requirement for the intracellular proline-rich domain of Caspr1 in controlling GluA1 post-transcriptionally by modulating ZBP1 function. This mechanism could be a means to regulate the availability of local pools of GluA1 mRNA in close range to where its synthesis is most required, and allow a prompt replenishment of novel AMPARs. On the other hand, although this post-transcriptional effect is clearly independent of the other intracellular GNP motif of Caspr1 (Figure 3.2B), we should not disregard a possible contribution of this juxtamembrane domain of Caspr1 in regulating the trafficking and synaptic insertion of GluA1-containing AMPARs. This intracellular GNP motif is capable of interacting with proteins encompassing FERM domains, such as the cytoskeleton adaptor 4.1N protein (Denisenko-Nehrbass *et al.*, 2003b). The 4.1N protein is known to bind to the C-tail of the GluA1 subunit to regulate its surface expression, being required for the maintenance of LTP mechanisms (Lin *et al.*, 2009; Shen *et al.*, 2000). Further studies using the deletion mutants of Caspr1 intracellular domains can be undertaken to fully comprehend the contribution of either the GNP motif or the proline-rich sequence in the regulation of AMPARs.

Requirement of Caspr1 and ZBP1 for homeostatic synaptic scaling of GluA1-containing AMPARs

Given the importance that AMPARs play in the mechanisms that underlie several forms of plasticity, we investigated whether Caspr1, and its downstream mechanism, is regulated by changes in neuronal activity. We observed that a prolonged period of neuronal inactivity induced by TTX in hippocampal neurons results in a significant upregulation of the levels of endogenous Caspr1 (Figure 3.6A - C). Moreover, this chronic manipulation of neuronal activity induces activation of the Src signalling pathway, increasing the phosphorylation levels of both Src and ZBP1 (Figure 3.7A, B). These results suggest that Caspr1, and the downstream mechanism it triggers, might be involved in the homeostatic regulation of AMPARs. It is possible that, following chronic changes in neuronal activity, the expression of endogenous Caspr1 is potentiated leading to the activation of the Src / ZBP1-mediated signalling mechanism that regulates GluA1 levels, thus contributing to the synaptic scaling of AMPARs and expression of homeostatic plasticity events. In agreement with this idea is the evidence showing a 50% decrease in the levels of GluA1 mRNA bound to ZBP1 following prolonged neuronal inactivity (Figure 3.7C). This result is in accordance with the described regulatory function of ZBP1, which binds to target transcripts in an active non-phosphorylated state, halting their translation. Following Src-mediated phosphorylation at Y396, ZBP1 becomes inactive and releases mRNAs from its grip, allowing activation of their translation (Huttelmaier *et al.*, 2005; Sasaki *et al.*, 2010; Welshhans & Bassell, 2011). Taking into account the fact that chronic inactivity is associated with the phosphorylation of ZBP1, likely as a consequence of increased Caspr1 expression, we hypothesize that the interaction between ZBP1 and GluA1 mRNA depends on activity; prolonged periods of low activity induce a release of GluA1 mRNA from ZBP1 to create a local pool of AMPAR transcripts readily available to undergo translation. Interestingly, previous studies have already reported an activity-dependent regulation of ZBP1, with increased movement of ZBP1 granules into dendrites and spines to transport β-actin mRNA in response to synaptic activity (Tiruchinapalli *et al.*, 2003).

Loss of ZBP1 expression results in a significant decrease in the surface expression and synaptic content of GluA1-containing AMPARs, a phenotype that is rescued following overexpression of WT ZBP1 (Figure 3.5). To better understand this role of ZBP1, we used a loss-of-function mutagenesis approach to impair the canonical function of ZBP1 as a RNA-binding protein. By overexpressing a phosphodead ZBP1.Y396F mutant of ZBP1, which cannot undergo Src-dependent phosphorylation at Y396, and consequently fails to de-repress bound transcripts, we determined that the translational competence of ZBP1 is necessary to regulate the overall cell surface and synaptic content of the GluA1 subunit (ZBP1.Y396F,

Figure 3.12). These results raise the exciting possibility of ZBP1 functioning as a “distributor and supplier” of GluA1 transcripts, serving local pools of newly synthesised AMPARs that can rapidly replenish their synaptic content.

Because both the expression of Caspr1 and ZBP1 function, shown to be required to regulate basal levels of GluA1, are homeostatically regulated by paradigms that induce prolonged periods of neuronal inactivity, we hypothesized that both proteins play an important part in AMPAR synaptic scaling. Resorting to an imaging approach, we observed that both the surface expression and synaptic content of GluA1 clusters are scaled in a multiplicative manner following prolonged neuronal inactivity, induced by parallel blockade of action potentials and NMDARs with TTX and APV, respectively (Figure 3.8). However, specific loss of the endogenous expression of either Caspr1 or ZBP1 completely prevents the scaling of total and synaptic GluA1 surface puncta (Figure 3.9; 3.11), suggesting that both proteins are fundamental for the homeostatic regulation of synaptic AMPARs. We also determined that the translational competence of ZBP1 is further implicated in its capacity to homeostatically regulate GluA1, since restoring the expression of ZBP1 with the phosphodead loss-of-function mutant fails to re-establish the multiplicative scaling of surface and synaptic GluA1-containing AMPARs following chronic neuronal inactivity (ZBP1.Y396F, Figure 3.13). Taking this into account, together with the fact that prolonged absence of activity increases the Src-dependent phosphorylation of ZBP1 at Y396 (Figure 3.7A, B), and consequently induces a release of GluA1 mRNA from ZBP1 (Figure 3.7C), our results strongly suggest that homeostatic scaling of AMPARs requires activity-dependent ZBP1-mediated translation of GluA1. Additional studies should still be performed to better understand if the translational competence of ZBP1 is required to mediate the local dendritic synthesis of GluA1.

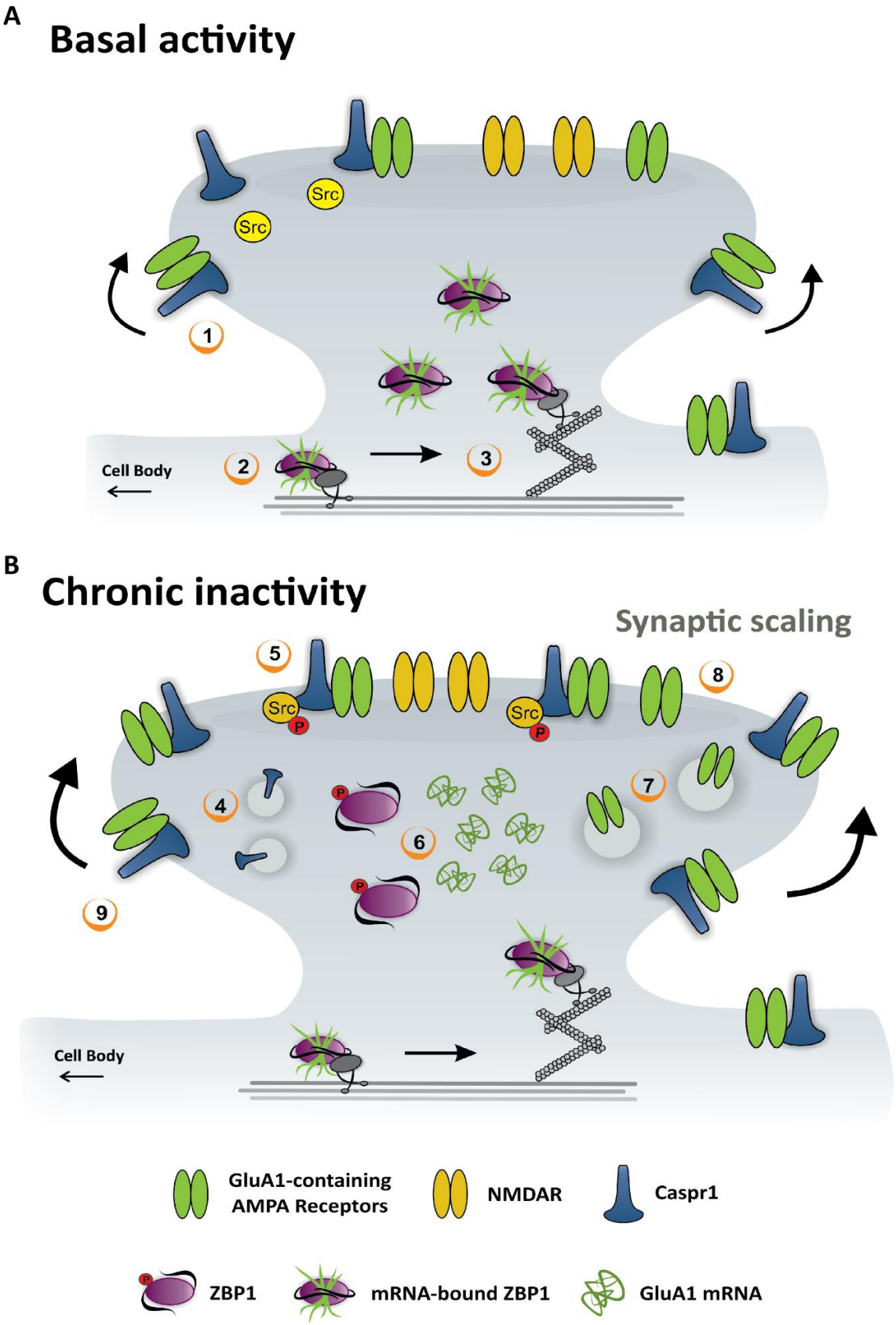
Indeed, our results gain further relevance in light of previous reports showing the translation of GluA1 occurring locally in dendrites and near synaptic sites upon activity and how this is crucial for plasticity events, including homeostatic synaptic scaling [(Aoto *et al.*, 2008; Grooms *et al.*, 2006; Ju *et al.*, 2004; Kacharmina *et al.*, 2000; Maghsoodi *et al.*, 2008; Poon & Chen, 2008; Poon *et al.*, 2006; Soden & Chen, 2010; Sutton *et al.*, 2006; Sutton *et al.*, 2004), and reviewed in (Buffington *et al.*, 2014; Swanger & Bassell, 2011; Wang *et al.*, 2010a)]. Some of the players known to be involved in this activity-dependent local regulation of GluA1 are retinoic acid and FMRP. RA induces homeostatic scaling of AMPARs by selectively increasing the local dendritic synthesis of GluA1, through signalling via the RA receptor RARα, which binds to and represses GluA1 mRNA translation in basal conditions (Aoto *et al.*, 2008; Maghsoodi *et al.*, 2008; Poon & Chen, 2008). Moreover, both inactivity- and RA-induced scaling of AMPARs further require the Fragile X-associated FMRP, which promotes the

stabilization and translation of GluA1 mRNA and its subsequent membrane insertion (Guo *et al.*, 2015; Muddashetty *et al.*, 2007; Soden & Chen, 2010). In this case, it has been further suggested that FMRP might work in concert with RARα to adequately target GluA1 mRNA, and other relevant synaptic transcripts such as PSD95 and CaMKII, to undergo translation in RA-response sites near synapses that have been "tagged" by previous activity (Henry, 2011; Soden & Chen, 2010), in what is defined as the synaptic tagging hypothesis (Frey & Morris, 1997; Redondo & Morris, 2011). Curiously, ZBP1 is thought to be a major component of large dendritic mRNA silencing *foci*, transitional assemblies of repressed mRNAs and associated proteins, which selectively disassemble in response to neuronal activity to putatively unmask repressed transcripts and release them for translation in specifically "tagged" active synapses [reviewed in (Buffington *et al.*, 2014; Doyle & Kiebler, 2011; Graber *et al.*, 2013; Krichevsky & Kosik, 2001; Pimentel & Boccaccio, 2014; Thomas *et al.*, 2014)]. Supporting this hypothesis, and taking advantage of single-molecule *in situ* hybridization approaches and transgenic animals to visualize mRNA *in vivo*, it was shown that β-actin mRNA is present in dendritic granules that are specifically unmasked following induction of chemical LTP or KCl-induced neuronal depolarization, and subsequently translated locally (Buxbaum *et al.*, 2014; Park *et al.*, 2014; Whalley, 2014).

In this chapter we demonstrate that 1) increased expression of Caspr1, which regulates GluA1 levels, induces a selective accumulation of phosphorylated ZBP1 clusters to synapses, 2) prolonged absence of neuronal activity results in increased phosphorylation of ZBP1, possibly downstream of Caspr1, and 3) ZBP1 phosphorylation, which modulates its translational competence, is required for homeostatic scaling of synaptic GluA1. Overall, these results fit with the hypothesis of ZBP1, activated downstream of Caspr1, being able to selectively restrict GluA1 translation to stimulated synapses undergoing prolonged activity deprivation. Taking this into consideration, it would be of the utmost importance to fully understand if ZBP1 is able to regulate the local availability of GluA1 mRNA in dendrites and synaptic sites and its subsequent translation in face of synaptic activity.

Current working model

Altogether, here we provide compelling evidence to suggest that the RNA-binding protein ZBP1 plays a role in underlying the mechanism responsible for the effect of Caspr1 in the regulation of GluA1-containing AMPARs. Furthermore, we demonstrate that Caspr1 and ZBP1 play in concert to regulate synaptic AMPARs in face of prolonged absence of neuronal activity, both being required for the expression of homeostatic plasticity events.



[Figure 3.14 - subtitle on the next page**]**

Figure 3.14 – Proposed model of regulation of GluA1-containing AMPARs by Caspr1 and ZBP1, in basal conditions (A) or in face of chronic absence of neuronal activity (B). **(A)** In baseline conditions, Caspr1 interacts with GluA1 and regulates its insertion and trafficking into synapses **(1)**. ZBP1 binds to the 3'UTR of GluA1 mRNA to repress its translation **(2)**, and incorporates it into RNP granules that translocate to dendritic sites **(3)**, creating a local storage of dormant GluA1 transcripts. **(B)** When neuronal activity is chronically deprived, by blockade of action potentials and synaptic inhibition of NMDARs, the expression of endogenous Caspr1 increases **(4)** and Caspr1 induces the phosphorylation and activation of the tyrosine kinase Src **(5)**. Src phosphorylates ZBP1 at Y396, which induces the de-repression and release of GluA1 mRNA from ZBP1 **(6)**. This will allow the creation of a local pool of GluA1 mRNA readily available to undergo translation and form novel AMPARs **(7)**, which can then be inserted and trafficked into synapses **(8)**. In turn, Caspr1 might also facilitate and increase the trafficking of already-expressed surface AMPARs into the synaptic membrane **(9)**. Altogether, the proposed mechanism underlies homeostatic synaptic scaling of GluA1-containing AMPARs to balance the absence of neuronal activity.

Taking into consideration the results presented in this chapter, together with previous evidence obtained in our lab and the current knowledge in the literature, we propose a model in which Caspr1, in basal conditions of activity, is able to interact with AMPAR subunits and regulate their surface expression and trafficking along the cell membrane (Figure 3.14A - 1). In addition, ZBP1 binds to the zipcode located in the 3'UTR of GluA1 mRNA, presumably temporarily halting its translation (Figure 3.14A - 2). Moreover, it is possible that ZBP1 allows the incorporation of GluA1 transcripts into ribonucleoprotein complexes that transport the mRNA in a dormant state into specific dendritic sites, creating a local mRNA silencing *foci* that transitionally stores GluA1 mRNA molecules (Figure 3.14A - 3).

Opposing basal conditions, when neurons face prolonged changes in their activity (chronic inactivity), there is a homeostatic compensation in the expression of Caspr1, which increases significantly (Figure 3.14B - 4). Because Caspr1 is able to interact with the tyrosine kinase Src, its increased expression will induce the phosphorylation and resulting activation of Src (Figure 3.14B - 5). In turn, the signalling pathway downstream of Src will then target ZBP1 for phosphorylation at Y396, which conformationally changes ZBP1 so that it de-represses and releases GluA1 mRNA from its grip (Figure 3.14B - 6). This unmasking of GluA1 mRNA will allow the creation of a local pool of GluA1 readily available to undergo translation and synthesis of novel GluA1-containing AMPARs (Figure 3.14B - 7), which can then be inserted and trafficked into deprived synapses (Figure 3.14B - 8). In parallel, it is possible that, because its expression is upregulated following chronic inactivity, Caspr1, by itself, further contributes to increase the insertion and trafficking of AMPARs to synapses (Figure 3.14B - 9). Altogether, the proposed mechanism will allow the homeostatic synaptic scaling of cell surface GluA1-containing AMPARs to counteract the effects of activity deprivation.

Physiological relevance of the proposed mechanism and potential implication in neurological disorders

We uncovered an interplay between the cell-adhesion molecule Caspr1 and the RNA-binding protein ZBP1 that is required to regulate the synaptic content of AMPARs in hippocampal neurons, both in basal conditions and upon prolonged neuronal inactivity. When Caspr1 is abrogated, or its underlying mechanism impaired, a failure of homeostatic scaling mechanisms occurs, revealing a requirement for both Caspr1 and ZBP1 in the regulation of homeostatic synaptic plasticity.

Because homeostatic synaptic plasticity keeps the activity of neuronal networks in-balance, the integrity of its underlying molecular mechanisms is crucial for maintenance of proper brain function. Alas, emerging evidence confirms that in some neurological and neuropsychiatric disorders homeostatic plasticity might be compromised, thus contributing to the pathophysiology of the disease [reviewed in (Ramocki & Zoghbi, 2008; Wondolowski & Dickman, 2013)]. Aberrations in homeostatic plasticity mechanisms have been implicated in neurodegenerative disorders such as Alzheimer's disease [(Kim *et al.*, 2015; Pratt *et al.*, 2011) and reviewed in (Jang & Chung, 2016)] and Huntington's disease (Rocher *et al.*, 2016), in epilepsy (Houweling *et al.*, 2005) and schizophrenia (Dickman & Davis, 2009), and most prominently in intellectual disability and autism spectrum disorders (ASDs) [extensively reviewed in (Huguet *et al.*, 2013; Mullins *et al.*, 2016; Toro *et al.*, 2010)], such as Rett's (Blackman *et al.*, 2012; Noutel *et al.*, 2011; Qiu *et al.*, 2012) and Fragile X syndromes (Soden & Chen, 2010). A link between homeostatic plasticity and ASDs, in particular, was first hypothesized following the realization that many of the molecular players known to be involved in synaptic homeostatic mechanisms have already been implicated as ASD-related susceptibility genes. Accordingly, emerging evidence from animal models with mutated/ablated ASD-related susceptibility genes show a failure of homeostatic processes and general neuronal homeostasis, with animals presenting behavioural phenotypes that recapitulate ASD-like symptoms (Bourgeron, 2015; Mullins *et al.*, 2016; Wondolowski & Dickman, 2013).

Recent mutations in the CASPR1-encoding gene *CNTNAP1* have been found in children with congenital hypomyelinating disorders (Hengel *et al.*, 2017; Lakhani *et al.*, 2017; Laquerriere *et al.*, 2014), however, thus far, evidence implicating Caspr1 or ZBP1 in neuropsychiatric disorders is scarce. The results presented in this chapter place Caspr1 and ZBP1 in the map of potential regulators of neuronal homeostasis in the human brain, and ultimately of proper brain function. Hence, it is not unlikely that either Caspr1 or ZBP1 could

be targets for dysfunction in the context of disease. Interestingly, a recent meta-analysis of transcriptomic profiling studies across several neuropsychiatric disorders, reveals that gene expression of *CNTNAP1* is significantly downregulated in psychiatric disorders such as ASD, SCZ, bipolar disorder and depression (Gandal *et al.*, 2018), giving a first prospect of the potential relevance of Caspr1 in brain function.

Caspr2, another member of the family of Contactin-associated proteins, is a susceptibility gene in autism, schizophrenia and epilepsy [(Alarcon *et al.*, 2008; Friedman *et al.*, 2008; Penagarikano *et al.*, 2011) and reviewed in (Poot, 2015)]. Interestingly, animal models lacking the *CNTNAP2* gene encoding Caspr2, besides displaying autistic-like behavioural phenotypes, also present reduced neuronal synchrony in the neocortex and altered balance of synaptic excitation and inhibition (E/I balance) (Hoffman *et al.*, 2016; Penagarikano *et al.*, 2011), which is reminiscent of aberrant neuronal homeostasis. Accordingly, in the next chapter we provide exciting new insights into the synaptic functions of Caspr2 and its requirement for the regulation of homeostatic synaptic scaling and experience-dependent homeostatic plasticity, which might account for the aberrations in the E/I balance of Caspr2 KO animals, and the ASD-core neuropsychiatric symptoms displayed by patients with *CNTNAP2* mutations. Because Caspr1 shares such a high degree of homology with Caspr2, and both seem to be implicated in the dynamic activity-dependent regulation of AMPARs, particularly in the context of homeostatic plasticity (for evidence on the role of Caspr2, please check the next chapters), it is conceivable that mutations in Caspr1, or aberrations in the Caspr1-dependent mechanism proposed here, might contribute to the pathophysiology of ASDs and other neuropsychiatric disorders.

Additionally, the mechanism presented in this chapter gains further emphasis in light of recent findings that ASDs frequently originate from aberrant local protein translation, such as in the case of Fragile X Syndrome, Rett's Syndrome and Tuberous Sclerosis Complex [extensively reviewed in (Kelleher & Bear, 2008; Mullins *et al.*, 2016)]. Indeed, the evidence we provide in the present study hint at a novel post-transcriptional mechanism mediated by the RNA-binding protein ZBP1 that can potentially be required to regulate the local translation of GluA1-containing AMPARs, which provides an efficient and energy-saving manner to fine-tune the local availability of synaptic AMPARs. If we take into consideration the requirement for local protein synthesis of AMPARs in the mechanisms that underlie homeostatic synaptic plasticity, together with the causal link between aberrant protein translation and ASDs, it becomes clear that a dysregulation of local translational processes, such as the one we propose here, might converge onto the disruption of neuronal homeostasis, and ultimately to the pathophysiology of ASDs.

Conclusions

The evidence presented in this chapter starts to shed light on the molecular mechanisms underlying the role of the cell-adhesion molecule Caspr1 in the dynamic regulation of synaptic AMPARs. Our findings uncover a novel interplay between Caspr1 and the RNA-binding protein ZBP1 that is crucial for the post-transcriptional regulation of the GluA1 subunit of AMPARs, and for the expression of synaptic scaling mechanisms. Further experiments are still required to understand if the mechanism conducted by Caspr1 and ZBP1 might be of relevance to fine-tune the local availability of new AMPARs near synaptic sites, particularly in the context of homeostatic synaptic plasticity. Importantly, our findings underscore the promising significance of both Caspr1 and ZBP1 in the regulation of neuronal homeostasis in the healthy brain, and raise the flag for potential targets for disruption in the context of neuropsychiatric disorders.

Chapter IV
Results & Discussion

Homeostatic and experience-dependent regulation of AMPA receptors by Caspr2

Part of the results in this chapter are included in the following manuscript in submission:

Dominique Fernandes, Sandra Santos, Ester Coutinho, Jessica L. Whitt, Tiago Rondão, M. Isabel Leite, Camilla Buckley, Hey-Kyoung Lee & Ana Luísa Carvalho (2018). Disrupted AMPA receptor function upon genetic- or antibody-mediated loss of autism-associated CASPR2. *In submission*

Summary

Throughout the past decade, mutations in the *CNTNAP2* gene have been recurrently implicated in neuropsychiatric disorders such as autism, schizophrenia and intellectual disability. Nevertheless, despite under intense research, the full-spectrum of cellular and molecular functions played by CASPR2 remains elusive. Importantly, it is still unclear how perturbations in CASPR2 function become pathogenic and drive the severe cognitive and psychiatric symptoms presented by patients.

Herein, we find that Caspr2 is expressed in cortical excitatory synapses, and identify Caspr2 as a novel AMPAR-interacting protein capable of regulating the trafficking of AMPARs to synapses. Moreover, we demonstrate that loss of Caspr2 impairs AMPA receptor function and *in vivo* excitatory synaptic transmission in the cortex, and reveal a requirement for Caspr2 in the regulation of homeostatic and visually-driven experience-dependent synaptic plasticity. Overall, our findings suggest that disruption of these mechanisms may underlie the pathogenesis of CASPR2-related disorders.

Introduction

The human brain is distinctively unique due to its remarkable ability for complex language, higher cognition, emotion regulation and executive control of behaviour; fundamentally the hallmark features of what makes us humans and not just high-order mammals. Yet, these are precisely the brain functions impaired in a plethora of distinctive neurodevelopmental and neuropsychiatric disorders that afflict a large percentage of the population worldwide, with significant impact on global disease burden (Trautmann *et al.*, 2016). Psychiatric disorders such as schizophrenia (SCZ), intellectual disability (ID) and autism-spectrum disorders (ASD), although clinically distinct, are often associated to manifestations of cognitive dysfunction, abnormal thoughts, and social and behavioural defects. Despite a complex aetiology that remains largely unknown to date, these disorders share particular defective features in brain structure, wiring and function that suggest a similar neurodevelopmental origin for their pathogenesis (de Lacy & King, 2013; Network & Pathway Analysis Subgroup of Psychiatric Genomics, 2015; Schubert *et al.*, 2015). Accordingly, recent genetic association studies have demonstrated that, in addition to often being of multigenic origin, distinct neuropsychiatric disorders can share the same susceptibility risk genes (O'Donovan & Owen, 2016; Vissers *et al.*, 2016).

One of the identified risk genes that has received considerable attention throughout the last decade, precisely because it has been implicated in a plethora of neuropsychiatric disorders, is the *CNTNAP2* gene [extensively reviewed in (Burbach & van der Zwaag, 2009; Penagarikano & Geschwind, 2012; Poot, 2015; Poot, 2017; Rodenas-Cuadrado *et al.*, 2014)]. In recent years, several linkage, association, gene expression analysis and imaging data studies have been able to identify a significant number of both rare and common variant mutations in the *CNTNAP2* gene that have been repeatedly implicated in the pathogenesis of ASD (Alarcon *et al.*, 2008; Arking *et al.*, 2008; Bakkaloglu *et al.*, 2008; O'Roak *et al.*, 2011; Poot *et al.*, 2010), SCZ and bipolar disorder (Friedman *et al.*, 2008; O'Dushlaine *et al.*, 2011; Wang *et al.*, 2010b), ID and language impairment (Mikhail *et al.*, 2011; Petrin *et al.*, 2010; Sehested *et al.*, 2010; Smogavec *et al.*, 2016; Vernes *et al.*, 2008; Zweier *et al.*, 2009), epilepsy and cortical dysplasia focal epilepsy (CFDE) syndrome (Friedman *et al.*, 2008; Mefford *et al.*, 2010; Smogavec *et al.*, 2016; Strauss *et al.*, 2006), Tourette syndrome (Belloso *et al.*, 2007; Verkerk *et al.*, 2003), among other disorders. Although with a complex phenotypic variability, patients carrying *CNTNAP2* mutations present a similar set of core features that include seizures, language impairments, intellectual disability and varying autistic-core behaviours [reviewed in (Penagarikano & Geschwind, 2012; Poot, 2017; Rodenas-Cuadrado *et al.*, 2014)].

Importantly, *Cntnap2* knockout (KO) mice develop epileptic and autism-related behavioural phenotypes that parallel patients' symptoms (Penagarikano *et al.*, 2011), thus emphasizing the relevance of the *CNTNAP2* gene in brain function. In accordance, recent human MRI studies revealed dramatic losses in frontal-lobe grey matter volume and alterations in functional connectivity in patients carrying *CNTNAP2* variations (Scott-Van Zeeland *et al.*, 2010; Tan *et al.*, 2010).

The *CNTNAP2* gene, located at the chromosomal region 7q35, encodes for Contactin-associated protein-like 2 (CASPR2), a neurexin-like cell-adhesion molecule first identified in the peripheral nervous system (PNS). Here, CASPR2 functions as a membrane scaffold for the clustering of *Shaker*-like K^+ channels (VGKC) in the juxtaparanodal domain region of the nodes of Ranvier, which facilitates the saltatory component of action potential propagation in myelinated nerves [(Poliak *et al.*, 1999; Poliak *et al.*, 2003), and reviewed in (Rasband & Peles, 2015; Simons & Trajkovic, 2006)]. Despite this fundamental role of CASPR2, it is clear that the pleiotropy of psychiatric symptoms presented by patients with *CNTNAP2* variations cannot be fully explained by a disruption of myelination processes (which occur only postnatally) or PNS dysfunction. Accordingly, CASPR2 is also highly expressed in the brain (Gordon *et al.*, 2016; Poliak *et al.*, 1999), early on during development into adulthood, in particular in the cerebral cortex (where there is an abundance of unmyelinated neurons) and hippocampus, thus hinting at additional crucial roles for CASPR2 in the CNS that still remain largely elusive and debatable. Loss of Caspr2 in mice, which present a striking ASD-like behavioural phenotype, results in striking neuropathological defects that include loss of inhibitory GABAergic interneurons and altered balance of excitation/inhibition (E/I balance), asynchronous neuronal firing, impaired neuronal migration, and reduced local and long-range prefrontal functional connectivity (Liska *et al.*, 2017; Penagarikano *et al.*, 2011). These latter phenotypes are in agreement with earlier brain histopathological and fMRI studies performed in patients with *CNTNAP2* mutations (Scott-Van Zeeland *et al.*, 2010; Strauss *et al.*, 2006). More recently, a few studies have provided valuable insights into a potential synaptic function mediated by CASPR2. One study reports that shRNA-mediated loss of Caspr2 in cortical neurons results in impaired dendritic arborisation, abnormal spine development and a reduced number of excitatory and inhibitory synapses, with consequent perturbations in basal synaptic transmission (Anderson *et al.*, 2012). In another study, *Cntnap2* KO-cultured cortical neurons were shown to display reduced spine density and altered spine morphology, with additional cytoplasmic aggregates of AMPARs, suggestive of altered glutamatergic function (Varea *et al.*, 2015).

Interestingly, synaptic abnormalities and glutamatergic dysfunction have been consistently pinpointed as common underlying pathologies in several psychiatric disorders, including ASD, ID and SCZ (Penzes *et al.*, 2011; Volk *et al.*, 2015). Indeed, a vast number of the susceptibility risk genes identified so far in genetic association studies encode for proteins in the glutamate receptor complex, including not only glutamate receptors, but also synaptic scaffolds, synaptic signalling complexes as well as other synaptic cell-adhesion molecules [thoroughly reviewed in (Pescosolido *et al.*, 2012; Volk *et al.*, 2015)]. Glutamatergic synapses mediate most of the fast excitatory neurotransmission in the brain, and experience-based changes in their strength and efficacy account for diverse forms of synaptic plasticity, the cellular correlates of higher cognitive functions, including learning and memory. Importantly, emerging findings link defects in synaptic plasticity phenomena to the pathogenesis of distinct psychiatric disorders (Ramocki & Zoghbi, 2008; Volk *et al.*, 2015; Wondolowski & Dickman, 2013). Indeed, increasing evidence suggest that a failure in neuronal synaptic homeostasis, in particular, may result in neurological outcomes that overlap with several neuropsychiatric symptoms distinctive of ASD, ID or SCZ (Mullins *et al.*, 2016; Ramocki & Zoghbi, 2008; Toro *et al.*, 2010), and some of the strongest candidate risk genes implicated so far in such cognitive disorders are actual important molecular players involved in the regulation of homeostatic plasticity mechanisms (Fernandes & Carvalho, 2016; Volk *et al.*, 2015; Wondolowski & Dickman, 2013). Homeostatic plasticity mechanisms are typically triggered by neurons in response to destabilizing activity changes, and through compensatory and bidirectional changes in the postsynaptic accumulation of AMPARs, they allow experience-based synaptic adaptations to occur while maintaining neuronal networks in-balance for proper brain function [extensively reviewed in (Fernandes & Carvalho, 2016; Turrigiano, 2008; Vitureira & Goda, 2013)]. Overall, it is clear that the molecular underpinnings of neuropsychiatric disorders such as ASD, ID or SCZ are still far from being completely understood. However, it is evident that glutamatergic synapse function and homeostatic plasticity processes are fundamental for proper brain function and cognition, and, if disrupted, can contribute to the pathogenesis of cognitive disorders.

Given the relevance CASPR2 has been assuming as a fundamental regulator of brain function, and its implication in several distinct neuropsychiatric disorders, it becomes imperative to fully comprehend the full-spectrum of molecular functions played by CASPR2, and how they can sustain such pleiotropic phenotypes if disrupted in the context of disease. In line with the recent synaptic functions described for CASPR2, in this chapter we address the hypothesis that CASPR2 regulates glutamatergic function and synaptic plasticity phenomena. Herein, we demonstrate that Caspr2 is not only present in axonal compartments, but it is also

expressed in dendrites, and enriched in postsynaptic densities of glutamatergic synapses. Moreover, we show that Caspr2 interacts with the GluA1 subunit of AMPARs and regulates the trafficking and synaptic content of cell surface GluA1-containing AMPARs. Furthermore, we demonstrate that this role of Caspr2 is physiologically relevant for the regulation of glutamatergic function *in vivo*, since silencing Caspr2 expression in the mouse primary visual cortex (V1) decreases the amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs). Interestingly, our findings reveal that the expression of Caspr2 is regulated by neuronal activity, in particular by sensory experience, since endogenous levels of Caspr2 are increased in the visual cortex of mice subjected to paradigms of visual deprivation. Finally, we uncover a requirement of Caspr2 for the regulation of homeostatic synaptic plasticity, since loss of Caspr2 hinders the triggering of homeostatic mechanisms that scale AMPAR expression and function following prolonged activity inhibition *in vitro* and visual deprivation *in vivo*. Importantly, our findings indicate that Caspr2 is required for visually-driven experience-dependent homeostatic plasticity *in vivo*, and ultimately for sensory integration of visual information, further emphasizing the physiological relevance of Caspr2 in the regulation of neuronal homeostasis and brain function. Altogether, our findings explore and uncover an important molecular function for Caspr2 in the regulation of synaptic AMPARs, glutamatergic function and experience-dependent homeostatic synaptic plasticity. Importantly, this study underpins a potential role for glutamatergic synapse dysfunction and impaired neuronal homeostasis in the pathogenesis of CASPR2-associated disorders, and helps to clarify some of the diverse cognitive symptoms presented by patients.

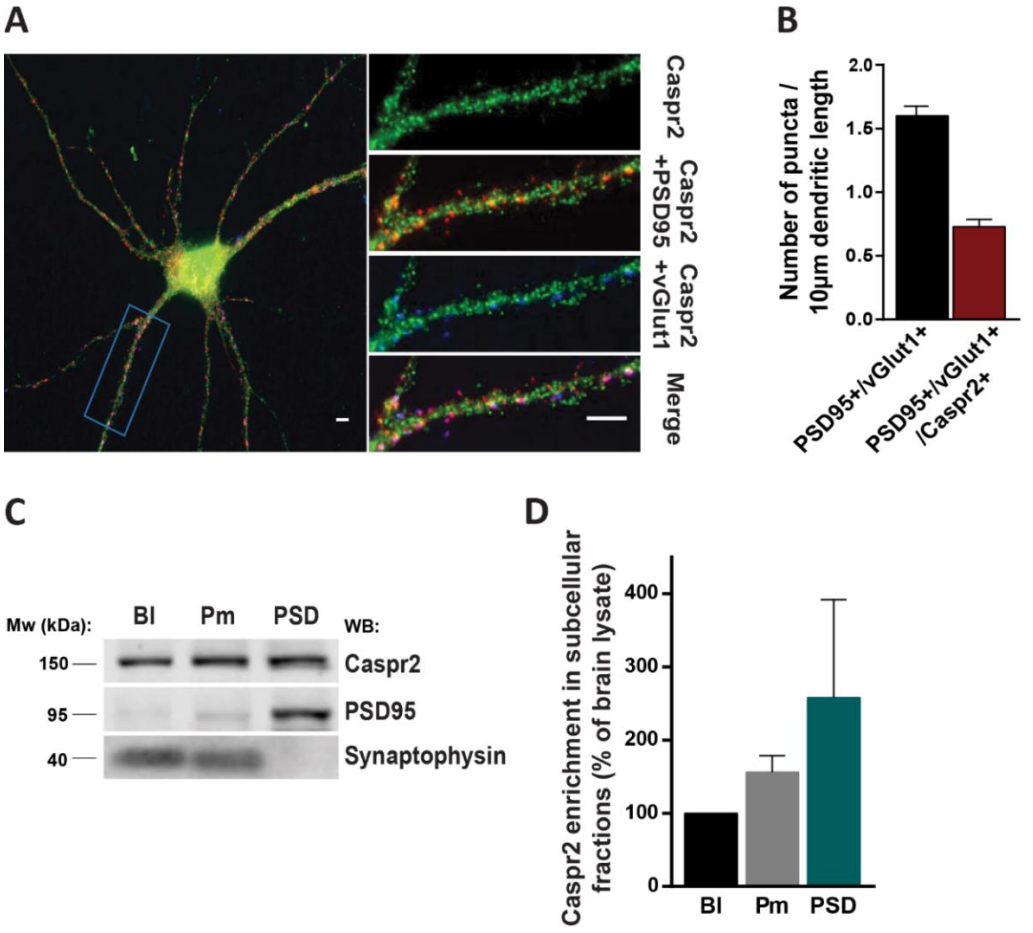
Results

Caspr2 localizes to excitatory synapses along dendrites, and it is present in isolated postsynaptic density fractions

Caspr2 has been thoroughly studied in peripheral axonal microdomains, where it is required for the clustering of *Shaker*-like K^+ channels in the juxtaparanodal region of the nodes of Ranvier, and for axonal excitability (Poliak *et al.*, 1999; Poliak *et al.*, 2003). Just recently, Caspr2 was additionally implicated in the regulation of dendritic arborization, and in the development of synapses and spines (Anderson *et al.*, 2012; Varea *et al.*, 2015). These novel functions attributed to Caspr2 suggest that its expression should not be limited to axonal compartments. Hence, in order to evaluate the neuronal and synaptic distribution of Caspr2, we immunolabelled low-density cultured rat cortical neurons with an antibody specific for Caspr2 and found an extensive punctuated distribution of Caspr2 along dendrites (Figure 4.1A). Neurons were also immunolabelled for the postsynaptic scaffolding protein PSD95 and for the presynaptic vesicular glutamate transporter vGluT1 to identify potentially functional glutamatergic synapses (Figure 4.1A), and to assess the presence of Caspr2 at or nearby these excitatory synapses. By determining the signal overlap of Caspr2 with synaptic clusters positive for both PSD95 and vGluT1, we found that 45.5 ± 3.6% of all cortical excitatory synaptic clusters contain Caspr2 (Figure 4.1B). Additionally, we observed a similar distribution of Caspr2 in approximately 60% of excitatory synapses in hippocampal neurons [Supplementary (Suppl.) Figure 4.S1]. If we consider that up until recently Caspr2 was not even studied outside axonal microdomains, these are rather impressive numbers that hint at potentially relevant roles played by Caspr2 at excitatory synapses.

