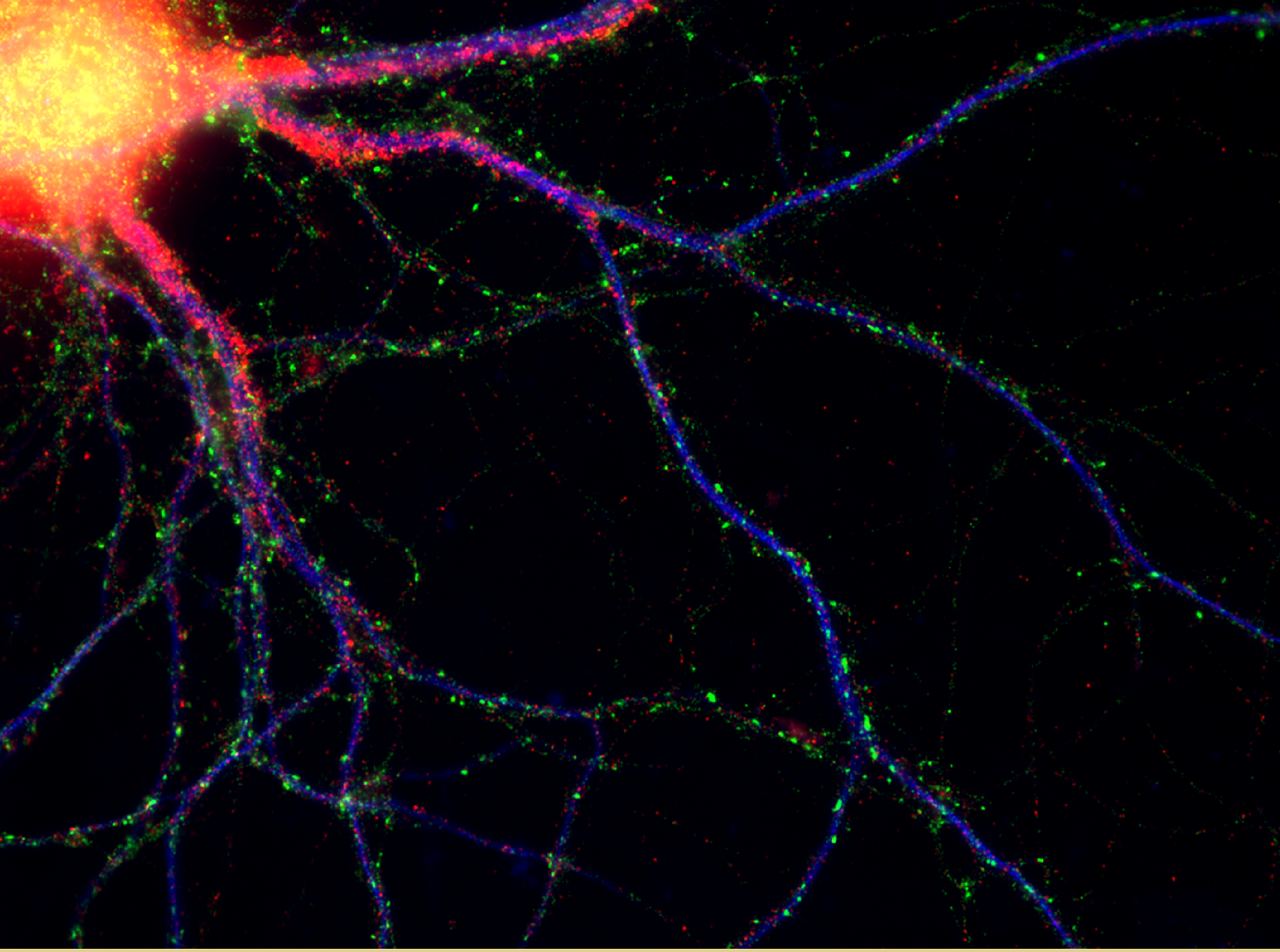

Graciano Silva Leal

REGULATION OF hnRNP A2/B1 AND hnRNP K BY SYNAPTIC ACTIVITY AND BDNF IN THE HIPPOCAMPUS

Tese de Doutoramento em Biociências, na especialidade de Neurociências, orientada pelo Professor Carlos B. Duarte, apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra

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UNIVERSIDADE DE COIMBRA

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Este trabalho foi realizado no Centro de Neurociências e Biologia Celular (Universidade de Coimbra) e no Centro K.G. Jebsen para a investigação de Doenças Neuropsiquiátricas (Departamento de Biomedicina, Universidade de Bergen, Bergen, Noruega), sob a supervisão do Professor Doutor Carlos Jorge Bandeira Duarte (Departamento de Ciências da Vida, Universidade de Coimbra) e co-supervisão do Professor Doutor Clive Bramham (Departamento de Biomedicina, Universidade de Bergen), e financiado pela bolsa de Doutoramento SFRH / BD / 66416 / 2009 da Fundação para a Ciência e Tecnologia, Portugal.

Graciano Silva Leal **Coimbra, 2013**

Cover note

Cover contains a picture of an hippocampal neuron from a primary culture maintained for 15 days and immunostained for hnRNP K, MAP2 and PSD95

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A todos os que me fazem feliz,

Table of contents

Abbreviations

4-AP	4-Aminopyridine
4EBPs	eIF4E binding proteins
A2RE	hnRNP A2 response element
Abi1	abelson-interacting protein 1
ADF	actin-depolymerizing factor
Akt	AKT8 virus oncogene cellular homolog
AMCA	aminomethylcoumarin acetate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	AMPA receptors
ANOVA	analysis of variance
Arc	activity-regulated cytoskeleton-associated protein
AS	antisense
Asap1	arfGAP with SH3 domain, ankyrin repeat and PH domain 1
BCA	bicinchoninic acid
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumine
CA	cornu ammonis
CaMKII	calcium/calmodulin-dependent protein kinase II
CaMKK	calcium/calmodulin-dependent protein kinase kinase
CBP80	cap-binding protein 80
Cds	coding sequence
CLAP	chymostatin, leupeptin, antipain, pepstatin
CNS	central nervous system
CPE	cytoplasmic polyadenylation element
CPEB	cytoplasmic polyadenylation element binding protein
CREB	[cyclic-adenosine monophosphate (AMP)-responsive element-binding protein]
CRK	CT10-regulated kinase
Ct	threshold cycle
CYFIP	cytoplasmic Fmr-interacting protein-1
DAG	diacylglycerol
DG	dentate gyrus
DICE	differentiation control element
DIV	days in vitro
DNA	deoxyribonucleic acid
DOC	sodium deoxycholate
DTT	dithiothreitol
EC	entorhinal cortex
ECF	enhanced chemifluorescence
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eEF	eukaryotic elongation factor
EGTA	ethylene glycol tetraacetic acid
eIF	eukaryotic initiation factor
EJC	exon junction complex
Elk-1	[E-twenty-six (ETS)-like transcription factor-1]
E-LTP	early LTP
EMSA	eletrophoretic mobility shift assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated protein kinase
fEPSP	field excitatory postsynaptic potential
FMRP	fragile X mental retardation protein
FRS2	fibroblast growth factor receptor substrate 2
GAB1	GRB2-associated binder-1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GIPC1	[PDZ (postsynaptic density-95/Discs large/zona occludens-1) domain-containing adaptor protein, type 1]
GluA	glutamate receptor, ionotropic, AMPA
GluN	glutamate receptor, ionotropic, NMDA
GO	gene ontology
GRB2	growth factor receptor-bound protein 2
GRIP1	glutamate receptor-interacting protein 1
HEPES	N-(2-hydroxyethyl)-1-piperazine-N''-(2-ethanesulfonic acid)
HFS	high-frequency stimulation

hnRNP	heterogeneous nuclear ribonucleoprotein
HRP	horseradish peroxidase
IEG	immediate-early gene
IGEPAL	octylphenoxypolyethoxyethanol
IgG	immunoglobulin G
IP	immunoprecipitation
IP3	inositol-1,4,5-trisphosphate
IRES	internal ribosomal entry site
IRS1	insulin-receptor substrate 1
KH	K homology domain
KI	K protein interactive domain
KNS	nuclear shuttling domain
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LE	localization element
LIMK1	LIM domain kinase-1
L-LTP	late LTP
LOX	15-lipoxygenase
LPP	lateral perforant path
Lsm1	sm-like protein 1
LTD	long-term depression
LTP	long-term potentiation
MAGE	melanoma-associated antigen
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MARTA1	MAP2-RNA trans-acting protein 1
MBP	myelin basic protein
Mef2	myocyte enhancer factor-2
MEK1/2	MAPK and ERK kinase, type 1/2
MEM	minimum essential medium eagle
MF	Mossy fibers
miRNA	microRNA
MMP9	matrix metalloproteinase 9
MPP	medial perforant path
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein complex
MSK1/2	mitogen- and stress-activated kinase 1/2
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor-κB
NGF	nerve growth factor
NLS	nuclear localization signal
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptors
NRIF	neurotrophin-interacting factor
NT3	neurotrophin 3
NT4	neurotrophin 4
N-WASP	neural Wiskott-Aldrich syndrome protein
ORF	open reading frame
$p75^{NTR}$	p75 neurotrophin receptor
PAGE	polyacrylamide gel electrophoresis
PAK	p21-activated kinase
P-bodies	processing bodies
PBS	phosphate-buffered saline
PDK1	3-phosphoinositide-dependent protein kinase 1
PET	polyethylene terephthalate
PH	pleckstrin homology domain
PI3-K	phosphatidylinositol 3-kinase
Pick1	protein interacting with C kinase 1
PKA	protein kinase A
PKC	protein kinase C
PKMζ	protein kinase M zeta
PLCγ	phospholipase C gamma
PMSF	phenylmethylsulfonyl fluoride
PP	perforant path
PSD	postsynaptic density
PSD95	postsynaptic density protein 95
PSF	polypyrimidine tract binding protein-associated splicing factor
Pum2	pumilio2

PVDF	polyvinylidene fluoride
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RBP	RNA-binding protein
RhoA	ras homolog family member A
RhoGDI	Rho GDP-dissociation inhibitor
Rim1α	Rab3a interacting molecular 1 α
RIP	RNP immunoprecipitation assays
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNG105	RNA granule protein 105
RNP	ribonucleoprotein
rpS6	ribosomal protein S6
RRM	RNA recognition motif
Sam68	Src-associated in mitosis of 68 kDa
SAP97	synapse-associated protein 97
SC	Shaffer collaterals
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SERT	serotonin transporter
SH2B	Src homology 2 domain-containing adaptor protein B
SH2B2	SH2B adaptor protein 2
Shank1	SH3 and multiple ankyrin repeat domains protein 1
Shc	Src homology 2-containing protein
SHP2	Src homology phosphatase 2
SIRT1	sirtuin1
SOS	son of sevenless
Src	Rous sarcoma oncogene cellular homolog
ssDNA	single-stranded DNA
Syngr1	synaptogyrin-1
TCF	ternary complex factor
TRAF6	[tumour necrosis factor (TNF) receptor-associated factor 6]
Trk	tropomyosin-related kinase
tRNA	aminoacyl-transfer RNA
TRPC	transient receptor-potential cation channel subfamily C
UTR	untranslated region
WAVE	WASP-family verprolin-homologous protein
ZBP	zipcode-binding protein

Keywords

Long-term potentiation

Hippocampus

BDNF

hnRNP A2/B1

hnRNP K

Palavras-chave

Potenciação de longa duração

Hipocampo

BDNF

hnRNP A2/B1

hnRNP K

Resumo

A plasticidade sináptica descreve um processo no qual a força da interacção entre neurónios, ou sinapses, é alterada. Por definição, é um termo funcional que se refere ao aumento ou decréscimo na eficácia sináptica, sendo acompanhado por alterações estruturais nas sinapses. A potenciação sináptica de longa duração (LTP) é a forma mais estudada de plasticidade sináptica e tem sido amplamente reconhecido que as alterações sinápticas responsáveis por certas formas de aprendizagem e memória podem ser semelhantes aquelas em que a expressão da LTP se baseia.

Algumas das modificações estruturais, bioquímicas e funcionais nas sinapses associadas à plasticidade sináptica dependem do transporte e da tradução de RNAs mensageiros (mRNAs) localizados nas dendrites, em resposta à actividade sináptica, com a concomitante alteração local do proteoma. É cada vez mais evidente que a síntese de proteínas nas dendrites desempenha um papel crucial em diversas formas de plasticidade sináptica, incluindo na potenciação de longa duração mediada pelo BDNF (factor neurotrófico derivado do cérebro).

Os transcritos dendríticos são transportados ao longo dos microtúbulos, numa forma em que a tradução está bloqueada, como componentes de complexos ribonucleoproteicos mensageiros (mRNPs). Para serem transportados, os mRNAs necessitam de conter na sua sequência um elemento *cis*-acting que é reconhecido por proteínas que ligam RNA e que, juntamente com outros factores, formam um complexo funcional que se liga a proteínas motoras para o transporte. Quando chegam ao seu destino, normalmente em sinapses ou na imediação de sinapses activadas, o bloqueio da tradução é libertado e os mRNAs são traduzidos após activação neuronal. Entre os constituintes moleculares que compõem os mRNPs em neurónios estão vários membros da família de proteínas hnRNP (ribonucleoproteinas nucleares heterogéneas). A proteína hnRNP A2/B1 é um dos mais bem descritos factores *trans*-acting envolvidos no transporte de mRNAs ao longo das dendrites em neurónios, contudo não se sabe se este é um processo constitutivo ou regulado. A hnRNP K também está presente em mRNPs em neurónios mas a sua função, e se desempenha algum papel no metabolismo de mRNAs nas dendrites, ainda está por determinar.

Neste trabalho mostramos que tanto a hnRNP A2/B1 como a hnRNP K se acumulam nas dendrites e nas sinapses após actividade sináptica em neurónios do hipocampo em cultura. Verificou-se também que o endereçamento de hnRNP A2/B1 para a sinapse em resposta à actividade neuronal depende de BDNF. De acordo com estes resultados, a estimulação de neurónios do hipocampo com BDNF também aumentou os níveis de hnRNP K e hnRNP A2/B1 nas dendrites e nas sinapses.

Estudos anteriores realizados no nosso laboratório identificaram diversos transcritos associados com a hnRNP K em neurónios do hipocampo, incluindo mRNAs que codificam proteínas importantes para a plasticidade sináptica como GluA1, GluN1, BDNF e CaMKIIß. De seguida verificou-se também que a neurotrofina BDNF induz a libertação desses transcritos de mRNPs que contêm hnRNP K em neurónios do hipocampo. Neste trabalho mostrámos que o BDNF também promove a dissociação dos mRNAs associados à hnRNP K localmente na sinapse. Estes resultados sugerem um papel fundamental da hnRNP K na tradução local de várias proteínas que contribuem para a fase tardia da LTP. De acordo com estas evidências experimentais, demonstrámos que os mRNAs associados à hnRNP K são regulados diferencialmente durante a potenciação de longa duração (LTP) induzida pela estimulação de alta frequência nas sinapses da via perforante - giro dentado em ratos vivos anestesiados. Demonstrámos também que esta forma de potenciação sináptica requer a activação dos receptores TrkB. Apesar de os resultados obtidos indicarem que o BDNF induz a libertação dos transcritos associados a mRNPs contendo a hnRNP K, não é possível excluir a contribuição de outras ribonucleoproteínas que co-imunoprecipitaram com a hnRNP K num estudo de proteómica efectuado.

No seu conjunto, os resultados obtidos apoiam o modelo que propõe uma função para a hnRNP A2/B1 na entrega de transcritos em regiões específicas das dendrites e sugerem que este é um processo regulado pela actividade sináptica e pelo BDNF. Demonstrámos também que a acumulação de hnRNP A2/B1 na sinapse após actividade sináptica depende de BDNF. A hnRNP K é outra hnRNP que parece desempenhar um papel crucial na regulação do metabolismo do mRNA localmente nas dendrites. Os nossos resultados demonstram que a interacção entre a hnRNP K e os mRNAs a ela associados é regulada pela actividade sináptica e

pelo BDNF, tanto *in vitro* como *in vivo*, o que sugere a hnRNP K como um importante regulador da função neuronal, em particular em fenómenos de plasticidade sináptica induzidos pelo BDNF.

Abstract

Synaptic plasticity describes the process by which connections between neurons, or synapses, change in strength. By definition, it is a functional term referring to an increase or decrease in synaptic efficacy, which is accompanied by structural changes at synapses. Long-term potentiation (LTP) is the most studied form of synaptic plasticity and it has been widely recognized that synaptic changes that underpin certain forms of learning and memory may be similar to those involved in the expression of LTP.

Some of the structural, biochemical and functional modifications at the synapse associated with synaptic plasticity require activity-dependent transport and translation of dendritic-localized mRNAs, with concomitant alterations in the synaptic proteome. It is becoming evident that dendritic protein synthesis has a crucial role in several forms of synaptic plasticity, including brain-derived neurotrophic factor (BDNF)-mediated LTP. Dendritic transcripts required for local protein synthesis are transported in a repressed state along the microtubule cytoskeleton as components of large messenger ribonucleoprotein complexes (mRNPs). In order to be transported, dendritic mRNAs must contain a *cis*-acting element in their sequence that is recognized by specific RNA binding proteins that, together with other factors, form a functional mRNP granule that engage with motor proteins for the transport. When they reach their destination, usually in, or in the vicinity of activated synapses, the translational-block is relieved and the mRNAs are translated upon neuronal activation. Among the molecular components of neuronal mRNPs, there are several members of the heterogeneous nuclear ribonucleoprotein (hnRNP) family of proteins. hnRNP A2/B1 is one of the best described *trans*-acting factor involved in the transport of dendritic-localized mRNAs in neurons, but whether this is a constitutive or a regulated process is unknown. hnRNP K is another member of the hnRNP family of proteins present in neuronal mRNPs, but the function of hnRNP K in neurons, and whether this protein plays a role in dendritic mRNA metabolism, remains to be determined.

