DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Post-transcriptional mechanisms of regulation of AMPA receptors:

Regulation of GluA1 expression by the Contactin associated protein 1

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutora Ana Luísa Carvalho (Universidade de Coimbra) e da Doutora Sandra Santos (Centro de Neurociências e Biologia Celular)

Dominique Moreira Fernandes

2011

Agradecimentos

Agora que chego ao fim de mais uma importante etapa da minha formação académica, gostaria de agradecer a todas as pessoas que foram fundamentais para tornar estes últimos anos tão enriquecedores de conhecimento não só científico, mas também pessoal…

Em primeiro lugar gostaria de agradecer à Professora Ana Luísa Carvalho por me ter dado esta oportunidade de realizar o meu projecto de mestrado no seu laboratório. Queria agradecer por toda a disponibilidade e preocupação que sempre demonstrou e por todos os conhecimentos que me transmitiu. Mas acima de tudo, por ser um verdadeiro exemplo de dedicação e paixão pela Ciência.

Queria agradecer também à Sandra Santos por toda a preocupação e toda a ajuda que sempre me prestou, por todos os conhecimentos e dicas para tudo funcionar mas, principalmente, por toda a inesgotável paciência sem nunca desistir de me 'enrijecer e formar o carácter'.

Ao Professor Carlos Duarte e ao Dr. Ramiro Almeida, gostaria de agradecer não só por toda a simpatia e boa-disposição, mas também por todos os conhecimentos partilhados e discussões científicas que ajudam no trabalho de todos.

Ao Luís Ribeiro, um agradecimento especial por me ter acompanhado nos primeiros tempos no laboratório, por todos os conhecimentos que me transmitiu e por toda a preocupação. Aos restantes membros do grupo, um muito obrigado por toda a entre-ajuda e apoio sempre disponível mas, principalmente, por toda a boa disposição que habitualmente reina no nosso laboratório.

Ao Luís Leitão, ao Pedro e ao Diogo agradeço por toda a amizade e boa-disposição, e por sempre me terem tentado animar, com sucesso, das formas mais incrivelmente parvas… no fundo, por toda a palermice a que me sujeitaram durante este ano. Em particular ao Leitão, um obrigado muito especial por toda a amizade e confiança, pelo apoio e preocupação constantes, mas, principalmente, por me aturar incansavelmente (quase) sem se queixar.

À Sara, ao João, ao Diogo, à Bia e ao Daniel, meus amigos de sempre, um obrigado por todos estes anos e pela amizade certa e constante, por muito afastados que estejamos em tempo e quilómetros.

Aos meus Pais, agradeço por tudo o que me ensinaram. Pela preocupação e paciência infinitas e pela ajuda constante. Por confiarem em mim e me apoiarem incondicionalmente em todas as minhas decisões. Por estarem sempre presentes. A toda a restante família, agradeço o apoio, preocupação e carinho.

E ao André, agradeço por tudo. Por toda a confiança, compreensão e ajuda constantes. Acima de tudo, por toda a paciência, companheirismo, carinho e amizade incondicional ao longo de todos estes anos.

Gostaria também de agradecer à Fundação para a Ciência e Tecnologia pelo financiamento atribuído a este projecto (PTDC/BIA-BCM/113738/2009).

Table of contents

Abbreviations

3'UTR	3'Untranslated region
λPP	Lambda protein phosphatase
AMCA	Aminomethylcoumarin Acetate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	AMPA receptors
ANOVA	Analysis of variance
APV	(2*R*)-amino-5-phosphonopentanoate
ARE	Adenosine and uridine-rich elements
Arp2/3	Actin related protein 2/3 complex
ATD	Amino terminal domain
AUF1	Adenosine and uridine-binding factor 1
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
Bicc	Bicuculline
BSA	Bovine serum albumin
CA1	*Cornu Ammonis* region 1
CaMKII	Calcium-calmodulin-dependent protein kinase II
Caspr1	Contactin associated protein 1
CLAP	Chymostatin, leupeptin, antipain and pepstatin
CNS	Central nervous system
CPE	Cytoplasmic polyadenylation element
CPEB	CPE binding protein
DIV	Days *in vitro*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Deoxycholic acid
DTT	Dithiothreitol
ECF	Enhanced chemifluorescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethyleneglycoltetraacetic acid
ELAV	Embryonic lethal abnormal vision
ER	Endoplasmic reticulum

ERK	Extracellular signal-regulated kinase
FERM	Four-point-one, ezrin, radixin, moesin
FMRP	Fragile X mental retardion protein
FXS	Fragile X Syndrome
GAP-43	Growth associated protein 43
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GluR	Glutamate receptors
GNP	Glycophorin C, neurexin, paranodin (GNP) motif
GPI	Glycosylphosphatidyl inositol
GRIP/ABP	Glutamate receptor interacting protein / AMPAR binding protein
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
GTPase	GTP hydrolase enzyme
HBSS	Hank's balanced salt solution
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HnRNP	Heterogenous nuclear ribonucleoproteins
IgG	Immunoglobulin G
kDa	kiloDalton
L1-CAM	L1-cell adhesion molecule
LBD	Ligand binding domain
LTD	Long-term depression
LTP	Long-term potentiation
MAG	Myelin associated glycoprotein
MAP2	Microtubule associated protein 2
MAPK	Mitogen-activated protein kinase
mEPSCs	Miniature excitatory postsynaptic currents
mRNA	Messenger RNA
N-CAM	Neural cell adhesion molecule
NMDA	*N*-methyl *D*-aspartate
P-bodies	Processing bodies
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDZ	Postsynaptic density-95 / disc large / zonula occludens-1 domain
PGY	Proline, glycine, tyrosine (PGY) repeats
PI3K	Phosphoinositide 3-kinase
PICK	Protein interacting with C-kinase 1

PKA	Protein kinase A
PKC	Protein kinase C
PLCγ	Phospholipase C gamma subunit
PMSF	Phenylmethylsulfonyl fluoride
PSD	Postsynaptic density
PSD-95	Postsynaptic density protein 95
PVDF	Polyvinylidene difluoride
RA	Retinoic acid
RARα	Retinoic acid receptor-α
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RRM	RNA recognition motif
SAP97	Synapse-associated protein 97
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylimide gel electrophoresis
SEM	Standard error of the mean
SFK	Src family of protein tyrosine kinases
SG	Stress granules
SH2	Src homology 2 domain
SH3	Src homology 3 domain
TARP	Transmembrane AMPAR regulatory protein
TBS	Tris-buffered saline
TBS-T	0.1% Tween-20 in TBS
TMD	Transmembrane domain
TNFα	Tumor necrosis factor-α
TTX	Tetrodotoxin
ZBP1	Zipcode-binding protein 1

Resumo

No sistema nervoso central, a maior parte da neurotransmissão excitatória é mediada por receptores de glutamato do tipo AMPA que possuem papéis fundamentais na plasticidade sináptica, o fenómeno celular na base de processos de aprendizagem e memória. Modificações no tráfego destes receptores e na sua inserção ao nível das sinapses, bem como na estabilidade do RNA mensageiro das subunidades dos receptores ou no seu decaimento, são cruciais para induzir alterações de longo prazo na força e eficiência sinápticas, o que permite a expressão de mecanismos de plasticidade. Tendo isto em conta, torna-se particularmente importante compreender a fundo como é que estes eventos são regulados, de forma a desvendar os mecanismos que estão na base de várias formas de plasticidade.

Um estudo recente realizado no nosso laboratório permitiu a identificação da proteína 1 associada à Contactina (Caspr1) como um novo interactor da subunidade GluA1 dos receptores AMPA. Esta proteína foi capaz de induzir um aumento nos níveis superficiais de GluA1, bem como mediar o seu endereçamento para a membrana sináptica. Para além disso, e duma maneira independente da transcrição, a Caspr1 foi capaz de induzir um aumento nos níveis de RNAm de GluA1, o que sugere um papel importante da Caspr1 na regulação da estabilidade destes transcriptos. Estas evidências propõem então a existência de um novo mecanismo de regulação pós-transcripcional dos receptores AMPA, desconhecido até agora. Ainda assim, o papel da Caspr1 na regulação da subunidade GluA1 está longe de ser compreendido, pelo que é importante continuar a caracterizar este mecanismo regulador.

Neste trabalho procurámos, em primeiro lugar, confirmar o papel da Caspr1 na regulação da subunidade GluA1. A sobreexpressão da Caspr1, tanto num sistema heterólogo como em neurónios primários de hipocampo, resultou num aumento significativo nos níveis totais de GluA1. Para além disso, conseguimos identificar o domínio intracelular da Caspr1 rico em prolinas como sendo o responsável por estes efeitos nos níveis de GluA1. De facto, uma forma da Caspr1 sem o domínio rico em prolinas não teve qualquer efeito nos níveis totais de GluA1, em células COS7.

Tendo em conta que o domínio rico em prolinas da Caspr1 é capaz de interagir com domínios SH3 de várias moléculas de sinalização, em particular com a tirosina cínase Src, colocámos a hipótese de o efeito da Caspr1 nos níveis de GluA1 ocorrer por activação, mediada pelo seu domínio de prolinas, de uma via de sinalização a jusante da Src. De facto, a expressão da Caspr1 em células COS7 foi capaz de induzir um grande aumento nos níveis de Src fosforilada, bem como nos níveis de ZBP1 (proteína 1 de ligação a ‘zipcodes’) fosforilada. Este alvo de fosforilação pela Src é uma proteína de ligação a RNAs, conhecida por regular a tradução de vários RNAm. Para além disso, a sobreexpressão da Caspr1 em neurónios de hipocampo induziu um aumento significativo e específico no número de agregados de Src e ZBP1 fosforiladas, ao nível da sinapse.

Por fim, tentámos investigar quais os estímulos fisiológicos capazes de regular a expressão endógena da Caspr1. Um pormenor interessante é que o efeito que a Caspr1 exerce sobre os níveis da subunidade GluA1 assemelha-se bastante ao efeito induzido por um bloqueio crónico da actividade neuronal, bloqueio esse que induz um fenómeno de plasticidade homeostática que depende do aumento da expressão de receptores AMPA, numa tentativa de restituir os níveis de actividade neuronal. De acordo com

isto, um bloqueio crónico da actividade neuronal induzido por TTX (bloqueador específico de canais de sódio dependentes de voltagem), foi capaz de promover um aumento significativo, não só nos níveis totais de GluA1, mas também nos níveis endógenos da Caspr1. Além disso, esta manipulação da actividade neuronal foi ainda capaz de induzir a activação da via de sinalização da Src, aumentando os níveis de Src e ZBP1 fosforiladas.

Em conclusão, este estudo contribuiu para caracterizar os mecanismos moleculares envolvidos na sobrerregulação da subunidade GluA1 pela Caspr1, bem como para identificar estímulos fisiológicos com impacto nestes mecanismos. Por fim, este estudo propõe um papel promissor para a Caspr1 na regulação de mecanismos na base da plasticidade homeostática.

Abstract

Fast excitatory synaptic transmission in the central nervous system is mediated by glutamate receptors of the AMPA-type, which play key roles in synaptic plasticity, the cellular correlate of learning and memory. Modulating the traffic and synaptic insertion of these receptors as well as their protein levels, e.g. through regulation of their mRNA stability and turnover, is crucial to induce long-term changes in synaptic strength and efficacy, which accounts for the expression of mechanisms of synaptic plasticity. Thus, understanding how these events are regulated is of major importance to fully unravel the mechanisms that underlie several forms of plasticity.

Recent data from our laboratory identified the integral membrane protein Contactin associated protein 1 (Caspr1) as a novel interactor of the GluA1 subunit of AMPARs. This protein was able to increase the cell surface expression of GluA1 and also, mediate its traffic to the synaptic membrane. Moreover, Caspr1 presented an upregulatory effect in GluA1 mRNA levels, in a transcription-independent manner, which suggests a role for Caspr1 in the regulation of GluA1 mRNA stability. These evidences suggest a novel post-transcriptional regulatory mechanism of AMPARs, unknown until now. Thus, it became important to further characterize the regulation of the GluA1 AMPAR subunit by Caspr1.

We firstly sought to confirm the role of Caspr1 in regulating the protein levels for the GluA1 subunit. Overexpression of Caspr1, both in a heterologous system and in cultured hippocampal neurons, resulted in a significant increase in the total levels of GluA1. Moreover, we identified the proline-rich region of Caspr1 as the molecular determinant responsible for its effect in GluA1 levels. Indeed, when expressing a

construct specifically deleted for the proline-rich domain, Caspr1 failed to increase GluA1 total levels, in COS7 cells.

Taking into account that the proline-rich domain of Caspr1 interacts with SH3 domains of various signaling molecules, particularly that of the tyrosine kinase Src, we hypothesized that the effect of Caspr1 in GluA1 levels occurs through a proline domain-mediated activation of a signaling pathway downstream of Src. Expression of Caspr1 in COS7 cells resulted in a marked increase in levels of phosphorylated Src as well as phosphorylated levels of its downstream target, Zipcode binding protein 1 (ZBP1), a RNA-binding protein known to regulate mRNA translation upon Src-dependent phosphorylation. Moreover, overexpression of Caspr1 in hippocampal neurons was able to induce a specific increase in the number of both phosphorylated Src and ZBP1 puncta at the synaptic level.

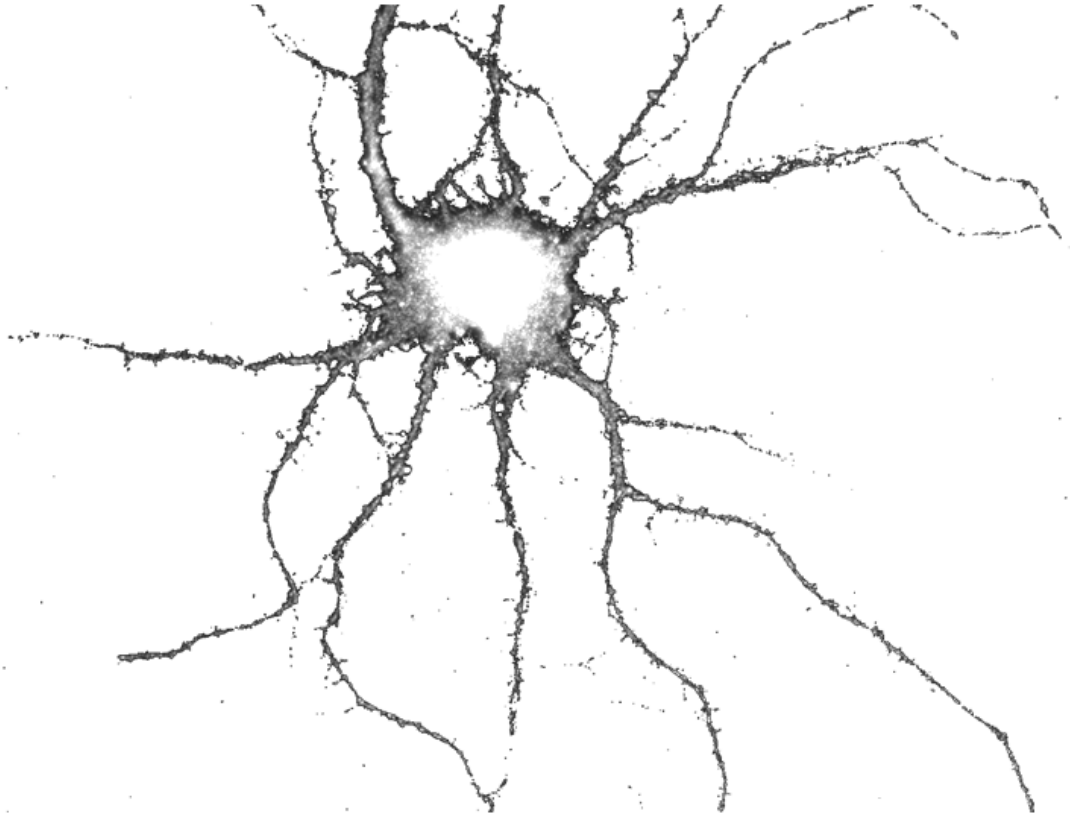
Furthermore, we sought to investigate physiological stimuli capable of regulating the endogenous expression of Caspr1. Interestingly, the upregulatory effect that Caspr1 exerts in levels of GluA1 subunit parallels that of chronically blocking neuronal activity, which results in a homeostatic synaptic scaling of GluA1. Accordingly, chronic blockade of activity induced by TTX, a blocker of voltage-gated sodium channels, was able to significantly increase not only GluA1 total levels, but also levels of endogenous Caspr1. Moreover, this manipulation of neuronal activity was able to induce an activation of the Src signaling pathway, with increases in phosphorylated levels of both Src and ZBP1.

In conclusion, this study contributed to characterize the molecular mechanisms involved in the upregulation of the GluA1 subunit by Caspr1, as well as the

physiological stimuli that impinge on those mechanisms. Moreover, it unveils a potentially promising role for Caspr1 in mediating homeostatic plasticity mechanisms.

Chapter 1

Introduction



The Glutamatergic Synapse

Most of the excitatory neurotransmission in the Central Nervous System (CNS) is mediated by the amino acid glutamate. After release from the presynaptic nerve terminal, glutamate binds to specific receptors on the postsynaptic membrane to conduct excitatory transmission. The effects of glutamate are mediated through its action on two distinct categories of glutamate receptors: metabotropic receptors and ionotropic receptors, which differ in their molecular, biochemical, pharmacological and physiological properties (Hollmann & Heinemann, 1994; Kew & Kemp, 2005; Santos *et al.*, 2009).

Metabotropic glutamate receptors are coupled to G proteins and their activation generates intracellular secondary messengers. Ionotropic glutamate receptors, on the other hand, form cation channels that open upon receptor activation by their specific agonists (Carvalho *et al.*, 2000). Ionotropic glutamate receptors exhibit great diversity and have been divided into three broad subtypes, according to their electrophysiological properties and most selective agonists: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors (Carvalho *et al.*, 2000; Watkins, 1981). Upon binding of synaptically released glutamate to the ionotropic receptors, a depolarization of the postsynaptic membrane is produced; nevertheless, these different types of glutamate receptors, particularly AMPA and NMDA, have very diverse roles in synaptic function (Carvalho *et al.*, 2000; Hollmann & Heinemann, 1994). AMPA receptors mediate the majority of fast excitatory currents in glutamate-mediated neurotransmission in conditions of basal neuronal activity, and thus, have a great influence in the strength of the synaptic response (Esteban, 2003; Ozawa *et al.*, 1998). On the other hand, NMDA receptors, at resting membrane

potential, remain silent with a voltage-dependent magnesium blockade. Nonetheless, their activation and subsequent calcium influx are critical for the induction of specific forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD) (Bear & Malenka, 1994; Esteban, 2003; Gomes *et al.*, 2003; Malenka & Nicoll, 1999).

AMPA Receptors

AMPA receptors are responsible for the primary depolarization in glutamatergic neurotransmission and are thought to play key roles in synaptic plasticity. Long-lasting and activity-dependent changes in synaptic strength and efficacy are associated with modifications in traffic and cellular distribution of AMPAR, post-translational and post-transcriptional modifications of AMPAR subunits, and are thought to underlie learning and memory formation (Morris, 2006; Rumpel *et al.*, 2005; Whitlock *et al.*, 2006).

Expression of AMPA Receptors

AMPA receptors 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 105kDa and assemble in tetramers in different stoichiometries to form AMPA receptors with different properties (Figure 1A) (desensitization/resensitization kinetics and conductance properties) (Ozawa *et al.*, 1998; Rosenmund *et al.*, 1998; Santos *et al.*, 2009). Several studies using *in situ* hybridization, receptor autoradiography and immunocytochemistry [reviewed in

(Hollmann & Heinemann, 1994)] reveal a widespread distribution of AMPA receptors throughout the brain, which is in conformity with their key role in excitatory neurotransmission (Santos *et al.*, 2009). While GluA1-GluA3 subunits are particularly enriched in the outer layers of the cerebral cortex, hippocampus and amygdala (Keinanen *et al.*, 1990), the GluA4 subunit is present in lower amounts throughout the CNS, except in the cerebellum, where this subunit is also abundant (Petralia & Wenthold, 1992). Interestingly, in the hippocampus, the expression of AMPAR subunits is also differentially regulated during development: in the mature hippocampus, AMPARs are mainly composed of GluA1 – GluA2 or GluA2 – GluA3 combinations (Wenthold *et al.*, 1996), whereas GluA4-containing AMPARs are primarily expressed in early postnatal development (Esteban, 2003; Zhu *et al.*, 2000).

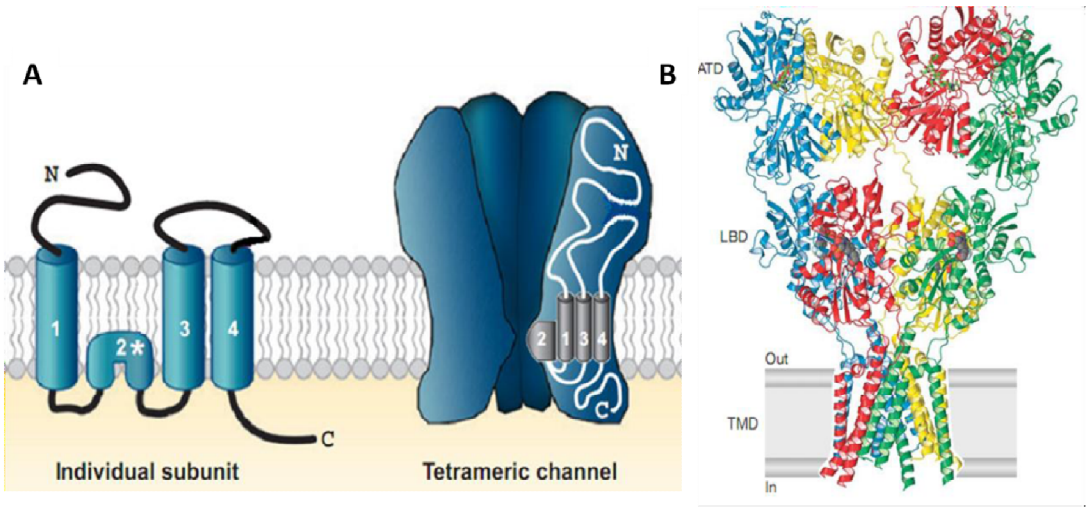


Figure 1 – Topology of AMPAR subunits and their assembly. (A) Membrane topology of the AMPAR subunit and schematic representation of the tetrameric channel. Each individual subunit is composed of an extracellular N-terminal, a ligand binding domain, four transmembrane domains that comprise the ionic channel of the receptor and a final highly variable intracellular C-terminal. The individual subunits assemble to form a heterotetrameric channel. Roles of each domain and the proposed mechanism of assembly are described in the

text. [Adapted from (Shepherd & Huganir, 2007)] (B) Structure of the full-length homotetrameric rat GluA2 receptor at 3.6Å obtained by X-ray crystallography [Adapted from (Sobolevsky *et al.*, 2009)].

AMPA receptor structure and diversity

AMPA receptors were initially predicted to assemble in a tetrameric stoichiometry for functional receptors, with a large extracellular N-terminal region followed by a membrane-spanning domain. The pore of the receptor appeared to be formed by a re-entrant hairpin loop within the transmembrane region, finally followed by a cytoplasmic C-tail (Bredt & Nicoll, 2003). Despite this plausible prediction, insights into the mechanism by which glutamate binding regulates AMPA receptor channel gating were not uncovered until 2009, when Gouaux and collaborators determined an accurate, atomic resolution description of AMPA receptor architecture and a definition of the arrangement of its subunits based on crystallographic studies (Figure 1B) (Sobolevsky *et al.*, 2009). Accordingly, AMPA subunits do indeed form functional homotetramers, although native receptors are almost exclusively heterotetramers. Each subunit has a modular composition that includes a large first extracellular amino terminal domain (ATD) that participates in subtype-specific receptor assembly, trafficking and modulation (Madden, 2002); followed by an adjacent ligand-binding domain (LBD) that is split into two segments, S1 and S2, whose structure had previously been determined by Gouaux and associates (Armstrong *et al.*, 1998). These segments show homology to the glutamine binding protein (QBP), which suggests a binding site for glutamate, granting the LBD a central role for agonist / competitive antagonist binding and to activation gating (Sobolevsky *et al.*, 2009). Next

comes the transmembrane domain (TMD) that, with the second hydrophobic hairpin loop, forms the membrane-spanning ion channel; and a final cytoplasmic carboxyl-terminal, the most structurally and functionally divergent region among subunits, which contains regulatory domains targeted by multiple intracellular signal transduction pathways and that mediate the involvement of the C-terminal in receptor localization and regulation (Figure 1) (Derkach *et al.*, 2007; Sobolevsky *et al.*, 2009).

In addition to the subunit stoichiometry, AMPAR function is further diversified by RNA processing events, including alternative splicing and RNA editing (Dingledine *et al.*, 1999). In the adult brain, particularly at the mature hippocampal excitatory synapses, AMPARs consist predominantly of GluA1 – GluA2 or GluA2 – GluA3 subunits. Most mature GluA2 proteins contain an arginine residue (R) within the re-entrant TM2 membrane loop region at position 586 in place of the genomically-encoded glutamine (Q) (Santos *et al.*, 2009; Sommer *et al.*, 1991). This Q/R discrepancy is explained by post-transcriptional mRNA editing and it occurs in more than 99% of GluA2 mRNA transcripts. The Q/R site controls various AMPAR properties including Ca^{2+} permeability, channel conductance, subunit assembly into functional receptors as well as endoplasmic reticulum (ER) export kinetics. Thus, GluA2-lacking receptors have a high Ca^{2+} permeability, channel conductance, open probability and rectification, that is, only allow ion flow into the cell, as opposed to GluA2-containing receptors that lack rectification and exhibit low channel conductance and open probability and are highly impermeable to Ca^{2+} (Bowie *et al.*, 1998; Derkach *et al.*, 2007; Geiger *et al.*, 1995; Greger *et al.*, 2003). Hence, the presence or absence of the GluA2 subunit in AMPARs can dramatically alter their properties and, consequently, synaptic transmission.

Another structural mechanism that determines channel properties is alternative splicing. Within the LBD, particularly at the C-terminal part of the S2 region, two mutually exclusive exons termed flip and flop can be expressed due to alternative RNA splicing. These two alternative exons are present in all four subunits and differ in a few amino acids only; nevertheless, their alternative splicing results in AMPARs with different desensitization and ER export kinetics (Santos *et al.*, 2009). Thus, the composition of subunits, post-transcriptional modifications, and especially RNA editing, endow AMPARs with substantial diversity and also determine the ion channel characteristics of the receptor (Jiang *et al.*, 2006).

Post-translational modifications of AMPA receptors

Properties and function of AMPARs may also be modulated by post-translational modifications such as glycosylation, palmitoylation and phosphorylation. Glycosylation is a protective modification that can occur at 4-6 different sites located in the extracellular domains of each AMPAR subunit. This N-glycosylation may facilitate the maturation of AMPARs and protect them from proteolytic degradation (Everts *et al.*, 1997; Jiang *et al.*, 2006). Palmitoylation is a reversible fatty acetylation that regulates protein trafficking and cellular localization. All AMPAR subunits can be palmitoylated on two cysteine residues in their transmembrane domain TM2 and in their intracellular C-terminal region. The first palmitoylation in TM2 leads to an accumulation of AMPAR in the Golgi apparatus, resulting in a decreased expression of the receptor in the cell surface. On the other hand, palmitoylation at the C-terminal domain contributes to receptor internalization by disrupting its interaction with the 4.1N protein, known to stabilize AMPAR expression on the surface (Hayashi *et al.*, 2005).

Up until today, four phosphorylation sites have been reported for the GluA1 subunit (Figure 2) (Lee *et al.*, 2010), all residing in the intracellular C-terminal of GluA1. 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 (CaMKII) (Mammen *et al.*, 1997). Also, Serine 845 (S845) was identified as a protein kinase A (PKA) phosphorylation substrate (Roche *et al.*, 1996). Serine 818 (S818) was only later discovered to be a PKC substrate (Boehm *et al.*, 2006) and only just recently Threonine 840 (T840) was found to be a major regulatory phosphorylation site of PKC in GluA1 (Figure 2) (Lee *et al.*, 2007). Phosphorylation of these residues is thought to be important for regulating the GluA1-containing AMPAR trafficking and synaptic insertion, and plays a role in two prototypic forms of synaptic plasticity: hippocampal NMDA-dependent Long-term Potentiation (LTP) and Depression (LTD). These plastic synaptic events, LTP and LTD, are associated with phosphorylation and dephosphorylation, respectively, of distinct GluA1 phosphorylation sites (Carvalho *et al.*, 2000; Jiang *et al.*, 2006; Lee *et al.*, 2000; Lee *et al.*, 2010).

The GluA2 subunit is also phosphorylated at two major sites (Figure 2). Serine 880 (S880) is the phosphorylation site for PKC located in the extreme C-terminal sequence within the PDZ (postsynaptic density 95/ disc large / zonula occludens-1) domain binding site, through which GluA2 binds to different proteins, namely the glutamate receptor interacting protein / AMPAR binding protein (GRIP/ABP) and the protein interacting with C-kinase-1 (PICK1). The other phosphorylation site, a tyrosine residue (Y876) in the GluA2 C-terminus, is a substrate for the Src family of protein tyrosine kinases that renders a similar effect as the phosphorylation in S880 (Hayashi & Huganir, 2004). Thus, phosphorylation at GluA2 plays a role in the differential binding

of GluA2 to interacting PDZ domain-containg proteins and is a critical means to regulate receptor trafficking (Gomes *et al.*, 2003). GluA2 phosphorylation at this region disrupts receptor binding to GRIP, recruits PICK1 to excitatory synapses and promotes a rapid internalization of surface receptors, which is a critical event for the induction of LTD (Chung *et al.*, 2003; Chung *et al.*, 2000; Matsuda *et al.*, 1999).

AMPA receptor biosynthesis

The biogenesis of oligomeric transmembrane proteins occurs at the ER membrane and commences with the co-translational insertion of nascent polypeptides in this organelle. AMPARs are modular polypeptides, divided into four distinct domains that engage in intersubunit interactions during assembly (Greger & Esteban, 2007; Greger *et al.*, 2007). Messages for AMPARs, just as for other oligomeric transmembrane proteins, are translated on the rough ER, and transit through the ER represents the first site for receptor assembly and regulation. In analogy to K^+ channels, AMPAR are thought to assemble as dimers of dimers (Madden, 2002; Tu & Deutsch, 1999). Within the ER, mechanisms that determine the combination of oligomers are not well understood but seem to depend on interactions between the luminal, N-terminal domains of the subunits that mainly mediate the initial dimer formation (Esteban, 2003; Greger *et al.*, 2007). This tight interaction constitutes a compatibility determinant, ensuring association between subunits of only a given GluR family (Greger & Esteban, 2007). The subsequent assembly step, tetramerization, involves the extracellular S2 loop and the transmembrane segments and appear to be guided by contacts within LBD dimers, generating the fourfold symmetry ion channel (Greger & Esteban, 2007; Greger *et al.*, 2007).

Following their synthesis in the ER, the assembled AMPARs transit to the Golgi apparatus and are trafficked by microtubule-based machinery either to dendrites or to axons, where they are inserted in membranes (Bredt & Nicoll, 2003). Nevertheless, tetramerization by itself does not seem to provide AMPARs with the ability to be exported from the ER. Interestingly, it has been suggested that binding of glutamate to the receptor within the ER may be required for ER exit by promoting the stabilization of different conformation states during folding and assembly (Fleck, 2006). Moreover, these conformational transition states appear to facilitate the co-assembly of AMPARs with auxiliary subunits or transport factors. The AMPAR-auxiliary subunit stargazin and its relatives, the transmembrane AMPAR regulatory proteins (TARPs), seem to be required for early secretory trafficking; also, they interact with the LBD, slowing gating kinetics and recognizing specific conformational states of this domain. Thus, they may contribute to render AMPAR competent for ER export (Tomita *et al.*, 2005b; Ziff, 2007). This may imply that the exit from the ER is under stringent quality control and that the efficiency of these processes impacts on ER export kinetics, determining the number of receptors available for expression at synapses (Greger & Esteban, 2007; Greger *et al.*, 2007).

AMPA receptor trafficking mechanisms

To ensure a correct neuronal communication, that requires the modulation of the excitatory neurotransmission, the presence of AMPARs at synapses has to be carefully regulated. Interestingly, these receptors are not static components of the synaptic membrane. On the contrary, they are continuously being delivered and removed in and out of the synapse in response to neuronal activity. Most AMPARs are likely to be

synthesized in the neuronal cell body, away from synapse. Therefore, newly synthesized receptors have to undergo a series of trafficking steps before being delivered to the synapse. This trafficking involves an intricate network of protein – protein interactions that, after the synthesis of receptors in the ER and transit through the Golgi apparatus, allows their transport along dendrites, ending with their local insertion and removal from synapses (Esteban, 2003).

AMPA receptor interaction partners

Unquestionably, the localization and trafficking of AMPARs are both extremely complicated and highly regulated processes. Because AMPARs themselves lack motor domains, they must associate with many protein partners that assist in their trafficking (Bredt & Nicoll, 2003; Henley, 2003; Jiang *et al.*, 2006). Indeed, accessory and scaffolding proteins interact with AMPARs at numerous subcellular domains. Particularly, the C-terminus of AMPARs, the most structurally and functionally divergent region among subunits, has been extensively investigated, mainly using yeast two-hybrid and GST pull-down assays, in the search for these interacting proteins (Figure 2). The large collection, almost certainly still incomplete, of AMPAR partners identified and characterized until today has begun to explain the details of AMPAR trafficking and surface expression and has given an insight on the roles of individual interactors in these mechanisms (Bredt & Nicoll, 2003; Henley, 2003).

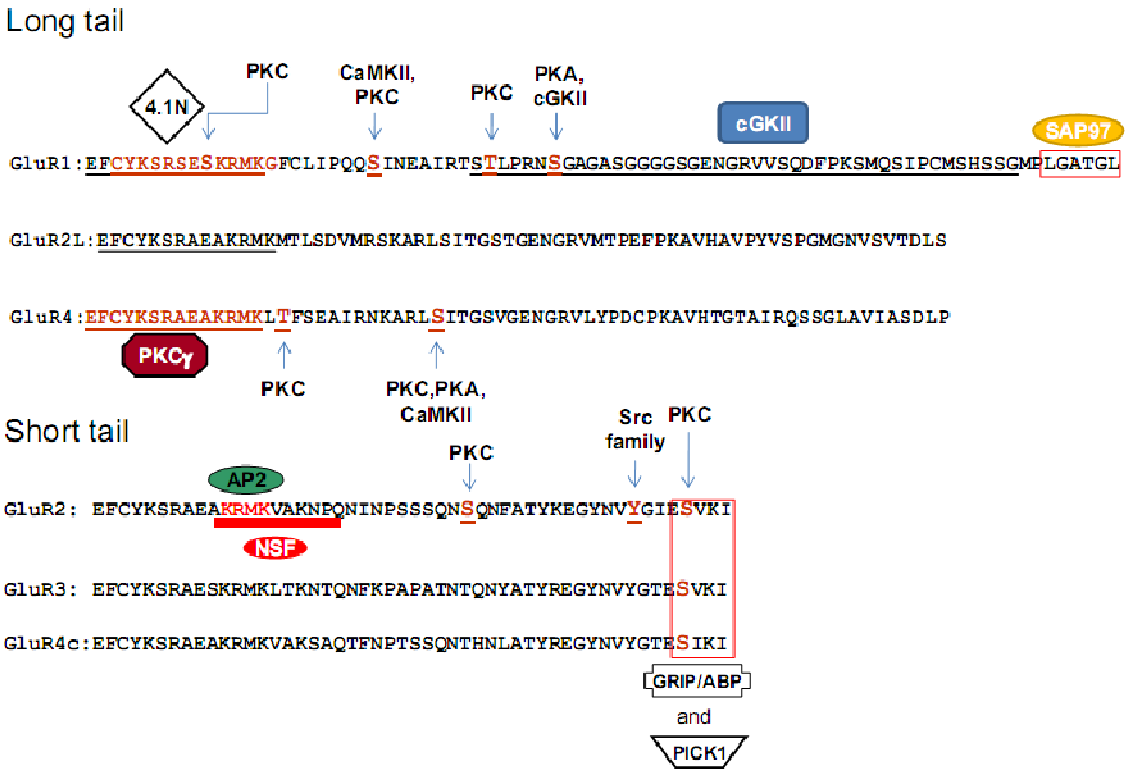


Figure 2 – Sequence alignment of the intracellular C-terminal region of the different AMPAR subunits. The different phosphorylation sites for each subunit and the various binding sites for interacting proteins are underlined and highlighted in red or with boxes, respectively. In the picture, the former nomenclature is used for the different subunits of AMPARs: GluR1, GluR2, GluR2L, GluR3, GluR4 and GluR4c correspond, respectively, to GluA1, GluA2, GluA2L, GluA3, GluA4 and GluA4c, in the new nomenclature (Santos *et al*., 2009).

AMPA receptor subunit-specific 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 the C-termini. Major insight into mechanisms that regulate trafficking of AMPARs came from the discovery that proteins containing PDZ domains play general roles in scaffolding membrane proteins (Bredt & Nicoll, 2003; Henley, 2003).

PDZ domains are modular protein – protein interaction motifs that contain three repeats of approximately 90 amino acids and are present in well over 100 otherwise unrelated proteins (Ponting *et al.*, 1997; Songyang *et al.*, 1997). Most of the interactions mediated by PDZ domains occur via the recognition of a short motif located at the extreme C-terminus of the binding protein, promoting both the clustering of ion channels and receptors at the plasma membrane, as well as the targeting of kinases and phosphatases towards their substrates (Garner *et al.*, 2000; Henley, 2003; Sheng & Sala, 2001).

Synapse-associated protein 97 (SAP97) was the first protein reported to directly interact with the GluA1 subunit (Figure 2). SAP97 has three PDZ domains and interacts with GluA1 C-terminal in its second PDZ domain (Leonard *et al.*, 1998). SAP97 highly accumulates at GluA1-containing synapses, which suggested that SAP97 could act as an anchoring molecule to help GluA1 reside stably in the synaptic surface (Valtschanoff *et al.*, 2000). The interaction between SAP97 and GluA1 first occurs in the receptor secretory pathway and is essential for the transport of the receptor from the ER to the *cis* face of the Golgi apparatus, with SAP97 dissociating from the complex at the plasma membrane (Sans *et al.*, 2001; Santos *et al.*, 2009). SAP97 was also reported to be important for the recruitment of PKA and PKC through the A Kinase Anchoring Protein 79 (AKAP79), which forms a complex that targets PKA to GluA1, facilitating its S845 phosphorylation (Colledge *et al.*, 2000; Jiang *et al.*, 2006).

Protein 4.1N is another GluA1-interacting protein that belongs to a family of multifunctional cytoskeleton components and that is essential for assembly and maintenance of the actin cytoskeleton. Protein 4.1N is highly expressed in excitatory synapses, where it interacts and binds to the intracellular membrane region of GluA1, colocalizing with AMPARs. Thus, 4.1N may serve as an adapter to link GluA1 to the

actin cytoskeleton in spines, which suggests that GluA1-containing AMPARs are delivered to synapses along actin filaments (Jiang *et al.*, 2006; Shen *et al.*, 2000).

PDZ proteins also interact with the C terminus of GluA2, which conforms to a type II PDZ binding site, as opposed to GluA1 that conforms to a type I PDZ ligand. Yeast two-hybrid screening with the C-terminal tail of GluA2 yielded two highly related proteins, previously mentioned, GRIP and ABP, both containing seven consecutive PDZ domains (Bredt & Nicoll, 2003; Dong *et al.*, 1997). Both proteins localize at synapses but also in post-Golgi vesicles, suggesting a possible role in AMPAR trafficking to dendrites. Mutations of the GluA2 PDZ binding site that selectively block its binding to ABP and GRIP accelerate GluA2 endocytosis at synapses (Osten *et al.*, 2000), identifying ABP and GRIP as anchors that are crucial to AMPARs synaptic accumulation but not to their synaptic targeting (Santos *et al.*, 2009). The C termini of GluA2/3 also associate with the PDZ domain of PICK1, a postsynaptic scaffold (Xia *et al.*, 1999). This protein was first described to interact with the catalytic domain of PKCα, and it has been proposed that dimeric PICK1 can act to chaperone activated PKC and AMPARs (Chung *et al.*, 2000). Several studies indicate that PICK1 regulates the surface expression of GluA2 (Terashima *et al.*, 2004), and it has recently been shown that the phosphorylation state of GluA2 S880, mediated by PKC, which promotes the binding of PICK1 to GluA2, regulates the rate of GluA2 recycling to the membrane (Lin & Huganir, 2007; Santos *et al.*, 2009).

AMPA receptor transmembrane interactors

AMPARs contain transmembrane AMPAR regulatory proteins (TARPs) as their auxiliary subunits (Ziff, 2007). These proteins are subdivided in two classes: class I that

comprises γ-2 (or stargazin), γ-3, γ-4 and γ-8, and γ-5 and γ-7 as class II (Sumioka *et al.*, 2010). Stargazin, the prototypical TARP, was the first identified auxiliary subunit of AMPARs and it was originally characterized as the mutant gene in the Stargazer mouse, which exhibits an ataxic and epileptic phenotype, resulting from the lack of functional AMPAR channels in cerebellar granule cells (Chen *et al.*, 2000; Osten & Stern-Bach, 2006). These proteins contain four transmembrane domains and their carboxyl terminus interacts with the PDZ domains of PSD-95 (Chen *et al.*, 2000). TARPs appear to coassemble with AMPARs early in the synthetic pathway and control their folding, assembly and maturation, stabilizing and facilitating their export from the ER. Furthermore, TARPs promote AMPAR surface expression and are critical for clustering AMPARs at excitatory synapses through their interaction with PSD-95 (Chen *et al.*, 2000; Santos *et al.*, 2009; Sumioka *et al.*, 2010; Tomita *et al.*, 2003).