In agreement with our results, some studies had previously reported the presence of Caspr2 clusters in dendritic spines (Varea *et al.*, 2015), as well as an enrichment in the synaptic plasma membrane (Bakkaloglu *et al.*, 2008; Chen *et al.*, 2015). Interestingly, the presence or enrichment of Caspr2 at the postsynaptic density (PSD), the functional specialization of the synapse, still remains controversial, with some studies suggesting that Caspr2 is merely perisynaptic and absent from the PSD (Chen *et al.*, 2015). Taking into consideration the functional relevance of proteins localized at the PSD, and how that localization often impacts their role in the regulation of synaptic plasticity mechanisms and synaptic signalling cascades, it is fundamental to confirm whether Caspr2 is indeed present at the PSD. To achieve this, we performed subcellular fractionation to isolate purified postsynaptic density fractions from the mouse whole brain (Figure 4.1C, D). Western blot analysis comparing the different subcellular fractions (Bl - brain lysate; Pm - plasma membrane; PSD - postsynaptic density) revealed an

enrichment of PSD95 from the Bl and Pm fractions to the PSD, whilst showing an absence of the presynaptic vesicular marker Synaptophysin in the PSD fraction (Figure 4.1C), indicating that we have successfully isolated purified PSD fractions. Importantly, we detected a 150 kDa band corresponding to the molecular weight of Caspr2 in all subcellular fractions (Figure 4.1C), and particularly enriched in the PSD (258.0 ± 133.8% when compared to the Bl fraction – Figure 4.1D), thus confirming the presence of Caspr2 in the postsynaptic density. This first set of results confirms that Caspr2 is expressed along dendritic neuronal compartments, where it localizes in great extent to sites at or nearby excitatory synapses. Moreover, these findings further refute previous evidence suggesting that Caspr2 is located only perisynaptically, and reveal the presence and enrichment of Caspr2 at the postsynaptic density. Although Caspr2 has been gaining considerable relevance as a novel regulator of synapses, its underlying mechanisms are still completely unknown. Hence, this privileged localization of Caspr2 at the PSD might give important clues about the potential roles it plays at the synapse, be it, for instance, as a canonical synaptic cell-adhesion molecule that bridges and stabilizes the synapse, or as a scaffolding protein that helps anchoring other molecules to the PSD, among other functions.



[Figure 4.1 - subtitle on the next page**]**

Figure 4.1 – Caspr2 is expressed along dendrites in cortical neurons, where it localizes to excitatory synapses, and it is present in isolated postsynaptic density fractions. (A, B) Caspr2 is localized at excitatory synapses along dendrites of cortical neurons. 13 DIV Banker cultured cortical neurons were fixed and stained against Caspr2, the postsynaptic marker PSD95 and the presynaptic marker VGluT1. **(A)** Representative immunofluorescence image of a cortical neuron showing significant co-localization between Caspr2 clusters and synaptic puncta. Blue square inset of a dendrite zoomed in on right panels. *Scale bars = 5 µm.* **(B)** Quantification of the number of glutamatergic synapses per dendritic length, identified as clusters with positive signal for both synaptic markers PSD95 and vGluT1 ($PSD95^+/vGluT1^+$), that contain Caspr2 ($PSD95^+/vGluT1^+/Caspr2^+$). Total number of analysed cells was obtained from at least three independent experiments. (N≥3, n≥ 30cells). Results are presented as mean ± S.E.M. **(C, D)** Caspr2 is present in isolated postsynaptic density fractions. Plasma membrane (Pm) and postsynaptic density fractions (PSD) were obtained by subcellular fractionation of mouse whole brain lysates (Bl). **(C)** The presence of PSD95, Synaptophysin and Caspr2 in the different fractions was determined by western blot (WB) analysis. **(D)** The enrichment of Caspr2 levels in the Pm and PSD fractions was quantified when compared to the Bl. Results were obtained from four independent experiments and are presented as mean ± S.E.M. (N=4).

Caspr2 interacts with the GluA1 subunit of AMPARs, and it is required to regulate surface GluA1-AMPARs in cortical synapses

Glutamate receptors of the AMPA-type are responsible for the fast component of excitatory synaptic transmission in the brain, and changes in the number or function of AMPARs at the synapse result in long-lasting modifications in synaptic strength and efficacy, which underlie synaptic plasticity events. Understandably, highly complex mechanisms are involved in the dynamic regulation of AMPARs, and numerous molecular players have already emerged as critical for AMPAR trafficking mechanisms, and for the expression of several forms of synaptic plasticity. Notably, we have previously identified Caspr1, a homologous family member of Caspr2, as an important regulator of AMPAR trafficking to synapses (Santos *et al.*, 2012). Taking into consideration the functional and structural homology between Caspr1 and Caspr2 (Einheber *et al.*, 1997; Peles *et al.*, 1997; Poliak *et al.*, 1999; Poliak *et al.*, 2003), and the strategic localization of Caspr2 at synapses, we decided to explore whether Caspr2 might also play a role in the regulation of synaptic AMPARs. To achieve this, we started by evaluating whether the dendritic and synaptic distribution of Caspr2 clusters might parallel that of surface GluA1-containing AMPARs (Figure 4.2A, B). To assess the surface expression of GluA1-containing AMPARs, low-density cultured cortical neurons at 13 DIV were live-stained with an antibody against an extracellular epitope in the GluA1 N-terminus. After fixation, neurons were further stained for Caspr2, and for PSD95 to visualize excitatory synapses. We observed that Caspr2 and GluA1 have a very similar punctuated distribution pattern along dendrites that partially overlaps (Figure 4.2A), and a quantitative analysis of the number of puncta per

dendritic length revealed that 23.0 ± 1.8% of all surface GluA1 clusters also contain Caspr2 (Figure 4.2B). A similar co-localization was also observed in hippocampal neurons (Suppl. Figure 4.S2).

This level of co-localization between GluA1 and Caspr2 clusters indicates a high degree of proximity between the two proteins, which is suggestive of a potential interaction between GluA1 and Caspr2. In order to confirm this hypothesis, we performed an immunoprecipitation assay with a specific antibody to pull-down Caspr2 from either mouse cortex (Figure 4.2C) or rat cerebellum lysates (Figure 4.2D), where Caspr2 is abundantly expressed (Poliak *et al.*, 1999). By WB analysis we confirmed that Caspr2 was successfully precipitated, and most importantly, we identified a 100 kDa-band corresponding to GluA1 that was co-precipitated with Caspr2, and that was absent when the immunoprecipitation was performed using non-immune immunoglobulins (IgGs) (Figure 4.2C, D). This result confirms that Caspr2 interacts with the GluA1 subunit of AMPARs, and it parallels previous evidence from the lab first identifying Caspr1 as an interactor of AMPAR subunits, including GluA1 (Santos *et al.*, 2012). This data hints at a potential shared role of Caspr1 and Caspr2 in AMPAR regulation.

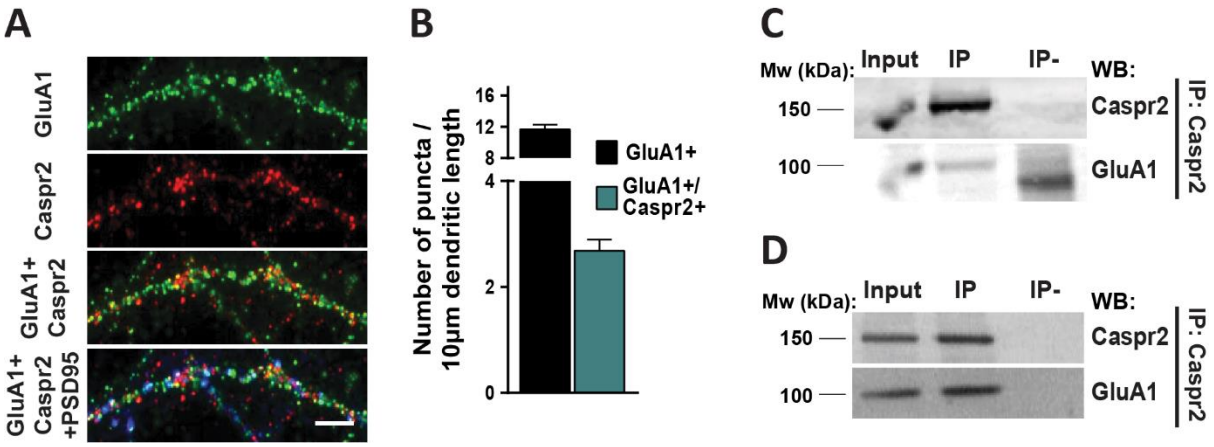


Figure 4.2 – Caspr2 interacts with the GluA1 subunit of AMPARs. (A, B) GluA1 and Caspr2 present a very similar dendritic distribution that substantially overlaps. 13 DIV low-density cultured cortical neurons were live-stained with an antibody against an extracellular epitope in the GluA1 N-terminus, fixed, and then stained for Caspr2 and the synaptic marker PSD95. **(A)** Representative immunofluorescence images showing the dendritic and synaptic distribution of GluA1 and Caspr2, with substantial cluster co-localization. *Scale bars = 5 µm*. **(B)** Quantification of the number of surface GluA1 clusters ($GluA1^+$) per dendritic length that are also positive for Caspr2 ($GluA1^+/Caspr2^+$). Total number of analysed cells was obtained from at least three independent experiments. Results are presented as means ± S.E.M. (N≥3, n≥ 30cells) **(C, D)** Caspr2 interacts with the GluA1 subunit of AMPARs. Caspr2 was immunoprecipitated from mouse cortex **(C)** or rat cerebellum **(D)** lysates using a specific antibody against the protein. Caspr2 and GluA1 levels were assessed by WB analysis. Immunoprecipitation (IP) of Caspr2, and co-IP of GluA1, were confirmed when compared to the initial protein sample (input) and a negative IP using non-immune IgGs (IP-).

Taking into account that 1) there is a high degree of structural and functional homology between Caspr1 and Caspr2, 2) both Caspr1 and Caspr2 are novel interactors of the GluA1 subunit of AMPARs, and 3) Caspr1 regulates the synaptic content of surface GluA1-containing AMPARs, we hypothesize that Caspr2 must be playing a similar role in the regulation of AMPAR trafficking. To investigate this, we transfected low-density cultured cortical neurons either with a control empty vector or with a bicistronic plasmid encoding mCherry and a specific shRNA sequence against Caspr2, previously validated to knock-down its endogenous expression (Anderson *et al.*, 2012). We then performed quantitative immunofluorescence analysis of the surface expression of GluA1-containing AMPARs and of the postsynaptic marker PSD95, as described above. Imaging fields were chosen by the presence of transfected, mCherry-positive cortical neurons (Figure 4.3A). We observed that shRNA-mediated loss of endogenous Caspr2 in cortical neurons resulted in a significant decrease of 18.8 ± 5.2% in the intensity of cell surface GluA1 total clusters, when compared to cells transfected with the control empty vector (Figure 4.3B - left). Furthermore, loss of Caspr2 also induced a significant decrease of 45.9 ± 5.6% in the fluorescence intensity of GluA1 clusters at the synapse, as determined by cluster co-localization with the postsynaptic marker PSD95 (Figure 4.3B - right). To exclude the contribution of off-target effects of the Caspr2 shRNA, neurons were co-transfected with the shRNA in parallel with a mutant construct of Caspr2 resistant to the shRNA (Anderson *et al.*, 2012). Expression of this refractory mutant rescued the KD-mediated decrease of total and synaptic GluA1 cluster intensities (Figure 4.3A, B), indicating that the defects in surface expression of GluA1 observed with the Caspr2 shRNA were specifically caused by the loss of endogenous Caspr2. Loss of Caspr2 in hippocampal neurons resulted in a similar disruption in the synaptic content of cell surface GluA1-containing AMPARs (Suppl. Figure 4.S3).

Although we have not yet tested for a potential interaction of Caspr2 with other AMPAR subunits other than GluA1, we have previously observed that Caspr1 does bind to other subunits including GluA2 and GluA4 (Santos *et al.*, 2012). AMPARs are composed of two out of four possible homologous subunits, from GluA1 to GluA4, that combine in heteromers to form functional ion channels with different properties, depending on the subunits present. In mature synapses, AMPARs are predominantly composed of GluA1/2 or GluA2/3 heteromers, and the GluA2 subunit is considered by many to be fundamental for the mechanisms that underlie several forms of synaptic plasticity. Curiously, we observed that shRNA-mediated loss of Caspr2 both in cortical and hippocampal neurons did not affect the cell surface and synaptic distribution of AMPARs containing the GluA2 subunit (Suppl. Figure 4.S4), although expression of the shRNA-resistant mutant construct of Caspr2 increased cell surface GluA2-AMPARs at the synapse (Suppl. Figure 4.S4D). These results suggest that, besides controlling

GluA1, Caspr2 may also play a role in the regulation of the GluA2 subunit of AMPARs, albeit not being required for maintaining its basal levels.

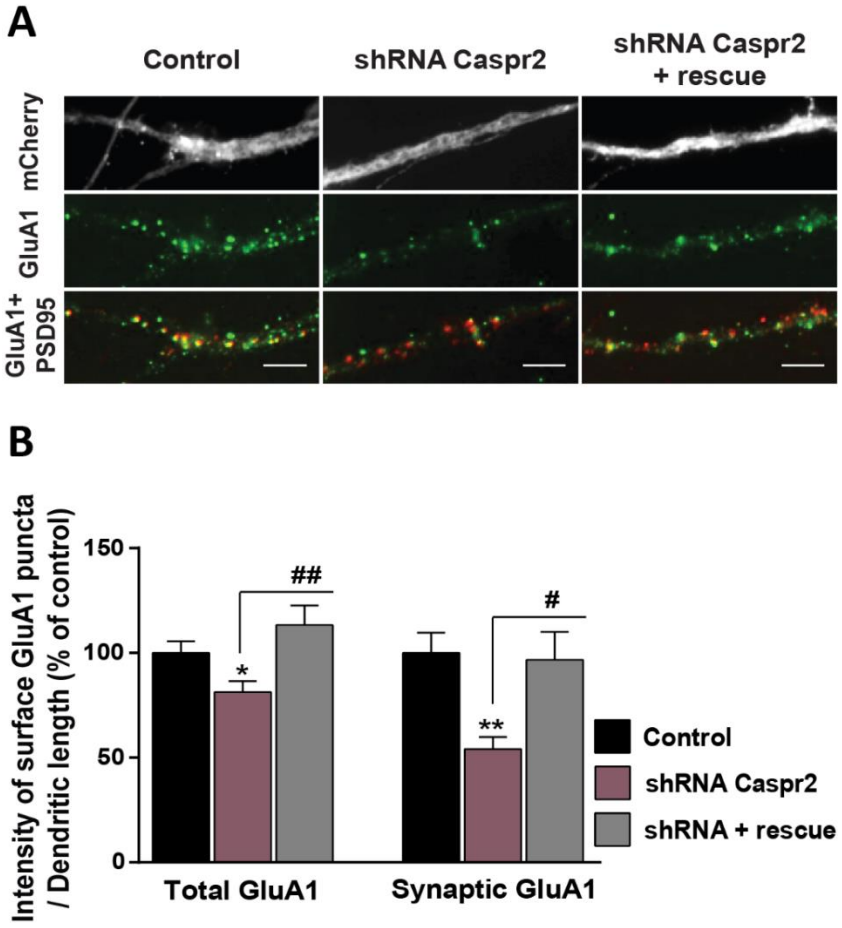


Figure 4.3 – Caspr2 is required to regulate the synaptic content of cell surface AMPARs. (A) Representative immunofluorescence images of mCherry-positive low-density cultured cortical neurons transfected at DIV 7 with a control empty vector or a plasmid encoding the Caspr2 shRNA, or co-transfected with the Caspr2 shRNA and a shRNA-resistant Caspr2 rescue mutant. At DIV 13, cells were live-stained against an extracellular epitope of GluA1, fixed, and then stained for the synaptic marker PSD95. *Scale bars = 5 μm*. **(B)** The fluorescence intensity of total and PSD95-colocalized synaptic clusters of surface GluA1 was analysed from mCherry-expressing transfected neurons. Total number of analysed cells was obtained from at least three independent experiments. (N≥3, n≥ 30cells). Results are presented as means ± S.E.M and the statistical significance was determined by the non-parametric Kruskal-Wallis test, followed by Dunn's Multiple Comparison post-hoc test, *p<0.05, **p<0.01 compared to control, and #p<0.05, ##p<0.01 relative to Caspr2 shRNA.

Overall, these pieces of evidence indicate that Caspr2, similarly to Caspr1, interacts with AMPARs, at least with the GluA1 subunit, and regulates their trafficking to the cell surface and into synapses, indicating that Caspr2 is important for the regulation of glutamatergic function. These findings are rather interesting and consistent with previous evidence showing the presence of cytoplasmic aggregates of AMPARs in *Cntnap2* KO cortical neurons (Varea *et al.*, 2015). It is possible that Caspr2 interacts with GluA1 early on during the secretory pathway to then promote the trafficking of AMPARs to the cell surface and into the synapse. In the absence of Caspr2 (or Caspr2 dysfunction in consequence of gene variations), these mechanisms of AMPAR trafficking might be compromised, hence giving origin to cytoplasmic aggregates of the receptors, and the consequent decrease in the cell surface expression and synaptic content of GluA1-containing AMPARs.

Caspr2 is required for *in vivo* regulation of AMPAR function in the mouse visual cortex

So far in the present chapter, we were able to demonstrate that the autism-related Caspr2, known to be enriched in axonal compartments, is localized in excitatory synapses along cortical dendrites, and present in purified postsynaptic density fractions. This pivotal localization at the synapse is suggestive of a fundamental synaptic role of Caspr2, and consistent with findings that implicate Caspr2 in spine development and maintenance, and dendritic arborisation (Anderson *et al.*, 2012; Gdalyahu *et al.*, 2015; Varea *et al.*, 2015). Additionally, we revealed that Caspr2 interacts with the GluA1 subunit of AMPARs and is necessary to regulate the surface expression of AMPARs and their trafficking into the synapse, thus confirming previous evidence suggestive of a role for Caspr2 in the control of glutamatergic function (Varea *et al.*, 2015). Taking into consideration the physiological importance of AMPARs and of the mechanisms that regulate their availability at the synapse, this novel function attributed to Caspr2 assumes particular relevance.

This potential Caspr2 role gains further significance in light of robust findings consistently pinpointing synaptic abnormalities and glutamatergic dysfunction as common underlying pathologies in several psychiatric disorders, including ASD, ID and SCZ (Bourgeron, 2015; Penzes *et al.*, 2011; Verpelli & Sala, 2012; Volk *et al.*, 2015). Indeed, a vast number of susceptibility risk genes associated to these disorders encode for proteins in the glutamate receptor complex, including not only glutamate receptors but also synaptic scaffolds, synaptic signalling complexes as well as other synaptic cell-adhesion molecules [thoroughly reviewed in (Pescosolido *et al.*, 2012; Volk *et al.*, 2015)]. Our data linking Caspr2 to the synapse and to

AMPAR regulation, together with evidence recurrently implicating the CASPR2-encoding *CNTNAP2* gene in several of the above psychiatric disorders (Penagarikano & Geschwind, 2012; Poot, 2017; Rodenas-Cuadrado *et al.*, 2014), flag *CNTNAP2* as one potential additional gene in this susceptibility hub of the glutamatergic synapse prone to dysfunction. Nevertheless, despite intense investigations, the pathogenic mechanisms ensuing from perturbations in Caspr2 function are still unknown.

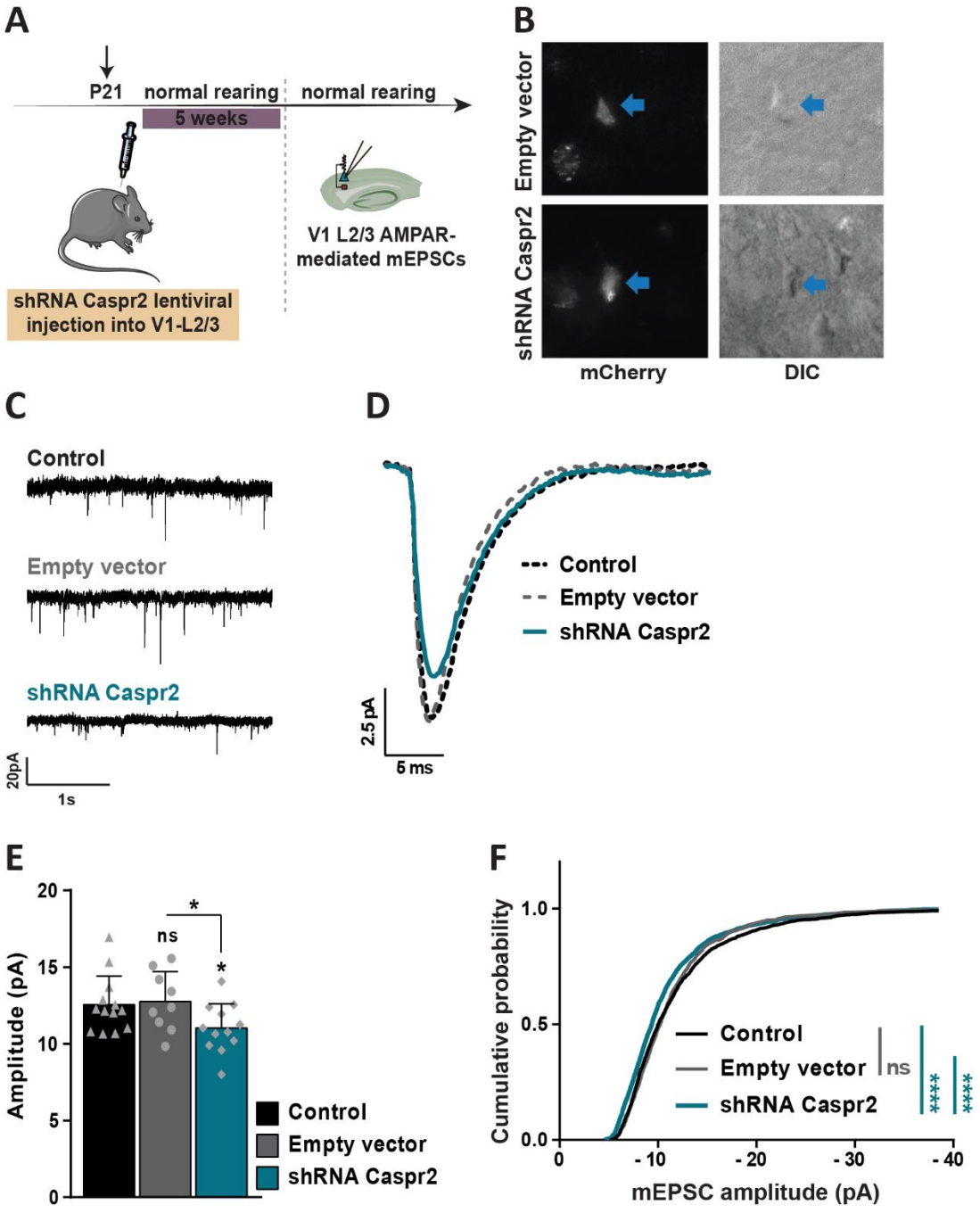


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Figure 4.4 – *In vivo* loss of Caspr2 in the mouse primary visual cortex (V1) significantly decreases the amplitude of AMPAR-mediated mEPSCs of layer 2/3 pyramidal neurons. **(A)** Lentivirus encoding either an empty-vector or the shRNA against Caspr2 were generated, and injected into layer2/3 of the V1 of normal reared P21-P28 C57BL/6J mice. Five weeks post-injection, AMPAR-mediated mEPSCs were recorded from mCherry-expressing infected V1-L2/3 pyramidal neurons. **(B)** Representative image of mCherry-expressing L2/3 pyramidal neurons in V1 acute slices from mice infected either with empty vector- or shRNA Caspr2-expressing lentivirus. **(C - E)** Comparison of representative mEPSC traces **(C)**, average mEPSC traces **(D)** and average mEPSC amplitudes **(E)** recorded from V1-L2/3 pyramidal cells of either control mice (black, n=13 cells), empty vector-infected (grey, n=9 cells) or shRNA Caspr2-infected mice (blue, n=12 cells). In **(E),** results are presented as mean ± S.E.M. and statistical significance was determined by one-way ANOVA test followed by Fisher's protected least significant difference (PLSD) post hoc test, *p<0.05; ns - non-significant. **(F)** Cumulative histograms showing that only the distribution of mEPSC amplitudes of shRNA Caspr2-infected mice (blue), but not of empty vector (grey), is significantly smaller than that of control littermates (black). Statistical significance was determined by the Kolmogorov-Smirnov Test, ****p<0.0001.

Clearly, our findings of a novel role for Caspr2 in the regulation of AMPAR trafficking warrant further exploration to determine whether such mechanism might be relevant for physiological glutamatergic function. Hence, we decided to investigate if Caspr2 is necessary for AMPAR function *in vivo*. To achieve this, we generated recombinant lentivirus encoding either the control empty vector, or the shRNA against Caspr2, and co-expressing mCherry from the bicistronic lentiviral vector used previously, and injected them into layer2/3 of the primary visual cortex (V1) of C57BL/6J mice to knockdown Caspr2 expression (Figure 4.4A). Infected pyramidal neurons in V1-layer2/3 of injected animals were identified by the pyramid-shaped soma with the apical dendrite pointing to the pia, and expression of mCherry fluorescence (Figure 4.4B). To determine changes in basal synaptic transmission, and assess if Caspr2 is required for basal AMPAR function, we measured AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs - Figure 4.4C). We observed that the average trace (Figure 4.4D - grey trace) and the average amplitude (Figure 4.4E - grey bar: 12.76 ± 0.65 pA) of mEPSCs recorded from animals injected with the empty vector virus remained unaltered when compared to mEPSCs recorded from non-infected control mice (in Figure 4.4E - black: 12.55 ± 0.52 pA). A cumulative histogram of mEPSC amplitudes further confirms that the distribution of mEPSC amplitudes of empty vector-infected mice does not differ significantly from control amplitudes (Figure 4.4F – grey trace).

On the other hand, there is a marked decrease in the amplitude of average trace of mEPSCs recorded from animals injected with the Caspr2 shRNA (Figure 4.4D - blue), and the average amplitude of mEPSCs is significantly smaller when compared to that of both control and empty vector-mice (Figure 4.4E - blue: 11.03 ± 0.46 pA). Moreover, the cumulative

distribution of mEPSC amplitudes of the Caspr2 shRNA-mice is significantly shifted towards smaller values than control or empty-vector amplitudes (Figure 4.4F - blue trace). No changes in average frequency or current kinetics of mEPSCs were observed between genotypes (Supplementary Table S1). These observations indicate that *in vivo* loss of Caspr2 impairs basal AMPAR-mediated synaptic transmission in the mouse visual cortex, revealing that Caspr2 is necessary for the regulation of glutamatergic function *in vivo*.

Caspr2 is regulated by neuronal activity and its loss prevents the homeostatic synaptic scaling of GluA1-AMPARs in cortical neurons

Rapid changes in AMPAR trafficking and function at the synapse occur in response to different patterns of neuronal activity, and are fundamental for the expression of various forms of synaptic plasticity (Anggono & Huganir, 2012; Shepherd & Huganir, 2007). In the present chapter, we were able to uncover a novel synaptic role for Caspr2 in the regulation of AMPAR trafficking to the cell surface and synapses, and, most importantly, we found Caspr2 to be physiologically relevant for the regulation of AMPAR function *in vivo* in the mouse visual cortex. Taking into consideration the significance of our findings, it is imperative to fully uncover the molecular mechanisms orchestrated by Caspr2 to control AMPAR trafficking, and determine whether such mechanisms may be activity-dependent and impinge on the regulation of synaptic plasticity events.

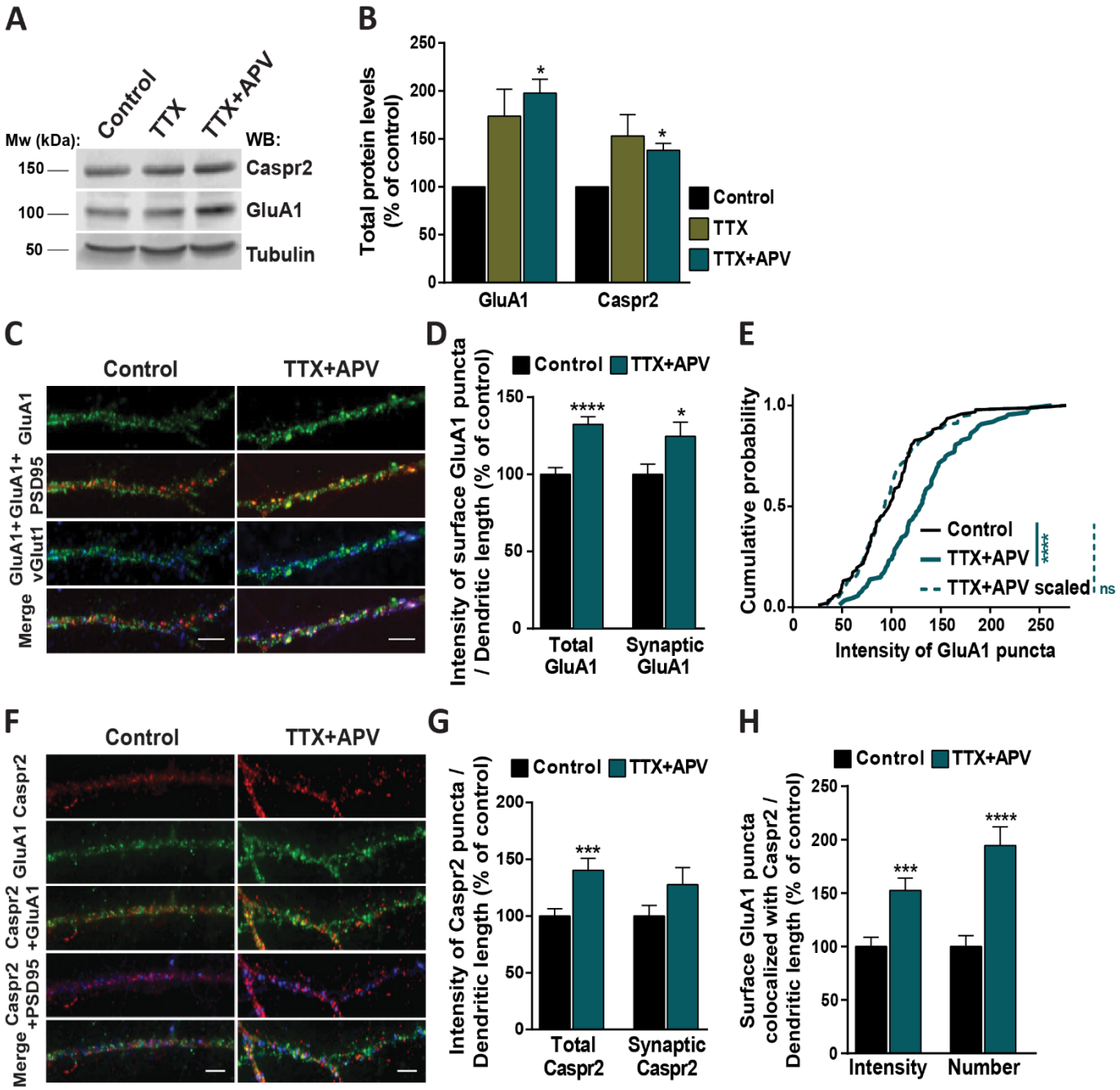
Interestingly, in the previous chapter we discovered that Caspr1, the homologous family member of Caspr2, is necessary for the regulation of hippocampal synaptic AMPARs in the context of homeostatic synaptic plasticity (please refer to Chapter III). This form of synaptic plasticity is able to maintain neuronal networks in-balance when in face of potentially destabilizing changes in activity that can compromise neuronal function (Fernandes & Carvalho, 2016; Turrigiano, 2008). Molecularly, the homeostatic adjustment of synaptic strengths can be achieved through bi-directional compensatory changes in the accumulation of AMPARs at the synapse, in a process commonly known as synaptic scaling. Although the mechanisms underlying homeostatic synaptic scaling are still far from understood, several molecular players involved in this process have already been identified, including Caspr1 (Fernandes & Carvalho, 2016). Most importantly, emerging findings now suggest that impaired neuronal synaptic homeostasis might be an underlying pathology in several cognitive disorders (Mullins *et al.*, 2016; Ramocki & Zoghbi, 2008; Volk *et al.*, 2015; Wondolowski & Dickman, 2013). Taking into consideration 1) the implication of the CASPR2-encoding *CNTNAP2* gene in neuropsychiatric disorders (Penagarikano & Geschwind, 2012; Poot, 2017; Rodenas-

Cuadrado *et al.*, 2014), together with 2) the newly-found role of Caspr2 in the regulation of AMPAR trafficking and function, and 3) our previous findings pinpointing the homologous Caspr1 as a molecular mediator of synaptic scaling, we hypothesized that Caspr2 plays a similar fundamental role in the regulation of neuronal homeostasis.

To explore this hypothesis, we first evaluated if the expression of endogenous Caspr2 is itself regulated by neuronal activity. Cultured cortical neurons were treated for 48 h with either the voltage-gated Na^+-channel blocker tetrodotoxin (TTX, 1 µM) to inhibit action potential firing, or TTX together with D-(-)-2-amino-5-phosphonopentanoic acid (APV, 100 µM) for additional blockade of NMDAR function, and total protein levels of both the GluA1 subunit of AMPARs and Caspr2 were evaluated by WB analysis (Figure 4.5A). As previously described (Ju *et al.*, 2004; Wierenga *et al.*, 2005), we observed that inhibition of neuronal activity with TTX or TTX+APV resulted in a marked increase of 73.7 ± 28.0% or 97.8 ± 14.4%, respectively, on total protein levels of GluA1 (Figure 4.5B), indicating that 48 h of activity blockade in cortical neurons are sufficient to induce homeostatic mechanisms that scale up GluA1-containing AMPARs. Importantly, we observed that the increase of GluA1 levels following activity inhibition with TTX and TTX+APV was paralleled by a clear upregulation of 53.0 ± 22.4% and 38.3 ± 7.1%, respectively, on total protein levels of endogenous Caspr2 (Figure 4.5B), demonstrating that the endogenous expression of Caspr2 can be regulated by neuronal activity. Interestingly, we found a similar effect in the expression of Caspr1 following activity inhibition in hippocampal neurons (see Chapter III).

We further evaluated the effect of prolonged inhibition of neuronal activity in surface GluA1-containing AMPARs and Caspr2 using an immunocytochemistry approach. We treated low-density cultured cortical neurons for 48 h with 1 µM TTX together with 100 µM APV for a parallel blockade of action potentials and NMDAR function, and performed quantitative immunofluorescence analysis of the surface expression of AMPARs by live-staining neurons with an antibody against an extracellular epitope in the GluA1 N-terminus (Figure 4.5C). After fixation, neurons were stained for MAP2 to visualize the dendritic structure (not shown), and for PSD95 (postsynaptic marker) and vGluT1 (presynaptic marker) to identify excitatory synapses (Figure 4.5C). We observed that prolonged inactivity induced by TTX+APV treatment of cortical neurons significantly increased the fluorescence intensity of both total (132.4 ± 4.9%) and synaptic (as defined by cluster colocalization with synaptic markers PSD95 and vGluT1 – 124.5 ± 9.3%) cell surface GluA1 clusters (Figure 4.5D). A cumulative histogram plot of cell surface GluA1 cluster intensities revealed that the entire distribution of TTX+APV-GluA1 intensities shifts uniformly and significantly towards larger values when compared to control (Figure 4.5E). Importantly, when transformed by a scaling factor, the scaled TTX+APV

distribution (Figure 4.5E – dashed trace) was completely superimposed with the control cumulative distribution, indicating that the observed scaling of cell surface GluA1 clusters following activity inhibition occurs in a multiplicative manner, which is a defining characteristic of synaptic scaling mechanisms that allows the conservation of the relative differences in strength across the entire synapse population of a given neuron (Turrigiano *et al.*, 1998).



[Figure 4.5 - subtitle on the next page**]**

Figure 4.5 – The endogenous expression of Caspr2 is regulated by neuronal activity. **(A-B)** Chronic blockade of activity in cortical neurons increases total protein levels of GluA1 and Caspr2. Cultured rat cortical neurons at 13 DIV were treated for 48 h either with 1 µM TTX alone, or together with 100 µM APV, to inhibit neuronal activity, after which protein extracts were obtained. **(A)** Total protein levels of GluA1 and Caspr2 were evaluated by WB analysis. **(B)** Results are presented as mean ± S.E.M., when compared to control and normalized to the loading control Tubulin. Statistical significance was determined by the non-parametric Kruskal-Wallis test, followed by the Dunn's post-hoc test, *p<0.05 (N=4). **(C - E)** Prolonged neuronal inactivity increases the superficial expression of synaptic GluA1-containing AMPARs in a multiplicative manner. Neuronal activity in 11 DIV low-density cultured cortical neurons was inhibited for 48 h either with 1 µM TTX together with 100 µM APV. Cells were then live-stained with a specific antibody against an extracellular epitope in GluA1 N-terminus, fixed, and then stained for the synaptic markers PSD95 and VGluT1. **(C)** Representative immunofluorescence images of cultured cortical neurons treated for 48 h with TTX + APV, and stained for superficial GluA1, PSD95 and VGluT1. *Scale bars = 5µm.* **(D)** The fluorescence intensity of total and synaptic (colocalized with PSD95 and vGlut1) clusters of surface GluA1 was quantified. Total number of analysed cells was obtained from at least three independent experiments. (N≥3, n≥30 cells). Results are presented as mean ± S.E.M. and statistical significance was determined by the non-parametric Mann-Whitney Test, *p<0.05, ****p<0.0001 compared to control. **(E)** Cumulative distribution of surface GluA1 cluster intensities is significantly shifted towards larger values following TTX+APV treatment (blue solid line). The scaled TTX+APV distribution (blue dashed line) superimposes with the control cumulative distribution, showing that prolonged neuronal inactivity upscaled GluA1 cluster intensities in a multiplicative manner. Statistical significance was determined by the Kolmogorov-Smirnov Test, ****p<0.0001 compared to control. **(F - H)** Prolonged neuronal inactivity increases total and synaptic clusters of Caspr2. Neuronal activity in cortical neurons was inhibited as described above, and cells were stained against surface GluA1, Caspr2 and the synaptic marker PSD95. **(F)** Representative immunofluorescence images of cultured cortical neurons treated for 48 h with TTX + APV, and stained for superficial GluA1, Caspr2 and PSD95. *Scale bars = 5µm.* **(G, H)** The fluorescence intensity of total and PSD95-colocalized synaptic clusters of Caspr2 **(G)**, and the intensity and number of surface GluA1 clusters that colocalize with Caspr2 **(H)** were quantified. Total number of analysed cells was obtained from at least three independent experiments. (N≥3, n≥30 cells). Results are presented as mean ± S.E.M. and statistical significance was determined by the non-parametric Mann-Whitney Test, ***p<0.001, ****p<0.0001 compared to control.