Here we show that both hnRNP A2/B1 and hnRNP K accumulate in dendrites and at the synapse following neuronal activation in hippocampal neurons. Importantly, the activity-dependent delivery of hnRNP A2/B1 into synaptic sites requires BDNF. In addition, we observed that this

neurotrophin also upregulates the dendritic and synaptic levels of hnRNP K and hnRNP A2/B1 in primary cultures of hippocampal neurons.

Previous studies from our laboratory identified several transcripts associated with hnRNP K in cultured hippocampal neurons, including mRNAs coding for proteins with roles in synaptic plasticity such as GluA1, GluN1, BDNF and CaMKIIß. In addition, we demonstrated that the neurotrophin BDNF induces the release of these transcripts from the hnRNP K-containing mRNPs in hippocampal neurons. Herein we show that BDNF also promotes the dissociation of hnRNP K-bound mRNAs locally at the synapse. These results suggest a key role for hnRNP K in the local translation of several proteins that contribute to the late phase of LTP. Accordingly, we demonstrate that hnRNP K-associated mRNAs are differentially regulated during high-frequency stimulation (HFS)-induced LTP in the perforant path - dentate gyrus synapse in live anesthetized rats. Importantly, we show that this form of synaptic potentiation requires TrkB (tropomyosin-related kinase B) signaling. Although our results indicate that BDNF induces the release of the transcripts bound to mRNPs containing hnRNP K, at this time point it is not possible to rule out the contribution of other RNA-binding proteins that were found to co-immunoprecipitate with hnRNP K in a proteomic screen.

Altogether, our data support the evidence available pointing to a prominent role of hnRNP A2/B1 in the delivery of transcripts into dendritic domains and suggest that this process is regulated by synaptic activity and by the neurotrophin BDNF. Furthermore, we show that BDNF is required for the activity-induced synaptic delivery of hnRNP A2/B1. We also show that hnRNP K is another hnRNP that is likely to have a major role in the regulation of local mRNA metabolism in dendrites. Our results show that hnRNP K and hnRNP K-associated mRNAs are regulated by synaptic activity and BDNF, both *in vitro* and *in vivo*, and suggest hnRNP K as an important regulator of neuronal function, in particular in BDNF-induced plasticity events.

Chapter 1

Introduction

Part of the content of this chapter is published in:

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An adult human brain is thought to have more than 100 billion neurons, being these cells considered the core information processing structure in the central nervous system. Neurons are different from most other cells in the body in that they are polarized and have distinct morphological regions, each with specific functions. The connection between neurons is called synapse. Neurons connect to each other to form neuronal networks, and most of the actions mediating information storage are thought to take place at synapses. Thus, it is evident that synaptic alterations are critically involved in several fundamental physiological processes. On the other hand, several disease-related phenomena also implicate synaptic dysfunction.

There are two distinct types of synaptic connections, chemical and electric synapses. The typical chemical synapse in the nervous system requires the following steps: the synthesis of a neurotransmitter molecule in the presynaptic cell and its storage in secretory vesicles, the activity-dependent fusion of the neurotransmitter-containing vesicles with the plasma membrane, with the concomitant release of the neurotransmitter from the presynaptic terminal, the diffusion of these molecules across the synaptic cleft (space between the pre- and postsynaptic neurons), the activation of specific receptors localized in the postsynaptic cell eliciting a biochemical/electric response, and finally a mechanism for termination of the action of the released neurotransmitter. In the vertebrate central nervous system (CNS), the predominant mode of excitatory transmission is mediated by the neurotransmitter glutamate and the ionotropic glutamate receptors (Traynelis et al., 2010). Upon activation, ionotropic receptors open a channel which is more or less permeable to Na^+ and K^+ ions, and some receptors are also Ca^{2+}-permeable. While α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors support fast excitatory transmission, N-methyl-D-aspartate (NMDA) receptors have been classically viewed as coincidence detectors for the induction of long-term plasticity expressed as changes in AMPA receptors-mediated transmission (Malenka and Nicoll, 1993).

Synaptic plasticity describes the process by which synapses change in strength. By definition, it is a functional term referring to an increase or decrease in synaptic efficacy, which is accompanied by structural changes at synapses. Plasticity at synapses can be mediated at the presynaptic level, by changing the release of neurotransmitter molecules, or postsynaptically by changing the number, type, or properties of neurotransmitter receptors and their coupling to the intracellular signaling machinery. Long-term potentiation (LTP) is the main form of synaptic

plasticity, reflecting the activity of synaptic information storage processes, and has been identified as the prime candidate to be the cellular correlate of learning and memory (Lynch, 2004; Malenka, 2003a).

The neurotrophin brain-derived neurotrophic factor (BDNF) has been shown to play a key role in LTP and learning (Minichiello, 2009). This neurotrophin was shown, for instance, to regulate the induction and maintenance of a stable LTP (Minichiello, 2009), to regulate neurotransmitter release, to modulate postsynaptic glutamate receptors, to regulate protein synthesis and transcription (Carvalho et al., 2008), to play a role in the delivery and translation of dendritic-localized mRNAs (Leal et al., 2013; Santos et al., 2010) and to modulate structural plasticity in dendritic spines (Waterhouse and Xu, 2009). In this chapter we discuss the role of BDNF in LTP, highlighting the modulatory actions induced by this neurotrophin on dendritic protein synthesis.

1.1. Hippocampal formation and the trisynaptic circuit

The hippocampal formation is a large functional unit of the brain that belongs to the limbic system and plays important roles in the consolidation of short- and long-term memory and spacial navigation. One of the most captivating features of the hippocampus is its neuroanatomy. The peculiar organization of its main cell layers coupled to the highly organized laminar distribution (most axons are oriented parallel to each other and develop nearly transversally to the hippocampus axis) of many of its inputs has encouraged the use of the hippocampus as a model system for modern neurobiology (Amaral and Lavenex, 2007; Whitter, 2011). The hippocampal formation comprises three cytoarchitechtonically distinct regions: the dentate gyrus (DG), the subiculum and the hippocampus proper (Cornu Ammonis, CA). The CA is subdivided into the CA1, CA2 and CA3 regions (Fig. 1.1). Other regions of the hippocampal formation include the presubiculum, parasubiculum, and entorhinal cortex (Amaral and Lavenex, 2007; Whitter, 2011).

One of the unique features of the hippocampal formation is that many of its connections are unidireccional (Fig. 1.1). The entorhinal cortex provides most of the input to the dentate gyrus via fibers named the perforant path. The perforant pathway can be divided into two parts based on the origin and pattern of termination of its fibers. The lateral perforant path, which originates in the lateral entorhinal area and terminates in the most superficial third of the molecular layer, and the medial perforant path, originating in the medial entorhinal area and terminating in the middle third

of the molecular layer (Fig. 1.1) (Amaral and Lavenex, 2007; Whitter, 2011). Since the entorhinal cortex is the major source of cortical sensory information and the dentate gyrus the major receiver of entorhinal cortex projections, the dentate gyrus represents the first step in the processing of information that may lead to the formation of a memory (Amaral et al., 2007). The dentate gyrus does not project back to the entorhinal cortex. The dentate granule cells, the major cell type in the dentate gyrus, project their axons (Mossy fibers) into the CA3 region in the hippocampus. Again, the CA3 cells do not project back; they are instead the major input into the CA1 region (Shaffer collaterals). The CA1 projections are to some extent more elaborated however; the CA1 cells constitute the major excitatory input into the subiculum region although they also project into the entorhinal cortex (Amaral and Lavenex, 2007; Whitter, 2011). Because of its "relatively" simple organization and architecture the hippocampus is the ideal place to study synaptic transmission in the mammalian brain.

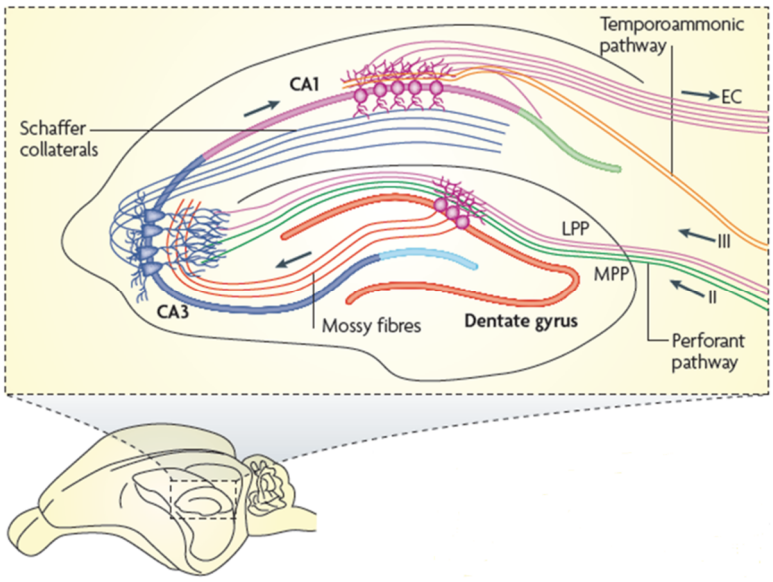

Figure 1.1 - The rodent hippocampus and the trisynaptic circuitry. The main input into the hippocampus comes from the entorhinal cortex (EC) and is named perforant path. The lateral perforant path (LPP) terminates in the most superficial third of the molecular layer whereas the medial perforant path (MPP) terminates in the middle third of the molecular layer. The granule cells of the dentate gyrus project to the CA3 region of the hippocampus via Mossy fiber projections. Pyramidal neurons in the CA3 region of the hippocampus project to CA1 via Schaffer collaterals. Pyramidal cells in CA1 project to the subiculum. Both CA1 and the subiculum project back to the deep layers of the entorhinal cortex. The entorhinal cortex also projects to the CA1 region through another pathway called temporoammonic pathway [Adapted from (Deng et al., 2010)].

1.2. Neurons: the functional units of the central nervous system

The idea that neurons constitute the functional units of the CNS arose after the extensive work on the structure of the nervous system performed by Santiago Ramón y Cajal in the late 19th and early 20th centuries. He postulated the neuron theory which has changed all the neuroscientific disciplines since then. He demonstrated that the relation between nerve cells was not of continuity but rather of contiguity (Lopez-Munoz et al., 2006) and that signal propagation is mediated by contacts at the level of certain apparatus (Lopez-Munoz et al., 2006). The concept of synapse emerged but from a semantic point of view. It was only several years later that the name synapse was adopted. We now know that the typical chemical synapse in the nervous system involves the activity-dependent release of a neurotransmitter molecule from the presynaptic cell that will act onto specific receptors localized in the postsynaptic cell.

In the vertebrate CNS, the predominant mode of excitatory transmission is mediated by the neurotransmitter glutamate and by ionotropic glutamate receptors (Traynelis et al., 2010). The dendritic spines constitute the primary recipient for the excitatory input and provide the biochemical compartments that locally control the signaling mechanisms at individual synapses (Bourne and Harris, 2008). Long-term changes in the efficacy of these synapses are coupled to changes in spine structure and morphology (Bourne and Harris, 2008). There is a considerable diversity in spine size and shape, and the following morphologies are typically found: stubby-, thin-, mushroom-shaped (Fig 1.2) (Bourne and Harris, 2008; Racz and Weinberg, 2013) and branched spines (Bourne and Harris, 2008). In contrast, inhibitory synapses are symmetric and typically located in the dendritic shaft (Bourne and Harris, 2011; Megias et al., 2001).

In the postsynaptic cell of an excitatory synapse there is a highly dense and specialized region attached to the postsynaptic membrane called postsynaptic density (PSD) that is composed by receptors, anchoring and scaffold proteins, as well as signaling molecules. Glutamatergic synapses mediate virtually all excitatory transmission in mammalian brains. Glutamate released from the presynaptic terminals activates several types of glutamate-gated ion channels on postsynaptic membranes, including AMPA and NMDA receptors (AMPAR; NMDAR) (Traynelis et al., 2010).

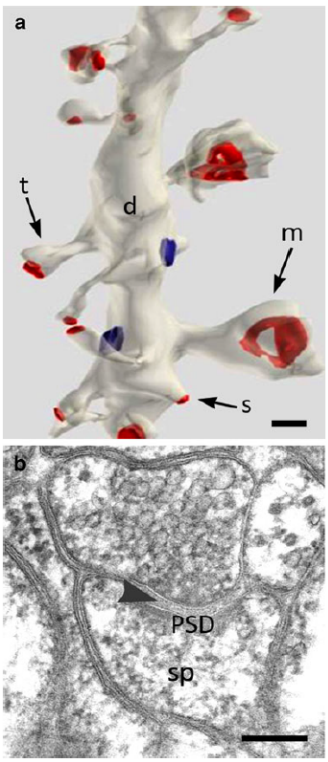

Figure 1.2 - Morphology of dendritic spines as observed by electron microscopy. (A) 3D reconstruction of serial thin sections from the *stratum radiatum* of the rat CA1 region. In the same dendritic shaft (d) it is possible to observe thin- (t), stubby- (s) and mushroom-shaped (m) spines. Excitatory synaptic contacts are colorized in red and symetric (presumably inhibitory) synapses in the dendritic shaft in blue. **(B)** Micrograph showing a typical mushroom-shaped spine from the rat hippocampus. Note the postsynaptic density (PSD) and the spine head (sp) with filamentous material that represent the actin cytoskeleton. The arrow indicates the synaptic cleft and at the top it is visible the presynaptic terminal with synaptic vesicles. Scale bars: 200 nm. From (Racz and Weinberg, 2013).

AMPAR are composed by several combinations of four subunits (GluA1-GluA4), and only AMPAR that lack GluA2 are permeable to Ca^{2+} (Cull-Candy et al., 2006). In contrast, all NMDAR are permeable to Ca^{2+} and are tetrameric complexes composed by two essential GluN1 subunits (8 isoforms) and by two GluN2 (from A-D) or GluN3 (A and B) subunits (Sanz-Clemente et al., 2013). In addition to glutamate, NMDAR require membrane depolarization to display a high opening probability, because of a voltage-dependent Mg^{2+} block. During bouts of synaptic activity AMPAR-mediated membrane depolarization of the postsynaptic membrane facilitates the the activation of NMDAR, which in turn initiate Ca^{2+} signaling pathways that modulate the presence of AMPAR at the membrane (Derkach et al., 2007; Kessels and Malinow, 2009; Malinow and Malenka, 2002; Rao and Finkbeiner, 2007; Scannevin and Huganir, 2000). Changes in the surface expression of AMPAR affect the synaptic strength, being LTP and long-term depression (LTD) two of the best described phenomena in which these changes are observed (Malenka and

Bear, 2004). Stability of LTP and LTD over time require synthesis of new gene products. The synaptic signals that culminate in gene transcription are tought to originate in the NMDAR-dependent activation of second messenger pathways (West et al., 2002). Thus, in a classical view of long-term changes in synaptic plasticity, the NMDA receptors have a regulatory role whereas AMPA receptors are the effectors.

1.3. Basic mechanisms of Long-Term Potentiation (LTP)

The concept of synaptic plasticity was postulated by Hebb in 1949 when he suggested that the experience can modify synapses favoring some neuronal pathways within a circuit and weakening others (Hebb, 1949). Since then, an enormous effort was made to understand the mechanisms that contribute to synaptic strengthening.

The first full description of LTP was published in 1973 by Bliss and Lomo (Bliss and Lomo, 1973). They reported that trains of high-frequency stimulation to the rabbit perforant path induced a sustained increase in efficiency of synaptic transmission in the granule cells of the dentate gyrus. This report, and others which followed during the 1970s, confirmed the Hebbian nature of this form of synaptic plasticity, and it was immediately recognized that the synaptic changes that occur during LTP may be similar and correlate with those that occur during certain forms of learning and memory. The LTP is characterized by three basic properties: i) the cooperativity, which describes the existence of an intensity threshold for induction; ii) LTP is associative in the sense that a weak input can be potentiated by other separated but convergent input; iii) finally, LTP is input-specific meaning that potentiation is not shared to other pathways distinct from the potentiated synapse (Bliss and Collingridge, 1993).

Since its discovery, LTP has been studied in detail in several brain regions and much is known about the molecular mechanisms underlying this form of plasticity. LTP in the hippocampus is the most studied form of synaptic plasticity and is typically divided into at least three distinct and sequential phases: short-term potentiation, early LTP (E-LTP), and late LTP (L-LTP). Short-term potentiation and E-LTP are transient and involve the modification of preexisting proteins, whereas L-LTP requires changes in gene expression and *de novo* protein synthesis, and lasts for hours, or even days (Kandel, 2001; Sweatt, 1999).

It is now well established that the postsynaptic influx of Ca^{2+} mediated by the activation of NMDA receptors is necessary and sufficient for the induction of E-LTP (Bliss and Collingridge, 1993). The opening of NMDA receptors requires glutamate binding and membrane depolarization which relieves the Mg^{2+} block. Activation of NMDA receptors allow the entry of Ca^{2+} into the cell and this rise in the intracellular Ca^{2+} concentration activates several enzymes that mediate E-LTP induction, including Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) (Sweatt, 1999). The sustained activation of these enzymes is also crucial for the maintenance of E-LTP [reviewed in (Bliss and Collingridge, 1993; Lynch, 2004)]. During the maintenance of E-LTP, the longer-term effects on synaptic strength are achieved due to generation of persistently active forms of PKC and CaMKII that become autonomously active and phosphorylate downstream targets that underlie E-LTP maintenance, such as AMPA receptors, resulting in an increase in their trafficking and insertion into the postsynaptic membrane, with a consequent upregulation in receptor activity (Derkach et al., 2007; Lin et al., 2009; Malenka, 2003a, b; Shepherd and Huganir, 2007). In addition, the increase in the surface expression of NMDA receptors was also observed upon E-LTP induction (Grosshans et al., 2002).