In a recent study, another family of transmembrane proteins, the cornichon proteins, was found to interact with AMPARs subunits (Schwenk *et al.*, 2009). According to this study, the majority of AMPARs in the rat brain are coassembled with two members of the cornichon family of transmembrane proteins (cornichon homologs 2 and 3), rather than with the TARPs. Cornichons increase surface expression of AMPARs in cultured cells and Xenopus oocytes. Moreover, electrophysiology recording from Xenopus oocytes showed that these proteins alter channel gating by markedly slowing deactivation and desensitization kinetics.

Vesicular / Cytoskeletal trafficking of AMPA receptors

Neurons pose many unique problems for the trafficking of membrane proteins because of their highly polarized and elaborate structure. Membrane proteins must

travel extremely long distances, and transmembrane proteins may be inserted at plasma membrane domains far from their final location (Shepherd & Huganir, 2007). Early in development, packets of receptors and scaffolding proteins travel along dendrites (Gerrow *et al.*, 2006). However, the precise cues that determine where these receptors stop and form synapses are not fully understood so far. Moreover, due to the primary localization of AMPAR mRNA at the neuronal cell body, the long-range dendritic transport of AMPARs is likely to depend on the microtubular cytoskeleton that runs along dendritic shafts (Esteban, 2003; Groc & Choquet, 2006). The transport of membrane organelles on microtubule tracks is an active process that relies on the motor proteins of the kinesin and dynein superfamilies (Hirokawa & Takemura, 2005). The PDZ domain-containing protein GRIP / ABP, besides binding to the C-terminal PDZ binding motif of GluA2 and GluA3, interacts directly with the heavy chain of conventional kinesin (KIF5), suggesting that GRIP may also serve as the link between AMPARs and microtubular motor proteins (Esteban, 2003; Setou *et al.*, 2002; Shepherd & Huganir, 2007).

Although dendrites contain microtubules along which most cargo is transported, dendrites also contain actin. Particularly, dendritic spines are devoid of microtubular cytoskeleton, but enriched of highly motile actin filaments (Fischer *et al.*, 1998). Therefore, at some point, AMPAR-containing organelles, trafficking along microtubular tracks, must be transferred to the actin-based cytoskeleton for their final delivery at synapses (Esteban, 2003). Myosins, the main actin-dependent motor proteins, have recently been implicated in AMPAR transport. Myosin Vb has been associated with GluA1 and the expression of the myosin Vb tail domain in developing hippocampal neurons enhances the accumulation of GluA1 in the soma, reducing the surface

expression of this subunit (Lisé *et al.*, 2006). Myosin VI has also been implicated in AMPAR trafficking, since myosin VI-deficient neurons exhibit deficits in activity-dependent AMPAR internalization as well as a decrease in the number of synapses and dendritic spines (Shepherd & Huganir, 2007). Also, protein 4.1N may act as an actin adaptor between AMPARs and the actin cytoskeleton, since it was shown to associate with AMPARs and stabilize the surface expression of GluA1 (Shen *et al.*, 2000; Shepherd & Huganir, 2007).

Exocytosis

AMPARs localization is a highly dynamic process, with AMPARs cycling in and out of synapses under a variety of situations related to synaptic plasticity and development. One of the last steps in the long journey of AMPARs to the synapse is their delivery into the specialized dendritic membrane that constitutes the postsynaptic terminal (Groc & Choquet, 2006). However, the precise targeting and insertion of receptors is extremely complicated. Despite intense study, it is still unclear whether AMPARs are first inserted into extrasynaptic plasma membrane or directly into synapses. Some theories support the idea that AMPARs first are exocytosed into the plasma membrane at extrasynaptic sites, followed by their lateral diffusion at the neuronal surface until they reach dendrites and are finally trapped at synapses through anchoring in the PSD (Figure 3B) (Groc & Choquet, 2006; Shepherd & Huganir, 2007). Initial studies with epitope-tagged transfected receptors have suggested that AMPARs are inserted along dendrites in a subunit-specific manner (Passafaro *et al.*, 2001) and a recent study using an innovative method to measure receptor insertion of AMPARs has suggested that most receptors are predominantly exocytosed at extrasynaptic sites

(Adesnik *et al.*, 2005). Silencing of surface AMPARs with a membrane-impermeable photoreactive AMPAR antagonist allowed measuring the real-time trafficking of native AMPARs electrophysiologically. The recovery of synaptic receptors measured was surprisingly slow, taking hours rather than minutes, and the fast cycling of surface AMPARs from intracellular pools occurred almost exclusively at extrasynaptic somatic sites, on a much smaller timescale, suggesting that newly inserted extrasynaptic AMPARs travel along dendrites to synapses by lateral diffusion (Adesnik *et al.*, 2005; Groc & Choquet, 2006; Santos *et al.*, 2009). More recently, in an attempt to understand the mechanisms underlying this lateral diffusion of AMPARs, several studies showed evidences that the exchange of receptors from extrasynaptic to synaptic sites depends on the interaction of Stargazin with PSD-95, since disruption of this interaction increases AMPAR surface diffusion and prevents AMPAR accumulation at synapses (Bats *et al.*, 2007; Santos *et al.*, 2009), suggesting that the stargazin-PSD-95 complex is critical for the retention of AMPARs at the synapse.

However, these data are inconsistent with many studies, including results from the same laboratory, that found more rapid insertion of AMPARs into the plasma membrane at dendrites and synapses (Lu *et al.*, 2001). This would be possible if AMPARs were trafficked intracellularly into dendrites via cytoskeleton-associated motors and then directly inserted at synaptic sites, a hypothesis recently supported by two studies (Figure 3A). Real-time measurements of receptor exocytosis in organotypic hippocampal slice cultures using time-lapse two-photon laser microscopy and AMPAR subunits tagged with super-ecliptic pHluorins (SEP) (green-fluorescent protein variants with strong pH-dependent fluorescence) showed that the strengthening of glutamatergic synapses by NMDAR activation selectively increased GluA1-containing receptors in

spine surfaces with no significant net change on the nearby dendrite (Groc & Choquet, 2006; Kopec *et al.*, 2006; Santos *et al.*, 2009).

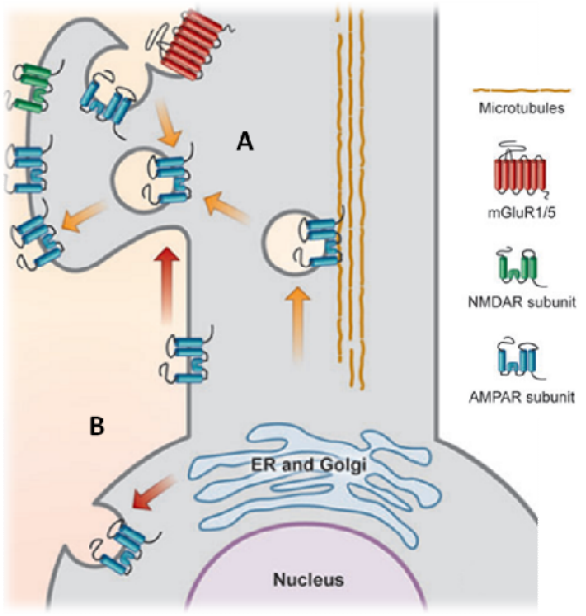


Figure 3 – Targeting and insertion of AMPARs in the synaptic membrane. Two hypotheses are proposed. (A) AMPARs are transported along dendrites by the cytoskeleton machinery into the synapses, where they are directly inserted by exocytosis. (B) The other possibility suggests that AMPARs are firstly exocytosed in extrasynaptic sites, travelling then by lateral diffusion to the synaptic membrane where they are ultimately anchored to the PSD [Adapted from (Shepherd & Huganir, 2007)].

Accordingly, another study found that overexpression of a dominant negative form of a subunit of the exocyst complex impaired the insertion of receptors at the spine surface, promoting their accumulation intracellularly, suggesting that Exo70 mediates AMPAR budding from intra-spine compartments directly within the PSD rather than at extrasynaptic membranes (Gerges *et al.*, 2006; Santos *et al.*, 2009). Most likely, a combination of all these mechanisms occurs, possibly depending on the subunit

composition of the receptors and the context of the neuron activity state (Shepherd & Huganir, 2007).

Moreover, many studies have shown that the mechanisms regulating AMPAR exocytosis are subunit specific. While GluA2 insertion is rapid and occurs constitutively under basal conditions, without the need for synaptic activity, GluA1 exocytosis is slow but inducible, requiring the activation of NMDARs or stimulation with insulin (Passafaro *et al.*, 2001). Although both subunits ultimately concentrate in synapses, GluA2 subunit accumulation in these structures is faster than that of the GluA1 subunit which, given the different hypothesis of exocytosis, suggests that either the GluA1 is inserted initially at extrasynaptic sites, whereas GluA2 subunit is inserted more directly at synapses, or that both subunits are inserted at extrasynaptic sites but the GluA2 subunit diffuses faster in the membrane and thus accumulate faster in synapses (Groc & Choquet, 2006). Hence, given that endogenous AMPARs consist mostly of either GluA1/2 or GluA2/3 heteromers, and that GluA1 trafficking signals dominate over GluA2 in controlling insertion (Shepherd & Huganir, 2007), a simple model has been proposed based on these subunit-specific trafficking rules: GluA2/3-containing receptors are continuously cycling in and out of synapses, preserving the total number of synaptic AMPARs (the constitutive pathway), whereas GluA1/2 receptors are added into synapses in an activity-dependent manner during synaptic plasticity (the regulated pathway) (Malinow *et al.*, 2000). Therefore, the constitutive pathway may maintain synaptic strength despite protein turnover, and the regulated pathway may act transiently upon the induction of synaptic plasticity (Shepherd & Huganir, 2007).

Endocytosis

In addition to their regulated trafficking and insertion into the synaptic membrane, the expression of AMPARs in the synapse is also dependent on their regulated internalization (Gomes *et al.*, 2003). This process is thought to occur through clathrin-mediated endocytosis (Man *et al.*, 2000) in specializations stably positioned adjacent to the postsynaptic membrane in dendritic spines. These endocytic zones lie in lateral domains of the spine, and they develop and persist independently of synaptic activity (Blanpied *et al.*, 2002). Furthermore, their localization in close proximity to the PSD was found to play an important role in maintaining synaptic AMPARs and in sustaining basal excitatory neurotransmission.

Endocytosis of AMPARs is similar to the stimulated endocytosis of G-protein coupled receptors (Carroll *et al.*, 2001) in that both processes occur via clathrin-coated pits and require analogous proteins of the core endocytic protein machinery, such as dynamin and endophilin, essential for endosome formation, as well as AP-2 that links specific cargo with the clathrin lattice. Indeed, the expression of a dominant-negative form of dynamin was shown to block clathrin-mediated endocytosis, and consequently, the endocytosis of AMPARs (Carroll *et al.*, 1999). However, postsynaptic endocytosis of receptors is thought to be mediated by specific protein isoforms. Dynamin 2 and 3, GTPases with a crucial role in severing the neck of invaginated clathrin-coated vesicles, are mostly postsynaptic and localized to the PSD via their interaction with the postsynaptic scaffolding proteins Shank and Homer, respectively (Gray *et al.*, 2003; Okamoto *et al.*, 2001). Likewise, distinct isoforms of endophilins (2 and 3) are localized to postsynaptic membranes (Shepherd & Huganir, 2007). Moreover, other postsynaptic proteins, such as the one encoded by the immediate-early gene CPG2 (Cottrell *et al.*,

2004), and the Arc/Arg3.1 protein, also encoded by an immediate-early gene (Chowdhury *et al.*, 2006), are thought to selectively promote the endocytosis of AMPARs.

Furthermore, the internalization of AMPARs can be caused upon several stimuli, such as the activation of NMDA or insulin receptors (Carroll *et al.*, 1999; Man *et al.*, 2000), with the subsequent activation of downstream signaling pathways. Thus, depending on the endocytic stimulus and activated signaling cascade, AMPARs can be differentially sorted between recycling and degradative pathways following endocytosis. These divergent pathways are in the basis for mechanisms of synaptic plasticity and in the regulation of basal neurotransmission. Following their incorporation in clathrin-coated pits, endocytic vesicles carrying membrane-bound receptors are targeted to the early endosome, from where they are sorted to either a recycling endosome or a late endosome. AMPARs sorted to the late endosome are usually fused with lysosomes and subsequently degraded, and this mechanism may underlie LTD phenomena. Receptors targeted to recycling endosomes can then be reinserted into the synaptic membrane, allowing the maintenance of a stable pool of AMPARs in synapses, which in turn underlies LTP phenomena. Moreover, these sorting cycles between recycling/degradation pathways can also underlie scaling mechanisms associated with homeostatic plasticity (Hanley, 2010).

AMPA receptors in synaptic plasticity

As previously mentioned, it is thought that the modulation of AMPARs, through mechanisms such as the regulation of the stoichiometry of subunits, RNA processing events, post-translational modifications, interacting proteins and other events that highly

impact and mediate the trafficking mechanisms and localization of AMPARs, underlies the phenomena of 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 is exquisitely regulated by highly intricate molecular mechanisms (Fleming & England, 2010; Shepherd & Huganir, 2007) and it occurs at the level of synapses that become stronger or weaker in response to specific patterns of activity. These changes mediate the efficiency of synaptic transmission and, consequently, the activity of neuronal networks, ultimately representing the cellular correlate of learning and memory. The basic mechanism for activity-dependent synaptic plasticity was first formally postulated by D.O. Hebb in 1949, who stated that when pre- and post-synaptic cells are repetitively active together, the efficiency of transmission between them improves (Hebb, 1949). Only in 1973, Hebb's theory gained experimental support with the impacting observation, by Bliss and Lømo, that repetitive activation of excitatory synapses in the hippocampus triggered a persistent increase in synaptic transmission lasting hours and even days (Bliss & Lomo, 1973). This long-lasting increase in synaptic strength, upon a short period of high-frequency stimulation, has been termed long-term potentiation (LTP), and the converse process, a persistent weakening of synaptic strength relative to baseline triggered by prolonged low-frequency stimulation, has been termed long-term depression (LTD) (Genoux & Montgomery, 2007; Santos *et al.*, 2009). Despite being the most thoroughly studied forms of synaptic plasticity, the molecular mechanisms mediating LTP and LTD are still unclear.

In general, two molecular mechanisms seem to underlie the changes in synaptic strength: either changes in the amount of neurotransmitters released by presynaptic neurons into the synaptic cleft or changes in the number and function of receptors on the postsynaptic neuron that respond to those neurotransmitters (Fleming & England, 2010). This second mechanism has gained particular support in the last decade, even though Lynch and Baudry had already proposed an increase in the number of synaptic GluRs during LTP more than twenty years ago (Lynch & Baudry, 1984). This idea came back to light after electrophysiological experiments suggested the existence of 'silent synapses' (Isaac *et al.*, 1995). These synapses, lacking AMPARs but with NMDARs, upon induction of LTP are converted to 'functional' synapses by delivery of AMPARs to the synaptic membrane, which prompted the idea that at excitatory synapses the insertion or removal of AMPARs from the PSD underlie the changes in synaptic strength associated with LTP and LTD, respectively (Santos *et al.*, 2009).

AMPA receptors in long-term potentiation (LTP)

AMPARs in the adult hippocampus contain GluA1/2 or GluA2/3 heteromers, but several lines of evidence point to a central role for the GluA1 subunit in hippocampal LTP, since knockout mice for GluA1 subunit were reported to be deficient in LTP (Zamanillo *et al.*, 1999). Accordingly, studies in organotypic hippocampal cultures transiently expressing GFP-tagged AMPARs showed a rapid translocation of GluA1-GFP to dendritic spines following LTP (Shi *et al.*, 1999). Moreover, the rapid translocation of this central subunit to the synaptic membrane requires a high-frequency stimulation and is highly dependent on the activation of NMDARs (Shi *et al.*, 1999), which is consistent with what was described earlier, suggesting the activity-dependent

insertion of GluA1-containing AMPARs in the synaptic membrane. Furthermore, it was shown that the re-insertion of GluA1-containing AMPARs into the plasma membrane from recycling endosomes is enhanced in response to LTP-inducing stimuli (Figure 4A), contributing not only to enhance synaptic efficacy but also to supply lipid membrane for the extension of dendritic spines during this phenomenon (Park *et al.*, 2004). Thus, these results seem to suggest the need of a stable pool of GluA1-containing AMPARs in close proximity to synaptic sites for the rapid modulation of the synaptic membrane upon LTP induction. Recent data by Isaac and collaborators suggest that GluA1 homomers are the first channels to be inserted during LTP, contributing to the early remodelling of synapses that occurs in the initial phases of this phenomenon, with a subsequent switch to GluA2-containing heteromers, thought to contribute to the consolidation of LTP (Figure 4A) (Plant *et al.*, 2006) although this finding remains controversial (Adesnik & Nicoll, 2007). Also, the changes in synaptic activity, based on the cycling of AMPARs in and out of synapses, are highly dependent on the phosphorylation of receptors and many studies support a critical role for CaMKII- and PKA-dependent phosphorylation of GluA1 at Ser831 and Ser845, respectively, in LTP. Particularly, while phosphorylation of Ser831 by CaMKII seems to be crucial for the induction of LTP (Lee *et al.*, 2000) but not required for the synaptic delivery of receptors (Hayashi *et al.*, 2000), PKA-mediated phosphorylation of Ser845 is necessary, although not sufficient, for this event (Figure 4A) (Malinow, 2003).

AMPA receptors in long-term depression (LTD)

Regarding LTD, many studies show that this phenomenon results from the endocytosis of AMPARs (Figure 4B) (Beattie *et al.*, 2000). Indeed, the activation of

NMDARs or insulin receptors can cause a loss of synaptically expressed AMPARs (Carroll *et al.*, 1999; Man *et al.*, 2000). Particularly, NMDAR-dependent LTD is known to require a moderate increase in postsynaptic calcium influx and activation of the calcium-dependent phosphatase calcineurin (Beattie *et al.*, 2000). The activation of this phosphatase mediates the regulation of the phosphorylation of AMPAR subunits, which is also important for LTD expression (Fig. 4B). Thus, LTD further requires the dephosphorylation of the GluA1 subunit in Ser831 and 845 (Lee *et al.*, 2000). The mechanisms by which these dephosphorylation states of the GluA1 subunit mediate the internalization of AMPARs are still unclear but may involve differential regulation of AMPAR binding partners (Shepherd & Huganir, 2007). Furthermore, the regulated endocytosis of AMPARs is also dependent of the GluA2 subunit. Interaction between the GluA2 subunit and the clathrin adaptor protein AP2 is required to AMPAR internalization, and also, phosphorylation of this subunit mediates the disruption of the stabilizer GluA2-GRIP interaction, resulting in the removal of synaptic AMPARs, by facilitation of the GluA2-PICK1 interaction (Perez *et al.*, 2001).

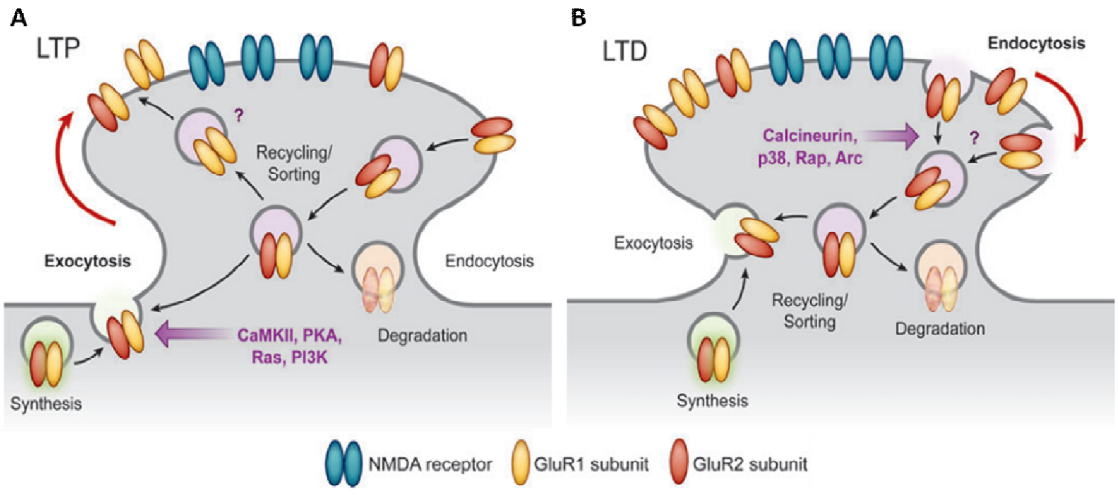


Figure 4 – Differential AMPAR trafficking during synaptic plasticity. (A) During LTP, AMPARs are supplied to the synapse either through exocytosis at extrasynaptic sites or through direct exocytosis at the synaptic membrane. AMPARs present in a stable recycling pool can also provide a source of receptors to be inserted during this phenomenon. It is thought that upon induction of LTP, homomers of GluA1 receptors are the first to be inserted into the synapse, occurring then a switch to heterotetramers of GluA2-containing AMPARs. Some kinases and downstream signaling molecules are involved in the induction of LTP and insertion of receptors to the synapse. (B) AMPAR endocytosis occurs during LTD. Receptors diffuse out of the PSD to lateral endocytic sites where they are internalized. Phosphatases such as calcineurin are required for the dephosphorylation of AMPAR subunits and subsequent endocytosis. [Adapted from (Shepherd & Huganir, 2007)].

AMPA receptors in homeostatic plasticity

Many of the plastic changes that underlie developmental and learning-related adaptations are thought to require synapse-specific changes in synaptic strength, yet these processes exert a powerful destabilizing influence on network function (Abbott & Nelson, 2000). Hebbian types of plasticity, like LTP or LTD, are able to strengthen synaptic inputs that are effective at depolarizing the postsynaptic neuron and weaken inputs that are not, thus reinforcing useful pathways in the brain (Abbott & Nelson, 2000; Malenka & Bear, 2004). However, these mechanisms pose a serious problem to the stability of neuronal networks, since synapses that are strengthened become more effective at depolarizing the postsynaptic neuron and will continue to be strengthened in an unconstrained positive feedback cycle, eventually driving neuronal activity to saturation (Abbott & Nelson, 2000; Turrigiano, 2008). These evidences imply that neurons must have some kind of sensor that detects these perturbations in their own

excitability and that triggers feedback mechanisms to counteract these destabilizing changes in activity, maintaining the homeostasis of the network.

Experimental evidence for these adaptive compensatory mechanisms started erupting over a decade ago [reviewed in (Turrigiano, 1999, 2008; Yu & Goda, 2009)]. Initial compelling evidences suggested that circuit activity is homeostatically regulated to maintain firing rates and/or firing patterns within certain functional boundaries. Studies carried by Turrigiano *et al.* and Burrone *et al.,* showed that when cortical or hippocampal neurons were induced to fire more than normal, over many hours, firing rates returned to baseline levels, and if neuronal firing was conversely reduced over time, neurons would also compensate and again firing rates would return to normal (Burrone *et al.,* 2002; Turrigiano *et al.,* 1998). These evidences, thus suggested that neuronal circuits possess mechanisms that maintain firing rates at a homeostatic set point.

Neuronal firing arises from the interplay between synaptic currents and the intrinsic firing properties of a neuron (Turrigiano, 2011). This would mean that the homeostatic regulation of excitability could impact on two major targets: intrinsic excitability and synaptic efficacy (Pozo & Goda, 2010). Neurons could slowly adjust synaptic strength and efficacy up and down in the right direction to stabilize average firing (Turrigiano *et al.*, 1998) or, conversely, they could modulate intrinsic excitability to shift the relationship between synaptic input and firing rate (Desai *et al.*, 1999; Turrigiano *et al.*, 1994). In principle, both of these processes could work, and many neurons appear able to undergo homeostatic regulation of firing via either mechanism (Desai *et al.*, 1999; Turrigiano *et al.*, 1994). In this review, we will only focus in the synaptic counterpart of these homeostatic mechanisms.

Homeostatic Synaptic Scaling

An important function of homeostatic synaptic plasticity in central neurons is to compensate for developmental or learning-induced changes in synaptic strength. This adaptation could, in theory, occur through changes either at the postsynaptic level, by altering postsynaptic receptors, or through changes in presynaptic neurotransmitter release. The first reports of synaptic homeostasis at excitatory synapses showed that a chronic blockade of activity resulted in an increased excitatory synaptic transmission, just by changing the postsynaptic accumulation of AMPA receptors (O'Brien *et al.*, 1998; Turrigiano *et al.*, 1998), with no changes in presynaptic function. Moreover, pharmacological manipulations of activity were shown to induce bidirectional compensatory changes that increased or decreased the amplitude of miniature excitatory postsynaptic currents (mEPSCs) mediated by AMPARs, resulting in an effective scaling of the postsynaptic strength up or down (Turrigiano & Nelson, 2004). Blockade of spiking with Tetrodotoxin (specific blocker of voltage-gated sodium channels) resulted in increased amplitude of mEPSCs, whereas blocking GABA-mediated inhibition with bicuculline (antagonist of $GABA_A$ receptors) resulted in an initial increase in firing rates that, over a time scale, returned to control values (Turrigiano *et al.*, 1998). Such a postsynaptic scaling process is predicted to stabilize activity without changing the relative strength of synaptic inputs, since neurons are able to detect their activity, possibly through calcium-dependent sensors, and regulate their own excitability while preserving the relative differences between individual synapses, thus avoiding disruption of information-storage mechanisms. Due to this proportional change in postsynaptic strength, this homeostatic mechanism was termed as synaptic scaling (Turrigiano *et al.*, 1998).

Following these original studies that first evidenced the scaling of AMPA-mediated minis upon chronic activity modulation, much effort has been made to clarify the postsynaptic mechanisms that underlie homeostatic synaptic plasticity (Pozo & Goda, 2010; Turrigiano, 2008). These homeostatic changes in postsynaptic strength result from alterations in the composition and abundance of synaptic AMPARs (O'Brien *et al.*, 1998; Wierenga *et al.*, 2005). Therefore, the postsynaptic expression of homeostatic plasticity possibly relies on a variety of mechanisms that mediate activity-dependent delivery and stabilization of AMPARs at synapses (Figure 5). Interestingly, it was already shown that, rather than being synthesized and delivered from the cell soma, AMPARs are locally translated at dendrites. Ju and colleagues showed that chronic blockade of activity with TTX resulted in an enhancement of dendritic synthesis of AMPARs, as new receptors continued to accumulate when dendrites were physically separated from the cell body (Ju *et al.*, 2004).

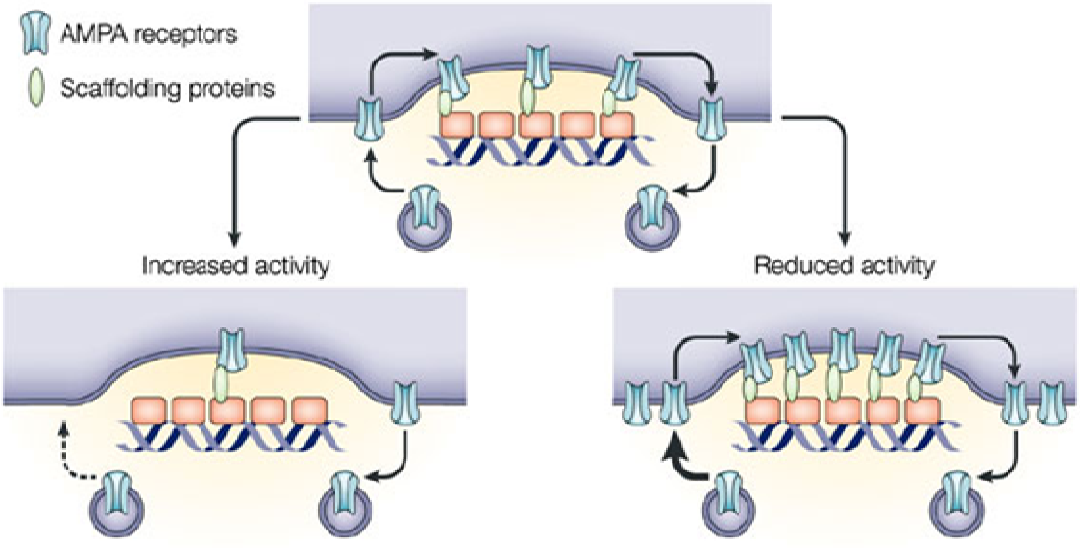


Figure 5 – Homeostatic synaptic scaling is accompanied by changes in the accumulation of AMPA receptors at synaptic sites. In response to chronic changes in activity, neurons trigger homeostatic mechanisms to counteract perturbations in their excitability and restore their

activity to a homeostatic set point. This adaptation can occur by adjusting the trafficking and insertion of receptors into synapses. While chronic enhancement of neuronal activity results in decreased accumulation of AMPARs, blockade of activity promoted the traffic and insertion of novel receptors into the synapse. [Adapted from (Turrigiano & Nelson, 2004)].

While studies agree that synaptic scaling is induced by changes in AMPAR accumulation at synapses, there is less agreement on the subunit composition of the newly inserted receptors. While some studies reported a proportional increase of both GluA1 and GluA2 subunits of AMPARs upon blockade of activity (O'Brien *et al.*, 1998; Wierenga *et al.*, 2005), other studies report only the postsynaptic recruitment of GluA1 but not GluA2 (Ju *et al.*, 2004; Sutton *et al.*, 2006). Moreover, Ju and colleagues further describe that this accumulation of GluA1 to the synapse upon blockade of activity is due to the specific enhancement of GluA1 dendritic synthesis. Altogether these evidences seem to propose that synaptic scaling may occur as a consequence of regulatory mechanisms that not only change AMPAR traffic and synaptic insertion upon changes in activity, but also regulate the dendritic availability of AMPARs transcripts and their local translation.

Molecular pathways underlying synaptic scaling

Since the first descriptions of homeostatic plasticity and synaptic scaling, the knowledge on the cellular properties of this type of plasticity has increased significantly. However, the molecular mechanisms that underlie this negative feedback, by which synaptic strength is adjusted, are still poorly understood (Pozo & Goda, 2010). Nevertheless, novel players, whole loss of function interferes with the expression of

homeostatic plasticity, have been identified and contribute to emphasize the complexity of possible signaling pathways that neurons use to maintain their homeostasis.

One of the first identified players was the immediate-early gene Arc/Arg3.1. Arc protein is rapidly induced by the chronic changes in activity used to promote synaptic scaling *in vitro*. Also, overexpression of the protein results in decreased AMPAR-mediated currents and prevents the increase in mEPSC amplitude induced by chronic TTX. Conversely, knockdown of Arc elevates AMPAR-mediated transmission and occludes the effect of TTX on mEPSCs (Rial Verde *et al.*, 2006; Shepherd *et al.*, 2006). Finally, the effect of Arc in AMPAR-mediated currents seems to occur via its ability to activate a novel and selective AMPAR endocytic pathway (Chowdhury *et al.*, 2006).

Other players involved in mechanisms that regulate the expression of homeostatic plasticity are β3 integrins. A recent study showed that, under basal conditions, β3 integrins act to stabilize synaptic AMPARs and loss of function of these cell adhesion molecules specifically impaired the homeostatic scaling of mEPSCs mediated by a TTX-induced blockade of activity. Thus, this study not only suggests a specific postsynaptic requirement for β3 integrins in scaling up of AMPARs but also an important contribution of the extracellular matrix in coordinating homeostatic plasticity (Cingolani *et al.*, 2008).

Several studies have also proposed a role for some secreted molecules in shaping homeostatic adaptations of synaptic strength. Stellwagen and Malenka showed that TNFα, a pro-inflammatory cytokine produced by glial cells, upregulated the cell surface expression of AMPARs as well as their synaptic insertion, upon pharmacological blockade of activity. This resulted in a compensatory increase in AMPAR-mediated

currents and thus, implicated TNFα in the regulation of the mechanisms inducing synaptic scaling (Stellwagen & Malenka, 2006).

Finally, Retinoic acid (RA) has just recently been included in the list of molecules that are implicated in mechanisms of homeostatic plasticity. In a recent study, blockade of activity with TTX and APV to induce synaptic scaling also resulted in an increased synthesis of RA (Aoto *et al.*, 2008). Moreover, applying RA by itself induced synaptic scaling of AMPARs, occluding the same effect induced by TTX. Also, this scaling of AMPARs induced by RA was due to an increased local synthesis of the GluA1 subunit, through signaling via the RA receptor RARα (Aoto *et al.*, 2008; Maghsoodi *et al.*, 2008).

The Caspr1/Contactin1 complex

Contactin 1 associated protein (Caspr1), also known as paranodin, is a type I integral membrane protein of 190kDa, highly expressed in the CNS and it was first described as an interactor for the cell adhesion molecule Contactin1 (Einheber *et al.*, 1997; Peles *et al.*, 1997). These two proteins form a heterodimer that localizes to neuronal membranes, particularly to paranodal junctions shortly after the onset of myelination (Einheber *et al.*, 1997).

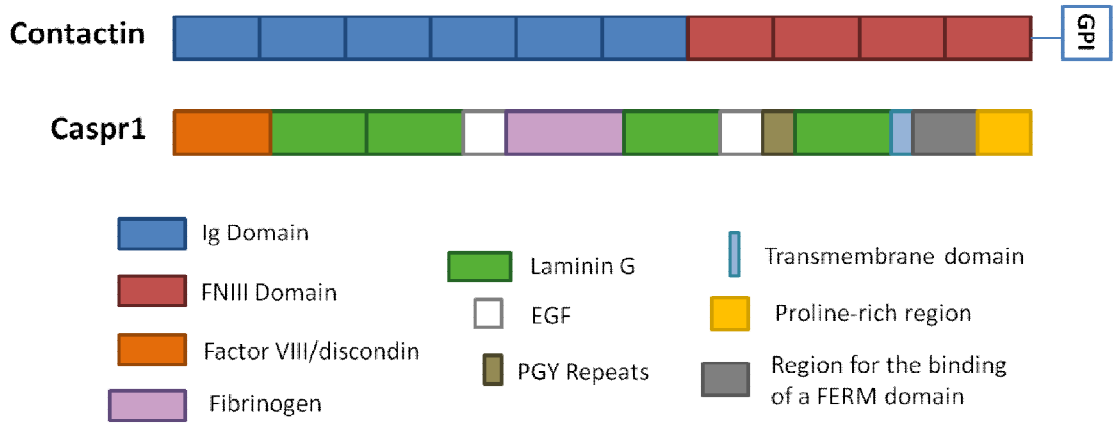


Figure 6 – Schematic representation of Contactin and Caspr1. The extracellular domain of Caspr1 has a discoidin domain next to the N-terminal, four laminin G domains, two repeats EGF-like and several PGY (Pro – Gly – Tyr) repeats. Its intracellular C-terminal has a region for the binding of FERM domains and a sequence rich in proline residues. Contactin presents a modular structure composed of 6 Ig domains and 4 repeats of the fibronectin domain.

Contactin1 is a glycoprotein of the immunoglobulin superfamily (Ig) and it presents a modular structure with repeats of the Ig domain and of a fibronectin domain (Figure 6). Caspr1, on its turn, is composed by a mosaic of domains, typically involved in protein-protein interactions. Since it belongs to the family of neurexins, its overall extracellular architecture is very similar to that of neurexins and it contains a discoidin domain next to its N-terminal, several sequences with homology to the extracellular matrix proteins laminin A, agrin, slit and perlecan, two epidermal growth factor (EGF)-like regions, a central fibrinogen domain and several PGY (proline – glycine – tyrosine) repeats (Figure 6).

Its intracellular C-terminal tail, on the other hand, possesses particular interest, since it contains a juxtamembrane region for the binding of molecules containing FERM domains, such as protein 4.1N (or 4.1B), schwannomin and β-integrin1

(Denisenko-Nehrbass *et al.*, 2003a; Denisenko-Nehrbass *et al.*, 2003b), and a sequence rich in proline residues, with at least one canonical SH3-domain binding site, which suggests the interaction of Caspr1 with several molecules typically involved in signaling pathways (Peles *et al.*, 1997). In fact, it has already been shown the selective interaction between the C-tail of Caspr1 and the SH3 domains of Src, Fyn, p85 and PLCγ in *in vitro* essays (Figure 6) (Peles *et al.*, 1997). Moreover, the interaction between Caspr1 and Src was further confirmed when both proteins were exogenously expressed in co-transfected COS7 cells: immunoprecipitation with several c-Src-specific antibodies was able to co-precipitate Caspr1 from lysates of transfected cells (Peles *et al.*, 1997).

Caspr1 was one of the first constituents of the paranodal junctions to be identified (Einheber *et al.*, 1997; Menegoz *et al.*, 1997). Biochemical studies showed that Caspr1 associates in *cis* with Contactin1 (Peles *et al.*, 1997), and this complex is highly enriched in the paranode junctions, septate-like junctions that seal the myelinizing sheath to the axonal membrane, and thus, permit the anchoring of myelin to axons (Rios *et al.*, 2000), exerting a quite well characterized function in the process of myelinization. Mice that lack Caspr1 exhibit tremor, ataxia and motor paresis (Bhat *et al.*, 2001). Moreover, in the absence of Caspr1 there is a perturbation of the paranodal organization and altered distribution of the junctional components, such as Contactin1 and neurofascin-155. Accordingly, the nerve conduction velocity is markedly reduced in Caspr1 mutant mice (Bhat *et al.*, 2001).

Intracellular traffic of the complex Caspr1/Contactin1

Up until now, the information available about the regulation of Caspr1 concerns its intracellular traffic. Apparently, the interaction between Contactin1 and Caspr1 is

essential for its expression at the cell surface. The interaction between these two proteins occurs in the ER and is mediated by the GPI anchor, the FNIII repeats and the IgG regions of Contactin1. This interaction is required for an efficient recruitment of Caspr1 to lipid rafts and sorting to the plasma membrane (Boyle *et al.*, 2001; Faivre-Sarrailh *et al.*, 2000).

Both Caspr1 and Contactin1 are essential for the generation of the axoglial junction, and their absence results in the disappearance of septa and widening of the space between the axon and the paranodal loop (Bhat *et al.*, 2001; Boyle *et al.*, 2001). In transgenic mice, the extracellular region of Caspr1 is sufficient for directing it to the paranodes and the retention of the Caspr1/Contactin1 complex at the junction depends on the presence of an intact cytoplasmic domain of Caspr1 (Gollan *et al.*, 2002). This region is able to bind to the cytoskeleton associated protein 4.1, allowing Caspr1 to stabilize the Caspr1/Contactin1 adhesion complex at the paranodal junction by connecting it to cytoskeletal components within the axon. Moreover, expression of Caspr1 on its own, in neuroblatoma cells, resulted in its ER retention, a phenomenon that seems to be dependent on an ER retention signal located in the extracellular PGY-repeat region of Caspr1 (Bonnon *et al.*, 2007; Bonnon *et al.*, 2003).

Synaptic role of Caspr1/Contactin1 complex

Both Contactin1 and Caspr1 have already been found in synaptic sites, throughout dendrites of CA1 area cultured hippocampal neurons, where they co-localize with synapsin-1 and synaptophysin (Murai *et al.*, 2002). Thus, by anchoring to the synaptic membrane, the complex Caspr1/Contactin1 might regulate intracellular interactions required for changes in synaptic strength, for example. Contactin1 was

shown to have a function in synaptic plasticity, particularly in LTD: whole-cell recordings from CA1 pyramidal cells revealed that mice with disrupted Contactin1 gene expression are specifically impaired in paired pulse facilitation (PPF) and LTD. Ablation of Contactin1 did not affect the development of the synaptic ultrastructure in the CA1 area (Murai *et al.*, 2002), but a molecular analysis indicated that Contactin1 is essential for the membrane and synaptic targeting of Caspr1 (Murai *et al.*, 2002). Another study investigated whether mice mutant for Caspr1 have deficits in synaptic plasticity and axonal organization similar to those observed in Contactin1 mutants, but according to this study, Caspr1 mutants have normal synaptic transmission and plasticity in the CA1 region of the hippocampus (Pillai *et al.*, 2007).

Role of Caspr1 in AMPA receptors regulation

Just recently, a study carried out in our laboratory suggests that the complex Caspr1/Contactin1 may regulate the transport of AMPA receptor subunits as well as their genetic expression. By combining affinity purification of protein complexes with mass spectrometric analysis of their composition, Santos *et al.*, were able to identify Caspr1 as a novel binding partner for AMPARs, particularly for the GluA4 subunit. Later on, performing immunoprecipitation and pull-down assays they found biochemical evidences suggesting Caspr1 as a strong interactor also for the GluA1, GluA2, GluA2L subunits (Santos, 2009; Santos *et al.*, 2010).

Also, biotinylation assays showed that overexpression of Caspr1 and Contactin1 leads to an increase in GluA1 cell surface levels and electrophysiology studies indicated that the C-terminal domain of Caspr1 increases the amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs), which suggests that the

intracellular region of Caspr1 increases the traffic of AMPARs to synapses (Santos, 2009).