These observations are in agreement with previous findings reporting a multiplicative scaling in the synaptic expression of GluA1-containing AMPARs following activity blockade in cortical neurons (Wierenga *et al.*, 2005). Using an identical approach as described above, we observed that TTX+APV treatment of cortical neurons further induced a marked increase in the fluorescence intensity of both total (140.3 ± 10.5%) and PSD95-colocalized synaptic (127.6 ± 15.0%) clusters of Caspr2 (Figure 4.5F, G), which complements our WB data showing an upregulation of Caspr2 total protein levels. Moreover, we found that the fluorescence intensity and number of cell surface GluA1 clusters that co-localize with Caspr2 were significantly upscaled (152.5 ± 11.5% and 194.5 ± 17.4%, respectively) following prolonged inhibition of neuronal activity with TTX+APV (Figure 4.5H), which might be suggestive of a stronger

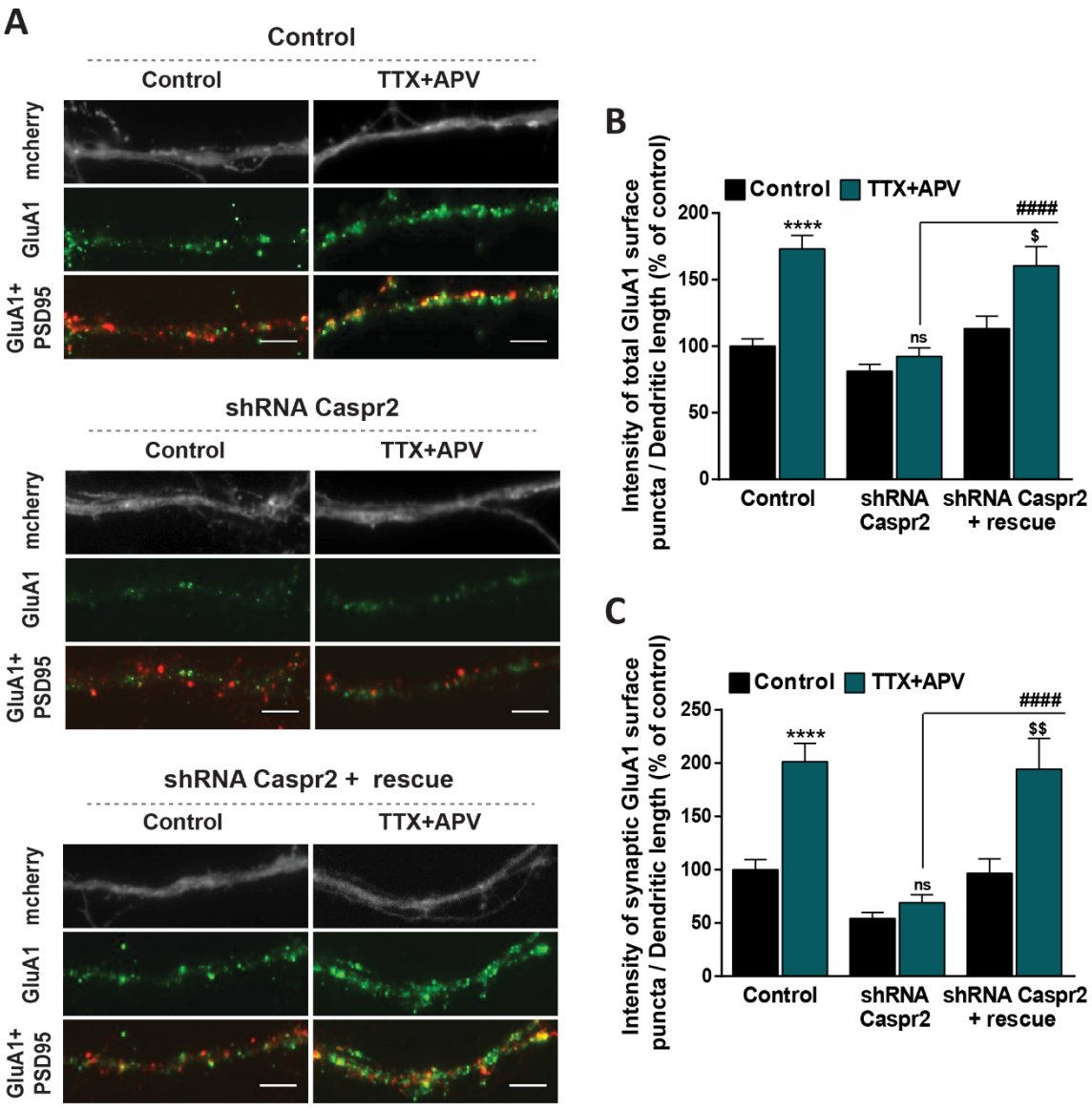
interaction between Caspr2 and GluA1 in such conditions. Prolonged inhibition of neuronal activity in hippocampal neurons elicited a similar homeostatic synaptic upscaling of Caspr2 endogenous expression (Figure 4.S5B), as well as an increase in the co-localization between GluA1 and Caspr2 clusters (Figure 4.S5C).

Overall, this set of results indicates that the endogenous expression of Caspr2 is regulated by neuronal activity, in particular by paradigms of activity inhibition that elicit the parallel scaling of surface AMPARs at the synapse. Importantly, these findings concur with recent evidence revealing an activity-dependent regulation of the expression or function of some of the already-identified molecular players underlying synaptic scaling mechanisms (Fernandes & Carvalho, 2016). Taking these pieces of evidence into account, together with the relevant role of Caspr2 in the regulation of AMPAR trafficking and function, it is possible that Caspr2 is required for the mechanisms underlying the synaptic scaling of surface GluA1-containing AMPARs. To investigate this, we transfected low-density cultured cortical neurons either with a control empty vector or with the bicistronic plasmid encoding mCherry and the shRNA sequence against Caspr2 to knock-down its endogenous expression, as described previously. We then treated neurons for 48 h with 1 µM TTX together with 100 µM APV, and live-stained them for cell surface GluA1-AMPARs, as described above. After fixation, neurons were stained for PSD95 as a postsynaptic marker of excitatory synapses, and imaging fields were chosen by the presence of transfected, mCherry-positive cortical neurons (Figure 4.6A). As shown earlier, we observed that TTX+APV-induced prolonged inhibition of neuronal activity in control cortical neurons resulted in a significant increase in the fluorescence intensity of both total (173.1 ± 10.1%; Figure 4.6B) and PSD95-colocalized synaptic (201.3 ± 17.3%; Figure 4.6C) cell surface GluA1 clusters. On the other hand, neurons expressing the shRNA to knock-down Caspr2 expression not only presented a marked decrease in the fluorescence intensity of GluA1 cell surface clusters in basal conditions, as shown previously in this chapter (please refer to Figure 4.3), but completely failed to trigger the homeostatic scaling of GluA1 following activity inhibition with TTX+APV (Figure 4.6B, C). Importantly, expression of a shRNA-resistant mutant form of Caspr2 in cortical neurons not only rescued the KD-mediated decrease of GluA1 surface levels, but it fully restored the scaling of both total (160.6 ± 14.4%; Figure 4.6B) and synaptic (194.6 ± 28.7%; Figure 4.6C) cell surface GluA1 clusters following activity blockade with TTX+APV. These results indicate that the defects in the surface expression of GluA1 observed with the Caspr2-shRNA were specifically caused by the loss of endogenous Caspr2, and reveal that Caspr2 is required for the synaptic scaling of cell surface GluA1-containing AMPARs in cortical neurons.

Altogether, these results uncover an activity-dependent control of Caspr2 expression, and most likely of its function, and reveal that the role played by Caspr2 in the regulation of AMPAR trafficking is fundamental for the expression of homeostatic synaptic plasticity, thus pinpointing Caspr2 as a novel molecular regulator of synaptic scaling. Given that in the previous chapter we uncovered an identical role for Caspr1 in the regulation of homeostatic synaptic scaling in the hippocampus, our findings attribute a novel set of unsuspected central functions to the family of CASPR proteins, and elevate it to a status of relevance in the context of neuronal synaptic homeostasis. Remarkably, however, there seems to be some region-specificity for the requirement of either Caspr1 or Caspr2 to underlie such scaling processes. Indeed, unlike what was observed in cortical neurons (Figure 4.6), synaptic scaling of cell surface AMPARs following prolonged activity inhibition was intact in hippocampal neurons lacking Caspr2 (Figure 4.S6), indicating that Caspr2 is required for homeostatic plasticity in the cortex, but not in the hippocampus. Instead, Caspr1 is the alternative required CASPR protein for homeostasis in the hippocampus. Confirming this duality of region-specific roles played by both CASPR proteins, we found no changes in the basal synaptic content of cell surface AMPARs (Figure 4.S7) nor in basal AMPAR-mediated currents (Figure 4.S8) in the absence of Caspr1 in cortical cultures or in the mouse visual cortex, respectively, suggesting that Caspr1, unlike Caspr2, is not required for the regulation of cortical glutamatergic function.

This dichotomy, despite being rather interesting, raises several questions. Firstly, both Caspr1 and Caspr2 are similarly expressed in the hippocampus and cortex (Peles *et al.*, 1997; Poliak *et al.*, 1999), which makes it unlikely that differences in region-specificity result from variations in protein expression. Secondly, Caspr1 and Caspr2 share a significantly high degree of homology, both in their structure and function (Peles *et al.*, 1997; Poliak *et al.*, 1999), which might indicate that they play fairly similar roles. Indeed, this is confirmed by the findings uncovered in the present study, together with observations previously collected in our laboratory (Santos *et al.*, 2012): both Caspr1 and Caspr2 are novel interactors of the GluA1 subunit of AMPARs, and are necessary for the regulation of cell surface AMPAR trafficking to the synapse and for the expression of homeostatic synaptic plasticity mechanisms. Interestingly, in the previous chapter we were able to dissect the molecular mechanism orchestrated by Caspr1, and identified the RNA-binding protein ZBP1 as the potential downstream mediator of Caspr1 in the regulation of AMPARs (please refer to Chapter III). Nevertheless, a similar mechanism is not likely to underlie Caspr2 function, since it is dependent on an intracellular proline-rich domain that is exclusive to Caspr1 structure, and absent from Caspr2. Hence, it is critical to further explore the role of Caspr2 in an attempt to dissect its underlying molecular mechanism. Such knowledge would be important to understand how Caspr1- or Caspr2-mediated mechanisms of AMPAR regulation differ from

one another, and how those differences can influence their relevance on activity-dependent processes either in the hippocampus or the cortex. Interestingly, although the mechanisms of AMPAR regulation and synaptic plasticity are thought to be common and fairly conserved across brain regions, important differences have already been found between the hippocampus and neocortex, for instance, which account for specific functional requirements of each region [reviewed in (Lee & Kirkwood, 2011)].



[Figure 4.6 - subtitle on the next page**]**

Figure 4.6 – Loss of endogenous Caspr2 prevents homeostatic upscaling of synaptic GluA1-containing AMPARs upon prolonged inactivity in cortical neurons. **(A)** Representative immunofluorescence images of mCherry-positive low-density cultured cortical neurons transfected at DIV 7 with a control empty vector (control - top) or a plasmid encoding the Caspr2 shRNA (middle), or co-transfected with the Caspr2 shRNA and a shRNA-resistant Caspr2 rescue mutant (bottom). At DIV 11, neuronal activity was blocked for 48 h with 1 µM TTX and 100 µM APV. Cells were then live-stained with an antibody against an extracellular epitope in GluA1 N-terminus, fixed, and then stained for the synaptic marker PSD95. *Scale bars = 5µm.* **(B, C)** The fluorescence intensity of total **(B)** and PSD95-colocalized synaptic **(C)** clusters of surface GluA1 was analysed from mCherry-expressing transfected neurons. Total number of analysed cells was obtained from at least three independent experiments (N≥3, n≥30 cells). Results are presented as mean ± S.E.M., and statistical significance was determined by the parametric two-way ANOVA Test, followed by Tukey's Multiple Comparison Test, ****p<0.0001 compared to control, ns relative to control Caspr2-shRNA, ####p<0.0001 compared to TTX+APV Caspr2-shRNA, $p<0.05, $$p<0.01 relative to control Caspr2-shRNA+rescue.

Caspr2 is regulated by visual experience and it is required for visually-driven experience-dependent homeostatic plasticity

Up until now in the present chapter, we were able to uncover a novel synaptic role for the autism-related Caspr2 in the regulation of AMPAR trafficking to the cell surface and synaptic sites, and revealed a requirement of Caspr2 for the triggering of mechanisms of homeostatic synaptic scaling following prolonged inhibition of neuronal activity. Importantly, we observed an impairment of AMPAR-mediated basal synaptic transmission in the visual cortex of mice lacking Caspr2, revealing that Caspr2 is necessary for the regulation of cortical glutamatergic function *in vivo*.

Taking this into consideration, we asked whether Caspr2 function *in vivo* might impinge on mechanisms of experience-dependent homeostatic plasticity that are fundamental for sensory processing (Whitt *et al.*, 2014). Such mechanisms have been widely characterized in the mouse visual cortex, since it is amenable to manipulations of neuronal activity *in vivo* through varied paradigms of sensory deprivation and, thus, an excellence model to investigate experience-driven homeostatic changes (Whitt *et al.*, 2014). It has been shown that just two days of visual deprivation (in the form of dark exposure - DE) causes a homeostatic increase of AMPAR-mediated mEPSCs in pyramidal neurons of layer 2/3 of the mouse primary visual cortex (V1-layer 2/3) that is accompanied by a specific upregulation in protein levels of the GluA1 subunit of AMPARs (Goel *et al.*, 2006; Goel *et al.*, 2011), suggesting that a prolonged absence of visually-driven activity scales up excitatory synapses in layer 2/3 of the V1. Since we previously observed an increase in GluA1 and Caspr2 protein levels following prolonged

inhibition of activity elicited by TTX+APV treatment of cultured cortical neurons (see Figure 4.5), we decided to evaluate if the endogenous expression of Caspr2 can also be regulated by visual experience *in vivo*. To investigate this, mice within the critical period for visual plasticity (Desai *et al.*, 2002; Whitt *et al.*, 2014) were visually deprived for two days, in the form of dark exposure (DE), after which primary visual cortices (V1) were collected for WB analysis (Figure 4.7A). As previously demonstrated (Goel *et al.*, 2006; Goel *et al.*, 2011), we observed a significant upregulation of total protein levels of GluA1 in the V1 of DE mice, when compared to normal reared (NR) littermates (Figure 4.7B, C). Additionally, DE induced a parallel significant increase in total protein levels of Caspr2 when compared to NR (Figure 4.7B, D), thus confirming that the expression of Caspr2 in the visual cortex is regulated by visual experience. This observation agrees with previous studies reporting visual experience-induced variations in the expression of several other known molecular players involved in mechanisms underlying synaptic scaling *in vivo* [reviewed in (Fernandes & Carvalho, 2016)]. Curiously, a recent study found that *in vivo* Caspr2 protein levels in mice change during the wake/sleep cycle, which is thought to be associated to homeostatic plasticity processes and fundamental for memory consolidation and learning (Diering *et al.*, 2017).

Given that Caspr2 is required for the regulation of basal AMPAR-mediated synaptic transmission *in vivo* in the visual cortex, and that its expression is altered in response to experience-dependent changes in neuronal activity, we asked whether Caspr2 might be required for visually-driven experience-dependent homeostatic plasticity in the visual cortex. To investigate this, C57BL/6J mice were injected into the V1 with recombinant lentivirus for the expression of the Caspr2 shRNA or the control empty vector, as described previously, and then visually deprived through dark exposure for two days (Figure 4.8A). Infected pyramidal neurons in V1-layer 2/3 of injected animals were identified by their structure and expression of mCherry fluorescence from the bicistronic lentiviral plasmid, as mentioned earlier (see Figure 4.4B), and AMPAR-mediated mEPSCs were recorded (Figure 4.8B). As expected, we observed that DE resulted in a marked increase of average traces [control - Figure 4.8C (NR - black, DE - green); empty vector - Figure 4.8F (NR - black, DE - pink)] and average amplitudes (control - Figure 4.8D [NR (black) = 12.55 ± 0.52 pA; DE (green) = 15.09 ± 1.01 pA]; empty vector - Figure 4.8G [NR (black) = 12.76 ± 0.74 pA; DE (pink) = 14.12 ± 4.64 pA]) of mEPSCs recorded from both non-infected control or empty vector-infected mice, when compared to their respective NR littermates. Additionally, the cumulative distribution of mEPSC amplitudes of both control (Figure 4.8E: NR - black; DE - green) and empty vector-infected (Figure 4.8H: NR - black; DE - pink) animals following DE was significantly shifted towards larger values than the respective NR distribution. Average frequency and current kinetics of mEPSCs remained unaltered following DE of both control and empty vector-infected mice (Suppl. Table S1).

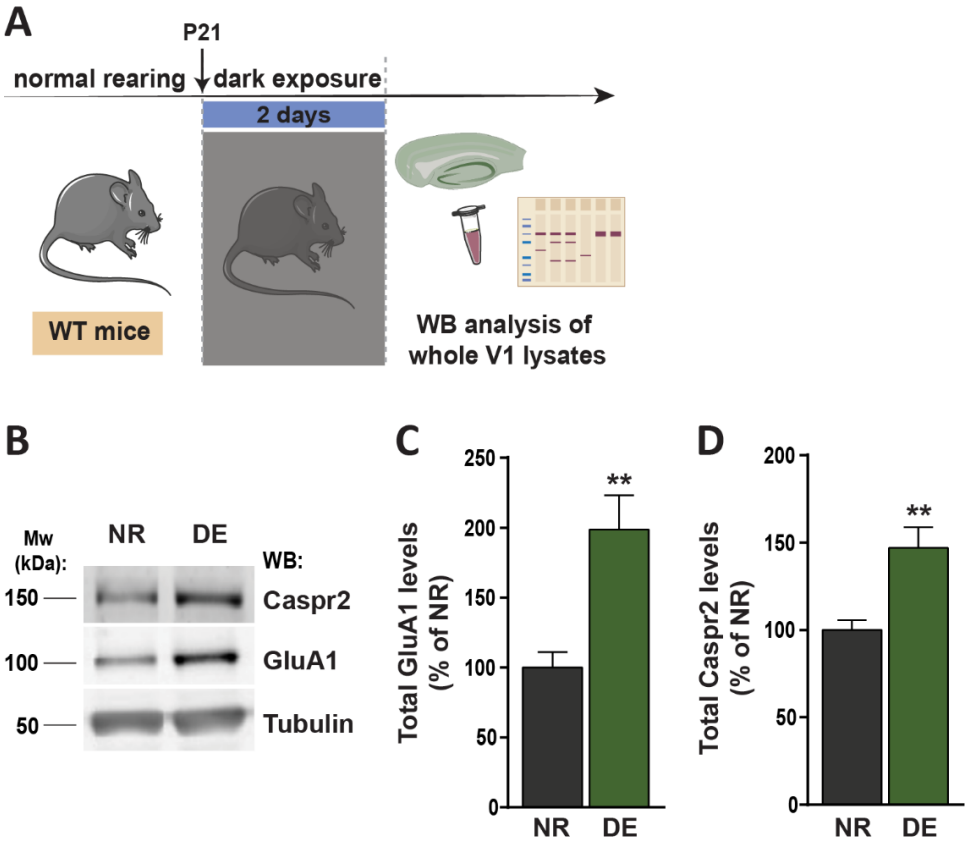
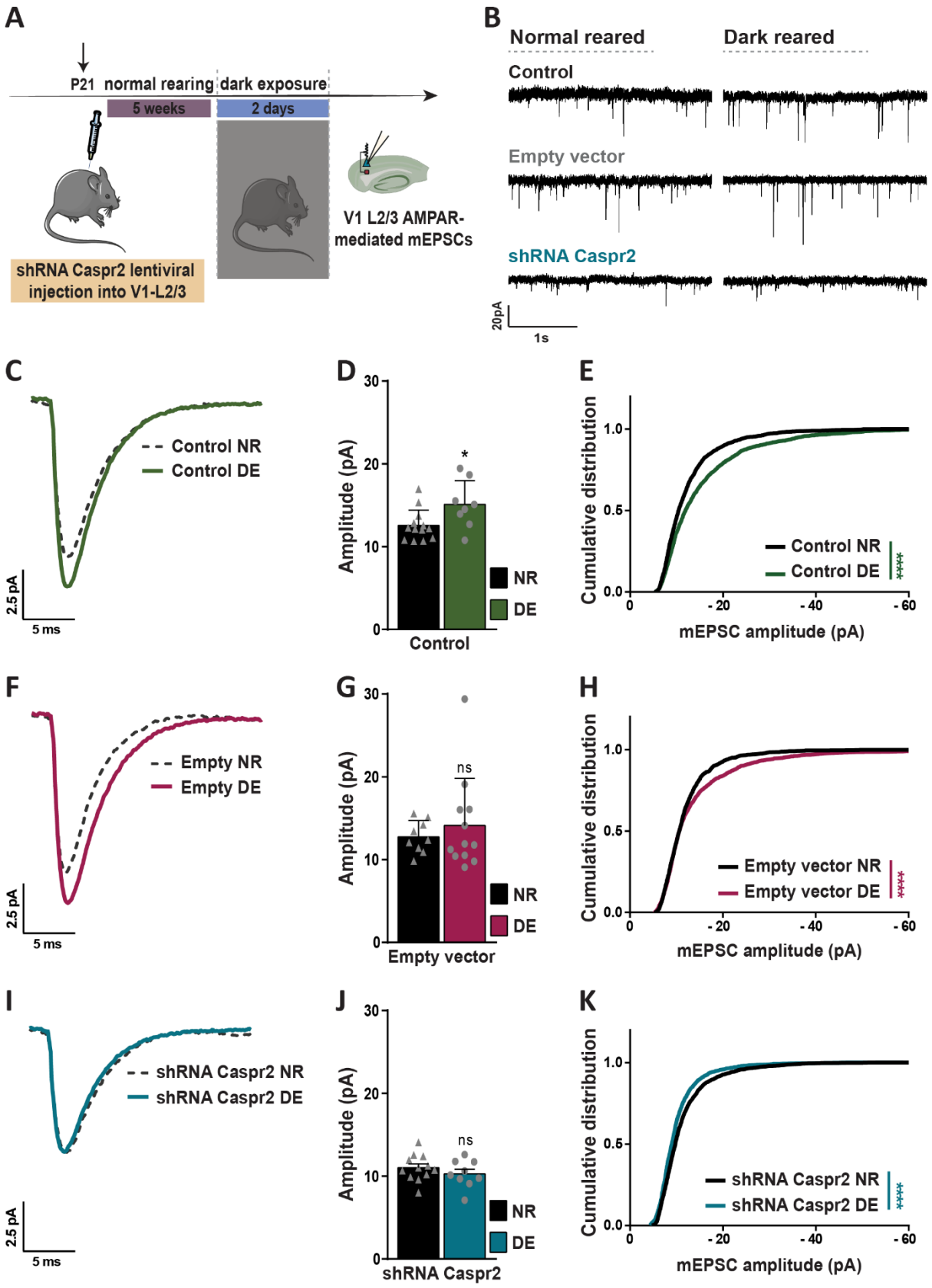


Figure 4.7 – Paradigms of visual deprivation significantly increase endogenous Caspr2 protein levels in the mouse primary visual cortex (V1). **(A)** Normal reared P21-P28 C57BL/6J mice were subjected to paradigms of visual deprivation, by being placed in a dark room for two days. Following dissection of whole chunks of the primary visual cortex (V1), V1 protein lysates were prepared for WB analysis. **(B)** Representative blots of GluA1 and Caspr2 levels in V1 lysates of normal reared (NR) or dark exposed (DE) mice. **(C, D)** Total protein levels of GluA1 **(C)** and Caspr2 **(D)** were determined. Results are presented as mean ± S.E.M, when compared to NR and normalized to the loading control Tubulin. Statistical significance was determined by the non-parametric Mann-Whitney Test, **p<0.01 relative to NR. (GluA1: N=5; Caspr2: N=7).



[Figure 4.8 - subtitle on the next page**]**

Figure 4.8 – *In vivo* loss of Caspr2 in the mouse primary visual cortex (V1) prevents experience-dependent homeostatic upscaling of AMPAR-mediated mEPSCs following paradigms of visual deprivation. **(A)** Lentivirus encoding either an empty-vector or the shRNA against Caspr2 were generated, and injected into layer2/3 of the V1 of normal reared P21-P28 WT C57BL/6J mice. Five weeks post-injection, mice were subjected to paradigms of visual deprivation, by being placed in a dark room for two days. AMPAR-mediated mEPSCs were then recorded from mCherry-expressing infected V1-L2/3 pyramidal neurons. **(B)** Representative mEPSC traces recorded from non-infected controls, or empty vector- or shRNA Caspr2-infected mice that were either normal reared (NR – left) or subjected to visual deprivation (DE – right). **(C - E)** Average mEPSC traces **(C)**, average mEPSC amplitudes **(D)**, and cumulative distribution of mEPSC amplitudes **(E)** recorded from V1-L2/3 pyramidal cells of either NR (black) or DE (green) control mice (NR, n=13 cells; DE, n=8 cells). **(F - H)** Average mEPSC traces **(F)**, average mEPSC amplitudes **(G)**, and cumulative distribution of mEPSC amplitudes **(H)** recorded from NR (black) or DE (pink) empty vector-infected mice (NR, n=9 cells; DE, n=12 cells). **(I - K)** Average mEPSC traces **(I)**, average mEPSC amplitudes **(J)**, and cumulative distribution of mEPSC amplitudes **(K)** recorded from NR (black) or DE (blue) shRNA Caspr2-infected mice (NR, n=12 cells; DE, n=9 cells). In **(D)**, **(G)** and **(J)**, results are presented as mean ± S.E.M. and statistical significance was determined by the non-parametric Mann-Whitney test, *p<0.05, ns – nonsignificant compared to NR. In **(E)**, **(H)** and **(K)**, statistical significance was determined by the Kolmogorov-Smirnov Test, ****p<0.0001.

As observed earlier, the average trace (Figure 4.8I: NR - black) and average amplitude (Figure 4.8J) of mEPSCs recorded from Caspr2 shRNA-infected mice was strikingly smaller than control non-infected or empty vector-infected mice. Importantly, dark exposure of Caspr2 shRNA-infected mice completely failed to upscale the average mEPSC trace (Figure 4.8I: DE - blue) and average mEPSC amplitudes (Figure 4.8J [NR (black) = 11.03 ± 0.45 pA; DE (blue) = 10.28 ± 0.55 pA]), and the cumulative distribution of DE amplitudes instead showed a small but statistically significant shift towards smaller values than that of NR Caspr2-shRNA (Figure 4.8K), suggesting a downscaling effect. This result is rather interesting as it indicates that animals lacking Caspr2, instead of counterbalancing the lack of visual experience, undergo further depotentiation after visual deprivation, thus confirming a complete failure in negative-feedback homeostatic processes in these animals. Taking into account these observations, it would be important to evaluate whether Caspr2 might also be required for homeostatic downscaling mechanisms, which act to balance-out firing rates during re-exposure to light following dark exposure, for instance (Whitt *et al.*, 2014). Homeostatic scaling-down processes have also been shown to occur during cross-modal plasticity of sensory modalities [(Goel *et al.*, 2006), and reviewed in (Lee & Whitt, 2015)], and during sleep [(de Vivo *et al.*, 2017; Diering *et al.*, 2017) and reviewed in (Cirelli, 2017; Tononi & Cirelli, 2014)], although their underlying mechanisms remain largely unknown. The recent discovery of varying protein levels of Caspr2 during the wake/sleep cycle in mice (Diering *et al.*, 2017) suggests that Caspr2 might be

relevant during sleep/wake physiology, and substantiates the importance of evaluating the role of Caspr2 in the regulation of homeostatic downscaling mechanisms.

Overall, this set of findings indicate that *in vivo* levels of Caspr2 in the mouse visual cortex can be regulated by visual experience, and that loss of Caspr2 in this region hinders the scaling of AMPAR function elicited by prolonged visual deprivation, suggesting that Caspr2 is essential for visually-driven experience-dependent homeostatic plasticity.

Discussion

Throughout the past decade, investigations of the *CNTNAP2* gene and its encoded protein CASPR2 have grown exponentially, on account of numerous reports implicating them in several neuropsychiatric disorders, including ASD, SCZ and ID [reviewed in (Penagarikano & Geschwind, 2012; Poot, 2015; Poot, 2017)]. Despite this, the full-spectrum of cellular and molecular functions played by CASPR2 remains unclear. Furthermore, it still remains to be answered how different sequence variations in the *CNTNAP2* gene disrupt CASPR2 function to the point of pathogenesis, and how that correlates to patient symptomatology.

In a previous study carried in our laboratory (Santos *et al.*, 2012), and followed up in the previous chapter, we were able to identify Caspr1, an homologous family member of Caspr2, as a novel interactor of AMPARs that regulates their trafficking to the synapse and is required for the expression of mechanisms underlying homeostatic synaptic plasticity. Interestingly, synaptic abnormalities, impaired glutamatergic signalling and disrupted homeostatic processes have been implicated in the pathogenesis of neuropsychiatric disorders. In line with recent synaptic functions described for Caspr2, in this chapter we addressed the hypothesis that Caspr2 regulates glutamatergic function and neuronal synaptic homeostasis, and ultimately uncovered an exciting requirement of Caspr2 to regulate sensory-driven experience-dependent homeostatic plasticity processes *in vivo*. We herein propose that these mechanisms can be disrupted in consequence of CASPR2 dysfunction in the context of disease, and thus contribute to disease pathogenesis.

Synaptic role of Caspr2 in the regulation of AMPAR trafficking and *in vivo* AMPAR-mediated synaptic transmission

In the present study we evaluated the subcellular distribution of Caspr2 and found that it is present in approximately half of all excitatory synapses in rat cortical neurons (Figure 4.1A, B), and enriched in PSD fractions from the mouse brain (Figure 4.1C, D). These findings are in agreement with reports indicating that Caspr2 is present in dendritic spines (Varea *et al.*, 2015) and enriched in the synaptic plasma membrane (Bakkaloglu *et al.*, 2008), and refute previous evidence suggesting that Caspr2 is merely perisynaptic and absent from the PSD (Chen *et al.*, 2015). Taking into consideration the functional relevance of proteins localized at the PSD, and how that localization often impacts their role in the regulation of synaptic signalling cascades and synaptic plasticity mechanisms, this privileged localization of Caspr2 gives out important clues about the potential roles it plays at the synapse. Being a cell-adhesion molecule, Caspr2 might act as a canonical synaptic CAM that bridges and stabilizes the synapse; alternatively, given its modular structure of domains involved in protein-protein interactions (Poliak *et al.*, 1999), Caspr2 can act as a scaffolding protein that helps anchoring other molecules to the PSD, namely other synaptic scaffolds, synaptic signalling molecules or proteins belonging to the glutamate receptor complex.

Indeed, in the present study we were able to establish a critical physiological role for Caspr2 in the regulation of glutamatergic function at excitatory synapses. When silencing Caspr2 expression, we observed a decrease in the cell surface expression of AMPARs *in vitro* at cortical synapses (Figure 4.3), and in the amplitude of AMPAR-mediated mEPSCs *in vivo* in the mouse visual cortex (Figure 4.4). Previous studies report defects in the dendritic arborisation and spine development of *Cntnap2* KO cortical neurons (Anderson *et al.*, 2012; Gdalyahu *et al.*, 2015). However, in our system we didn't observe any changes in synapse number (data not shown) nor a decrease in the frequency of mEPSCs (Suppl. Table S1), indicating that our effects are purely postsynaptic. Furthermore, our observations are accordant with the presence of large cytoplasmic aggregates of AMPARs found in *Cntnap2* KO cortical neurons (Varea *et al.*, 2015), and, together, suggest an impairment in AMPAR trafficking and AMPAR-mediated synaptic transmission upon Caspr2 loss of function. The dynamic regulation of AMPAR trafficking and localization to synapses is a fundamental mechanism for the expression of multiples forms of synaptic plasticity, and it is known to be modulated by several mechanisms ranging from post-transcriptional and post-translational modifications of AMPAR subunits, to specific interacting partners (Anggono & Huganir, 2012; Jiang *et al.*, 2006; Santos *et al.*, 2009). In particular, several auxiliary AMPAR-interacting intracellular scaffolds (PSD95, SAP97 and other MAGUKs, PICK1, GRIP1, protein 4.1N, etc.)

and transmembrane proteins (Stargazin and other TARPs, cornichons, CKAMPs, SynDIG1, etc.) are able to control not only the targeting and insertion (or removal) of AMPARs to the cell membrane, but also their membrane lateral diffusion to the synapse and anchoring to the PSD (Jackson & Nicoll, 2011; Shepherd & Huganir, 2007). Importantly, although the mechanisms whereby Caspr2 regulates AMPAR trafficking are still unclear, in this study we found that Caspr2 immunoprecipitates the GluA1 subunit of AMPARs from cerebellum and cortical lysates (Figure 4.2C, D), and thus identify Caspr2 as a novel AMPAR-interacting protein.

Interestingly, we have previously identified Caspr1, the homologous family member of Caspr2, as a novel interactor of AMPARs capable of regulating their trafficking and synaptic content (Santos *et al.*, 2012), indicating that both CASPR proteins play similar roles in AMPAR regulation. Accordingly, Caspr1 and Caspr2 share a significant degree of homology, and their overall molecular architecture is very similar (Peles *et al.*, 1997; Poliak *et al.*, 1999). Both proteins are single-pass transmembrane molecules with a long extracellular region of different domains that mediate cell adhesion roles and extracellular matrix interactions. Additionally, Caspr1 and Caspr2 encompass a short cytoplasmic tail with regulatory domains typically involved in protein-protein interactions that could be accountable for the binding with AMPARs. Indeed, we were able to demonstrate that Caspr1 interacts with AMPARs through its intracellular C-tail (Santos *et al.*, 2012), although it remains undetermined through which domain, and whether the interaction occurs directly or through other binding partners. Importantly, however, we were able to dissect the molecular determinants behind Caspr1 function and identified the RNA-binding protein ZBP1 as the potential downstream mediator of Caspr1 in AMPAR regulation, through a post-transcriptional mechanism probably dependent on a C-tail proline-rich domain that is exclusive to Caspr1 structure (please refer to Chapter III); thus, it is unlikely that this mechanism also underlies Caspr2 function.

We haven't yet determined whether Caspr2 interacts with GluA1-AMPARs through its C-tail, but this small intracellular portion of Caspr2, whilst not containing the Caspr1-exclusive proline-rich domain (Peles *et al.*, 1997), encompasses a common GNP motif for the binding of FERM domain-containing proteins, and an additional type-II PDZ binding domain (Poliak *et al.*, 1999), both involved in important protein-protein interactions. Recent biochemical and proteomic studies have partially uncovered the interactome of Caspr2, identifying several novel interactors that bind to its intracellular tail, including the cytoskeleton adaptor 4.1N protein (Denisenko-Nehrbass *et al.*, 2003b), subunits of the Kv1 family of K^+-channels (Horresh *et al.*, 2008), G protein-coupled receptor 37 [(GPR37), (Tanabe *et al.*, 2015)], members of the ADAM (Disintegrin and metalloproteinase domain-containing protein) family of proteins, in particular ADAM22 in complex with the secreted LGI1 (Leucine-rich glioma-inactivated 1) protein (Chen

et al., 2015), as well as PSD95, SAP97, CASK and other PSD-MAGUKs (Chen *et al.*, 2015; Horresh *et al.*, 2008; Poliak *et al.*, 1999). It is quite impressive that the majority of these intracellular Caspr2 interactors typically locate at the synapse, which argues in favour of Caspr2 playing a pivotal synaptic function. Importantly, most of these Caspr2 interactors have already established functions in the regulation of AMPAR trafficking [(Fukata *et al.*, 2006; Shen *et al.*, 2000) and reviewed in (Henley & Wilkinson, 2016; Shepherd & Huganir, 2007)], playing fundamental roles in virtually all steps of the AMPAR secretory pathway, all the way from their biogenesis in the ER to their insertion in the synaptic membrane and anchoring at the PSD.

Further investigation to fully understand the synaptic function of Caspr2 in AMPAR regulation will be crucial, but taking into consideration the evidence available so far, and the Caspr2 interactome, we can start to speculate in which step of AMPAR trafficking Caspr2 intervenes. Given the intracellular interaction of Caspr2 to PSD-MAGUKs, and its extracellular binding to partners such as Contactin-1 and Contactin-2 (Poliak *et al.*, 2003; Rubio-Marrero *et al.*, 2016), typically involved in cell-adhesion complexes, one first tantalizing hypothesis is that Caspr2 could serve as a bridge between the pre- and postsynaptic sides of the synapse, promoting the anchoring and stabilization of AMPARs in postsynaptic nanodomains that align with presynaptic sites of glutamate release in functional trans-synaptic molecular nanocolumns. Such modular organization of the synapse, which has just recently been demonstrated experimentally (MacGillavry *et al.*, 2013; Nair *et al.*, 2013; Tang *et al.*, 2016), enables a higher resolution of spatio-temporal control of synaptic strength, and hence, a more efficient transmission of information [thoroughly reviewed in (Biederer *et al.*, 2017)]. Although the mechanisms underlying this specialized alignment of the synapse are still unclear, several other CAMs, including Neuroligins and members of the LRRTM family, have been proposed to be important organizers of these trans-synaptic nanocolumns (Biederer *et al.*, 2017), hence, a potential similar role could be played by Caspr2. An alternative hypothesis it that Caspr2 interacts with GluA1 early on during the secretory pathway to then promote the intracellular trafficking of AMPARs and their insertion to the cell surface and into the synapse. In the absence of Caspr2 (or Caspr2 dysfunction in consequence of gene variations), these mechanisms of AMPAR trafficking might be compromised, hence giving origin to cytoplasmic aggregates of the receptors [as observed in (Varea *et al.*, 2015)], and the consequent decrease in their cell surface expression and synaptic content.