The E-LTP is transient and is followed by the L-LTP which is believed to depend on dendritic protein synthesis (Sutton and Schuman, 2006) and transcription activity [reviewed in (Adams and Dudek, 2005; Lynch, 2004)] (Fig. 1.3). Several signaling molecules such as PKA, CaMKIV and extracellular signal-regulated protein kinase (ERK) are involved in the activation of key transcription factors, including cyclic-AMP-responsive element-binding protein (CREB) and the ternary complex factor (TCF) Elk-1 [reviewed in (Adams and Dudek, 2005; Lynch, 2004; Platenik et al., 2000)], which will lead to an increase in the synthesis of proteins that mediate changes in the structure and/or function of the synapses, presumably required for the maintenance of L- LTP. In addition, the maintenance of L-LTP is also thought to require structural changes in dendritic spines. In particular, the actin-mediated enlargement of spine head and increase in spine number is believed to support the long-term changes in synaptic efficacy (Fukazawa et al., 2003; Krucker et al., 2000).

Several studies now indicate that neurotrophins, in particular brain-derived neurotrophic factor (BDNF), are potent regulators of hippocampal LTP [for a review see (Bramham and Messaoudi, 2005; Lu et al., 2008; Minichiello, 2009)]. BDNF is one of the downstream genes induced by L-

LTP and the protein has been shown to be a crucial player in the induction and maintenance of LTP, as discussed in the next sections.

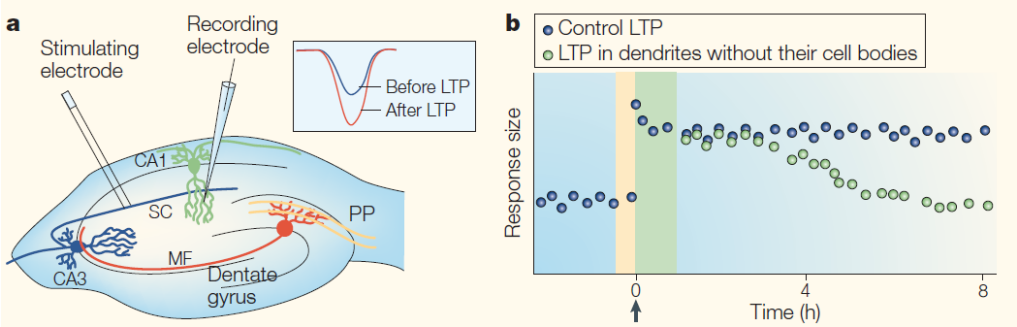

Figure 1.3 - LTP in the Shaffer collateral-CA1 synapses and the dependence on transcription of its late phase. (A) Illustration of a coronal slice of the rodent hippocampus. A stimulating electrode placed in the *stratum radiatum* (pyramidal cell dendrites) activates the fibers of the Schaffer collateral-commissural pathway that forms synapses on CA1 pyramidal cells. Synaptic responses can be recorded extracellularly with a recording electrode, also placed in the *stratum radiatum*. Inset: extracellular responses before and after the induction of long-term potentiation (LTP). CA3 and CA1, CA3 and CA1 hippocampal region; MF, Mossy fibers; PP, Perforant path; SC Shaffer collaterals. **(B)** LTP induced with high-frequency stimulation (arrow) often lasts more than 8 h in a slice preparation (blue dots). If dendrites are severed from their cell bodies, LTP decays to baseline level within 4 h (green dots). From (Adams and Dudek, 2005).

1.4. Dendritic protein synthesis and dendritic transcripts

The hypothesis of local protein synthesis at neuronal sites distant from the soma was raised after the work of Steward and Levy, who showed that polyribosomes can accumulate at the base of the dendritic spines forming a rosette-like structure (Steward and Levy, 1982), suggesting that they were bound to mRNA and involved in protein synthesis. This observation lead to the search for the mRNAs present in dendrites, and to the study of the mechanisms involved in their transport and how local protein synthesis is regulated at the synapse.

In highly polarized cells such as neurons, the transport of mRNAs coupled with local translation provides an important mechanism for spatial and temporal control of protein synthesis. Dendritic-localized mRNAs are usually packaged into large messenger ribonucleoprotein complexes (mRNPs) that engage with motor proteins for the microtubule-dependent transport along dendrites. In order to be transported, the transcripts must contain a *cis*-acting element (also called zipcode or localization element) in their sequence, typically present in the 3'-untranslated region (UTR), which is recognized by the proper RNA-binding proteins (RBPs) present in mRNPs together with other factors (Fig. 1.4). These transcripts are generally kept in a dormant state

during the transport and are then translated upon stimulation at or near activated synapses (Bramham and Wells, 2007). Several mRNAs were found to localize in dendritic processes under different physiological conditions. One of the major challenges of identifying dendritic mRNAs is the concentration of mRNAs in dendrites which is several times lower when compared with cell body-localized mRNAs, making somatic contamination a real issue.

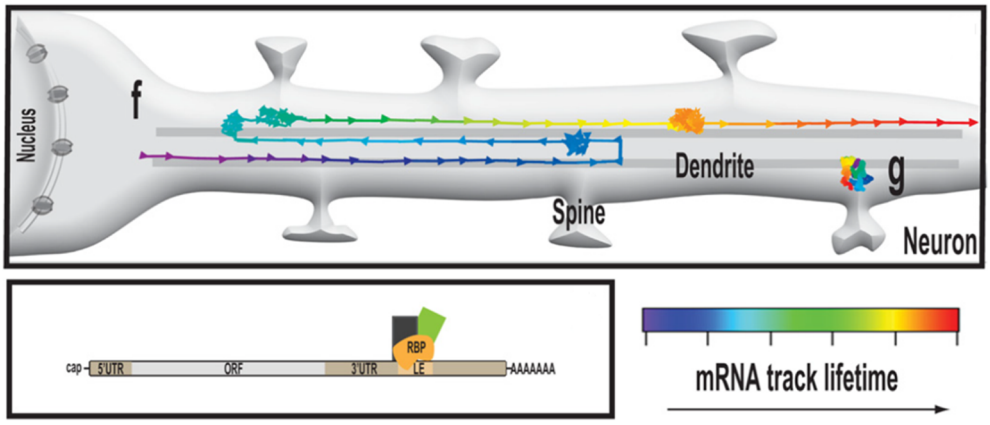

Figure 1.4 - Dendritic mRNA transport in mammalian neurons. Bottom panel- Schematic representation of a mRNA. The cap structure in the 5'-end, the poly(A) tail in the 3'-end, the 5'UTR, the ORF, the 3'UTR containing a localization element (LE), and the associated RBPs are represented. Top panel- Simulation of mRNA motility in neurons. mRNA tracks represent mRNA movement as a function of time, coded from purple/blue to red. (f) Neuronal mRNAs largely depend on microtubule-based transport for localization into dendrites. (g) mRNAs are seen to be docked at specific domains at or near activated synapses. Adapted from (Eliscovich et al., 2013).

The dendritic transcriptome is not yet fully characterized. The first estimations predicted that approximately 400 dendritic mRNAs could be present in the dendrites of cultured rat hippocampal neurons (Eberwine et al., 2001). A similar number of transcripts was identified in two other studies: i) in neurites of hippocampal neurons, using microarray analysis and a culture system that allows mechanical separation of axons and dendrites (129 mRNAs) (Poon et al., 2006); ii) in the *stratum radiatum* (dendritic lamina) from the rat hippocampal CA1 region (156 mRNAs) (Zhong et al., 2006). A different approach used consisted in the identification of conserved sequence elements in the transcripts that may explain their cellular localization and intracellular transport specificity (Lein et al., 2007). This strategy also predicted a low number of dendritic mRNAs (59 transcripts). A much higher number of transcripts was recently identified using deep RNA sequencing in microdissected synaptic neuropil (*stratum radiatum* and *lacunosum moleculare*) segments from the CA1 region of the adult rat hippocampus (Cajigas et al., 2012).

Since the neuropil tissue is comprised of dendrites, axons, glial cells, interneurons and blood vessels, the data obtained for the full neuropil transcriptome was filtered and 2550 mRNAs were attributed to dendrites and/or axons (Cajigas et al., 2012).

1.5. The neurotrophin family and its receptors

Nerve growth factor (NGF) was the first member of the neurotrophin family to be discovered in the early 50's as a target-derived protein that promotes the growth and survival of sympathetic and sensory neurons during development (Cohen et al., 1954). The establishment of the neurotrophin family came when in the early 80's brain-derived neurotrophic factor (BDNF) was purified from pig brain, and showed similar neurotrophic actions in sensory neurons (Barde et al., 1982). Two more neurotrophins were discovered since then, neurotrophin 3 (NT3) and neurotrophin 4 (NT4) (Lewin and Barde, 1996).

A major advance in our understanding of neurotrophins came when it was suggested that neurotrophins can be released in response to neuronal activity (Thoenen, 1991). This inspired future investigation addressing the roles of the neurotrophins in plasticity-related events. It is now well established that neurotrophins have pleiotropic functions in the nervous system, including roles in the control of neuronal survival and differentiation, in synaptogenesis and as mediators of activity-dependent synaptic plasticity (Park and Poo, 2013).

The physiological responses to neurotrophins are mediated by the activation of two distinct classes of membrane-bound receptors. Neurotrophins bind to $p75^{NTR}$ receptors and to one of the three tropomyosin-related kinase (Trk) receptors. NGF binds to TrkA, BDNF and NT4 bind to TrkB and NT3 binds to TrkC (Chao, 2003; Reichardt, 2006). In contrast with Trk receptors, activation of $p75^{NTR}$ does not display the same specificity. $p75^{NTR}$ binds both the mature and the uncleaved forms (pro-neurotrophins) of neurotrophins (Lee et al., 2001; Teng et al., 2005).

1.5.1. BDNF synthesis and secretion

The subcellular localization and the nature of the secreted BDNF protein is still very unclear, in part due to the very low levels of endogenous BDNF. Like many other secreted proteins, BDNF is initially synthetized in the endoplasmic reticulum as a precursor protein, pre-pro-BDNF, which is

then converted into pro-BDNF by removal of the signal peptide. Pro-BDNF is then cleaved to generate the mature form of this neurotrophin, but whether this occurs before the neurotrophin is released remains controversial (see below). BDNF produced in the cell body is targeted to vesicles of the regulated secretory pathway before being transported to postsynaptic dendrites in secretory granules (Adachi et al., 2005; Hartmann et al., 2001; Kohara et al., 2001; Matsuda et al., 2009). Alternatively, the neurotrophin may be delivered to presynaptic terminals in large dense core vesicles through anterograde transport (Adachi et al., 2005; Dieni et al., 2012; Kohara et al., 2001; Matsuda et al., 2009). BDNF stands out among all neurotrophins in the activity-dependent regulation of its expression and secretion [reviewed in (Lessmann and Brigadski, 2009)]. Upon high-frequency stimulation, BDNF is secreted in response to the influx of Ca^{2+} through NMDA receptors and/or voltage-gated Ca^{2+} channels (Aicardi et al., 2004; Balkowiec and Katz, 2002; Gartner and Staiger, 2002; Hartmann et al., 2001). Synaptic stimulation may also induce the release of the dendritic pool of BDNF by activating N-type Ca^{2+} channels and the release of Ca^{2+} from intracellular stores (Balkowiec and Katz, 2002).

Several lines of evidence suggest BDNF is released mainly in the precursor form (pro-BDNF), and the molecule is further processed into the mature form extracellularly, via the action of mettaloproteinases and the extracellular plasmin (Pang et al., 2004; Woo et al., 2005). A recent study using a BDNF hemagglutinin knock-in mice showed the presence of a significant amount of endogenous pro-BDNF during the perinatal period in mice, and both BDNF and pro-BDNF were found to be secreted in hippocampal cultures (Yang et al., 2009). In contrast, another study demonstrated that pro-BDNF is rapidly converted intracellularly into mature BDNF being the later stored and released by excitatory input (Matsumoto et al., 2008). Accordingly, a recent study showed the presence of BDNF and its cleaved pro-peptide in large dense core vesicles located at the presynaptic terminals of excitatory neurons (Dieni et al., 2012). These findings suggest that, although the cleavage of pro-BDNF may occur in secretory granules, both forms of the neurotrophin may be released from the presynaptic terminals.

As mentioned above, several studies performed in primary cultures demonstrated that BDNF may be stored and released from both axonal and dendritic compartments [reviewed in (Lessmann and Brigadski, 2009)]. However, a recent study using a pH-sensitive fluorescent tagged BDNF showed that axonal secretion requires much higher levels of neuronal spiking than

dendritic secretion (Matsuda et al., 2009), supporting the model proposing that dendritic-BDNF release represents the predominant form of activity-dependent BDNF secretion in these neurons. A large number of findings suggest that BDNF mRNA is transported to dendrites to allow the local synthesis of the protein [reviewed in (Leal et al., 2013)]. The release of this pool of BDNF from dendrites with concomitant activation of dendritic TrkB receptors (Baj et al., 2011) further indicates that BDNF can be released from dendritic processes. Given that the majority of the studies investigating the release of BDNF were performed in cultured neurons, future studies should address the nature and the source of secreted BDNF *in vivo,* and explore the physiological relevance of those findings.

1.5.2. TrkB localization and signaling pathways

TrkB receptors have been found in nerve terminals, axons and dendritic spines of glutamatergic pyramidal and granule cells in the hippocampus (Drake et al., 1999), and in dendritic spines of cerebrocortical neurons (Aoki et al., 2000). Another study demonstrated that TrkB receptors are present at glutamatergic nerve terminals in the hippocampus and evenly distributed between the presynaptic active zone and the postsynaptic density (Pereira et al., 2006).

The binding of neurotrophins to TrkB receptors induces ligand-receptor dimerization and autophosphorylation of tyrosine residues in the intracellular kinase domain of the receptor. This leads in turn to the phosphorylation of tyrosine residues in the intracellular juxtamembrane domain as well as in the carboxyl terminus of the receptor, which act as docking sites for adaptor molecules (Fig. 1.5). Phosphorylation of these two tyrosine residues, located outside the kinase activation domain of the Trk receptors, mediates the interaction with Shc (Src homology 2-containing protein) and phospholipase Cγ (PLCγ), respectively (Reichardt, 2006). Another adaptor molecule, fibroblast growth factor receptor substrate 2 (FRS2), competes with Shc adaptor molecules for the binding to Trk receptors (Kouhara et al., 1997; Meakin et al., 1999). Additional adaptor proteins containing pleckstrin homology and SH2 domains, such as SH2B and SH2B2, bind the phosphotyrosine residues on the catalytic domain of Trk receptors and can activate Trk signaling (Qian et al., 1998).

Due to the high homology of the intracellular domains, the signaling cascades are highly conserved among Trk receptors (Atwal et al., 2000). The three main intracellular signaling cascades activated by TrkB receptors are the Ras-mitogen activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3-K)-Akt pathway and the PLCγ-Ca^{2+} pathway (Kaplan and Miller, 2000).

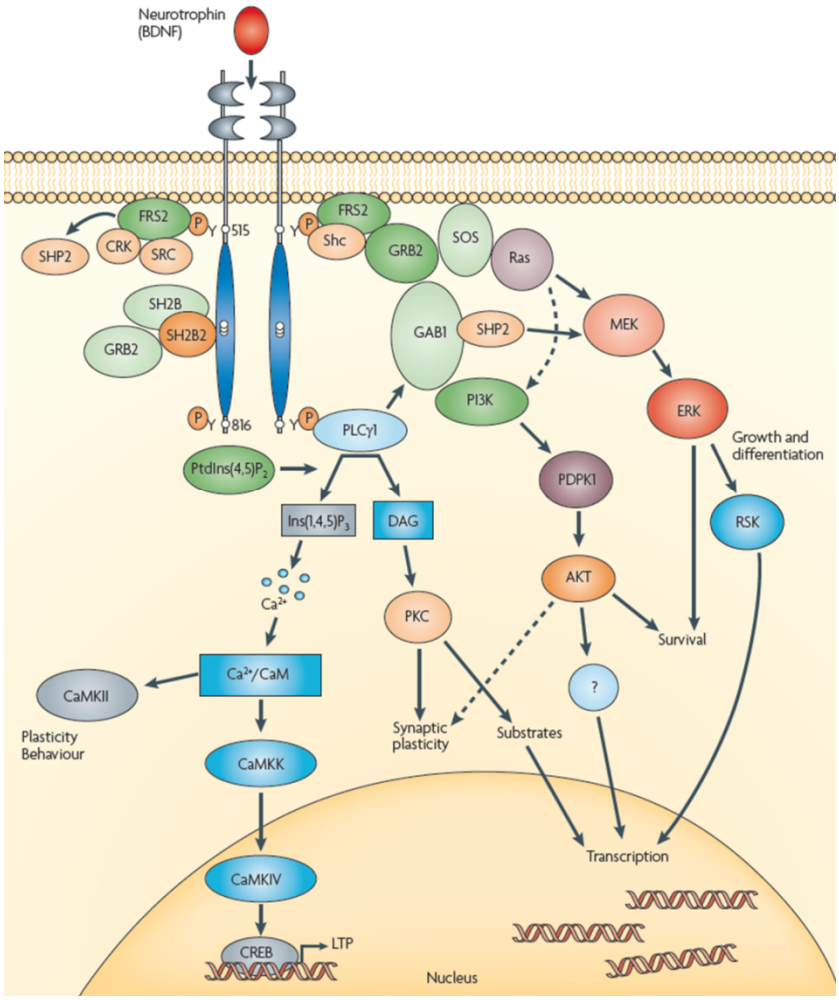

Figure 1.5 - Major signaling cascades activated by TrkB receptors. Binding of BDNF to TrkB receptors induces the dimerization and transphosphorylation of the receptors on tyrosine residues localized in the intracellular kinase domain. This increases the tyrosine kinase activity of the receptors and leads to the phosphorylation of other tyrosine residues that recruit several adaptor and signaling molecules such as Shc, FRS2 and PLCγ (more details in the text). TrkB signaling activates three major signaling pathways: the Ras-MAPK, the PI3-kinase and the PLCγ pathways. The activation of these pathways is coupled to several physiological actions that control neuronal survival, differentiation and synaptic plasticity. From (Minichiello, 2009).