Furthermore, in addition to affecting AMPAR traffic, preliminary results indicated that Caspr1 slightly increases GluA1 total protein levels, without affecting protein stability. This result prompted the idea that the increase in GluA1 levels could be due to an effect of Caspr1 in GluA1 mRNA levels, which was confirmed by quantitative real-time PCR experiments. These preliminary results suggest, then, that the complex Caspr1/Contactin1 may regulate the expression and, possibly, the stability of GluA1 AMPAR subunit and, hence, participate in the modulation of synaptic strength involving changes in AMPARs (Santos, 2009).

Nevertheless, how Caspr1 promotes this upregulation of GluA1 mRNA levels is still not known. One hypothesis is that the effect of Caspr1 is mediated through a direct interaction with RNA-binding proteins. A similar mechanism has been described for the transmembrane stargazin-related protein γ7 that regulates the stability of the calcium channel $Ca_v2.2$ mRNA by binding through its C-terminal to the hnRNP A2, and preventing hnRNPA2 from binding to the $Ca_v2.2$ mRNA (Ferron *et al.*, 2008). Moreover, given the structure of the C-terminus of Caspr1, another possibility is that the upregulatory effect of Caspr1 is through the activation of signaling cascades that result in altered RNA binding protein activity.

Post-transcriptional regulation of the GluA1 AMPA receptor subunit

The regulation of transcription and translation is an obvious mechanism to control gene expression. In neurons, these mechanisms are highly dynamic and complex and have become essential regulatory mechanisms for proper neuronal function.

Local Protein Synthesis in Dendrites

Nowadays, it is well established that the consolidation of LTP (late-phase LTP) requires gene transcription and is highly dependent on new protein synthesis. This set of newly synthesized proteins can include not only AMPAR subunits, but also proteins necessary for the trafficking of the receptors or for their anchoring in the synapse, as well as proteins involved in the structural remodelling of dendritic spines that occurs during LTP (Derkach *et al.*, 2007). Indeed, the modulation of synaptic strength is also dependent on the modulation of AMPARs, either by changing the composition in subunits of receptors already expressed at the synapse or by the rapid recruitment of new receptors to this structure. However, the complex polarized structure of neurons poses a problem for this rapid synthesis and transport of subunits and receptors, required for neuronal plasticity, all the way from the cell body to dendritic spines. Thus, the exclusive dependence of plasticity phenomena in somatic protein synthesis would probably result in low efficiency of these mechanisms and impair synaptic plasticity, and consequently, memory formation and storage. This idea prompted the possibility of the occurrence of local dendritic protein synthesis underlying the many forms of long-term synaptic plasticity.

Up until recently, the lack of evidences for such a mechanism as local dendritic translation led neuroscientists to believe that proteins were only made in the cell body. mRNAs would be translated in the ER and translocated to the Golgi apparatus and, only then, the resulting protein would be transported to its target, the dendritic spine, for example, through the cytoskeleton machinery. Only when Steward and Levy, back in 1982, detected the presence of polyribosomes in the distal dendrites of dentate granule cell neurons (Steward & Levy, 1982), did the possibility of local protein synthesis start to be taken seriously (Sutton & Schuman, 2006). Later studies further showed that the entire translational machinery is present in dendrites, since, besides ribosomes, initiation and elongation factors for translation have also been found in these neuronal processes (Asaki *et al.*, 2003). Throughout these most recent years, several were the mRNAs found to be localized in dendrites, most of them coding for synaptic proteins involved in the modulation of synaptic strengthening and synaptic transmission, such as mRNA encoding for the microtubule-associated protein 2 (MAP2), the alpha-subunit of calcium-calmodulin-dependent protein kinase II (CaMKII), the brain-derived neurotrophic factor (BDNF), the activity-regulated cytoskeleton-associated protein (Arc), the tyrosine-related kinase B receptor (TrkB), the IP_3 receptor, the atypical protein kinase Mζ, the NMDAR GluN1 subunit and the glycine receptor alpha-subunit, among many others (for a review see [(Martin & Zukin, 2006; Schuman *et al.*, 2006)]. Thus, the subcellular targeting and localization of specific mRNAs to distinct regions provides an important means to regulate the gene expression in neurons and the activity-induced translational control of these localized mRNAs allows neurons to alter protein composition of specific synapses with great temporal and spatial resolution (Holt & Bullock, 2009; Poon *et al.*, 2006).

Since more mRNAs are continually being found in dendrites, suggesting that the number of different mRNA species in these neurites could reach the several hundred, it is difficult to evaluate the full spectrum of synaptic functions that this local translation might be regulating (Sutton & Schuman, 2006). Furthermore, the mechanism by which a specific mRNA is transported to a specific synaptic site is still largely a mystery. Still, neurons have somehow developed specific pathways that enable the transport of mRNAs to dendrites, where subsequently local translation can occur. Nevertheless, a model for mRNA transport and localization in dendrites is emerging (Figure 7). Not every step in this model has been verified for any single mRNA; rather, the model is a combination of data generated from observations of multiple different mRNAs (Bramham & Wells, 2007).

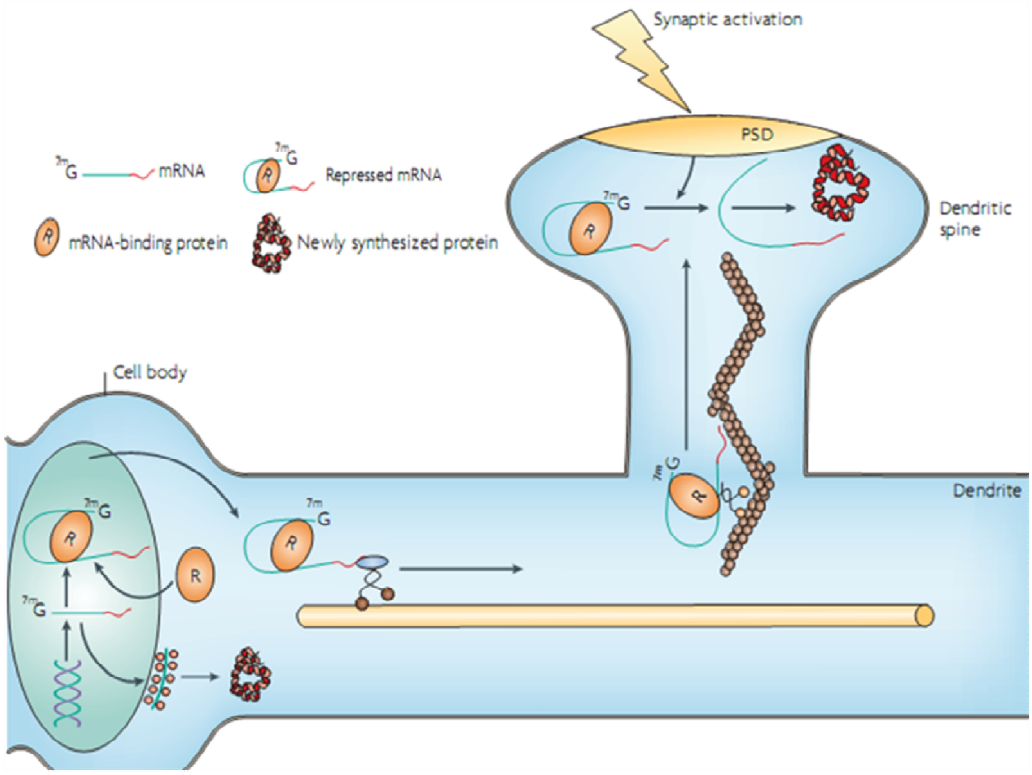


Figure 7 – Proposed model for mRNA transport and local translation in neuronal dendrites. Specific mRNAs can bind to mRNA-binding proteins that inhibit the translation of the mRNA in the protein-synthetic apparatus in the cell body. The repressed mRNA can then be

packaged and sequestered into transport granules that travel into the dendrites by kinesin motors on the microtubule-based cytoskeleton. Following synaptic activation, the granules are dispersed and the mRNA is localized to spines by the actin-based myosin motor proteins, where, after the repressive RNA-binding protein is neutralized, the mRNA is free to be translated [Adapted from (Bramham & Wells, 2007)].

Thus, it is widely accepted that most mRNAs are transported as part of large RNA-containing granules, namely large ribonucleoprotein particles (RNPs) containing proteins involved in RNA transport, protein synthesis, RNA helicases, heterogeneous nuclear ribonucleoproteins (hnRNPs), and RNA-associated proteins (Hirokawa, 2006). Moreover, it is thought that the mRNAs present in these granules are in a translationally dormant state, until a specific stimulus is able to activate translation (Martin & Zukin, 2006). This translational repression is likely to occur in the nucleus, where the mRNAs may bind to RNA-binding proteins that sequester the transcripts from the translational machinery in the cell body and mediate their inclusion into the RNPs. Following this incorporation, the granules travel along dendrites through the interaction with kinesin motors on the microtubule-based cytoskeleton. Upon synaptic activation, the granules can be dispersed and the repressed mRNAs are incorporated by the actin-based myosin motor proteins into spines, where the repressor binding protein is neutralized, leaving mRNAs free to be translated (Figure 7) (Bramham & Wells, 2007). Besides RNPs, different cytoplasmic macromolecular RNA structures exist in neurons, such as processing bodies (P-bodies) and stress granules (Sossin & DesGroseillers, 2006). The relationship between transport RNPs, P-bodies and stress granules is not currently understood, but these different granules are thought to be functionally distinct. P-bodies are mostly known to be foci of mRNA degradation, since they recruit mRNAs targeted

for deadenylation and degradation. Nonetheless, they are also thought to have a dual function: they can harbour translationally silenced mRNAs that can later exit again from P-bodies and re-engage in translation (Kulkarni *et al.*, 2010). As for stress granules, they are generated in response to stress, sequestering mRNAs from translation as a means to adapt the mRNA metabolism to the changing conditions (Sossin & DesGroseillers, 2006). Despite this model, the actual mechanism of regulation of local dendritic translation is still far from being completely understood. What determines the targeting of certain specific mRNAs to specific synaptic sites? How exactly is this mechanism regulated and what are the RNA-binding proteins that associate specifically with each mRNA? Do they have a role only in the translational control of these transcripts or do they also regulate their stability? These are some questions that are still not fully answered and that will be focused later.

Local Dendritic Synthesis of AMPA Receptor Subunits

One of the possible mechanisms to control the composition of AMPARs during the modulation of synaptic strength, upon neuronal activity, is through local translation at dendrites, in close proximity to synaptic sites, of mRNA molecules for specific AMPAR subunits. Accordingly, quite recently, a couple of studies showed that a substantial fraction of synaptic sites contain mRNA molecules for the GluA1 and GluA2 AMPAR subunits, which is consistent with a strategic positioning of these molecules for their local translation, and thus, allowing the regulation of the local abundance of receptors, as well as their composition and insertion to the membrane (Figure 8) (Grooms *et al.*, 2006; Ju *et al.*, 2004; Kacharmina *et al.*, 2000).

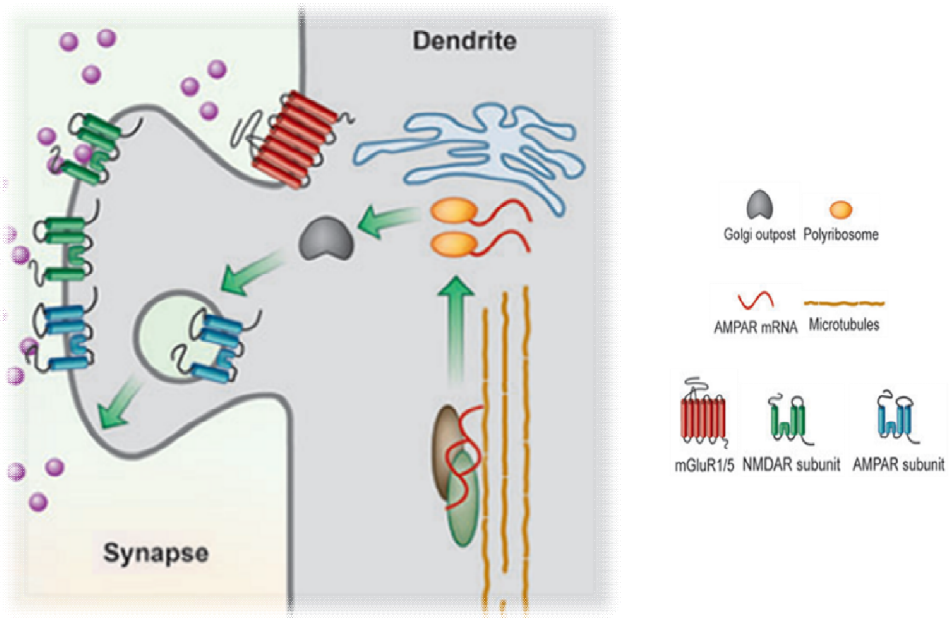


Figure 8 – Local dendritic synthesis of AMPARs. Several studies propose the local synthesis of GluA1/2 subunits in dendrites. These mRNA molecules can be trafficked out into dendrites via a RNA-protein complex travelling along the cytoskeleton. In synapses, mRNA can be translated upon neuronal activity [Adapted from (Shepherd & Huganir, 2007)].

Using biarsenical dyes, FlAsH and ReAsH, Malenka and collaborators showed that exogenously expressed GluA1 and GluA2 subunits can be synthesized in specific dendritic compartments, independently of the cell soma (Ju *et al.*, 2004). Moreover, they demonstrated that the pharmacological manipulation of neuronal activity, such as the activation of metabotropic glutamate receptors or the acute depolarization with KCl led to an increase in the synthesis of both GluA1 and GluA2 subunits, whereas a chronic blockade of synaptic activity, known to increase the strength of synapses by an accumulation of AMPARs, could specifically increase the dendritic synthesis of the GluA1 subunit (Ju *et al.*, 2004). Zukin and associates were also able to find endogenous mRNA molecules for GluA1 and GluA2 subunits localized to distal and proximal

dendrites in hippocampal neurons (Grooms *et al.*, 2006). They also suggested that the local abundance and localization of these mRNAs can be controlled by paradigms that manipulate glutamatergic signaling. That is, the activation of NMDARs results in a decrease of mRNA abundance, dependent on a higher influx of intracellular calcium and on the activation of the ERK (extracellular signal-regulated kinase) / MAPK (mitogen-activated protein kinase) signaling pathway, whereas the activation of metabotropic glutamate receptors increases GluA1 dendritic mRNA, by upregulating the anterograde transport of mRNA through the cytoskeleton machinery (Grooms *et al.*, 2006). These results, as it happens for the general mechanism of local dendritic translation, raise some doubts concerning the regulation of AMPAR subunits local synthesis. How are GluA1 and GluA2 transcripts targeted to specific synaptic sites? How is their transport regulated? Which proteins are binding to the mRNAs and repressing their translation? Is this regulatory mechanism also influencing the decay and stability of the transcripts?

Pathways of mRNA stability and turnover

In all organisms, the process of protein synthesis must be precisely regulated in order to minimize the energy costs for cells, and in many circumstances cells need to rapidly adapt the amount of specific proteins being produced. To achieve this, cells have developed the ability to degrade mRNAs so that patterns of protein synthesis can be altered rapidly, and thus, mRNA degradation directly affects protein synthesis through its impact on the concentration of mRNA available for translation (Belasco, 2010). This requirement may explain why mRNA stability is such a highly regulated post-transcriptional step, tightly coordinated with mRNA translation. mRNAs can either be

translated immediately or be sequestered such that they can later undergo translation or be degraded. In the latter case, several factors can modulate the decay rate of a certain mRNA and mediate the interrelationship between degradation and translation (De Rubeis & Bagni, 2010).

Pathways of mRNA turnover

In mammals, two major pathways for mRNA degradation have been described, being the most important the deadenylation-dependent mRNA decay. mRNAs are created with two integral stability determinants – the 5' 7-methylguanosine cap and the 3' poly(A) tail – that are incorporated co-transcriptionally. These two structures can protect transcripts from exonucleases and enhance the initiation of translation of the transcripts. Thus, in order to initiate decay, either one of these structures must be compromised or the mRNA must be cleaved internally by endonucleolytic attack (Garneau *et al.*, 2007; Wilusz *et al.*, 2001). In higher eukaryotes and also in yeasts, most mRNAs undergo decay by a pathway that is initiated by poly(A)-tail shortening. Once an mRNA is targeted to be destroyed by the cell, one of two irreversible routes can take place. Either the unprotected 3' end is attacked by the exosome, a large complex with 3' → 5' exonucleolytic activity, or the 5' cap is cleaved by a process known as decapping, which allows the mRNA body to be degraded in the 5' → 3' direction. However, despite the intense study dedicated to mRNA turnover, there are still some aspects concerning the enzymes, pathways and regulation of this mechanism that remain unclear. Apart from this, mRNA decay is now considered a key player in the regulation of gene expression (Garneau *et al.*, 2007; Wilusz *et al.*, 2001).

mRNA Stability and Turnover – Role of RNA-binding Proteins

As it was discussed until this point, post-transcriptional gene regulation, including mRNA turnover and translation, is an important way of regulating gene expression. In the CNS, there is a growing number of genes whose expression levels are controlled by changes in the rate of mRNA decay, and thus, involve a balance between mRNA stability and turnover (Perrone-Bizzozero & Bolognani, 2002). This balance may involve the interaction of transcripts with specific RNA-binding proteins. Examples of neuronal genes regulated via mRNA stability involving the interaction with a RNA-binding protein include developmentally regulated proteins, such as GAP-43 (Benowitz & Routtenberg, 1997); signaling molecules, such as calmodulin and CAMKII (Wu *et al.*, 1998); receptors, such as the β2-adrenergic receptor (Tholanikunnel *et al.*, 1999), the m4 muscarinic receptor (Lee & Malek, 1998) and the dopamine receptor D2 (Duan *et al.*, 2003); as well as channels, such as the $Ca_v2.2$ calcium channel (Ferron *et al.*, 2008).

In most cases, it is thought that the decay rate of mRNAs is regulated by interactions of the aforementioned sequence-specific RNA-binding proteins to *cis*-acting elements frequently located in the 3'untranslated region (3'UTR) of the transcripts. Several examples of this type of regulation over mRNA stability dependent on *cis*-elements on the 3'UTR of the transcripts can be pointed out. One first example focuses on the translational control of several mRNAs correlated with increases in their poly(A) tail length. This process, termed cytoplasmic polyadenylation, requires a polyadenylation hexanucleotide sequence (typically AAUAAA) as well as additional 3'UTR regulatory sequences, including cytoplasmic polyadenylation elements (CPE) with the general structure UUUUUAU. To be functionally active, the CPE sequence

requires an interaction with the CPE-binding protein (CPEB), a highly conserved zinc finger and RNA-recognition motif (RRM)-type RNA-binding protein, necessary for the cytoplasmic polyadenylation-induced translation. This phenomenon was initially observed in *Xenopus laevis* oocytes where Mos, cyclin B1 and several other mRNAs with short tails were translationally dormant, and only upon the poly(A) tail elongation translation would take place (Charlesworth *et al.*, 2004; Prasad *et al.*, 2008). Moreover, CPEB has already been shown to be present in dendritic layers of the hippocampus, at synapses in cultured neurons and in PSDs of adult brains, which suggests that CPEB is not only involved in the regulation of early development, but may also be potentially important in the regulation of mRNAs crucial for synaptic plasticity. Accordingly, translation of the α-CaMKII mRNA, which contains two CPEs in its 3'UTR and is localized to dendrites, being crucial for the induction of LTP, has been shown to be regulated by CPEB (type I) via cytoplasmic polyadenylation in the synaptic region, in response to synaptic activity (Wu *et al.*, 1998). Moreover, regarding AMPARs, it was recently demonstrated that the translation of mRNA for the GluA2 subunit is under the control of CPEB3, a CPEB (type 1) like protein. Richter and collaborators showed that CPEB3, in absence of synaptic stimulation, represses the translation of GluA2 mRNA, and that this repression is reverted upon NMDARs activation; also, in knockdowns for CPEB3, GluA2 mRNA translation is stimulated, suggesting, then, that CPEB3 may be a specific translational repressor for GluA2 mRNA (Huang *et al.*, 2006).

Another well-characterized sequence involved in mRNA stabilization is a 50- to 150-nucleotide sequence rich in adenosine and uridine, the so-called AU-rich element (ARE). These sequences are, like CPEs, located in the 3'UTR of mRNAs, however, they appear to have a very significant importance in the regulation of gene expression,

since 5-8% of human genes encode ARE-containing transcripts (Bakheet *et al.*, 2001). Although AREs were originally defined as an AUUUA core associated with instability, it became clear over the years that ARE motifs can vary and regulate mRNA stability in both directions (Barreau *et al.*, 2005). In fact, the interaction between ARE sequences and ARE-binding proteins can either block or enhance the recruitment of the mRNA decay machinery and lead to a rapid modification of gene expression (De Rubeis & Bagni, 2010). One of the first RNA-binding proteins known to bind AREs and to have a destabilizing function was the AU-binding factor 1 (AUF1), also known as hnRNP D. This factor was shown to modulate the decay of ARE-containing transcripts, namely the mRNA encoding the α2-subunit of the nitric oxide-sensitive guanylyl cyclase in the cerebellar granule cells: in basal conditions the transcript is bound to AUF, but upon NMDAR activation, AUF1 is downregulated and the α2 mRNA stabilized (Jurado *et al.*, 2006). Moreover, the mRNAs for the AT1 receptor (Berger *et al.*, 2005) and the β2-adrenergic receptor (Tholanikunnel *et al.*, 1999) have already been shown to be destabilized via AUUUUA hexamers in the 3'UTRs of the transcripts, consistent with ARE-like sequences.

One rather interesting example concerns the fragile X mental retardation protein (FMRP), absent in patients with Fragile X Syndrome (FXS), characterized by mental retardation. This protein is also an RNA-binding protein thought to regulate translation and subcellular localization of several transcripts (Zalfa *et al.*, 2003). Just recently, however, this protein was described to have a role in the regulation of mRNA decay, particularly for transcripts encoding the crucial synaptic protein PSD-95 (De Rubeis & Bagni, 2010; Zalfa *et al.*, 2007). The FMRP-binding site in the 3'UTR of the PSD-95 mRNA is close to three U-rich tracts, two of them actually containing two AREs. Thus,

the interaction of FMRP can be preventing the action/binding of other destabilizing proteins and protecting the PSD-95 mRNA from decay (Zalfa *et al.*, 2007).

Zipcode binding protein1 (ZBP1)

Phenomena such as axonal guidance and neurite outgrowth in developing neurons are highly dependent on the reorganization of the cytoskeleton. Moreover, these phenomena are also mediated by guidance cues that induce an asymmetric targeting and translation of β-actin mRNA in the growth cone (Huttelmaier *et al.*, 2005; Ming, 2006; Sasaki *et al.*, 2010; Welshhans & Bassell, 2011). The Zipcode binding protein1 (ZBP1) is involved in the translational control and transport of β-actin mRNA. ZBP1 contains two RNA recognition motifs (RRMs) and four heterogeneous ribonucleoprotein particle K-homology (hnRNP KH) domains, which are also known RNA-binding motifs.

ZBP1 recruits β-actin mRNA at the site where it is transcribed, by binding to a conserved 54-nucleotide element, known as the 'zipcode', located in the 3'UTR of β-actin mRNA (Ross *et al.*, 1997). ZBP1 binding to the β-actin mRNA induces both translational silencing of the transcript and its incorporation into RNPs. Following this incorporation, the granules travel along dendrites through the interaction with kinesin motors on the microtubule-based cytoskeleton (Figure 9). Upon particular stimuli, the tyrosine kinase Src is able to phosphorylate ZBP1 at a key tyrosine residue that is required for its binding to RNA, thus relieving the repression of translation of β-actin mRNA once the transcript reaches its destination (Figure 9) (Huttelmaier *et al.*, 2005). These sequential events provide both temporal and spatial control over β-actin translation and are necessary to its localization to the cell periphery.

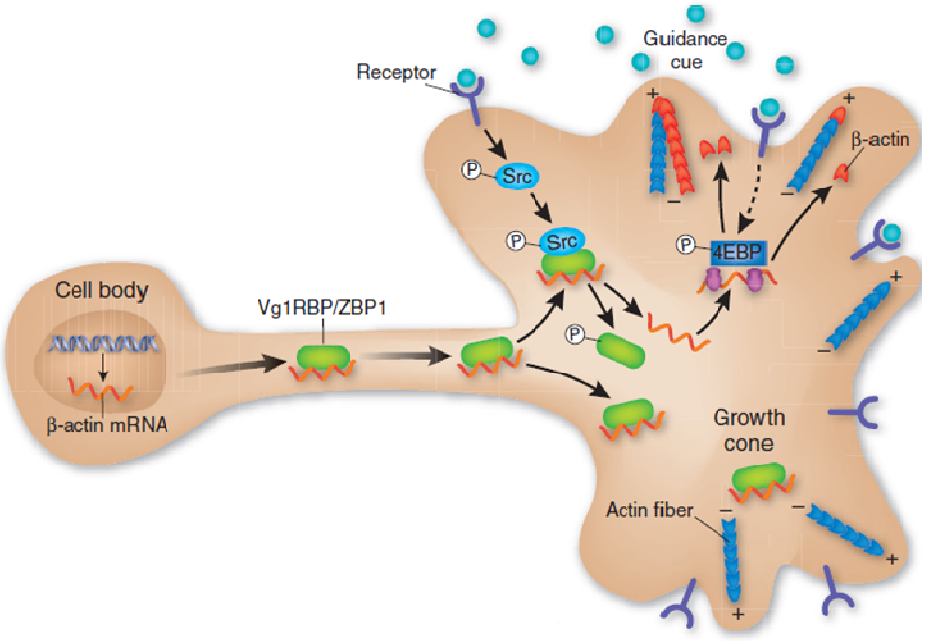


Figure 9 - A model for guidance cue–induced asymmetric transport and translation of β-actin mRNA mediated by ZBP1. Translocation of β-actin mRNA is controlled by ZBP1. ZBP1 associates with the β-actin mRNA in the nucleus and transports it into neuronal growth cones while blocking its translation initiation. A gradient of guidance cues activates yet unknown pathways to promote the asymmetric transport of granules containing ZBP1/β-actin mRNA complex into the growth cone periphery. Translation of β-actin mRNA is regulated by phosphorylation events. Phosphorylation of 4EBP promotes cap-dependent translation initiation, whereas Src kinase phosphorylates ZBP1 to release its block of translation initiation. Together, these cellular events lead to asymmetric distribution of newly synthesized β-actin and the preferential incorporation of this nascent β-actin into the cytoskeleton at one side of the growth cone, contributing to growth cone turning [Adapted from (Ming, 2006)].

Particularly at developing growth cones ZBP1 activity is important for neurotrophin-induced neurite outgrowth (Zhang *et al.*, 2001) and its Src-dependent phosphorylation is required for the assymetrical and local synthesis of β-actin and growth cone turning and guidance, induced by external cues such as BNDF and Netrin1

(Leung *et al.*, 2006; Sasaki *et al.*, 2010; Welshhans & Bassell, 2011; Yao *et al.*, 2006). Apart from its role in axonal guidance, ZBP1 also regulates the dendritic arboring of hippocampal neurons (Perycz *et al.*, 2011) and localizes to dendritic spines, regulating their density and structure through the local translation of β-actin (Eom *et al.*, 2003; Tiruchinapalli *et al.*, 2003). Moreover, ZBP1 has also been shown to bind to the mRNA of cofilin, an actin-depolymerizing factor, and mediate Slit2-induced local translation during growth cone collapse (Piper *et al.*, 2006). Thus, it is evident that ZBP1 plays a key role in the regulation of some transcripts with major importance in neuronal development and function, which endows ZBP1 with the potential to functionally regulate other transcripts.

Furthermore, ZBP1 has already been implicated in the regulation of stability of several other target mRNAs, such as c-Myc, CD44, βTrCP1 and β-catenin (Gu *et al.*, 2008; Leeds *et al.*, 1997; Noubissi *et al.*, 2006; Vikesaa *et al.*, 2006; Yisraeli, 2005)

RNA-binding proteins interacting with the GluA1 mRNA

The importance of the regulation of mRNA stability, particularly of transcripts involved in synaptic plasticity, raises the tantalizing question of how does this regulation occur for AMPAR subunits. It is already known that transcripts of GluA1 and GluA2 subunits localize to dendrites and specific synaptic sites and that their translation is controlled by paradigms of synaptic activation. But how exactly are these transcripts targeted and transported to dendritic spines? How is the translation of these transcripts repressed during their transport? What are the RNA-binding proteins either promoting or disrupting the stability of these mRNAs? It is already known that CPEB3 may act as a translational repressor for the GluA2 subunit mRNA. Also, it has recently

been shown that retinoic acid (RA) receptor α associates with the GluA1 mRNA in dendrites through the 5'UTR of the transcript, repressing its translation; when RA binds to the receptor, its association with GluA1 mRNA is decreased, relieving the translational repression (Poon & Chen, 2008). However, there is no knowledge of any RNA-binding protein stabilizing the GluA1 mRNA. Nevertheless, in recent studies by Santos *et al*. (unpublished data) a detailed screening of the 3'UTR of the GluA1 mRNA was performed to find possible conserved mRNA binding motifs. Indeed, multiple conserved canonical *cis* elements were found, among which several binding sequences for the RNA-binding protein HuR and for other RNA-binding proteins of the embryonic lethal abnormal vision (ELAV) family. In fact, proteins belonging to the Hu family are some of the few specific proteins thought to be key regulators of mRNA turnover in the brain.

Hu proteins were first identified as autoantigens in patients affected by paraneoplastic encephalomyelitis (Dalmau *et al.*, 1990). Later on, after the cloning and sequencing of these proteins, it was found that they are human homologues of the Drosophila ELAV, an RNA-binding protein whose deletion results in an embryonic lethal abnormal vision phenotype in flies (Robinow *et al.*, 1988). In mammals, four ELAV / Hu proteins have been described: HuR (also known as HuA) that is ubiquitously expressed, and HuB, HuC and HuD that are neuronal-specific. Structurally, the four proteins possess a high degree of sequence homology and they contain three ~ 90 amino acid-long RNA recognition motif-type (RRM) domains. These RRM domains are able to bind to AREs located in the 3'UTR of the transcripts. The first two RRMs, positioned at the N-terminal end, are separated from the third motif by a hinge region, thought to contain sequences that mediate the nuclear export of these

proteins. Indeed, Hu proteins have a relative similar expression both in the nucleus and the cytoplasm, which suggests that these proteins have a nucleocytoplasmic shuttling activity (Pascale *et al.*, 2008). Their export to the cytoplasm may be considered a main prerequisite for the protective effects from mRNA decay these proteins confer to their cognate target mRNAs (Fan & Steitz, 1998) and thus, they can modulate the post-transcriptional fate of mRNAs from birth to death, including the regulation of mRNA stability, localization and translation (Pascale *et al.*, 2008). Experimental support for this supposed stabilizing function of Hu proteins has already been provided. For example, overexpression of HuR increases the stability of several ARE-containing mRNAs (Fan & Steitz, 1998), as opposed to the knockdown of the protein that promotes a decrease of stability in target mRNAs (Rodriguez-Pascual *et al.*, 2000). Moreover, the interaction and stabilizing effect of Hu proteins has also already been reported for the mitogen-activated protein kinase phosphatase 1 (MKP-1) mRNA (Kuwano *et al.*, 2008) and for the neuronal growth-associated protein 43 (GAP-43) mRNA (De Rubeis & Bagni, 2010). These recent evidences implicate a role of ELAV like proteins in spatial memory formation, and suggest a model in which the RNA-binding proteins locally stabilize mRNAs whose products could be important for memory storage (Pascale *et al.*, 2008). Thus, considering this and having in account the canonical *cis* binding sites for ELAV proteins found in the 3'UTR of GluA1 mRNA, we hypothesize that these proteins may be binding to the GluA1 transcripts, allowing their stabilization and preventing their decay.

The objective of this introductory section was to review current knowledge regarding the structure and function of AMPA receptors as well as their regulatory

mechanisms. One of the questions in the field concerns the mechanisms that regulate the localized expression of AMPA receptor subunits, namely in the context of homeostatic synaptic plasticity. The work presented in this thesis contributes to elucidate some aspects of this emergent question.

Objectives of the present study

Glutamate receptors of the AMPA-type mediate most of the excitatory neurotransmission in the brain and play a major role in the expression of synaptic plasticity mechanisms that underlie learning and memory in the hippocampus (Santos *et al.*, 2009). Modulation of these receptors by alterations of receptor traffic and synaptic insertion, mRNA stability and turnover is crucial to induce long-term changes in synaptic strength and efficacy (Kessels & Malinow, 2009; Sutton & Schuman, 2006). Even though mechanisms of AMPAR trafficking are now very well characterized, the molecular mechanisms regulating AMPAR transcripts are still poorly understood.

A previous study carried out in our laboratory allowed the identification of the cell adhesion molecule Caspr1 has a novel interactor of the GluA1 subunit of AMPARs. The biochemical characterization of this molecule has shown that, besides its wide expression throughout dendrites, Caspr1 plays a major role in the cell surface expression of GluA1 as well as in its synaptic tagging. Moreover, preliminary data suggested an effect of Caspr1 in total levels of the GluA1 subunit, trough upregulation of its mRNA (Santos, 2009). Given the tantalizing hypothesis that these evidences raise of a novel post-transcriptional regulatory mechanism of AMPARs, it became important to further characterize the regulation of the GluA1 AMPAR subunit by Caspr1.

1. Preliminary results by Sandra Santos show that Caspr1 upregulates GluA1 protein and mRNA levels, with no effect in GluA1 protein stability. Thus, the primary goal of this study is to further confirm the effect of Caspr1 in total levels of GluA1. Moreover, we further intend to map the molecular determinants in Caspr1 responsible for its effect. Santos and colleagues found that the intracellular C-terminal domain of Caspr1 was sufficient for its effect in GluA1 expression and that deletion of

its proline-rich region abolished the effect of Caspr1 in GluA1 mRNA. By overexpressing a construct of Caspr1 that lacks its proline domain, we will evaluate the requirement of this region in the upregulatory effect of total protein levels of GluA1.

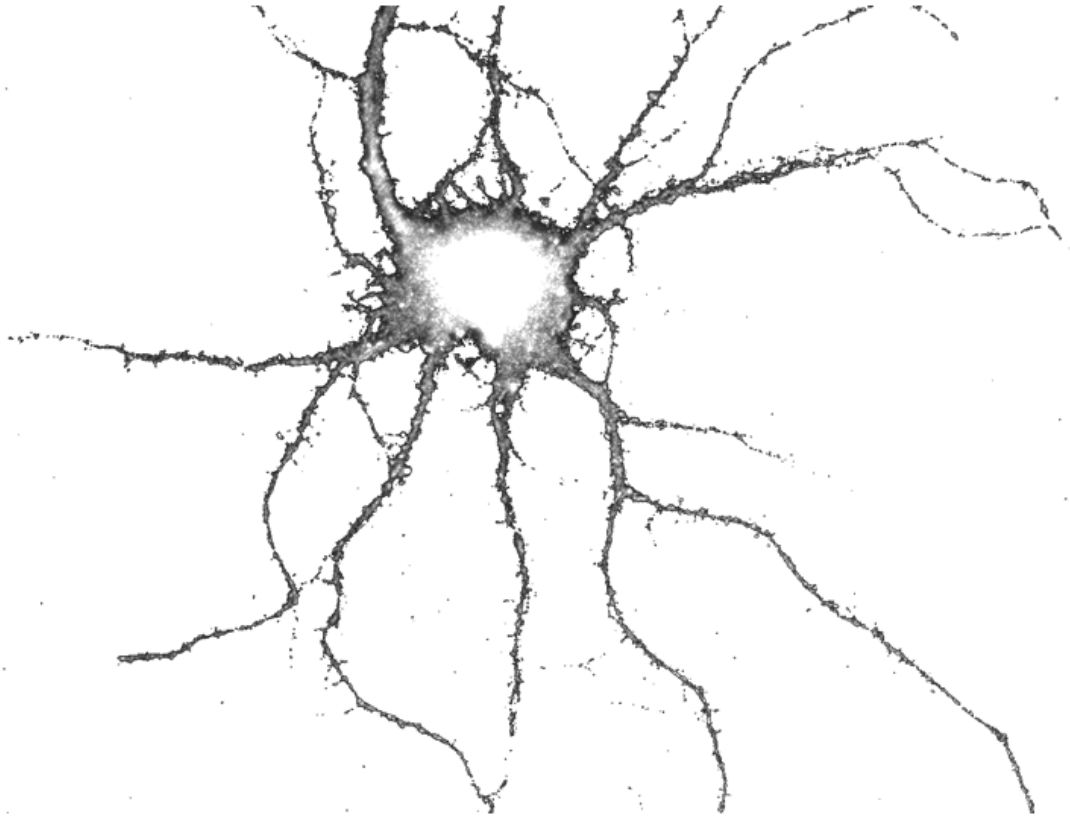
2. We further hypothesize that the effect of Caspr1 in GluA1 levels occurs through the Caspr1-mediated activation of a signaling pathway. The proline-rich region of Caspr1 has been shown to interact with SH3 domains of several signaling molecules, particularly with that of the tyrosine kinase Src (Peles *et al.*, 1997). Thus, we propose to evaluate the effect of Caspr1 in the activation of a signaling pathway downstream of Src, by assessing levels of phosphorylated Src as well as of phosphorylated ZBP1, a RNA-binding protein known to regulate mRNA translation upon Src-dependent phosphorylation.

3. Given the importance of dendritically localized GluA1 transcripts to the expression of synaptic plasticity and the role that Caspr1 may exert in these mechanisms, it is of crucial importance to understand which physiological stimuli regulate the endogenous expression of Caspr1. Up until now, the only information available on the regulation of Caspr1 concerns its intracellular traffic. However, Caspr1 effect in GluA1 levels parallels that of chronically blocking neuronal activity, which results in homeostatic synaptic scaling by increasing GluA1 dendritic synthesis and accumulation at synapses. Thus, our final aim is to evaluate how chronic blockade of neuronal activity with TTX, a blocker of voltage-gated sodium channels, affects the endogenous expression of Caspr1. Moreover, we will evaluate if these manipulations of activity also activate a Src-dependent signaling pathway, which is of particular interest to unveil the role that Caspr1 plays in the mechanisms of homeostatic plasticity.

Overall, in this project we will clarify the Caspr1-mediated molecular mechanisms that regulate the GluA1 subunit of AMPARs, as well as characterize physiological stimuli that impinge on these mechanisms. The results obtained will be relevant to understand how neuronal activity regulates glutamatergic synapses.

Chapter 2

Materials & Methods



Materials

Dulbecco's modified Eagle's medium (DMEM), Minimum essential medium Eagle (MEM), kynurenic acid, dithiothreitol (DTT) as well as the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and chymostatin, leupeptin, antipain and pepstatin (CLAP, stock solution 1mg/ml in dimethyl sulfoxide – DMSO) were purchased from Sigma-Aldrich (Sintra, Portugal). Neurobasal medium, OptiMEM reduced serum medium, fetal bovine serum (FBS), horse serum (HS), penicillin-streptomycin, trypsin and glutamine were acquired from Gibco, as part of Invitrogen Life Technologies (Barcelona, Spain). Lipofectamine reagent was also from Invitrogen Life Technologies (Barcelona, Spain) and Neurocult® SM1 neuronal supplement was bought from Stemcell Technologies (Grenoble, France). The enhanced chemifluorescence substrate (ECF) was acquired from Amersham, as part of GE HealthCare (Carnaxide, Portugal) and the fluorescent mounting medium was from DAKO (Glostrup, Denmark). Tetrodotoxin (TTX) and Bicuculline (Bic) were both purchased from Tocris Bioscience (Bristol, UK) and Lambda protein phosphatase (λPP) was acquired from New England BioLabs (through Izasa, Lisbon, Portugal). Polyvinylidene difluoride (PVDF) membranes were from Millipore (Madrid, Spain) and the BCA kit for protein quantification from Pierce, as part of Thermo Fisher Scientific (Rockford, Illinois, USA). The QIAGEN Plasmid mini kit was obtained from QIAGEN (QIAGEN GmbH, Hilden, Germany) and the PureLink^{TM} HiPure Plasmid Filter maxiprep kit was from Invitrogen Life Technologies (Barcelona, Spain). QuickChange II XL-site-directed mutagenesis kit was purchased from Stratagene (Cambridge, UK). All other reagents were from Sigma (Sintra, Portugal), BioRad (Amadora, Portugal) or from Merck (Darmstadt, Germany).

Antibodies

Anti-GluA1 C-terminal polyclonal (rabbit) antibody was purchased from Millipore (Chemicon - Massachusetts, USA), anti-GFP monoclonal (mouse) antibody was acquired from Roche Applied Science (Carnaxide, Portugal), anti-Caspr1 polyclonal (rabbit) and anti-GAPDH monoclonal (mouse) antibodies were bought from Abcam (Cambridge, UK). Anti-Phospho Src (Tyr416) monoclonal (rabbit) and anti-Src monoclonal (mouse) antibodies were acquired from Cell Signaling Technologies (Massachusetts, USA) and anti-phospho-ZBP1 (Tyr396) polyclonal (rabbit) antibody was a kind gift from Dr. Gary Bassell (Emory University, Atlanta, USA). Anti-human transferrin receptor monoclonal (mouse) antibody was purchased from Invitrogen Life Technologies (Barcelona, Spain) and anti-β-tubulin monoclonal (mouse) antibody bought from Sigma-Aldrich (Sintra, Portugal). The alkaline phosphatase-conjugated anti-rabbit and anti-mouse whole secondary antibodies (from goat) were obtained from GE Healthcare (Carnaxide, Portugal).