Interestingly, defects in AMPAR trafficking consequent to disruptions in Caspr2 function could, in principle, be explained by some of the identified sequence variations in the *CNTNAP2* gene [thoroughly reviewed in (Penagarikano & Geschwind, 2012; Poot, 2017)]. For instance, a rare frame-shift homozygous mutation of *CNTNAP2* (3709delG - single-base G deletion at

nucleotide 3709 in exon 22), found in individuals from an Old Order Amish family that presented cortical dysplasia and focal epilepsy, ASD-core behaviours with language regression, and mental retardation (Strauss *et al.*, 2006), is predicted to result in a premature stop codon that produces a truncated form of the CASPR2 protein that lacks its transmembrane and cytoplasmic domains, resulting in a loss of function of the protein and shedding to the extracellular space (Falivelli *et al.*, 2012; Strauss *et al.*, 2006). These observations suggest that the intracellular region of CASPR2 must be a relevant molecular determinant underlying CASPR2-mediated functions in the brain, and fit adequately with our earlier hypothesis of a CASPR2 C-tail-mediated interaction to mediate AMPAR trafficking and anchoring in the synapse. One other rare nonsynonymous *CNTNAP2* mutation found in monozygotic twins affected with autism [CASPR2 - D1129H, exon 21; (Bakkaloglu *et al.*, 2008)], was shown to cause a local misfolding of the protein that is largely retained in the ER and Golgi apparatus, with consequent severe trafficking abnormalities of CASPR2 to the cell membrane (Falivelli *et al.*, 2012). In accordance to what we hypothesized above, if CASPR2 interacts with GluA1 early on during the AMPAR secretory pathway, then this deleterious mutation in *CNTNAP2* could disrupt AMPAR trafficking to the cell membrane, thus contributing to the formation of the cytoplasmic aggregates of AMPARs.

Unfortunately, these hypothesis are all still speculative, as little information is yet available regarding the molecular and cellular defects resulting from these and several other *CNTNAP2* mutations [for a comprehensive review on all sequence variations found in the *CNTNAP2* gene, please see (Penagarikano & Geschwind, 2012; Poot, 2017)]. Evidently, it is of the utmost importance to clarify the molecular determinants of the interaction of Caspr2 with AMPARs, in order to fully uncover potential regulatory mechanisms orchestrated by Caspr2, and comprehend how they can be affected by deleterious mutations in the *CNTNAP2* gene. Furthermore, future experiments will be required to fully explore the role of Caspr2 in the regulation of AMPAR trafficking. Taking advantage of super-resolution microscopy techniques and single-particle tracking methods, further imaging experiments should be undertaken to determine whether Caspr2 regulates the intracellular trafficking of AMPARs to the cell membrane, or the dynamics of their lateral diffusion at the cell surface; as well as evaluate the potential of Caspr2 to function as an organizer of the modular nanodomain alignment of AMPARs at the synapse. Furthermore, coupling these experiments to a molecular replacement strategy with mutant forms of Caspr2 that mimic the genetic variations found in patients will provide invaluable knowledge on the mechanisms that underlie the pathogenesis of *CNTNAP2*-related neuropsychiatric disorders.

Requirement of Caspr2 in the regulation of homeostatic synaptic scaling and *in vivo* experience-dependent homeostatic plasticity

Synaptic plasticity mechanisms, of either Hebbian or homeostatic type, highly depend on rapid changes in the synaptic content of AMPARs to modulate activity-dependent alterations in synaptic strength. Interestingly, the molecular players underlying such AMPAR trafficking mechanisms are often themselves activity-regulated (Fernandes & Carvalho, 2016; Shepherd & Huganir, 2007). Taking into account the new role we attribute to Caspr2 in the regulation of basal AMPAR trafficking, we assessed whether Caspr2 could be regulated by neuronal activity. Indeed, we observed a marked upregulation in the expression of Caspr2, including at the synapse, following prolonged inhibition of neuronal activity in cortical neurons *in vitro* (Figure 4.5A, B - F, G). Of relevance, we observed a significant increase of Caspr2 levels in the primary visual cortex of mice that were visually deprived for two days (Figure 4.7), indicating that the expression of Caspr2 is regulated *in vivo* by sensory experience. This result is rather interesting as it suits well with the enriched expression of Caspr2 in all primary sensory cortical areas and in other brain regions involved in sensory signal processing (Gordon *et al.*, 2016). Moreover, *Cntnap2*-null mice were shown to have impaired responses to olfactory sensory stimuli and anomalies in auditory processing (Gordon *et al.*, 2016; Truong *et al.*, 2015), which, together with our evidence, suggest that Caspr2 may be necessary for the integration of sensory information in response to sensory experience.

In agreement, further experiments in this study revealed that loss of Caspr2, induced by shRNA-mediated knockdown, prevents the homeostatic synaptic scaling of surface AMPARs following prolonged inhibition of neuronal activity *in vitro* (Figure 4.6), indicating that Caspr2 is required for the regulation of homeostatic synaptic plasticity. To our knowledge, this is the first conclusive evidence to establish a relevant role for Caspr2 in the modulation of synaptic plasticity phenomena, in particular of neuronal synaptic homeostasis. These findings are rather important as they contribute to increase the knowledge on the mechanisms underlying homeostatic plasticity, which remain poorly characterized to date. Our laboratory has contributed significantly to this field, identifying both the TARP Stargazin (Louros *et al.*, 2014) and Caspr1 (please refer to Chapter III), as well as its downstream target ZBP1, as novel molecular players underlying the expression of synaptic scaling mechanisms.

Given the requirement of Caspr2 to regulate AMPAR function *in vivo*, we took advantage of the amenability of the visual cortex for *in vivo* manipulations of neuronal activity (Whitt *et al.*, 2014) and further explored whether the role of Caspr2 in regulating homeostatic synaptic scaling was relevant *in vivo*. Accordingly, we observed that *in vivo* loss of Caspr2 in the primary

visual cortex hinders the scaling of AMPAR-mediated currents following prolonged visual deprivation (Figure 4.8), thus revealing a critical requirement of Caspr2 for the regulation of visually-driven experience-dependent plasticity. These findings confirm our earlier hypothesis that Caspr2 is necessary for the integration of sensory information in response to experience. Indeed, homeostatic plasticity mechanisms *in vivo* are thought to be of particular importance during periods of heightened plasticity, such as occurring not only during development, but also during sensory processing or adaptation to sensory environment changes, when synaptic contacts and neuronal circuits are massively remodelled (Fernandes & Carvalho, 2016; Turrigiano, 2008; Whitt *et al.*, 2014). Homeostatic scaling processes were also shown to occur during cross-modal plasticity of sensory modalities [(Goel *et al.*, 2006; Petrus *et al.*, 2014), and reviewed in (Lee & Whitt, 2015)], a process elicited to compensate for the loss of function of an affected modality in either deaf or blind people, for instance. Furthermore, a relevant role for homeostatic plasticity has just been recently established in the regulation of sleep/wake physiology (de Vivo *et al.*, 2017; Diering *et al.*, 2017; Hengen *et al.*, 2016), considered to be fundamental for memory consolidation and learning [reviewed in (Cirelli, 2017; Tononi & Cirelli, 2014)]. Curiously, Caspr2 protein levels in the mouse brain were shown to vary during the wake/sleep cycle (Diering *et al.*, 2017), suggesting that Caspr2 might be relevant for the expression of homeostatic scaling mechanisms that regulate the sleep/wake physiology.

Most important, it has been proposed that a failure in neuronal synaptic homeostasis may result in neurological outcomes that overlap with symptoms of several neurological and neuropsychiatric disorders [reviewed in (Nelson & Valakh, 2015; Ramocki & Zoghbi, 2008; Wondolowski & Dickman, 2013)], suggesting that compromised homeostatic processes could contribute to disease pathophysiology. Consistent with this hypothesis is the observation that some of the strongest candidate risk genes implicated so far in such cognitive disorders are actual important molecular players involved in the mechanisms that regulate homeostatic plasticity, such as Stargazin, MeCP2, FMRP, SynGAP1, etc. [reviewed extensively in (Fernandes & Carvalho, 2016; Mullins *et al.*, 2016; Volk *et al.*, 2015; Wondolowski & Dickman, 2013)]. Accordingly, emerging evidence from disease animal models with mutated/ablated susceptibility genes show alterations in homeostatic signalling, with striking E/I imbalances and loss of general neuronal homeostasis, with animals presenting behavioural phenotypes that recapitulate disease symptoms [reviewed in (Huguet *et al.*, 2013; Mullins *et al.*, 2016; Wondolowski & Dickman, 2013)]. Interestingly, animal models lacking the *Cntnap2* gene, besides displaying autistic-like behavioural phenotypes, also present reduced neuronal synchrony in the neocortex and altered E/I balance (Hoffman *et al.*, 2016; Penagarikano *et al.*, 2011), which is reminiscent of aberrant neuronal homeostasis. Accordingly, a recent report

revealed a striking amelioration of abnormal behavioural phenotypes of *Cntnap2* KO mice following optogenetic manipulation of E/I balance (Selimbeyoglu *et al.*, 2017).

A role for disrupted homeostatic plasticity underlying disease pathogenesis is further supported by evidence of a wide percentage of neuropsychiatric patients presenting abnormal behavioural responses to sensory stimulation [reviewed in (LeBlanc & Fagiolini, 2011; Marco *et al.*, 2011)]. Reports from autistic patients indicate the occurrence of both hyper- and hyposensitivities at multiple domains, as well as a disruption in the integration and processing of information across primary sensory modalities. Indeed, it is believed that autistic-like behavioural defects in communication and socialization skills might originate from abnormalities in auditory, visual and somatosensory processing, since higher cognitive processes require a parallel integration of information from these primary sensory areas [reviewed in (LeBlanc & Fagiolini, 2011; Marco *et al.*, 2011)]. Our findings showing that animals lacking Caspr2 in their primary visual cortex cannot elicit homeostatic scaling mechanisms in response to visual experience, together with the studies revealing abnormal responses to olfactory sensory stimuli and anomalies in auditory processing of *Cntnap2* KO mice (Gordon *et al.*, 2016; Truong *et al.*, 2015), may suggest that the integration and processing of information in primary sensory areas is modulated by Caspr2-dependent homeostatic processes. Importantly, we can speculate that deleterious *CNTNAP2* mutations that disrupt this CASPR2 function could, in principle, impair the processing of sensory information and its integration into higher cognitive functions, which would, in turn, translate to abnormal output behaviours such as defective communication and social skills, hallmarks of autistic-core phenotypes. Overall, we herein propose that a failure in CASPR2-dependent mechanisms of neuronal synaptic homeostasis may underlie the pathogenesis of *CNTNAP2*/CASPR2-associated neuropsychiatric disorders by driving hallmark defects in E/I balance and sensory integration and processing.

How do the proposed mechanisms integrate with other known CASPR2 functions?

In the present study we uncover an exciting and unanticipated novel function of Caspr2 in the regulation of glutamatergic function and homeostatic and experience-dependent synaptic plasticity, and propose that a disruption of such mechanisms might underlie the pathogenesis of *CNTNAP2*-associated neuropsychiatric disorders. Whilst our findings provide a plausible molecular underpinning that can, by itself, explain most of the psychiatric and cognitive phenotypes presented by patients, they must be integrated with other already

described functions of CASPR2, in order to get a full picture of the overall role of CASPR2 in the regulation of brain function.

CASPR2 was first identified as a membrane scaffold required for the clustering of Shaker-like K^+ channels along the nodes of Ranvier of peripheral and, most recently, central myelinated neurons (Poliak *et al.*, 1999; Poliak *et al.*, 2003; Scott *et al.*, 2017), in a process thought to be important for the regulation of myelination and axonal excitability. However, experimental evidence on this matter have been controversial, with some studies finding no gross alterations in myelination despite K^+ channel disorganization (Liska *et al.*, 2017; Poliak *et al.*, 2003), whilst others report defects in nodal formation and myelin ensheathing, as well as abnormal action potential conductance in *Cntnap2* KO mice (Scott *et al.*, 2017). One other overarching phenotype reported in *Cntnap2* KO animal models is a loss of GABAergic interneurons and impaired GABAergic inhibition (Bridi *et al.*, 2017; Hoffman *et al.*, 2016; Jurgensen & Castillo, 2015; Penagarikano *et al.*, 2011; Vogt *et al.*, 2017). Nevertheless, once again, these findings are not unanimous between studies, including our own. Indeed, several studies either find no changes in GABAergic neurotransmission (Scott *et al.*, 2017), report converse alterations in glutamatergic and excitatory function [our own study, and (Gdalyahu *et al.*, 2015; Varea *et al.*, 2015)], or even uncover parallel disruption of both inhibitory and excitatory neurotransmission (Anderson *et al.*, 2012), indicating that alterations in either excitation or inhibition upon loss of CASPR2 may not be mutually exclusive, and reflecting a most likely potential bidirectional effect in E/I balance. Finally, several other studies uncovered abnormalities in dendritic arborization and spine development and maintenance, as well as a decrease in synapse number (Anderson *et al.*, 2012; Gdalyahu *et al.*, 2015; Varea *et al.*, 2015). These observations, together with the reported changes in the migration of excitatory projection neurons and cortical layer patterning (Penagarikano *et al.*, 2011) can help explain the reduced local and long-range prefrontal functional connectivity observed both in *Cntnap2* KO mice and in patients with *CNTNAP2* variations (Liska *et al.*, 2017; Scott-Van Zeeland *et al.*, 2010).

Although the integration of all phenotypes subsequent to the loss of Caspr2 might suggest a multilevel regulatory role of Caspr2 in brain function, it is possible that some of the observed effects are secondary to others. Indeed, most studies performed so far to uncover CASPR2 functions should take into account possible masking/confounding effects that can arise from the use of a full KO model that presents such a wide-ranging spectrum of severe phenotypes. The advantage of using a transient model of genetic deletion such as shRNA-mediated knockdown is that any defects consequent to the loss of Caspr2 are occurring in a cell-autonomous manner, thus allowing us to pinpoint specific Caspr2-dependent molecular

mechanisms more easily. Finally, it is likely that divergent or dissimilar reports between studies can be explained by differences in the age of the animals or the brain region under study. Indeed, in our experiments we came across one clear example of this: although loss of Caspr2 decreases the trafficking of surface AMPARs in both hippocampal and cortical neurons (Figure 4.3 vs. Figure 4.S3), Caspr2 is only required for the regulation of homeostatic synaptic scaling in the cortex (Figure 4.6 vs. Figure 4.S6), whilst in the hippocampus these mechanisms depend on Caspr1 (see Chapter III).

How far have we come in understanding the pathogenesis of *CNTNAP2*-related neuropsychiatric disorders?

Mutations in the *CNTNAP2* gene have been recurrently associated to an impressive number of distinct neuropsychiatric disorders, with patients presenting a wide variability in phenotypes, even within the same disease spectrum [reviewed in (Penagarikano & Geschwind, 2012; Poot, 2017; Rodenas-Cuadrado *et al.*, 2014)]. Indeed, several of the identified sequence variations associated to either ASD, ID or SCZ, among others, are scattered throughout the entire *CNTNAP2* gene, such that no specific gene domain can be correlated to a single disorder. These observations agree with the idea that different mutations in the same gene may cause distinct molecular, cellular and functional phenotypes, an effect that was just recently observed for ASD- and SCZ-related mutations in the *SHANK3* gene (Zhou *et al.*, 2016), and for SCZ- and ID-associated mutations in the Stargazin-encoding *CACNG2* gene (unpublished data from our laboratory by Caldeira, G. *et al.*).

The only molecular studies to date that evaluate the deleterious effect of specific *CNTNAP*2 mutations exclusively explore effects in CASPR2 itself: one rare frame-shift homozygous mutation (3709delG) originates a non-functional truncated form of CASPR2 that is secreted to the extracellular space (Falivelli *et al.*, 2012; Strauss *et al.*, 2006); one other nonsynonymous mutation (D1129H) produces a misfolded protein that is largely retained in the ER (Bakkaloglu *et al.*, 2008; Falivelli *et al.*, 2012); whilst another homozygous deletion introduces a premature stop codon that elicits nonsense-mediated mRNA decay, thus producing no protein at all (Rodenas-Cuadrado *et al.*, 2016; Watson *et al.*, 2014). Alas, no information is yet available regarding the cellular or functional outcomes arising from any particular *CNTNAP2* mutation.

Given the modular structure of CASPR2, it is likely that different mutations, independently of the disease spectrum, will give rise to specific cellular and functional phenotypes, depending on which protein domains of CASPR2 are affected. If holding true, such specific phenotypic clusters from unrelated mutations would be indicative of a common pathophysiology of psychiatric disorders. Now that several putative functions have been described for CASPR2, it is of the utmost importance that additional molecular studies are performed to explore the cellular and functional phenotypes of specific *CNTNAP2* mutations, evaluate how they differ from one another, and assess how they contribute to disease pathogenesis and correlate with patient symptomatology.

Conclusions

In this chapter, we identify the autism-related Caspr2 as a novel interactor of AMPARs that regulates their trafficking and function at the synapse, and reveal a specific requirement of Caspr2 for the expression of mechanisms underlying homeostatic synaptic plasticity. Importantly, our observations uncover an exciting and unsuspected physiological role for Caspr2 in the regulation of cortical glutamatergic function and sensory-driven experience-dependent plasticity *in vivo.* Future experiments will be required to uncover the downstream mechanisms underlying the proposed function of Caspr2, and to explore if glutamatergic function and neuronal synaptic homeostasis can be disrupted by specific *CNTNAP2* mutations. Overall, our findings underscore potential targets for dysfunction in disease, and propose that they contribute to the pathogenesis and underlie the cognitive and psychiatric symptoms of *CNTNAP2*-related psychiatric disorders.

Supplementary Figures

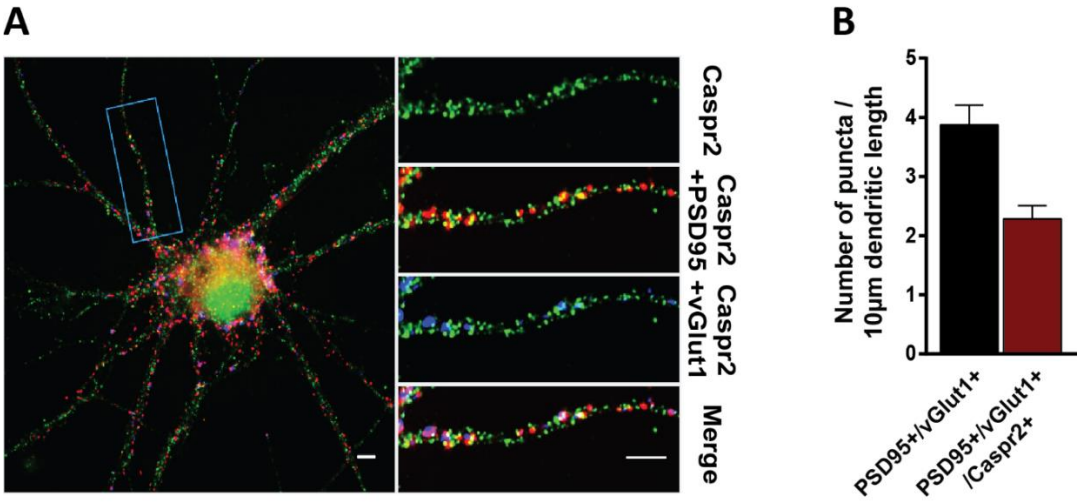


Figure 4.S1 – Caspr2 is expressed along dendrites in hippocampal neurons, where it localizes to excitatory synapses. (A, B) 15 DIV Banker cultured hippocampal neurons were fixed and stained against Caspr2, the postsynaptic marker PSD95 and the presynaptic marker vGluT1. **(A)** Representative immunofluorescence image of a hippocampal neuron showing significant co-localization between Caspr2 clusters and synaptic puncta. Blue square inset of a dendrite zoomed in on right panels. *Scale bars = 5 µm.* **(B)** Quantification of the number of glutamatergic synapses per dendritic length, identified as clusters with double positive signal for both synaptic markers PSD95 and vGluT1 ($PSD95^+/vGluT1^+$), that contain Caspr2 ($PSD95^+/vGluT1^+/Caspr2^+$). Total number of analysed cells was obtained from three independent experiments. Results are presented as means ± S.E.M. (N=3, n=30 cells).

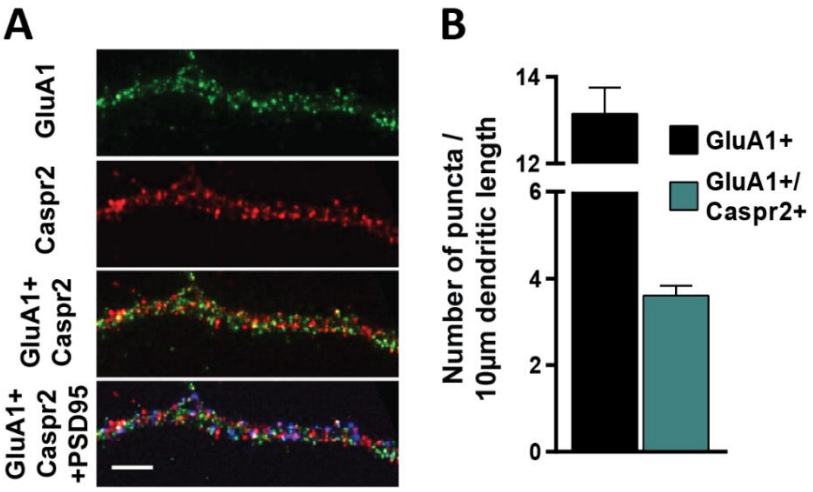


Figure 4.S2 – The dendritic distribution pattern of Caspr2 substantially overlaps with the GluA1 subunit of AMPARs. (A, B) 15 DIV low-density cultured hippocampal neurons were live-stained with an antibody against an extracellular epitope in the GluA1 N-terminus, fixed, and then stained for Caspr2 and the synaptic marker PSD95. **(A)** Representative immunofluorescence images showing the dendritic and synaptic distribution of GluA1 and Caspr2, with substantial cluster co-localization. *Scale bars = 5 µm*. **(B)** Quantification of the number of surface GluA1 clusters ($GluA1^+$) per dendritic length that are also positive for Caspr2 ($GluA1^+/Caspr2^+$). Total number of analysed cells was obtained from three independent experiments. Results are presented as means ± S.E.M. (N=3; n=30 cells).

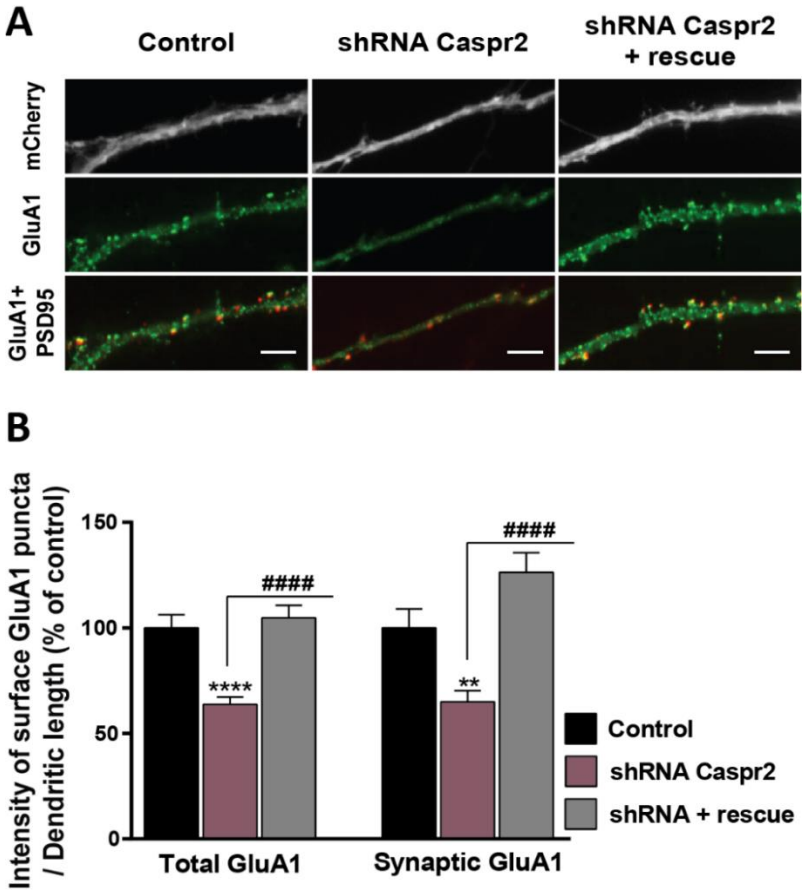


Figure 4.S3 – Caspr2 is required to regulate the synaptic content of cell surface AMPARs in hippocampal neurons. (A) Representative immunofluorescence images of mCherry-positive low-density cultured hippocampal neurons transfected at DIV 7 with a control empty vector or a plasmid encoding the Caspr2 shRNA, or co-transfected with the Caspr2 shRNA and a shRNA-resistant Caspr2 rescue mutant. At DIV 15, cells were live-stained against an extracellular epitope of GluA1, fixed, and then stained for the synaptic marker PSD95. *Scale bars = 5 µm*. **(B)** The fluorescence intensity of total and PSD95-colocalized synaptic clusters of surface GluA1 was analysed from mCherry-expressing transfected neurons. Total number of analysed cells was obtained from at least three independent experiments. (N≥3, n≥ 30cells). Results are presented as means ± S.E.M and the statistical significance was determined by the non-parametric Kruskal-Wallis test, followed by Dunn's Multiple Comparison post-hoc test, **p<0.01, ****p<0.0001 compared to control, and ####p<0.0001 relative to Caspr2-shRNA.

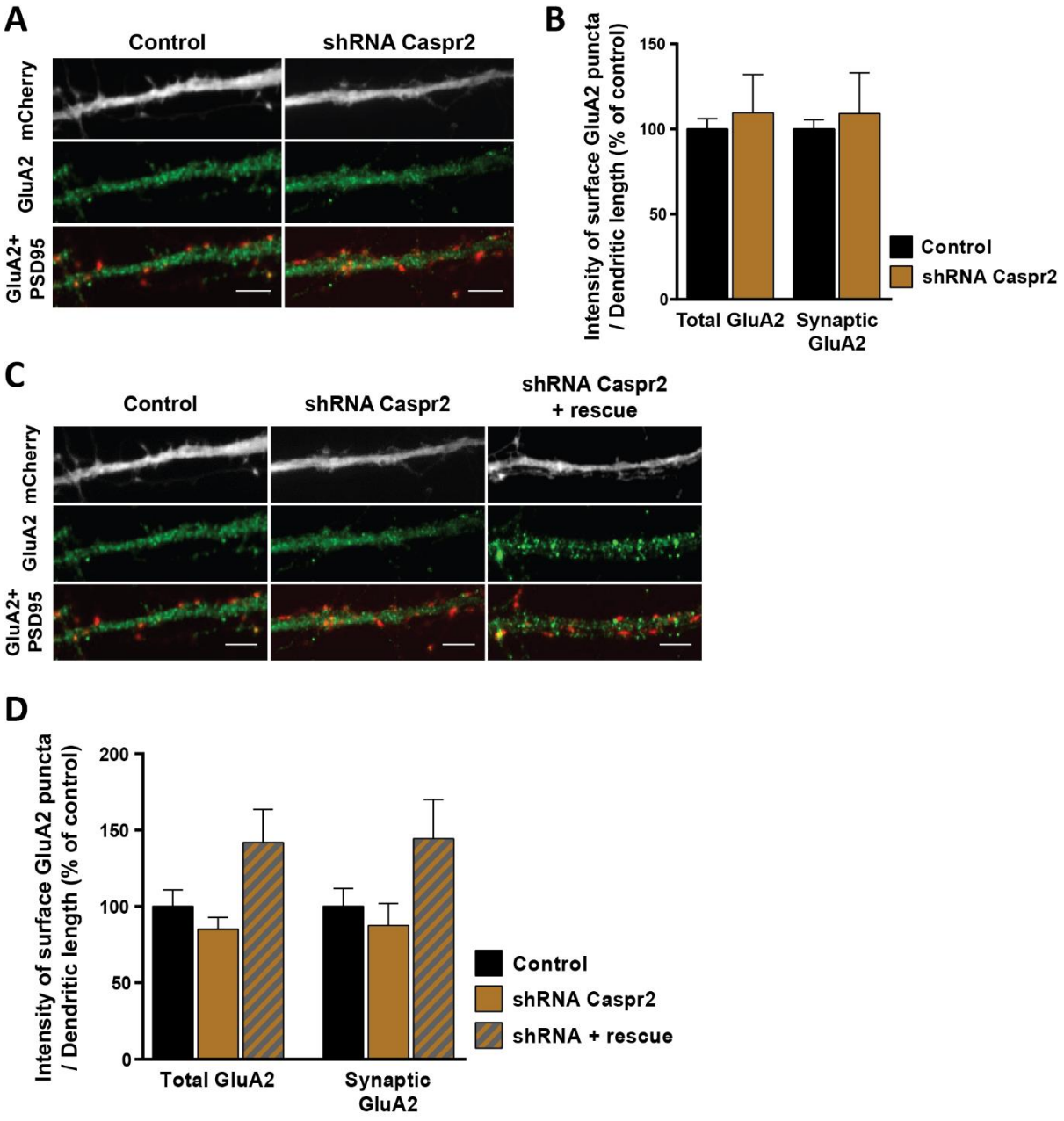


Figure 4.S4 – Caspr2 is not required for maintaining basal levels of surface GluA2-containing AMPARs. Loss of Caspr2 expression in either cortical **(A, B)** or hippocampal neurons **(C, D)** has no effect in the expression of surface GluA2-containing AMPARs. **(A, C)** Representative immunofluorescence images of mCherry-positive low-density cultured cortical **(A)** and hippocampal **(C)** neurons transfected at DIV 7 with a control empty vector or a plasmid encoding the Caspr2 shRNA, or co-transfected with the Caspr2 shRNA and a shRNA-resistant Caspr2 rescue mutant. At DIV 13/15, cells were stained against an extracellular epitope of GluA2 and for the synaptic marker PSD95. *Scale bars = 5 µm*. **(B, D)** The fluorescence intensity of total and PSD95-colocalized synaptic clusters of surface GluA2 was analysed from mCherry-expressing transfected cortical **(B)** and hippocampal **(D)** neurons. Results are presented as means ± S.E.M. [In **(B)**, N=2, n≥ 15cells; in **(D)**, N=3, n≥ 30cells].

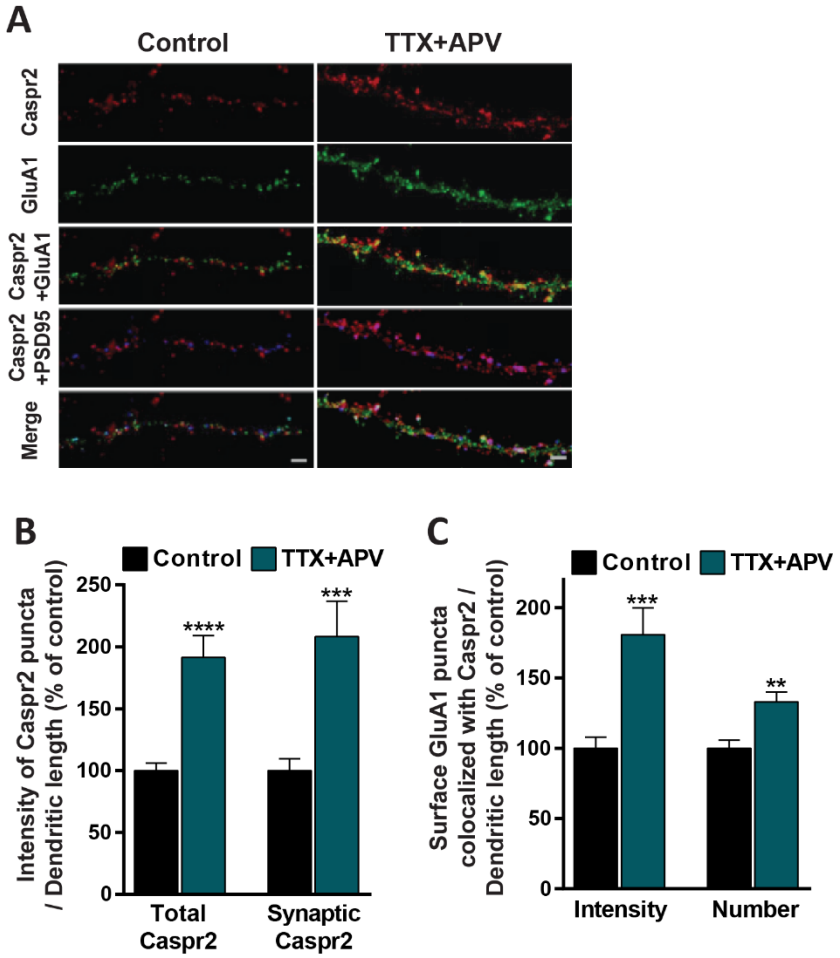


Figure 4.S5 – The endogenous expression of Caspr2 in hippocampal neurons is regulated by neuronal activity. (A-C) Prolonged neuronal inactivity in hippocampal neurons increases total and synaptic clusters of Caspr2. Neuronal activity in 13 DIV low-density cultured hippocampal neurons was inhibited for 48 h either with 1 µM TTX together with 100 µM APV. Cells were then live-stained with a specific antibody against an extracellular epitope in GluA1 N-terminus, fixed, and then stained for Caspr2 and the synaptic marker PSD95. **(A)** Representative immunofluorescence images of cultured hippocampal neurons treated for 48 h with TTX + APV, and stained for superficial GluA1, Caspr2 and PSD95. *Scale bars = 5µm.* **(B, C)** The fluorescence intensity of total and PSD95-colocalized synaptic clusters of Caspr2 **(B)**, and the intensity and number of surface GluA1 clusters that colocalize with Caspr2 **(C)** were quantified. Total number of analysed cells was obtained from three independent experiments. (N≥3, n≥30 cells). Results are presented as mean ± S.E.M. and statistical significance was determined by the non-parametric Mann-Whitney Test, **p<0.01, ***P<0.001, ****p<0.0001 compared to control.

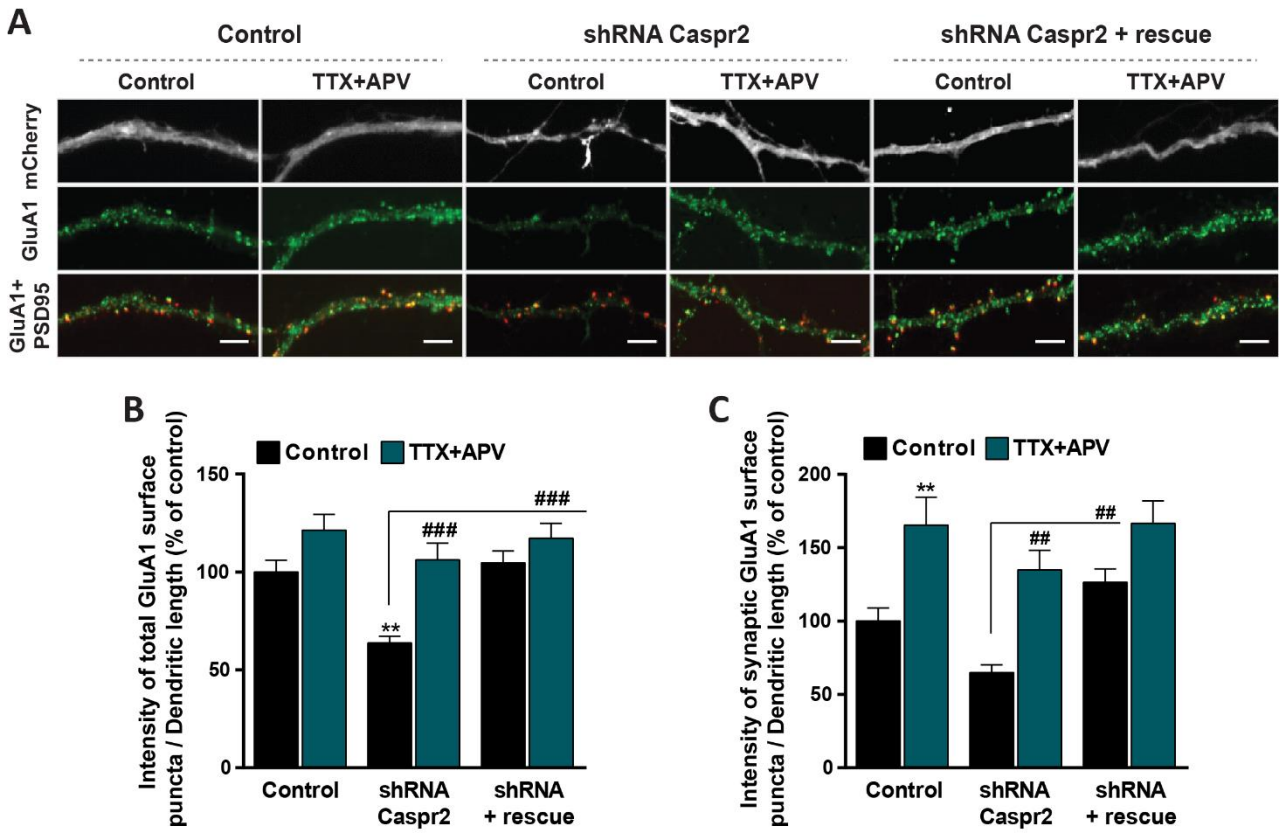


Figure 4.S6 – Caspr2 is not necessary for homeostatic upscaling of synaptic GluA1-containing AMPARs in hippocampal neurons. (A) Representative immunofluorescence images of mCherry-positive low-density cultured hippocampal neurons transfected at DIV 7 with a control empty vector (control) or a plasmid encoding the Caspr2 shRNA, or co-transfected with the Caspr2 shRNA and a shRNA-resistant Caspr2 rescue mutant. At DIV 13, neuronal activity was blocked for 48 h with 1 µM TTX and 100 µM APV. Cells were then live-stained with an antibody against an extracellular epitope in GluA1 N-terminus, fixed, and then stained for the synaptic marker PSD95. *Scale bars = 5µm.* **(B, C)** The fluorescence intensity of total **(B)** and PSD95-colocalized synaptic **(C)** clusters of surface GluA1 was analysed from mCherry-expressing transfected neurons. Total number of analysed cells was obtained from at least three independent experiments (N≥3, n≥30 cells). Results are presented as mean ± S.E.M., and statistical significance was determined by the parametric two-way ANOVA Test, followed by Tukey's Multiple Comparison Test, **p<0.01 compared to control, ##p<0.01, ###p<0.001 relative to control Caspr2-shRNA.