1.5.2.1. ERK/MAPK pathway

The recruitment and phosphorylation of Shc adaptors results in the binding of growth factor receptor-bound protein 2 (GRB2) and son of sevenless (SOS), with the consequent activation of the Ras-MAPK pathway (Reichardt, 2006). FRS2 is also phosphorylated and can recruit GRB2 providing an Shc-independent mechanism for the activation of Ras through the GRB2-SOS complex (Hadari et al., 1998; Kouhara et al., 1997; Wright et al., 1997). FRS2 may also recruit additional signaling proteins such as CRK, the tyrosine kinase Src and a protein phosphatase, Src homology phosphatase 2 (SHP2), which are thought to mediate sustained activation of the MAPK pathway upon neurotrophin binding (Reichardt, 2006). The activation of this pathway leads, for instance, to the activation of CREB which activates the transcription of essential pro-survival genes [reviewed in (Reichardt, 2006)].

1.5.2.2. PI3-kinase pathway

Several adaptor proteins mediate the association and activation of PI3-K by TrkB receptors. GRB2-associated binder-1 (GAB1), insulin-receptor substrate 1 (IRS1) and IRS2 bind to the activated TrkB receptors through GRB2 to activate the PI3-K pathway (Holgado-Madruga et al., 1997; Yamada et al., 1997). Activation of PI3-K generates 3-phosphoinositides that activate 3-phosphoinositide-dependent protein kinase 1 (PDK1) which in turn, together with the 3-phosphoinositides, activate protein kinase Akt (Crowder and Freeman, 1998). Among the targets of Akt is SHP2 which enhances MAPK signaling (Liu and Rohrschneider, 2002). The PI3-kinase pathway has several targets that promote axon growth and pathfinding as well as neuronal differentiation [reviewed in (Reichardt, 2006)].

1.5.2.3. PLCγ pathway

Phosphorylation of the TrkB receptor on tyrosine 816 recruits PLCγ1 which is phosphorylated by the active receptor (Kaplan and Miller, 2000). Activated PLCγ1 hydrolyses phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) to generate inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG). Ins(1,4,5)P_3 promotes the release of Ca^{2+} from internal stores, which results in the activation of Ca^{2+}-dependent enzymes such as Ca^{2+}/calmodulin-dependent protein kinases. Signaling through this pathway controls the expression and/or activity

of many proteins, including ion channels and transcription factors, and plays an important role in TrkB-mediated long-term synaptic potentiation (Minichiello et al., 2002).

1.5.3. Activation of $p75^{NTR}$ by pro-neurotrophins

Several lines of evidence show that the activation of $p75^{NTR}$ by pro-neurotrophins induces physiological responses distinct from those initiated by TrkB receptors. Activation of the complex of $p75^{NTR}$-sortilin by pro-BDNF induces neuronal apoptosis (Teng et al., 2005). In addition, several studies demonstrated that the binding of pro-BDNF to $p75^{NTR}$ is required for LTD induction at CA3-CA1 synapses (Pang et al., 2004; Woo et al., 2005). However, a distinct study using conditional *Bdnf*-knockout mice showed that LTD induction was normal in these synapses, suggesting that neither the mature BDNF nor the pro-BDNF is involved in LTD (Matsumoto et al., 2008).

$p75^{NTR}$ is a member of the tumour necrosis factor receptor superfamily with a cytoplasmic region that contains a “death” domain similar to those present in other members of this family (He and Garcia, 2004). Although the receptor does not have catalytic activity, several signaling pathways are activated following neurotrophin binding to $p75^{NTR}$. These are mediated through the binding of adaptor proteins, including TRAF6, neurotrophin-interacting factor (NRIF), melanoma-associated antigen (MAGE), RhoGDI, among others (Reichardt, 2006). Activation of $p75^{NTR}$ may activate the Jun kinase signaling cascade (Reichardt, 2006). Neurotrophin binding to $p75^{NTR}$ also promotes the activation of NF-κB, thereby inducing NF-κB-dependent neuronal survival (Hamanoue et al., 1999). Activation of $p75^{NTR}$ by neurotrophins also controls Rho family GTPase activity and neurite outgrowth (Yamashita et al., 1999). Interestingly, it was suggested that Trk receptor function may be regulated by the presence of $p75^{NTR}$. For example, activation of $p75^{NTR}$ suppresses the ubiquitination of TrkB and thereby delaying receptor internalization and degradation (Makkerh et al., 2005).

1.6. BDNF and regulation of mRNA transport along dendrites

1.6.1. *Cis*-acting elements and mRNA transport into dendrites

The evidence discussed in the section 1.4 demonstrate that dendritic mRNA targeting is not a feature of a small subset of mRNAs as initially thought and confirms the enormous potential of localized translation in synaptic regulation. Among the dendritic mRNAs identified there are several transcripts encoding proteins with synaptic roles such as ionotropic and metabotropic receptors, scaffolding proteins, adhesion molecules, signaling molecules, and components of the translational machinery (Cajigas et al., 2012). The use of high-resolution RNA *in situ* hybridization revealed the mRNA for CaMKIIα (Ca^{2+}/calmodulin-dependent protein kinase II, α subunit) as the most abundant in the neuropil region. The dendritic trafficking of this transcript and the role of local translated CaMKIIα in synaptic plasticity events has been widely studied (Mayford et al., 1996; Miller et al., 2002; Ouyang et al., 1999). Other relatively abundant mRNAs in the neuropil region included the transcripts encoding for Shank1, PSD95 (postsynaptic density protein 95), Dendrin and MAP (microtubule-associated protein) 1A (Cajigas et al., 2012), all of them previously reported as dendritic mRNAs (Bockers et al., 2004; Herb et al., 1997; Muddashetty et al., 2007; Tucker et al., 1989).

The selectivity of dendritic mRNA transport results from a complex and orchestrated series of events and is determined by the presence of *cis*-acting RNA elements which are recognized by *trans*-acting RNA-binding proteins. These ribonucleoprotein complexes may combine with other factors to form a functional complex which is transported along the microtubule cytoskeleton to its final destination (Doyle and Kiebler, 2011; Martin and Ephrussi, 2009). The *cis*-acting elements on the mRNAs (also called zipcodes or localization elements) can vary in length and in spatial arrangement and, with few exceptions, are contained in the 3'UTR (Andreassi and Riccio, 2009). Nevertheless, the presence of localization elements in the 5'UTR and in the coding sequence has also been reported (Chiaruttini et al., 2009; Pal et al., 2003). One of the shortest, well described, zipcodes was found in myelin basic protein (MBP) mRNA and is only 11-nucleotide long (Munro et al., 1999). This localization element was identified after mutational analysis of a 21-nucleotide *cis*-acting element previously identified in MBP mRNA (Ainger et al., 1997). The later is called hnRNP A2 response element (A2RE) because it is recognized by hnRNP A2/B1, a *trans*-acting

factor responsible for MBP mRNA trafficking in oligodendrocytes (Ainger et al., 1997; Munro et al., 1999) The A2RE-dependent targeting of mRNAs is also involved in the dendritic delivery of Arc, CaMKIIα, and Neurogranin mRNAs, which appear to cluster in the same hnRNP A2/B1-containing granules in hippocampal neurons (Gao et al., 2008). Moreover, hnRNP A2/B1 was also shown to be necessary for the delivery of the noncoding BC1 RNA and PKMζ mRNA to distal dendritic domains (Muslimov et al., 2006; Muslimov et al., 2011). Surprisingly, the interaction of the latter RNAs with hnRNP A2/B1 depends on a non-canonical purine*purine interaction within BC1 and PKMζ zipcodes, suggesting the existence of a spatial code mediating the targeting of these transcripts to dendrites by hnRNP A2/B1 (Muslimov et al., 2011). In a recent study, the guanine (G)-quadruplex structures present in the 3'UTRs of CaMKIIα and PSD95 mRNAs were shown to direct these transcripts into dendrites (Subramanian et al., 2011). Taken together, these results demonstrate that the RNA structure adopted by the zipcodes may be essential for the localization of the transcripts in the cell. Interestingly, several other well established dendritic mRNAs possess G-quadruplex structures in their 3'UTR (Subramanian et al., 2011), which may indicate the presence of a common signature for the recognition of the *cis*-acting elements by the proper RNA-binding proteins. In fact, given the number of localized mRNAs, it is plausible to think that several localization elements could share structural characteristics that allow the recruitment of the appropriate transport apparatus.

The identification of localization elements within dendritic mRNAs can sometimes generate conflicting results. An early study identified a 94-nucleotide element in the 3'UTR of CaMKIIα mRNA that was sufficient for the delivery of the transcript into dendrites (Mori et al., 2000). However, additional studies showed that CaMKIIα mRNA containing this element but lacking most of the 3'UTR fail to be targeted to dendrites *in vivo* (Miller et al., 2002). Furthermore, another localization element was identified in the middle of the 3'UTR of CaMKIIα transcript (Blichenberg et al., 2001), and the CaMKIIα transcript also contains an hnRNP A2 response element (A2RE) (Gao et al., 2008). Taken together, these findings suggest that the dendritic localization of CaMKIIα mRNA depends on diverse *cis*-acting elements. Similarly, two different localization elements were described for the Arc mRNA (Gao et al., 2008; Kobayashi et al., 2005) but surprisingly little is known about the mechanism of Arc mRNA transport into dendrites. In hippocampal neurons the Arc transcripts are transported along dendrites in hnRNP A2/B1-

containing granules (Gao et al., 2008) and a *cis*-acting element on the 3'UTR of the Arc mRNA showed a moderate ability to target these transcripts to dendrites (Kobayashi et al., 2005). However, it is currently unknown whether hnRNP A2/B1 is required for Arc mRNA trafficking along dendrites *in vivo* and the identity of the *trans*-acting factors that recognize the localization element present in the Arc transcripts described by Kobayashi and coworkers also remains to be determined.

Another well described dendritic mRNA is the β-actin transcript. β-actin mRNA contains a *cis*-acting element 54-nucleotide long in the 3'UTR, which is recognized by ZBP1 (zipcode-binding protein 1) and is essential for the localization of the transcript in the cytoplasm of cultured chick embryo fibroblasts (Kislauskis et al., 1994; Ross et al., 1997) and in neurites and growth cones of cultured chick forebrain neurons (Zhang et al., 2001). The complex ZBP1-β-actin mRNA accumulates in dendritic spines upon synaptic activity (Tiruchinapalli et al., 2003). In developing neurons the localization of ZBP1-β-actin mRNA in growth cones, which is coupled to localized β-actin translation, critically regulates growth cone navigation by mediating responses to external cues (Lin and Holt, 2007). In addition to the role in the localization of β-actin mRNA, ZBP1 also represses its translation (Huttelmaier et al., 2005). This is abrogated through Src-dependent phosphorylation of ZBP1, which results in the release of β-actin mRNA from ZBP1-containing granules and local translation of β-actin (Huttelmaier et al., 2005). Recent findings implicated the RNA-binding protein Src-associated in mitosis of 68 kDa (Sam68) in the metabolism of β-actin mRNA in dendrites (Klein et al., 2013). The authors showed that knockdown of Sam68 or blocking the interaction of Sam68 with β-actin mRNA decreases the levels of β-actin transcript in dendrites and results in fewer dendritic spines (Klein et al., 2013). In addition, absence of Sam68 results in a decreased number of functional synapses *in vivo* and reduces the amount of β-actin mRNA in synaptic polysomal fractions (Klein et al., 2013), suggesting that Sam68 may also be involved in the translational control of β-actin locally at the synapse.

A role for BDNF in the regulation of mRNA localization in dendrites has been suggested by several studies. Intrahippocampal infusion of BDNF resulted in the accumulation of Arc transcripts in dendrites and triggered long-term potentiation (BDNF-LTP) at medial perforant path-granule cell synapses *in vivo* (Messaoudi et al., 2007; Ying et al., 2002), and similar effects were observed in cultured cerebrocortical neurons (Rao et al., 2006), but the mechanisms involved

have not been clarified. BDNF signaling induces the phosphorylation of ZBP1 and local synthesis of β-actin in growth cones, contributing to growth cone turning (Sasaki et al., 2010). In a recent study, ZBP1 was shown to regulate dendritic branching (Perycz et al., 2011). Interestingly, knockdown of ZBP1 inhibited the growth of dendritic protrusions in response to BDNF stimulation (Eom et al., 2003), suggesting that under limiting amounts of ZBP1 and β-actin mRNA in dendrites, neurons are not able to induce robust actin growth following stimulation with BDNF. Future studies should address the mechanisms underlying ZBP1-mediated transport of β-actin mRNA into dendrites, and possibly other mRNAs, and explore the molecular mechanisms by which BDNF regulates β-actin mRNA locally.

The effect of BDNF on the abundance of dendritic mRNAs was further investigated using a culture system that allows the mechanical separation of neurites from cell bodies (Manadas et al., 2009; Poon et al., 2006). BDNF differentially regulated the dendritic localization of transcripts encoding for several translation-related proteins, including initiation and elongation factors, and aminoacyl-tRNA synthases (Manadas et al., 2009). However, it is not clear if BDNF regulates the delivery of these transcripts to dendrites and/or alters their stability.

1.6.2. Dendritic targeting of BDNF mRNA

The nuclear events, such as splicing and alternative polyadenylation site selection, may be essential determinants for the differential localization of mRNA in the cytoplasm (Giorgi and Moore, 2007). Perhaps the best example in mammalian neurons is the targeting of BDNF mRNA into dendrites. The presence of two polyadenylation sites in the BDNF transcript results in two distinct pools of mRNAs in the brain containing either a short or a long 3'UTR (Timmusk et al., 1993). The short 3'UTR-carrying mRNAs apparently are restricted to the soma whereas the long 3'UTR-containing mRNAs are also found in dendrites (An et al., 2008). Nevertheless, BDNF constructs carrying a short 3'UTR were shown to be targeted to dendrites in cultured hippocampal neurons (Baj et al., 2011; Chiaruttini et al., 2009; Oe and Yoneda, 2010). The short 3'UTR-mediated dendritic targeting of the transcript may rely on the presence of cytoplasmic polyadenylation element (CPE)-like motifs in its sequence (Oe and Yoneda, 2010). The CPE-like elements are apparently required for both constitutive and activity-dependent localization of the transcript in dendrites (Oe and Yoneda, 2010). In separated studies, the destination of BDNF

mRNA was, ultimately, ruled by the presence of 5'UTR exons (Baj et al., 2011; Chiaruttini et al., 2009). The selective localization of several 5'UTR splice variants in dendrites was coupled to local TrkB activation and differentially modulates dendritic complexity (Baj et al., 2011). Importantly, blocking the dendritic localization of BDNF mRNA *in vivo*, reduced BDNF protein levels in dendrites and resulted in deficits in dendritic spine pruning and enlargement (An et al., 2008). Deficits in dendritic synthesis of BDNF also lead to the selective impairment of LTP in dendrites (An et al., 2008). Together, these results suggest a critical role for dendritically synthesized BDNF in synaptic plasticity. It was recently demonstrated in hippocampal neurons that somatically synthetized BDNF promotes the formation of new dendritic spines whereas dendritically synthetized BDNF is a key regulator of spine head growth and pruning (Orefice et al., 2013), suggesting differential roles for distinct pools of synthetized BDNF in the regulation of dendritic spine number and morphology.

BDNF multiple transcripts can also be generated by alternative splicing of the 5'UTR region. As many as 11 different splice-variants of BDNF mRNA were described in both humans and rodents (Aid et al., 2007). Some of these variants show a differential distribution in hippocampal laminae in response to different incoming stimuli (Chiaruttini et al., 2008). It is not clear though, how the different forms of BDNF mRNA are sorted into distinct subcellular compartments. This process most likely requires additional and at the moment unknown factors. Translin binds to the coding sequence of BDNF mRNA regulating both constitutive and activity-induced transport of the transcript to dendrites (Chiaruttini et al., 2009). It was further demonstrated that, depending on the stimuli, translin may be required or not for BDNF mRNA trafficking (Wu et al., 2011). Interestingly, exogenous application of BDNF is sufficient to induce the transport of BDNF and TrkB mRNAs into dendrites in hippocampal neurons through the activation of the phosphatidylinositol 3-kinase (PI3-K) pathway (Righi et al., 2000).

1.6.3. Nuclear history of dendritic-localized mRNPs

Several RNA-binding proteins that compose the dendritic mRNP complexes are nucleo-shuttling proteins highly expressed in the nucleus (Giorgi and Moore, 2007). These observations *per se* suggest that some *trans*-acting factors accompany the mRNA from transcription sites and ultimately regulate their localization in dendrites (Giorgi and Moore, 2007). One of the best

examples of nuclear-acquired RNA-binding proteins implicated in transcript localization comes from the exon junction complex (EJC), a set of proteins involved in mRNA splicing. Several proteins of the EJC, including Magoh, Y14, MLN14 and Barentsz, were detected in dendrites (Glanzer et al., 2005; Macchi et al., 2003). In particular the EJC protein eukaryotic initiation factor (eIF) 4AIII is present in neuronal RNA granules and binds several dendritic mRNAs such as the Arc transcript (Giorgi et al., 2007). However, EJC was not involved in the trafficking of Arc mRNA, but instead directed the transcript for degradation after translation (Giorgi et al., 2007).

Another example is related with the RNA-binding protein ZBP1. ZBP1 is mainly cytoplasmic, but its association with β-actin mRNA is likely to occur at transcription sites (Oleynikov and Singer, 2003; Pan et al., 2007). Interestingly, this interaction appears to be facilitated by ZBP2 (Pan et al., 2007). ZBP2 is predominantly nuclear but a small fraction of the protein colocalizes with cytoplasmic β-actin mRNA in both fibroblasts and neurons (Gu et al., 2002). Overexpression of a truncated form of ZBP2 disrupts the dendritic localization of β-actin transcripts (Gu et al., 2002). Altogether, these results suggest a cooperative role for ZBP1 and ZBP2 in the formation of functional nuclear mRNPs, and ultimately in the regulation of β-actin mRNA localization. Accordingly, the ZBP2-related protein MARTA1 binds with high affinity the dendritic targeting element in MAP2 mRNA (Rehbein et al., 2002) and it is suggested to have a role in both nuclear-export and dendritic trafficking of MAP2 mRNAs. The importance of nuclear-acquired factors in dendritic mRNA localization is also shown by the role played by the nuclear CAP-binding complex. The mRNA degradation factor LSm1 was shown to form dendritic-localized mRNPs that also contain the nuclear CAP-binding protein CBP80, which associates with mRNA percursors in the nucleus (di Penta et al., 2009). These results demonstrate that the LSm1-containing mRNPs are assembled in the nucleus.