The antibodies used for immunocytochemistry are listed below. Besides the antibodies against GluA1, Caspr1, phospho-ZBP1 and phospho-Src mentioned above, anti-PSD95 monoclonal (mouse) antibody was acquired from Affinity BioReagents, as part of Thermo Fisher Scientific (Rockford, Illinois, USA) and anti-MAP2 polyclonal (chicken) antibody purchased from Abcam (Cambridge, UK). The secondary fluorescent antibodies anti-rabbit IgG AlexaFluor 594 (goat), anti-mouse IgG AlexaFluor 488 (goat) and anti-mouse IgG AlexaFluor 647 (goat) were both acquired from Molecular Probes, as part of Invitrogen Life Sciences (Barcelona, Spain) and AMCA-conjugated anti-chicken IgG (goat) secondary antibody was from Jackson ImmunoResearch (Porto, Portugal).

Constructs for transfection of hippocampal neurons and COS7 cells

GluA1 construct was a kind gift from Dr. Juna Lerma (Instituto de Neurociencias de Alicante, Spain). 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 chimeric construct Caspr1ΔPro, deleted for the proline-rich region of Caspr1 C-terminal, was prepared with the QuickChange II XL-site-directed mutagenesis kit (Stratagene). Caspr1 construct was used as template and primers (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') were designed to match the upstream and downstream regions of the proline-rich domain (between nucleotides 4138 and 4251 of the Caspr1 construct), in order to specifically delete only this region. The constructs pEGFP-N1 (Clontech - Saint-Germain-en-Laye, France) and pBK-CMV (Stratagene, Cambridge, UK) were used as controls for transfection. All DNA constructs were verified by DNA sequencing.

COS7 cells culture – maintenance and transfection

COS7 cells, a cell line derived from immortalized kidney cells of the African green monkey, were maintained at 37°C in a humidified incubator with 5% CO_2 / 95% air, incubated in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 44mM $NaHCO_3$ at pH 7.2, to a subconfluence of 60-80% and diluted 1:5 every three days.

COS7 cells were transiently transfected with lipofectamine reagent as follows: lipofectamine was diluted in OptiMEM reduced serum medium, to which a total of 12µg 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 and incubated at room temperature for 20 minutes to allow the formation of complexes. Precipitates were then added to cells cultured on $10cm^2$ wells and incubated for 5 hours at 37°C. After the transfection period, fresh culture medium was added to the culture and cells were returned to the incubator and allowed to express the transfected constructs for 48 hrs.

Hippocampal cultures (high density cultures)

Primary cultures of rat hippocampal neurons were prepared from the hippocampus of E18-E19 Wistar rat embryos. After dissection, hippocampi were treated for 15 min at 37°C with trypsin (0.06%), in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS: 5.36mM KCl, 0.44mM KH_2PO_4, 137mM NaCl, 4.16mM $NaHCO_3$, 0.34mM $Na_2HPO_4.2H_2O$, 5mM glucose, 1mM sodium pyruvate, 10mM HEPES and 0.001% phenol red). To stop trypsin activity, hippocampal cells were washed with HBSS containing 10% fetal bovine serum, and then washed again with HBSS to remove serum and avoid glia growth. Finally, hippocampal cells were transferred to Neurobasal medium (supplemented with SM1 neuronal supplement (1:50 dilution), 25µM glutamate, 0.5mM glutamine and 0.12mg/ml gentamycin), mechanically dissociated and then plated at a density of 8.9×10^5 cells/cm^2 in 6-well plates, coated with poly-D-lysine (0.1mg/ml). Cultures were maintained at 37°C in a humidified incubator of 5% CO2 / 95% air, for seven or fourteen days.

Hippocampal Banker cultures (low density cultures)

Banker cultures were prepared from hippocampal neurons as previously described by Banker and colleagues (Banker & Goslin, 1998). Briefly, hippocampal neurons, dissected from E18 rat embryos and dissociated using trypsin (0.25%) and trituration, were plated in neuronal plating medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1mM pyruvic acid) onto poly-D-lysine-coated coverslips in 60mm culture dishes, at a final density of 1-5 x 10^5 cells/dish. After 2-4 hrs, coverslips were flipped over an astroglial feeder layer in Neurobasal medium (supplemented with SM1 neuronal supplement (1:50 dilution), 25µM glutamate, 0.5mM glutamine and 0.12mg/ml gentamycin). 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 5µM 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 3 weeks.

Transfection of hippocampal neurons – calcium phosphate protocol

Constructs were recombinantly expressed in both high- and low-density cultures of hippocampal neurons at DIV7 using a calcium phosphate transfection protocol adapted from Jiang and collaborators (Jiang *et al.*, 2004). Plasmid DNAs were diluted in Tris-EDTA transfection buffer (10mM Tris-HCl, 2.5mM EDTA, pH 7.3). A $CaCl_2$ solution (2.5M in 10mM HEPES) was added, drop-wise, to the DNA solution to a final concentration of 250mM $CaCl_2$, and added to an equivalent volume of HEPES-buffered solution (274mM NaCl, 10mM KCl, 1.4mM Na_2HPO_4, 11mM dextrose and 42mM HEPES, pH 7.2). The final transfection solution was then gently mixed for 2-3 sec and

incubated, protected from light, at room temperature for 30 min to allow the formation of precipitates, and vortexed every 5 min. Precipitates were added, drop-wise, to DIV7 hippocampal neurons and cultures were incubated at 37°C for 1-3 hrs in the presence of kynurenic acid (2mM in Neurobasal medium). After this period, transfection medium was removed and cells were incubated at 37°C for 20 min with culture medium containing kynurenic acid, slightly acidified with HCl. Finally, reserved, conditioned medium was added to the cells, which returned to the incubator to allow expression of the transfected constructs, until DIV15.

Stimulation of primary hippocampal cultures

To look for physiological stimuli regulating the expression of Caspr1, activity in hippocampal neurons was either blocked or enhanced with tetrodotoxin (TTX) or bicuculline (Bic), respectively. Hippocampal neurons at DIV14 (from primary high density cultures) or at DIV20 (hippocampal neurons from Banker cultures) were stimulated for 24 hrs, at 37°C, in conditioned medium. TTX was used at 1µM (stock solution – 2mM in aqueous solution) and Bic was used at 40µM (stock solution – 40mM in DMSO). After 24 hrs, cells were either extracted for Western blot analysis (high density cultures) or fixed for immunocytochemistry experiments (Banker cultures).

COS7 cells and hippocampal neurons total extracts

Total extracts of COS7 cells and high density hippocampal cultures were prepared for Western Blot (WB) analysis. Total extracts of COS7 cells were prepared at 48 hrs after transfection and total cell extracts of hippocampal neurons were prepared at DIV15. Cells were washed once with cold phosphate buffered saline (PBS – 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.4) and then ice-cold lysis buffer RIPA (150mM NaCl, 50mM Tris-HCl (pH 7.4), 5mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5, with phosphatase inhibitors (1mM sodium orthovanadate (Na_3VO_4) and 50mM sodium fluoride (NaF)) supplemented immediately before use with 1mM DTT and a cocktail of protease inhibitors (200µM PMSF and 1µg/ml each of chymostatin, leupeptin, antipain and pepstatin) was added to the cells, which were then scraped. The lysates obtained were sonicated with an ultrasonic probe, on ice, for 30 sec (6 pulses of 5 sec each). After centrifugation at maximum speed for 10 min at 4°C, cell debris and insoluble material were discarded and the supernatant submitted to protein quantification by the BCA method (Pierce). The protein was aliquoted and frozen at -20°C, until needed.

For the dephosphorylation assays using the Lambda protein phosphatase (λPP), total cell extracts of hippocampal neurons at DIV15 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 150µg of protein of each sample to be dephosphorylated were incubated with 2µl of λPP in 1xNEBuffer for protein metallophosphatases (PMP) (50mM HEPES, 100mM NaCl, 2mM DTT, 0.01% Brij 35,

pH 7.5) supplemented with 1mM $MnCl_2$, for 2 hrs at 30°C. Following this period, samples were denatured at 95°C for 5 min, aliquoted and stored at -20°C, until needed.

Gel electrophoresis and Western blot

Samples were denatured at 95°C for 5 min and resolved by SDS-PAGE in Tris-glycine-SDS (TGS) buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3) in 7.5% polyacrylamide gels 1.5mm thick at 60-80V. Approximately 130-150µg of each sample was loaded to the gels. For Western blot analysis, proteins were transferred onto a PVDF membrane by electroblotting overnight at 40V and 4°C. Membranes were washed once with 0.1% Tween 20 in Tris-buffered saline (TBS) (20mM Tris, 137mM NaCl, pH 7.6 (TBS-T)) and then blocked for 1 hr at room temperature either with 5% (w/v) BSA or 5% (w/v) non-fat dry milk in TBS-T. Membranes were washed again once with TBS-T and probed during 1 hr, at room temperature, or overnight, at 4°C, with the primary antibody diluted in 5% BSA in TBS-T (the antibody against total Src was prepared in 5% non-fat milk in TBS-T and all washes were done with 0.5% milk in TBS-T). The following dilutions of primary antibodies were used: anti-GluA1 1:1000, anti-Caspr1 1:250, anti-phospho-ZBP1 1:500, anti-phospho-Src 1:1000, anti-Src 1:1000, anti-GFP 1:1000, anti-human transferrin receptor 1:1000, anti-GAPDH 1:20000 and anti-β-tubulin 1:25000.

Following several washes with TBS-T, the membranes were incubated for 1 hr, at room temperature, with alkaline phosphatase-conjugated IgG secondary antibody (1:20000 - anti-rabbit or anti-mouse, depending on the primary antibody host-species). Membranes were then washed again and immunostaining was resolved with the enhanced chemifluorescence (ECF) substrate for a maximum of 5 min or until protein

bands were visible and membranes were scanned on a VersaDoc Imaging System (Model3000 – BioRad, Amadora, Portugal). Bands corresponding to the proteins of interest were quantified using Image J 1.43 software and normalized with the loading controls (Gapdh, Human transferrin receptor or β-tubulin) indicated in figure captions.

Stripping and reprobing

The first antibodies to be probed in membranes were the ones against phospho-ZBP1 and phospho-Src. In order to reprobe the membranes for the other antibodies, membranes were re-activated and ECF was removed with a solution of 40% (v/v) methanol for 30 min. After a 5 min wash with distilled water, membranes were stripped for 10 min with 0.2M NaOH and washed once again with water. Membranes were blocked for 1 hr, at room temperature, with 5% BSA or 5% non-fat dry milk in TBS-T, incubated in the primary and secondary antibodies and resolved, as previously described.

Immunocytochemistry

Hippocampal neurons from Banker cultures at DIV15 or DIV21 were fixed for 15 min, at room temperature, in 4% sucrose / 4% paraformaldehyde in PBS, washed 3 times in PBS 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 primary antibodies, diluted in 3% BSA in PBS, overnight at 4°C. The following dilutions of primary antibodies were used: anti-GluA1 C-terminal 1:100, anti-Caspr1 1:50, anti-phospho-ZBP1 1:250, anti-phospho-Src 1:250, anti-PSD95 1:300 and anti-MAP2 1:10000.

After several washes with PBS to remove primary antibodies, cells were incubated with the appropriate secondary antibodies (anti-mouse IgG AlexaFluor 488 (1:500), anti-rabbit IgG AlexaFluor 594 (1:500), anti-mouse IgG AlexaFluor 647 (1:500) or AMCA-conjugated anti-chicken IgG (1:200)), diluted in 3% BSA in PBS, for 1 hr at 37°C. Coverslips were then washed with PBS and mounted using fluorescent mounting medium from DAKO. Preparations were cured overnight at 4°C, protected from light, sealed with nailpolish and kept at 4°C until microscopy analysis.

Fluorescence microscopy and quantification

Fluorescent imaging was performed on a Zeiss Axiovert 200M inverted microscope, with an AxioCam HRm camera and with AxioVision 4.8 software. Images were acquired with a Plan-Neofluor 63 x oil objective (numerical aperture 1.4). In single experiments, cells were cultured and stained simultaneously and imaged using identical settings. In experiments where cells had been previously transfected, fields for imaging were chosen by the GFP channel, for the presence of transfected, GFP-positive, neurons.

GluA1, phospho-ZBP1 and phospho-Src clustering was analyzed and quantified with Image J 1.43 analysis software. Dendrites were randomly chosen from exported 16-bit images and their lengths determined. Selected dendrites had similar thickness and appearance and their selection was carried out in MAP2 images, without observing the labeling of interest. The GluA1, phospho-ZBP1 and phospho-Src signals were analyzed after thresholds were set, such that recognizable clusters were included in the analysis, and the background intensity of each image was subtracted. Synaptic GluA1, phospho-ZBP1 or phospho-Src puncta were selected by colocalization with PSD95. Regions

around thresholded puncta were overlaid as a mask in the PSD95 channel, and colocalization was determined. To evaluate the percentage of synapses containing phospho-ZBP1 or phospho-Src clusters, PSD95 puncta were thresholded and overlaid as a mask in phospho-ZBP1 or phospho-Src channels to determine colocalization.

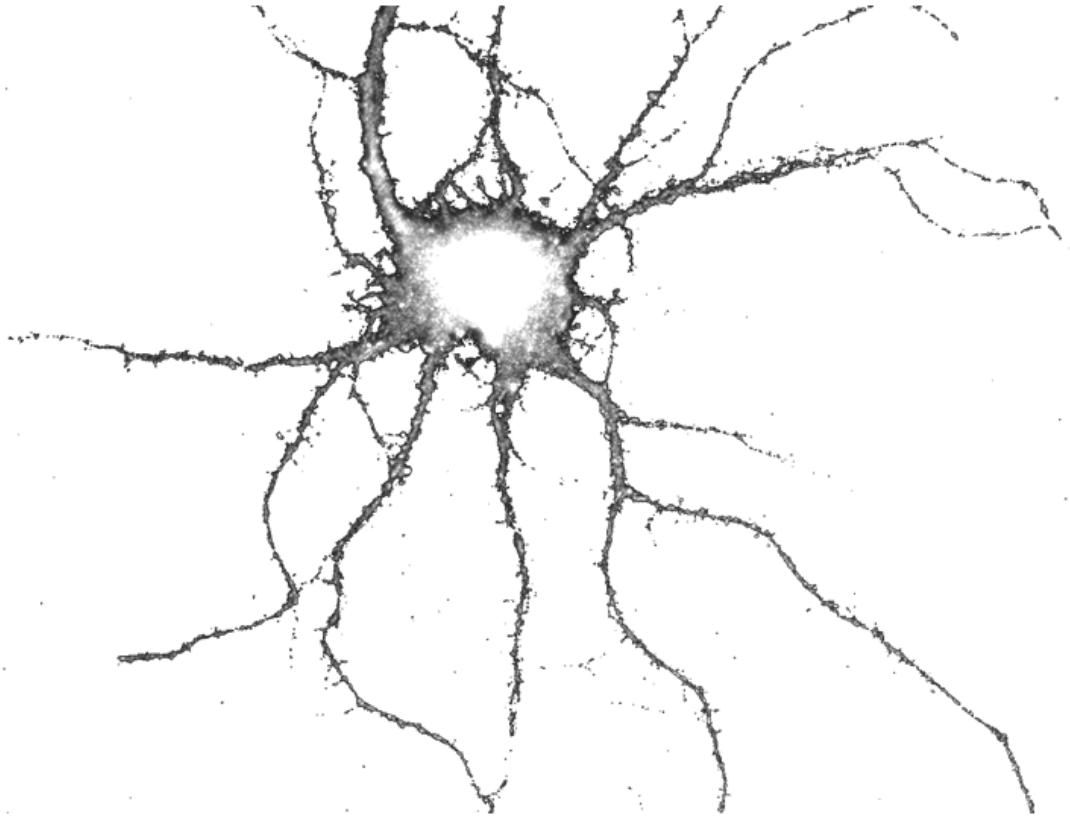
Per each selected cell, intensity, area and number of total and synaptic puncta per dendritic length was determined. The values obtained per cell were normalized against the control mean of that single experiment. Measurements were performed in three independent experiments (except for phospho-Src, with a single experiment) and at least 12 cells per condition were analyzed for each preparation.

Statistical analysis

Graphs and statistical analysis were performed using Graph Pad Prism 5 software. Results are presented as normalized means ± S.E.M. and statistical analysis was assessed by unpaired *t*-test or one-way ANOVA analysis of variance followed by the Dunnett's post test, as indicated in figure captions.

Chapter 3

Results & Discussion



Caspr1 overexpression increases total protein levels of GluA1 in COS7 cells and in hippocampal neurons in culture

Throughout the last years, many efforts have been made to understand the molecular mechanisms underlying synaptic plasticity, the cellular correlate of learning and memory. These two 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 postsynaptic alterations in AMPAR number or function, through tight regulation of receptor trafficking, are crucial to induce long-term changes in synaptic strength and efficacy (Kessels & Malinow, 2009; Malinow & Malenka, 2002; Newpher & Ehlers, 2008; Song & Huganir, 2002). Moreover, AMPARs are not static components of the synaptic membrane but, instead, they are continuously being delivered and removed in and out of synapses in response to neuronal activity. Indeed, synaptic plasticity mechanisms involve the modulation of AMPARs that are already expressed at the synapse and/or the rapid recruitment of new AMPARs to the synaptic membrane to alter receptor number and complement (Santos *et al.*, 2009). During long-term potentiation (LTP), an increase in AMPA-mediated currents occurs following the activity-dependent recruitment and insertion of AMPARs into the postsynaptic membrane, whereas long-term depression (LTD) occurs through the internalization of AMPARs, culminating in a decreased synaptic function of the receptors (Malenka & Bear, 2004). Thus, it is evident that these phenomena of synaptic plasticity require an exquisite regulation of the trafficking of AMPARs and of the rate of their synaptic insertion or removal. Events such as the regulation of the stoichiometry

of receptor subunits, RNA processing events, posttranslational modifications, such as phosphorylation, and, particularly, interacting partners of AMPARs, are major contributors to the regulation of AMPAR traffic and localization.

AMPAR subunits, through their C-terminus, are able to bind to a variety of accessory and scaffolding proteins. These interacting proteins form highly dynamic macromolecular complexes thought to anchor AMPARs to the synaptic membrane, and ultimately contributing to the strengthening of synapses. Moreover, it is now well established that the final steps in the synaptic delivery of AMPARs are subunit-specific, that is, they highly depend on AMPAR subunit composition and, specifically, on *cis*-signals contained within their intracellular C-termini (Passafaro *et al.*, 2001; Shi *et al.*, 2001). Thus, in the search for these AMPAR binding partners, the cytosolic carboxy terminal of AMPAR subunits has been intensively studied, mainly using yeast two-hybrid and GST pull-down assays. Some of the already known interactors of AMPARs include several PDZ-domain containing proteins, such as SAP97, the first protein reported to directly interact with the GluA1 subunit of AMPARs (Leonard *et al.*, 1998). Protein 4.1N is another well described GluA1-interacting protein that serves as an adapter to link GluA1 to the actin cytoskeleton in spines, thus stabilizing the surface expression of GluA1-containing AMPARs (Shen *et al.*, 2000). Several transmembrane proteins, such as stargazin, belonging to the transmembrane AMPAR regulatory proteins (TARPs) family, have also been characterized as auxiliary subunits of AMPARs. TARPs coassemble with AMPARs and promote their surface expression and clustering at excitatory synapses, through direct binding to PSD-95 (Chen *et al.*, 2000; Sumioka *et al.*, 2010; Ziff, 2007). More recently, proteins of the cornichon family were identified as novel transmembrane interactors of AMPARs and are thought not only to increase surface expression of AMPARs but also to alter channel gating properties

(Schwenk *et al.*, 2009). Although the composition of this intricate protein network that binds to AMPARs is yet not fully characterized, the large collection of AMPAR partners identified so far has already started to shed a light on the details of AMPAR trafficking and surface expression and has given some insight on the roles of individual interactors in these mechanisms (Bredt & Nicoll, 2003; Henley, 2003).

Nevertheless, in order to further understand the mechanisms governing AMPAR trafficking and unravel new interactors of these receptors, a recent proteomic study was carried out in our laboratory. By combining affinity purification of protein complexes with mass spectrometric analysis of their composition, the identification of several novel binding partners for AMPARs was possible (Santos *et al.*, 2010). Several cytoskeleton proteins, such as spectrin and α-internexin; motor proteins, such as myosin 9 and Va; RNA processing proteins, such as heterogeneous nuclear ribonucleoprotein M and RNA helicases, among others, were some of the identified AMPAR interacting proteins. The glycosylphosphatidyl inositol (GPI)-anchored Contactin1, a cell adhesion molecule previously implicated in synaptic plasticity (Murai *et al.*, 2002), was also found to interact with AMPARs. However, one of the most interesting findings in this proteomic screening was the identification of a novel transmembrane protein, Contactin associated protein 1 (Caspr1), as an interactor of AMPARs. This cell adhesion molecule, also known as paranodin, together with Contactin1, exerts important functions in the process of axonal myelination, particularly at septate-like paranodal junctions (Einheber *et al.*, 1997). With an extracellular architecture similar to that of neurexins, it is the intracellular C-terminal tail of Caspr1 that possesses particular interest since it contains a region for binding of FERM domains (like protein 4.1N) and a sequence rich in proline residues, with potential binding sites for SH3 domains (Peles *et al.*, 1997).

The previous evidences implicating Contactin1 in synaptic plasticity, the localization of the complex Caspr1 / Contactin1 in synaptic sites throughout dendrites of CA1 hippocampal neurons (Murai *et al.*, 2002), the fact that both proteins appear to be binding partners of AMPARs and, also, the complex architecture of Caspr1, typical of molecules involved in protein-protein interactions, led Santos and colleagues to perform the biochemical characterization of the interactions of Caspr1 and Contactin1 with AMPARs, and understand the role of these novel interactors in the trafficking of the receptors. Immunoprecipitation experiments and pull-down assays further confirmed the interaction of Caspr1 and Contactin1 with AMPARs, particularly with the GluA1 and GluA4 subunits, both in a heterologous system and in rat brain synaptosomes. Also, biotinylation assays showed that overexpression of Caspr1 and Contactin1 leads to an increase in GluA1 cell surface levels. Furthermore, in addition to affecting AMPAR traffic, preliminary results indicated that Caspr1 slightly increases GluA1 total protein levels, without affecting protein stability. This result prompted the idea that the increase in GluA1 levels could be due to an effect of Caspr1 in GluA1 mRNA levels, which was confirmed by quantitative real-time PCR experiments, suggesting a role for Caspr1 in the stability of GluA1 mRNA (Santos, 2009).

In the course of the experiments mentioned above and given the tantalizing hypothesis that they raise of a possible novel mechanism of regulation for AMPARs, it became important to further confirm the effect of Caspr1 in the total levels of GluA1, which was, then, the primary objective of the present study. To achieve this, a heterologous system was firstly used to express GluA1 in COS7 cells, alone or together with Caspr1. At 48 hrs post-transfection, total cell extracts were obtained and the total levels of GluA1 were assessed by western blot analysis (Figure 10A).

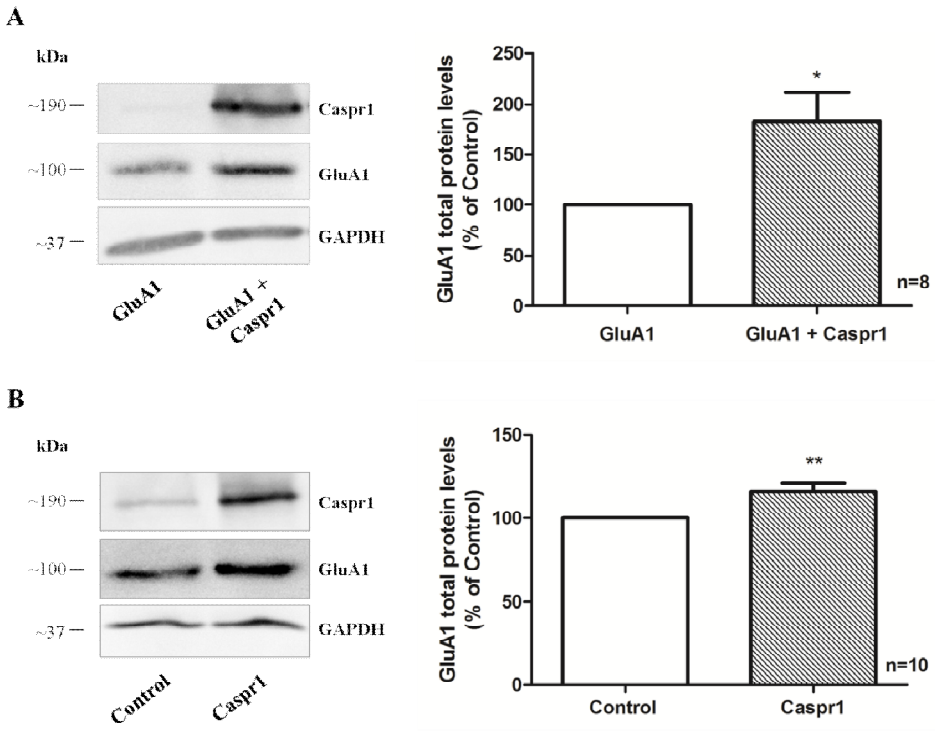


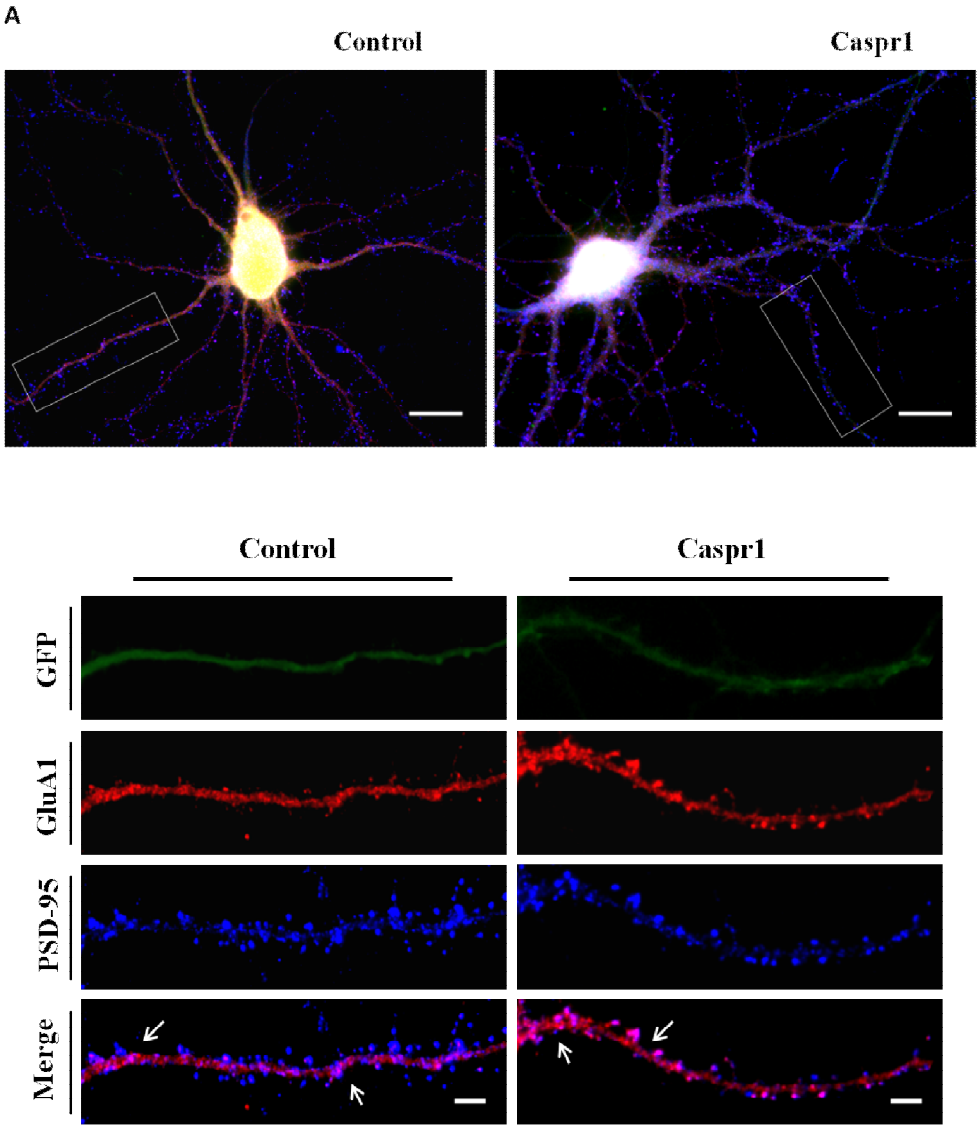
Figure 10 – Caspr1 increases GluA1 total protein levels in transfected COS7 cells and hippocampal neurons. (A) Cultured COS7 cells were co-transfected either with GluA1 + CMV empty vector + GFP or with GluA1 + Caspr1 + GFP, as indicated. Cells were allowed to express the constructs for 48 hrs, after which total cell extracts were obtained and GluA1 and Caspr1 levels assessed by western blot. (B) High-density cultured hippocampal neurons were transfected at DIV7 with GFP (Control) or with GFP + Caspr1, as indicated. Cells were allowed to express the proteins until DIV15, when total cells extracts were obtained and GluA1 and Caspr1 levels assessed by western blot. (A-B) Results are presented as means ± S.E.M. when compared to control and normalized to the loading control GAPDH. Statistical analysis was determined by the unpaired *t*-test, (A) *$p<0.05$ and (B) **$p<0.01$.

Indeed, when GluA1 is co-transfected with Caspr1 in COS7 cells, there is a significant increase of 83 ± 29 % (to 183 ± 29 % of control, *$p<0.05$) in GluA1 total protein levels, compared to control values (Figure 10A). In order to confirm if a similar

effect can be observed in a neuronal system, Caspr1 was overexpressed in hippocampal neurons in culture, by transfecting high-density cultures at DIV7, using the calcium phosphate method. At DIV15, total extracts were obtained and GluA1 levels were assessed by western blot (Figure 10B). According to the results obtained in the heterologous system, overexpression of Caspr1 in hippocampal neurons led to a small but statistically significant increase of 16 ± 5 % (**$p<0.01$) in GluA1 total protein levels, when compared to control values (Figure 10B).

When comparing the results obtained in COS7 cells and hippocampal neurons, overexpression of Caspr1 in hippocampal neurons leads to a consistent but smaller upregulation of GluA1 levels when compared to the effect observed in COS7 cells, even though results are statistically significant in both situations. This discrepancy may be explained by the fact that Caspr1 is endogenously expressed in neurons, and therefore overexpression of Caspr1 in the neuronal system is not as efficient in promoting GluA1 upregulation as in COS7 cells, where endogenous Caspr1 is absent. The other aspect to be taken into account is differences in the transfection efficiency between both expression systems. COS7 cells have a higher rate of transfection, since they are more easily transfected and more resistant to this process, with little consequent cellular death. Hippocampal neurons, however, are not only more difficult to transfect but also very sensitive to transfection protocols. Thus, using a calcium phosphate method will possibly prevent a higher percentage of cell death following transfection but it will not be as efficient as other protocols. Having this into account, one has to consider that total cell lysates of high-density-cultured hippocampal neurons are probably extracted from a majority of non-transfected cells, which, when assessing GluA1 levels by western blot, will possibly dilute the effect of Caspr1 overexpression in upregulating total levels of the GluA1 subunit. One possible alternative to overcome this problem is to perform

immunocytochemistry experiments and evaluate the effect of Caspr1 in the GluA1 subunit at the single cell level, exclusively from transfected cells. To achieve this, Banker type low density cultured hippocampal neurons were transfected at DIV7 either with GFP (control) or GFP + Caspr1, using the calcium phosphate protocol. Cells were allowed to express the proteins until DIV15, when they were fixed and double-labeled with antibodies for the GluA1 subunit as well as for the post-synaptic marker PSD-95. To specifically evaluate the effect of Caspr1 overexpression in GluA1 levels, fields for imaging were chosen by the GFP channel, for the presence of transfected, GFP-positive, neurons (Figure 11).



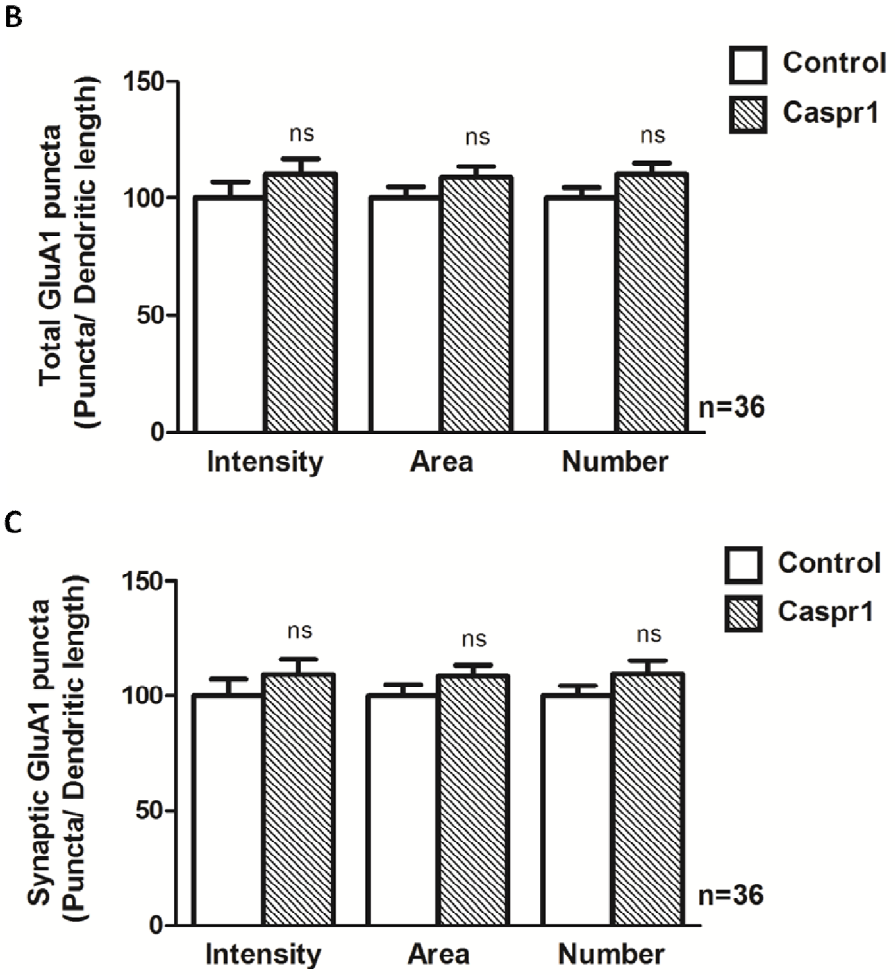


Figure 11 – Caspr1 slightly increases intensity, area and number of total and synaptic GluA1 puncta in transfected hippocampal neurons. (A) Primary hippocampal neurons from low-density Banker cultures were transfected at DIV7 with GFP (Control) or with GFP + Caspr1, as indicated. Cells were allowed to express the proteins until DIV15, after which they were fixed and immunolabeled with antibodies for GluA1 (red) and PSD-95 (blue). Images were taken using a Zeiss Axiovert 200 fluorescent microscope. Dendrites were visualized via MAP2 staining and fields for imaging were chosen by the GFP channel, for the presence of transfected, GFP-positive, neurons. Scale bar: 20µm; Scale bar (crops): 5µm. Arrows indicate colocalization with PSD-95 puncta. (B - C) Quantification of the results shown in (A). GluA1 clustering was analyzed and quantified with Image J 1.43 analysis software. Dendrites were randomly chosen and their lengths determined. (B) Total GluA1 signal was analyzed after

thresholds were set, such that recognizable clusters were included in the analysis, and the background intensity of each image was subtracted. (C) Synaptic GluA1 puncta were selected by colocalization with PSD-95 (see arrows). (B-C) Per each selected cell, intensity, area and number of total (B) and synaptic (C) puncta per dendritic length was determined. Measurements were performed in three independent experiments with at least 12 cells per condition, and values obtained per cell were normalized against the control mean of that single experiment. Results are presented as means ± S.E.M. Statistical analysis was determined by the unpaired *t*-test, although no statistical significance was achieved, ns - $p>0.05$.

Surprisingly, when analyzing GluA1 clustering from single transfected cells, the expected upregulatory effect of Caspr1 in GluA1 levels was not evident and a more robust response was expected (Figure 11). Overexpression of Caspr1 was only able to slightly increase in 10 ± 6 % the total intensity of GluA1 puncta. A similar effect was obtained for the total area and number of GluA1 puncta (109 ± 5 % and 110 ± 5 % of control, respectively) (Figure 11B). Moreover, Caspr1 was not able to significantly increase the intensity, area or number of synaptic (assessed by PSD-95 colocalization) GluA1 clusters (109 ± 6 %, 109 ± 5 % and 110 ± 5 % of control, respectively), although there is a clear upregulatory tendency (Figure 11C). Indeed, despite the fact that these results do not reach statistical significance, they are comparable to what was observed by western blot analysis, since a 10 to 16 % increase in GluA1 levels was obtained in both experiments.

Altogether, these results are in accordance to what was presented by Santos and colleagues. In both COS7 cells and hippocampal neurons, Caspr1 appears to be upregulating total protein levels of the GluA1 subunit. Analyzing the results at the single cell level, there appears to be an incremental tendency in GluA1 levels when

Caspr1 is overexpressed, in the intensity, area and number of GluA1 puncta, although values do not reach statistical significance. Regarding synaptic levels of the GluA1 subunit, the results observed are far from what was expected. Santos and colleagues reported a marked increase in the cell surface expression of GluA1 when Caspr1 is overexpressed, suggesting a role for this protein in the regulation of AMPAR traffic (Santos, 2009). Thus, it would be expectable that, when overexpressing Caspr1, a significant increase in synaptic GluA1 puncta was observed, which did not occur. Still, Caspr1 was able to slightly increase this synaptic subset of GluA1 clusters, and perhaps a significant difference in results can be achieved if more experiments are performed. Hence, these results, together with the ones presented by Santos, endow Caspr1 with particular importance in regard to the regulation of AMPARs, proposing a novel player to control their expression and trafficking.

Nevertheless, the mechanisms underlying this effect of Caspr1 in GluA1 levels are still unknown. Given the overall architecture of Caspr1, composed of domains typically involved in protein-protein interactions, it is a natural assumption to think that the interaction of Caspr1 with GluA1 and its upregulatory effect in this AMPAR subunit are mediated by one of its regions. The extracellular domain of Caspr1 is similar to the extracellular structure of neurexins, a family of highly polymorphic cell surface molecules (Peles *et al.*, 1997). It contains a discoidin domain next to its N-terminal, several sequences with homology to the extracellular matrix proteins laminin A, agrin, slit and perlecan, two epidermal growth factor (EGF)-like regions, a central fibrinogen domain and several PGY (proline – glycine – tyrosine) repeats (Figure 6). Its intracellular C-terminal tail, on the other hand, possesses particular interest, since it contains a juxtamembrane region for the binding of molecules containing FERM domains, such as protein 4.1N (or 4.1B), schwannomin and β-integrin1 (Denisenko-

Nehrbass *et al.*, 2003a; Denisenko-Nehrbass *et al.*, 2003b), and a sequence rich in proline residues, with at least one canonical SH3-domain binding site, which suggests the interaction of Caspr1 with several molecules typically involved in signaling pathways (Peles *et al.*, 1997). Still, despite the knowledge on its architecture, very little is known about Caspr1 and its interactors: extracellularly, Contactin1 is the only known partner of Caspr1, and its intracellular partners, despite several already described, are very poorly characterized. Given this, in order to shed some light onto the mechanism underlying the effect of Caspr1 in GluA1 levels, Santos and colleagues firstly sought to dissect the region of Caspr1 involved in the interaction with AMPAR subunits. By expressing the C-terminal of Caspr1 as a GST-fusion protein, they were able to pull-down GluA1, GluA2 and GluA4 subunits from rat cerebellum lysates and, thus, confirming a role for the C-terminal region of Caspr1 in the interaction with AMPARs. However, they also showed that, besides the C-terminal region of both AMPAR subunits and Caspr1, other molecular determinants must be involved in their interaction, since immunoprecipitation of a truncated form of GluA1, lacking its intracellular C-terminal, was able to co-immunoprecipitate Caspr1, thus, suggesting that the C-terminal tail of Caspr1 is not essential for the interaction with GluA1, and that other regions of the molecule are also involved (Santos, 2009). Interestingly, similar evidences have been described for the interaction between AMPARs and stargazin, with both extracellular and intracellular regions of stargazin participating in the interaction and differentially controlling AMPAR channel properties and trafficking, respectively (Tomita *et al.*, 2005a; Tomita *et al.*, 2004).