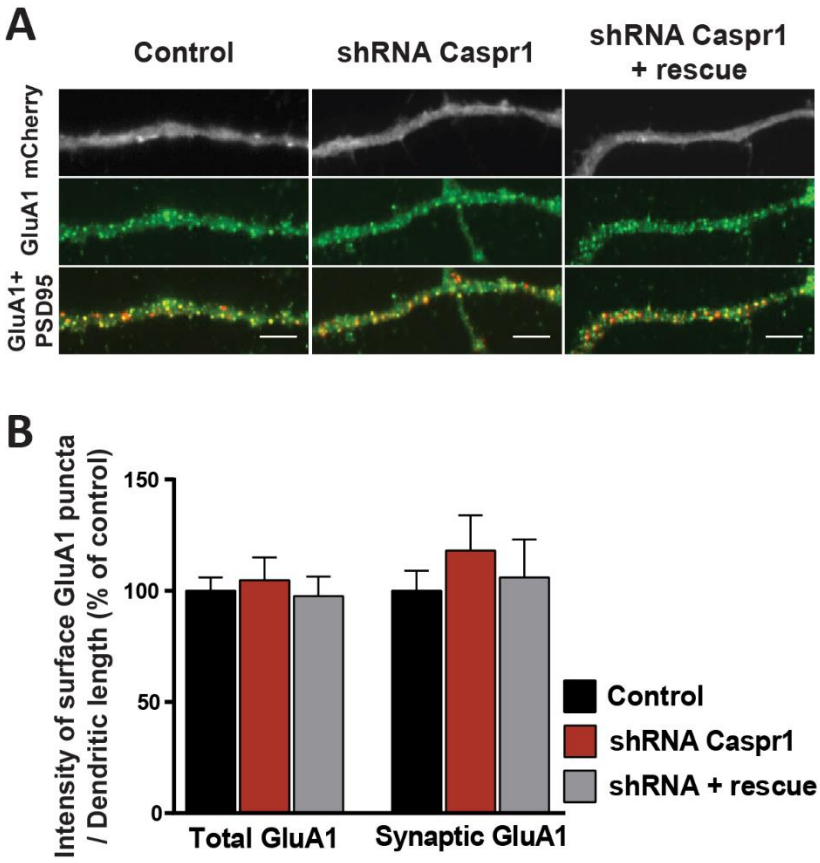


Figure 4.S7 – Caspr1 does not regulate the synaptic content of cell surface GluA1-containing AMPARs in cortical neurons. **(A)** Representative immunofluorescence images of mCherry-positive low-density cultured cortical neurons transfected at DIV 7 with a control empty vector or a plasmid encoding the Caspr1 shRNA, or co-transfected with the Caspr1 shRNA and a shRNA-resistant Caspr1 rescue mutant. At DIV 13, cells were live-stained against an extracellular epitope of GluA1, fixed, and then stained for the synaptic marker PSD95. *Scale bars = 5 µm*. **(B)** The fluorescence intensity of total and PSD95-colocalized synaptic clusters of surface GluA1 was analysed from mCherry-expressing transfected neurons. Total number of analysed cells was obtained from at least three independent experiments. (N≥3, n≥ 30cells). Results are presented as mean ± S.E.M and statistical significance was determined by the non-parametric Kruskal-Wallis test, followed by Dunn's Multiple Comparison test.

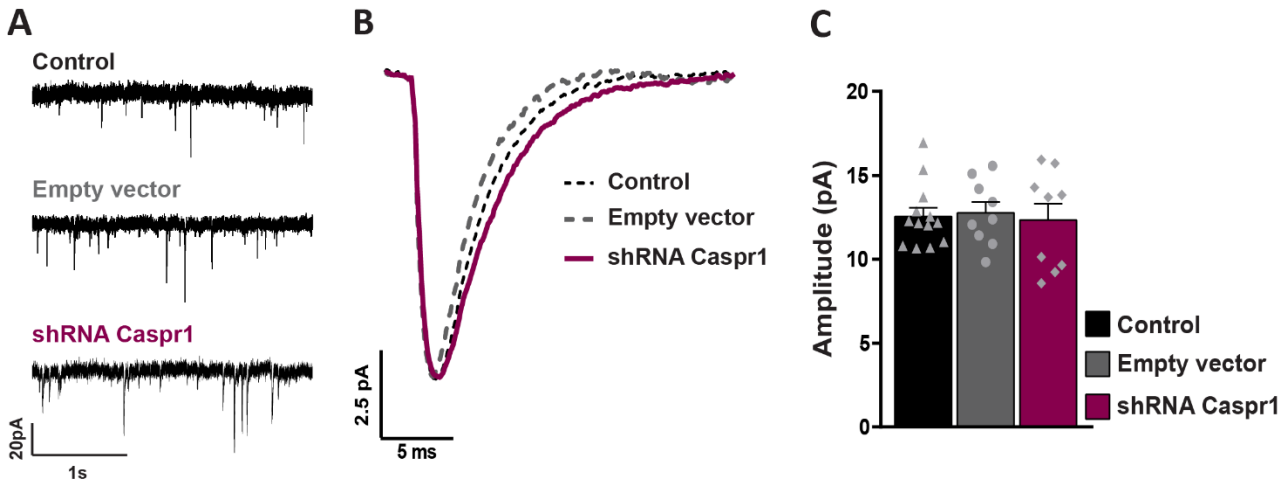


Figure 4.S8 – Caspr1 is not required for *in vivo* regulation of AMPAR function in the mouse primary visual cortex (V1). Lentivirus encoding either an empty-vector or the shRNA against Caspr1 were generated, and injected into layer2/3 of the V1 of normal reared P21-P28 C57BL/6J mice. Five weeks post-injection, AMPAR-mediated mEPSCs were recorded from mCherry-expressing infected V1-L2/3 pyramidal neurons. **(A - C)** Comparison of representative mEPSC traces **(A)**, average mEPSC traces **(B)** and average mEPSC amplitudes **(C)** recorded from V1-L2/3 pyramidal cells of either control mice (black, n=13 cells), empty vector-infected (grey, n=9 cells) or shRNA Caspr1-infected mice (pink, n=9 cells). In **(C),** results are presented as mean ± S.E.M. and statistical significance was determined by one-way ANOVA test followed by Fisher's protected least significant difference (PLSD) post hoc test.

Supplementary Tables

Table S1 – Comparison of mEPSCs and neuronal parameters across experimental groups of lentiviral-infected mice subject to visual paradigms.

Experiment Group	Paradigm	Amplitude (pA)	Frequency (Hz)	Rise time (ms)	Decay (τ, ms)	RMS Noise	Series R (MΩ)	Input R (MΩ)
Non-infected	NR	12.6±0.52	5.5±0.54	1.22±0.05	3.49±0.16	1.9±0.02	16.5±0.8	252.2±14.3
	DE	15.1±1.02*	5.6±0.94	1.18±0.06	3.55±0.20	1.8±0.05	15.8±0.7	223.6±21.6
Empty vector	NR	12.8±0.65	8.1±0.62	1.10±0.08	3.07±0.17	1.9±0.04	16.5±1.3	241.3±14.0
	DE	14.1±1.63	4.4±0.48	1.28±0.09	3.75±0.26	1.8±0.04	19.8±1.2	424.5±49.8
ShRNA Caspr2	NR	11.0±0.46*#	7.0±0.72	1.35±0.04	3.99±0.29	1.8±0.05	21.0±1.6	334.4±36.4
	DE	10.3±0.55	5.5±0.64	1.21±0.05	3.71±0.22	1.8±0.06	19.4±1.4	353.6±47.4

Results are presented as mean ± S.E.M. of each measured parameter from neurons. (R: resistance). When comparing NR conditions across different genotypes, statistical significance was determined by one-way ANOVA test, followed by Fisher's PLSD post-hoc test: *p<0.05 relative to control NR, and #p<0.05 relative to empty-vector NR. For comparison of NR vs. DE within genotypes statistical significance was determined by Mann-Whitney test, *p<0.05 compared to WT

Chapter V
Results & Discussion

Disrupted AMPA receptor function upon autoantibody-mediated loss of Caspr2

Part of the results in this chapter are included in the following manuscript in submission:

Dominique Fernandes, Sandra Santos, Ester Coutinho, Jessica L. Whitt, Tiago Rondão, M. Isabel Leite, Camilla Buckley, Hey-Kyoung Lee & Ana Luísa Carvalho (2018). Disrupted AMPA receptor function upon genetic- or antibody-mediated loss of autism-associated CASPR2. *In submission*

Summary

Autoimmune synaptic encephalitides are recently described neurological rare diseases thought to develop in consequence of aberrant autoantibody production against surface neuronal proteins, including CASPR2. Autoantibodies against CASPR2 (CASPR2-Abs) were first identified in the serum and cerebrospinal fluid of patients featuring diverse clinical presentations, including Morvan's syndrome, limbic encephalitis, memory and cognitive impairments and psychosis. However, definitive evidence for a pathogenic role of CASPR2-Abs remains elusive, and the mechanisms underlying disease symptoms are still uncharacterized.

Herein, we set out to explore the pathogenic mechanisms elicited by CASPR2-Abs, by using human immunoglobulins (IgGs) purified from the plasma of a patient with CASPR2 encephalitis. We found that patient IgGs significantly decrease the synaptic content of endogenous Caspr2 and cell surface AMPARs in cortical neurons, suggesting that CASPR2-Abs exert pathogenesis by disrupting the role we previously described for Caspr2 in the regulation of AMPAR trafficking. Moreover, we discovered that patient IgGs hamper Caspr2 function *in vivo* and perturb basal glutamatergic synaptic transmission in the visual cortex of mice. Additionally, patient IgGs prevent the triggering of long-term potentiation, whilst sparing homeostatic synaptic scaling mechanisms. Finally, we reveal that patient IgGs bound to the neuronal surface can undergo time-dependent internalization, thus underpinning a likely mechanism of pathogenesis elicited by CASPR2-Abs. Altogether, our findings pinpoint the glutamatergic system as a likely target for pathogenesis ensuing from an antibody-mediated disruption of CASPR2 function, and elucidate the mechanisms driving the cognitive and psychiatric symptoms presented by patients.

Introduction

For a long time, the brain was thought to be an immunologically privileged organ, mostly owing to the blood-brain barrier (BBB) and its capacity to limit the access of antibodies, immune mediators and immune cells from the systemic circulation into the brain. The discovery, around 10 years ago, of autoantibodies against cell surface neuronal proteins in immunotherapy-responsive patients manifesting with severe, but reversible, neurological and neuropsychiatric symptoms, drove a new paradigm shift in the establishment and understanding of CNS autoimmunity [reviewed in (Coutinho *et al.*, 2014; Crisp *et al.*, 2016; Dalmau, 2016; Jain & Balice-Gordon, 2016)]. This seminal finding boosted a massive expansion in the clinical diagnosis of many new patients or of patients previously misdiagnosed with some kind of idiopathic neurological syndrome or psychosis, and in the development of better therapies that significantly improved the outcome of patients, otherwise at life risk [reviewed in (Chefdeville *et al.*, 2016; Coutinho *et al.*, 2014; Dalmau, 2016; Lancaster & Dalmau, 2012)].

Throughout the past decade, several surface antigenic epitopes have been identified to be targeted for autoantibody-binding in a disorder now generally termed autoimmune synaptic encephalitis, given the significant proportion of targeted neuronal receptors and synaptic proteins, including glutamate receptors of both NMDA- (Dalmau *et al.*, 2008; Dalmau *et al.*, 2007) and AMPA-type (Lai *et al.*, 2009), GABAergic (Lancaster *et al.*, 2010; Petit-Pedrol *et al.*, 2014), glycinergic (Hutchinson *et al.*, 2008) and metabotropic receptors (Lancaster *et al.*, 2011b; Marignier *et al.*, 2010), as well as proteins of the VGKC complex (Vincent *et al.*, 2004), including the leucine-rich glioma-inactivated 1 protein [LGI1; (Irani *et al.*, 2010; Lai *et al.*, 2010)] and Contactin-associated protein 2 [CASPR2; (Irani *et al.*, 2010; Lancaster *et al.*, 2011a)], among others [extensively reviewed in (Chefdeville *et al.*, 2016; Coutinho *et al.*, 2014; Crisp *et al.*, 2016; Dalmau, 2016)].

Autoantibodies against the synaptic cell-adhesion molecule CASPR2 (CASPR2-Abs) were first identified in the serum and cerebrospinal fluid of patients with autoimmune encephalitis less than a decade ago [(Irani *et al.*, 2010; Lancaster *et al.*, 2011a), and reviewed in (Bastiaansen *et al.*, 2017; Pruss & Lennox, 2016; van Sonderen *et al.*, 2017)], and have since been associated with the development of a very diverse clinical spectrum of neurological syndromes that can include neuromyotonia, Morvan's syndrome and limbic encephalitis [reviewed in (Bastiaansen *et al.*, 2017; Binks *et al.*, 2017; Pruss & Lennox, 2016; van Sonderen *et al.*, 2017)]. These syndromes frequently overlap, presenting with peripheral disturbances including peripheral nerve hyperexcitability and neuropathic pain, but also central nervous system dysfunctions such as sleep alterations, seizures, memory impairment, cognitive deficits

and psychosis (Bien *et al.*, 2017; Irani *et al.*, 2010; Irani *et al.*, 2012; Joubert *et al.*, 2016; Klein *et al.*, 2013; Lancaster *et al.*, 2011a; Somers *et al.*, 2011; Sunwoo *et al.*, 2015; van Sonderen *et al.*, 2016). In most reported cases of CASPR2-Ab encephalitis, there is a favourable response to timely immunotherapy, with robust patient improvement. Nevertheless, the risk for relapse is particularly high and most patients require continuous immunosuppression throughout life, with some retaining permanent cognitive sequelae [reviewed in (Bastiaansen *et al.*, 2017; Binks *et al.*, 2017; Pruss & Lennox, 2016; van Sonderen *et al.*, 2017)]. Interestingly, the severity of symptoms in CASPR2-Ab encephalitis, particularly those of psychiatric nature, is associated with higher CASPR2-Ab titres in the patient serum and CSF, whereas symptom recovery following immunotherapy correlates with decreasing circulating titres [reviewed in (Bastiaansen *et al.*, 2017; Pruss & Lennox, 2016; van Sonderen *et al.*, 2017)], which is suggestive of a potential direct pathogenic effect of CASPR2-Abs.

Indeed, the cell surface localization of synaptic antigens such as CASPR2 can, in principle, allow autoantibodies to directly modulate the structure and function of the target antigen, hinting at a direct antibody-mediated pathogenic effect. In agreement, several studies have recently emerged reporting synaptic and cellular disruptive mechanisms induced by NMDAR- (Hughes *et al.*, 2010; Mikasova *et al.*, 2012; Moscato *et al.*, 2014; Zhang *et al.*, 2012), AMPAR- (Gleichman *et al.*, 2014; Lai *et al.*, 2009; Peng *et al.*, 2015) and LGI1-Abs (Ohkawa *et al.*, 2013), that closely mimic pharmacological or genetic disruption models of the corresponding antigens [reviewed in (Chefdeville *et al.*, 2016; Crisp *et al.*, 2016; Dalmau, 2016; Masdeu *et al.*, 2016)]. Furthermore, definite proof of antibody pathogenicity has just been established for NMDAR-Abs: mice subjected to an antibody passive transfer protocol with chronic ventricular infusion of patients' CSF samples develop a transient phenotype recapitulating patients' symptoms that is gradually resolved once infusion is stopped (Planaguma *et al.*, 2015).

To date, very few studies have explored potential disruptive cellular mechanisms induced by CASPR2-Abs, and their pathogenicity hasn't been fully established yet. Curiously, the cognitive and psychiatric symptoms of CASPR2-Ab encephalitis patients substantially overlap with those of neuropsychiatric patients carrying mutations in the CASPR2-encoding gene *CNTNAP2* [reviewed in (Penagarikano & Geschwind, 2012; Poot, 2017; Rodenas-Cuadrado *et al.*, 2014)], and with behavioural phenotypes presented by *Cntnap2* KO animal models (Hoffman *et al.*, 2016; Penagarikano *et al.*, 2011), which further argues in favour of the pathogenicity of CASPR2-Abs. Importantly, circulating CASPR2-Abs were recently detected during pregnancy in mothers of children with autism-spectrum disorders (Brimberg *et al.*, 2016) or intellectual disability (Coutinho *et al.*, 2017a), and recent studies reveal that offspring mice

exposed *in utero* to CASPR2-Abs develop behavioural abnormalities that relate to neuropsychiatric phenotypes (Brimberg *et al.*, 2016; Coutinho *et al.*, 2017b), thus hinting that gestational transfer of maternal CASPR2-Abs to the foetus could be pathogenic and likely contribute to the development of neuropsychiatric disorders in the progeny.

Taking into consideration the expanding clinical spectrum of anti-CASPR2 autoimmune encephalitis, and the lack of definite proof of a direct CASPR2-Ab-induced pathogenesis underlying the disease and patients' symptoms, we believe it is of the utmost importance to explore potential synaptic, cellular and functional phenotypes triggered by CASPR2-Abs. In the previous chapter of this study (Please refer to Chapter IV – Homeostatic and experience-dependent regulation of AMPA receptors by Caspr2), we uncovered a novel synaptic role for Caspr2 in the regulation of AMPAR trafficking to synapses, as well as a physiological function in the control of excitatory synaptic transmission in the mouse primary visual cortex. Furthermore, we revealed an unanticipated requirement of this Caspr2 function in the regulation of synaptic scaling mechanisms underlying experience-dependent homeostatic synaptic plasticity. In line with these findings, in the present chapter we address the hypothesis that CASPR2-Abs perturb this novel synaptic role of CASPR2 and consequently impair AMPAR trafficking and function, and disrupt synaptic plasticity phenomena. Herein, we demonstrate that human immunoglobulins (IgGs) purified from serum samples of a patient with CASPR2-Ab encephalitis specifically recognize and bind to Caspr2, altering its dendritic and synaptic distribution in rat cortical neurons. Additionally, we observe a parallel decrease in the synaptic content of cell surface GluA1-containing AMPARs in cortical neurons incubated with patient IgGs, confirming our hypothesis that CASPR2-Abs may exert their pathogenicity by impeding the function of Caspr2 in the regulation of AMPAR synaptic trafficking. Furthermore, we reveal a significant perturbation in cortical excitatory synaptic transmission induced by CASPR2-Abs, since *in vivo* incubation of patient IgGs in the mouse primary visual cortex (V1) decreases the amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs). Although mechanisms of homeostatic synaptic scaling remain intact, we further demonstrate that patient IgGs impair the trafficking and synaptic insertion of AMPARs induced by a chemical protocol of long-term potentiation (cLTP), suggesting a failure in activity-dependent changes of synaptic strength that are necessary for learning and memory processes. Finally, preliminary evidence indicate that CASPR2-Abs bound to cortical neurons are internalized from the cell surface, hinting at a likely preferential mechanism of pathogenesis in CASPR2-Ab encephalitis.

Overall, our findings uncover striking defects in AMPAR trafficking and function occurring subsequently to an autoantibody-mediated disruption of Caspr2 function, suggesting that dysregulation of glutamatergic synaptic transmission is a likely mechanism underlying the pathogenesis of CASPR2-Ab encephalitis. Importantly, these observations help clarify some of the severe cognitive and psychiatric symptoms presented by patients and may enable the development of more specific and efficient therapies for this type of encephalitis.

Results

CASPR2 autoantibodies purified from a patient with autoimmune encephalitis specifically recognize and bind to Caspr2, altering its synaptic distribution

Autoantibodies targeting surface epitopes of CASPR2 (CASPR2-Abs) have just recently been found in patients with autoimmune synaptic encephalitis that present with severe neurological and neuropsychiatric symptoms. However, to date, it is unclear whether CASPR2-Abs play a direct role in disease pathogenesis and the mechanisms underlying patients' symptoms still remain elusive. In collaboration with the John Radcliffe Hospital at Oxford University, we collected serum samples from a male patient with anti-CASPR2 autoimmune encephalitis presenting with severe cognitive symptoms (for details of the patient and his clinical history, please refer to Chapter II – Material and Methods) in order to explore the mechanisms underlying the pathogenesis of CASPR2 autoantibodies. Antibodies in the patient serum, at a titration of 1:6400 as determined by a live cell-based assay as previously described in (Coutinho *et al.*, 2017b), were shown to bind strongly to the surface of rat hippocampal neurons after 1 h incubation (Supplementary Figure 5.S1A). To test for specificity of binding to CASPR2, patient serum was first pre-absorbed in either untransfected HEK293 cells (Suppl. Figure 5.S1B) or in cells transfected with human CASPR2 (Suppl. Figure 5.S1C), and then re-incubated in hippocampal cultures. We observed that staining of hippocampal neurons was lost after pre-absorption of serum in CASPR2-transfected cells (Suppl. Figure 5.S1C). Human immunoglobulins (IgGs) were then purified from samples through an ammonium sulphate precipitation method, as previously described in (Coutinho *et al.*, 2017b), and their specificity of binding to CASPR2 was again confirmed (Figure 5.1A). Cultured hippocampal neurons from either WT or *Cntnap2* KO mice were incubated for 1 h with human IgGs purified from the patient serum (patient pIgG), fixed and then immunolabeled with an appropriate anti-human secondary antibody to detect human IgGs. After permeabilization, neurons were further stained against the neuronal marker MAP2 (Figure 5.1A). We observed that whilst patient pIgGs bind strongly to the surface of WT hippocampal neurons, they fail to do so on *Cntnap2* KO neurons, indicating that the human IgGs present in the patient plasma specifically recognize and bind to Caspr2. Moreover, this finding indicates that it is unlikely that the patient serum contains antibodies against other target antigens, otherwise patient IgGs would still bind the surface of neurons lacking Caspr2.

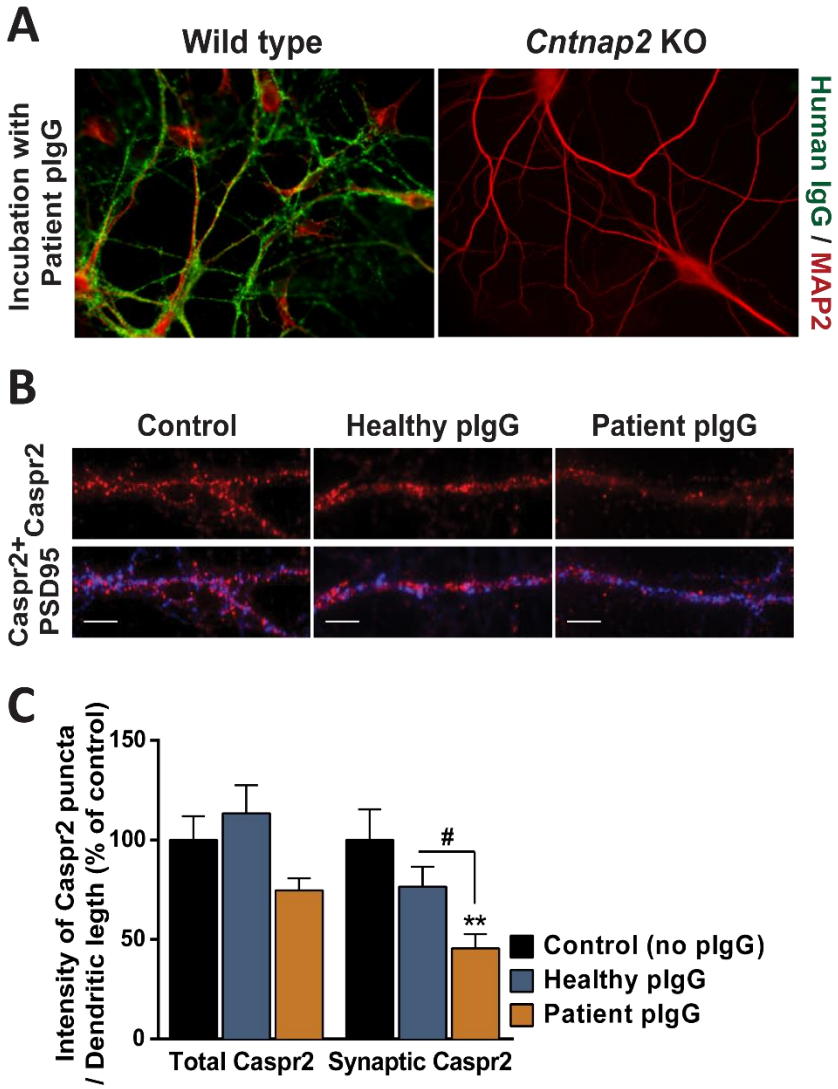


Figure 5.1 – CASPR2 autoantibodies specifically recognize and bind to Caspr2, altering its synaptic content. **(A)** CASPR2 autoantibodies fail to bind to *Cntnap2* KO hippocampal neurons. 14 - 18 DIV WT or *Cntnap2* KO mouse hippocampal neurons were incubated for 1 h with human immunoglobulins (IgGs) purified from the plasma of a patient with anti-CASPR2 encephalitis (Patient pIgGs). Neurons were then fixed and immunolabelled against human IgGs and the neuronal/dendritic marker MAP2. Patient pIgGs bind strongly to the surface of WT, but not of *Cntnap2* KO neurons. Images kindly provided by Ester Coutinho (Oxford University, UK). **(B, C)** CASPR2 autoantibodies significantly decrease the synaptic expression of Caspr2 in WT rat cortical neurons. 13 DIV low-density cultured cortical neurons were incubated for 7 h with 200 ηg/mL of human IgGs purified from the plasma of either the patient, or of a sex- and age-matched healthy subject (Healthy pIgG). Cells were then fixed and stained for Caspr2 and the synaptic marker PSD95. **(B)** Representative fluorescence images of cultured cortical neurons incubated for 7 h with either healthy or patient-purified human IgGs, and stained for Caspr2 and PSD95. *Scale bars = 5μm.* **(C)** The fluorescence intensity of total and synaptic (co-localized with PSD95) clusters of Caspr2 was quantified. Total number of analysed cells was obtained from three independent experiments. (N=3, n≥30 cells). Results are presented as mean ± S.E.M. and statistical significance was determined by the non-parametric Kruskal-Wallis test, followed by Dunn's Multiple Comparison post-hoc test, **p<0.01 compared to control, #p<0.05 relative to healthy pIgG.

Given the specificity of the human IgGs purified from the patient serum to bind Caspr2, we asked whether these CASPR2-Abs may have a direct modulatory effect on the structure and function of their target antigen. To investigate this, we started by evaluating the effect of CASPR2-Abs in the dendritic and synaptic distribution of Caspr2. For that, low-density cultured rat cortical neurons were incubated for 7 h with 200 ηg/mL of pIgGs purified from either the patient, or from an age- and sex-matched healthy control subject, and then immunolabeled with an antibody specific for Caspr2, and for the postsynaptic scaffolding protein PSD95 to identify glutamatergic synapses (Figure 5.1B, C). Firstly, we observed that only the patient pIgGs, but not IgGs purified from the healthy control, are able to bind to dendritic compartments of cortical neurons (Supplementary Figure 5.S2), which once again argues for the specificity of binding of CASPR2-Abs. Quantitative immunofluorescence analysis revealed that, whilst incubation of neurons with healthy pIgGs did not affect the distribution and expression of Caspr2 when compared to non-incubated control cells, incubation with patient pIgGs resulted in a marked decrease of 25.4 ± 6.1% in the fluorescence intensity of total dendritic clusters of Caspr2 (Figure 5.1C - left). Moreover, the fluorescence intensity of Caspr2 clusters located at the synapse, as determined by cluster co-localization with the postsynaptic marker PSD95, was also significantly decreased by 54.5 ± 7.2% upon incubation with patient pIgGs (Figure 5.1C - right).

Overall, these results demonstrate that autoantibodies in the serum of a patient with autoimmune encephalitis specifically recognize and bind to Caspr2, resulting in a significant disruption of the dendritic distribution of Caspr2, as well as of its synaptic content. To our knowledge, this is the first study reporting a CASPR2-Ab-mediated perturbation in the levels of endogenous Caspr2, thus indicating that CASPR2-Abs, similarly to NMDAR- (Hughes *et al.*, 2010; Mikasova *et al.*, 2012; Moscato *et al.*, 2014; Zhang *et al.*, 2012), AMPAR- (Gleichman *et al.*, 2014; Lai *et al.*, 2009; Peng *et al.*, 2015) and LGI1-Abs (Ohkawa *et al.*, 2013), can directly modulate the expression/distribution of their target antigen, and most likely its function, hinting at a direct antibody-mediated pathogenic effect of CASPR2-Abs underlying the patient's symptoms.

CASPR2 autoantibodies disrupt cortical synaptic AMPAR trafficking and glutamatergic synaptic transmission in the mouse visual cortex

Patients with anti-CASPR2 autoimmune encephalitis typically present with a very diverse clinical spectrum of neurological syndromes that can include neuromyotonia, Morvan's Syndrome and limbic encephalitis. Patients often manifest a clinical condition that overlaps the different syndromes, presenting with autonomic disturbances, peripheral nerve hyperexcitability and neuropathic pain, but also sleep alterations and insomnia, seizures, confusion, memory impairment, cognitive deficits and psychosis, reflecting a complex dysfunction of both the peripheral and central nervous systems underlying disease pathophysiology [reviewed in (Bastiaansen *et al.*, 2017; Binks *et al.*, 2017; Pruss & Lennox, 2016; van Sonderen *et al.*, 2017)]. Such dysregulation can be correlated with the complex spectrum of cellular and molecular functions recently attributed to CASPR2. Initial reports following the identification of CASPR2 pinpointed a fundamental role of the protein in the regulation of voltage-gated K^+-channel clustering in axonal microdomains of peripheral myelinated neurons and, thus, in the modulation of action potential propagation and axonal excitability. This sole role of CASPR2 could easily sustain the peripheral nature of patients' symptoms, but falls short on clarifying the pathogenesis for such a striking CNS dysfunction. The development of *Cntnap2* KO animal models, which present aberrant behavioural phenotypes that remarkably recapitulate the symptoms of *CNTNAP2*-related neuropsychiatric patients, enabled a better understanding of the role of CASPR2 in the regulation of brain function. Accordingly, numerous studies from recent years report hallmark defects in neuronal migration, dysregulated balance of excitation and inhibition, altered dendritic complexity, as well as synapse abnormalities [extensively reviewed in (Penagarikano & Geschwind, 2012; Poot, 2017; Rodenas-Cuadrado *et al.*, 2014)].

Furthermore, in the previous chapter of this study (please refer to Chapter IV), we uncovered an additional unsuspected role for Caspr2 in the regulation of glutamate AMPAR trafficking and function. Indeed, we found that loss of Caspr2 decreases the synaptic content of cell surface AMPARs, and disrupts glutamatergic synaptic transmission *in vivo*. Moreover, we revealed an activity-dependent requirement of Caspr2 to regulate homeostatic mechanisms of synaptic scaling underlying the expression of sensory-driven experience-dependent synaptic plasticity in the mouse visual cortex. Importantly, the dynamic regulation of AMPAR trafficking and function at the synapse is necessary for the expression of different forms of synaptic plasticity (Anggono & Huganir, 2012; Shepherd & Huganir, 2007), considered the cellular correlates of higher cognitive processes, including learning and memory, and a failure in such mechanisms has already been proposed to underlie the pathogenesis of several

neuropsychiatric disorders in which CASPR2 has been implicated (Volk *et al.*, 2015). Thus, given the vast cognitive and psychiatric symptoms also presented by patients with anti-CASPR2 encephalitis, a plausible antibody-mediated mechanism of disease pathogenesis could be the dysregulation of AMPAR function and glutamatergic synaptic transmission.

Taking all this into consideration, we decided to evaluate whether the CASPR2-Ab-mediated disruption of Caspr2, described in the previous set of results, might perturb its function in the regulation of AMPAR trafficking. To investigate this, low-density cultured cortical neurons were incubated for 7 h with either healthy or patient pIgGs, as described above. To assess the surface expression of GluA1-containing AMPARs, neurons were fixed and immunolabelled with an antibody against an extracellular epitope in the GluA1 N-terminus. Following permeabilization, neurons were further stained for Caspr2, and for PSD95 to visualize excitatory synapses (Figure 5.2A). We observed that incubation of patient pIgGs in cortical neurons resulted in a significant decrease of 28.7 ± 5.4% in the fluorescence intensity of total clusters of cell surface GluA1-containing AMPARs (Figure 5.2B - left), when compared to either control neurons or cells incubated with healthy pIgGs. Moreover, patient pIgGs also significantly decreased the fluorescence intensity of PSD95-colocalized synaptic clusters of cell surface GluA1 (60.3 ± 7.1%; Figure 5.2B - right). The decreases in GluA1 cluster intensities that we observe with the patient pIgGs are strikingly similar to those resulting from a shRNA-mediated loss of Caspr2 expression (please refer to Figure 4.3 in Chapter IV), which suggests that CASPR2-Abs perturb the trafficking and synaptic content of AMPARs in consequence of a severe disruption of Caspr2 function. In agreement, we also found that patient pIgGs significantly decrease the fluorescence intensity of synaptic GluA1 clusters that overlap with Caspr2 (45.4 ± 7.2%; Figure 5.2C), hinting at the possibility that CASPR2-Abs may disrupt the interaction between Caspr2 and GluA1, which we found in the previous chapter (please refer to Figure 4.2 in Chapter IV). Importantly, we found no changes in either the fluorescence intensity, area or number of PSD95 clusters upon incubation with patient pIgGs (Supplementary Figure 5.S3), indicating that CASPR2-Abs have no effect in synapse development/maintenance, and ruling out the possibility that the observed defects in AMPAR synaptic content are secondary to synapse loss.

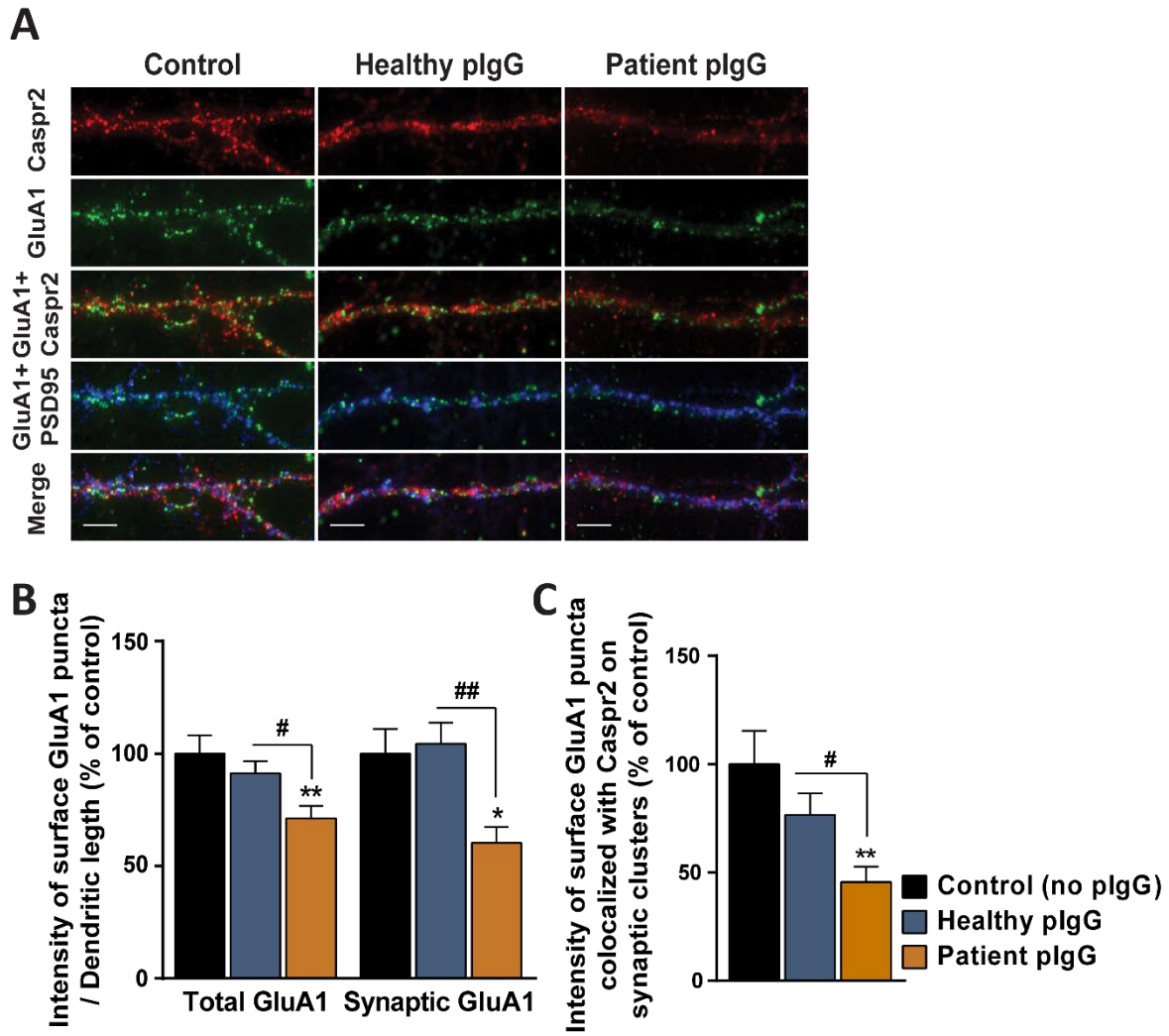


Figure 5.2 – CASPR2 autoantibodies decrease the trafficking and synaptic content of cell surface AMPARs. (A - C) 13 DIV low-density cultured cortical neurons were incubated for 7 h with 200 ηg/mL of human IgGs purified from the plasma of either the CASPR2-encephalitis patient or a healthy subject. Cells were then fixed and immunolabeled with an antibody against an extracellular epitope in the GluA1 N-terminus for staining of surface GluA1-containing AMPARs. After permeabilization, cells were additionally stained for Caspr2 and the synaptic marker PSD95. **(A)** Representative immunofluorescence images of cultured cortical neurons incubated for 7 h with either healthy or patient-purified human IgGs, and stained for superficial GluA1, Caspr2 and PSD95. *Scale bars = 5μm.* **(B, C)** The fluorescence intensity of total and PSD95-colocalized synaptic clusters of surface GluA1 **(B)** and of synaptic GluA1 cluster co-localized with Caspr2 puncta **(C)** was quantified. Total number of analysed cells was obtained from three independent experiments. (N=3, n≥ 30 cells). Results are presented as means ± S.E.M and the statistical significance was determined by the non-parametric Kruskal-Wallis test, followed by Dunn's Multiple Comparison post-hoc test, *p<0.05, **p<0.01 compared to control, #p<0.05, ##p<0.01 relative to healthy pIgGs.