The role of the nuclear compartment in the delivery of RNAs to dendrites was also addressed in experiments in which labeled RNAs were microinjected in the cytoplasm of hippocampal neurons. The transcripts were found along dendrites, showing that the delivery to this compartment does not require any nuclear event (Tubing et al., 2010). In most cases though, it is not clear how the nuclear history of endogenous dendritically targeted mRNAs regulates their localization.

1.6.4. *Trans*-acting factors

Among the best studied *trans*-acting factors that contribute to dendritic mRNA localization in neurons are the RNA-binding proteins ZBP1, Staufen, Fragile X mental retardation protein (FMRP), hnRNP A2/B1, and the cytoplasmic polyadenylation element binding protein (CPEB). The ultimate function of the *trans*-acting factors is to recognize *cis*-acting elements in the mRNAs and target the transcripts to distal dendrites. However, some of these RNA-binding proteins can also act as translation repressors (Huttelmaier et al., 2005; Jung et al., 2006; Napoli et al., 2008; Richter, 2007), linkers to motor proteins (Dictenberg et al., 2008; Falley et al., 2009) or even regulators of mRNA decay (Kim et al., 2005).

Staufen proteins are among the best characterized proteins regulating mRNA localization in many species. In mammals, Staufen is involved in the binding and targeting of mRNAs into dendrites (Kiebler et al., 1999; Tang et al., 2001). The Staufen family of proteins comprises two members, Staufen1 and Staufen2, which are components of different RNA granules in neurons (Duchaine et al., 2002). Staufen2 is mainly expressed in the brain and is necessary for the microtubule-dependent delivery of CaMKIIα mRNA to dendrites (Jeong et al., 2007). Staufen1 is required for the late phase of long-term potentiation (L-LTP) in the hippocampus (Lebeau et al., 2008) whereas Staufen2 regulates mGluR-dependent long-term depression (mGluR-LTD) (Lebeau et al., 2011), suggesting distinct roles for the two Staufen proteins at the synapse.

Another important regulator of mRNA localization in neurons is FMRP. FMRP associates with several well described dendritic mRNAs, such as CaMKIIα, Arc, MAP1b, PSD95, as well as its own mRNA (Bassell and Warren, 2008). Furthermore, FMRP interacts directly with the motor protein kinesin to promote the transport of FMRP and cognate mRNAs along dendrites, further suggesting a role in the transport of ribonucleoprotein complexes along dendrites (Dictenberg et al., 2008). This study, together with the purification of KIF5-associated granules (Kanai et al., 2004), provided the best evidence for motor-based transport of RNA granules along the microtubule cytoskeleton. Another evidence for an interaction of *trans*-acting proteins and motor-based transport systems came from studies showing that the dendritic transport of Shank1 mRNA requires KIF5C and KIF5-associated protein Staufen1 (Falley et al., 2009). FMRP is also one of the best described factors that regulate the translation of mRNAs at the synapse. For instance, FMRP can recruit 4E-BP-like cytoplasmic Fmr-interacting protein-1 (CYFIP1) to repress the

translation of target mRNAs (Napoli et al., 2008). Recent findings indicate that FMRP can also repress the translation of cognate mRNAs through the association with miRNAs (Edbauer et al., 2010; Muddashetty et al., 2011) and through the reversible stalling of the ribosomes on polyribosomes containing FMRP-target mRNAs (Darnell et al., 2011).

RNG105 is an RNA-binding protein present in discrete RNA granules in dendrites of hippocampal neurons where it colocalizes with Staufen and CaMKIIα mRNA (Shiina et al., 2005). BDNF induces the release of RNG105 from RNA granules which is coincident with the translation of an mRNA reporter near the granules (Shiina et al., 2005). However, the translation of RNG105-associated mRNAs was still suppressed in the absence of RNG105 (Shiina et al., 2010), suggesting that translation repression is not the primary role of RNG105. In fact, the RNG105-deficient mice showed reduced levels of RNG105-associated mRNAs in dendrites indicating a role for RNG105 in the dendritic localization of these mRNAs.

Another *trans*-acting factor required for dendritic mRNA transport is the CPEB. CPEB and the cytoplasmic polyadenylation complex are well known regulators of translation [(For a review see (Richter, 2007)] but less is known about the role of CPEB in mRNA trafficking. CPEB was shown to facilitate the transport of CPE (cytoplasmic polyadenylation element)-binding mRNAs to distal dendrites (Huang et al., 2003). It was recently shown that CPEB-associated proteins bidirectionally regulate the translation of specific mRNAs in dendrites resulting in a bidirectional regulation of LTP at hippocampal synapses (Udagawa et al., 2012), but whether CPEB is involved in the dendritic localization of those transcripts remains to be investigated.

1.6.5. Molecular composition of RNA granules

In the recent years, a great effort has been made to characterize the molecular composition of the granules responsible for the transport of RNAs. Hirokawa and coworkers purified large RNA granules from the mouse brain which were associated with the tail of conventional kinesin KIF5 (Kanai et al., 2004). These granules contained CaMKIIα and Arc mRNAs as well as 42 different proteins. Among the identified proteins there are several well established *trans*-acting factors involved in mRNA localization such as Staufen, FMRP and Pur-α. This proteomic approach also led to the identification of new players in mRNA transport such as hnRNP U and the Polypyrimidine tract binding protein-associated splicing factor (PSF) (Kanai et al., 2004).

Interestingly, several members of the hnRNP family of proteins that were detected in this RNA granule preparation were found to accumulate at postsynaptic sites upon neuronal activation (Zhang et al., 2012), further supporting their role in local mRNA regulation.

In a separated study, subcellular fractionation was used to obtain fractions enriched in ribonucleoprotein complexes from developing rodent cortex (Elvira et al., 2006). The composition of these granules was to some extent different from the KIF5-associated granules described by Hirokawa and coworkers (Kanai et al., 2004). First, these granules were enriched in β-actin and did not contain CaMKIIα or Arc transcripts. Second, proteomic analysis of these fractions revealed several RNA-binding proteins that were not detected in the KIF5-associated granules (Elvira et al., 2006). Nevertheless, the two granule preparations had many common components, including several hnRNPs, Staufen2, and DEAD-box3 helicase. Interestingly, it was further demonstrated that BDNF treatment increased the number of motile DEAD-box3-carrying granules in hippocampal neurons (Elvira et al., 2006). Taken together, these results suggest that RNA granules are heterogeneous complexes and that some of their components might constitute general machinery for mRNA transport whereas others can be added specifically according to the mRNA/mRNAs that are transported.

One of the most important questions remaining in the field is related to the nature and number of mRNAs present in each ribonucleoprotein granule. The mRNAs encoding for Arc, CaMKIIα, and Neurogranin coassemble in the same set of hnRNP A2/B1-containing granules in cultured hippocampal neurons (Gao et al., 2008). Studies using labeled RNAs microinjected into the cytoplasm of hippocampal neurons showed that MAP2 and CaMKIIα transcripts were sorted into distinct RNA granules in dendrites (Tubing et al., 2010). It was further demonstrated that endogenous MAP2, CaMKIIα, and β-actin mRNAs localize in distinct ribonucleoprotein particles which, unexpectedly, contained very few mRNA molecules (Mikl et al., 2011). Accordingly, *in situ* hybridization experiments with single molecule sensitivity to study 8 different and well established dendritic mRNAs showed that different mRNAs had little or no tendency to cluster together in the same granules and most likely were transported singly into dendrites (Batish et al., 2012). These recent findings suggest a simpler model in which solitary mRNAs can recruit their specific set of proteins and travel to distal dendrites, which may provide finer regulation of distinct mRNAs at individual synapses. Nevertheless, multiplexed mRNA trafficking has been observed in

mammalian neurons (Gao et al., 2008) as well as in other biological contexts (Martin and Ephrussi, 2009). Moreover, RNA granules were observed with the RNA staining dye SYTO14 which indicates the presence of high RNA content (Knowles et al., 1996; Tang et al., 2001).

Further research is required to determine the molecular composition of single RNA granules and to unravel the mechanisms orchestrating the assembly and the transport of these complexes along dendrites.

1.6.6. BDNF regulation of P-bodies

The population of RNA-containing granules in neurons is heterogeneous. They can be classified in RNA transport granules, stress granules, and RNA processing bodies (P-bodies) [(for further reading see (Anderson and Kedersha, 2006; Kiebler and Bassell, 2006)]. P-bodies are distinct cytoplasmic RNA granules that contain the components of the 5'-3' mRNA decay, nonsense-mediated decay pathway and RNA-induced silencing complex (RISC). It was recently shown that BDNF treatment increased the formation of dendritic and somatic P-bodies that contain untranslated RNA targeted for repression or degradation (Huang et al., 2012). Furthermore, BDNF was shown to induce the translocation of dendritic P-body-like structures towards the distal regions of dendrites in hypothalamic neurons (Cougot et al., 2008). These P-body-like structures often contained ZBP1 and FMRP, two well described RNA-binding proteins involved in mRNA transport (Cougot et al., 2008). These findings, together with the emerging roles of miRNAs in the regulation of local mRNAs, suggest a close interplay between P-bodies and RNA transport granules. Stimulation of hippocampal neurons with BDNF induced the dissociation of P-bodies (Zeitelhofer et al., 2008), suggesting a model in which BDNF may relief P-bodies-mediated repression or decay. On the other hand, a general increase in miRNA biogenesis and in the formation of P-bodies was observed upon BDNF induction of Dicer in hippocampal neurons (Huang et al., 2012). Despite the apparent conflicting results, both studies demonstrated that P-bodies are dynamic entities that respond to BDNF treatment. Interestingly, it was recently reported that P-bodies accumulate at the synapse and release mRNA upon neuronal activation (Oh et al., 2013). Future research is needed to investigate whether BDNF regulates the mRNA content of this type of granules.

1.7. Regulation of hippocampal LTP by BDNF

Work performed with *trkB*- or *Bdnf*-deficient mice showed that an impairment of the BDNF-TrkB signaling leads to a significant downregulation of early and late phases of LTP in the Schaffer collateral-CA1 hippocampal synapses (Korte et al., 1995; Korte et al., 1998; Korte et al., 1996; Minichiello et al., 1999), which was correlated with a reduction in the performance in certain learning tasks (Gruart et al., 2007; Linnarsson et al., 1997; Minichiello et al., 1999; Pozzo-Miller et al., 1999; Xu et al., 2000). Importantly, the effects on LTP observed in mice carrying a null mutation in the *Bdnf* gene were reverted upon restoring the expression of the *Bdnf* gene or following treatment with recombinant BDNF (Korte et al., 1996; Patterson et al., 1996), further suggesting a role for BDNF in long-term synaptic potentiation in the hippocampus. In agreement with these findings, LTP is significantly inhibited when high-frequency stimulation (HFS) is performed in the presence of the BDNF and NT4 (neurotrophin-4) scavenger TrkB-IgG or with anti-TrkB antibodies (Chen et al., 1999; Figurov et al., 1996; Kang et al., 1997; Korte et al., 1998). The *Val66Met* polymorphism in the *Bdnf* gene which results in a defect in regulated release of BDNF and affects episodic memory (Egan et al., 2003) also leads to an impairment of LTP (Ninan et al., 2010), further indicating a role for BDNF in this form of plasticity in the hippocampus. However, at this point the timing and relative role of the pre- and postsynaptic region in the release of BDNF still remains to be elucidated.

Facilitatory effects of BDNF in the induction of LTP have also been reported under conditions of weak synaptic stimulation that would not normally induce synaptic potentiation in the hippocampal CA1 region (Kovalchuk et al., 2002). Bath application of BDNF also triggers a sustained enhancement of synaptic transmission in the CA1 region of the hippocampus by a mechanism dependent on protein synthesis (Ji et al., 2010; Kang and Schuman, 1996). Transcription and translation activities are also required for BDNF-induced long-term potentiation at medial perforant path-granule cell synapses *in vivo* following intrahippocampal infusion of the neurotrophin (Messaoudi et al., 2007; Ying et al., 2002).

The TrkB receptors for BDNF are expressed in axons, nerve terminals and dendritic spines of the hippocampal granule and pyramidal neurons of the adult rat hippocampus (as previously discussed) raising the question of whether the neurotrophin acts at the pre- and/or postsynaptic level in LTP, and the signaling mechanisms involved. The TrkB receptors are coupled to the

activation of multiple signaling pathways, including the Ras/ERK pathway and the PI3-K/Akt pathway, initiated by interaction of Shc with the phosphorylated receptor, and the phospholipase Cγ (PLCγ) (as previously discussed). Studies performed in mice with targeted mutations in either the Shc or the PLCγ binding sites of TrkB showed that the latter mechanism is preferentially coupled to potentiation of the CA3-CA1 synapse and associative learning (Gruart et al., 2007; Minichiello et al., 2002). Furthermore, inhibition of the PLCγ pathway at the pre- and or postsynaptic level, by overexpression of the PLCγ pleckstrin homology domain (PH) with a viral vector, showed a role for pre- and postsynaptic TrkB receptors in LTP in the hippocampal CA1 region (Gartner et al., 2006). Interestingly, in this study concurrent inhibition of pre- and postsynaptic PLCγ signaling was required to reduce LTP to levels similar to those observed in *trkB* and *Bdnf* knockout mice. In contrast, blockade of presynaptic or postsynaptic signaling alone did not result in a significant reduction of LTP (Gartner et al., 2006). Recruitment of Ca^{2+} from inositol 1,4,5-trisphosphate (IP3)-sensitive intracellular stores was suggested to play a key role in LTP downstream of PLCγ activation (Gartner et al., 2006).

A different study addressed the relative role of pre- and postsynaptic TrkB receptors in LTP using mutant mice with reduced expression of the receptor throughout the brain, including the CA3 and CA1 regions, and mice lacking TrkB in the hippocampus CA1 region (Xu et al., 2000). The LTP was only affected in the former animal model but not in mice lacking TrkB receptors in the CA1 region (postsynapse), suggesting that BDNF apparently acts through TrkB presynaptically, but not postsynaptically to modulate LTP.

In contrast with the reports suggesting a concurrent role of pre- and postsynaptic TrkB receptors in LTP in the CA1 region of the hippocampus, a postsynaptic effect was proposed to account for the robust induction of LTP at the synapses of the medial perforant path fibers when BDNF was applied together with a weak synaptic stimulation that would not normally induce synaptic potentiation (Kovalchuk et al., 2002). Although a presynaptic effect of BDNF was not ruled out in this paradigm, the differences may be due to the distinct synapses investigated.

1.7.1. Transcription- and translation-independent synaptic regulation by BDNF

The role of BDNF in the early phase of LTP is likely dependent on the post-translational regulation of pre- and postsynaptic proteins. At the presynaptic level the activation of TrkB

receptors was shown to upregulate depolarization-evoked glutamate release from isolated hippocampal and cerebrocortical synaptosomes (Jovanovic et al., 2000; Pascual et al., 2001; Pereira et al., 2006; Simsek-Duran and Lonart, 2008) (Fig. 1.6). Studies performed in cultured hippocampal neurons showed that BDNF increases the frequency of miniature excitatory postsynaptic currents, further supporting a presynaptic effect of the neurotrophin (Lessmann and Heumann, 1998; Li et al., 1998; Schinder et al., 2000; Tyler and Pozzo-Miller, 2001). The rapidly recycling pool of synaptic vesicles is targeted by a BDNF-dependent mechanism in the hippocampal CA1 region, and this modulation was shown to be necessary for the enhancement of exocytosis caused by induction of LTP (Tyler et al., 2006). The recycling of synaptic vesicles induced by BDNF requires the presence myosin VI, a minus end-directed actin-based motor, and the adaptor protein GIPC1 [PDZ (postsynaptic density-95/Discs large/zona occludens-1) domain-containing adaptor protein, type 1], which form a complex that can engage TrkB receptors (Yano et al., 2006). However, how myosin VI contributes to the BDNF-induced regulation of the exocytotic release of glutamate remains to be elucidated.

The potentiation of glutamate release by BDNF was not observed is cerebrocortical synaptosomes isolated from synapsin I and synapsin II deficient mice (Jovanovic et al., 2000), an ERK substrate that interacts with small synaptic vesicles. These results suggest that TrkB receptor activation coupled to synapsin phosphorylation may dissociate the synaptic vesicles from actin filaments, thereby increasing vesicle docking and glutamate release. Accordingly, BDNF increased the number of docked vesicles per active zone at CA1 spine synapses in hippocampal slice cultures, but since in these experiments the treatment with the neurotrophin was performed for 48 h, the effect may involve, at least in part, translation activity (Tartaglia et al., 2001).

Synaptic potentiation by BDNF is also dependent on the small GTP-binding protein Rab3a, which is known to play a role in vesicular trafficking. Rab3a is associated with synaptic vesicles and the BDNF-induced upregulation of neurotransmitter release was shown to be impaired in cultured hippocampal neurons form *Rab3a* knockout mice (Alder et al., 2005; Thakker-Varia et al., 2001). A role for Rab3a in the presynaptic effects of BDNF is further suggested by the results showing an impairment of the BDNF-induced potentiation of glutamate release in synaptosomes isolated from the CA1 region of Rim1α (Rab3a interacting molecular 1 α) knockout mice (Simsek-Duran and Lonart, 2008). Rim1α is a component of Rab3a molecular pathway which is

phosphorylated by an ERK-dependent mechanism following stimulation of CA1 nerve terminals with BDNF. However, the Rim1α downstream targets in BDNF-induced potentiation of glutamate release remain to be identified.