Despite defining that both extracellular and intracellular regions of Caspr1 are involved in the interaction between this protein and AMPARs, the previously described evidences by Santos and colleagues are not clear regarding the actual region responsible

for the upregulatory effect in GluA1 levels. Accordingly, further experiments were performed indicating a prominent role for the intracellular C-terminal domain of Caspr1 in increasing GluA1 subunit. Electrophysiology studies indicated that the C-terminal domain of Caspr1 increases the amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs), which suggests that the intracellular region of Caspr1 increases the traffic of AMPARs to synapses. Moreover, overexpression of the same construct encoding the C-terminal domain of Caspr1 led to a significant increase both in GluA1 total protein levels, in hippocampal neurons, and mRNA levels in COS7 cells, further confirming the role of the C-terminal tail of Caspr1 in the overall upregulatory effect in the GluA1 subunit (Santos, 2009).

In the present study, we sought to further explore and map the molecular determinants responsible for the effect of Caspr1 in the GluA1 subunit. Based on the evidences presented by Santos and colleagues, the intracellular C-terminal region of Caspr1 assumes an important role in the regulation of AMPARs. As previously described, this intracellular domain of Caspr1 presents a rather interesting architecture, common in molecules involved in protein-protein interactions. Its membrane proximal intracellular region comprises a short peptide with a motif conserved in glycophorin C, neurexin IV and paranodin (GNP motif) and is capable to interact with protein encompassing FERM domains (Denisenko-Nehrbass *et al.*, 2003a; Denisenko-Nehrbass *et al.*, 2003b). Interestingly, the GNP motif is also found in proteins such as SynCAM, a protein capable to drive synaptic assembly (Biederer *et al.*, 2002). In Caspr1, this GNP motif allows its interaction with the protein 4.1B, a major physiological partner of Caspr1 at paranodes. This association could provide a link between intercellular complexes and the cytoskeleton, allowing for the stabilization of Caspr1 at the plasma membrane (Gollan *et al.*, 2002; Menegoz *et al.*, 1997). One possible model is that

Caspr1, through binding to proteins containing the 4.1 domain and which serve as actin adapters, may indirectly modulate the interaction of AMPAR subunits with specialized components of the cytoskeleton, eventually present at the postsynaptic scaffold, and thus, contribute to the stabilization of receptors at the cell surface and to their synaptic targeting. Curiously, the synaptic targeting of the GluA1 subunit by SAP97 has already been described to be dependent on the interaction of SAP97 with the protein 4.1 (Rumbaugh *et al.*, 2003). As for the tail of Caspr1 C-terminal domain, it contains a sequence rich in proline residues that comprises a SH3-domain binding site, which suggests the interaction of Caspr1 with several molecules typically involved in signaling pathways (Peles *et al.*, 1997). Indeed, the proline-rich region of Caspr1 has already been found to selectively interact with the SH3 domains of Src, Fyn, PLCγ and the p85 subunit of PI3K. Moreover, the interaction between Caspr1 and Src was further confirmed when both proteins were exogenously expressed in co-transfected COS7 cells: immunoprecipitation with several c-Src-specific antibodies was able to co-precipitate Caspr1 from lysates of transfected cells (Peles *et al.*, 1997). Nevertheless, the physiological relevance of the described interactions remains unclear.

Thus, having in consideration all the results suggesting an important role for the C-terminal of Caspr1 in the overall upregulation of GluA1 levels and all the evidences regarding possible binding partners for Caspr1 that can be mediating this effect, we decided to further dissect the molecular determinants of Caspr1 responsible for its effect on GluA1 levels. For that, cultured COS7 cells were transfected either with GluA1 alone, together with the full-length Caspr1 construct or together with a deletion mutant of Caspr1, designed to selectively lack its intracellular C-terminal proline-rich region (Caspr1ΔPro). Cells were allowed to express the proteins for 48 hrs, after which total

cell extracts were obtained and total levels of GluA1 assessed by western blot analysis (Figure 12).

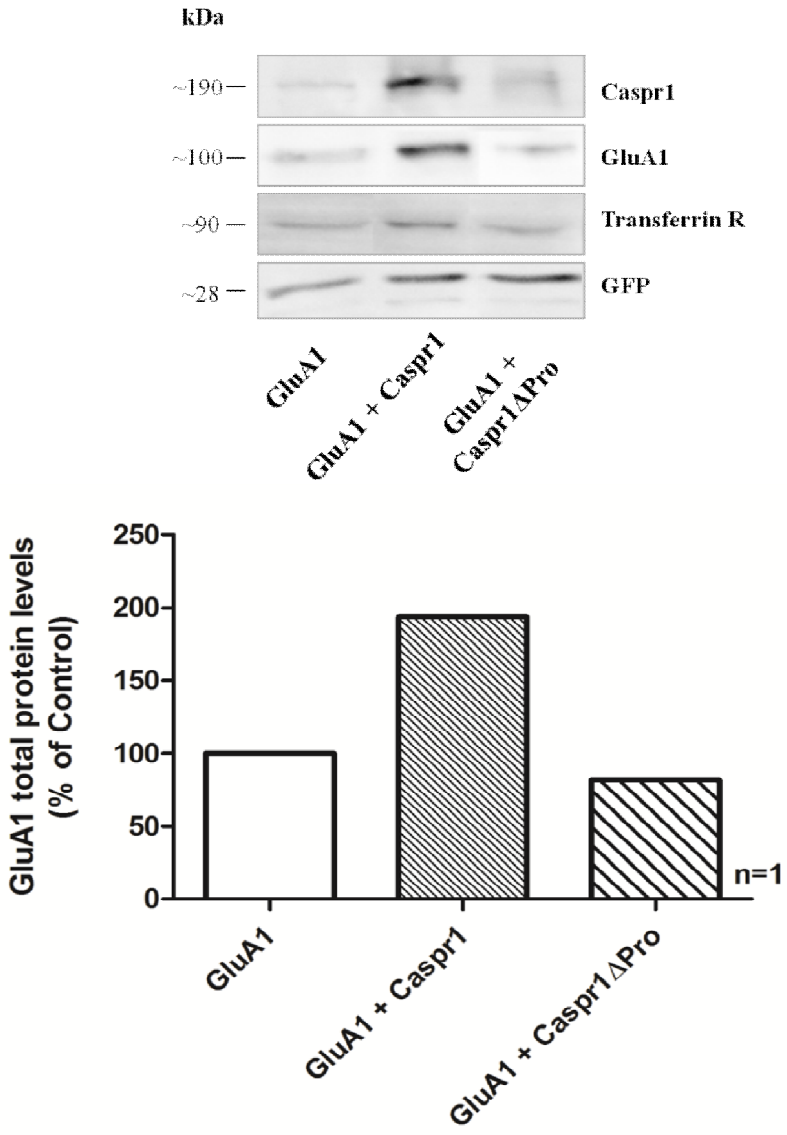


Figure 12 – The proline (PRO)-rich region of the C-terminal intracellular domain of Caspr1 plays a role in upregulating GluA1 total protein levels, in COS7 cells. Cultured COS7 cells were co-transfected either with GluA1 + CMV empty vector + GFP**,** GluA1 + Caspr1 (full-length) + GFP or with GluA1 + Caspr1ΔPro + GFP, as indicated. Cells were allowed to express the constructs for 48 hrs, after which total cell extracts were obtained and GluA1 levels assessed by western blot. Values corresponding to GluA1 levels were normalized

to the loading control Transferrin R (human transferrin receptor) and the results presented correspond to a single independent experiment.

As expected, the exogenous expression of Caspr1 in GluA1-co-transfected COS7 cells resulted in a marked increase of approximately 94% in GluA1 total protein levels, when compared to control values (Figure 12). However, when COS7 cells were co-transfected with the deletion mutant for the proline-rich region of Caspr1 (Caspr1ΔPro), designed to specifically lack this region, the upregulatory effect of Caspr1 in GluA1 total proteins levels was completely lost, with GluA1 levels decreasing to values similar to the control situation (88% of control) (Figure 12). Despite the fact that these results are very preliminary (they are the result of a single independent experiment), they are extremely interesting. The abolishment of the upregulatory effect in GluA1 levels when the proline-rich region is deleted from the intracellular C-terminal of Caspr1 indicates that this domain may be necessary for Caspr1 to exert its effect. Accordingly, preliminary results obtained in our laboratory further confirm this possibility. GluA1 mRNA levels are significantly upregulated when COS7 cells are co-transfected with Caspr1 and a similar result is verified when cells are transfected with another deletion mutant of Caspr1, this time lacking the GNP motif of its intracellular C-terminal. Conversely, expression of the mutant for the proline-rich region, Caspr1ΔPro, resulted in GluA1 mRNA levels comparable to the control (unpublished data). These results further confirm the hypothesis that the upregulatory effect of Caspr1 in GluA1 protein and mRNA levels is specifically mediated by the proline-rich region of Caspr1 intracellular C-terminal tail and that this domain is necessary for the effect to be verified, whilst deletion of the GNP motif of Caspr1 did

not significantly alter the effect of Caspr1 on the GluA1 mRNA levels, indicating that this domain of Caspr1 is not required for its effect on GluA1 levels.

As previously described, Santos and colleagues proposed a role for the C-terminal domain of Caspr1 not only in the upregulation of GluA1 levels and its stabilization at the cell surface, but, specifically, in its synaptic targeting (Santos, 2009). However, the results shown in the present study, as well as results regarding the effect of the deletions mutants, Caspr1ΔPro and Caspr1ΔGNP, in GluA1 mRNA levels, go further into understanding the molecular determinants of Caspr1 responsible for its effect and describe the proline-rich region of Caspr1 intracellular domain as the major player in the regulation of the GluA1 subunit. Given the knowledge on the architecture of Caspr1, particularly of its intracellular C-terminal, and of its binding partners, it is conceivable that Caspr1, through its proline-rich region, interacts with binding proteins involved in the activation of several signaling pathways that, somehow, mediate an upregulation in GluA1 levels. Nonetheless, despite the apparent specific requirement for the proline-rich region of Caspr1 in mediating its effect in the regulation of the GluA1 subunit, it does not mean that its GNP motif is obsolete. Through interaction with 4.1 proteins, and, indirectly, to the actin cytoskeleton, this motif of Caspr1 may promote the stabilization of the GluA1 subunit at the cell surface, as proposed above, and thus, further contribute to an overall role of Caspr1 in the regulation of the GluA1 subunit. Future experiments, using both deletion mutants for the proline-rich region and the GNP motif, should be carried to unveil, with clarity, the role that each domain of the intracellular C-terminal region of Caspr1 plays in this proposed regulatory mechanism of the GluA1 subunit, either in its mRNA and protein stability, as well as in its surface expression and synaptic targeting.

Overexpression of Caspr1 upregulates the phosphorylation of Src and of its downstream target, ZBP1, in cultured COS7 cells and hippocampal neurons

Up until now, in the present study we described an important role for the intracellular C-terminus of Caspr1, particularly for its proline-rich region, in the regulation of the GluA1 subunit of AMPARs. The evidences presented, although still preliminary, are extremely interesting and raise a whole set of possibilities regarding the mechanisms underlying the effect of Caspr1 in GluA1 levels. Contributing to this is the distinct structure of the intracellular C-terminal domain of Caspr1, typical of molecules implicated in protein-protein interactions. In particular, its proline-rich region, containing at least one canonical SH3 domain-binding site, is thought to interact with proteins involved in signaling pathways. Indeed, Peles and colleagues were able to selectively pull-down Caspr1 bound to SH3 domains of several signaling molecules, namely PLCγ, the p85 subunit of PI3K and members of the Src family of protein tyrosine kinases (SFK), Src and Fyn (Peles *et al.*, 1997). All these molecules are able to transduce signals from the extracellular environment to intracellular biochemical pathways involved in controlling an array of signaling networks regulating metabolism, viability, proliferation, differentiation and migration, among several other physiological functions (Guarino, 2010; Ingley, 2008). Moreover, it was proposed that members of the Src family are involved in the transmission of signals generated by cell recognition molecules, present at the cell surface, such as L1- and N-CAM, MAG and, particularly, Contactin1 (Beggs *et al.*, 1994; Ignelzi *et al.*, 1994; Umemori *et al.*, 1994; Zisch *et al.*, 1995). Being a GPI-anchored protein lacking a cytoplasmic domain, Contactin1, in order to mediate its biological responses, has to interact with a transmembrane protein,

such as Caspr1, that contains an appropriate cytoplasmic domain capable of recruiting and activating intracellular signaling molecules.

Altogether, these evidences prompted us to hypothesize that, through its intracellular proline-rich region, Caspr1 interacts with signaling molecules that trigger the activation of biochemical responses that lead to the upregulation of the GluA1 subunit. Indeed, further immunoprecipitation studies by Peles and colleagues confirmed an interaction between Caspr1 and Src, when both proteins were exogenously expressed in co-transfected COS7 cells (Peles *et al.*, 1997). Thus, the confirmation of such a specific interaction between Caspr1 and Src further supports the idea of a possible upregulation of GluA1 levels through a Caspr1-mediated activation of Src signaling pathways. To confirm this hypothesis, Caspr1 was exogenously expressed in COS7 cells, together with the GluA1 subunit. Cells were allowed to express the constructs for 48 hrs, after which total protein levels of phosphorylated Src were assessed by western blot analysis (Figure 13).

A slight increase in levels of phosphorylated Src seems to occur when Caspr1 is expressed in COS7 cells (Figure 13A). However, the band corresponding to phospho-Src appears at a molecular weight of approximately 75 kDa, which is not in accordance to what was expected. The monoclonal antibody used to label phospho-Src detects endogenous Src phosphorylated in its tyrosine residue 416 (Tyr416), located in the activation loop of the kinase domain of Src, which renders activity to the enzyme (Boggon & Eck, 2004; Thomas & Brugge, 1997). This form or Src would be expected to result in a protein band migrating at 60 kDa. One hypothesis to explain this difference in molecular weight is the occurrence of a shift in the molecular mass of Src after its phosphorylation and consequent activation. Also, several other posttranslational

modifications, such as myristoylation, palmitoylation (Koegl *et al.*, 1994; Resh, 1999) etc., may be occurring at the same time of phosphorylation and thus, altogether, be contributing to a significant shift in the molecular weight of Src. In order to test for the specificity of the band observed, a dephosphorylation assay was performed in the same samples of Caspr1-transfected COS7 cells used before. Lysates were incubated for 2 hrs, at 30°C, with the protein phosphatase Lambda (λPP) and levels of phosphorylated Src were assessed by western blot analysis, as described above (Figure 13A). As stated, expression of Caspr1 in COS7 cells appears to promote a slight increase in a 75 kDa protein, thought to correspond to phospho-Src. When the same sample was subjected to a dephosphorylation assay with the lambda phosphatase (GluA1 + Caspr1 + λPP), the 75 kDa band corresponding to phosphorylated Src specifically disappeared, whilst labeling for Caspr1 and the human transferrin receptor (Transferrin R) remains distinguishable (although some proteolysis may have occurred) (Figure 13A). Hence, this assay suggests that expression of Caspr1 in COS7 cells results in an activation of Src and a consequent increase in its phosphorylated levels.

Additionally, we further confirmed that this Caspr1-mediated activation of Src occurs through the intracellular proline-rich region of Caspr1 (Figure 13B and 13C). Expression of Caspr1 in COS7 cells does, indeed, increase the phosphorylated levels of Src in 36 ± 15 %, when compared to control values. The deletion mutant of Caspr1 lacking its proline-rich region (Caspr1ΔPro), however, failed to have a similar effect, resulting in levels of phospho-Src to values comparable to the control (101 ± 50 % - Figure 13B and 13C). (Of note, this result corresponds to three independent experiments: in two of them levels of phospho-Src with Caspr1ΔPro were similar to control, whereas in the third we observed an increase in phosphorylated Src, for the same condition of transfection). This result is in agreement to results presented by Peles

and colleagues, which confirmed an interaction between Caspr1 and Src, possibly through the proline-rich region of Caspr1 (Peles *et al.*, 1997). In the present study, we further show that this intracellular proline-rich region of Caspr1 is responsible for the activation of Src, since the deletion mutant for that region, Caspr1ΔPro, had no effect in phosphorylated levels of Src (Figure 13B and 13C).

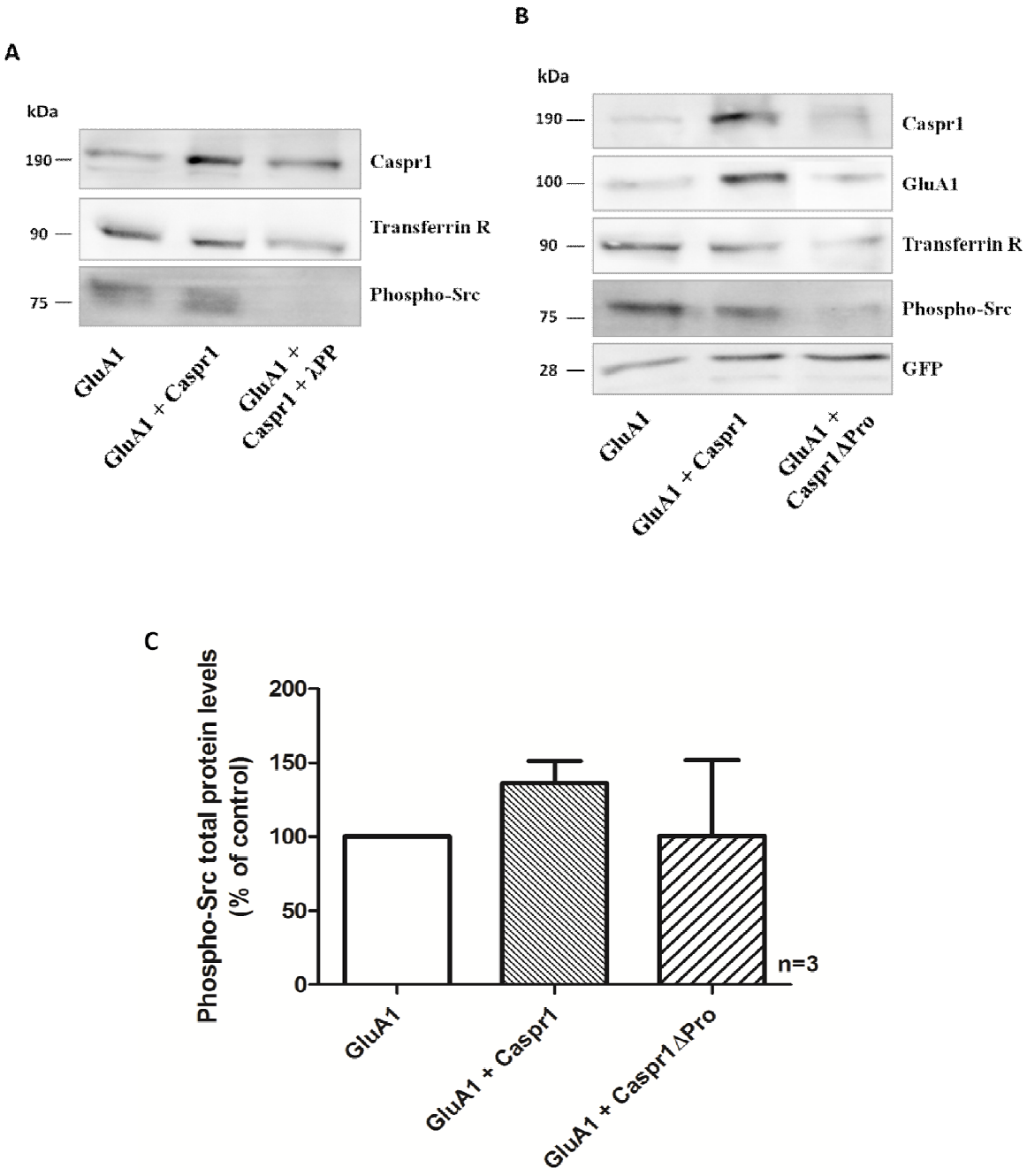
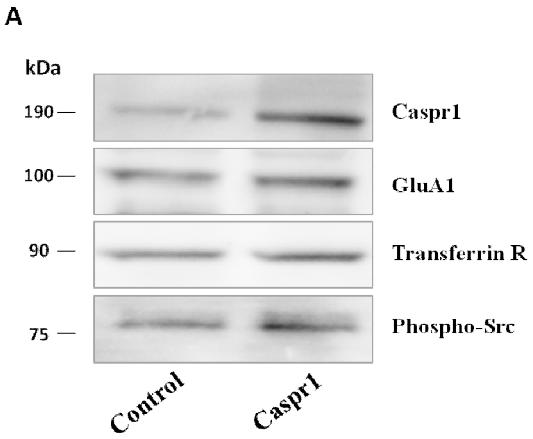
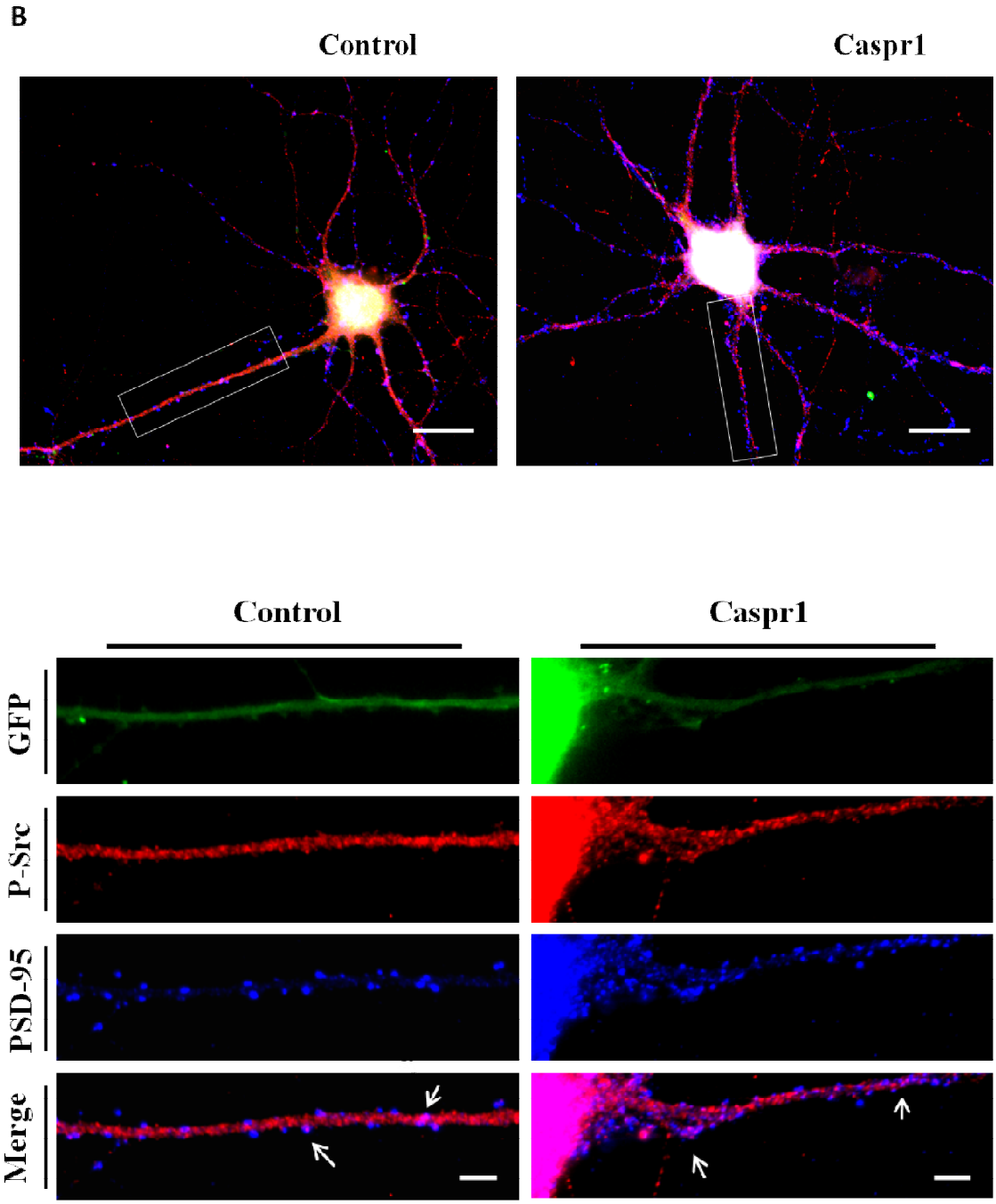


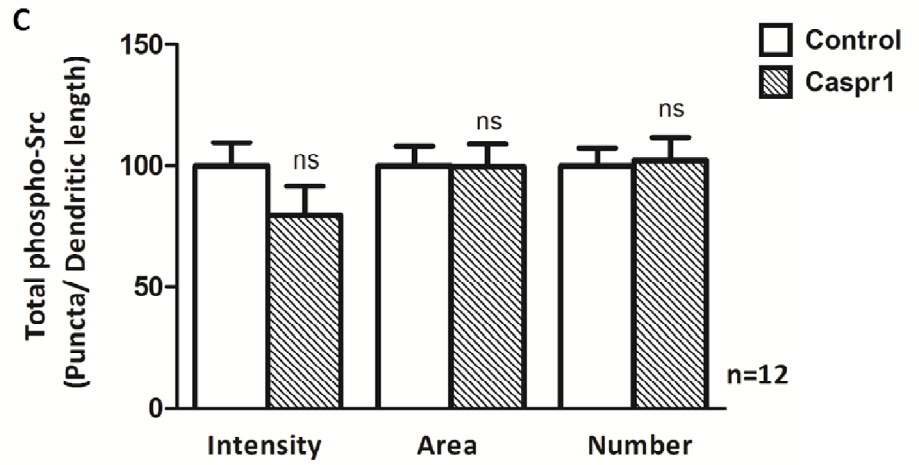
Figure 13 – Caspr1, through its intracellular proline-rich region, is able to induce a specific increase in phosphorylated levels of the tyrosine kinase Src, in COS7 cells. (A) Cultured COS7 cells were co-transfected either with GluA1 + CMV empty vector + GFP or with GluA1 + Caspr1 (full-length) + GFP, as indicated. Cells were allowed to express the constructs for 48 hrs, after which total cell extracts were obtained. Approximately 150µg of protein from lysates of Caspr1-transfected cells were subjected to a dephosphorylation assay with lambda protein phosphatase (GluA1 + Caspr1 + λPP) for 2 hrs, at 30ºC. Levels of phosphorylated Src were then assessed by western blot. (B-C) Cultured COS7 cells were co-transfected either with GluA1 + CMV empty vector + GFP**,** GluA1 + Caspr1 (full-length) + GFP or with GluA1 + Caspr1ΔPro + GFP, as indicated. Cells were allowed to express the constructs for 48 hrs, after which total cell extracts were obtained and levels of GluA1 and phosphorylated Src assessed by western blot. Results are presented as means ± S.E.M. when compared to control and normalized to the loading control Transferrin R (human transferrin receptor). Statistical analysis was determined by the one-way ANOVA analysis of variance followed by the Dunnett's post test, although no statistical significance was achieved, $p>0.05$.

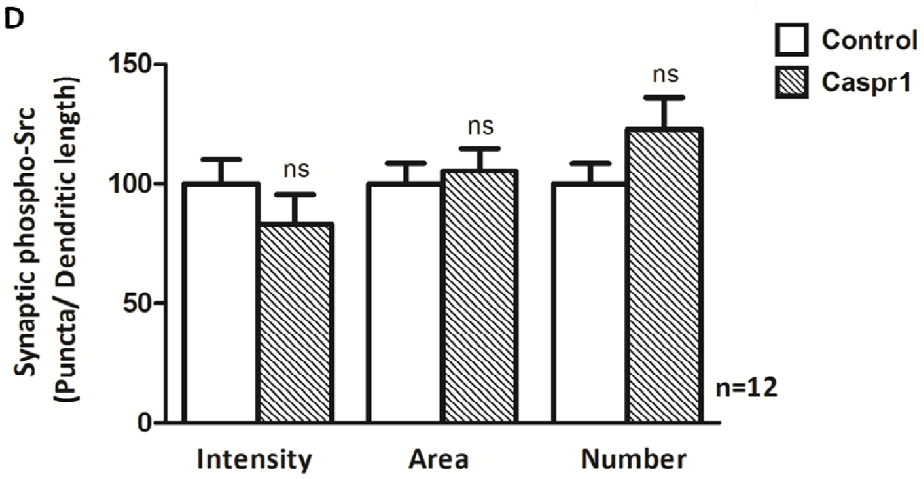
To test for a Caspr1-mediated activation of Src in a neuronal system, Caspr1 was overexpressed in hippocampal neurons in culture, by transfecting high-density cultures at DIV7. At DIV15, total extracts were obtained and levels of phosphorylated Src were assessed by western blot (Figure 14A). According to the results obtained using the heterologous system, this preliminary result indicates that overexpression of Caspr1 in hippocampal neurons also appears to induce a distinct increase in the levels of phospho-Src, when compared to the control situation (Figure 14A). This evidence in hippocampal neurons not only confirms the hypothesis of a role for Caspr1 in activating the Src signaling pathway, but also suggests an interaction between these two proteins

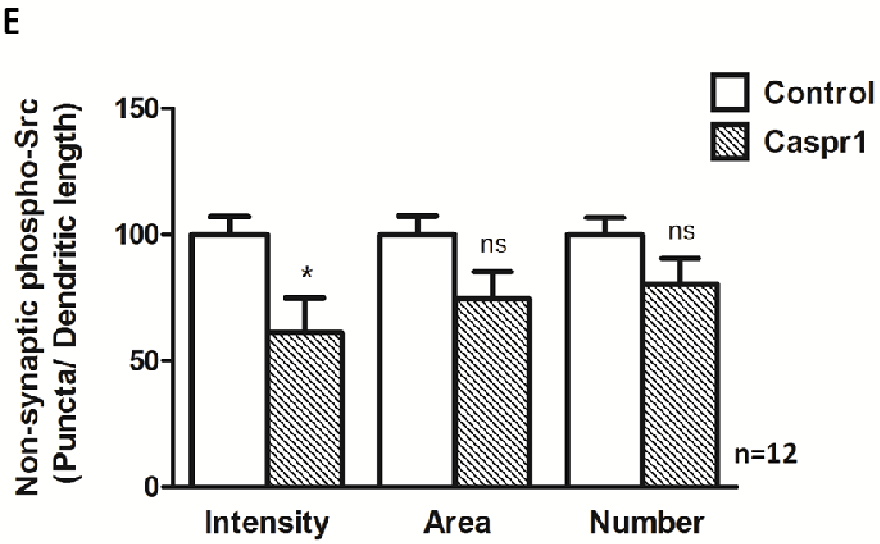
in a neuronal system, which has not been shown until now. In Src, several factors contribute to its inactive conformation: besides the phosphorylation on a tyrosine residue (Tyr527) present in the negative regulatory C-terminal tail of Src, intramolecular interactions between its modular SH3 and SH2 domains restrain the catalytic domain in such a way that phosphorylation of Tyr416, in the activating loop, is blocked (Boggon & Eck, 2004; Guarino, 2010; Ingley, 2008; Thomas & Brugge, 1997). Given our results, it is likely that the proline-rich region of Caspr1, a high affinity ligand of SH3 domains, competes for the SH3 domain of Src, displacing and relieving the intramolecular inhibition between the SH2/SH3 domains and causing a shift to the active form of the kinase domain of Src. Consequently, Tyr416 in the exposed activation loop is allowed to undergo phosphorylation, which leads to a maximal kinase activity, thus triggering the activation of a downstream signaling cascade. Indeed, what is also interesting in our results is that this Caspr1-mediated increase in phospho-Src appears to occur along with an increase in GluA1 levels both in COS7 cells (Figure 13B) and in hippocampal neurons (Figure 14A), at least in the one experiment performed in neurons. Thus, overall, these results suggest that the upregulatory effect of Caspr1 in GluA1 levels may occur through a Src-dependent signaling pathway.




B
Control
Caspr1
Control
Caspr1
GFP
P-Src
PSD-95
Merge


C
Total phospho-Src
(Puncta/ Dendritic length)
150
100
50
0
ns
ns
ns
Control
Caspr1
n=12
Intensity
Area
Number


D
Synaptic phospho-Src
(Puncta/ Dendritic length)
150
100
50
0
ns
ns
ns
Control
Caspr1
n=12
Intensity
Area
Number


E
Non-synaptic phospho-Src
(Puncta/ Dendritic length)
150
100
50
0
*
ns
ns
Control
Caspr1
n=12
Intensity
Area
Number

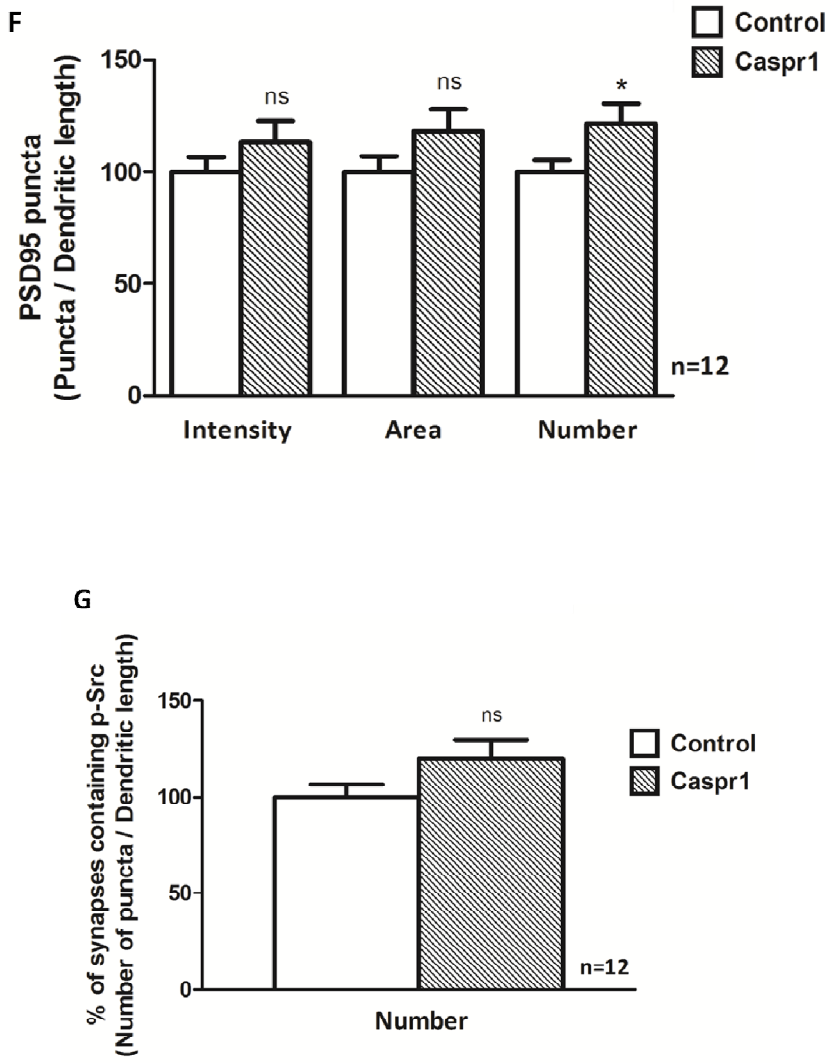


Figure 14 – Caspr1 induces an increase in total levels of phosphorylated Src, as well as a specific increase in the number of synaptic puncta of the phosphorylated kinase, in hippocampal neurons. (A) High-density cultured hippocampal neurons were transfected at DIV7 with GFP (Control) or with GFP + Caspr1, as indicated. Cells were allowed to express the proteins until DIV15, when total cell extracts were obtained and levels of GluA1 and phosphorylated Src assessed by western blot. (B) Primary hippocampal neurons from low-density Banker cultures were transfected at DIV7 with GFP (Control) or with GFP + Caspr1, as indicated. Cells were allowed to express the proteins until DIV15, after which they were fixed and immunolabeled with antibodies for phosphorylated Src (red) and PSD-95 (blue). Images

were taken using a Zeiss Axiovert 200 fluorescent microscope. Neuronal dendrites were visualized via MAP2 staining and fields for imaging were chosen by the GFP channel, for the presence of transfected, GFP-positive, neurons. Scale bar: 20μm; Scale bar (crops): 5μm. Arrows indicate colocalization with PSD95 puncta (C - G) Quantification data are shown in (C-G). Phosphorylated Src clustering was analyzed and quantified with Image J 1.43 analysis software. Dendrites were randomly chosen and their lengths determined. (C) The signal for total phospho-Src was analyzed after thresholds were set, such that recognizable clusters were included in the analysis, and the background intensity of each image was subtracted. (D-E) Synaptic (D) phospho-Src puncta were selected by colocalization with PSD-95 (see arrows), whereas non-synaptic (E) clusters of phospho-Src correspond to puncta that did not colocalize with PSD-95. (F) PSD-95 puncta were analyzed after thresholds were set, such that recognizable clusters were included in the analysis, and the background intensity of each image subtracted. (G) The percentage of synapses containing phospho-Src was determined by colocalization of PSD-95 puncta as a mask overlaid on the channel for phospho-Src. (C-G) Per each selected cell, intensity, area and number of total (C), synaptic (D) and non-synaptic (E) puncta of phosphorylated Src per dendritic length was determined. The same was determined regarding puncta of PSD-95 (F). Measurements were performed in a single independent neuronal preparation, with 12 cells per condition, and values obtained per cell were normalized against the control mean of the experiment. Results are presented as means ± S.E.M. Statistical analysis was determined by the unpaired *t*-test, (E-F) *$p<0.05$; ns - $p>0.05$.

Immunocytochemistry experiments were also performed to further confirm the activation of Src, at the single-cell level. Low-density hippocampal neurons were transfected at DIV7 either with GFP (control) or GFP + Caspr1 and cells were allowed to express the proteins until DIV15, when they were fixed and double-labeled with antibodies for phosphorylated Src as well as for the postsynaptic marker PSD-95. To specifically evaluate the effect of Caspr1 overexpression in the activation of Src, fields

for imaging were chosen by the GFP channel, for the presence of transfected, GFP-positive, neurons (Figure 14B). Surprisingly, overexpression of Caspr1 did not induce an increase in the intensity of total puncta of phosphorylated Src. Indeed, and contrary to what was observed in the western blot analysis, the total intensity of phospho-Src puncta is decreased in about 20% (to 79.5 ± 12 % of control), when compared to control values, although differences do not reach a statistical significance (Figure 14C). Moreover, the same effect was observed for the intensity of phosphorylated Src puncta, at the synaptic level (colocalized with PSD-95 – see arrows), which also decreased to approximately 83 ± 12 %, when compared to the control (Figure 14D). Regarding the area of phospho-Src puncta, comparing to control, overexpression of Caspr1 did not exert any differences for either total or synaptic pools. However, comparison between the total and synaptic number of phospho-Src puncta shows that Caspr1 induces an increase of about 23 % (123 ± 13 % of control) in phospho-Src puncta present at synapses but not in total clusters, which remain with values similar to the control (Figure 14C and D). Interestingly, we were able to observe that the increase in the number of phospho-Src puncta seems to be a specific effect on their synaptic pool. Overexpression of Caspr1 not only significantly decreased the intensity of non-synaptic phospho-Src puncta (61 ± 13 % of control, *$p<0.05$) and their area (74 ± 11 % of control, ns - $p>0.05$), but also had an effect in decreasing the number of non-synaptic puncta in about 20 % (to 80 ± 10 % of control), when compared to control values, although not reaching a statistical significance (Figure 14E). To further understand the significance of such a disparate effect in the number of synaptic and non-synaptic puncta of phosphorylated Src, we investigated whether Caspr1 was having an effect in PSD-95 puncta (Figure 14F). Indeed, overexpression of Caspr1 resulted in an increased intensity (113 ± 9 % of control) and area (118 ± 9 % of control) of PSD-95 puncta, as

well as a significant increase in their number (122 ± 8 % of control), indicating that there are more synapses when Caspr1 is overexpressed. This evidence is, by itself, very interesting, suggesting a possible role for Caspr1 in the regulation of this scaffolding protein. However, it compromises, in a certain way, the quantification of synaptic phospho-Src puncta, determined by colocalization with PSD-95 as a synaptic marker. Despite this, we further investigated if the totality of synapses (PSD-95 puncta per dendritic length) contains more phosphorylated Src, when Caspr1 is overexpressed (Figure 14G). Comparing with the control, there seems to be an incremental tendency in the percentage of synapses containing phospho-Src, when Caspr1 is overexpressed, although no statistical significance was attained (120 ± 9 % of control).