Taking into consideration that some patients with anti-CASPR2 autoimmune encephalitis can develop a partial atrophy of the hippocampus (Bien *et al.*, 2017; Joubert *et al.*, 2016; Kortvelyessy *et al.*, 2015), a core brain region for memory and learning processes, we evaluated whether CASPR2-Abs also disrupt Caspr2 and impair AMPAR trafficking in this region, but found no gross differences in the fluorescence intensity of either Caspr2 or cell surface AMPAR clusters when cultured hippocampal neurons were incubated for 7 h with patient pIgGs (Supplementary Figure 5.S4). Curiously, this finding agrees with the region-specific modulatory effect of Caspr2 we observed in the previous chapter. Indeed, although loss of Caspr2 in the hippocampus decreases the basal trafficking of cell surface AMPARs to the synapse, similarly to what occurs in cortical neurons, the activity-dependent requirement of Caspr2 for the regulation of homeostatic synaptic scaling mechanisms is restricted to the cortex (please refer to supplementary data in Chapter IV). Surprisingly, when hippocampal neurons were incubated for either 2 or 21 h with a higher concentration of patient pIgGs (50 µg/mL), the fluorescence intensity of surface GluA1 clusters increased significantly (Supplementary Figure 5.S5). This result, although contradictory to what we have reported so far, may suggest that increasing titres of CASPR2-Abs could elicit alternative pathogenic mechanisms to the ones triggered with lower concentrations, which would also help explain the wide phenotypic variability presented by patients with differing autoantibody titres. This hypothesis indicates that future studies to explore the pathogenicity of CASPR2-Abs should be performed with varying concentrations of autoantibodies to better understand their underlying mechanisms. Most importantly, they warrant a more thorough characterization of each patient individually in order to establish a potential correlation between antibody titres and specific phenotypic clusters and, thus, enable a more specific and efficient approach of treatment.

Our observations of a deleterious effect of CASPR2-Abs in the novel synaptic function that we have previously proposed for Caspr2 start to shed light on the potential pathogenic mechanisms triggered by CASPR2-Abs that can elicit the disease pathophysiology and underlie patients' symptoms. Clearly, the altered synaptic distribution of Caspr2 and subsequent disruption of AMPAR trafficking mechanisms triggered by the CASPR2-Abs warrant further exploration to determine whether such a potentially pathogenic mechanism might be relevant for physiological glutamatergic function. Hence, to investigate if the disruptive effect of CASPR2-Abs in the regulation of AMPAR trafficking is relevant for AMPAR function *in vivo*, we injected 200 ηg of either healthy or patient IgGs into layer 2/3 of the primary visual cortex (V1) of C57BL/6J mice (Figure 5.3A). To determine changes in basal glutamatergic synaptic transmission, and assess if CASPR2-Abs perturb basal AMPAR function, we measured AMPAR-mediated miniature excitatory postsynaptic currents of V1-

layer2/3 pyramidal neurons (mEPSCs - Figure 5.3A, B). We observed that the average trace (Figure 5.3C - blue trace) and the average amplitude (Figure 5.3D - blue bar: 14.87 ± 0.81 pA) of mEPSCs recorded from mice injected with healthy IgGs remained unaltered when compared to mEPSCs recorded from non-injected control mice (Figure 5.3D - black bar: 14.27 ± 0.84 pA). A cumulative histogram of mEPSC amplitudes further confirms that the distribution of mEPSC amplitudes of mice injected with healthy IgGs does not differ significantly from control amplitudes (Figure 5.3E - blue trace).

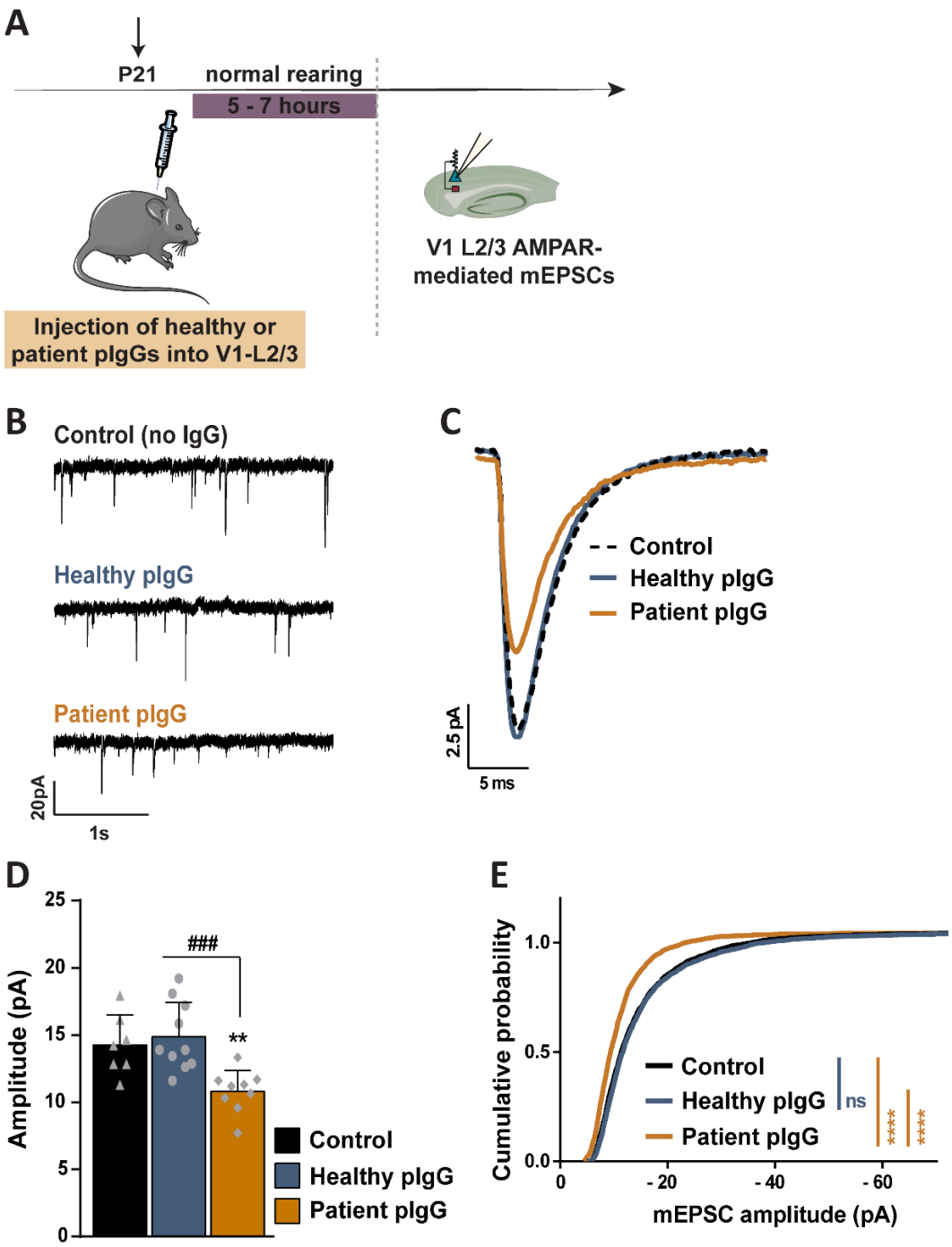


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Figure 5.3 – *In vivo* incubation of CASPR2 autoantibodies in the mouse visual cortex significantly decreases the amplitude of AMPAR-mediated mEPSCs of layer 2/3 pyramidal neurons. **(A)** 200ηg of either healthy- or patient-purified human IgGs were injected into layer2/3 of the primary visual cortex (V1-L2/3) of normal reared P21-P28 C57BL/6J mice. 7 h post-injection, AMPAR-mediated mEPSCs were recorded from V1-L2/3 pyramidal neurons. **(B - D)** Comparison of representative traces **(B)**, average traces **(C)** and average amplitudes **(D)** of mEPSCs recorded from V1-L2/3 pyramidal cells of control non-injected mice (black, n=7 cells), or of mice injected with either healthy (blue, n=10 cells) or patient pIgGs (yellow, n=9 cells). In **(D),** results are presented as mean ± S.E.M. and statistical significance was determined by one-way ANOVA test followed by Fisher's protected least significant difference (PLSD) post hoc test, **p<0.01 when compared to control, ###p<0.001 relative to healthy pIgGs. **(E)** Cumulative histograms showing that only the distribution of mEPSC amplitudes recorded from mice injected with patient pIgGs (yellow), but not with healthy pIgGs (blue), is significantly smaller than that of control littermates (black). Statistical significance was determined by the Kolmogorov-Smirnov Test, ****p<0.0001.

Conversely, there is a marked decrease in the amplitude of average trace of mEPSCs recorded from mice injected with the patient pIgGs (Figure 5.3C - yellow), and the average amplitude of mEPSCs is significantly smaller when compared to that of both control non-injected littermates and mice injected with healthy pIgGs (Figure 5.3D - yellow: 10.82 ± 0.52 pA). Moreover, the cumulative distribution of mEPSC amplitudes recorded from the mice injected with the patient IgGs is significantly shifted towards smaller values than that of control or healthy pIgGs-amplitudes (Figure 5.3E - yellow trace). No changes in mEPSC average frequency or current kinetics were observed between treatment groups (Suppl. Table S1).

Altogether, these findings reveal that CASPR2-Abs not only impair the localization of Caspr2 to excitatory synapses, but also have an impact in the regulation of AMPAR trafficking that perturbs AMPAR-mediated synaptic transmission *in vivo*. Importantly, these findings start to uncover the pathogenic mechanisms of CASPR2-Abs and propose a disruption of glutamatergic synaptic transmission as a potential molecular underpinning for the psychiatric and cognitive symptoms presented by autoimmune encephalitis patients.

CASPR2 autoantibodies prevent chemical LTP-, but not homeostatic synaptic scaling-induced AMPAR trafficking and synaptic insertion

Rapid changes in the postsynaptic accumulation and function of AMPARs occur in response to different patterns of neuronal activity, resulting in long-lasting modifications in synaptic strength and efficacy. Hence, the dynamic regulation of AMPAR trafficking to the synapse has emerged as a critical mechanisms for the expression of multiple forms of synaptic plasticity in different areas of the brain (Anggono & Huganir, 2012; Fernandes & Carvalho,

2016; Shepherd & Huganir, 2007). In the previous chapter (please refer to Chapter IV), we were able to uncover a novel role for Caspr2 in the regulation of AMPAR trafficking to the cell surface and synapses that is physiologically relevant for proper glutamatergic synaptic transmission *in vivo* in the mouse visual cortex. Importantly, we found this Caspr2 function to be fundamental for the regulation of neuronal synaptic homeostasis *in vivo*. Indeed, we observed that loss of Caspr2 impairs the homeostatic synaptic scaling of AMPARs following prolonged blockade of neuronal activity *in vitro*, and hinders the triggering of sensory-driven experience-dependent homeostatic plasticity in the visual cortex following prolonged visual deprivation in mice (please refer to Figures 4.6 & 4.8 in Chapter IV).

Homeostatic synaptic scaling, molecularly expressed as a set core of mechanisms that bi-directionally control the postsynaptic accumulation of AMPARs, is a compensatory modality of plasticity that prevents potentially destabilizing changes in synaptic strength to occur, while maintaining a well fine-tuned balance of the overall activity of neuronal networks [reviewed in (Fernandes & Carvalho, 2016; Turrigiano, 2008)]. Normal cognition and behaviour are thought to depend on such type of modulatory mechanisms, which have been shown to occur in compensation for periods of heightened plasticity such as occurring during development, learning-related processes, processing and integration of sensory inputs and adaptation to sensory environment changes, sleep, or even as a coping restorative process in cases of cross-modal plasticity of sensory modalities or in disease-states (Fernandes & Carvalho, 2016; Turrigiano, 2008; Whitt *et al.*, 2014). Understandably, emerging findings suggest that a failure in neuronal synaptic homeostasis might be an underlying pathology in several cognitive and psychiatric disorders [reviewed in (Mullins *et al.*, 2016; Ramocki & Zoghbi, 2008; Volk *et al.*, 2015; Wondolowski & Dickman, 2013)]. Interestingly, patients with anti-CASPR2 encephalitis have been shown to develop insomnia and sleep disturbances, vision loss, as well as altered perceptual processing and sensory hallucinations (visual and auditory), which suggest potential defects in sensory integration and processing (Bastiaansen *et al.*, 2017; Binks *et al.*, 2017; Pruss & Lennox, 2016; van Sonderen *et al.*, 2017).

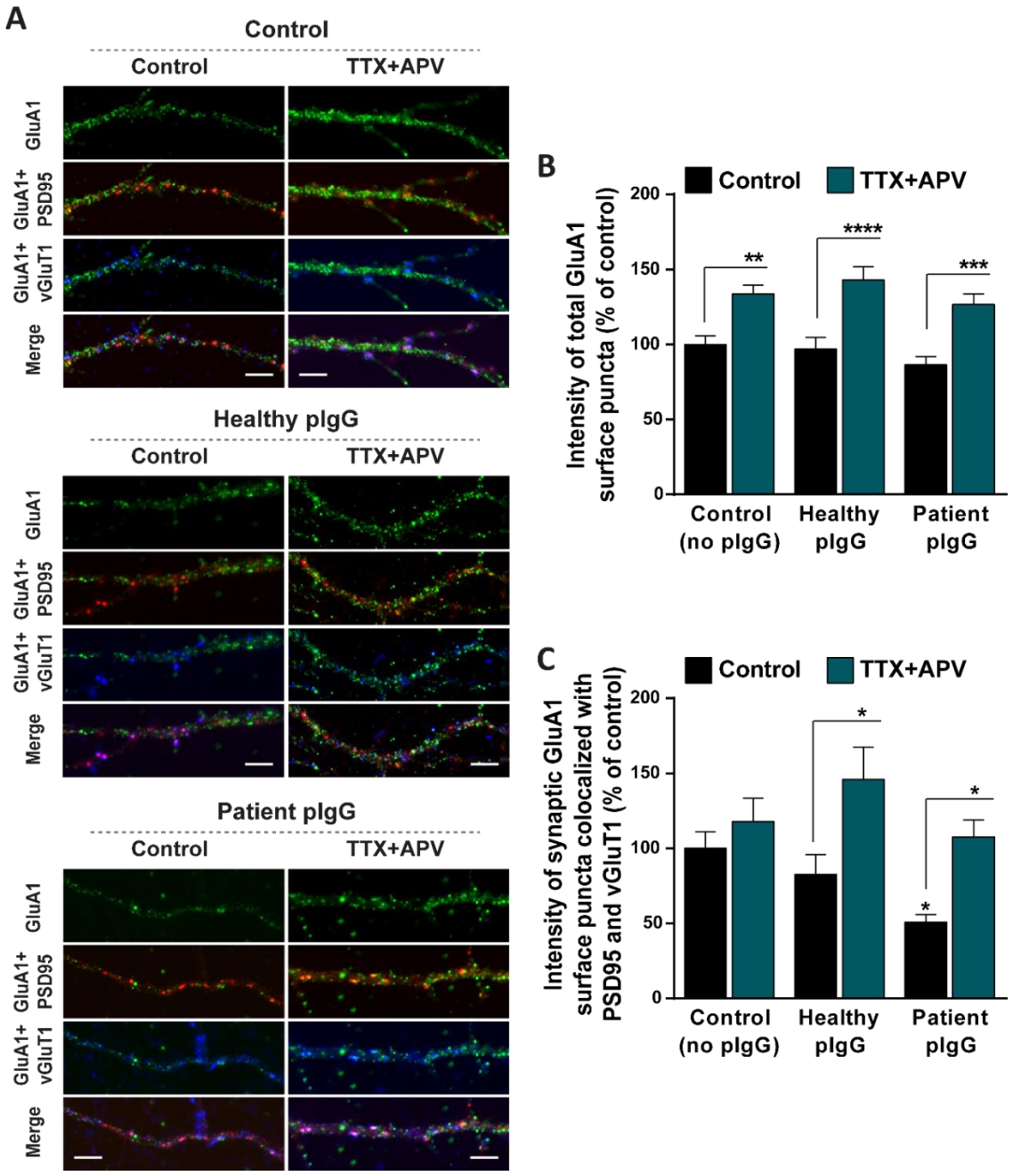
Taking into consideration 1) the cognitive and psychiatric nature of some of the symptoms presented by patients with CASPR2-Ab encephalitis, together with 2) the disruption of Caspr2-dependent mechanisms underlying AMPAR trafficking and function mediated by a potential pathogenic effect of CASPR2-Abs, and 3) our previous findings pinpointing Caspr2 as a novel molecular player fundamental for the expression of homeostatic mechanisms of synaptic scaling, we hypothesized that the pathogenic effects elicited by CASPR2-Abs on Caspr2 function are likely to perturb the normal triggering of homeostatic plasticity mechanisms

and, thus propose a failure in neuronal homeostasis as a potential pathophysiology of CASPR2 autoimmune encephalitis.

To investigate whether CASPR2-Abs disrupt the homeostatic synaptic scaling of AMPARs, we induced a prolonged inhibition of neuronal activity in low-density cultured cortical neurons for 48 h with 1 µM TTX together with 100 µM APV, which block action potential generation and NMDAR function, respectively. In parallel, neurons were incubated with 200 ηg/mL of either healthy- or patient-purified IgGs. After fixation, neurons were immunolabelled for surface GluA1-containing AMPARs as described above, and then stained for the postsynaptic marker PSD95 and the presynaptic marker vGluT1 for identification of potentially functional glutamatergic synapses (Figure 5.4A). Similarly to what we observed earlier, we found that incubation of cortical neurons with patient pIgGs for 48 h resulted in a significant decrease of 49.1 ± 5.0% in the fluorescence intensity of PSD95- and vGluT1-colocalized synaptic clusters of cell surface GluA1 (Figure 5.4C), when compared to both control or healthy pIgG-incubated cells. Moreover, as expected, both control cells or cells incubated with healthy pIgGs were able to scale up the fluorescence intensity of both total (Figure 5.4B; control TTX+APV: 133.7 ± 5.8%; healthy pIgGs TTX+APV: 142.9 ± 9.0%) and synaptic (Figure 5.4C; control TTX+APV: 117.8 ± 15.5%; healthy pIgGs TTX+APV: 146.0 ± 21.4%) clusters of cell surface GluA1 following prolonged activity inhibition with TTX+APV. Surprisingly, we observed that TTX+APV treatment of cortical neurons incubated with the patient pIgGs also significantly increased the fluorescence intensity of both total (126.6 ± 7.1%; Figure 5.4B) and synaptic (107.7 ± 11.3%; Figure 5.4C) cell surface GluA1 clusters, when compared to the respective basal patient pIgG-incubation condition. These results are quite unexpected, since we had previously seen that 1) the role of Caspr2 in the regulation of AMPAR trafficking is required for the mechanisms underlying homeostatic synaptic scaling (please refer to Chapter IV), and 2) CASPR2-Abs disrupt Caspr2 and consequently impair AMPAR trafficking and function; thus, we expected that CASPR2-Abs would abrogate the synaptic scaling of cell surface GluA1 following activity inhibition. On the contrary, these findings indicate that CASPR2-Abs do not disrupt the role of Caspr2 in regulating homeostatic synaptic scaling, and that these mechanisms remain intact with CASPR2-Abs. Importantly, they rule out the potential contribution of failed neuronal homeostatic processes for disease pathogenesis.

In spite of these results, CASPR2-Abs do significantly affect the basal trafficking and synaptic content of cell surface AMPARs. One would assume that such a striking dysregulation in AMPAR trafficking would impinge on the synaptic delivery and incorporation mechanisms of these receptors in response to different patterns of neuronal activity. Given the relevance of activity-dependent mechanisms of AMPAR regulation for different forms of synaptic plasticity,

we cannot disregard a potential for CASPR2-Abs to perturb forms of plasticity, such as Hebbian plasticity, other than homeostatic synaptic scaling. Unlike homeostatic plasticity mechanisms, which are of negative-feedback nature, Hebbian plasticity is a positive-feedback process that typically functions in an input-specific manner, is rapidly induced and associative as it requires correlated firing of the pre- and postsynaptic neurons. Because these hallmark features facilitate the reinforcement of precise synaptic connections, which is fundamental for information storage in the brain, Hebbian mechanisms are thought to be the cellular correlates of learning and memory [reviewed in (Huganir & Nicoll, 2013; Luscher & Malenka, 2012; Malenka & Bear, 2004)].



[Figure 5.4 - subtitle on the next page**]**

Figure 5.4 – Homeostatic mechanisms of AMPAR synaptic scaling are unaffected by CASPR2 autoantibodies. 11 DIV low-density cultured cortical neurons were incubated with 200 ηg/mL of either healthy- or patient-purified human pIgGs for 48 h, whilst their neuronal activity was inhibited with 1 µM TTX together with 100 µM APV. At 13 DIV, cells were fixed and immunolabeled with a specific antibody against an extracellular epitope in GluA1 N-terminus, permeabilized and then stained for the synaptic markers PSD95 and VGluT1. **(A)** Representative immunofluorescence images of both control and TTX+APV-treated cortical neurons incubated for 48 h with either healthy or patient-purified human IgGs, and stained for superficial GluA1, PSD95 and vGluT1. *Scale bars = 5µm.* **(B, C)** The fluorescence intensity of total **(B)** and synaptic **(C –** colocalized with PSD95 and vGluT1**)** clusters of surface GluA1 was quantified. Total number of analysed cells was obtained from at least three independent experiments (N≥3, n≥30 cells). Results are presented as mean ± S.E.M., and statistical significance was determined by the parametric two-way ANOVA Test, followed by Tukey's Multiple Comparison Test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The classical Hebbian paradigm for activity-dependent synaptic delivery of AMPARs is NMDAR-dependent long-term potentiation (LTP). Although Hebbian LTP and synaptic upscaling are thought to operate under different computational rules (for e.g., NMDAR-LTP requires NMDAR activation and subsequent Ca^{2+}-dependent triggering of several kinases to phosphorylate AMPARs and promote their synaptic incorporation [reviewed in (Huganir & Nicoll, 2013; Luscher & Malenka, 2012; Malenka & Bear, 2004)], whereas homeostatic mechanisms can operate under NMDAR inhibition and induce transcriptional and translational programmes to regulate AMPAR scaling [reviewed in (Fernandes & Carvalho, 2016; Turrigiano, 2008; Vitureira & Goda, 2013)]), mounting evidence suggests that homeostatic and Hebbian forms of plasticity are likely to share particular molecular mechanisms and converge to regulate common effectors at the synapse. Accordingly, several of the molecular players identified so far in the regulation of homeostatic plasticity mechanisms have previously been implicated in the regulation of Hebbian forms of synaptic plasticity (Fernandes & Carvalho, 2016; Vitureira & Goda, 2013).

Taking all this into account, together with the striking effect of CASPR2-Abs in the trafficking and synaptic content of cell surface AMPARs, we hypothesized that CASPR2-Abs might perturb the synaptic incorporation of AMPARs triggered by NMDAR-dependent LTP. To directly test this, we used a neuronal culture model of LTP (chemical LTP, cLTP), in which pharmacological activation of NMDARs promotes an increase in the surface expression of GluA1-containing AMPARs (Diering *et al.*, 2016; Hussain *et al.*, 2014; Lu *et al.*, 2001; Passafaro *et al.*, 2001; Ribeiro *et al.*, 2014). In a preliminary experiment, low-density cultured cortical neurons were incubated for 7 h with either healthy- or patient pIgGs, followed by cLTP induction (Diering *et al.*, 2016; Hussain *et al.*, 2014). Surface GluA1-containing AMPARs were immunolabelled as described above, and the postsynaptic marker PSD95 and the presynaptic

marker vGluT1 were also stained for identification of potentially functional glutamatergic synapses (Figure 5.5A). Consistent with previous studies, we observed that cLTP induction caused a marked increase in the fluorescence intensity of both total (136.4 ± 12.4%; Figure 5.5B) and synaptic clusters (162.9 ± 46.3%; Figure 5.5C) of cell surface GluA1. Similarly to control cells, cLTP induction in neurons incubated with healthy pIgGs also induced the incorporation of cell surface GluA1 clusters (total: 125.5 ± 5.0% - Figure 5.5B; synaptic: 180.4 ± 19.0% - Figure 5.5C). On the other hand, in neurons incubated with the patient pIgGs, in which the fluorescence intensity of cell surface GluA1 clusters was already markedly decreased in basal conditions, cLTP induction completely failed to increase both total (50.9 ± 9.3%; Figure 5.5B) and synaptic (40.7 ± 9.4%; Figure 5.5C) GluA1 clusters.

These preliminary findings indicate that the previously observed CASPR2-Ab-mediated disruption of AMPAR trafficking perturbs their activity-dependent recruitment and synaptic incorporation upon cLTP induction, which suggests that LTP phenomena may be impaired in patients with CASPR2-Abs and contribute for disease pathogenesis, explaining the memory complaints and cognitive deficits presented by patients. Furthermore, these observations hint at the possibility that Caspr2 plays a fundamental role in general activity-dependent mechanisms of AMPAR regulation that facilitate the expression of different forms of synaptic plasticity. Because these results are very preliminary, future experiments need to be done to confirm them. Nevertheless, if holding true, these findings warrant a more thorough characterization of the molecular mechanisms underlying the function of Caspr2 in response to different patterns of activity. Such characterization would provide a better understanding into how Caspr2 might function as a common synaptic effector to integrate divergent experience-dependent changes in activity, and would provide valuable insights regarding this dynamic interplay between different forms of plasticity.

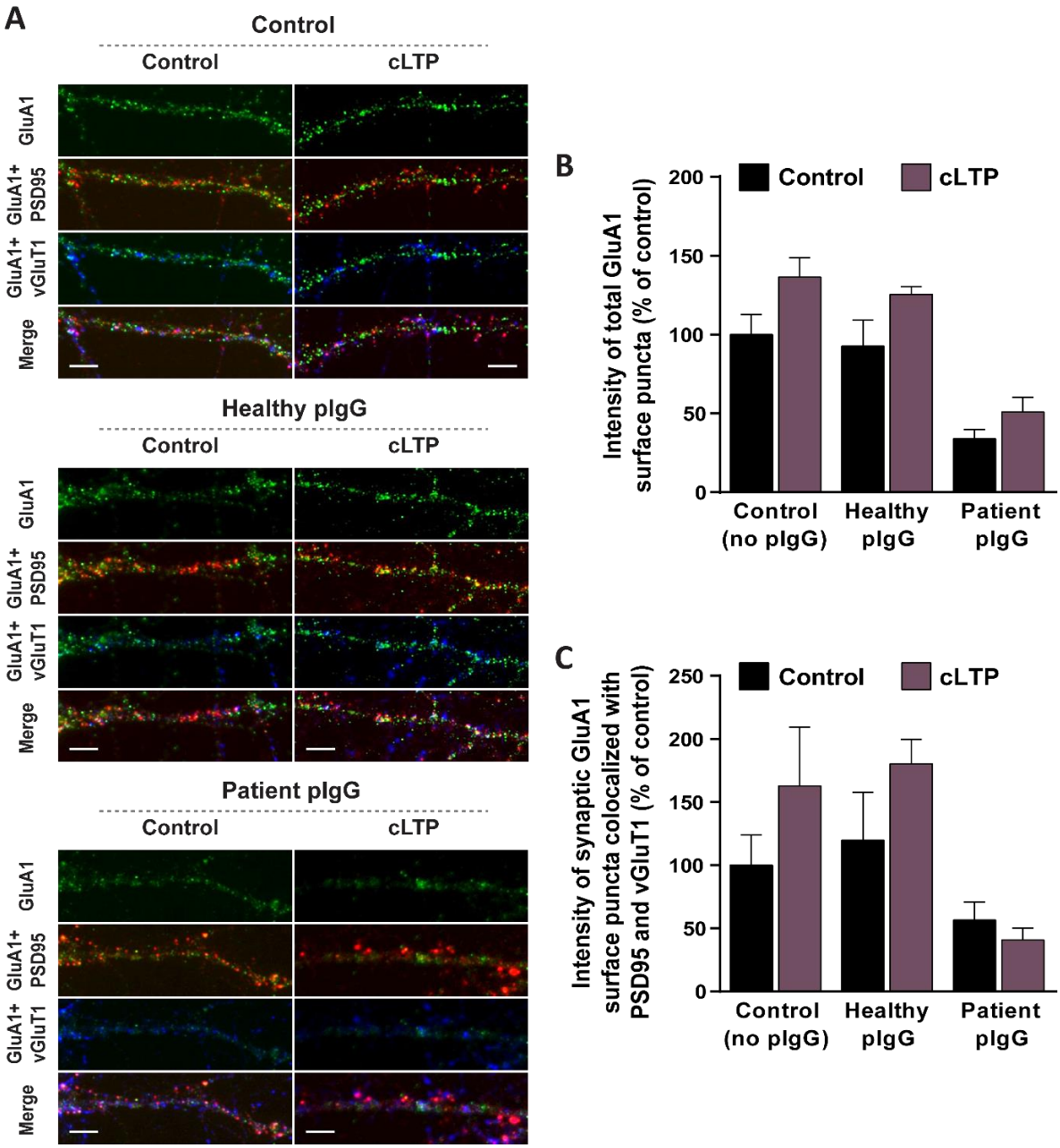


Figure 5.5 – CASPR2 autoantibodies disrupt cLTP-mediated trafficking and synaptic incorporation of cell surface AMPARs. 13 DIV low-density cultured cortical neurons were incubated for 7 h with 200 ηg/mL of either healthy- or patient human pIgGs. A cLTP protocol was then induced as follows: cells were pre-incubated for 15 min in extracellular solution (ECS) containing Mg^{2+}, followed by cLTP induction with 200 µM glycine for 5 min in the absence of Mg^{2+}. Neurons were then recovered at 37°C for 20 min (without glycine) to allow AMPAR incorporation. Cells were fixed and immunolabeled with a specific antibody against an extracellular epitope in GluA1 N-terminus, permeabilized and then stained for the synaptic markers PSD95 and vGluT1. **(A)** Representative immunofluorescence images of both control and cLTP cortical neurons incubated for 7 h with either healthy or patient-purified human IgGs, and stained for superficial GluA1, PSD95 and vGluT1. *Scale bars = 5µm.* **(B, C)** The fluorescence intensity of total **(B)** and synaptic **(C -** colocalized with PSD95 and vGluT1**)** clusters of surface GluA1 was quantified. Total number of analysed cells was obtained from a single independent experiment (N=1, n=10 cells). Results are presented as mean ± S.E.M.

Patient-purified human immunoglobulins at the cell surface undergo time-dependent internalization.

So far in this chapter we have been able to demonstrate that CASPR2-Abs present in the plasma of a patient with autoimmune encephalitis bind to the cell surface of cortical neurons to alter the synaptic distribution of Caspr2 and perturb its function in the regulation of AMPARs. Indeed, we observed that incubation of cortical neurons with human IgGs from the patient disrupts the trafficking and synaptic content of cell surface GluA1-containing AMPARs and perturbs the regulation of glutamatergic synaptic transmission *in vivo* in the mouse visual cortex. Importantly, the CASPR2-Ab-mediated perturbation of AMPAR trafficking and function that we reveal here is strikingly similar to the findings observed upon shRNA-mediated loss of Caspr2 expression, presented in the previous chapter. This parallelism confirms that the pathogenic effect of CASPR2-Abs is most likely mediated by a loss of function of Caspr2. Interestingly, we observed that, whilst homeostatic synaptic scaling mechanisms (shown to be regulated by Caspr2 in previous chapter) remain intact in the presence of CASPR2-Abs, preliminary evidence shows alterations in the synaptic incorporation of AMPARs following cLTP induction, giving a first hint that mechanisms of LTP might be perturbed in the presence of CASPR2-Abs. Overall, our findings provide seminal understanding of the potential mechanism of pathogenesis induced by CASPR2-Abs, and identify the glutamatergic system as a likely target for disease pathophysiology. Nevertheless, our findings still fall short on explaining how the pathogenic mechanism is triggered and how CASPR2-Abs manage to disrupt Caspr2 and its underlying functions.

One important consideration to take into account regarding the potential mechanism of pathogenesis of autoantibodies is their IgG subtyping. Immunoglobulins typically occur in four subclasses, IgG1 to IgG4, being IgG1 the most predominant and IgG4 the rarest. Importantly, their mode of action differs significantly [reviewed in (Vidarsson *et al.*, 2014)]. Antibodies of the classical IgG1 subtype can fix the complement and crosslink their target antigens, promoting their internalization [reviewed in (Crisp *et al.*, 2016; Jain & Balice-Gordon, 2016; Leypoldt *et al.*, 2015; Vidarsson *et al.*, 2014)]. Such mechanism of pathogenesis has already been proposed for NMDAR- (Hughes *et al.*, 2010; Moscato *et al.*, 2014) and AMPAR- (Lai *et al.*, 2009; Peng *et al.*, 2015) autoantibodies as they are predominantly of the IgG1 subclass. On the other hand, IgG4 antibodies cannot activate complement, and do not bind Fc receptors on effector cells, indicating that they most likely mediate a monovalent disruption of the target antigen function, rather than promoting its internalization [reviewed in (Bastiaansen *et al.*, 2017; Crisp *et al.*, 2016; van Sonderen *et al.*, 2017; Vidarsson *et al.*, 2014)]. The pathogenesis

of LGI1 autoantibodies, which are predominantly IgG4, has recently been proposed to occur in such manner (Ohkawa *et al.*, 2013).

In several recent case series of patients with CASPR2-encephalitis a subtyping analysis of IgG subclasses was performed, revealing that in a vast majority of patients, antibodies were of both IgG1 and IgG4 subclasses, with a higher preponderance of the latter (Bien *et al.*, 2017; Joubert *et al.*, 2016; van Sonderen *et al.*, 2016). In order to characterize the IgG subclasses of the CASPR2-Abs used in this study, we also subtyped the patient plasma and found a similar IgG specificity as described in other studies, although with a predominance of the IgG1 subclass (subtyping: IgG1≥IgG4>IgG2; Supplementary Figure 5.S6). Unlike the other types of encephalitis, such as the NMDAR- and LGI1-encephalitis, in which the characterization of the antibody IgGs is very well defined with a predominant subclass, this finding of a mixed contribution of CASPR2-Ab IgG subclasses with completely alternate modes of action, hampers the identification of a precise pathogenic mechanism triggered by CASPR2-Abs.

Despite this, the disruption of the synaptic distribution of Caspr2 induced by CASPR2-Abs, together with the perturbed trafficking and decreased synaptic content of cell surface AMPARs is suggestive of a potential internalization of Caspr2 and AMPARs upon CASPR2-Ab-binding, likely mediated by IgG1 antibodies. To explore this hypothesis, we performed a preliminary experiment to assess whether CASPR2-Abs can be themselves internalized over time. Low-density cultured cortical neurons were incubated during 1 or 7 h with either healthy or patient pIgGs, fixed and then immunolabelled with an excess concentration of an anti-human fluorophore-conjugated secondary antibody to stain human IgGs bound to the cell surface. After permeabilization, cells were immunolabelled with a second anti-human fluorophore-conjugated secondary antibody to stain potentially internalized human IgGs, and further stained against MAP2 to visualize dendritic processes (Figure 5.6A). Firstly, we found human IgG clusters at the cell surface of neurons incubated with the patient pIgGs (1h: 228.0 ± 28.1%; 7h: 203.7 ± 23.5%), which were absent in control cells or healthy IgG-incubated neurons (1h: 120.1 ± 19.7%; 7h: 118.3 ± 14.0%), confirming once again that only the patient IgGs bind to antigen epitopes expressed at the cell surface (Figure 5.6B). Moreover, in neurons incubated with the patient pIgGs, we observed a slight decrease in the fluorescence intensity of IgG clusters after a 7 h incubation (203.7 ± 23.5%), when compared to a shorter time-point (228.0 ± 28.1%), which might suggest a time-dependent internalization of CASPR2-Abs at the cell surface (Figure 5.6B).