BDNF-induced modulation of glutamate receptors is likely to contribute to the effects of this neurotrophin on E-LTP (Fig. 1.6). BDNF activation of postsynaptic TrkB receptors was shown to induce the phosphorylation of plasma membrane-associated NMDA receptors, as shown for the GluN1 and GluN2B subunits (Lin et al., 1998; Suen et al., 1997), which may account for BDNF-induced increase in the receptor open probability observed in cultured hippocampal neurons (Levine et al., 1998). Accordingly, the effects of BDNF on the electrophysiological properties of NMDA receptors depend on GluN2B subunits (Levine and Kolb, 2000). Later, evidence was provided for a direct link between TrkB and NMDA receptors phosphorylation by a tyrosine kinase Fyn (Mizuno et al., 2003). More recently, it was demonstrated that BDNF modulates the expression and trafficking of NMDA receptors in hippocampal neurons (Caldeira et al., 2007b).

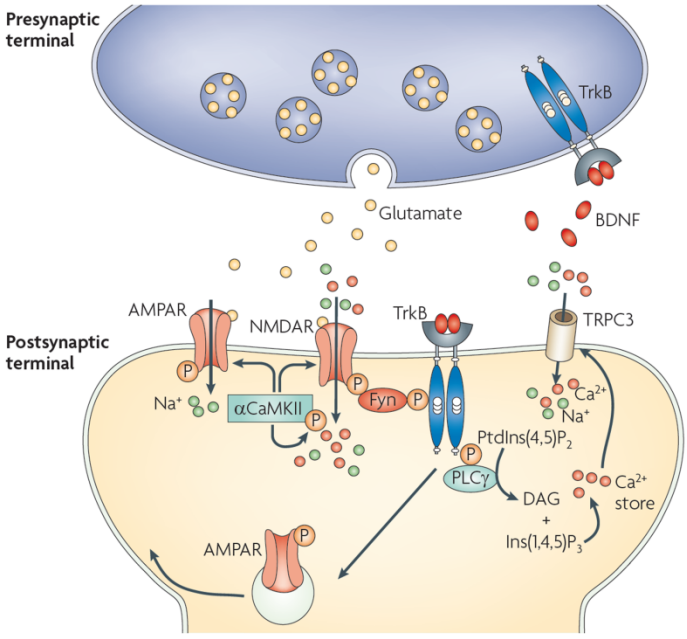

Figure 1.6 - Modulation of glutamatergic synapses by BDNF. BDNF may activate pre- or postsynaptic TrkB receptors with distinct effects on the glutamatergic synaptic transmission. At the presynaptic level, BDNF upregulates glutamate release. Activation of postsynaptic TrkB receptors also upregulate NMDA and AMPA receptor activity by different mechanisms. In particular Fyn kinase activated by TrkB receptors interacts with NMDAR and increases the open probability of the ion channel. BDNF-TrkB can also induce cation influx through TRPC3 channels facilitating the entry of Ca^{2+} through voltage-gated calcium channels and the activity of NMDA receptors. Ca^{2+} influx through NMDAR activates CamKIIα which in turn contributes to the positive regulation of AMPAR and NMDAR with a consequent induction and expression of LTP. BDNF also modulates the trafficking and membrane insertion of AMPA receptors. From (Minichiello, 2009).

It is widely accepted that trafficking of AMPA receptors to the synaptic plasma membrane has an essential role in LTP (Malenka, 2003b). BDNF was also shown to increase the phosphorylation of GluA1 AMPA receptor subunits in cultured organotypic hippocampal slices and increased synaptic delivery of AMPA receptors within about 30 min (Caldeira et al., 2007a), further contributing to an upregulation of the postsynaptic response to glutamate release. Similar effects of BDNF on the synaptic accumulation of GluA1 containing AMPA receptors were reported in cultured cortical (Nakata and Nakamura, 2007) and hippocampal neurons (Fortin et al., 2012).

The rapid pre- and postsynaptic events induced by BDNF, mediated by phosphorylation of existing proteins, are likely to be transient and therefore may contribute to E-LTP. Additional mechanisms should contribute to the sustained synaptic changes that occur during L-LTP.

1.7.2. BDNF and regulation of translation machinery

BDNF promotes translation of specific mRNAs by regulating the activity of the protein synthesis machinery. Translation is initiated with the formation of the eIF4F complex, followed by recruitment of the ribosome and the mRNA molecule. The eIF4F is comprised by the eIF4E subunit, which binds the 5'-capped mRNAs, the eIF4A, responsible for unwinding the secondary structure of mRNAs, and the eIF4G, which bridges the transcript to the 43S pre-initiation complex. The formation of this complex and induction of translation activity is promoted by phosphorylation of eIF4E-binding proteins (4EBPs) (Pestova et al., 2007), which prevents their interaction of eIF4e. The initiation steps of translation are followed by recruitment of elongation factors, including the eukaryotic elongation factor (eEF) 2, which promote the translocation of the new protein chain from the A-site to the P-site of the ribosome (Taylor et al., 2007).

The initiation and elongation steps of translation are considered rate-limiting, being subjected to regulation at multiple sites (Herbert and Proud, 2007; Santos et al., 2010). BDNF is thought to act at different levels to increase translation activity, by altering the phosphorylation of proteins involved in the initiation and elongation steps of protein synthesis. Stimulation of cultured cerebrocortical neurons with BDNF was shown to induce the phosphorylation of eIF4E and 4EBP1, by activating the ERK and PI3-K signaling pathways, respectively (Takei et al., 2004). Furthermore, the BDNF-induced 4EBP1 phosphorylation was sensitive to mTOR inhibition with rapamycin (Takei et al., 2004). A BDNF-induced increase in the phosphorylation of 4EBP1,

p70S6 kinase and its substrate ribosomal S6 protein was also demonstrated in the dendrites of cultured cortical neurons and in synaptoneurosomes (subcellular fraction containing the pre- and postsynaptic regions) isolated from the brain cortex (Takei et al., 2004). The mTOR-dependent phosphorylation of 4EBP1 enhances cap-dependent translation, by inducing eIF4F complex formation, whereas the activation of the S6 kinase pathway enhances translation initiation of 5'-oligopyrimidine tract-containing mRNAs, such as those coding for ribosomal proteins and elongation factors (Fumagalli and Thomas, 2000). Therefore, the effects of BDNF on protein synthesis in dendrites are likely to be mediated by at least two translation initiation pathways.

An additional effect of BDNF on translation initiation is mediated by phosphorylation of the guanine nucleotide exchange factor eIF2B (Takei et al., 2001). This initiation factor catalyzes the exchange of eIF2.GDP to eIF2.GTP, which is required for the assembly of the eIF2.GTP.Met.tRNAi complex (Rhoads, 1999) and for priming each 40S ribosomal subunit. Studies performed in cultured cortical neurons showed that BDNF increases the phosphorylation of glycogen synthase kinase-3β, which is known to regulate the eIF2B activity, and this may constitute an additional mechanism to promote translation activity following activation of the TrkB receptors by BDNF (Takei et al., 2001).

Several lines of evidence suggest that the effects of BDNF in synaptic potentiation may be mediated, at least in part, by promoting translation initiation. A BDNF-induced increase in the phosphorylation of eIF4E, which is correlated with enhanced rates of translation (Gingras et al., 2004), was also observed in dentate gyrus (DG) following infusion of BDNF to induce LTP (BDNF-LTP) and in DG synaptoneurosomes stimulated with BDNF (Kanhema et al., 2006). Furthermore, BDNF infusion into the DG of anesthetized animals was found to increase eIF4E total protein levels, further suggesting that eIF4E upregulation induces local protein synthesis at the synapse, and may contribute to the effects of BDNF in synaptic potentiation. A role for mTOR was also shown in the late phase LTP at hippocampal CA1 synapses induced by presynaptic tetanic stimulation and in the BDNF-induced synaptic potentiation (Tang et al., 2002). Based on studies with conditional expression of a dominant negative form of MEK1 in the postnatal murine forebrain it was suggested that the translation efficiency necessary for establishing long-term synaptic plasticity may involve the functional interplay between mTOR- and the ERK-dependent signaling pathways (Kelleher et al., 2004).

BDNF also regulates translation activity by acting at the elongation step. In cultured cortical neurons BDNF was shown to increase the phosphorylation (activation) of eukaryotic elongation factor 1A (eEF1A), which is known to recruit aminoacyl-transfer RNA (tRNA) to the A-site of ribosomes when bound to GTP (Inamura et al., 2005). eEF2, a GTP-binding protein required for the second step of elongation (ribosomal translation), is also regulated by BDNF, although contrasting results were obtained in different preparations. Local infusion of BDNF was shown to induce a rapid and transient increase in eEF2 phosphorylation in the hippocampal dentate gyrus, under conditions that induce LTP (BDNF-LTP) (Kanhema et al., 2006). This alteration in eEF2 phosphorylation at Thr56 is typically associated with a reduction in the interaction of the elongation factor with ribosomes, thereby decreasing global protein synthesis (Nairn and Palfrey, 1987; Ryazanov et al., 1991; Ryazanov et al., 1988), but an increase in the translation of some dendritic mRNAs (e.g. αCaMKII and Arc) upon eEF2 phosphorylation has also been reported (Belelovsky et al., 2005; Chotiner et al., 2003; Marin et al., 1997; Park et al., 2008; Scheetz et al., 2000). However, in contrast with the results obtained following injection of BDNF in the dentate gyrus, the neurotrophin was without effect on eEF2 phosphorylation in synaptoneurosomes isolated from the same region, suggesting that the arresting of elongation may be limited to non-synaptic sites. At the synapse, BDNF induces the phosphorylation of eIF4E (see above), thereby increasing protein synthesis, whereas in non-synaptic regions translation activity may be 5'-cap-independent and mediated by IRES [internal ribosomal entry site; (Costa-Mattioli et al., 2009)]. The regulation of eEF2 in neurons may be more complex and will require further investigation, since studies performed in cultured cerebrocortical neurons showed a decrease in the phosphorylation of this elongation factor following acute and chronic stimulation with BDNF, which favors the interaction with the ribosomes and protein synthesis (Inamura et al., 2005; Nairn and Palfrey, 1987; Ryazanov et al., 1988; Takei et al., 2009). Interestingly, neuronal activity in hippocampal cultures was also shown to have differential effects on eEF2 phosphorylation in dendrites and in the soma (Verpelli et al., 2010). The activity-induced eEF2 phosphorylation in dendrites is mediated via activation of metabotropic glutamate receptors and correlates with protein synthesis (e.g. dendritic synthesis of BDNF). Furthermore, in this model the activity-induced synthesis of BDNF increases spine maturation and density (Verpelli et al., 2010), but

direct *in vivo* evidence for local synthesis of BDNF in response to LTP-inducing activity is still not available.

1.7.3. BDNF and spine plasticity

Synaptic stimulation under conditions that induce LTP of excitatory synapses is also associated with sustained structural alterations in the postsynaptic region, including an increase in the number of dendritic spines and their volume (Kasai et al., 2010). BDNF may contribute to some of these alterations since the sustained enlargement of synaptic spines at the hippocampal CA3-CA1 synapses in response to LTP-inducing synaptic stimulation was shown to depend on endogenous BDNF and required protein synthesis (Tanaka et al., 2008). A role for BDNF in structural spine plasticity is further supported by studies with exogenous application of the neurotrophin to dissociated cultures of hippocampal neurons and hippocampal slice cultures (Alonso et al., 2004; Amaral and Pozzo-Miller, 2007; Ji et al., 2010; Tyler and Pozzo-Miller, 2003; Tyler and Pozzo-Miller, 2001). BDNF stimulation increases synaptic spine density by a mechanism dependent on the Ras/ERK pathway (Alonso et al., 2004) and the transient receptor-potential cation channel subfamily C (TRPC) type 3 (Amaral and Pozzo-Miller, 2007). Studies performed in hippocampal slices from adult rats showed that BDNF mediates the theta-burst stimulation-induced increase in actin polymerization in dendritic spines, through regulation of p21-activated kinase (PAK) and ADF (actin-depolymerising factor)/cofilin (Rex et al., 2007). An increase in actin polymerization in spines may also arise from activation of m-calpain through ERK-dependent phosphorylation, as shown in cultured neurons (Zadran et al., 2010). F-actin polymerization in spines plays a key role in LTP maintenance *in vivo* (Fukazawa et al., 2003) and, therefore, these alterations may underlie some of the effects of BDNF in long-term synaptic potentiation.

1.7.4. BDNF-induced changes in the neuronal proteome

The analysis of the neuronal proteome in a given brain region or in cell cultures is a useful approach to evaluate which are the proteins regulated by certain stimuli, but it does not provide information regarding the subcellular localization of the nascent proteins. A gel-based proteome profiling of the long-term (12 h) effects of BDNF in cultured hippocampal neurons identified 29

proteins that were upregulated and 17 proteins downregulated, for a total of 46 proteins altered by BDNF (Manadas et al., 2009). Bioinformatic analysis of the proteins using GOMiner showed an upregulation of proteins related to RNA metabolic processes and cellular protein metabolic processes, such as proteolysis (related to the ubiquitin-proteasome system) and translation (regulators of translation initiation and elongation) (Manadas et al., 2009). These results suggest that BDNF regulates the proteome by acting both on *de novo* protein synthesis and by affecting the rate of degradation of proteins targeted by the ubiquitin-proteasome system (Manadas et al., 2009), but provide no evidence regarding local translation at the synapse.

The first experimental findings suggesting a role for BDNF in the activation of local translation in dendrites of hippocampal were obtained using a reporter in which the coding sequence of GFP was flanked by the 5'- and 3'UTR regions from CaMKIIα, providing both dendritic mRNA localization and translational regulation (Aakalu et al., 2001). This was later supported by studies showing effects of BDNF on the translation machinery in dendrites of cultured cerebrocortical neurons (Takei et al., 2004) as well as in synaptoneurosome preparations (Kanhema et al., 2006; Takei et al., 2004). The effect of BDNF on the synaptic proteome was analysed in more detail in synaptoneurosomes isolated from cultured cortical neurons, using multidimensional protein identification technology (MudPIT) and relative quantification by spectra counting. Since synaptoneurosomes were isolated 30 min after stimulation of the neurons with BDNF it was suggested that the alterations observed were due to local protein synthesis at the synapse. This study showed 410 proteins regulated by BDNF in synaptoneurosomes, 214 of which were upregulated and 196 were found to be downregulated (Liao et al., 2007).

In addition to the proteins identified in the proteomics analysis discussed above, target oriented studies have shown effects of BDNF in total expression of AMPA and NMDA receptor subunits in cultured hippocampal and cortical neurons (Caldeira et al., 2007a; Caldeira et al., 2007b; Fortin et al., 2012; Guire et al., 2008; Narisawa-Saito et al., 1999; Small et al., 1998). Interestingly, stimulation of hippocampal neurons with BDNF also increased the synaptic accumulation of GluA1 AMPA receptor subunit, by a mechanism dependent on mTOR and Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) (Caldeira et al., 2007a; Fortin et al., 2012; Guire et al., 2008). BDNF also upregulated GluA1 protein levels in rat forebrain

synaptoneurosomes, further indicating that the neurotrophin induces local translation of AMPA receptors at the synapse (Schratt et al., 2004).

The anchoring and stabilization of glutamate receptors at the synapse is controlled by scaffold proteins which interact with other postsynaptic density (PSD) components, including signaling molecules. BDNF was also shown to upregulate the PSD scaffold protein Homer2 in rat forebrain synaptoneurosomes (Schratt et al., 2004), suggesting an effect of the neurotrophin in strengthening the postsynaptic machinery involved in the response to glutamate. Furthermore, BDNF increased the protein levels of the PSD scaffold proteins SAP97 (Synapse-associated protein 97), GRIP1 (glutamate receptor-interacting protein 1) and Pick1 (protein interacting with C kinase 1) in cultured cerebrocortical neurons (Jourdi et al., 2003). BDNF also induces the translation of signaling proteins, as shown for CaMKIIα (Takei et al., 2004). The Ca^{2+}/calmodulin-dependent protein kinase II is a major constituent of the PSD and plays a structural role in enlarging and strengthening the synapse in the late phases of LTP (Lisman et al., 2012).

Arc is a postsynaptic protein encoded by a gene belonging to the class of immediate-early genes (IEGs), which are rapidly and transiently transcribed in response to synaptic activity (Link et al., 1995; Steward et al., 1998), playing an important role in synaptic plasticity in the hippocampus [reviewed in (Bramham et al., 2010)]. The dendritic localization and translation of the Arc mRNA has been extensively studied (Messaoudi et al., 2007; Rao et al., 2006; Steward et al., 1998; Steward and Worley, 2001; Yin et al., 2002; Ying et al., 2002), and BDNF was shown to increase dendritic Arc mRNA and protein levels in cultured cortical neurons (Rao et al., 2006). A role for Arc in LTP was shown in experiments with intrahippocampal infusion of antisense (AS) oligodeoxynucleotides to inhibit Arc protein expression, which impaired the maintenance phase of LTP without affecting its induction (Guzowski et al., 2000). Furthermore, a sustained local Arc synthesis in dendrites is necessary for the maintenance of LTP (Messaoudi et al., 2007). Interestingly, intrahippocampal infusion of BDNF also resulted in the accumulation of Arc transcripts in dendrites and triggered long-term potentiation (BDNF-LTP) at medial perforant path-granule cell synapses *in vivo* by an Arc-dependent mechanism (Messaoudi et al., 2007; Ying et al., 2002). Despite the evidence indicating an effect of BDNF in the Arc translation, Arc mRNA was not present in the group of transcripts identified in the polysomal fraction following stimulation of cerebrocortical neurons with BDNF (Schratt et al., 2004). This apparent discrepancy may

suggest that BDNF is preferentially coupled to the translation of the Arc mRNA at the synapse. In agreement with this hypothesis, BDNF was shown to upregulate Arc protein levels in synaptoneurosomes in a rapid and relatively selective manner without increasing overall protein synthesis (Yin et al., 2002).