Altogether, these results suggest that Caspr1 plays a role in the activation of the tyrosine kinase Src, increasing its phosphorylated levels. Results obtained by western blot analysis, both in COS7 cells and hippocampal neurons, are consistent with this hypothesis. On the contrary, the decrease in the intensity of total and synaptic puncta of phospho-Src, obtained from imaging experiments, indicates otherwise. Nevertheless, one must bear in mind that, while immunocytochemistry data refer to the effect of Caspr1 in phospho-Src exclusively in dendrites, results from western blot experiments are obtained from whole cell lysates, thus reflecting the contribution of cell bodies and axons, in which the effect of Caspr1 in the activation of Src remains unknown. It is possible that the major effect of Caspr1 in the phosphorylation of Src occurs at the cell body level, which would account for the diminished intensity of Src puncta, observed in the imaging experiments. Despite this, one hypothesis that arises from the increased number of phosphorylated Src puncta in synapses with the overexpression of Caspr1, which is further supported by the reduction in area and number of phospho-Src puncta from a non-synaptic pool, is that overexpression of Caspr1, abundant in synapses,

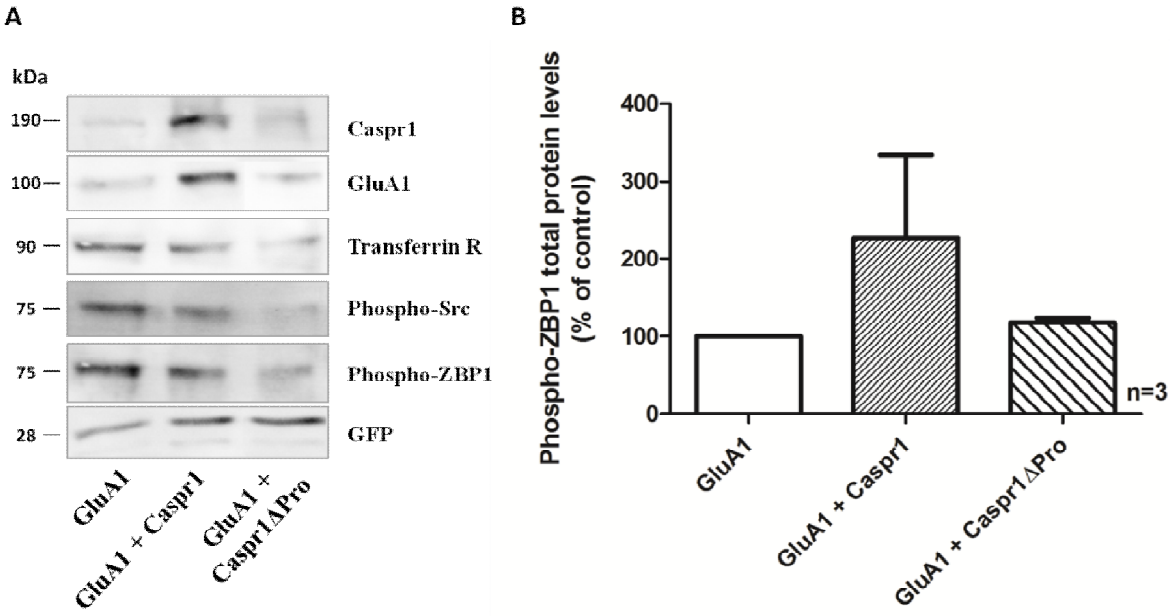
accounts for more synaptic binding sites for phosphorylated Src. Moreover, interaction of Src with Caspr1 may induce a fragmentation of non-synaptic puncta, leading to a delocalization of phosphorylated Src to more synapses, justifying the increased number of synaptic puncta. Thus, this would mean that more synapses would contain more activated Src, capable of triggering downstream signaling cascades.

These rather interesting evidences proposing the upregulation of GluA1 levels by Caspr1 as well as the Caspr1-mediated activation of a signaling pathway downstream of Src, particularly at the synaptic level, are extremely promising. However, they raise one critical question. Which signaling cascade is specifically being triggered following the Caspr1-mediated activation of Src and which are the specific efectors involved in it? Src has already been thoroughly described as a regulator of many fundamental cellular processes including cell growth and survival, proliferation, cell communication, shape, differentiation and migration, among several other physiological functions (Huveneers & Danen, 2009; Ingley, 2008; Parsons & Parsons, 2004; Thomas & Brugge, 1997). One of its best described and interesting roles regards the reorganization of the cytoskeleton, which highly impacts axonal guidance and neurite outgrowth in developing neurons. The downstream target of Src in these events is the oncofetal protein Zipcode binding protein (ZBP1). This RNA-binding protein binds to a conserved 54-nucleotide element, known as the 'zipcode', located in the 3'UTR of β-actin mRNA (Ross *et al.*, 1997), repressing its premature translation. Upon particular stimuli, Src is able to promote translation of β-actin transcripts by phosphorylating a key tyrosine residue in ZBP1 that is required for its binding to RNA (Huttelmaier *et al.*, 2005). These sequential events provide both temporal and spatial control over β-actin translation and are necessary to its localization to the cell periphery. Particularly at developing growth cones ZBP1 activity is important for neurotrophin-

induced neurite outgrowth (Zhang *et al.*, 2001) and its Src-dependent phosphorylation is required for the assymetrical and local synthesis of β-actin and growth cone turning and guidance, induced by external cues such as BNDF and Netrin1 (Leung *et al.*, 2006; Sasaki *et al.*, 2010; Welshhans & Bassell, 2011; Yao *et al.*, 2006). Apart from its role in axonal guidance, ZBP1 also regulates the dendritic arboring of hippocampal neurons (Perycz *et al.*, 2011) and localizes to dendritic spines, regulating their density and structure through the local translation of β-actin (Eom *et al.*, 2003; Tiruchinapalli *et al.*, 2003). Moreover, ZBP1 has also been shown to bind to the mRNA of cofilin, an actin-depolymerizing factor, and mediate Slit2-induced local translation during growth cone collapse (Piper *et al.*, 2006). Thus, it is evident that ZBP1 plays a key role in the regulation of some transcripts with major importance in neuronal development and function, which endows ZBP1 with the potential to functionally regulate other transcripts.

Interestingly, a study carried out in our laboratory indicated that Caspr1 significantly upregulates GluA1 mRNA levels in COS7 cells, and that this effect is specifically mediated by Caspr1 intracellular proline-rich region, since expression of the deletion mutant for this domain (Caspr1ΔPro) resulted in GluA1 mRNA levels comparable to the control (unpublished data). The new data that we are now reporting show that Caspr1 promotes activation of the Src signaling pathway in a proline-rich domain dependent manner. Given that ZBP1 is a downstream target of Src capable of regulating several transcripts, it would be extremely interesting to clarify if Caspr1 affects ZBP1 as a downstream effect of the activation of Src and if so, understand if ZBP1 binds to GluA1 mRNA and regulates the levels of this transcript.

In order to evaluate if the activation of Src by Caspr1 results in a downstream effect in ZBP1, Caspr1 or its deletion mutant for the proline-rich region (Caspr1ΔPro) were exogenously expressed in COS7 cells, together with the GluA1 subunit. Cells were allowed to express the constructs for 48 hrs, after which total protein levels of phosphorylated Src, phosphorylated ZBP1 and GluA1 were assessed by western blot analysis (Figure 15).



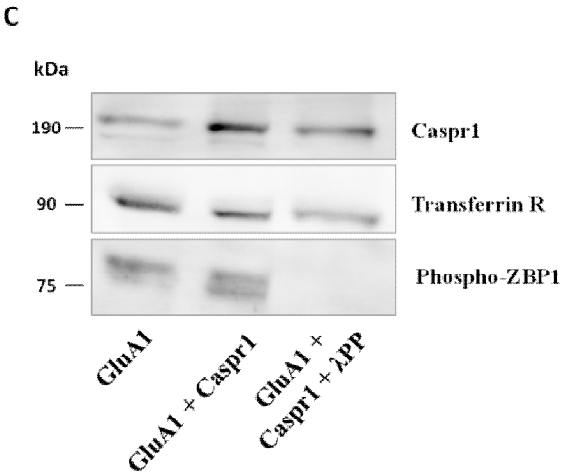


Figure 15 – Caspr1, through its intracellular proline-rich region, induces a specific increase in phosphorylated levels of the Zipcode binding protein (ZBP1), in COS7 cells. (A-B) Cultured COS7 cells were co-transfected either with GluA1 + CMV empty vector + GFP**,** GluA1 + Caspr1 (full-length) + GFP or with GluA1 + Caspr1ΔPro + GFP, as indicated. Cells were allowed to express the constructs for 48 hrs, after which total cell extracts were obtained and levels of phosphorylated ZBP1, phosphorylated Src and GluA1 were assessed by western blot. Results are presented as means ± S.E.M. when compared to control and normalized to the loading control Transferrin R (human transferrin receptor). Statistical analysis was determined by the one-way ANOVA analysis of variance followed by the Dunnett's post test, although no statistical significance was achieved, $p>0.05$. (C) Cultured COS7 cells were co-transfected either with GluA1 + CMV empty vector + GFP or with GluA1 + Caspr1 (full-length) + GFP, as indicated. Cells were allowed to express the constructs for 48 hrs, after which total cell extracts were obtained. Approximately 150µg of protein from lysates of Caspr1-transfected cells were subjected to a dephosphorylation assay with lambda protein phosphatase (GluA1 + Caspr1 + λPP) for 2 hrs, at 30ºC. Levels of phosphorylated ZBP1 were then assessed by western blot.

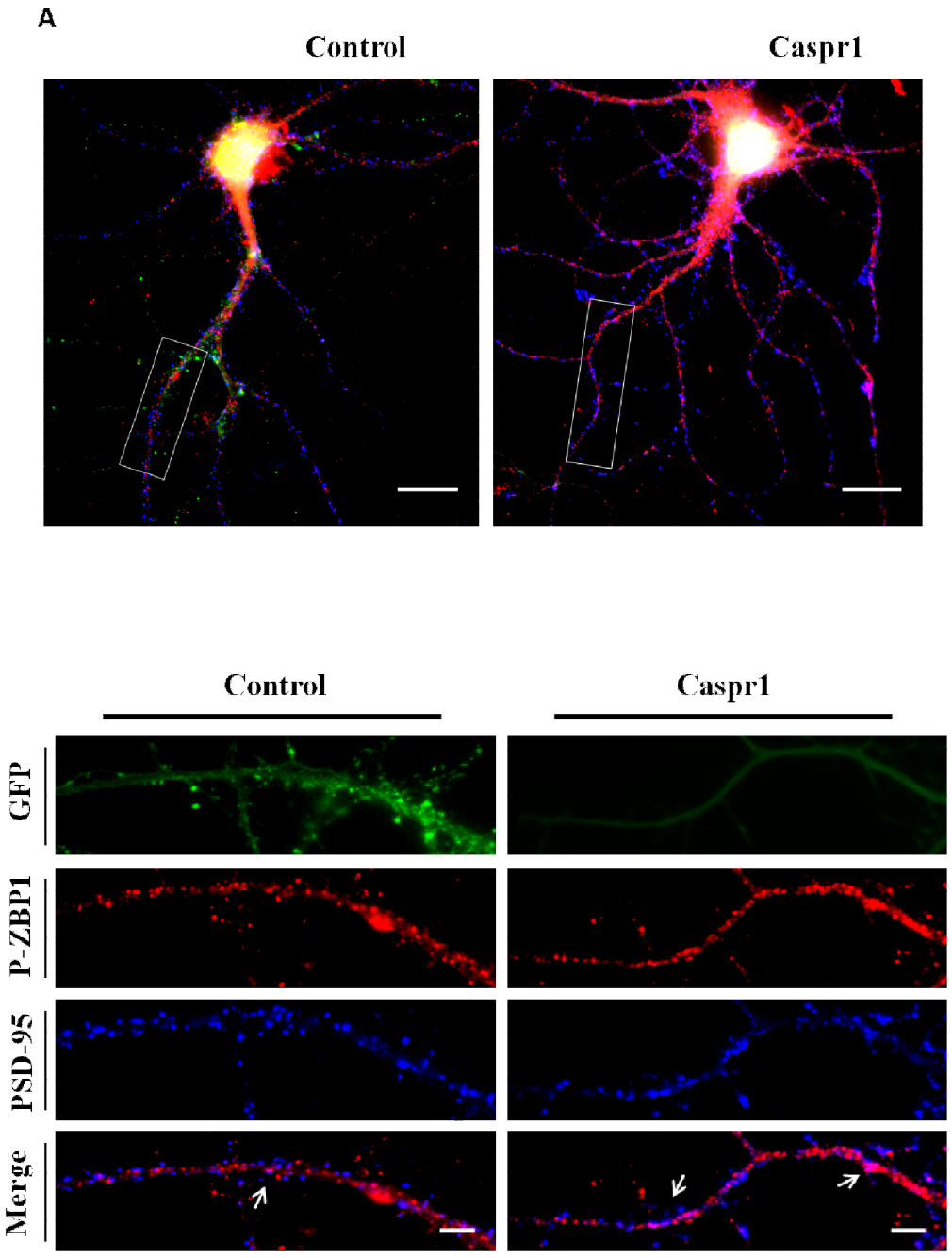
In parallel to the increase in levels of phosphorylated Src (see Figure 13), expression of Caspr1 in COS7 cells resulted in an evident upregulation in levels of phosphorylated ZBP1 (227 ± 107 % of control), when compared to control (Figure 15A and 15B), although values do not reach statistical significance. On the other hand, expression of the deletion mutant of Caspr1 lacking its proline-rich domain (Caspr1ΔPro) resulted in a marked decrease of phospho-ZBP1 levels to control values (117 ± 5 % of control – Figure 15A and 15B). However, ahead of any preliminary conclusions, it is of notice that, similarly to what happened to phospho-Src, the polyclonal antibody raised against phospho-ZBP1 detected a band which mobility in

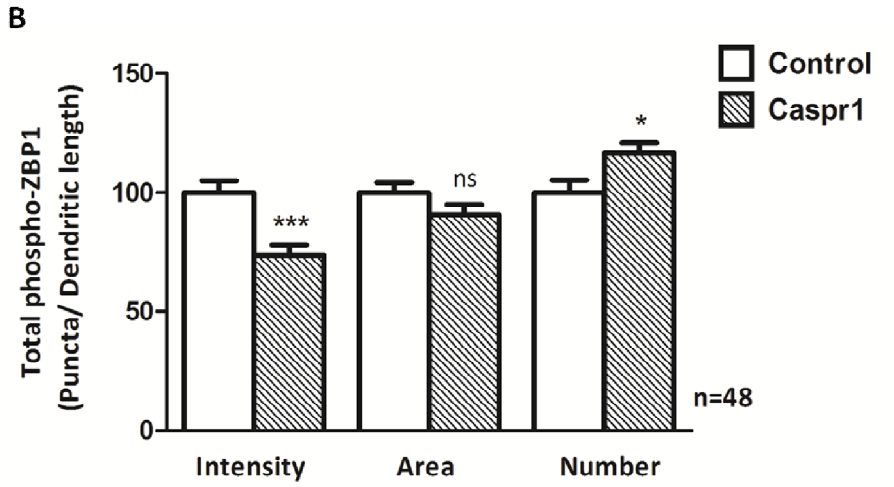
SDS-PAGE is compatible with a protein of 75kDa, which does not correspond to the 65kDa expected for phospho-ZBP1. Thus, to evaluate the specificity of these results, lysates from Caspr1-transfected COS7 cells were subjected to a dephosphorylation assay with λPP, for 2 hrs at 30ºC (Figure 15C), which resulted in an almost complete disappearance of the 75kDa band, indicating that this band corresponds to a specific increase in phospho-ZBP1 by Caspr1. Overall, this upregulatory effect in phospho-ZBP1, occurring in parallel with an increase of phospho-Src, suggests that Caspr1 is, indeed, activating a downstream signaling pathway of Src, which, in turn, promotes an increase in the phosphorylation of its target, ZBP1. Moreover, these results provide yet another confirmation on the major role of the intracellular proline-rich region of Caspr1, which is necessary for its upregulatory effect in levels of phosphorylated ZBP1. What is also interesting is the consistent increase in levels of phosphorylated Src and phosphorylated ZBP1 in parallel to an increase in GluA1 levels, in COS7 cells expressing Caspr1 (Figure 15A and B). Moreover, the parallel upregulation in the three evaluated proteins is not observed in the absence of the proline-rich region of Caspr1.

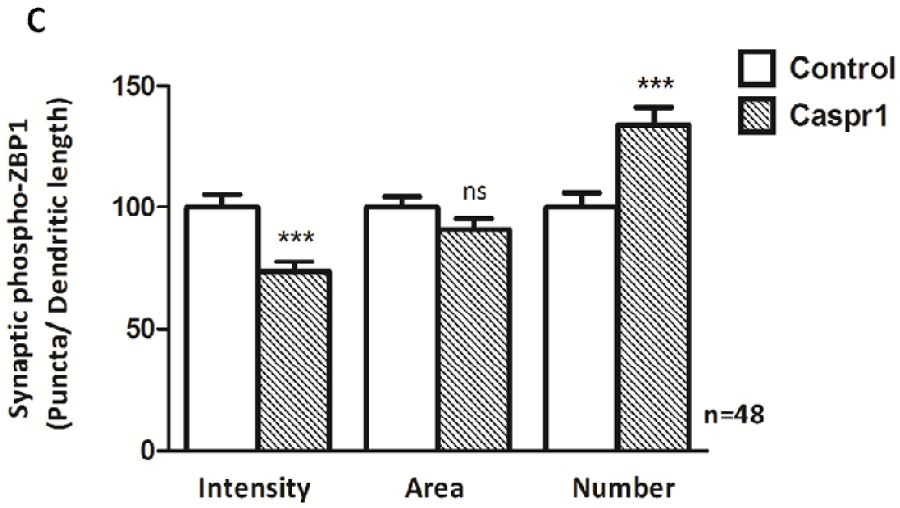
To further confirm these results in a neuronal system, levels of phosphorylated ZBP1 in the presence of Caspr1 were assessed at the single cell level, by transfecting low-density hippocampal neurons at DIV7, either with GFP alone (control) or GFP + Caspr1. After expressing proteins until DIV15, cells were fixed and immunolabeled with antibodies for phosphorylated ZBP1 and for PSD-95 as a postsynaptic marker. To specifically evaluate the effect of Caspr1 in phospho-ZBP1, imaging fields were chosen by the GFP channel, for the presence of transfected, GFP-positive neurons (Figure 16A). Curiously, once again, overexpression of Caspr1 did not exert the expected upregulatory effect in the intensity of phosphorylated ZBP1 puncta. Similarly to what

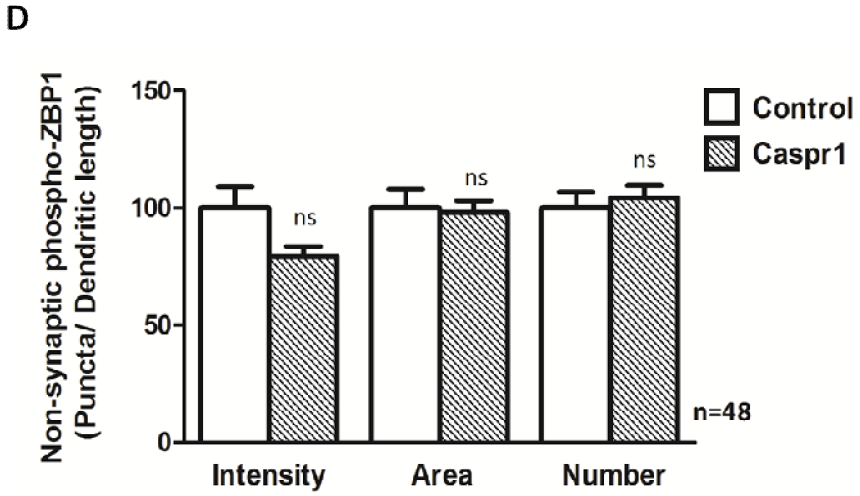
happened to phospho-Src, Caspr1 overexpression resulted in a significant decrease both in the total and synaptic intensity of phospho-ZBP1 puncta to 74 ± 4 % (***p<0.001) and 73 ± 4 % (***p<0.001), respectively, when compared to control values (Figure 17B and 16C). Moreover, the area of phospho-ZBP1 puncta, total or synaptic, seems to have a tendency to decrease in about 10% (approximately 91 ± 4 % of control, in both cases), when Caspr1 is overexpressed, although no significant difference is reached (Figure 16B and 16C). However, the effect of Caspr1 in the number of phosphorylated ZBP1 puncta is in opposite direction to its effect in the other analyzed parameters. Indeed, as it happened for the number of phospho-Src puncta, Caspr1 was able to induce not only a significant increase (116 ± 4 % of control, *p<0.05) in the number of total phospho-ZBP1 puncta, but also a robust upregulation to 134 ± 7 % (***p<0.001) in the number of phospho-ZBP1 puncta, at the synapse level (Figure 16B and C). Furthermore, this increase in the number of phospho-ZBP1 puncta occurred specifically on their synaptic pool, since the same effect was not observed in the number of non-synaptic phospho-ZBP1 puncta. Indeed, the number of phosphorylated ZBP1 puncta at non-synaptic sites remained with values similar to control, when Caspr1 was overexpressed (104 ± 5 % of control – Figure 16D). Also, the area of non-synaptic phospho-ZBP1 puncta was not altered in the presence of Caspr1, whereas their intensity was slightly decreased, although not to significant values (98 ± 5 % and 80 ± 5 % of control, respectively - Figure 16D). Additionally, an analysis on the intensity, area and number of PSD-95 puncta was also performed in these experiments, allowing the confirmation of a possible role of Caspr1 in PSD-95 clustering and in the number of synapses. Once again (see Figure 14F), Caspr1 was able to induce a small but statistically significant increase in the number of PSD-95 puncta (113 ± 5 % of control), while not having any effect in the intensity or area of the clusters (Figure 16E). Moreover, the evaluation of the total

percentage of synapses (PSD-95 puncta per dendritic length) containing phosphorylated ZBP1 showed that, when compared to control, overexpression of Caspr1 seems to induce an increasing tendency in the percentage of synapses containing phosphorylated ZBP1 (110 ± 4 % of control), although no statistical significance was determined (Figure 16F).




B
Total phospho-ZBP1
(Puncta/ Dendritic length)
150
100
50
0
***
ns
*
Control
Caspr1
n=48
Intensity
Area
Number


C
Synaptic phospho-ZBP1
(Puncta/ Dendritic length)
150
100
50
0
***
ns
***
Control
Caspr1
n=48
Intensity
Area
Number


D
Non-synaptic phospho-ZBP1
(Puncta/ Dendritic length)
150
100
50
0
ns
ns
ns
Control
Caspr1
n=48
Intensity
Area
Number

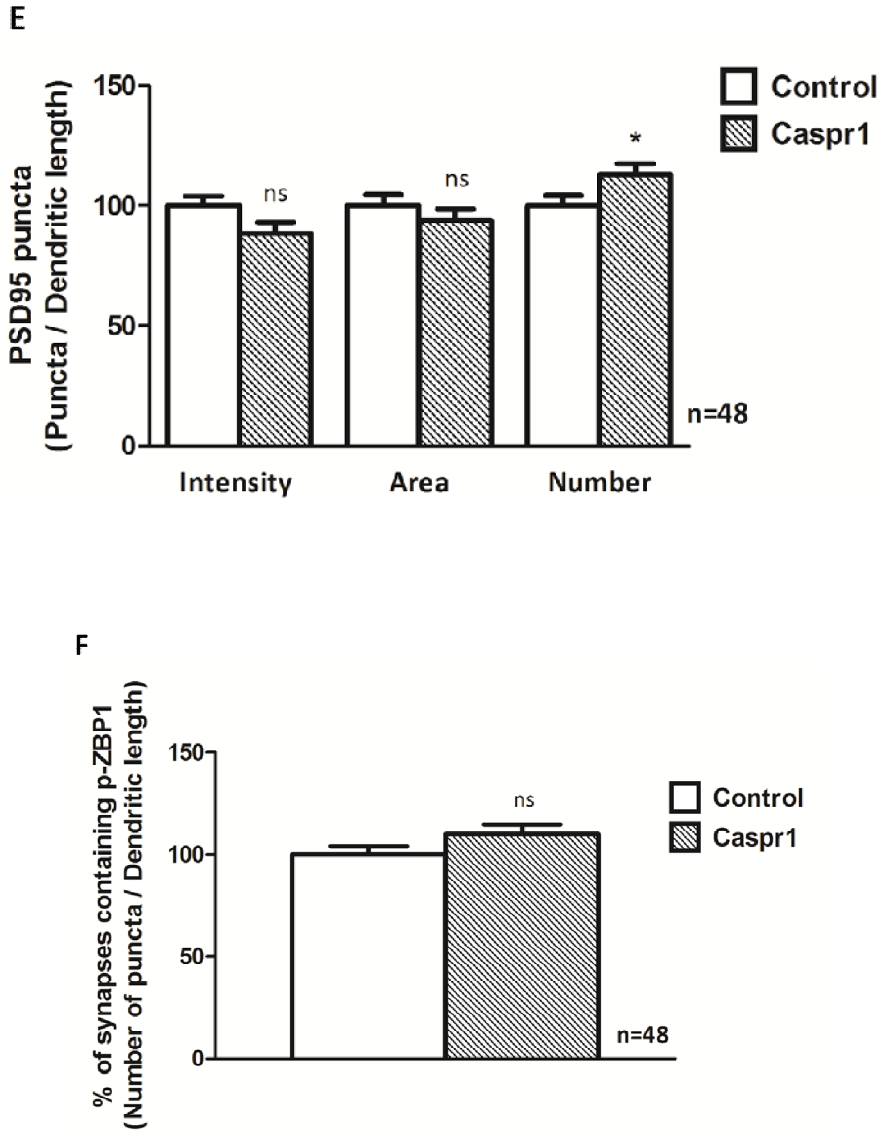


Figure 16 – Caspr1 is able to induce a specific significant increase in the number of phosphorylated ZBP1 synaptic puncta, in hippocampal neurons. (A) Primary hippocampal neurons from low-density Banker cultures were transfected at DIV7 with GFP (Control) or with GFP + Caspr1, as indicated. Cells were allowed to express the proteins until DIV15, after which they were fixed and immunolabeled with antibodies for phosphorylated ZBP1 (red) and PSD-95 (blue). Images were taken using a Zeiss Axiovert 200 fluorescent microscope. Dendrites were visualized via MAP2 staining and fields for imaging were chosen by the GFP channel, for the presence of transfected, GFP-positive, neurons. Scale bar: 20µm; Scale bar (crops): 5µm. Arrows represent colocalization with PSD95 puncta. (B - F) Quantification of the results shown

in (A). Phosphorylated ZBP1 clustering was analyzed and quantified with Image J 1.43 analysis software. Dendrites were randomly chosen and their lengths determined. (B) The signal for total phospho-ZBP1 was analyzed after thresholds were set, such that recognizable clusters were included in the analysis, and the background intensity of each image was subtracted. (C - D) Synaptic (C) phospho-ZBP1 puncta were selected by colocalization with PSD-95 (see arrows), whereas non-synaptic (D) clusters of phospho-ZBP1 correspond to puncta that did not colocalize with PSD-95. (E) PSD-95 puncta were analyzed after thresholds were set, such that recognizable clusters were included in the analysis, and the background intensity of each image subtracted. (F) The percentage of synapses containing phospho-ZBP1 was determined by colocalization of PSD-95 puncta as mask overlaid on the channel for phospho-ZBP1. (B-F) Per each selected cell, intensity, area and number of total (B), synaptic (C) and non-synaptic (D) puncta of phosphorylated ZBP1 per dendritic length was determined. PSD-95 clusters were also analysed (E). Measurements were performed in three independent experiments, with at least 16 cells per condition, and values obtained per cell were normalized against the control mean of that single experiment. Results are presented as means ± S.E.M. Statistical analysis was determined by the unpaired *t*-test, (B – F) ***$p<0.001$; *$p<0.05$; ns - $p>0.05$.

Taken together, these results tend to confirm the hypothesis of an important role of Caspr1 in the activation of a signaling pathway downstream of Src that results in an increased phosphorylation of its target ZBP1. Despite the decrease in the intensity of total and synaptic puncta of phospho-ZBP1, which we cannot explain, Caspr1 induces a specific, significant and robust increase in the number of phosphorylated ZBP1 synaptic puncta, an effect that does not occur in puncta present in the non-synaptic pool. A possible explanation for these results comes from evidences showing the presence of large ZBP1 granules localized to dendritic shafts and spine compartments (Tiruchinapalli *et al.*, 2003). Moreover, ZBP1 colocalizes with β-actin mRNA into

individual dendritic RNPs or stress granules, where it promotes a transient translational repression of this transcript (Eom *et al.*, 2003; Stohr *et al.*, 2006). It is conceivable that ZBP1 is contained in these synaptic granules in an active, RNA-bound and unphosphorylated state and that, upon Src-mediated phosphorylation, granules disperse, promoting translation of localized transcripts. Thus, this would account for the increased number of phosphorylated ZBP1 synaptic puncta that results from a Caspr1-mediated activation of the Src signaling pathway.

So far, together with evidences from previous studies carried out in our laboratory, we have seen that Caspr1 is able to not only increase total GluA1 protein levels, but also their surface expression and synaptic tagging [through biotinylation and electrophysiology experiments carried by Sandra Santos (Santos, 2009)]. Moreover, the increase in protein levels appears to occur through an upregulation of Caspr1 in GluA1 mRNA levels, dependent on the proline-rich region of Caspr1 (data not shown). Regulation of neuronal transcripts frequently occurs through interaction of *cis*-acting elements located in their 3'UTR with specific *trans*-acting RNA-binding proteins. Indeed, analysis of the 3'UTR of GluA1, performed by Santos and colleagues, further indicates the presence of regulatory elements in the 3'UTR of the GluA1 transcript that are essential for Caspr1 to exert its effect, since Caspr1 completely lost its upregulatory effect in GluA1 mRNA, when the 3'UTR of GluA1 was deleted. Furthermore, a sequence analysis of the 3'UTR of GluA1 revealed several putative binding sites for RNA-binding proteins, particularly of the embryonic lethal abnormal vision (ELAV) family, known to regulate the stability of a subset of mRNAs (Santos, 2009). Overall, these evidences propose a novel way to control the GluA1 subunit, through regulation of its mRNA stability. If Caspr1 is regulating the stability of GluA1 mRNA levels through the activation of a signaling pathway downstream of Src that ends up with an

increase in phosphorylated levels of ZBP1, it is reasonable to propose that this stabilizing effect is mediated through an interaction of ZBP1, an RNA-binding protein that recognizes specific *cis*-elements in the 3'UTR of transcripts, with the mRNA of the GluA1 subunit. Indeed, ZBP1 has already been implicated in the regulation of stability of various target mRNAs, such as c-Myc, CD44, βTrCP1 and β-catenin (Gu *et al.*, 2008; Leeds *et al.*, 1997; Noubissi *et al.*, 2006; Vikesaa *et al.*, 2006; Yisraeli, 2005). Future experiments should be carried out to confirm a possible interaction between ZBP1 and the GluA1 mRNA and to evaluate a possible regulation of the stability of GluA1 transcripts by this RNA-binding protein.

Chronic blockade of neuronal activity increases levels of endogenous Caspr1

Given the physiological importance of AMPA receptors, comprehending how dendritic transcripts of the GluA1 subunit are regulated would provide further insight into the mechanisms underlying synaptic plasticity. So far, we have seen that the new interactor of the GluA1 subunit, Caspr1, may play a major role in the regulation of GluA1 protein, and particularly of its mRNA stability. Moreover, we proposed that this effect occurs through a Caspr1-mediated activation of a Src signaling pathway that ends up in the phosphorylation of the downstream target ZBP1, a RNA-binding protein that could stabilize GluA1 transcripts. Thus, since Caspr1 is the most upstream effector in this mechanism, it would be important to understand which physiological stimuli regulate endogenous Caspr1, and in particular to test if it is regulated by neuronal activity.

The information available about the regulation of Caspr1 concerns its intracellular traffic. Apparently, the interaction between Contactin1 and Caspr1 is essential for its expression at the cell surface. This interaction occurs at the ER and it is required for an efficient recruitment of Caspr1 to lipid rafts and sorting to the plasma membrane (Boyle *et al.*, 2001; Faivre-Sarrailh *et al.*, 2000). Moreover, expression of Caspr1 on its own, in neuroblatoma cells, resulted in its ER retention, a phenomenon that seems to be dependent on an ER retention signal located in the extracellular PGY-repeat region of Caspr1 (Bonnon *et al.*, 2007; Bonnon *et al.*, 2003). Further evidences regarding the control of endogenous Caspr1 in neurons, particularly in what concerns its synaptic function, have not been described so far, although Caspr1 has been described to localize to postsynaptic regions (Murai *et al.*, 2002).

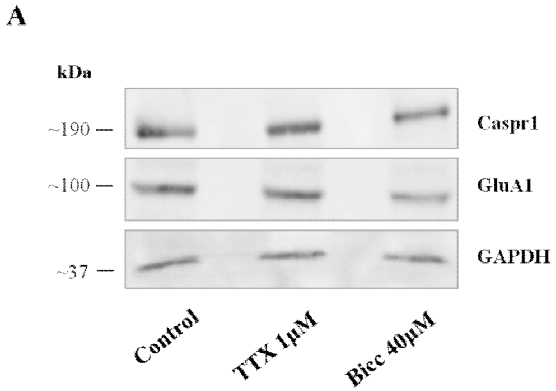
In an attempt to uncover mechanisms that regulate the endogenous levels of Caspr1, we decided to evaluate physiological stimuli that exert an effect in the GluA1 subunit of AMPARs similar to the effect we have observed when we overexpress Caspr1, that is an upregulation of the GluA1 protein levels. Neurons use homeostatic mechanisms to elicit changes in the strength of excitatory synapses in response to prolonged increases or decreases in activity (Turrigiano & Nelson, 2000). Indeed, accumulating evidences from recent years indicate that manipulations that chronically affect neuronal activity have a major impact on the total levels of GluA1 and particularly in the subunit content in synapses (O'Brien *et al.*, 1998; Wierenga *et al.*, 2005). This synaptic scaling, through changes in AMPAR accumulation, comprises one form of homeostatic plasticity, which adjusts the overall synaptic strength of a neuron to compensate for unrestrained changes in activity that would saturate synapses and take them out of the physiological range of neuronal activation (Turrigiano, 2008). Accordingly, Ju and colleagues showed that the chronic blockade of synaptic activity in hippocampal neurons, either with TTX (blocker of voltage-gated sodium channels) or APV (selective antagonist of NMDA receptors) for 3 days, resulted in a selective enhancement of the dendritic synthesis of the GluA1 subunit, presumably to increase GluA1 levels and compensate for the lack of activity (Ju *et al.*, 2004). Conversely, Turrigiano and colleagues showed that, in cortical neurons, blocking GABA-mediated inhibition with bicuculline decreased the amplitude of mESPCs, possibly through a postsynaptic change in glutamate responsiveness or a presynaptic change in the glutamate content of synaptic vesicles (Turrigiano *et al.*, 1998).

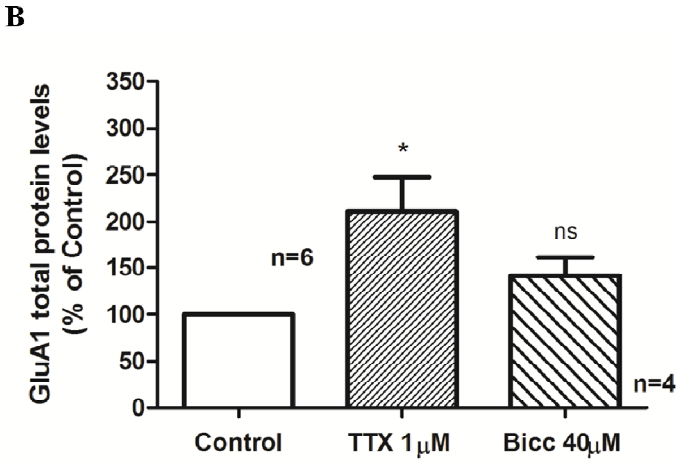
Despite the evidences regarding the cellular properties of homeostatic plasticity, the molecular mechanisms underlying this negative feedback by which synaptic strength is adjusted are still poorly understood. Nevertheless, novel players, whose loss

of function interferes with the expression of homeostatic plasticity, have been identified and contribute to emphasize the complexity of possible signaling pathways that neurons use to maintain their homeostasis (Pozo & Goda, 2010). One of the first identified players was the immediate-early gene Arc/Arg3.1 that is rapidly induced by neuronal activity and mediates homeostatic synaptic scaling of AMPARs via its ability to activate a novel and selective AMPAR endocytic pathway (Shepherd *et al.*, 2006). Also, a recent study showed a specific postsynaptic requirement for β3 integrins in scaling up of synaptic AMPARs induced by activity suppression: under basal condition, β3 integrins act to stabilize synaptic AMPARs and loss of function of these cell adhesion molecules specifically impaired the homeostatic scaling up of mEPSCs mediated by a TTX-induced blockade of activity (Cingolani *et al.*, 2008). The β3-mediated homeostatic scaling suggests an important contribution of the extracellular matrix and surrounding environment in the coordination of homeostatic changes. Furthermore, in another study, TNFα, a pro-inflammatory cytokine produced in glial cells, was implicated in homeostatic synaptic scaling through upregulation of the cell surface expression of AMPARs and their synaptic insertion upon pharmacological blockade of neuronal activity in hippocampal neurons with TTX, leading to a compensatory increase in AMPAR-mediated currents (Stellwagen & Malenka, 2006). These evidences confirm an active participation of the extracellular environment, particularly of glial cells, in the homeostatic activity-dependent regulation of synaptic connectivity (Stellwagen & Malenka, 2006). Interestingly, besides exerting an effect in the GluA1 subunit similar to that of TNF-α, Caspr1, complexed with Contactin1, localizes to the cell membrane and interacts with RPTPβ (receptor protein tyrosine phosphatase β) expressed on the surface of glial cells. This interaction allows for the bidirectional exchange of signals between

glia and neurons and contributes to the homeostasis between them and their local environment (Peles *et al.*, 1997).

Thus, to evaluate if chronic changes in neuronal activity can regulate the endogenous levels of Caspr1, activity in hippocampal neurons was bidirectionally manipulated. Hippocampal neurons at DIV14 were stimulated for 24 hrs, at 37ºC with TTX or bicuculline (Bicc), to either block or enhance, respectively, neuronal activity. After 24 hrs, cell lysates were obtained and levels of GluA1 and Caspr1 were assessed by western blot (Figure 17).





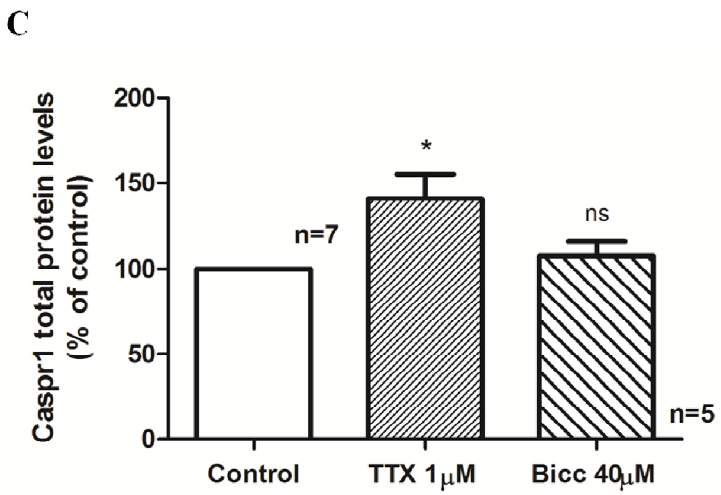


Figure 17 – TTX-induced blockade of activity increases both GluA1 subunit and Caspr1 total levels in cultured hippocampal neurons. (A) High-density cultured hippocampal neurons at DIV14 were treated for 24 hrs, at 37ºC, either with Tetrodotoxin (TTX) 1μM or with Bicuculline (Bicc) 40μM, as indicated. After this period, total GluA1 (B) and Caspr1 (C) levels were assessed by western blot and quantified. (B – C) Results are presented as means ± S.E.M. when compared to control and normalized to the loading control GAPDH. Statistical significance was determined by One-Way ANOVA followed by the Dunnett's Multiple Comparison post-test. (B – C) *$p<0.05$; ns - $p>0.05$.

As expected, chronic blockade of activity induced by a 24 hrs-treatment of hippocampal neurons with TTX, resulted in a significant increase of 111% (to 211 ± 90 % of control, *$p<0.05$) in total levels of the GluA1 subunit (Figure 17B), in agreement with previous reports (O'Brien *et al.*, 1998; Wierenga *et al.*, 2005). This result indicates that stimulating hippocampal neurons for 24 hrs with TTX at a concentration of 1μM is enough to chronically block activity and induce mechanisms of homeostatic plasticity that result in synaptic scaling of GluA1 levels. Conversely, the chronic enhancement of neuronal activity with bicuculline did not result in a decrease of total GluA1 levels, as

would be expected. Instead, bicuculline promoted a slight but not significant increase in GluA1, when compared to control levels (140 ± 38 % of control) (Figure 17B). It is unclear at this point why bicuculline did not exert the expected effect in regulating the levels of GluA1 upon enhancement of activity, as previously reported (Turrigiano *et al.*, 1998). Either bicuculline is not exerting its effect in the blockade of GABA receptors or its effect is occluded by a contribution from the vehicle (DMSO). More experiments should be performed using DMSO as a control for bicuculline, in order to exclude a possible contribution from the vehicle.

With an established protocol to induce homeostatic mechanisms in hippocampal neurons that results in the synaptic scaling of GluA1, we could now test if this type of physiological stimuli has an effect in the regulation of endogenous levels of Caspr1. Indeed, when hippocampal neurons were stimulated with TTX for 24 hrs to chronically block activity, levels of endogenous Caspr1 increased significantly to 141 ± 37 % (*$p<0.05$), when compared to control values (Figure 17C). When neurons were treated with bicuculline, levels of Caspr1 were very similar to the control (107 ± 8 % of control). The changes in Caspr1, induced by chronic blockade of neuronal activity were also confirmed by imaging experiments. Hippocampal neurons at DIV20 were stimulated with TTX for 24 hrs, at 37ºC, to chronically block neuronal activity. After 24 hrs, cells were fixed and immunolabeled with antibodies against Caspr1 and the synaptic marker PSD-95 (Figure 18).