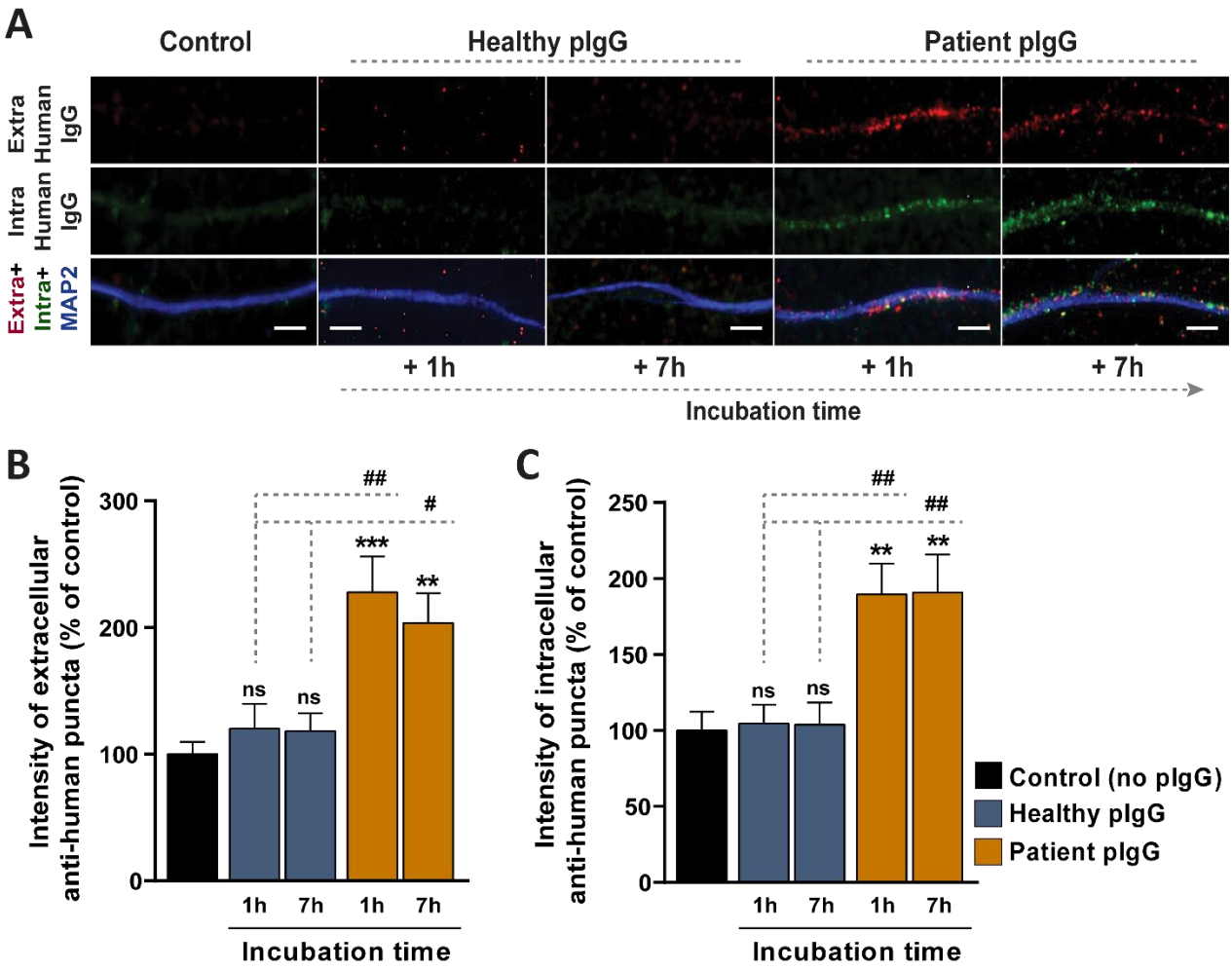


Figure 5.6 – CASPR2 autoantibodies bound to the cell surface undergo time-dependent internalization. 13 DIV low-density cultured cortical neurons were incubated with 200 ηg/mL of either healthy- or patient pIgGs for the duration of 1 or 7 h. Cells were fixed and immunolabelled with an excess concentration of anti-human fluorophore-tagged secondary antibody (red) to stain human IgGs bound to the cell surface. After permeabilization, neurons were again immunolabelled with a regular concentration of a second anti-human fluorophore-tagged (green) secondary antibody to evaluate if human IgGs underwent internalization. Cells were further stained against MAP2 for visualization of dendritic compartments. **(A)** Representative immunofluorescence images of neurons incubated for either 1 or 7 h with either healthy or patient-purified human IgGs, and stained for surface (red) or internalized (green) human IgGs, and for MAP2. *Scale bars = 5μm.* **(B, C)** The fluorescence intensity of extracellular/surface **(B)** and intracellular/internalized **(C)** clusters of human IgGs was quantified. Total number of analysed cells was obtained from three independent experiment (N=3, n≥30 cells). Results are presented as mean ± S.E.M., and statistical significance was determined by the non-parametric Kruskal-Wallis test, followed by Dunn's Multiple Comparison post-hoc test, **p<0.01, ***p<0.001 compared to control, #p<0.05, ##p<0.01 relative to healthy pIgGs.

Quantitative immunofluorescence analysis of intracellular human IgG clusters again revealed a significant increase in the fluorescence intensity of intracellular human IgG clusters in neurons incubated with the patient pIgGs (1h: 189.5 ± 20.4%; 7h: 190.8 ± 25.0%), when compared to control cells or neurons incubated with healthy IgGs (1h: 104.6 ± 12.3%; 7h: 103.7 ± 14.7%), confirming our hypothesis that CASPR2-Abs at the cell surface can be internalized (Figure 5.6C). When comparing different time-points of incubation with patient pIgGs, it is not clear whether CASPR2-Abs undergo a time-dependent internalization since the fluorescence intensity of intracellular patient IgG clusters at 7 h incubation does not increase significantly from 1 h-incubation (Figure 5.6C). Nevertheless, this is a preliminary finding and further experiments, with a time-point series of shorter and longer periods of incubation with the patient pIgGs, should be performed to clarify the possibility of a time-dependent internalization of CASPR2-Abs.

Overall, these results indicate that CASPR2-Abs bound to the cell surface can be internalized, which, together with our previous findings revealing an interaction between Caspr2 and the GluA1 AMPAR subunit, suggest that CASPR2-IgG1-Abs possibly crosslink Caspr2 and induce the internalization of Caspr2/AMPAR synaptic complexes, thus explaining their decreased synaptic content. However, because we characterized CASPR2-Abs to be of a mixed predominance of both IgG1 and IgG4 subclasses, we cannot disregard the possibility of a CASPR2-IgG4-Ab-mediated monovalent disruption of the interaction of Caspr2 with AMPARs. Importantly, this set of results starts hinting at the potential mode of pathogenesis mediated by CASPR2-Abs that underlies the disruption of Caspr2 and perturbed AMPAR trafficking, but also highlights that a thorough characterization of CASPR2-Ab IgG subtyping is fundamental for a full understanding of the mechanisms mediated by CASPR2-Abs, and how they can contribute for disease pathogenesis.

Discussion

In recent years, autoantibodies against CASPR2 have been identified in patients with autoimmune encephalitis that present with severe neurological syndromes, including neuromyotonia, Morvan's Syndrome and limbic encephalitis. Patients often manifest a clinical condition that overlaps the different syndromes, presenting with autonomic disturbances, peripheral nerve hyperexcitability and neuropathic pain, but also gross sleep disturbances, memory impairment and cognitive deficits and severe psychosis, thus reflecting a complex dysfunction of both the peripheral and central nervous systems underlying disease pathophysiology. However, despite intense clinical research, with an expanding number of case series being reported, definite proof for a pathogenic role of CASPR2-Abs is still missing, and the mechanisms underlying the patients' symptoms remain elusive.

In the previous chapter we uncovered an unsuspected and physiologically relevant role for Caspr2 in the regulation of AMPAR trafficking to synapses, and for proper glutamatergic synaptic transmission *in vivo*. Furthermore, we revealed a fundamental requirement for Caspr2 in the regulation of homeostatic synaptic scaling mechanisms that are relevant *in vivo* for visually-driven experience-dependent synaptic plasticity. In line with the clinical symptoms presented by patients, in this chapter we hypothesized that CASPR2 autoantibodies mediate disease pathogenesis by disrupting this novel role of Caspr2 in AMPAR regulation. Using serum and purified IgG samples from a patient with anti-CASPR2 encephalitis, we investigated the cellular, synaptic and functional effects of the patient CASPR2-Abs and found a decrease of Caspr2 and surface AMPAR levels in cortical synapses, ultimately leading to a remarkable perturbation of glutamatergic synaptic transmission *in vivo*. We herein propose the glutamatergic system as a likely target for pathogenesis ensuing from an antibody-mediated disruption of CASPR2 function, and correlate these findings with the cognitive and psychiatric nature of the symptoms presented by patients.

Disrupted AMPAR function and glutamatergic synaptic transmission upon autoantibody-mediated loss of CASPR2 function.

In collaboration with the John Radcliffe Hospital at the Oxford University, we had access to serum and purified IgGs from a patient with anti-CASPR2 autoimmune encephalitis. Initial experiments demonstrated that human IgGs present in the patient plasma bound strongly to the surface of cultured neurons (Figure 5.1; Suppl. Figure 5.S1, S2). We then confirmed that these IgGs specifically targeted Caspr2, but no other antigens: patient serum previously pre-absorbed in HEK cells expressing human CASPR2 did not bind to the surface of cultured

neurons (Suppl.Figure 5.S1), and human IgGs purified from the patient plasma failed to stain *Cntnap2* KO neurons (Figure 5.1A). With this in mind, we then investigated whether patient IgGs had any effect on their target antigen, and found a significant decrease in synaptic clusters of Caspr2 (Figure 5.1B). This finding is in agreement with evidence from other autoimmune encephalitides, in which specific autoantibodies have been shown to perturb, in some extent, their own synaptic target antigens: NMDAR-, AMPAR-, GABAR- and GlyR-Abs have all been shown to decrease the synaptic expression of their respective receptors, which, in some cases, are inclusively targeted for lysosomal-dependent degradation [reviewed in (Chefdeville *et al.*, 2016; Crisp *et al.*, 2016; Jain & Balice-Gordon, 2016)]. Although other studies have been performed to explore the cellular and molecular effects of CASPR2-Abs (Coutinho *et al.*, 2017b; Olsen *et al.*, 2015; Patterson *et al.*, 2017; Pinatel *et al.*, 2015), to our knowledge, this is the first report of a direct effect of CASPR2-Abs on endogenous Caspr2 levels. Importantly, these observations suggest a potential disruption of Caspr2 function upon antibody-binding. Accordingly, in the previous chapter we found a pivotal localization of Caspr2 at the PSD of excitatory synapses, where it fulfils a fundamental role in the regulation of AMPAR function and glutamatergic synaptic transmission. The de-localization of Caspr2 from the synapse upon CASPR2-Ab binding suggests that this synaptic role of Caspr2 is most likely disrupted.

Indeed, we found that CASPR2-Abs significantly decrease the cell surface expression of AMPARs in cortical synapses (Figure 5.2), and the amplitude of AMPAR-mediated mEPSCs when incubated *in vivo* in the mouse visual cortex (Figure 5.3), indicating a relevant pathophysiological impact of CASPR2-Abs in AMPAR trafficking and glutamatergic function *in vivo*. Importantly, these findings remarkably parallel what we observed in the previous chapter upon shRNA-mediated loss of Caspr2 endogenous expression, thus confirming that the effect of CASPR2-Abs in AMPAR trafficking and glutamatergic transmission is secondary to a CASPR2-Ab-mediated loss of Caspr2 function. Besides the obvious examples of NMDAR- and AMPAR-Abs that directly target their respective receptors and significantly decrease their synaptic expression and mediated currents (Hughes *et al.*, 2010; Peng *et al.*, 2015), antibodies against the VGKC complex-associated LGI1 protein were also reported to induce a secondary effect in the trafficking and synaptic expression of AMPARs (Ohkawa *et al.*, 2013). Interestingly, LGI1-Abs were shown to alter the interaction of LGI1 with ADAM22 and ADAM23 proteins (Ohkawa *et al.*, 2013), consequently disrupting a LGI1/ADAM trans-synaptic complex that is required for the synaptic stabilization of AMPARs and proper synaptic transmission (Fukata *et al.*, 2006; Fukata *et al.*, 2010). Curiously, a recent proteome study identified ADAM22 as a novel Caspr2 binding partner (Chen *et al.*, 2015), and both proteins are known to regulate the clustering of VGKCs (Poliak *et al.*, 1999; Poliak *et al.*, 2003; Schulte *et al.*, 2006;

Seagar *et al.*, 2017). Probably not coincidental, LGI1- and CASPR2-Abs were first identified, and are often reported together in several encephalitis patients previously diagnosed with anti-VGKC autoimmune encephalitis [reviewed in (Bastiaansen *et al.*, 2017; Binks *et al.*, 2017; van Sonderen *et al.*, 2017)]. Clearly, one cannot disregard the remarkable parallelism between LGI1 and Caspr2 molecular mechanisms, nor the consequent effects resulting from an antibody-mediated disruption of their function, which suggests that the mechanisms of pathogenesis triggered by each type of autoantibody should be similar, which warrants further investigation.

Overall, our findings provide definite proof for a dysregulation of AMPARs and glutamatergic synaptic transmission underlying the pathogenic mechanisms triggered by a CASPR2-Ab-mediated disruption of Caspr2 function. Nevertheless, we cannot disregard the possibility of CASPR2-Abs also impairing other known functions of Caspr2. Like in other types of autoimmune encephalitis, the full-spectrum of synaptic and functional phenotypes induced by CASPR2-Abs seem to closely mimic those induced by genetic disruption of Caspr2, including by disease-related mutations in the human *CNTNAP2* gene [reviewed in (Penagarikano & Geschwind, 2012; Poot, 2017)]. Indeed, recent studies have shown that CASPR2-Abs can promote defects in cortical thickness and layer patterning, loss of GABAergic interneurons and inhibitory synapses, as well as abnormal dendritic complexity and spine density (Brimberg *et al.*, 2016; Coutinho *et al.*, 2017b; Pinatel *et al.*, 2015). Interestingly, one of the studies reports a decrease in the density of GluA1-AMPAR clusters and in the number of glutamatergic synapses, although this is most likely due to an antibody-mediated complement-induced activation of microglia that results in synaptic pruning (Coutinho *et al.*, 2017b). In our experiments, incubation with patient IgGs for 7 h (or 48 h – data not shown) either *in vitro* or *in vivo* did not induce any observable changes in PSD95 clusters (Suppl. Figure 5.S4), or a decrease in the frequency of AMPAR-mediated mEPSCs (Suppl. Table), indicating that the number of excitatory synapses remained intact. Importantly, the studies referred above use chronic maternal-to-foetal antibody transfer models to evaluate effects of CASPR2-Abs in the progeny that most likely are occurring during development. Given the acute nature and late-onset age of CASPR2 autoimmune encephalitis, it is unlikely that developmental functions of CASPR2 contribute significantly to disease pathogenesis.

CASPR2-Ab-mediated perturbation of synaptic plasticity events

In the previous chapter we discovered that Caspr2 not only regulates the basal trafficking of AMPARs, but that it is necessary for activity-dependent mechanisms of AMPAR regulation in the context of homeostatic synaptic plasticity. Furthermore, we found this unsuspected role of Caspr2 to be relevant *in vivo* and fundamental for the expression of visually-driven experience-dependent synaptic plasticity (please refer to Chapter IV). Importantly, this form of plasticity is crucial for maintaining the balance of a wide-range of brain processes required for an adequate brain function [reviewed in (Fernandes & Carvalho, 2016; Turrigiano, 2008; Whitt *et al.*, 2014)]. Understandably, emerging evidence now suggests that compromised neuronal synaptic homeostasis, resulting most likely from altered molecular players required for the regulation of its underlying mechanisms, can contribute to the pathophysiology of several cognitive and neuropsychiatric disorders, including ASD, ID and SCZ in which CASPR2 has already been thoroughly implicated [reviewed in (Nelson & Valakh, 2015; Ramocki & Zoghbi, 2008; Wondolowski & Dickman, 2013)].

In line with the cognitive and psychiatric symptoms typically presented by patients with CASPR2 autoimmune encephalitis, including sleep disturbances, altered sensory perception and visual and auditory hallucinations, processes in which homeostatic plasticity has been implicated, we decided to investigate whether CASPR2-Abs would impinge on the role Caspr2 plays on this form of plasticity. Surprisingly, we observed no changes in the synaptic scaling of cell surface AMPARs following prolonged inhibition of neuronal activity in cortical neurons incubated with the patient pIgGs (Figure 5.4), indicating that homeostatic plasticity mechanisms remain intact in the presence of CASPR2-Abs. Curiously, a couple of studies exploring the synaptic and cellular effects of NMDAR- and AMPAR-Abs suggest that homeostatic synaptic plasticity can be triggered as a secondary mechanism to cope with the decrease in NMDAR- or AMPAR surface expression and synaptic currents. NMDAR- and AMPAR-Abs induce an exacerbated compensatory decrease in inhibitory currents and inhibitory synapse number, as well as an upregulation of the intrinsic neuronal excitability, which, altogether can contribute to the pathophysiology of the disease and explain seizure-like episodes in these encephalitis patients (Moscato *et al.*, 2014; Peng *et al.*, 2015). However, given our findings showing that CASPR2-Abs at the neuronal cell surface are internalized after just 1 h of incubation, potentially in a time-dependent manner, it is likely that after 48 h (time-scale used in the experiments to evaluate synaptic scaling) of incubation, most IgGs have been internalized and cells have managed to re-establish the cell surface trafficking of Caspr2 and AMPARs, thus allowing the expression of synaptic scaling mechanisms.

Conversely, preliminary experiments showed that CASPR2-Abs prevent the increase of cell surface AMPARs upon induction of a chemical LTP protocol (Figure 5.5), indicating that CASPR2-Abs perturb the trafficking and synaptic incorporation of AMPARs induced by NMDAR-dependent LTP instead. Due to their associative nature that reinforces precise synaptic connections, Hebbian forms of synaptic plasticity such as LTP are considered to be the cellular correlates of learning and memory processes. Hence, compromised mechanisms of LTP would help explain the severe memory complaints and cognitive impairments presented by patients with CASPR2 autoantibodies. In agreement, a couple of recent studies have elegantly demonstrated that LTP mechanisms are severely impaired in consequence of NMDAR autoantibody-mediated pathogenic disruption of the surface diffusion and synaptic retention of GluN2A- and GluN2B-NMDAR subunits (Dupuis *et al.*, 2014; Mikasova *et al.*, 2012).

Although preliminary, our findings of a potential parallel role for Caspr2 in the regulation of LTP are not unexpected. In agreement, several of the molecular players required for the regulation of homeostatic synaptic scaling had previously been implicated in Hebbian forms of plasticity, including Stargazin, PICK1 and GRIP1, type I mGluRs, BDNF and Arc, among others [reviewed in (Fernandes & Carvalho, 2016; Vitureira & Goda, 2013)]. Our findings simply further argue in favour of the elegant interplay between Hebbian and homeostatic plasticity in sharing common mechanisms that elicit common synaptic effects in response to completely opposing patterns of neuronal activity. Future experiments will have to be done to confirm the preliminary observation of a pathogenic effect of CASPR2-Abs in LTP induction, but also to establish that this effect occurs from a direct disruption of a Caspr2-mediated regulation of LTP, which we have not evaluated so far. If holding true, though, it will be interesting to understand in the future how (and when) Caspr2 can be recruited to elicit one type of plasticity or the other. Furthermore, if Caspr2 is confirmed to be required for both modalities of synaptic plasticity, it will be important to understand why CASPR2-Abs perturb LTP but not synaptic scaling. One likely possibility is that loss of function of Caspr2 has a more deleterious effect in LTP mechanisms than loss of expression of the protein itself (which we still have to test), and most likely, homeostatic synaptic scaling mechanisms can more easily cope with an altered function of the protein, thus only being disrupted in the absence of Caspr2.

What are the underlying mechanisms behind the pathogenic effect of CASPR2-autoantibodies in CASPR2 and AMPAR function?

Although we have been able to pinpoint the pathogenic effects of CASPR2-Abs to a disruption of Caspr2 function and consequent perturbation of AMPAR trafficking, basal glutamatergic synaptic transmission and synaptic plasticity phenomena, the pathogenic mechanisms underlying these effects of CASPR2-Abs still remain elusive. One important consideration regarding the potential mechanism of pathogenesis of autoantibodies is their IgG subtyping, which can occur in four subclasses, from the most common IgG1 to the rarest IgG4, and mediate their effects through significantly different modes of action [reviewed in (Vidarsson *et al.*, 2014)]. In several recent case series of patients with CASPR2-encephalitis a subtyping analysis of IgG subclasses was performed, revealing that in a vast majority of patients, antibodies were of both IgG1 and IgG4 subclasses, with a predominance of IgG4 (Bien *et al.*, 2017; Joubert *et al.*, 2016; van Sonderen *et al.*, 2016). Subtyping analysis of the CASPR2-Abs used in this study revealed a similar mixed contribution of both IgG1 and IgG4, although with a slight predominance of the IgG1 subclass (Suppl. Figure 5.S6).

Antibodies of the classical IgG1 subtype can activate the complement and, because they bind their target antigens in a divalent manner, they can crosslink them and promote their internalization. Such mechanism of pathogenesis has already been proposed for most autoantibodies identified in different encephalitides, since their IgGs are predominantly of the IgG1 subclass [reviewed in (Crisp *et al.*, 2016; Jain & Balice-Gordon, 2016; Leypoldt *et al.*, 2015; Vidarsson *et al.*, 2014)], and experimentally confirmed for NMDAR-Abs in a very elegant experiment: whilst intact patient NMDAR-Abs induce a significant decrease in the surface expression of NMDARs, the enzymatic digestion of the patient IgG Fc domains to generate Fab fragments (which bind in monovalent manner) blocks NMDAR crosslinking and subsequent internalization (Hughes *et al.*, 2010). Although IgG1 antibodies have been frequently found in serum samples of CASPR2-Ab encephalitis patients, no study has yet explored whether they are relevant for disease pathogenesis. Since we observed a significant decrease in the synaptic distribution of Caspr2 and surface AMPARs induced by CASPR2-Abs, and a predominance of the IgG1 subclass, we hypothesized that their pathogenic mechanism could be similar to that of NMDAR-Abs. Accordingly, in a preliminary experiment we found that CASPR2-Abs bound to the surface of cortical neurons undergo internalization (Figure 5.6). Moreover, human IgG clusters at the cell surface seem to decrease over the time of incubation, which hints that CASPR2-Abs undergo internalization in a time-dependent manner. Further experiments, with a time-point series of shorter and longer periods of incubation with the patient pIgGs, should be performed to clarify this possibility. Differing time-

dependent dynamics of internalization have been proposed for both NMDAR- and AMPAR-Abs and their respective target receptors. Whilst surface NMDARs undergo continuous time-dependent internalization throughout a period of 48 h (Moscato *et al.*, 2014), AMPAR-Abs reach a fast peak of internalization at 4 h and then undergo lysosomal-mediated degradation (Peng *et al.*, 2015). Importantly, our findings suggest that the potential mechanism of CASPR2-Abs for induction of pathogenesis is through crosslinking and internalization of Caspr2. Taking into consideration the interaction between Caspr2 and the GluA1 AMPAR subunit, it is likely that surface AMPARs are also internalized from the synapse in complex with Caspr2, thus explaining their decreased synaptic levels. Curiously, when incubating hippocampal neurons with a higher concentration of patient IgGs, we observed a striking contradictory effect in the synaptic accumulation of cell surface AMPARs, which increased significantly just after 2 h of incubation (Suppl. Figure 5.S5). It is possible that higher IgG concentrations induce a bulk of crosslinked Caspr2/AMPAR synaptic complexes that simply hinder the surface dynamics and constitutive endocytic pathways of AMPARs, resulting in a massive synaptic accumulation. Future experiments using varying titres of CASPR2-antibodies should be undertaken to tackle this question.

CASPR2-Abs, including the ones used in this study, are also of the IgG4 subclass, which functions in a completely different mode of action than that of IgG1 subtype. IgG4 antibodies are heterobispecific due to a constant exchange of Fab arms, indicating that they do not bind Fc receptors on effector cells, and most likely mediate a monovalent disruption of the target antigen function, rather than promoting its internalization [reviewed in (Bastiaansen *et al.*, 2017; Crisp *et al.*, 2016; van Sonderen *et al.*, 2017; Vidarsson *et al.*, 2014)]. Interestingly, a recent study has reported that CASPR2-Abs, predominantly of the IgG4 subclass, perturb the interaction of Caspr2 with its extracellular binding partner Contactin-2 (Patterson *et al.*, 2017). Taking this into consideration, it is possible that CASPR2-Abs disrupt the interaction of Caspr2 with AMPARs, which could promote a destabilization of AMPARs at the synapse and consequent internalization. The pathogenesis of LGI1 autoantibodies, which are almost exclusively of the IgG4 type, has recently been proposed to occur in such manner: LGI1-IgG4-Abs directly disrupt the interaction between LGI1 and ADAM22 (curiously, ADAM22 also interacts with Caspr2), which is required for the stabilization of AMPARs at the synapse, and significantly decrease the synaptic expression of surface AMPARs (Ohkawa *et al.*, 2013).

Another aspect that most likely will influence the pathogenic mechanisms triggered by CASPR2-Abs is the modular multi-domain extracellular structure of CASPR2. CASPR2 is a single-pass transmembrane protein whose overall extracellular structure resembles that of neurexins: it contains a signal peptide domain right next to the N-terminal, a discoidin I

homology domain, several laminin G neurexin-like motifs, two epidermal growth factor-like repeats and a fibrinogen-like region (Poliak *et al.*, 1999). Importantly, a couple of studies have recently reported that serum from different patients recognize different epitope domains of Caspr2, although most patient IgGs, particularly of the IgG4 subtype, seem to require the discoidin I and the first Laminin G domains of Caspr2 for recognition and binding (Olsen *et al.*, 2015; Pinatel *et al.*, 2015). Interestingly, a homozygous (exon 2-9 deletion) mutation in the *CNTNAP2* gene, recurrently implicated in patients presenting with severe ID, epilepsy, language impairments and autistic features, originates a truncated form of Caspr2 that retains its membrane-anchoring structure but lacks 2 N-terminal Laminin G and the discoidin I domains (Zweier *et al.*, 2009). Overall, given the modular structure of Caspr2 with multiple domains involved in different functions, it is likely that antibody-mediated targeting of different epitopes will result in varying outcomes for Caspr2 function, and consequently for disease pathogenesis.

Other types of encephalitis, such as the NMDAR- and LGI1-encephalitis, have a very well-defined characterization of antibody IgG subclasses and target antigen epitopes, which renders the identification of precise pathogenic mechanisms relatively easy. That is definitely not the case for CASPR2-Abs: they present a mixed contribution of IgG subclasses with alternate modes of action; patient sera can target different antigen epitopes; and varying Ab titres elicit opposite pathogenic effects. These findings hamper the identification of a precise pathogenic mechanism triggered by CASPR2-Abs, but do explain the wide phenotypic variability presented by patients. Most importantly, they warrant a more thorough characterization of each patient individually. The establishment of a potential correlation between antibody titres, IgG subclasses and antigen targets with specific phenotypic clusters would enable the development of more specific and efficient approaches of treatment.

How do the proposed pathogenic mechanisms correlate with the pathophysiology of CASPR2 autoimmune encephalitis, and of other *CNTNAP2*-related neuropsychiatric disorders?

Patients with CASPR2 autoantibodies typically present a clinical phenotype that overlaps the pathophysiology of different neurological syndromes that include neuromyotonia, Morvan's Syndrome and limbic encephalitis. The overarching symptoms manifest a complex dysfunction of the peripheral and central nervous systems and can include autonomic disturbances, peripheral nerve hyperexcitability, neuropathic pain, gross sleep disturbances, severe memory complaints, cognitive deficits and severe psychosis, often with hallucinations. In the present study, we found that CASPR2-Abs alter the synaptic distribution of Caspr2, induce aberrations

in AMPAR trafficking and function and impair synaptic plasticity phenomena. Certainly, other CASPR2 functions, namely its role in VGKC clustering, contribute to the pathophysiology of peripheral symptoms presented by patients. However, the synaptic, cellular and functional phenotypes we found in consequence of CASPR2-Ab-binding to Caspr2 are likely to underlie disease pathogenesis, and sustain the central symptoms of memory complaints, cognitive deficits and psychotic features presented by patients.

Importantly, identification of Caspr2-dependent mechanisms impaired in consequence of CASPR2-Abs may also provide invaluable insights into the understanding of the pathogenic effects resulting from variations in the *CNTNAP2* gene that have been recurrently implicated in several neuropsychiatric disorders such as ASD, ID and SCZ. Indeed, the psychiatric symptoms of CASPR2-Ab encephalitis patients substantially overlap with those of neuropsychiatric patients carrying *CNTNAP2* mutations [reviewed in (Penagarikano & Geschwind, 2012; Poot, 2017; Rodenas-Cuadrado *et al.*, 2014)], and with behavioural phenotypes presented by *Cntnap2* KO animal models (Hoffman *et al.*, 2016; Penagarikano *et al.*, 2011). Interestingly, recent maternal-to-foetal CASPR2-antibody transfer models reveal that offspring mice exposed *in utero* to CASPR2-Abs develop synaptic, cellular and behavioural abnormalities that relate to *CNTNAP2*-associated neuropsychiatric phenotypes, thus not only confirming the pathogenicity of CASPR2-Abs, but also hinting that gestational transfer of maternal CASPR2-Abs to the foetus could likely contribute to the development of neuropsychiatric disorders in the progeny. In agreement, circulating CASPR2-Abs were recently detected during pregnancy in mothers of children with autism-spectrum disorders (Brimberg *et al.*, 2016) or intellectual disability (Coutinho *et al.*, 2017a). The identification of the molecular underpinnings of CASPR2-Ab-mediated pathogenesis might additionally foster better diagnosis and the development of more efficient therapy approaches in cases of idiopathic psychosis, first-episode schizophrenia, postpartum illness or even Creutzfeldt-Jakob Disease, to which CASPR2-Abs (and others) have been associated, although without indication of pathogenesis [reviewed in (Coutinho *et al.*, 2014; Pruss & Lennox, 2016)].

Conclusions

In this chapter, we aimed at studying the potential synaptic, cellular and functional pathogenic phenotypes induced by CASPR2-Abs commonly found in psychiatric patients with autoimmune synaptic encephalitis. We discovered that CASPR2-Abs trigger a direct pathogenic mechanism that disrupts the synaptic function we previously found for CASPR2 in the regulation of AMPARs. CASPR2-Abs significantly altered the synaptic content of Caspr2 and cell surface AMPARs, indicating severe aberrations in AMPAR trafficking that ultimately

compromise proper glutamatergic synaptic transmission and synaptic plasticity phenomena. Importantly, our findings underscore the glutamatergic system as a potential target for dysfunction mediated by CASPR2-Abs, and propose that it contributes to the pathophysiology of CASPR2-Ab encephalitis, likely underlying the severe cognitive and psychiatric symptoms presented by patients. Furthermore, understanding the pathogenic mechanisms triggered by CASPR2-Abs will provide better knowledge on the full spectrum of cellular and molecular functions played by CASPR2, and how they can be perturbed by mutations in the *CNTNAP2* gene, implicated in genic neuropsychiatric disorders such as ASD, ID, and SCZ.

Supplementary Figures

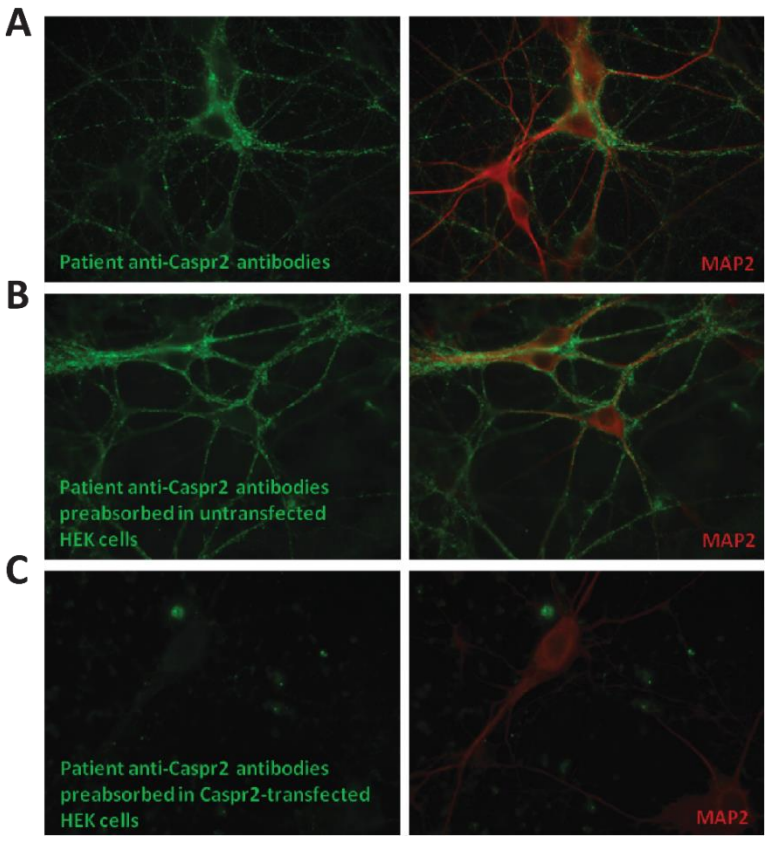


Figure 5.S1 – CASPR2 autoantibodies are present in the plasma of a patient with autoimmune synaptic encephalitis. (A - C) Representative immunofluorescence images of 14-18 DIV hippocampal neurons incubated for 1 h with the plasma of a patient with anti-CASPR2 encephalitis and stained for surface human immunoglobulins (IgGs; green) and the neuronal marker MAP2 (red). **(A)** Staining of hippocampal neurons incubated for 1 h with normal serum of a patient with CASPR2-encephalitis shows that human IgGs in the patient plasma bind strongly to the surface of neurons. **(B, C)** To confirm the specificity of patient IgGs for CASPR2, and test for the presence of autoantibodies against other neuronal protein targets, patient plasma was pre-absorbed either in **(B)** untransfected HEK293 cells, or **(C)** HEK293 cells expressing human CASPR2, collected and re-incubated for 1 h in 14-18 DIV hippocampal neurons. Plasma pre-absorbed in CASPR2-expressing cells fails to stain hippocampal neurons. Images in this panel were kindly provided by Ester Coutinho (Oxford University, UK).

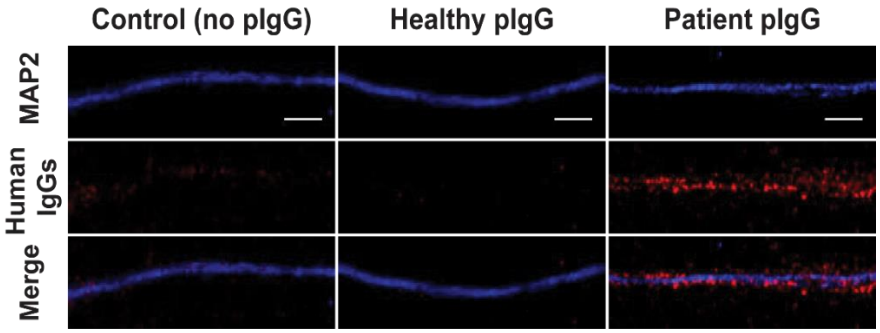


Figure 5.S2 – CASPR2 autoantibodies bind to the surface of neuronal dendritic compartments. Representative immunofluorescence images of 13 DIV low-density cultured cortical neurons incubated for 7 h with 200 ηg/mL of human IgGs purified from either a sex- and aged-matched healthy control (healthy pIgG) or from a patient with anti-CASPR2 autoimmune encephalitis (patient pIgG), and immunolabeled for surface human IgGs and for the dendritic neuronal marker MAP2. Staining for human IgGs bound to the surface of cortical neurons is only visible with patient pIgGs. *Scale bars = 5 µm*.

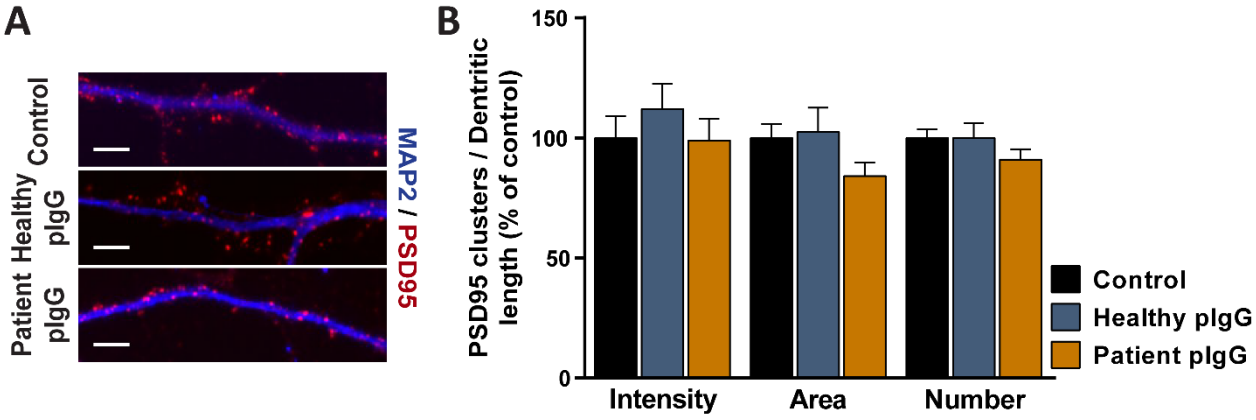


Figure 5.S3 – CASPR2 autoantibodies do not affect the development or maintenance of excitatory synapses. (A, B) 13 DIV low-density cultured cortical neurons were incubated for 7 h with 200 ηg/mL of human IgGs purified from the plasma of either the CASPR2-encephalitis patient or a healthy subject. Cells were then fixed and stained for the postsynaptic marker PSD95 and the dendritic neuronal marker MAP2. **(A)** Representative immunofluorescence images of cultured cortical neurons incubated for 7 h with either healthy or patient-purified human IgGs, and stained for PSD95 and MAP2. *Scale bars = 5µm.* **(B)** The fluorescence intensity, area and number of PSD95 clusters was quantified. Total number of analysed cells was obtained from three independent experiments. (N=3, n≥ 30cells).