Although it is presently clear that Arc plays a role in LTP induced by HFS and in BDNF-LTP, the mechanism(s) involved are not fully elucidated. The upregulation of Arc may act in synaptic potentiation by promoting the phosphorylation and consequent inhibition of cofilin, an actin-binding protein which regulates actin polimerization. Accordingly, the induction of LTP in the hippocampal CA1 region (in brain slices) and in the dentate gyrus (*in vivo*) was shown to promote the phosphorylation of cofilin, thereby inhibiting its activity and increasing actin polimerization (Chen et al., 2007; Fukazawa et al., 2003). The infusion of Arc AS 2 h post-HFS was shown to decrease the amount of phospho-cofilin present in dentate gyrus homogenates, which correlates with the loss of nascent F-actin at medial perforant path synapses and blockade of LTP consolidation (Messaoudi et al., 2007). The same study showed that jasplakinolide, an F-actin-stabilizing drug, blocked the Arc AS effect on LTP (Messaoudi et al., 2007), further suggesting that Arc couples translation activation to F-actin expansion and LTP stabilization. These findings suggest that Arc-induced cytoskeleton remodeling is a key event in the formation of a stable L-LTP, which is characterized by an expansion of the PSD and enlargement of the dendritic spines (Bosch and Hayashi, 2012). In addition to the effects on the cytoskeleton mediated by Arc, BDNF may also regulate the actin cytoskeleton through upregulation of RhoA protein levels, as described in synaptoneurosomes isolated from the mouse forebrain (Troca-Marin et al., 2010). RhoA is a regulator of actin polymerization (Luo, 2000), but it is not known whether the BDNF-induced upregulation of this member of the Rho GTPase family contributes to the effects of BDNF in synaptic plasticity.

Synaptic vesicle proteins and proteins related to their traffic were also shown to be upregulated by BDNF (Melo et al., 2013; Tartaglia et al., 2001; Thakker-Varia et al., 2001) but additional studies are required to determine whether some of these alterations are extended to the synapse. Taken together, the BDNF-induced changes in the neuronal proteome described above suggest several putative mechanisms that may contribute to synaptic potentiation by BDNF.

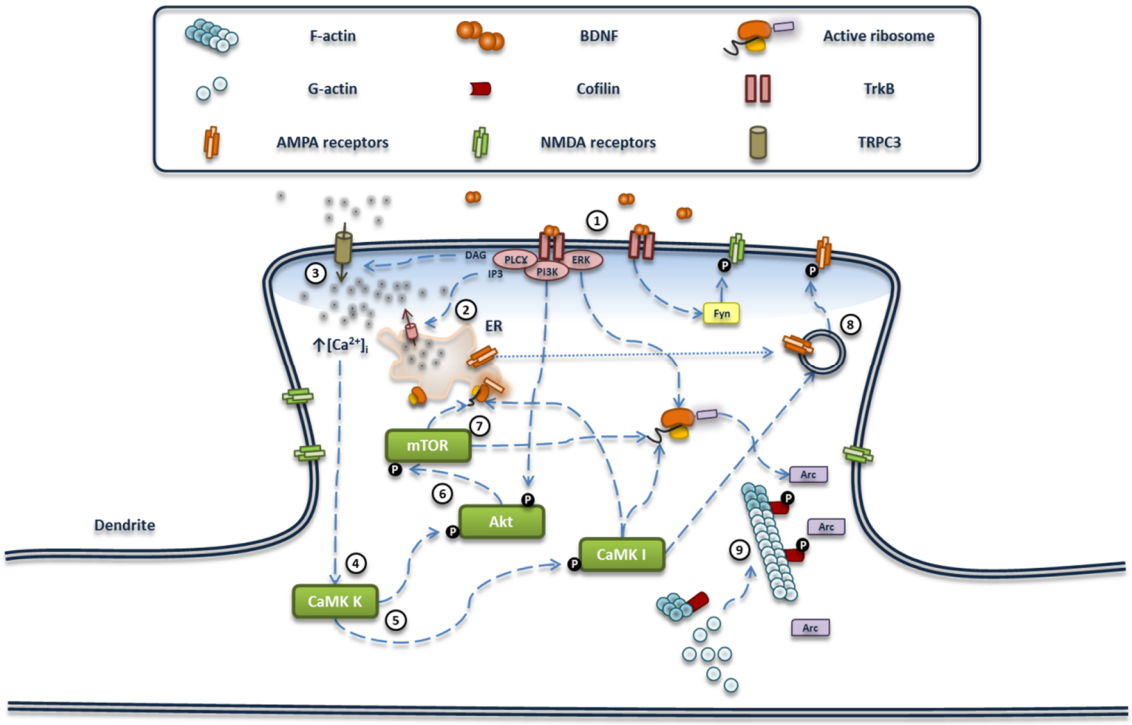

Figure 1.7 - BDNF-induced changes in synaptic proteome and cytoskeleton alterations. Activation of TrkB receptors with BDNF stimulates the Src-family tyrosine kinase Fyn, which phosphorylates the GluN2B subunits of NMDA receptors, thereby increasing their activity and synaptic transmission (1). TrkB receptor activation triggers three different signaling pathways with different actions on the postsynaptic site (1). PLCγ converts phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and IP3; the latter activates IP3 receptors localized in the endoplasmic reticulum (ER), releasing Ca^{2+} from intracellular stores (2). DAG activates the TRPC3 channels which further increase the intracellular calcium concentration (3) leading to activation of CaMKK (4). This kinase phosphorylates and activates both Akt and its effector CaMKI (5). Akt is also activated by the PI3-K signaling pathway downstream of TrkB receptor stimulation (1). Consequently, Akt upregulates mTOR activity (6), and this is followed by activation of the translational machinery (7). BDNF was shown to regulate the translation of GluA1 subunits locally and to induce the insertion of GluA1-containing AMPA receptors in the membrane (8). CaMKI enhances the incorporation of these receptors in the postsynaptic membrane (8), which was shown to depend on actin polymerization (9). Arc is an IEG rapidly translated in dendrites upon BDNF stimulation (7), being necessary to keep cofilin in the phosphorylated state and making possible F-actin elongation (9).

1.7.4.1. miRNAs as mediators of BDNF-induced alteration in the proteome

miRNAs are non-coding RNAs that, together with RISC, repress the translation of target transcripts [for a review see (Chekulaeva and Filipowicz, 2009)]. It is increasingly evident that miRNAs post-transcriptionally regulate localized mRNAs (Konecna et al., 2009) and that some of the effects of BDNF in the central nervous system are mediated through alteration of their expression and/or activity (Table 1.1). The BDNF-induced synthesis of LIM domain kinase-1 (LIMK1) in dendrites through local relief of the activity of miR-134 in translation repression was the first demonstration that the synaptic effects of the neurotrophin could be mediated by regulation of miRNAs (Schratt et al., 2006). Interestingly, there is still residual BDNF induction of a

reporter mRNA translation when miR-134 cannot bind LIMK1, suggesting the presence of additional mechanisms for regulation of BDNF-induced translation of LIMK1 (Schratt et al., 2006).

BDNF and synaptic activity were shown to increase the transcription of miR-134, which was coupled to the down-regulation of Pumilio2 (Pum2) mRNA to promote dendritogenesis in hippocampal neurons (Fiore et al., 2009). Accordingly, neurons lacking Pum2 show enhanced dendritic arborization (Vessey et al., 2010). Pum2 is an RNA-binding protein present in discrete RNA-containing particles, which negatively regulates eIF4E translation (Vessey et al., 2010). Interestingly, loss of *Pumilio* impairs long-term memory in *Drosophila* (Dubnau et al., 2003). In a separated study, miR-134 function was associated with synaptic plasticity and memory formation in rodents (Gao et al., 2010). The authors showed a role for SIRT1 in synaptic plasticity via miR-134-dependent regulation of cAMP-response element-binding protein (CREB) and BDNF expression (Gao et al., 2010). Altogether, miR-134 appears to be an important mediator of BDNF actions in the nervous system. BDNF-induced upregulation of miR-134 promotes dendritogenesis via down-regulation of Pum2 mRNA (Fiore et al., 2009) whereas miR-134 inactivation in dendrites upon BDNF stimulation is coupled to LIMK1 translation and spine growth (Schratt et al., 2006). Furthermore, miR-134 itself is able to regulate BDNF levels (Gao et al., 2010).

More recently, an embracing study demonstrated that the dual regulation of miRNAs biogenesis by the neurotrophin BDNF plays an important role in BDNF-induced protein synthesis (Huang et al., 2012). BDNF treatment induced a downregulation of the Let7-family of miRNAs through Lin28, and relieved the repression prompted by these miRNAs (Huang et al., 2012). In contrast, a general increase in miRNA biogenesis through Dicer was reported in response to BDNF stimulation and this dual regulation of miRNA biogenesis determines and confers selectivity to BDNF-mediated protein synthesis (Huang et al., 2012). Although the presented mechanism occurs globally in the cell, the regulation of BDNF-induced translation by miRNAs most likely affect protein synthesis in different subcellular compartments such as in dendrites.

Additional studies identified other miRNAs regulated by BDNF. Application of this neurotrophin induces miR-125b and ectopic expression of miR-125b promotes neurite outgrowth in a human neuroblastoma cell line (Le et al., 2009). In a similar system, BDNF-induced neurite outgrowth was inhibited after silencing HuD, a neuronal RNA-binding protein, or overexpressing miR-375 (Abdelmohsen et al., 2010). In addition, it was recently shown that miR-9 represses MAP1b

translation and responds locally to BDNF to promote axon branching (Dajas-Bailador et al., 2012). Another miRNA under the regulation of BDNF is miR-132. This miRNA is induced in cultured cortical neurons by BDNF through the activation of CREB and enhance dendritic growth by inhibiting the translation of a GTPase-activating protein, P250GAP (Kawashima et al., 2010; Remenyi et al., 2010; Vo et al., 2005). Similarly, miR-212 was shown to be upregulated in cortical neurons upon BDNF stimulation (Remenyi et al., 2010). The effect of BDNF on the upregulation of miR-132 and miR-212 was dependent on the activation of ERK pathway and the downstream MSK1 (mitogen- and stress-activated kinase 1) (Remenyi et al., 2010). Importantly, blocking the endogenous miR-132 results in a decrease of BDNF-dependent upregulation of several synaptic proteins such as the glutamate receptor subunits GluN2A, GluN2B and GluA1 (Kawashima et al., 2010). The direct targets of miR-132 that contribute for BDNF regulation of glutamate receptors remain to be identified.

Together, the available data suggest a critical role for BDNF-regulated miRNAs in the control of neuronal development and function. Future research is needed to clarify the mechanisms mediating miRNAs synthesis, trafficking and actions in central nervous system. It will be of great interest to develop methodologies that allow the simultaneous observation of miRNAs and their targets in living systems. Interestingly, it was reported that the entire molecular machinery necessary for miRNAs biogenesis is present near synapses (Lugli et al., 2012) supporting the idea that miRNA synthesis may occur locally. If so, it would be important to investigate the nature of the different pools of miRNAs at the synapse (locally synthesized vs transported) and determine which of those, or if both, account for the role of BDNF in synaptic regulation.

Table 1.1 – Effects of BDNF on miRNA expression and functional implications

miRNA	Target mRNA	BDNF regulation and neuronal function	Ref.
miR-134	LIMK1	• Inactivated in dendrites • Regulates spine growth in hippocampal neurons	1
miR379-410 cluster	Pum2 (via miR-134)	• Increased transcription through Mef2 activation • Promotes dendritogenesis in cultured hippocampal neurons	2
miR-132	P250GAP	• Highly induced • Promotes neurite outgrowth in cultured cortical neurons	3
miR-212/132 locus	-	• Increased transcription via ERK1/2- and MSK1/2-dependent and -independent mechanisms	4
miR-132	-	• Highly induced • Contributes to BDNF-dependent upregulation of glutamate receptors subunits GluN2A, GluN2B and GluA1 in cultured cortical neurons	5
miR-9	MAP1b	• Short- or long-term stimulation of axons with BDNF decreases or increases axonal miR-9 levels, respectively • Regulates axon extension and branching in cultured cortical neurons	6
Let-7a, Let-7b, Let- 7f, miR-107, miR-143 and others not described	-	• General increase in miRNA biogenesis through activation of Dicer • Downregulation of Let-7 family members as well as miR-107 and miR-143 • Dual regulation of BDNF-induced protein synthesis • Regulation of BDNF-dependent dendrite arborization	7
miR-375	HuD	• Inhibition of BDNF-induced neurite outgrowth in BE(2)-M17 cells	8
miR-125b	10 putative targets identified	• Upregulated • Promotes neurite outgrowth in SH-SY5Y cells	9
miR-124a	-	• Upregulated • Promotes neurite outgrowth in SH-SY5Y cells	9

(1) (Schratt et al., 2006); (2) (Fiore et al., 2009); (3) (Vo et al., 2005); (4) (Remenyi et al., 2010); (5) (Kawashima et al., 2010); (6) (Dajas-Bailador et al., 2012); (7) (Huang et al., 2012); (8) (Abdelmohsen et al., 2010); (9) (Le et al., 2009)

1.7.4.2. BDNF-induced changes in the transcription activity and synaptic proteome

The role of BDNF in the L-LTP, which is dependent on transcription activity, suggests that changes in gene expression underlie some of the effects of the neurotrophin. This hypothesis is based on studies showing induction of LTP by exogenously applied BDNF at the medial perforant

path-granule cell synapses *in vivo* (Messaoudi et al., 1998), an effect that is blocked by the transcription inhibitor actinomycin D (Messaoudi et al., 2002). Furthermore, BDNF induces the expression of genes coding for regulators of synaptic activity, such as Arc (Ying et al., 2002; Zheng et al., 2009) and several synaptic vesicle proteins, including vesicular glutamate transporters (Melo et al., 2013; Tartaglia et al., 2001). In 2006 Wibrand and colleagues identified several genes co-upregulated together with Arc during BDNF-induced LTP in the dentate gyrus, including genes that code proteins with functions in excitatory synaptogenesis, axon guidance and glutamate receptor clustering (Wibrand et al., 2006).

A recent study using a microfluidic device that allows the isolation of the dendritic compartment showed that BDNF acts on dendrites of cultured cortical neurons to induce a dendrite-to-nucleus signal that upregulates the expression of the IEGs *Arc* and *c-Fos* (Cohen et al., 2011). The effect of BDNF in the signaling from dendrites to the nucleus depends on MEK1/2 (MAPK and ERK kinase, type 1/2) and did not require glutamate signaling. Intriguingly, the effects of dendritic BDNF on gene expression require TrkB activity mainly on the soma compartment suggesting the existence of an endosome-like signaling pathway (Cohen et al., 2011). Furthermore, BDNF induces c-Fos and Arc by different mechanisms since the Ca^{2+} concentration in the soma and in the dendritic compartments influenced the expression of Arc but not c-Fos (Cohen et al., 2011). Additional studies are required to identify other genes that are specifically regulated following activation of dendritic receptors for BDNF.

To contribute to the long-term potentiation of the synapse that was initially stimulated, the newly synthesized mRNAs or the translated proteins should be targeted to a specific synapse. The proteins locally translated at the synapse in response to BDNF stimulation could function in the capture of the neurotrophin-induced RNA granules or proteins traveling along dendrites, as predicted by the synaptic tagging and capture hypothesis (Redondo and Morris, 2011), but this remains to be investigated.

Long-term treatment of hippocampal slice cultures with BDNF was shown to upregulate synaptophysin, synaptobrevin and synaptotagmin protein levels (Tartaglia et al., 2001). However, it remains to be determined whether activation of presynaptic TrkB receptors by BDNF, which is followed by the transport of TrkB-BDNF complexes as part of signaling endosomes (Ha et al., 2008; Watson et al., 2001; Xie et al., 2012; Ye et al., 2003), upregulates the expression of genes

coding for presynaptic proteins. If this is the case, the newly synthesized proteins could be then delivered to the synapse, thereby contributing to an upregulating of glutamate release. The anterograde axonal transport of the proteins may be further enhanced by upregulating KIF1A (Kondo et al., 2012), a motor protein involved in the transport of synaptic vesicle precursors, including synaptophysin, synaptotagmin and Rab3A (Okada et al., 1995; Yonekawa et al., 1998). Future studies are required to fully understand the role of this pathway in the L-LTP and the mechanisms involved.