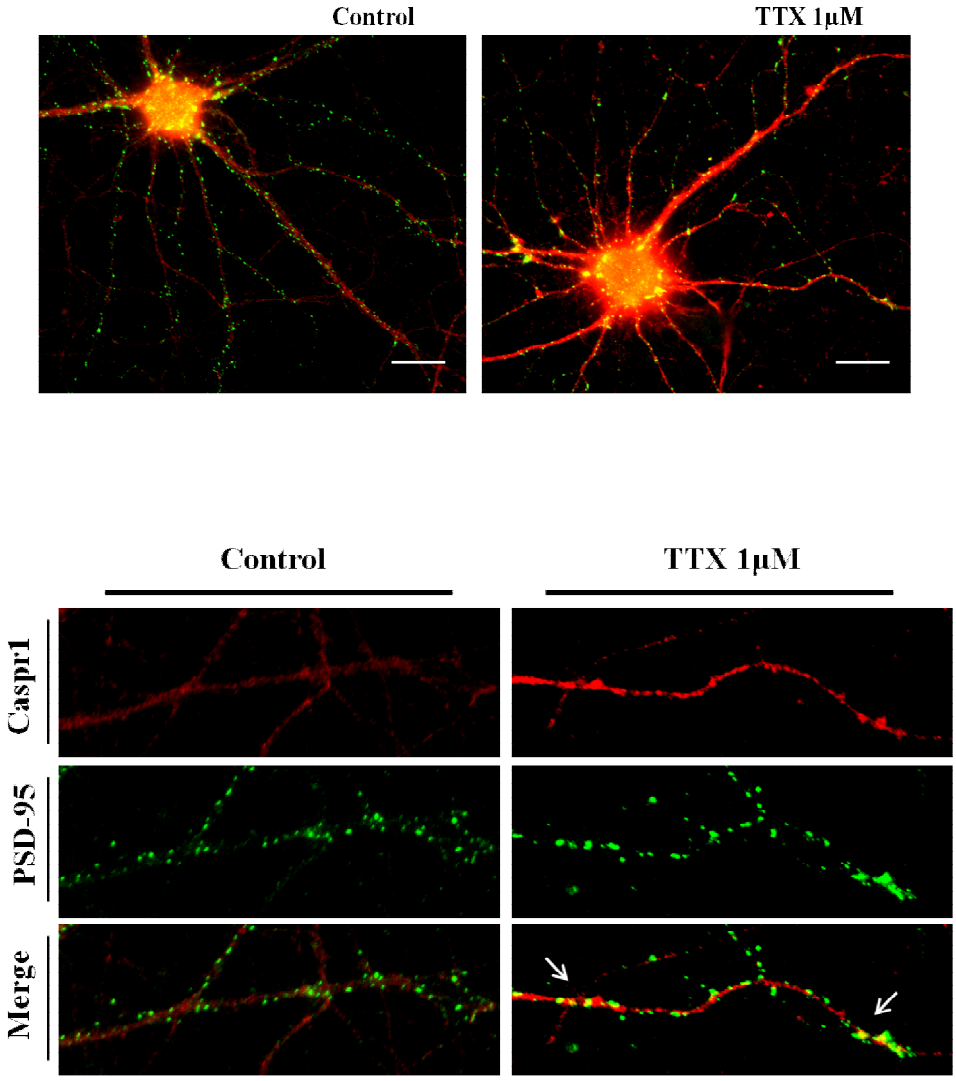


Figure 18 – Caspr1 is significantly increased in hippocampal neurons where activity has been chronically blocked with TTX. Primary hippocampal neurons from low-density Banker cultures were stimulated at DIV20 with TTX 1µM for 24 hours, at 37ºC, in order to chronically block neuronal activity. After the period of stimulus, cells were fixed and immunolabeled with antibodies for Caspr1 (red) and PSD-95 (green) as a postsynaptic marker. Images were taken using a Zeiss Axiovert 200 fluorescent microscope. Fields for imaging were chosen by the MAP2 channel. Images were obtained using identical settings. Scale Bar: 20µm. Scale Bar (crops): 5µm. Arrows represent colocalization with PSD95 puncta.

These representative images clearly show that chronic stimulation of hippocampal neurons with TTX induces a major upregulation in levels of endogenous Caspr1. Moreover, it also appears that this stimulus promotes an increase, at least in number, in clusters of Caspr1 at the synaptic level, which can be evaluated by the increase in colocalization with PSD-95 puncta (see arrows - Figure 18). Quantitative analysis of this effect should now be performed.

Altogether, these evidences indicate that chronic blockade of neuronal activity induced by TTX not only increases GluA1 levels but also the expression of Caspr1, thus allowing the identification of a novel physiological stimulus regulating the endogenous levels of Caspr1. Moreover, if we correlate the synaptic scaling of the GluA1 subunit, which occurs upon induction of homeostatic changes, with our results concerning the overall effect of Caspr1, we can pinpoint a possible role for the Caspr1-mediated upregulation of GluA1 mRNA in the mechanisms underlying homeostatic plasticity. Interestingly, a similar description of regulation and function has been proposed for retinoic acid (RA). In a recent study, synaptic scaling in hippocampal neurons induced by activity blockade with TTX and APV also resulted in an increased RA synthesis (Aoto *et al.*, 2008). Moreover, exogenous application of RA promoted a rapid scale up of AMPARs that occluded the same effect induced by TTX and APV. Furthermore, the synaptic scaling induced by RA occurred through the local synthesis of GluA1, through signaling via the RA receptor, RARα, which is dendritically localized (Aoto *et al.*, 2008; Maghsoodi *et al.*, 2008). Overall, these evidences propose a possible role for Caspr1 in mediating GluA1 synaptic scaling upon homeostatic changes, and further suggest a role for dendritic protein synthesis in the onset of homeostatic plasticity.

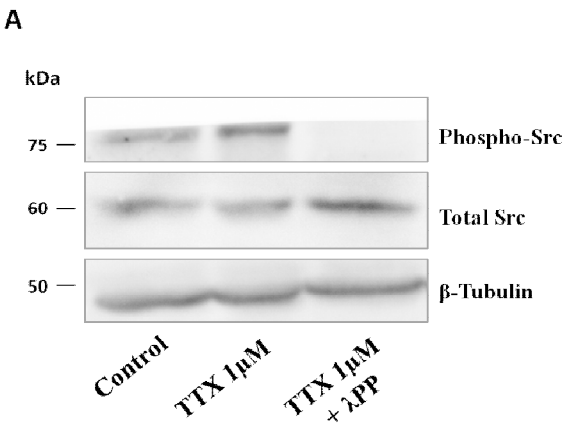
Chronic blockade of neuronal activity increases levels of phosphorylated Src and phosphorylated ZBP1

Summarizing what was described so far, we have seen that Caspr1 is able to promote an increase in total levels of the GluA1 subunit of AMPARs, both in COS7 cells and in hippocampal neurons. Also, this effect seems to require the intracellular proline-rich region of Caspr1, since Caspr1 lacking this domain fails to upregulate GluA1. Moreover, preliminary results obtained in our laboratory further confirm the role of proline-rich region of Caspr1 in mediating its effect in the upregulation of GluA1, particularly at the mRNA level. This region is highly implicated in protein-protein interaction, which prompted the hypothesis that Caspr1 would be exerting its effect through activation of some signaling pathway, via the proline-rich region. In agreement with previous evidence suggesting a possible interaction between the tyrosine kinase Src and the proline domain of Caspr1, we found that Caspr1, particularly at the synaptic level, activates a signaling pathway downstream of Src, which involves the RNA-binding protein ZBP1. This signaling pathway culminates in the Src-mediated phosphorylation of ZBP1 and loss of its RNA-binding activity. Correlation of all these results raises the hypothesis of a mechanism in which ZBP1 binds GluA1 mRNA. Upon Caspr1-mediated activation of the downstream pathway of Src, ZBP1 is phosphorylated and GluA1 mRNA molecules are free to be locally translated at synaptic sites.

This rationale is consistent with the homeostatic synaptic scaling of GluA1 that occurs in response to chronic changes in activity. Accordingly, we have already proposed a role for the Caspr1-mediated upregulation of GluA1 mRNA in the mechanisms that underlie homeostatic plasticity, since chronic blockade of activity with

TTX resulted in an increase of endogenous Caspr1. Thus, it would be interesting to evaluate if such a manipulation of neuronal activity induces an activation of the Src signaling pathway that parallels that of Caspr1.

To achieve this goal, hippocampal neurons at DIV14 were stimulated for 24 hrs with TTX to chronically block activity. After this period, cell lysates were obtained and levels of phosphorylated Src and phosphorylated ZBP1 were assessed by western blot (Figure 10). Moreover, to assess the specificity of the results, lysates from hippocampal neurons treated with TTX were subjected to a dephosphorylation assay with the Lambda protein phosphatase (Figure 19A and 19C). In both control and TTX-stimulated conditions, labeling either with an antibody for phospho-Src (A) or phospho-ZBP1 (C) detects bands of approximately 75kDa, previously identified in Caspr1-transfected lysates as corresponding either to phospho-Src (Figure 13A) or phospho-ZBP1 (Figure 15C). These bands specifically disappear when samples are dephosphorylated with λPP (A and C – TTX 1μM + λPP), which ensures the specificity of the results further presented.



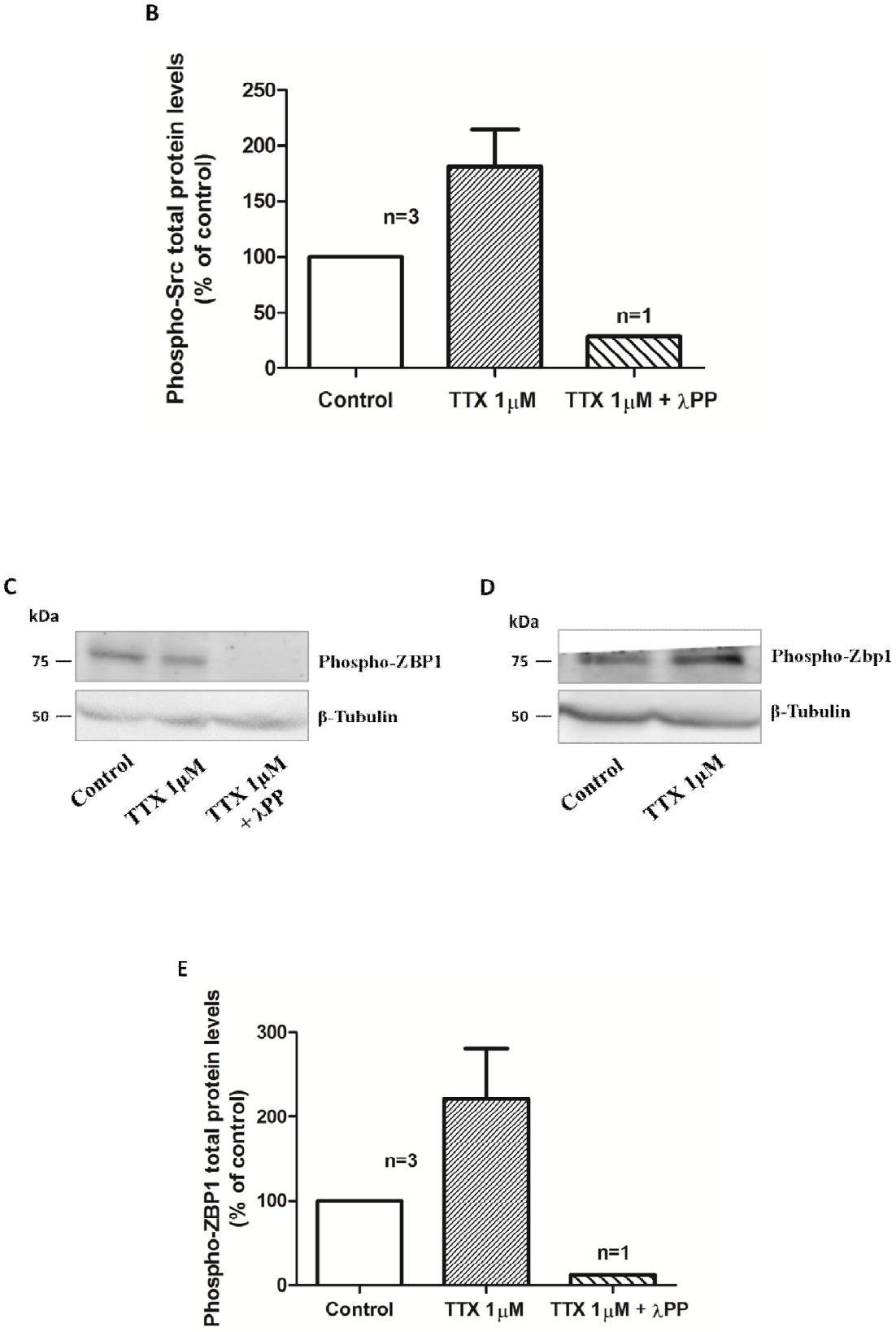

B
Phospho-Src total protein levels (% of control)
250
200
150
100
50
0
n=3
n=1
Control
TTX 1μM
TTX 1μM + λPP
C
kDa
75
50
Phospho-ZBP1
β-Tubulin
Control
TTX 1μM
TTX 1μM + λPP
D
kDa
75
50
Phospho-Zbp1
β-Tubulin
Control
TTX 1μM
E
Phospho-ZBP1 total protein levels (% of control)
300
200
100
0
n=3
n=1
Control
TTX 1μM
TTX 1μM + λPP

Figure 19 – Chronic blockade of activity, induced by TTX, results in an increase of total levels of phosphorylated Src and phosphorylated ZBP1, in cultured hippocampal neurons. (A, C-D) Primary high-density hippocampal neurons at DIV14 were treated for 24 hrs, at 37ºC, with Tetrodotoxin (TTX) 1μM, as indicated. After this period, whole cells lysates were obtained. Approximately 150μg of protein from TTX-stimulated lysates were subjected to a dephosphorylation assay with lambda protein phosphatase (TTX 1μM + λPP) for 2 hrs, at 30ºC. Levels of total Src and of its phosphorylated counterpart (A - B) and phosphorylated ZBP1 (C - E) were assessed by western blot and quantified. (B, E) Results are presented as means ± S.E.M. when compared to control and normalized to the loading control β-Tubulin. Statistical analysis was determined by the unpaired t-test, although no statistical significance was achieved, $p>0.05$.

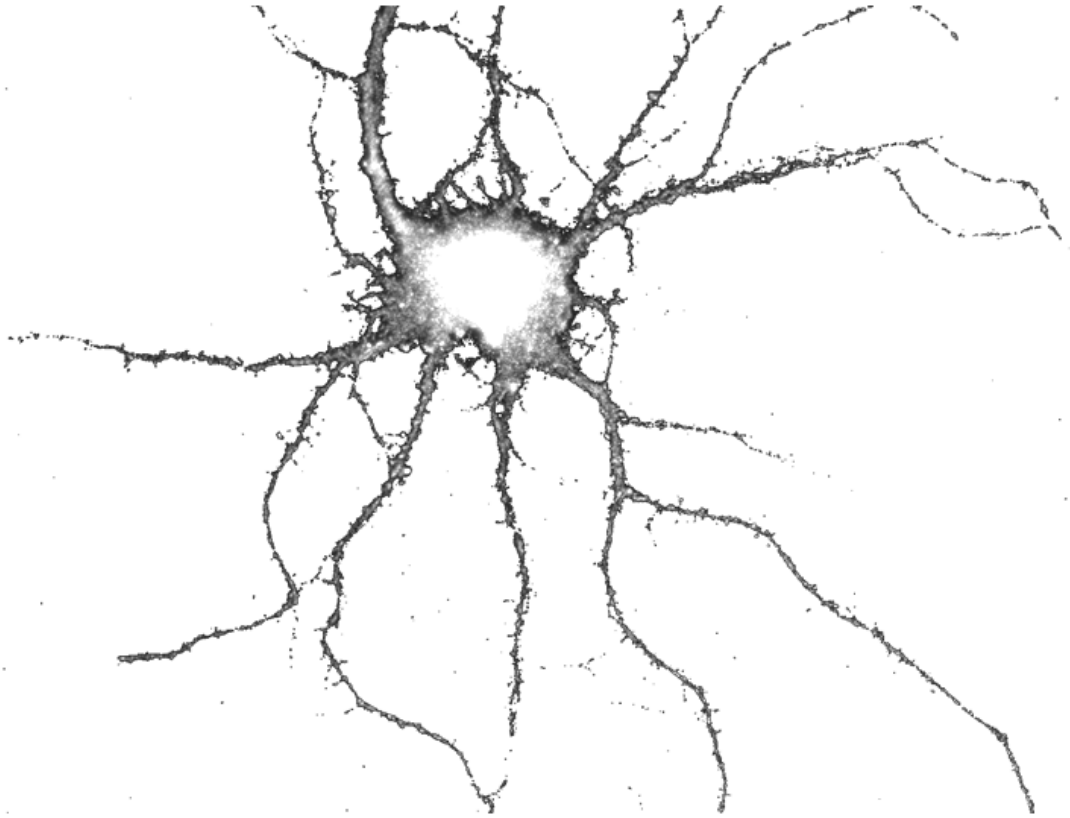
Interestingly, when neuronal activity in hippocampal neurons was chronically blocked for 24 hrs with TTX, levels of phosphorylated Src suffered a major increase to 181 ± 34 %, when compared to control values (Figure 19A and B). A similar effect was observed for the levels of phosphorylated ZBP1 (Figure 19D and E). TTX-induced blockade of activity resulted in a marked increase of about 120 % (220 ± 60 % of control) in levels of phosphorylated ZBP1, when compared to the control situation. Overall, TTX-induced chronic blockade of activity appears to play a role in the activation of the Src signaling pathway, increasing levels of its phosphorylated counterpart as well as of its downstream target, phospho-ZBP1.

These evidences correlate to the upregulatory effect that TTX-induced blockade of activity has in synaptic scaling of GluA1 and expression of Caspr1. And altogether, they further contribute to the tantalizing hypothesis of a Caspr1-mediated upregulation of GluA1 mRNA that may play a role for the induction of a form of synaptic scaling that requires dendritic protein synthesis (Sutton *et al.*, 2006). Just recently, the FMRP

RNA-binding protein has been implicated in mechanisms underlying homeostatic plasticity. By binding to target mRNAs, FMRP is thought to act as a downstream mediator of RA-signaling, crucial for the dendritic protein synthesis triggered by this signaling molecule, unveiling a novel role for FMRP in the induction of homeostatic plasticity. All evidences taken into account, it is reasonable to propose that upon chronic changes in activity the expression of endogenous Caspr1 is increased. In turn, Caspr1 mediates the activation of a signaling pathway downstream of Src that culminates in the phosphorylation of ZBP1. This event promotes the release of ZBP1 target mRNAs, creating an on-site pool of transcripts ready to undergo translation. It is possible that ZBP1 interacts with GluA1 transcripts. ZBP1 is a RNA-binding protein that recognizes specific 'zipcodes' located in the 3'UTR of its target mRNAs. Conversely, the 3'UTR of GluA1 is thought to contain not only endogenous regulatory elements, but also multiple putative binding sites for *trans*-acting proteins. If so, the Src-dependent phosphorylation of ZBP1, as a downstream consequence of higher Caspr1 expression, will contribute to increase the availability of GluA1 transcripts on-site. This will increase the synthesis of novel AMPARs and their synaptic insertion and ultimately contribute to the homeostatic synaptic scaling that counteracts massive changes in activity.

Chapter 4

Concluding Remarks



Conclusion

Recent data from our laboratory identified the integral membrane protein Contactin associated protein1 (Caspr1) as a novel interactor of the GluA1 subunit, capable of upregulating the expression of this AMPAR subunit, particularly at the mRNA level. This evidence suggested a novel post-transcriptional regulatory mechanism for AMPARs that could be of crucial importance to the induction of mechanisms underlying several forms of synaptic plasticity. This possibility prompted us to further characterize the regulation of the GluA1 subunit by Caspr1.

Firstly, we aimed at characterizing the role of Caspr1 in the total protein levels of the GluA1 subunit and found that, both in a heterologous system and in hippocampal neurons, protein levels of GluA1 are significantly increased when Caspr1 is overexpressed (Figure 10). Moreover, we were able to map the molecular determinant of Caspr1 responsible for its effect in GluA1 levels. A Caspr1 mutant specifically deleted for its intracellular proline-rich domain failed to increase GluA1 levels (Figure 12), which pinpoints a specific requirement for this region in the upregulatory effect that Caspr1 exerts. Taken together, these results are consistent with previous evidences by Sandra Santos proposing a major role for the C-terminal of Caspr1 not only in the upregulation of GluA1 levels and its stabilization at the cell surface, but specifically, in its synaptic targeting (Santos, 2009).

Secondly, we sought to identify and characterize the molecular mechanisms underlying the effect of Caspr1 in GluA1 levels. With the proline-rich region of Caspr1 identified as the major player in this effect, it was reasonable to consider that the effect of Caspr1 would occur through triggering of some signaling pathway, since the proline region of Caspr1 had previously been described to interact with SH3 domains of several

signaling molecules, particularly with that of the tyrosine kinase Src (Peles *et al.*, 1997). This led us to hypothesize that the effect in GluA1 levels would be mediated by Caspr1-dependent activation of the Src signaling cascade and, indeed, we were able to confirm this hypothesis. Expression of Caspr1 in COS7 cells resulted not only in increased levels of phosphorylated Src (Figure 13), but also of phosphorylated ZBP1 (Figure 15), a downstream target of Src. Moreover, in hippocampal neurons, Caspr1 induced a significant increase in the number of both phosphorylated Src and ZBP1 puncta, specifically at the synaptic level (Figures 14 and 16). Also, this Caspr1-mediated increase in phospho-Src and phospho-ZBP1 paralleled that of GluA1, and all three effects were absent when the proline domain was deleted from Caspr1. These evidences could point to a role for ZBP1 in mediating the upregulation of GluA1. ZBP1 is a RNA-binding protein known to regulate mRNA translation upon Src-dependent phosphorylation, by binding to specific zipcodes located in the 3'UTR of its target mRNAs (Huttelmaier *et al.*, 2005; Ross *et al.*, 1997). Moreover, ZBP1 has already been described to localize to dendritic RNPs and stress granules where it promotes the stabilization of several transcripts (Gu *et al.*, 2008; Leeds *et al.*, 1997; Noubissi *et al.*, 2006; Stohr *et al.*, 2006; Vikesaa *et al.*, 2006). Accordingly, evidences from our laboratory indicate not only, the presence of several regulatory *cis*-elements located in the 3'UTR of GluA1 mRNA that are required for the upregulatory effect of Caspr1, but also several putative binding sites for RNA-binding proteins. These evidences, thus suggest the possibility a post-transcriptional regulation of GluA1 at the level of mRNA stability, mediated by binding of ZBP1 to GluA1 transcripts.

Finally, given the importance of dendritically localized GluA1 transcripts to the expression of synaptic plasticity and the role that Caspr1 may exert in these mechanisms, we tried to investigate how the endogenous expression of Caspr1 is

regulated by neuronal activity. Interestingly, the effect of Caspr1 in GluA1 levels parallels that of chronically blocking neuronal activity, which results in the expression of homeostatic plasticity mechanisms, such as the synaptic scaling of AMPARs, increasing GluA1 dendritic synthesis and accumulation at synapses (Turrigiano, 2008). Accordingly, when chronically blocking neuronal activity with TTX we observed not only a significant increase in GluA1 levels, but also a significant increase in total levels of endogenous Caspr1 (Figure 17 and 18), which suggests that upon changes in neuronal activity the expression of Caspr1 is altered, consequently inducing upregulatory mechanisms in GluA1 levels that contribute to AMPAR synaptic scaling and expression of homeostatic plasticity. Moreover, this manipulation of neuronal activity also resulted in the activation of the Src signaling pathway, since chronic blockade of activity resulted in increased levels for both phosphorylated Src and ZBP1 (Figure 19). Altogether, these evidences propose a possible Caspr1-mediated mechanism that leads to the upregulation of GluA1 mRNA, through activation of Src signaling cascade, and suggest that this mechanism is induced by chronic activity blockade.

Based on the current knowledge and on the evidences in this study, our current hypothesis is that after transcription ZBP1 binds to *cis*-elements located in the 3'UTR of GluA1 transcripts, incorporating them in ribonucleoproteins that localize to specific dendritic sites, exerting a stabilizing effect in GluA1 mRNA. Upon chronic changes in activity the expression of Caspr1 is upregulated. In turn, Caspr1 interacts, through its proline-rich region, with the tyrosine kinase Src, rendering it active and triggering its downstream signaling pathway that culminates in the phosphorylation of ZBP1. This event promotes the release of the GluA1 mRNA from its binding to ZBP1, thus creating an on-site pool of GluA1 transcripts ready to undergo translation. This will then

increase local synthesis of novel AMPARs and promote their synaptic insertion, contributing to the expression of homeostatic synaptic scaling that, ultimately, counteracts the initial change in activity.

Overall, with this study we were able to highlight a possible post-transcriptional role for Caspr1, involving the RNA-binding protein ZBP1, in the regulation of the GluA1 subunit of AMPARs. Post-transcriptional gene regulation, including regulation of mRNA translation and stability, has gained a significant amount of attention in these last years and is now regarded as a major mechanism to regulate eukaryotic gene expression. Particularly in the CNS, this level of regulation appears to have a critical role in mediating events that underlie the phenomena of synaptic plasticity. Alterations in the accumulation of AMPARs at synapses are critical to induce long-term changes in the strength and efficacy of glutamatergic synapses. Thus, regulation of AMPARs at a post-transcriptional level could be of major importance in altering synaptic AMPAR composition and number, ultimately underlying the expression of synaptic plasticity mechanisms. Moreover, this type of regulation at the mRNA level must be crucial to the induction of homeostatic mechanisms that rely on dendritic protein synthesis, such as the synaptic scaling of AMPARs. Accordingly, based in the present study we propose that the Caspr1-mediated post-transcriptional regulation of GluA1 may contribute to the expression of mechanisms in the basis of homeostatic plasticity.

Nevertheless, future work will be necessary to elucidate the role of Caspr1 in regulating GluA1 mRNA, possibly through a stabilizing mechanism, and further comprehend how this level of post-transcriptional regulation of AMPARs impacts the onset of homeostatic plasticity.

Future perspectives

In this work, together with previous evidences obtained in our laboratory, we have demonstrated that Caspr1 induces an upregulatory effect in the GluA1 subunit of AMPARs. We have shown that Caspr1, through its proline-rich domain, is able to induce an increase both in protein (present study) and mRNA (Luís Ribeiro and Sandra Santos, personal communication) levels of GluA1, possibly through a stabilizing effect in transcripts for GluA1. Nevertheless, further studies are required in order to understand the mechanisms underlying this effect.

Firstly, one of the essential milestones that needs to be achieved in the future to fully understand the regulation of the GluA1 subunit by Caspr1 is to determine how Caspr1 affects the stability of GluA1 transcripts, possibly by regulating their degradation or translation rate. This could be tested by evaluating levels of GluA1 mRNA, in the presence or absence of Caspr1, at several time points after the application of the transcription inhibitor actinomycin D. Moreover, using the same approach in hippocampal neurons transduced with lentivirus to either overexpress Caspr1 or silence its expression with specific shRNAs for Caspr1, would allow us to determine the physiological role of endogenous Caspr1 in regulating the half-life of GluA1 mRNA.

Moreover, given the role of dendritically localized GluA1 mRNA in mechanisms of synaptic plasticity, and the localization of Caspr1 to dendrites and postsynaptic densities, we could assess the importance of Caspr1 in regulating dendritic GluA1 mRNA by overexpressing or knocking down the expression of endogenous Caspr1 in hippocampal neurons grown on filters through which axons and dendrites, but not cell bodies, can penetrate (Manadas *et al.*, 2009), and compare the GluA1 mRNA harvested from the different subcellular compartments. Another approach to confirm

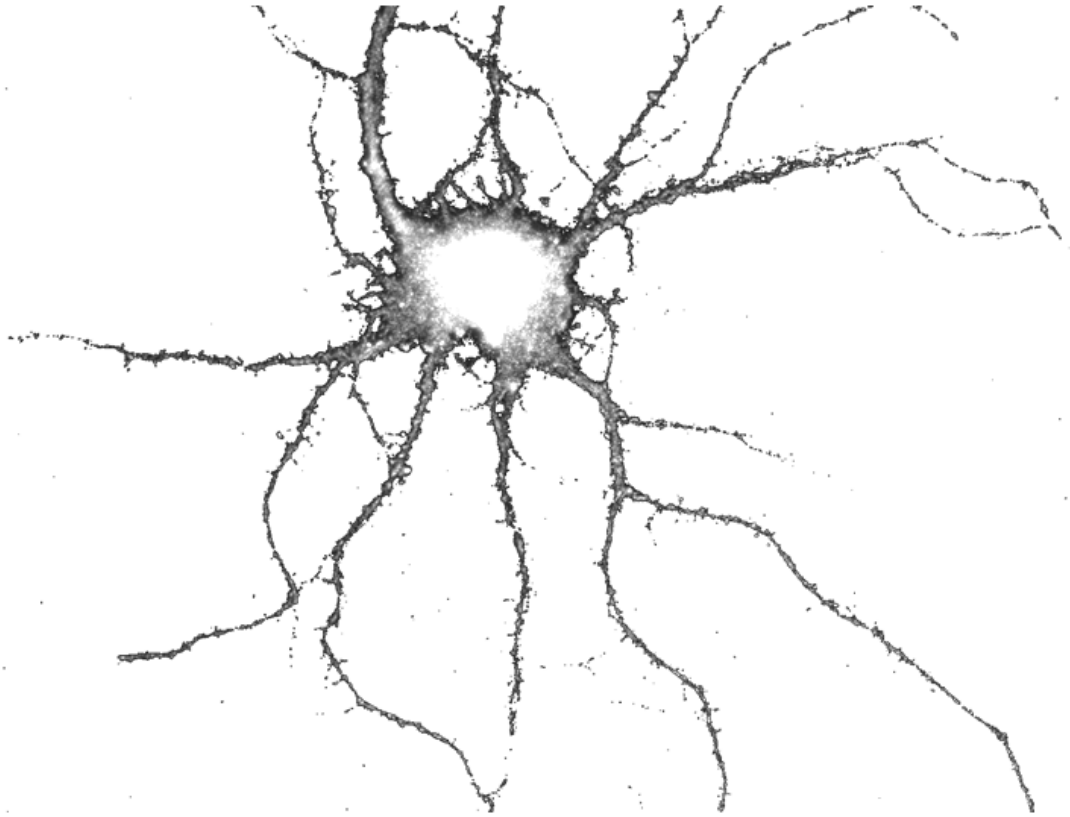
the role of Caspr1 in the regulation of dendritic GluA1 transcripts would be to specifically evaluate GluA1 mRNA exclusively in isolated dendrites of neurons where Caspr1 had been overexpressed or silenced by performing fluorescence *in situ* hybridization assays using oligonucleotide probes for GluA1.

In this study we have also shown that the overexpression of Caspr1 induces the activation of a downstream signaling cascade of Src that culminates in the phosphorylation of ZBP1, a RNA-binding protein known to bind to the 3'UTR of several transcripts to regulate their stability and translation. This would point to a possible role for ZBP1 in regulating the mechanisms activated by Caspr1 that induce a stabilization of GluA1 mRNA. Thus, it would be interesting to search for putative binding sites for ZBP1 in GluA1 mRNA and use biotinylated RNA oligonucleotides corresponding to these sequences, or the full length 3'UTR of GluA1, to test for the pulldown of ZBP1. Also, immunoprecipitation of ZBP1 or non-radioactive electrophoretic mobility shift assays, using probes against the zipcodes found in GluA1 mRNA could be performed to evaluate bound GluA1 mRNA or ZBP1, respectively. Furthermore, we could also evaluate the half-life of GluA1 mRNA, following transcription inhibition with actinomycin D, in hippocampal neurons where the expression of ZBP1 is silenced with RNA interference. These experiments would be crucial to confirm the interaction between both ZBP1 and GluA1 mRNA and unravel a ZBP1-dependent regulation of GluA1 mRNA stability. Moreover, to further confirm the importance of the Caspr1-activated Src signaling pathway and Src-dependent phosphorylation of ZBP1 in the upregulation of GluA1 levels we could measure total and synaptic levels of GluA1 in neurons overexpressing Caspr1 but treated with a specific inhibitor of Src, which we would expect to block the increase in GluA1 levels seen with Caspr1 overexpression.

Furthermore, in the present work we were also able to observe that chronic blockade of neuronal activity, a manipulation known to induce the expression of homeostatic plasticity mechanisms, resulted not only in increased levels of GluA1, but also of Caspr1, phosphorylated Src and phosphorylated ZBP1, suggesting a role for the Caspr1-mediated upregulation of GluA1, possibly through activation of the Src pathway, in the induction of homeostatic mechanisms, such as the synaptic scaling of AMPARs. Thus, it would be interesting to confirm that Caspr1 plays an important role in the induction of these homeostatic mechanisms. If so, it is expectable that knocking down the expression of endogenous Caspr1 blocks the synaptic scaling of GluA1 levels induced by chronic blockade of activity, whereas Caspr1 overexpression occludes this effect. If confirmed, these effects would support the idea that a Caspr1-dependent mechanism is necessary for the induction of AMPARs synaptic scaling. Also, if by blocking the activation of Src pathway, with a specific Src inhibitor, or knocking down the expression of ZBP1 we would block the effect of chronic blockade of neuronal activity in GluA1 levels, we would further confirm that the Src signaling pathway possibly mediates the effect of Caspr1 in these homeostatic mechanisms. Most importantly, we would contribute with knowledge to the possibility of a form of homeostatic synaptic scaling that is dependent on post-transcriptional mechanisms to regulate availability and local dendritic protein synthesis of transcripts important to synaptic plasticity.

Chapter 5

References



References

Abbott, L.F. & Nelson, S.B. (2000). Synaptic plasticity: taming the beast. *Nat Neurosci*. **3 Suppl**: 1178-1183.

Adesnik, H. & Nicoll, R.A. (2007). Conservation of glutamate receptor 2-containing AMPA receptors during long-term potentiation. *J Neurosci*. **27**: 4598-4602.

Adesnik, H., Nicoll, R.A. & England, P.M. (2005). Photoinactivation of native AMPA receptors reveals their real-time trafficking. *Neuron*. **48**: 977-985.

Aoto, J., Nam, C.I., Poon, M.M., Ting, P. & Chen, L. (2008). Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity. *Neuron*. **60**: 308-320.

Armstrong, N., Sun, Y., Chen, G.Q. & Gouaux, E. (1998). Structure of a glutamate-receptor ligand-binding core in complex with kainate. *Nature*. **395**: 913-917.

Asaki, C., Usuda, N., Nakazawa, A., Kametani, K. & Suzuki, T. (2003). Localization of translational components at the ultramicroscopic level at postsynaptic sites of the rat brain. *Brain Res*. **972**: 168-176.

Bakheet, T., Frevel, M., Williams, B.R., Greer, W. & Khabar, K.S. (2001). ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. *Nucleic Acids Res*. **29**: 246-254.

Banker, G. & Goslin, K. (1998). Culturing nerve cells, 2nd edn (Cambridge, Mass.: MIT Press).

Barreau, C., Paillard, L. & Osborne, H.B. (2005). AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res*. **33**: 7138-7150.

Bats, C., Groc, L. & Choquet, D. (2007). The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. *Neuron*. **53**: 719-734.

Bear, M.F. & Malenka, R.C. (1994). Synaptic plasticity: LTP and LTD. *Curr Opin Neurobiol*. **4**: 389-399.

Beattie, E.C., Carroll, R.C., Yu, X., Morishita, W., Yasuda, H., von Zastrow, M. & Malenka, R.C. (2000). Regulation of AMPA receptor endocytosis by a signaling mechanism shared with LTD. *Nat Neurosci*. **3**: 1291-1300.

Beggs, H.E., Soriano, P. & Maness, P.F. (1994). NCAM-dependent neurite outgrowth is inhibited in neurons from Fyn-minus mice. *J Cell Biol*. **127**: 825-833.

Belasco, J.G. (2010). All things must pass: contrasts and commonalities in eukaryotic and bacterial mRNA decay. *Nat Rev Mol Cell Biol*. **11**: 467-478.

Benowitz, L.I. & Routtenberg, A. (1997). GAP-43: an intrinsic determinant of neuronal development and plasticity. *Trends Neurosci*. **20**: 84-91.

Berger, A., Stierkorb, E. & Nickenig, G. (2005). The role of the AUUUUA hexamer for the posttranscriptional regulation of the AT1 receptor mRNA stability. *Biochem Biophys Res Commun*. **330**: 805-812.

Bhat, M.A., Rios, J.C., Lu, Y., Garcia-Fresco, G.P., Ching, W., St Martin, M., Li, J., Einheber, S., Chesler, M., Rosenbluth, J., Salzer, J.L. & Bellen, H.J. (2001). Axon-glia interactions and the domain organization of myelinated axons requires neurexin IV/Caspr/Paranodin. *Neuron*. **30**: 369-383.

Biederer, T., Sara, Y., Mozhayeva, M., Atasoy, D., Liu, X., Kavalali, E.T. & Sudhof, T.C. (2002). SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science*. **297**: 1525-1531.

Blanpied, T.A., Scott, D.B. & Ehlers, M.D. (2002). Dynamics and regulation of clathrin coats at specialized endocytic zones of dendrites and spines. *Neuron*. **36**: 435-449.

Bliss, T.V. & Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol*. **232**: 331-356.

Boehm, J., Kang, M.G., Johnson, R.C., Esteban, J., Huganir, R.L. & Malinow, R. (2006). Synaptic incorporation of AMPA receptors during LTP is controlled by a PKC phosphorylation site on GluR1. *Neuron.* **51**: 213-225.

Boggon, T.J. & Eck, M.J. (2004). Structure and regulation of Src family kinases. *Oncogene*. **23**: 7918-7927.

Bonnon, C., Bel, C., Goutebroze, L., Maigret, B., Girault, J.A. & Faivre-Sarrailh, C. (2007). PGY repeats and N-glycans govern the trafficking of paranodin and its selective association with contactin and neurofascin-155. *Mol Biol Cell*. **18**: 229-241.

Bonnon, C., Goutebroze, L., Denisenko-Nehrbass, N., Girault, J.A. & Faivre-Sarrailh, C. (2003). The paranodal complex of F3/contactin and caspr/paranodin traffics to the cell surface via a non-conventional pathway. *J Biol Chem*. **278**: 48339-48347.

Bowie, D., Lange, G.D. & Mayer, M.L. (1998). Activity-dependent modulation of glutamate receptors by polyamines. *J Neurosci*. **18**: 8175-8185.

Boyle, M.E., Berglund, E.O., Murai, K.K., Weber, L., Peles, E. & Ranscht, B. (2001). Contactin orchestrates assembly of the septate-like junctions at the paranode in myelinated peripheral nerve. *Neuron*. **30**: 385-397.

Bramham, C.R. & Wells, D.G. (2007). Dendritic mRNA: transport, translation and function. *Nat Rev Neurosci*. **8**: 776-789.

Bredt, D.S. & Nicoll, R.A. (2003). AMPA receptor trafficking at excitatory synapses. *Neuron*. **40**: 361-379.

Burrone, J., O'Byrne, M. & Murthy, V.N. (2002). Multiple forms of synaptic plasticity triggered by selective suppression of activity in individual neurons. *Nature.* **420**: 414-418.

Carroll, R.C., Beattie, E.C., von Zastrow, M. & Malenka, R.C. (2001). Role of AMPA receptor endocytosis in synaptic plasticity. *Nat Rev Neurosci*. **2**: 315-324.

Carroll, R.C., Beattie, E.C., Xia, H., Luscher, C., Altschuler, Y., Nicoll, R.A., Malenka, R.C. & von Zastrow, M. (1999). Dynamin-dependent endocytosis of ionotropic glutamate receptors. *Proc Natl Acad Sci U S A.* **96**: 14112-14117.

Carvalho, A.L., Duarte, C.B. & Carvalho, A.P. (2000). Regulation of AMPA receptors by phosphorylation. *Neurochem Res*. **25**: 1245-1255.

Charlesworth, A., Cox, L.L. & MacNicol, A.M. (2004). Cytoplasmic polyadenylation element (CPE)- and CPE-binding protein (CPEB)-independent mechanisms regulate early class maternal mRNA translational activation in Xenopus oocytes. *J Biol Chem*. **279**: 17650-17659.

Chen, L., Chetkovich, D.M., Petralia, R.S., Sweeney, N.T., Kawasaki, Y., Wenthold, R.J., Bredt, D.S. & Nicoll, R.A. (2000). Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature*. **408**: 936-943.

Chowdhury, S., Shepherd, J.D., Okuno, H., Lyford, G., Petralia, R.S., Plath, N., Kuhl, D., Huganir, R.L. & Worley, P.F. (2006). Arc/Arg3.1 interacts with the endocytic machinery to regulate AMPA receptor trafficking. *Neuron*. **52**: 445-459.

Chung, H.J., Steinberg, J.P., Huganir, R.L. & Linden, D.J. (2003). Requirement of AMPA receptor GluR2 phosphorylation for cerebellar long-term depression. *Science*. **300**: 1751-1755.

Chung, H.J., Xia, J., Scannevin, R.H., Zhang, X. & Huganir, R.L. (2000). Phosphorylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain-containing proteins. *J Neurosci*. **20**: 7258-7267.