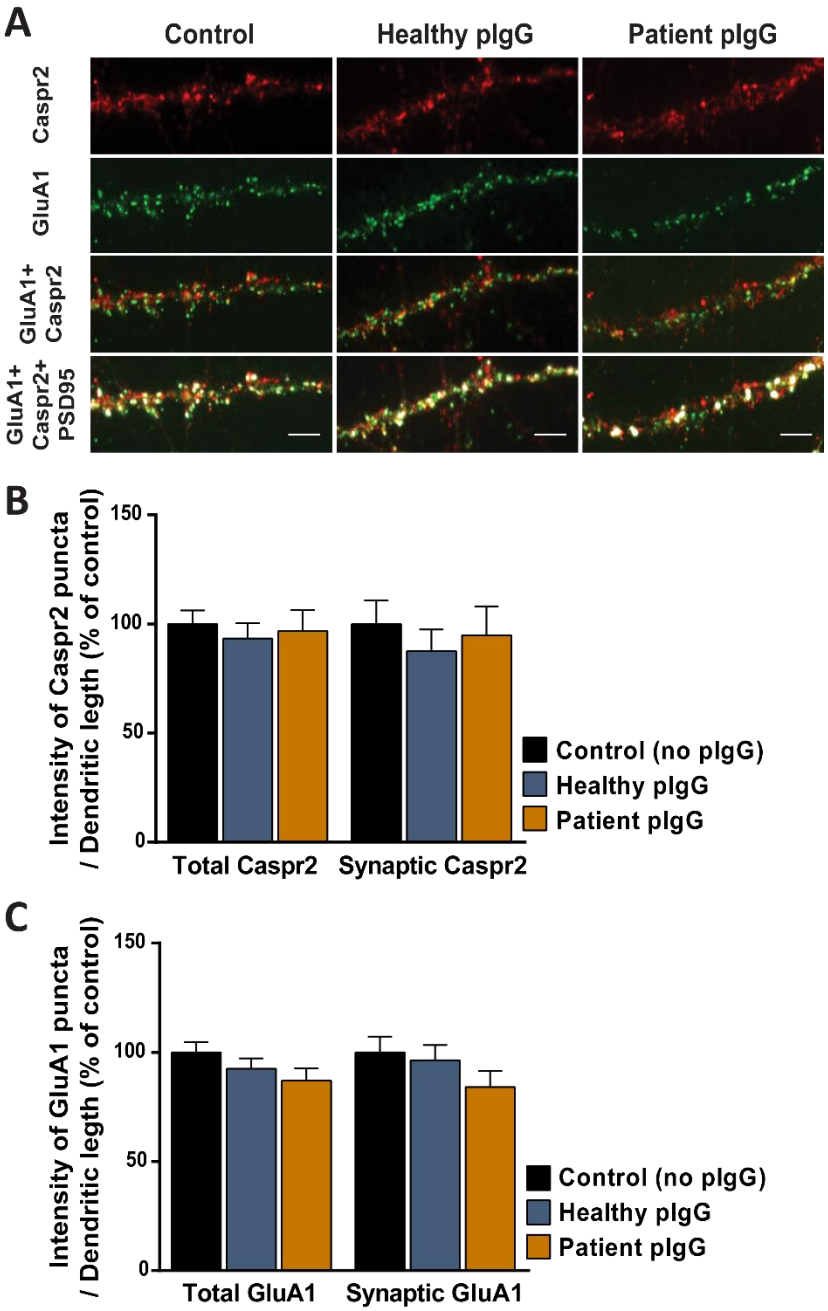


Figure 5.S4 – CASPR2 autoantibodies have no effects in the dendritic and synaptic distribution of Caspr2 nor do they alter the trafficking and synaptic content of cell surface AMPARs. **(A - C)** 15 DIV low-density cultured hippocampal neurons were incubated for 7 h with 200 ηg/mL of human IgGs purified from the plasma of either the CASPR2-encephalitis patient or a healthy subject. Cells were then fixed and immunolabeled with an antibody against an extracellular epitope in the GluA1 N-terminus for staining of surface GluA1-containing AMPARs. After permeabilization, cells were stained for Caspr2 and the synaptic marker PSD95. **(A)** Representative immunofluorescence images of hippocampal neurons incubated for 7 h with either healthy or patient-purified human IgGs, and stained for superficial GluA1, Caspr2 and PSD95. *Scale bars = 5μm.* **(B, C)** The fluorescence intensity of total and PSD95-colocalized synaptic clusters of both Caspr2 **(B)** and cell surface GluA1 **(C)** was quantified. Total number of analysed cells was obtained from at least three independent experiments. (N≥3, n≥ 30cells). Results are presented as means ± S.E.M.

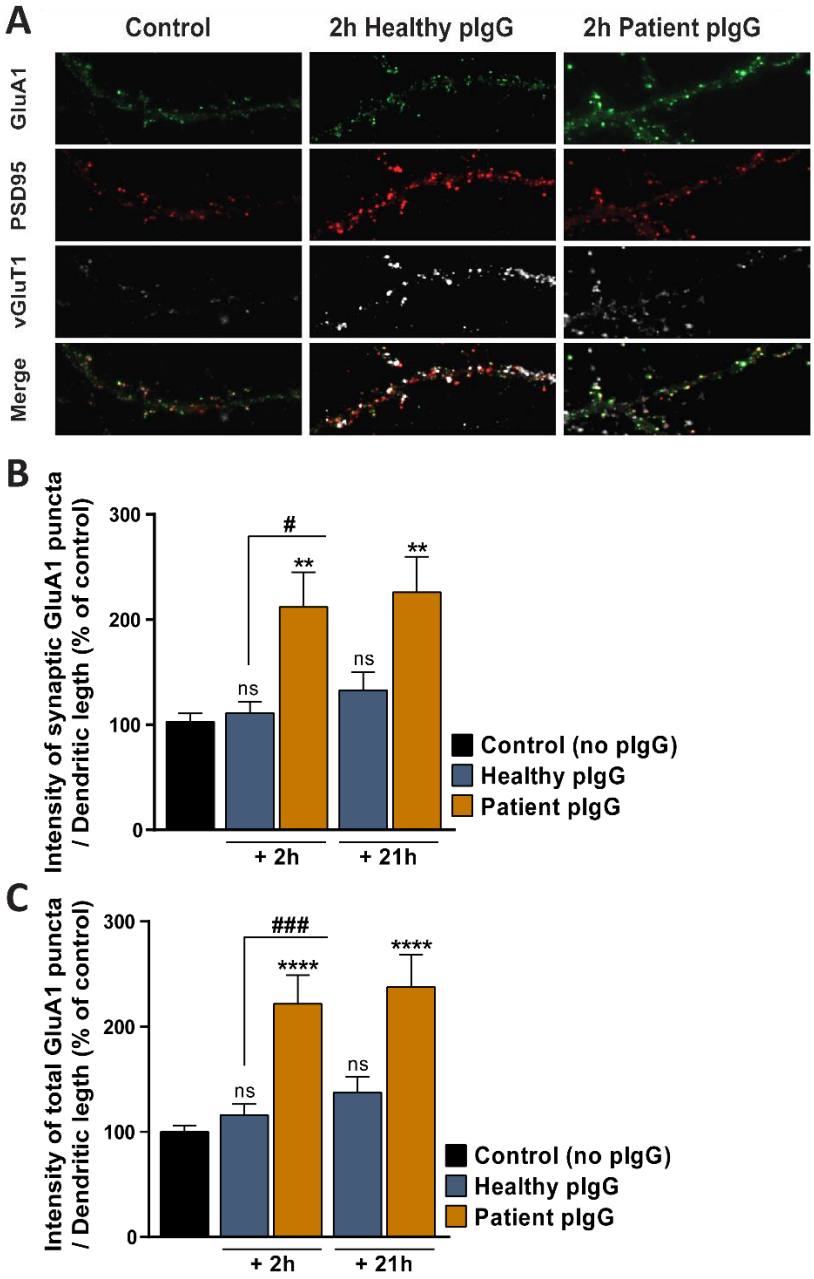


Figure 5.S5 – Higher concentration of CASPR2 autoantibodies significantly increases the trafficking and synaptic content of cell surface AMPARs. (A) 14-15 DIV low-density cultured hippocampal neurons were incubated for either 2 or 21 h with 50 µg/mL of human IgGs purified from the plasma of either the CASPR2-encephalitis patient or a healthy subject. Cells were then fixed and immunolabeled with an antibody against an extracellular epitope in the GluA1 N-terminus for staining of surface GluA1-containing AMPARs. After permeabilization, cells were stained for the postsynaptic marker PSD95 and the presynaptic marker vGluT1 for identification of excitatory synapses. **(B, C)** The fluorescence intensity of total **(B)** and synaptic **(C)** clusters (co-localized with both PSD95 and vGluT1) of cell surface GluA1 was quantified. Total number of analysed cells was obtained from at least two independent experiments. (2 h: N≥3, n≥ 30cells; 21 h: N=2, n≥ 20cells). Results are presented as means ± S.E.M. and the statistical significance was determined by the non-parametric Kruskal-Wallis test, followed by Dunn's Multiple Comparison post-hoc test, **p<0.01, ****p<0.0001 compared to control, #p<0.05, ###p<0.001 relative to healthy pIgGs.

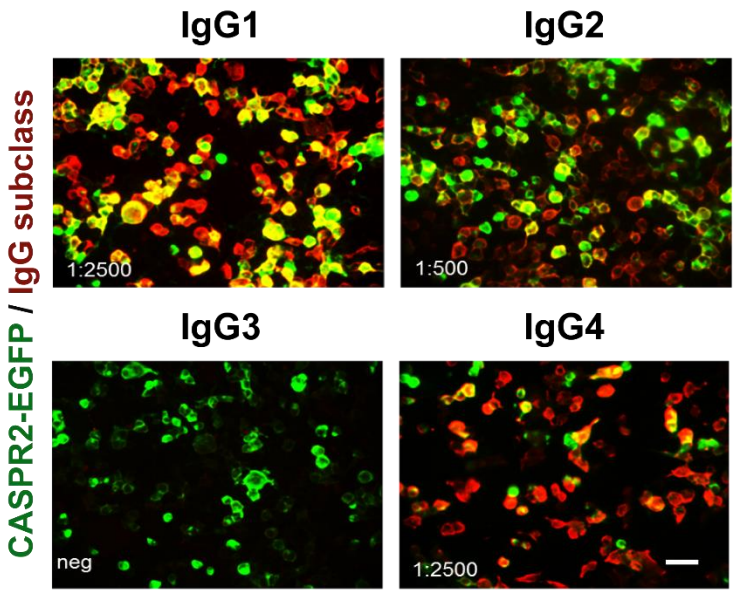


Figure 5.S6 – Subtyping of CASPR2 autoantibody IgG subclasses present in the plasma of a patient with CASPR2 autoimmune encephalitis. Representative immunofluorescence images of CASPR2-EGFP-transfected HEK293 cells incubated for 1 h with the patient plasma. Cells were then fixed and immunolabelled with a non-tagged mouse anti-human IgG antibody specific for each IgG subclass, followed by an appropriate anti-mouse fluorophore-conjugated secondary antibody. End-point CBA titrations for each subclass is given in the lower left corner of each panel. Images in this panel were kindly provided by Ester Coutinho (Oxford University, UK).

Supplementary Tables

Table 5.S1 - Comparison of mEPSCs and neuronal parameters across experimental groups of mice injected with purified human IgGs from healthy subjects or patients with CASPR2 encephalitis.

Experimental Group	Amplitude (pA)	Frequency (Hz)	Rise time (ms)	Decay (τ, ms)	RMS Noise	Series R (MΩ)	Input R (MΩ)
WT	14.3±0.84	7.0±1.16	1.27±0.04	3.50±0.18	1.8±0.05	20.5±0.9	225.4±13.7
Healthy pIgGs	14.9±0.83	5.2±1.26	1.14±0.04	3.28±0.16	1.9±0.05	17.0±1.1	308.4±47.8
Patient pIgGs	10.8±0.52*/##	8.1±1.30	1.29±0.07	3.69±0.42	1.8±0.07	18.9±0.9	402.8±62.3

Results are presented as mean ± S.E.M. of each measured parameter from neurons. (R: resistance). Statistical significance determined by Kruskal-Wallis test, followed by Dunn's post-hoc test: *p<0.05 relative to WT, ##p<0.01 compared to healthy IgGs.

Chapter VI
Concluding remarks & Future Perspectives

Concluding remarks

The human brain is distinctively unique due to its remarkable ability for complex language, higher cognition, emotion regulation and executive control of behaviour. The glutamatergic system is a major workhorse underlying most of these processes, hence it undergoes constant regulation in order to maintain the brain working properly. Perhaps conflicting with the definition of the word “maintain”, the hallmark feature of a brain working properly is plasticity. Indeed, the brain is ever-changing, either during development and refinement of the neuronal circuitry, or in response to different sensory inputs or pathological insults. Evidently, the brain does not change without restrains; instead it is capable of *keeping its cool* and adapt, preventing disruption of certain brain processes. Strongly contributing to this is homeostatic synaptic plasticity, which is capable of maintaining neuronal activity within dynamic, yet stable boundaries that keep neuronal networks in-balance, even in face of constant changes of activity. Hence, homeostatic synaptic plasticity seems to be inherently crucial for the maintenance of proper brain function, and ultimately behaviour and cognition.

Certainly, it comes as no surprise that emerging evidence implicate glutamatergic synaptic abnormalities and failure in homeostatic synaptic plasticity mechanisms in the pathogenesis of several cognitive and neuropsychiatric disorders, including ASD, SCZ and ID. Unfortunately, the molecular mechanisms underlying homeostatic synaptic plasticity are still far from being completely understood, and evidence on which and how such mechanisms become compromised in the context of disease is still scarce.

In the present study we set out to explore the role of Contactin-associated proteins 1 and 2 in the regulation of glutamate AMPARs, the synaptic effectors of long-lasting changes in synaptic efficacy and strength that underlie the expression of various modalities of synaptic plasticity, including homeostatic synaptic scaling. Together with previous results from our laboratory (Santos *et al.*, 2012), we herein identify Caspr1 and Caspr2 as novel AMPAR-interacting proteins capable of regulating activity-dependent mechanisms of AMPAR trafficking and glutamatergic synaptic transmission, and uncover an unsuspected requirement of these proteins to trigger homeostatic and sensory-driven experience-dependent synaptic plasticity processes, otherwise compromised in their absence (Figure 6.1 and 6.2). The recent implication of these proteins in neurological and neuropsychiatric disorders opens the path for understanding how neuronal synaptic homeostasis can falter in the context of disease, and identify the pathogenic underpinnings that drive the cognitive and psychiatric symptoms presented by patients.

Heads or tails - are Caspr1 and Caspr2 two sides of the same coin?

At first sight, the similarity of functions mediated by Caspr1 and Caspr2 is quite striking, suggesting that they play redundant roles in AMPAR regulation and synaptic plasticity. However, although their effects converge to the same end-point, they seem to trigger distinct mechanisms to achieve so. This divergence is quite remarkable if we consider the significant degree of structural and functional homology shared between them (Peles *et al.*, 1997; Poliak *et al.*, 1999). Such difference most likely stems from two key intracellular domains exclusive to either Caspr1 or Caspr2 C-tails.

Caspr1 encompasses a proline-rich region, absent from Caspr2, that binds to SH3 domains (Peles *et al.*, 1997), and which we found to be important for the role played by Caspr1 in AMPAR regulation. Through this domain, Caspr1 activates a Src-dependent signalling pathway that regulates the translational capacity of the RNA-binding protein ZBP1, which we found to bind to the mRNA of the GluA1 subunit of AMPARs. It is possible that ZBP1, downstream of a Caspr1-dependent pathway, mediates an activity-dependent posttranscriptional mechanism that fine-tunes the availability of a synaptic pool of GluA1 mRNA readily available to undergo translation on demand upon prolonged inhibition of neuronal activity. Such mechanism could easily sustain the expression of homeostatic synaptic plasticity, which has been shown to depend on local GluA1 protein synthesis. Because Caspr2 lacks this proline-rich motif, it is unlikely that it participates at such a posttranscriptional level of AMPAR regulation.

Whilst lacking the proline-rich region, Caspr2 contains a PDZ-binding domain (Poliak *et al.*, 1999) through which it interacts with several known regulators of AMPAR trafficking, including PSD95 and SAP97 (Chen *et al.*, 2015). Additionally, Caspr2 contains a common FERM motif that binds the cytoskeleton adaptor 4.1N protein (Denisenko-Nehrbass *et al.*, 2003b), which also has a recognised role in AMPAR trafficking. Although we haven't yet established the relevance of any Caspr2 molecular determinant on its function, it is possible that these two domains play a concerted action. The binding of Caspr2, through these domains, to SAP97, 4.1N protein and PSD95, which act on different steps of AMPAR trafficking [reviewed in (Anggono & Huganir, 2012; Shepherd & Huganir, 2007)], suggests that Caspr2 could, in principle, orchestrate a modular and step-wise pathway to traffic AMPARs all the way from their synthesis at the ER and Golgi apparatus, through binding to SAP97; to their exocytic insertion in the cell membrane and surface lateral diffusion, through binding to 4.1N protein; until their synaptic retention and stabilization at the PSD, through binding to PSD95.

These observations argue in favour of Caspr1 and Caspr2 mediating complementary, instead of redundant functions in the regulation of AMPARs. Further contributing to this hypothesis is the region-specificity of the effects elicited by either Caspr1 or Caspr2. Although they control the basal synaptic expression of AMPARs regardless of brain region (particularly Caspr2), the activity-dependence of their mechanisms is restricted to a specific region, with Caspr1 being required in the hippocampus, whilst Caspr2 in necessary in the cortex. Because the expression of both proteins does not differ significantly across these brain regions, it is likely that differences in region-specificity stem instead from varying expression of a downstream effector of one of the proteins.

CASPR1 and CASPR2 dysfunction underlying the pathogenesis of neurological and neuropsychiatric disorders

Throughout the past decade, several mutations in the *CNTNAP2* gene have been recurrently implicated in the pathogenesis of various neurological and psychiatric disorders, including ASD, SCZ and bipolar disorder, ID and language impairment, as well as epilepsy and syndromic forms of epilepsy, among others [reviewed in (Penagarikano & Geschwind, 2012; Poot, 2017)]. Overarching phenotypes of patients carrying *CNTNAP2* mutations include seizures, language impairments, intellectual disability and varying autistic-core behavioural and social defects. Furthermore, autoantibodies targeting CASPR2 have also been recently found in association with autoimmune synaptic encephalitis. Patients develop neurological syndromes that can include neuromyotonia, Morvan's Syndrome and limbic encephalitis, manifesting a clinical condition that overlaps a complex dysfunction of both peripheral and central nervous systems. Importantly, symptoms of cognitive / psychiatric nature presented by these patients substantially overlap with those of genic neuropsychiatric disorders, suggesting a common mechanism of pathogenesis ensuing from both genetic- and antibody-mediated disruptions of CASPR2 function.

Therapeutic approaches used to date to treat autistic or schizophrenic patients often only ameliorate or dampen a reduced scope of the behavioural phenotypes, and elicit severe secondary effects. Moreover, most immunotherapies used to treat CASPR2 autoimmune encephalitis are unspecific, and although there is usually a good clinical outcome, most patients require sustained immunosuppression, with several retaining permanent cognitive sequelae. Understanding how genetic- and antibody-mediated perturbations in CASPR2 function underlie the pathogenesis of distinct disorders may enable the development of more efficient and targeted therapies. Potential therapeutic approaches worthy of note are oxytocin

and oestrogen, which have been shown to revert the behavioural phenotypes of *Cntnap2* KO animal models (Hoffman *et al.*, 2016; Penagarikano *et al.*, 2015). Interestingly, oestrogen is known to regulate oxytocin (McCarthy, 1995), and oestrogen-mediated effects in glutamatergic signalling have been reported (Smith *et al.*, 2009). Concurring with the potential therapeutic effect of oestrogen is the clear gender bias in patients with CASPR2-related disorders: *CNTNAP2* mutation-related phenotypes are more frequent in males, and there is an extremely marked preponderance of males (around 90%) in CASPR2 encephalitis (Binks *et al.*, 2017; van Sonderen *et al.*, 2017), suggesting a sex-linked susceptibility to perturbations in CASPR2 function and hinting at the potential protective effect of oestrogen in females.

Although association of CASPR1 with disease is not vast, mutations in the CASPR1-encoding gene *CNTNAP1* have been recently implicated in the pathogenesis of congenital forms of hypomyelinating disorders and neuropathy (Hengel *et al.*, 2017; Lakhani *et al.*, 2017; Laquerriere *et al.*, 2014; Mehta *et al.*, 2017; Nizon *et al.*, 2016; Vallat *et al.*, 2016), and autoantibodies targeting CASPR1 were just found in association with inflammatory neuropathy (Doppler *et al.*, 2016). The implication of CASPR1 in the pathogenesis of these disorders indicates a potential dysregulation of its known function in paranodes of peripheral nerves, suggesting that a contribution of the role of CASPR1 in AMPAR regulation is unlikely to occur is such cases. Interestingly, a recent meta-analysis study of the transcriptomic profiling across several neuropsychiatric disorders indicates that *CNTNAP1* gene expression is downregulated in ASD, SCZ and bipolar disorder (Gandal *et al.*, 2018), thus giving a first hint that the role of CASPR1 in AMPAR regulation and homeostatic synaptic plasticity could be implicated in the pathogenesis of neuropsychiatric disorders, alongside CASPR2.

Interestingly, several other CASPR proteins and members of the CONTACTIN family, to which CASPR1 and CASPR2 typically bind, have been recurrently found to both harbour gene mutations implicated in genic neuropsychiatric disorders (Cottrell *et al.*, 2011; Fernandez *et al.*, 2008; Fromer *et al.*, 2016; Glessner *et al.*, 2009; Hirata *et al.*, 2016; Hu *et al.*, 2015; Karayannis *et al.*, 2014; Kashevarova *et al.*, 2014; Mercati *et al.*, 2016; Okita *et al.*, 2017; Pagnamenta *et al.*, 2010; Shangguan *et al.*, 2017; Stogmann *et al.*, 2013; Zhao *et al.*, 2013), and be targeted by autoantibodies in autoimmune pathologies (Boronat *et al.*, 2012; Derfuss *et al.*, 2009; Manso *et al.*, 2016), thus making this CASPR/CONTACTIN system an interesting susceptibility hub for dysfunction in neurological and psychiatric disorders.

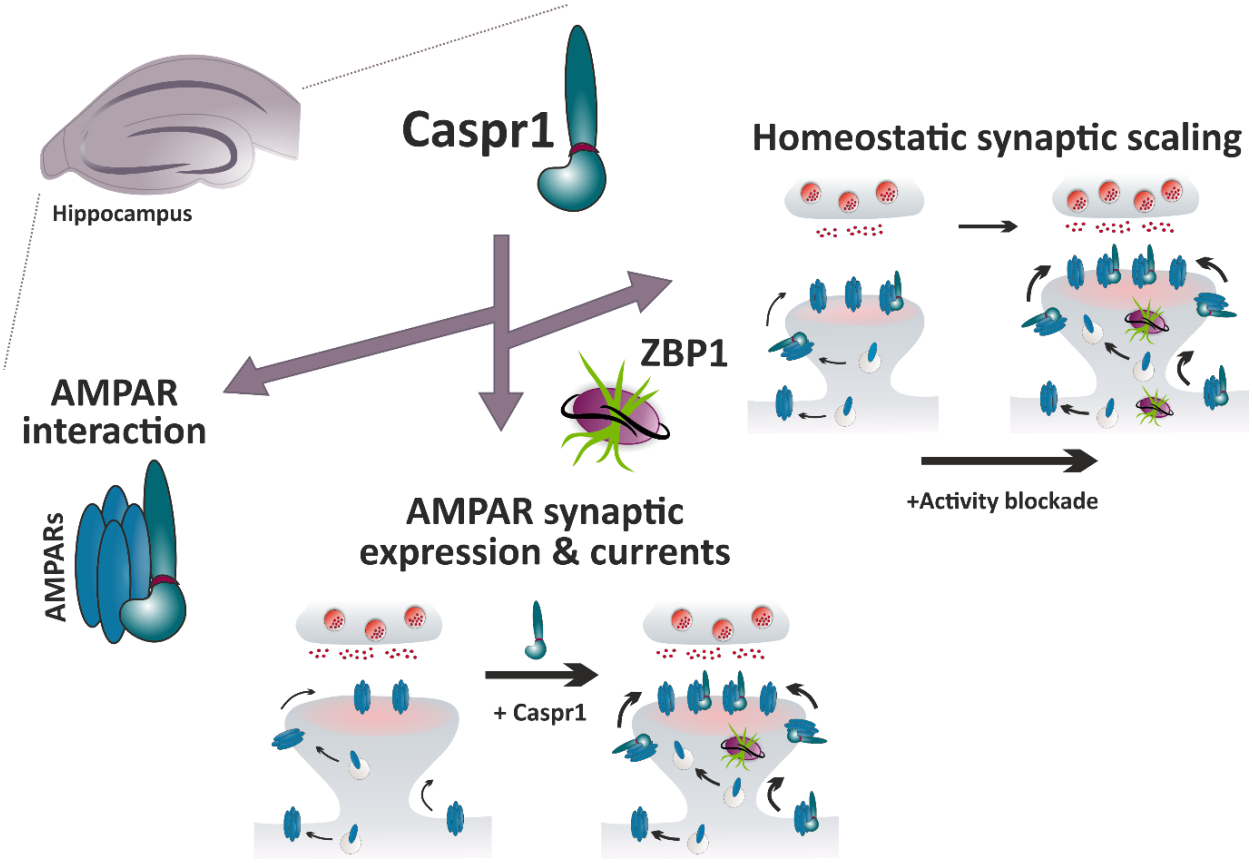


Figure 6.1 – Graphical representation of the molecular and synaptic functions mediated by Caspr1 and its underlying ZBP1-dependent mechanism. In a previous study from the laboratory we identified Caspr1 as a novel AMPAR-interacting protein necessary for the regulation of AMPAR synaptic expression and AMPAR-mediated currents. In the present study we uncovered a ZBP1-dependent posttranscriptional mechanism underlying the function of Caspr1 in AMPAR regulation, and further uncovered a requirement of this Caspr1/ZBP1 pathway for the expression of homeostatic synaptic scaling mechanisms, specifically in the hippocampus.

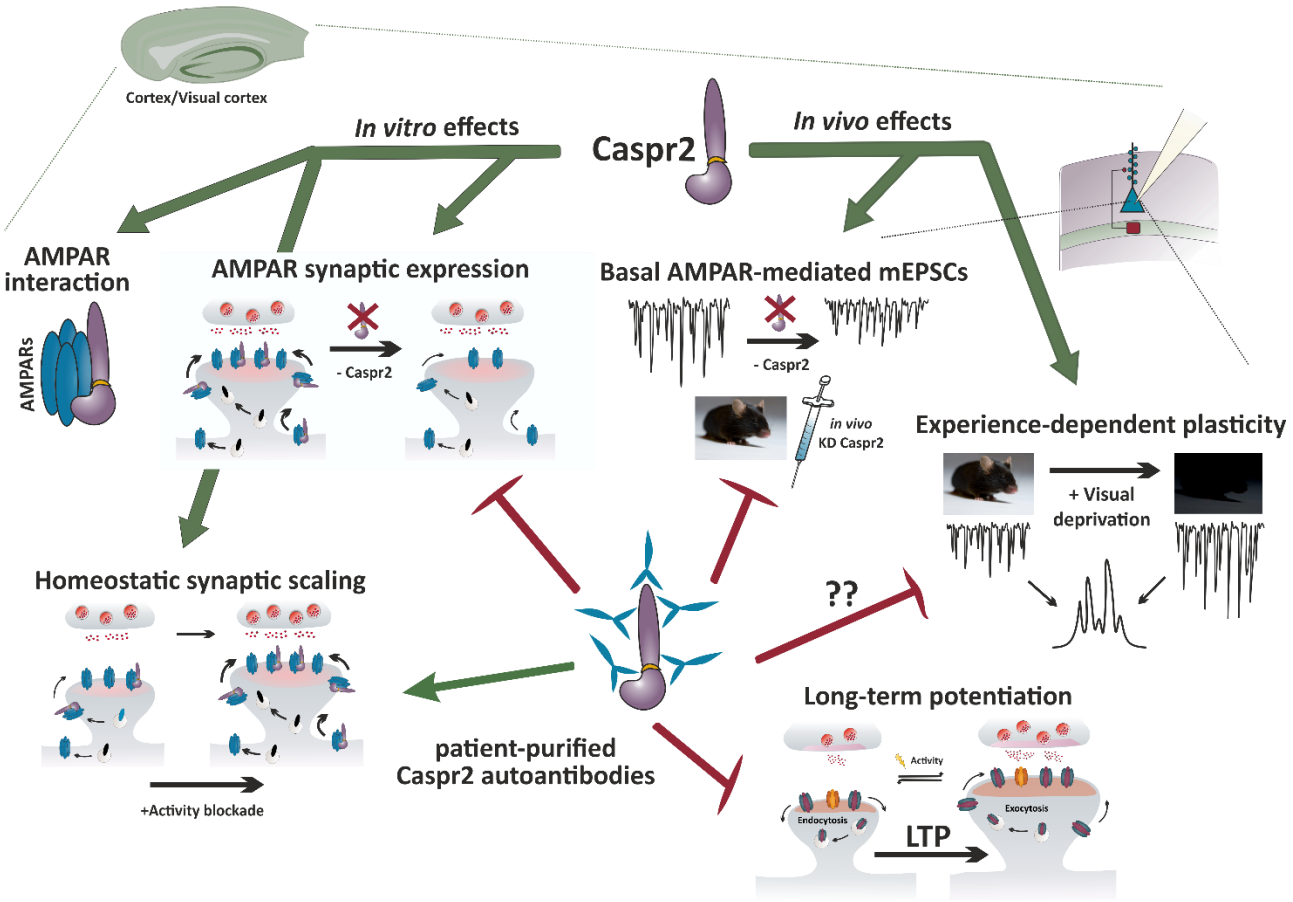


Figure 6.2 – Graphical representation of the molecular and synaptic functions mediated by Caspr2 and the pathogenic effects of CASPR2 autoantibodies. Herein, we identified Caspr2 as a novel AMPAR-interacting protein necessary for the regulation of AMPAR synaptic expression and AMPAR-mediated currents *in vivo*. Moreover, we uncovered a requirement of Caspr2 for the regulation of homeostatic synaptic scaling, and for the expression of visually-driven experience-dependent plasticity in the visual cortex. Furthermore, we explored the pathogenic effects of CASPR2 autoantibodies in Caspr2 functions and revealed that CASPR2-Abs perturb the synaptic expression of AMPARs and impair excitatory synaptic transmission in the visual cortex. Finally, we found that, whilst synaptic scaling mechanisms remain intact, CASPR2-Abs hinder NMDAR-dependent LTP.

Future perspectives

In this study we managed to unveil and characterize novel important functions mediated by the cell-adhesion molecules Caspr1 and Caspr2 in the regulation of AMPARs and synaptic plasticity phenomena. Nonetheless, our findings raise fundamental questions regarding the implication of these proteins, and their underlying mechanisms, in the regulation of fundamental physiological brain processes. Importantly, they warrant further investigation to explore how disease-related perturbations in the proposed function of Caspr1 and Caspr2 can contribute to disease pathogenesis.

Role of Caspr1 and ZBP1 in the regulation of activity-dependent local protein synthesis of AMPARs at dendrites and nearby synapses

In chapter III we uncovered an unanticipated posttranscriptional mechanism mediated by the RNA-binding protein ZBP1 underlying the role of Caspr1 in the regulation of AMPAR trafficking. We found that ZBP1 binds to GluA1 mRNA in basal conditions, in a process that likely hampers GluA1 translation. However, upon prolonged inhibition of neuronal activity, which promotes a Src-dependent de-activating phosphorylation of ZBP1, GluA1 mRNA molecules are substantially released from ZBP1. ZBP1 is well-recognized for providing an efficient and energy-saving mechanism to temporally and spatially control the local translation of β-actin mRNA in sites where actin dynamics is most required (Huttelmaier *et al.*, 2005; Oleynikov & Singer, 2003; Sasaki *et al.*, 2010; Welshhans & Bassell, 2011). Additionally, studies have already reported the presence of dendritic ZBP1/β-actin RNA granules nearby synapses that undergo disassembly with subsequent local translation of β-actin upon neuronal activity (Buxbaum *et al.*, 2014; Park *et al.*, 2014; Whalley, 2014). These findings prompted us to hypothesize that ZBP1 mediates an activity-dependent role to fine-tune the availability of a synaptic pool of GluA1 mRNA readily available to undergo translation on demand upon prolonged inhibition of neuronal activity. Performing live-imaging assays using translation reporters or single-RNA molecule tracking methods would allow us to visualize GluA1 local protein synthesis occurring at dendrites and nearby synapses. Combining this strategy with KD-mediated loss of expression of both ZBP1 and Caspr1, or with a loss-of-function molecular replacement approach, would allow us to fully understand whether ZBP1, downstream of Caspr1, is necessary to regulate local protein synthesis of GluA1.

The expression of homeostatic synaptic plasticity has been shown to depend on local protein synthesis of GluA1-AMPARs, in a mechanism mediated by retinoic acid (Aoto *et al.*, 2008; Ju *et al.*, 2004; Maghsoodi *et al.*, 2008; Poon & Chen, 2008; Sutton *et al.*, 2006). Given that loss of ZBP1 expression, or blockade of its Src-dependent phosphorylation, alters the synaptic content of surface AMPARs and hinders homeostatic synaptic plasticity, it is likely that these synaptic scaling mechanisms require a ZBP1-dependent GluA1 translation to replenish surface AMPARs at the synapse. Performing the study proposed above in conditions of prolonged activity blockade, or upon stimulation with retinoic acid, would allow us to elucidate this hypothesis.

Pathogenic effect of disease-related *CNTNAP2* mutations in the regulation of AMPARs mediated by CASPR2

In chapter IV, we found that Caspr2 is present at excitatory cortical synapses and identified Caspr2 as a novel AMPAR-interacting protein capable of regulating the synaptic content of surface AMPARs. Furthermore, we provide compelling evidence indicating that this Caspr2 function is relevant for the regulation of basal glutamatergic synaptic transmission, and to elicit homeostatic synaptic scaling mechanisms that are required for the expression of sensory experience-dependent synaptic plasticity *in vivo*. Despite these unsuspected findings, the molecular mechanisms underlying the role of Caspr2 remain uncharacterized. Given the known C-tail-mediated interaction of Caspr2 with important regulators of AMPAR trafficking, namely SAP97, 4.1N protein and PSD95 (Chen *et al.*, 2015; Denisenko-Nehrbass *et al.*, 2003b), we hypothesize that Caspr2 also binds to GluA1 through this region to mediate its role in AMPAR trafficking. Future molecular replacement experiments, using Caspr2 deletion mutants (whole C-tail or either one of the FERM or PDZ-binding domains), will be important to elucidate the molecular determinants of Caspr2 underlying its function. Assessing how different mutants impinge either on the intracellular trafficking of AMPARs, or in their surface diffusion and retention/anchoring at the PSD, as well as in the expression of synaptic plasticity events will elucidate the molecular mechanism behind Caspr2.

Undoubtedly, studies using *Cntnap2* KO animal models have provided invaluable insights into the cellular and molecular functions of Caspr2, and shed light on the potential effects of Caspr2 disruption in the context of disease. However, save few exceptions, most *CNTNAP2* mutations identified to date result in potentially deleterious forms of the protein, instead of an absence of it. Molecular studies evaluating specific *CNTNAP2* mutations have exclusively explored the mutational effect on Caspr2 itself. We believe that one major milestone still to be achieved in the field is thus understanding how specific potentially deleterious *CNTNAP2* mutations perturb the synaptic and cellular functions of CASPR2. By performing *in vitro* molecular replacement experiments with specific disease-related *CNTNAP2* mutations, we will be able to investigate their deleterious effects and determine how they disrupt CASPR2 function. Exploring the pathogenic effect of different mutations on AMPAR intracellular trafficking and surface dynamics, synaptic plasticity mechanisms, as well as on dendritic complexity and spine density and dynamics will elucidate specific-disease mechanisms of pathogenesis and help explain the phenotypic variability of patients carrying *CNTNAP2* mutations. Finally, it will be of the utmost importance to generate a *knockin* mouse model with the most relevant disease-related *CNTNAP2* mutation and assess its biochemical, electrophysiological and behavioural phenotypes. This will provide seminal insights on the

synaptic, cellular and circuitry functions of Caspr2 hampered *in vivo* by a disease-related mutation and serve as a corollary to understand disease pathogenesis in human patients.

Causal link for pathogenesis mediated by CASPR2 autoantibodies - insights from an *in vivo* mouse model of passive antibody transfer

In chapter V we aimed at exploring the pathogenic mechanisms of CASPR2 autoantibodies. We revealed that CASPR2-Abs perturb the synaptic content of endogenous Caspr2 and of surface AMPARs, ultimately impairing glutamatergic synaptic transmission *in vivo* and synaptic plasticity phenomena. In a preliminary experiment, we found that CASPR2-Abs bound to the surface of neurons potentially undergo time-dependent internalization themselves, which suggests they can exert their pathogenesis through internalization of Caspr2/AMPAR synaptic complexes from the cell surface. CASPR2-Abs are of the IgG1 but also of the rare IgG4 subtype, which do not activate the complement and are thus, thought to mediate a more direct disruption of their target antigens, instead of internalizing them. Indeed, CASPR2-IgG4-Abs were shown to perturb the interaction of Caspr2 with its extracellular binding partner Contactin-2 (Patterson *et al.*, 2017). Future experiments should be performed to evaluate whether CASPR2-Abs promote a time-dependent internalization of both Caspr2 and surface AMPARs from the synapse, or if they disrupt the interaction of Caspr2 with GluA1, thus promoting a destabilization of AMPARs at the synapse and an increase of their surface lateral diffusion. These experiments will shed light on the synaptic and cellular phenotypes elicited by CASPR2-Abs and underpin their exact mechanism of action to disrupt Caspr2 function.

Despite symptom severity, most patients undergo substantial recovery following immunotherapy, and a good clinical outcome typically correlates with a decrease in circulating CASPR2-Ab titres in the patient serum, which suggests a direct antibody-mediated pathogenesis underlying disease. To date, only a couple of studies tried to establish a causal link of CASPR2-Abs to disease pathogenesis: they report that mice exposed *in utero* to CASPR2-Abs, in a mouse model of chronic maternal-to-foetal antibody transfer, develop synaptic, cellular and behavioural abnormalities that relate to *CNTNAP2*-associated neuropsychiatric phenotypes (Brimberg *et al.*, 2016; Coutinho *et al.*, 2017b), thus confirming the pathogenicity of CASPR2-Abs. However, because these studies refer to pathogenic effects of CASPR2-Abs in the progeny that most likely occur during development, they fall short on serving as a corollary for autoimmune encephalitis patients. The development of a mouse model of passive antibody transfer through continuous cerebroventricular infusion of patient

CSF or IgGs to replicate the CNS symptoms associated with the disease will allow the formal assertion of a direct association of CASPR2-Abs to pathology. Behavioural, electrophysiological, morphological and synaptic characterization of the mouse model will provide not only a causal link for CASPR2-Abs in disease pathogenesis, but help elucidate the cognitive and psychiatric symptoms presented by patients. A similar approach was recently used to develop a mouse model of anti-NMDAR encephalitis and establish the pathogenicity of anti-NMDAR antibodies (Planaguma *et al.*, 2015).

Chapter VII
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