Cingolani, L.A., Thalhammer, A., Yu, L.M., Catalano, M., Ramos, T., Colicos, M.A. & Goda, Y. (2008). Activity-dependent regulation of synaptic AMPA receptor composition and abundance by beta3 integrins. *Neuron*. **58**: 749-762.

Colledge, M., Dean, R.A., Scott, G.K., Langeberg, L.K., Huganir, R.L. & Scott, J.D. (2000). Targeting of PKA to glutamate receptors through a MAGUK-AKAP complex. *Neuron*. **27**: 107-119.

Collingridge, G.L., Isaac, J.T. & Wang, Y.T. (2004). Receptor trafficking and synaptic plasticity. *Nat Rev Neurosci*. **5**: 952-962.

Cottrell, J.R., Borok, E., Horvath, T.L. & Nedivi, E. (2004). CPG2: a brain- and synapse-specific protein that regulates the endocytosis of glutamate receptors. *Neuron*. **44**: 677-690.

Dalmau, J., Furneaux, H.M., Gralla, R.J., Kris, M.G. & Posner, J.B. (1990). Detection of the anti-Hu antibody in the serum of patients with small cell lung cancer--a quantitative western blot analysis. *Ann Neurol*. **27**: 544-552.

De Rubeis, S. & Bagni, C. (2010). Fragile X mental retardation protein control of neuronal mRNA metabolism: Insights into mRNA stability. *Mol Cell Neurosci*. **43**: 43-50.

Denisenko-Nehrbass, N., Goutebroze, L., Galvez, T., Bonnon, C., Stankoff, B., Ezan, P., Giovannini, M., Faivre-Sarrailh, C. & Girault, J.A. (2003a). Association of Caspr/paranodin with tumour suppressor schwannomin/merlin and beta1 integrin in the central nervous system. *J Neurochem*. **84**: 209-221.

Denisenko-Nehrbass, N., Oguievetskaia, K., Goutebroze, L., Galvez, T., Yamakawa, H., Ohara, O., Carnaud, M. & Girault, J.A. (2003b). Protein 4.1B associates with both Caspr/paranodin and Caspr2 at paranodes and juxtaparanodes of myelinated fibres. *Eur J Neurosci*. **17**: 411-416.

Derkach, V.A., Oh, M.C., Guire, E.S. & Soderling, T.R. (2007). Regulatory mechanisms of AMPA receptors in synaptic plasticity. *Nat Rev Neurosci*. **8**: 101-113.

Desai, N.S., Rutherford, L.C. & Turrigiano, G.G. (1999). Plasticity in the intrinsic excitability of cortical pyramidal neurons. *Nat Neurosci.* **2**: 515-520.

Dingledine, R., Borges, K., Bowie, D. & Traynelis, S.F. (1999). The glutamate receptor ion channels. *Pharmacol Rev.* **51**: 7-61.

Dong, H., O'Brien, R.J., Fung, E.T., Lanahan, A.A., Worley, P.F. & Huganir, R.L. (1997). GRIP: a synaptic PDZ domain-containing protein that interacts with AMPA receptors. *Nature.* **386**: 279-284.

Duan, J., Wainwright, M.S., Comeron, J.M., Saitou, N., Sanders, A.R., Gelernter, J. & Gejman, P.V. (2003). Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor. *Hum Mol Genet.* **12**: 205-216.

Einheber, S., Zanazzi, G., Ching, W., Scherer, S., Milner, T.A., Peles, E. & Salzer, J.L. (1997). The axonal membrane protein Caspr, a homologue of neurexin IV, is a component of the septate-like paranodal junctions that assemble during myelination. *J Cell Biol.* **139**: 1495-1506.

Eom, T., Antar, L.N., Singer, R.H. & Bassell, G.J. (2003). Localization of a beta-actin messenger ribonucleoprotein complex with zipcode-binding protein modulates the density of dendritic filopodia and filopodial synapses. *J Neurosci.* **23**: 10433-10444.

Esteban, J.A. (2003). AMPA receptor trafficking: a road map for synaptic plasticity. *Mol Interv.* **3**: 375-385.

Everts, I., Villmann, C. & Hollmann, M. (1997). N-Glycosylation is not a prerequisite for glutamate receptor function but Is essential for lectin modulation. *Mol Pharmacol.* **52**: 861-873.

Faivre-Sarrailh, C., Gauthier, F., Denisenko-Nehrbass, N., Le Bivic, A., Rougon, G. & Girault, J.A. (2000). The glycosylphosphatidyl inositol-anchored adhesion molecule F3/contactin is required for surface transport of paranodin/contactin-associated protein (caspr). *J Cell Biol.* **149**: 491-502.

Fan, X.C. & Steitz, J.A. (1998). Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. *EMBO J.* **17**: 3448-3460.

Ferron, L., Davies, A., Page, K.M., Cox, D.J., Leroy, J., Waithe, D., Butcher, A.J., Sellaturay, P., Bolsover, S., Pratt, W.S., Moss, F.J. & Dolphin, A.C. (2008). The stargazin-related protein gamma 7 interacts with the mRNA-binding protein heterogeneous nuclear ribonucleoprotein A2 and regulates the stability of specific mRNAs, including CaV2.2. *J Neurosci.* **28**: 10604-10617.

Fischer, M., Kaech, S., Knutti, D. & Matus, A. (1998). Rapid actin-based plasticity in dendritic spines. *Neuron.* **20**: 847-854.

Fleck, M.W. (2006). Glutamate receptors and endoplasmic reticulum quality control: looking beneath the surface. *Neuroscientist.* **12**: 232-244.

Fleming, J.J. & England, P.M. (2010). AMPA receptors and synaptic plasticity: a chemist's perspective. *Nat Chem Biol.* **6**: 89-97.

Garneau, N.L., Wilusz, J. & Wilusz, C.J. (2007). The highways and byways of mRNA decay. *Nat Rev Mol Cell Biol.* **8**: 113-126.

Garner, C.C., Nash, J. & Huganir, R.L. (2000). PDZ domains in synapse assembly and signalling. *Trends Cell Biol.* **10**: 274-280.

Geiger, J.R., Melcher, T., Koh, D.S., Sakmann, B., Seeburg, P.H., Jonas, P. & Monyer, H. (1995). Relative abundance of subunit mRNAs determines gating and Ca2+ permeability of AMPA receptors in principal neurons and interneurons in rat CNS. *Neuron*. **15**: 193-204.

Genoux, D. & Montgomery, J.M. (2007). Glutamate receptor plasticity at excitatory synapses in the brain. *Clin Exp Pharmacol Physiol*. **34**: 1058-1063.

Gerges, N.Z., Backos, D.S., Rupasinghe, C.N., Spaller, M.R. & Esteban, J.A. (2006). Dual role of the exocyst in AMPA receptor targeting and insertion into the postsynaptic membrane. *EMBO J*. **25**: 1623-1634.

Gerrow, K., Romorini, S., Nabi, S.M., Colicos, M.A., Sala, C. & El-Husseini, A. (2006). A preformed complex of postsynaptic proteins is involved in excitatory synapse development. *Neuron*. **49**: 547-562.

Gollan, L., Sabanay, H., Poliak, S., Berglund, E.O., Ranscht, B. & Peles, E. (2002). Retention of a cell adhesion complex at the paranodal junction requires the cytoplasmic region of Caspr. *J Cell Biol*. **157**: 1247-1256.

Gomes, A.R., Correia, S.S., Carvalho, A.L. & Duarte, C.B. (2003). Regulation of AMPA receptor activity, synaptic targeting and recycling: role in synaptic plasticity. *Neurochem Res*. **28**: 1459-1473.

Gray, N.W., Fourgeaud, L., Huang, B., Chen, J., Cao, H., Oswald, B.J., Hemar, A. & McNiven, M.A. (2003). Dynamin 3 is a component of the postsynapse, where it interacts with mGluR5 and Homer. *Curr Biol*. **13**: 510-515.

Greger, I.H. & Esteban, J.A. (2007). AMPA receptor biogenesis and trafficking. *Curr Opin Neurobiol*. **17**: 289-297.

Greger, I.H., Khatri, L., Kong, X. & Ziff, E.B. (2003). AMPA receptor tetramerization is mediated by Q/R editing. *Neuron*. **40**: 763-774.

Greger, I.H., Ziff, E.B. & Penn, A.C. (2007). Molecular determinants of AMPA receptor subunit assembly. *Trends Neurosci*. **30**: 407-416.

Groc, L. & Choquet, D. (2006). AMPA and NMDA glutamate receptor trafficking: multiple roads for reaching and leaving the synapse. *Cell Tissue Res*. **326**: 423-438.

Grooms, S.Y., Noh, K.M., Regis, R., Bassell, G.J., Bryan, M.K., Carroll, R.C. & Zukin, R.S. (2006). Activity bidirectionally regulates AMPA receptor mRNA abundance in dendrites of hippocampal neurons. *J Neurosci*. **26**: 8339-8351.

Gu, W., Wells, A.L., Pan, F. & Singer, R.H. (2008). Feedback regulation between zipcode binding protein 1 and beta-catenin mRNAs in breast cancer cells. *Mol Cell Biol*. **28**: 4963-4974.

Guarino, M. (2010). Src signaling in cancer invasion. *J Cell Physiol*. **223**: 14-26.

Hanley, J.G. (2010). Endosomal sorting of AMPA receptors in hippocampal neurons. *Biochem Soc Trans*. **38**: 460-465.

Hayashi, T. & Huganir, R.L. (2004). Tyrosine phosphorylation and regulation of the AMPA receptor by SRC family tyrosine kinases. *J Neurosci*. **24**: 6152-6160.

Hayashi, T., Rumbaugh, G. & Huganir, R.L. (2005). Differential regulation of AMPA receptor subunit trafficking by palmitoylation of two distinct sites. *Neuron*. **47**: 709-723.

Hayashi, Y., Shi, S.H., Esteban, J.A., Piccini, A., Poncer, J.C. & Malinow, R. (2000). Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science*. **287**: 2262-2267.

Hebb, D.O. (1949). The organization of behavior; a neuropsychological theory (New York,: Wiley).

Henley, J.M. (2003). Proteins interactions implicated in AMPA receptor trafficking: a clear destination and an improving route map. *Neurosci Res*. **45**: 243-254.

Hirokawa, N. (2006). mRNA transport in dendrites: RNA granules, motors, and tracks. *J Neurosci*. **26**: 7139-7142.

Hirokawa, N. & Takemura, R. (2005). Molecular motors and mechanisms of directional transport in neurons. *Nat Rev Neurosci*. **6**: 201-214.

Hollmann, M. & Heinemann, S. (1994). Cloned glutamate receptors. *Annu Rev Neurosci*. **17**: 31-108.

Holt, C.E. & Bullock, S.L. (2009). Subcellular mRNA localization in animal cells and why it matters. *Science*. **326**: 1212-1216.

Huang, Y.S., Kan, M.C., Lin, C.L. & Richter, J.D. (2006). CPEB3 and CPEB4 in neurons: analysis of RNA-binding specificity and translational control of AMPA receptor GluR2 mRNA. *EMBO J*. **25**: 4865-4876.

Huttelmaier, S., Zenklusen, D., Lederer, M., Dictenberg, J., Lorenz, M., Meng, X., Bassell, G.J., Condeelis, J. & Singer, R.H. (2005). Spatial regulation of beta-actin translation by Src-dependent phosphorylation of ZBP1. *Nature*. **438**: 512-515.

Huveneers, S. & Danen, E.H. (2009). Adhesion signaling - crosstalk between integrins, Src and Rho. *J Cell Sci*. **122**: 1059-1069.

Ignelzi, M.A., Jr., Miller, D.R., Soriano, P. & Maness, P.F. (1994). Impaired neurite outgrowth of src-minus cerebellar neurons on the cell adhesion molecule L1. *Neuron*. **12**: 873-884.

Ingley, E. (2008). Src family kinases: regulation of their activities, levels and identification of new pathways. *Biochim Biophys Acta*. **1784**: 56-65.

Isaac, J.T., Nicoll, R.A. & Malenka, R.C. (1995). Evidence for silent synapses: implications for the expression of LTP. *Neuron*. **15**: 427-434.

Jiang, J., Suppiramaniam, V. & Wooten, M.W. (2006). Posttranslational modifications and receptor-associated proteins in AMPA receptor trafficking and synaptic plasticity. *Neurosignals*. **15**: 266-282.

Jiang, M., Deng, L. & Chen, G. (2004). High Ca(2+)-phosphate transfection efficiency enables single neuron gene analysis. *Gene Ther*. **11**: 1303-1311.

Ju, W., Morishita, W., Tsui, J., Gaietta, G., Deerinck, T.J., Adams, S.R., Garner, C.C., Tsien, R.Y., Ellisman, M.H. & Malenka, R.C. (2004). Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. *Nat Neurosci*. **7**: 244-253.

Jurado, S., Rodriguez-Pascual, F., Sanchez-Prieto, J., Reimunde, F.M., Lamas, S. & Torres, M. (2006). NMDA induces post-transcriptional regulation of alpha2-guanylyl-cyclase-subunit expression in cerebellar granule cells. *J Cell Sci*. **119**: 1622-1631.

Kacharmina, J.E., Job, C., Crino, P. & Eberwine, J. (2000). Stimulation of glutamate receptor protein synthesis and membrane insertion within isolated neuronal dendrites. *Proc Natl Acad Sci U S A*. **97**: 11545-11550.

Keinanen, K., Wisden, W., Sommer, B., Werner, P., Herb, A., Verdoorn, T.A., Sakmann, B. & Seeburg, P.H. (1990). A family of AMPA-selective glutamate receptors. *Science*. **249**: 556-560.

Kessels, H.W. & Malinow, R. (2009). Synaptic AMPA receptor plasticity and behavior. *Neuron*. **61**: 340-350.

Kew, J.N. & Kemp, J.A. (2005). Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmacology (Berl)*. **179**: 4-29.

Koegl, M., Zlatkine, P., Ley, S.C., Courtneidge, S.A. & Magee, A.I. (1994). Palmitoylation of multiple Src-family kinases at a homologous N-terminal motif. *Biochem J*. **303 (Pt 3)**: 749-753.

Kopec, C.D., Li, B., Wei, W., Boehm, J. & Malinow, R. (2006). Glutamate receptor exocytosis and spine enlargement during chemically induced long-term potentiation. *J Neurosci*. **26**: 2000-2009.

Kulkarni, M., Ozgur, S. & Stoecklin, G. (2010). On track with P-bodies. *Biochem Soc Trans*. **38**: 242-251.

Kuwano, Y., Kim, H.H., Abdelmohsen, K., Pullmann, R., Jr., Martindale, J.L., Yang, X. & Gorospe, M. (2008). MKP-1 mRNA stabilization and translational control by RNA-binding proteins HuR and NF90. *Mol Cell Biol*. **28**: 4562-4575.

Lee, H.K., Barbarosie, M., Kameyama, K., Bear, M.F. & Huganir, R.L. (2000). Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. *Nature*. **405**: 955-959.

Lee, H.K., Takamiya, K., He, K., Song, L. & Huganir, R.L. (2010). Specific roles of AMPA receptor subunit GluR1 (GluA1) phosphorylation sites in regulating synaptic plasticity in the CA1 region of hippocampus. *J Neurophysiol*. **103**: 479-489.

Lee, H.K., Takamiya, K., Kameyama, K., He, K., Yu, S., Rossetti, L., Wilen, D. & Huganir, R.L. (2007). Identification and characterization of a novel phosphorylation site on the GluR1 subunit of AMPA receptors. *Mol Cell Neurosci*. **36**: 86-94.

Lee, N.H. & Malek, R.L. (1998). Nerve growth factor regulation of m4 muscarinic receptor mRNA stability but not gene transcription requires mitogen-activated protein kinase activity. *J Biol Chem*. **273**: 22317-22325.

Leeds, P., Kren, B.T., Boylan, J.M., Betz, N.A., Steer, C.J., Gruppuso, P.A. & Ross, J. (1997). Developmental regulation of CRD-BP, an RNA-binding protein that stabilizes c-myc mRNA in vitro. *Oncogene*. **14**: 1279-1286.

Leonard, A.S., Davare, M.A., Horne, M.C., Garner, C.C. & Hell, J.W. (1998). SAP97 is associated with the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor GluR1 subunit. *J Biol Chem*. **273**: 19518-19524.

Leung, K.M., van Horck, F.P., Lin, A.C., Allison, R., Standart, N. & Holt, C.E. (2006). Asymmetrical beta-actin mRNA translation in growth cones mediates attractive turning to netrin-1. *Nat Neurosci*. **9**: 1247-1256.

Lin, D.T. & Huganir, R.L. (2007). PICK1 and phosphorylation of the glutamate receptor 2 (GluR2) AMPA receptor subunit regulates GluR2 recycling after NMDA receptor-induced internalization. *J Neurosci*. **27**: 13903-13908.

Lu, W., Man, H., Ju, W., Trimble, W.S., MacDonald, J.F. & Wang, Y.T. (2001). Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. *Neuron*. **29**: 243-254.

Lynch, G. & Baudry, M. (1984). The biochemistry of memory: a new and specific hypothesis. *Science*. **224**: 1057-1063.

Madden, D.R. (2002). The structure and function of glutamate receptor ion channels. *Nat Rev Neurosci*. **3**: 91-101.

Maghsoodi, B., Poon, M.M., Nam, C.I., Aoto, J., Ting, P. & Chen, L. (2008). Retinoic acid regulates RARalpha-mediated control of translation in dendritic RNA granules during homeostatic synaptic plasticity. *Proc Natl Acad Sci U S A*. **105**: 16015-16020.

Malenka, R.C. & Bear, M.F. (2004). LTP and LTD: an embarrassment of riches. *Neuron*. **44**: 5-21.

Malenka, R.C. & Nicoll, R.A. (1999). Long-term potentiation--a decade of progress? *Science*. **285**: 1870-1874.

Malinow, R. (2003). AMPA receptor trafficking and long-term potentiation. *Philos Trans R Soc Lond B Biol Sci*. **358**: 707-714.

Malinow, R., Mainen, Z.F. & Hayashi, Y. (2000). LTP mechanisms: from silence to four-lane traffic. *Curr Opin Neurobiol*. **10**: 352-357.

Malinow, R. & Malenka, R.C. (2002). AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci*. **25**: 103-126.

Mammen, A.L., Kameyama, K., Roche, K.W. & Huganir, R.L. (1997). Phosphorylation of the alpha-amino-3-hydroxy-5-methylisoxazole4-propionic acid receptor GluR1 subunit by calcium/calmodulin-dependent kinase II. *J Biol Chem*. **272**: 32528-32533.

Man, H.Y., Lin, J.W., Ju, W.H., Ahmadian, G., Liu, L., Becker, L.E., Sheng, M. & Wang, Y.T. (2000). Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. *Neuron*. **25**: 649-662.

Manadas, B., Santos, A.R., Szabadfi, K., Gomes, J.R., Garbis, S.D., Fountoulakis, M. & Duarte, C.B. (2009). BDNF-induced changes in the expression of the translation machinery in hippocampal neurons: protein levels and dendritic mRNA. *J Proteome Res*. **8**: 4536-4552.

Martin, K.C. & Zukin, R.S. (2006). RNA trafficking and local protein synthesis in dendrites: an overview. *J Neurosci*. **26**: 7131-7134.

Matsuda, S., Mikawa, S. & Hirai, H. (1999). Phosphorylation of serine-880 in GluR2 by protein kinase C prevents its C terminus from binding with glutamate receptor-interacting protein. *J Neurochem*. **73**: 1765-1768.

Menegoz, M., Gaspar, P., Le Bert, M., Galvez, T., Burgaya, F., Palfrey, C., Ezan, P., Arnos, F. & Girault, J.A. (1997). Paranodin, a glycoprotein of neuronal paranodal membranes. *Neuron*. **19**: 319-331.

Ming, G.L. (2006). Turning by asymmetric actin. *Nat Neurosci*. **9**: 1201-1203.

Morris, R.G. (2006). Elements of a neurobiological theory of hippocampal function: the role of synaptic plasticity, synaptic tagging and schemas. *Eur J Neurosci*. **23**: 2829-2846.

Murai, K.K., Misner, D. & Ranscht, B. (2002). Contactin supports synaptic plasticity associated with hippocampal long-term depression but not potentiation. *Curr Biol*. **12**: 181-190.

Newpher, T.M. & Ehlers, M.D. (2008). Glutamate receptor dynamics in dendritic microdomains. *Neuron*. **58**: 472-497.

Noubissi, F.K., Elcheva, I., Bhatia, N., Shakoori, A., Ougolkov, A., Liu, J., Minamoto, T., Ross, J., Fuchs, S.Y. & Spiegelman, V.S. (2006). CRD-BP mediates stabilization of betaTrCP1 and c-myc mRNA in response to beta-catenin signalling. *Nature*. **441**: 898-901.

O'Brien, R.J., Kamboj, S., Ehlers, M.D., Rosen, K.R., Fischbach, G.D. & Huganir, R.L. (1998). Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron*. **21**: 1067-1078.

Okamoto, P.M., Gamby, C., Wells, D., Fallon, J. & Vallee, R.B. (2001). Dynamin isoform-specific interaction with the shank/ProSAP scaffolding proteins of the postsynaptic density and actin cytoskeleton. *J Biol Chem*. **276**: 48458-48465.

Osten, P., Khatri, L., Perez, J.L., Kohr, G., Giese, G., Daly, C., Schulz, T.W., Wensky, A., Lee, L.M. & Ziff, E.B. (2000). Mutagenesis reveals a role for ABP/GRIP binding to GluR2 in synaptic surface accumulation of the AMPA receptor. *Neuron*. **27**: 313-325.

Osten, P. & Stern-Bach, Y. (2006). Learning from stargazin: the mouse, the phenotype and the unexpected. *Curr Opin Neurobiol*. **16**: 275-280.

Ozawa, S., Kamiya, H. & Tsuzuki, K. (1998). Glutamate receptors in the mammalian central nervous system. *Prog Neurobiol*. **54**: 581-618.

Park, M., Penick, E.C., Edwards, J.G., Kauer, J.A. & Ehlers, M.D. (2004). Recycling endosomes supply AMPA receptors for LTP. *Science*. **305**: 1972-1975.

Parsons, S.J. & Parsons, J.T. (2004). Src family kinases, key regulators of signal transduction. *Oncogene*. **23**: 7906-7909.

Pascale, A., Amadio, M. & Quattrone, A. (2008). Defining a neuron: neuronal ELAV proteins. *Cell Mol Life Sci*. **65**: 128-140.

Passafaro, M., Piech, V. & Sheng, M. (2001). Subunit-specific temporal and spatial patterns of AMPA receptor exocytosis in hippocampal neurons. *Nat Neurosci*. **4**: 917-926.

Peles, E., Nativ, M., Lustig, M., Grumet, M., Schilling, J., Martinez, R., Plowman, G.D. & Schlessinger, J. (1997). Identification of a novel contactin-associated transmembrane receptor with multiple domains implicated in protein-protein interactions. *EMBO J*. **16**: 978-988.

Perez, J.L., Khatri, L., Chang, C., Srivastava, S., Osten, P. & Ziff, E.B. (2001). PICK1 targets activated protein kinase Calpha to AMPA receptor clusters in spines of hippocampal neurons

and reduces surface levels of the AMPA-type glutamate receptor subunit 2. *J Neurosci*. **21**: 5417-5428.

Perrone-Bizzozero, N. & Bolognani, F. (2002). Role of HuD and other RNA-binding proteins in neural development and plasticity. *J Neurosci Res*. **68**: 121-126.

Perycz, M., Urbanska, A.S., Krawczyk, P.S., Parobczak, K. & Jaworski, J. (2011). Zipcode binding protein 1 regulates the development of dendritic arbors in hippocampal neurons. *J Neurosci*. **31**: 5271-5285.

Petralia, R.S. & Wenthold, R.J. (1992). Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. *J Comp Neurol*. **318**: 329-354.

Pillai, A.M., Garcia-Fresco, G.P., Sousa, A.D., Dupree, J.L., Philpot, B.D. & Bhat, M.A. (2007). No effect of genetic deletion of contactin-associated protein (CASPR) on axonal orientation and synaptic plasticity. *J Neurosci Res*. **85**: 2318-2331.

Piper, M., Anderson, R., Dwivedy, A., Weinl, C., van Horck, F., Leung, K.M., Cogill, E. & Holt, C. (2006). Signaling mechanisms underlying Slit2-induced collapse of Xenopus retinal growth cones. *Neuron.* **49**: 215-228.

Plant, K., Pelkey, K.A., Bortolotto, Z.A., Morita, D., Terashima, A., McBain, C.J., Collingridge, G.L. & Isaac, J.T. (2006). Transient incorporation of native GluR2-lacking AMPA receptors during hippocampal long-term potentiation. *Nat Neurosci*. **9**: 602-604.

Ponting, C.P., Phillips, C., Davies, K.E. & Blake, D.J. (1997). PDZ domains: targeting signalling molecules to sub-membranous sites. *Bioessays*. **19**: 469-479.

Poon, M.M. & Chen, L. (2008). Retinoic acid-gated sequence-specific translational control by RARalpha. *Proc Natl Acad Sci U S A*. **105**: 20303-20308.

Poon, M.M., Choi, S.H., Jamieson, C.A., Geschwind, D.H. & Martin, K.C. (2006). Identification of process-localized mRNAs from cultured rodent hippocampal neurons. *J Neurosci*. **26**: 13390-13399.

Pozo, K. & Goda, Y. (2010). Unraveling mechanisms of homeostatic synaptic plasticity. *Neuron*. **66**: 337-351.

Prasad, C.K., Mahadevan, M., MacNicol, M.C. & MacNicol, A.M. (2008). Mos 3' UTR regulatory differences underlie species-specific temporal patterns of Mos mRNA cytoplasmic polyadenylation and translational recruitment during oocyte maturation. *Mol Reprod Dev*. **75**: 1258-1268.

Resh, M.D. (1999). Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. *Biochim Biophys Acta*. **1451**: 1-16.

Rial Verde, E.M., Lee-Osbourne, J., Worley, P.F., Malinow, R. & Cline, H.T. (2006). Increased expression of the immediate-early gene arc/arg3.1 reduces AMPA receptor-mediated synaptic transmission. *Neuron*. **52**: 461-474.

Rios, J.C., Melendez-Vasquez, C.V., Einheber, S., Lustig, M., Grumet, M., Hemperly, J., Peles, E. & Salzer, J.L. (2000). Contactin-associated protein (Caspr) and contactin form a complex that is targeted to the paranodal junctions during myelination. *J Neurosci*. **20**: 8354-8364.

Robinow, S., Campos, A.R., Yao, K.M. & White, K. (1988). The elav gene product of Drosophila, required in neurons, has three RNP consensus motifs. *Science*. **242**: 1570-1572.

Roche, K.W., O'Brien, R.J., Mammen, A.L., Bernhardt, J. & Huganir, R.L. (1996). Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. *Neuron*. **16**: 1179-1188.

Rodriguez-Pascual, F., Hausding, M., Ihrig-Biedert, I., Furneaux, H., Levy, A.P., Forstermann, U. & Kleinert, H. (2000). Complex contribution of the 3'-untranslated region to the expressional regulation of the human inducible nitric-oxide synthase gene. Involvement of the RNA-binding protein HuR. *J Biol Chem*. **275**: 26040-26049.

Rosenmund, C., Stern-Bach, Y. & Stevens, C.F. (1998). The tetrameric structure of a glutamate receptor channel. *Science*. **280**: 1596-1599.

Ross, A.F., Oleynikov, Y., Kislauskis, E.H., Taneja, K.L. & Singer, R.H. (1997). Characterization of a beta-actin mRNA zipcode-binding protein. *Mol Cell Biol*. **17**: 2158-2165.

Rumbaugh, G., Sia, G.M., Garner, C.C. & Huganir, R.L. (2003). Synapse-associated protein-97 isoform-specific regulation of surface AMPA receptors and synaptic function in cultured neurons. *J Neurosci*. **23**: 4567-4576.

Rumpel, S., LeDoux, J., Zador, A. & Malinow, R. (2005). Postsynaptic receptor trafficking underlying a form of associative learning. *Science*. **308**: 83-88.

Sans, N., Racca, C., Petralia, R.S., Wang, Y.X., McCallum, J. & Wenthold, R.J. (2001). Synapse-associated protein 97 selectively associates with a subset of AMPA receptors early in their biosynthetic pathway. *J Neurosci*. **21**: 7506-7516.

Santos, S.D. (2009). Proteomic Analysis of the Interactome of Glutamate Receptors of the AMPA Type: Contactin Associated Protein 1 as a Regulator of AMPA Receptors. In Faculty of Sciences and Technology (University of Coimbra).

Santos, S.D., Carvalho, A.L., Caldeira, M.V. & Duarte, C.B. (2009). Regulation of AMPA receptors and synaptic plasticity. *Neuroscience*. **158**: 105-125.

Santos, S.D., Manadas, B., Duarte, C.B. & Carvalho, A.L. (2010). Proteomic analysis of an interactome for long-form AMPA receptor subunits. *J Proteome Res*. **9**: 1670-1682.

Sasaki, Y., Welshhans, K., Wen, Z., Yao, J., Xu, M., Goshima, Y., Zheng, J.Q. & Bassell, G.J. (2010). Phosphorylation of zipcode binding protein 1 is required for brain-derived neurotrophic factor signaling of local beta-actin synthesis and growth cone turning. *J Neurosci*. **30**: 9349-9358.

Schuman, E.M., Dynes, J.L. & Steward, O. (2006). Synaptic regulation of translation of dendritic mRNAs. *J Neurosci*. **26**: 7143-7146.

Schwenk, J., Harmel, N., Zolles, G., Bildl, W., Kulik, A., Heimrich, B., Chisaka, O., Jonas, P., Schulte, U., Fakler, B. & Klocker, N. (2009). Functional proteomics identify cornichon proteins as auxiliary subunits of AMPA receptors. *Science*. **323**: 1313-1319.

Setou, M., Seog, D.H., Tanaka, Y., Kanai, Y., Takei, Y., Kawagishi, M. & Hirokawa, N. (2002). Glutamate-receptor-interacting protein GRIP1 directly steers kinesin to dendrites. *Nature*. **417**: 83-87.

Shen, L., Liang, F., Walensky, L.D. & Huganir, R.L. (2000). Regulation of AMPA receptor GluR1 subunit surface expression by a 4. 1N-linked actin cytoskeletal association. *J Neurosci*. **20**: 7932-7940.

Sheng, M. & Sala, C. (2001). PDZ domains and the organization of supramolecular complexes. *Annu Rev Neurosci*. **24**: 1-29.

Shepherd, J.D. & Huganir, R.L. (2007). The cell biology of synaptic plasticity: AMPA receptor trafficking. *Annu Rev Cell Dev Biol*. **23**: 613-643.

Shepherd, J.D., Rumbaugh, G., Wu, J., Chowdhury, S., Plath, N., Kuhl, D., Huganir, R.L. & Worley, P.F. (2006). Arc/Arg3.1 mediates homeostatic synaptic scaling of AMPA receptors. *Neuron*. **52**: 475-484.

Shi, S., Hayashi, Y., Esteban, J.A. & Malinow, R. (2001). Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell*. **105**: 331-343.

Shi, S.H., Hayashi, Y., Petralia, R.S., Zaman, S.H., Wenthold, R.J., Svoboda, K. & Malinow, R. (1999). Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science*. **284**: 1811-1816.

Sobolevsky, A.I., Rosconi, M.P. & Gouaux, E. (2009). X-ray structure, symmetry and mechanism of an AMPA-subtype glutamate receptor. *Nature*. **462**: 745-756.

Sommer, B., Kohler, M., Sprengel, R. & Seeburg, P.H. (1991). RNA editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell*. **67**: 11-19.

Song, I. & Huganir, R.L. (2002). Regulation of AMPA receptors during synaptic plasticity. *Trends Neurosci*. **25**: 578-588.

Songyang, Z., Fanning, A.S., Fu, C., Xu, J., Marfatia, S.M., Chishti, A.H., Crompton, A., Chan, A.C., Anderson, J.M. & Cantley, L.C. (1997). Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science*. **275**: 73-77.

Sossin, W.S. & DesGroseillers, L. (2006). Intracellular trafficking of RNA in neurons. *Traffic*. **7**: 1581-1589.

Stellwagen, D. & Malenka, R.C. (2006). Synaptic scaling mediated by glial TNF-alpha. *Nature*. **440**: 1054-1059.

Steward, O. & Levy, W.B. (1982). Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. *J Neurosci*. **2**: 284-291.

Stohr, N., Lederer, M., Reinke, C., Meyer, S., Hatzfeld, M., Singer, R.H. & Huttelmaier, S. (2006). ZBP1 regulates mRNA stability during cellular stress. *J Cell Biol*. **175**: 527-534.

Sumioka, A., Yan, D. & Tomita, S. (2010). TARP phosphorylation regulates synaptic AMPA receptors through lipid bilayers. *Neuron*. **66**: 755-767.

Sutton, M.A., Ito, H.T., Cressy, P., Kempf, C., Woo, J.C. & Schuman, E.M. (2006). Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. *Cell*. **125**: 785-799.

Sutton, M.A. & Schuman, E.M. (2006). Dendritic protein synthesis, synaptic plasticity, and memory. *Cell*. **127**: 49-58.

Terashima, A., Cotton, L., Dev, K.K., Meyer, G., Zaman, S., Duprat, F., Henley, J.M., Collingridge, G.L. & Isaac, J.T. (2004). Regulation of synaptic strength and AMPA receptor subunit composition by PICK1. *J Neurosci*. **24**: 5381-5390.

Tholanikunnel, B.G., Raymond, J.R. & Malbon, C.C. (1999). Analysis of the AU-rich elements in the 3'-untranslated region of beta 2-adrenergic receptor mRNA by mutagenesis and identification of the homologous AU-rich region from different species. *Biochemistry*. **38**: 15564-15572.

Thomas, S.M. & Brugge, J.S. (1997). Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol*. **13**: 513-609.

Tiruchinapalli, D.M., Oleynikov, Y., Kelic, S., Shenoy, S.M., Hartley, A., Stanton, P.K., Singer, R.H. & Bassell, G.J. (2003). Activity-dependent trafficking and dynamic localization of zipcode binding protein 1 and beta-actin mRNA in dendrites and spines of hippocampal neurons. *J Neurosci*. **23**: 3251-3261.

Tomita, S., Adesnik, H., Sekiguchi, M., Zhang, W., Wada, K., Howe, J.R., Nicoll, R.A. & Bredt, D.S. (2005a). Stargazin modulates AMPA receptor gating and trafficking by distinct domains. *Nature*. **435**: 1052-1058.

Tomita, S., Chen, L., Kawasaki, Y., Petralia, R.S., Wenthold, R.J., Nicoll, R.A. & Bredt, D.S. (2003). Functional studies and distribution define a family of transmembrane AMPA receptor regulatory proteins. *J Cell Biol*. **161**: 805-816.

Tomita, S., Fukata, M., Nicoll, R.A. & Bredt, D.S. (2004). Dynamic interaction of stargazin-like TARPs with cycling AMPA receptors at synapses. *Science*. **303**: 1508-1511.

Tomita, S., Stein, V., Stocker, T.J., Nicoll, R.A. & Bredt, D.S. (2005b). Bidirectional synaptic plasticity regulated by phosphorylation of stargazin-like TARPs. *Neuron*. **45**: 269-277.

Tu, L. & Deutsch, C. (1999). Evidence for dimerization of dimers in K+ channel assembly. *Biophys J*. **76**: 2004-2017.

Turrigiano, G. (2011). Too many cooks? Intrinsic and synaptic homeostatic mechanisms in cortical circuit refinement. *Annu Rev Neurosci*. **34**: 89-103.

Turrigiano, G., Abbott, L.F. & Marder, E. (1994). Activity-dependent changes in the intrinsic properties of cultured neurons. *Science*. **264**: 974-977.

Turrigiano, G.G. (1999). Homeostatic plasticity in neuronal networks: the more things change, the more they stay the same. *Trends Neurosci*. **22**: 221-227.

Turrigiano, G.G. (2008). The self-tuning neuron: synaptic scaling of excitatory synapses. *Cell*. **135**: 422-435.

Turrigiano, G.G., Leslie, K.R., Desai, N.S., Rutherford, L.C. & Nelson, S.B. (1998). Activity-dependent scaling of quantal amplitude in neocortical neurons. *Nature*. **391**: 892-896.

Turrigiano, G.G. & Nelson, S.B. (2000). Hebb and homeostasis in neuronal plasticity. *Curr Opin Neurobiol*. **10**: 358-364.

Turrigiano, G.G. & Nelson, S.B. (2004). Homeostatic plasticity in the developing nervous system. *Nat Rev Neurosci*. **5**: 97-107.

Umemori, H., Sato, S., Yagi, T., Aizawa, S. & Yamamoto, T. (1994). Initial events of myelination involve Fyn tyrosine kinase signalling. *Nature*. **367**: 572-576.

Valtschanoff, J.G., Burette, A., Davare, M.A., Leonard, A.S., Hell, J.W. & Weinberg, R.J. (2000). SAP97 concentrates at the postsynaptic density in cerebral cortex. *Eur J Neurosci*. **12**: 3605-3614.

Vikesaa, J., Hansen, T.V., Jonson, L., Borup, R., Wewer, U.M., Christiansen, J. & Nielsen, F.C. (2006). RNA-binding IMPs promote cell adhesion and invadopodia formation. *EMBO J*. **25**: 1456-1468.

Watkins, J.C. (1981). Pharmacology of excitatory amino acid transmitters. *Adv Biochem Psychopharmacol*. **29**: 205-212.

Welshhans, K. & Bassell, G.J. (2011). Netrin-1-Induced Local {beta}-Actin Synthesis and Growth Cone Guidance Requires Zipcode Binding Protein 1. *J Neurosci*. **31**: 9800-9813.

Wenthold, R.J., Petralia, R.S., Blahos, J., II & Niedzielski, A.S. (1996). Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. *J Neurosci*. **16**: 1982-1989.

Whitlock, J.R., Heynen, A.J., Shuler, M.G. & Bear, M.F. (2006). Learning induces long-term potentiation in the hippocampus. *Science*. **313**: 1093-1097.

Wierenga, C.J., Ibata, K. & Turrigiano, G.G. (2005). Postsynaptic expression of homeostatic plasticity at neocortical synapses. *J Neurosci*. **25**: 2895-2905.

Wilusz, C.J., Wormington, M. & Peltz, S.W. (2001). The cap-to-tail guide to mRNA turnover. *Nat Rev Mol Cell Biol*. **2**: 237-246.

Wu, L., Wells, D., Tay, J., Mendis, D., Abbott, M.A., Barnitt, A., Quinlan, E., Heynen, A., Fallon, J.R. & Richter, J.D. (1998). CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of alpha-CaMKII mRNA at synapses. *Neuron*. **21**: 1129-1139.

Xia, J., Zhang, X., Staudinger, J. & Huganir, R.L. (1999). Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. *Neuron*. **22**: 179-187.

Yao, J., Sasaki, Y., Wen, Z., Bassell, G.J. & Zheng, J.Q. (2006). An essential role for beta-actin mRNA localization and translation in Ca2+-dependent growth cone guidance. *Nat Neurosci*. **9**: 1265-1273.

Yisraeli, J.K. (2005). VICKZ proteins: a multi-talented family of regulatory RNA-binding proteins. *Biol Cell*. **97**: 87-96.

Yu, L.M. & Goda, Y. (2009). Dendritic signalling and homeostatic adaptation. *Curr Opin Neurobiol*. **19**: 327-335.

Zalfa, F., Eleuteri, B., Dickson, K.S., Mercaldo, V., De Rubeis, S., di Penta, A., Tabolacci, E., Chiurazzi, P., Neri, G., Grant, S.G. & Bagni, C. (2007). A new function for the fragile X mental retardation protein in regulation of PSD-95 mRNA stability. *Nat Neurosci*. **10**: 578-587.

Zalfa, F., Giorgi, M., Primerano, B., Moro, A., Di Penta, A., Reis, S., Oostra, B. & Bagni, C. (2003). The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. *Cell*. **112**: 317-327.

Zamanillo, D., Sprengel, R., Hvalby, O., Jensen, V., Burnashev, N., Rozov, A., Kaiser, K.M., Koster, H.J., Borchardt, T., Worley, P., Lubke, J., Frotscher, M., Kelly, P.H., Sommer, B., Andersen, P., Seeburg, P.H. & Sakmann, B. (1999). Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. *Science*. **284**: 1805-1811.

Zhang, H.L., Eom, T., Oleynikov, Y., Shenoy, S.M., Liebelt, D.A., Dictenberg, J.B., Singer, R.H. & Bassell, G.J. (2001). Neurotrophin-induced transport of a beta-actin mRNP complex increases beta-actin levels and stimulates growth cone motility. *Neuron*. **31**: 261-275.

Zhu, J.J., Esteban, J.A., Hayashi, Y. & Malinow, R. (2000). Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. *Nat Neurosci*. **3**: 1098-1106.

Ziff, E.B. (2007). TARPs and the AMPA receptor trafficking paradox. *Neuron*. **53**: 627-633.

Zisch, A.H., D'Alessandri, L., Amrein, K., Ranscht, B., Winterhalter, K.H. & Vaughan, L. (1995). The glypiated neuronal cell adhesion molecule contactin/F11 complexes with src-family protein tyrosine kinase Fyn. *Mol Cell Neurosci*. **6**: 263-279.