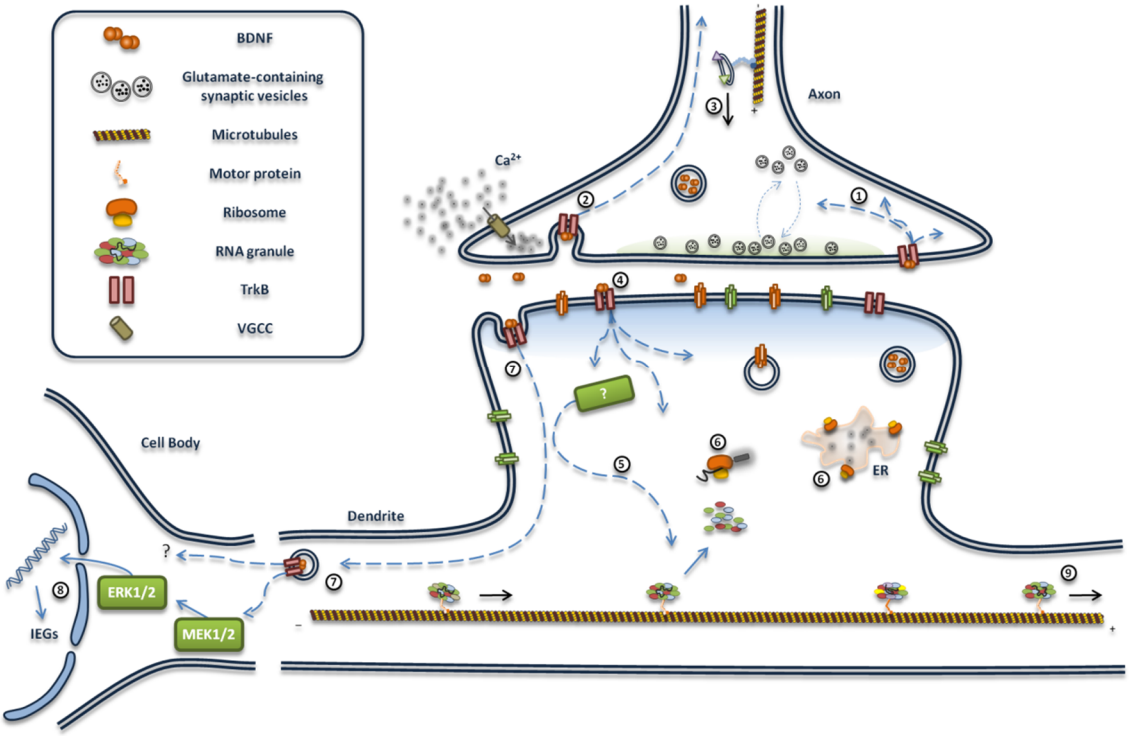

Figure 1.8 - BDNF-induced local translation at the synapse and upregulation of gene expression. TrkB receptor activation upon BDNF binding increases the accumulation of synaptic vesicles at the active zone in the presynaptic region, thereby potentiating synaptic transmission (1). BDNF-TrkB complex can be internalized and retrogradely transported towards the cell body (2). Once in the soma the active receptors may change gene expression and mRNA translation, and newly synthesized proteins may be then transported along the axon together with preexisting proteins (3). The BDNF-TrkB complex may also induce postsynaptic responses (4), including the disassembly of the RNA granules (5), through activation of different signaling pathways. RBPs-associated mRNAs become therefore available for translation, either at free polysomes or at the ER-associated ribosomes (6). BDNF-TrkB "signaling endosomes" are also transported from the dendritic spine to the cell body (7), where it leads to the transcription of IEGs in a MEK1/2 and ERK1/2 dependent manner (8). In dendrites, RNA granules containing mRNAs are transported along microtubules and can be "recruited" by an active dendritic spine or they may continue the movement towards more distal sites (9).

1.8. hnRNP family of proteins

Heterogeneous nuclear ribonucleoproteins (hnRNPs) constitute a large family of proteins that bind nascent transcripts and package them into hnRNP particles (Dreyfuss et al., 1993; Pinol-Roma et al., 1988; Pullman and Martin, 1983). This family includes 20 proteins with sizes ranging from 34 to 120 kDa that were named hnRNP A1 to hnRNP U (Pinol-Roma et al., 1988). Each protein contains at least one RNA-binding motif such as the RNA recognition motif (RRM), hnRNP K homology domain (KH) or the arginine/glycine rich domain (Han et al., 2010; Krecic and Swanson, 1999) (Fig. 1.9). Several hnRNPs possess other non-standard RRM referred as atypical RRMs (aRRMs) (Han et al., 2010).

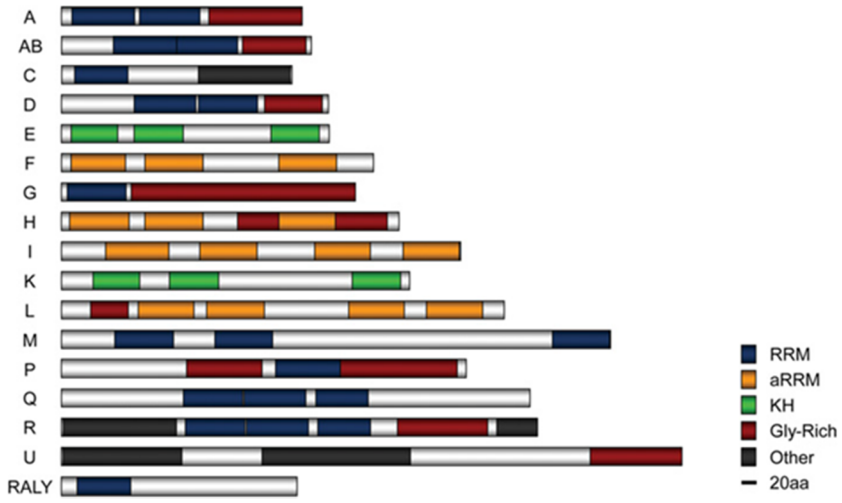

Figure 1.9 - Structural domains of the hnRNP proteins. RRM, RNA recognition motif; aRRM, atypical RNA recognition motif; KH, K homology domain; Gly-Rich, Glycine-Rich domain. 'A' represents hnRNP A0, A1, A2/B1 and A3 that are structurally similar. From (Han et al., 2010).

Another key characteristic of hnRNPs is the fact that they undergo nucleocytoplasmic shuttling (Pinol-Roma and Dreyfuss, 1992). Although hnRNPs are mainly expressed in the nucleus there are few exceptions. For example, an isoform of hnRNP M is a membrane-bound receptor (Bajenova et al., 2003). Also, hnRNP Q is mainly found in the cytoplasm (Mizutani et al., 2000) and is known to be involved in the transport of mRNA in neuronal dendrites (Bannai et al., 2004). Furthermore, hnRNP A2/B1 is one of the best described *trans*-acting factors that regulates mRNA localization in oligodendrocytes (Ainger et al., 1997; Munro et al., 1999) and neurons (Gao et al., 2008; Muslimov et al., 2006; Muslimov et al., 2011; Shan et al., 2003). The members of the

hnRNP family of proteins regulate several aspects of nucleic acid metabolism, including packaging of nascent transcripts, splicing, mRNA transport, stability and translation (Han et al., 2010).

1.8.1. The *trans*-acting factor hnRNP A2/B1 and mRNA transport

hnRNP A2/B1 is perhaps the best described *trans*-acting protein involved in the mRNA trafficking in oligodendrocytes and neurons. This member of the hnRNP family was originally described as the *trans*-acting factor that recognizes a *cis*-acting element present in the myelin basic protein (MBP) (Ainger et al., 1997). Because it is recognized by hnRNP A2/B1, the 21-nucleotide *cis*-acting element is called A2 response element (A2RE). Further studies demonstrated that the MBP mRNA is recruited and transported in RNA granules through association with hnRNP A2/B1 (Ainger et al., 1997; Carson et al., 1998; Hoek et al., 1998; Munro et al., 1999). The first evidence supporting a role for hnRNP A2/B1 in the trafficking of mRNAs in neuronal dendrites was provided by studies where A2RE-carrying mRNAs were injected in hippocampal neurons (Shan et al., 2003). In this study, mutations that abrogate the binding of injected mRNAs to hnRNP A2/B1 were found to compromise their dendritic localization (Shan et al., 2003). It was further demonstrated that hnRNP A2/B1 is required for the dendritic localization of Arc, CaMKIIα and Neurogranin mRNAs, which appear to cluster in the same hnRNP A2/B1-containing granules (Gao et al., 2008). Moreover, hnRNP A2/B1 is necessary for the delivery of the noncoding BC1 RNA and PKMζ mRNA to distal dendritic domains (Muslimov et al., 2006; Muslimov et al., 2011). Interestingly, it was shown that hnRNP A2/B1 directly binds and recruits hnRNP E1 to RNA transport granules, inhibiting the translation of A2RE-containing mRNAs in oligodendrocytes (Kosturko et al., 2006). On the other hand, activation of Fyn kinase results in the phosphorylation of hnRNP A2/B1 and stimulates the translation of a MBP A2RE-containing reporter (White et al., 2008).

1.8.2. hnRNP K structure and function

hnRNP K has a wide variety of nuclear and cytoplasmic functions, including mRNA transcription (Lynch et al., 2005; Ostrowski et al., 2003; Stains et al., 2005), splicing (Cao et al.,

2012; Expert-Bezancon et al., 2002), and regulation of mRNA stability (Fukuda et al., 2009; Skalweit et al., 2003) and translation (Feliers et al., 2007; Habelhah et al., 2001; Ostareck-Lederer et al., 2002; Ostareck et al., 1997; Yano et al., 2005; Yoon et al., 2013). hnRNP K binds single or double stranded nucleic acids, especially in CU/CT rich regions via its three K homology (KH) domains (Bomsztyk et al., 2004; Fenn et al., 2007; Grishin, 2001). Furthermore, it contains a nuclear localization signal (NLS) and a nuclear shuttling domain (KNS) (Michael et al., 1997) (Fig. 1.10). The functional versatility of hnRNP K arises from its modular structure with the three KH domains that can interact with RNA and ssDNA, and its K interactive (KI) region that can recruit a wide variety of factors, including kinases and factors that regulate splicing, mRNA stability and translation [for a review see (Bomsztyk et al., 2004)]. In fact, hnRNP K seems to be a centre for a vast network of interactions, with 114 binding partners identified until now (Mikula et al., 2006). The protein is thought to act as a "docking plattform" to integrate signaling cascades by promoting the cross-talk between kinases and factors involved in nucleic acid metabolism (Bomsztyk et al., 2004).

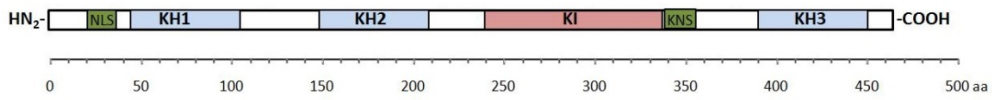

Figure 1.10 - Structure of hnRNP K. The three K homogy domains (KH), the localization signal (NLS), the nuclear shuttling domain (KNS) and the K interactive region (KI) are represented. Adapted from (Mikula and Ostrowski, 2010).

hnRNP K function may also be modulated by phosphorylation. In particular, two studies demonstrated how the hnRNP K-mediated translational control may be regulated by phosphorylation. First, the binding of hnRNP K to c-Src activates this enzyme and leads to the phosphorylation of hnRNP K. The c-Src-mediated phosphorylation of hnRNP K results in the translational activation of silenced mRNAs (Ostareck-Lederer et al., 2002). Second, the activation of ERK1/2 resulted in the phosphorylation and cytoplasmic accumulation of hnRNP K. The cytoplasmic increase of hnRNP K protein represses differentiation control element (DICE)-mediated translation (Habelhah et al., 2001). Another study showed that tyrosine phosphorylation of the hnRNP K protein regulates hnRNP K protein-protein and protein-RNA interactions (Ostrowski et al., 2000). Accordingly, the hnRNP K-mRNA complexes were disrupted by tyrosine

phosphorylation of hnRNP K, suggesting that the binding of hnRNP K to RNA may be responsive to the extracellular stimulus that activate tyrosine kinases (Ostrowski et al., 2000).

Several findings also suggest hnRNP K as a key player in the regulation of p53, cell cycle arrest, and in the response to DNA damage (Huarte et al., 2010; Lee et al., 2012; Moumen et al., 2005; Pelisch et al., 2012). Accordingly, a recent study demonstrate that phosphorylation of hnRNP K is likely to have a major role in integrating DNA damage signals to fine-tune p53-dependent transcriptional responses (Moumen et al., 2013).

hnRNP K was also shown to play a role in the regulation of actin dynamics. The protein interacts directly with the regulator of actin dynamics N-WASP via its KI domain (Yoo et al., 2006), regulating N-WASP-induced cell spreading and filopodia formation in mouse fibroblasts (Yoo et al., 2006).

1.8.2.1. Roles of hnRNP K in neuronal physiology

The function of hnRNP K in neurons is now starting to be unravelled. A recent study demonstrated that hnRNP K interacts with the Abelson-interacting protein 1 (Abi1) at postsynaptic sites in hippocampal neurons (Proepper et al., 2011). Abi1 belongs to a multiprotein complex that activates the WAVE protein family (Le Clainche and Carlier, 2008) and is localized in dendritic spines and postsynaptic densities (PSDs) where it plays an important role in regulating cytoskeleton reorganization and synaptic maturation (Courtney et al., 2000). Accordingly, hnRNP K knockdown results in a significant increase in filopodia formation and decreases the number of mature synapses, an effect that mimics the neuronal morphology in the absence of Abi1 (Proepper et al., 2011). In a separated study, hnRNP K knockdown was shown to disrupt forskolin-induced neurite outgrowth in PC12 cells (Cao et al., 2012). However, in this case the effect was attributed to the role of hnRNP K in the control of alternative splicing induced by forskolin. In addition, hnRNP K interacts with the RNA-binding protein Hu and represses the translation of p21 mRNA, antagonizing Hu function and promoting neurite outgrowth in neuroblastoma cells (Yano et al., 2005). Another study showed the association of endogenous neurofilament mRNAs with hnRNP K in developing cortex (Thyagarajan and Szaro, 2008), suggesting that hnRNP K and neurofilament mRNAs may constitute a post-transcriptional module that regulates the cytoskeleton compositions in neurons.

Recently, hnRNP K was shown to bind the serotonin transporter (SERT) mRNA in immortalized serotoninergic rat embryonic mid-brain cells (RN46A) and in mouse brain tissue, and evidence was provided for the translational control conferred by this ribonucleoprotein (Yoon et al., 2013). In *Xenopus laevis*, hnRNP K is also required for the efficient nuclear export and translation of multiple cytoskeletal-related mRNAs that are essential for axonal-development (Liu et al., 2008; Liu and Szaro, 2011) and optic axon regeneration (Liu et al., 2012). Furthermore, hnRNP K (among other hnRNPs) was identified as a component of transport RNA granules in neurons (Elvira et al., 2006), detected in synaptoneurosomal fractions (Liao et al., 2007) and at the postsynaptic densities in hippocampal neurons (Proepper et al., 2011). Given the nucleo-shuttling nature of hnRNP K and its ability to act as a scaffold protein integrating mRNA and proteins in the same ribonucleoprotein complex, it is tempting to speculate a major role for hnRNP K in mRNA transport to distal domains in neurons. However, despite the evidence here presented, the precise function of hnRNP K in neurons is still poorly understood.

Objectives of the present work

The main goal of this work is to investigate the regulation of the transport of hnRNP A2/B1 and hnRNP K in hippocampal neurons, and the interaction of the latter protein with selected transcripts, which is likely to play a role in synaptic plasticity in the hippocampus.

As previously mentioned, hnRNP A2/B1 plays a major role in the trafficking of mRNAs along dendrites but whether and how this process is regulated remains to be determined. Since several forms of synaptic plasticity require dendritic protein synthesis, including BDNF-mediated long-term potentiation, in the work described in chapter 3 we investigated how synaptic activity and BDNF regulate the distribution of hnRNP A2/B1 in cultured hippocampal neurons. Given its function in the control of mRNA localization in the dendritic compartment, and the role of local translation at the synapse in plasticity events, we focused our attention in the study of the dendritic- and synaptic-localized hnRNP A2/B1. Furthermore, the use of polysomal fractions obtained from synaptoneurosomes isolated from adult rat hippocampus allowed us to investigate whether hnRNP A2/B1 plays a role in translational control of synaptic-localized mRNAs.

Recent evidence led us to hypothesize that hnRNP K may also be a component of ribonucleoprotein complexes involved in the dendritic transport of mRNAs. In the work reported in chapter 4 we investigated the cytoplasmic distribution of the protein in hippocampal neurons under resting conditions and upon neuronal activation or in response to BDNF. We have previously shown that hnRNP K associates with several transcripts that encode proteins with roles in synaptic plasticity. Furthermore, previous results from our laboratory showed that BDNF stimulation induces the dissociation of hnRNP K-bound transcripts from the ribonucleoprotein complexes in cultured hippocampal neurons. Thus, here we studied the role of BDNF in the regulation of hnRNP K-bound mRNAs in synaptic preparations obtained from adult rat hippocampus. In addition, using a model of LTP induced in the perforant path-dentate gyrus synapse in live anesthetized rats, we determined whether hnRNP K and hnRNP K-associated mRNAs are regulated *in vivo*. Importantly, we show that this form of synaptic potentiation requires TrkB activation, presumably by BDNF. Furthermore, we also used a proteomic approach to identify hnRNP K-interacting proteins in the rat hippocampus.

Chapter 2

Experimental Procedures

2.1. Hippocampal Cultures

High-density hippocampal cultures were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (0.06%; 15 min incubation at 37°C; GIBCO - Life Technologies) and deoxyribonuclease I (5.36 mg/ml) in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4 \cdot 2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES, and 0.001% phenol red). The hippocampi were then washed with Hanks' balanced salt solution containing 10% fetal bovine serum (GIBCO - Life Technologies), to stop trypsin activity, and transferred to Neurobasal medium (GIBCO - Life Technologies) supplemented with SM1 supplement (1:50 dilution, STEMCELL Technologies), 25 µM glutamate, 0.5 mM glutamine, and 0.12 mg/ml gentamycin (GIBCO - Life Technologies). The cells were dissociated in this solution and then plated in 6-well plates (85.5 x 10^3 cells/cm^2) coated with poly-D-lysine (0.1 mg/ml). The cultures were maintained in a humidified incubator of 5% CO_2/95% air at 37°C for 14-15 days, and then stimulated with 100 ng/ml BDNF (Peprotech) for the indicated periods of time. Where indicated, neurons were stimulated with 50 µM bicuculline (Tocris), 2.5 mM 4-AP (Tocris) and 10 µM glycine (Sigma-Aldrich) to increase synaptic activity, or depolarized with KCl (30 mM). The experiments were performed in a basal saline solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 2.5 mM $CaCl_2$, 6 mM glucose, and 10 mM HEPES at final pH of 7.4). For KCl depolarization experiments, neurons were treated with a saline solution containing 102 mM NaCl, 34 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, and 10 mM HEPES at final pH of 7.4.

Low-density hippocampal cultures were prepared as previously described (Kaech and Banker, 2006). Briefly, hippocampi were dissected from E18 rat embryos and the cells were dissociated using trypsin (0.25%). Neurons were plated at a final density of 1-5 x 10^4 cells/dish on poly-D-lysine-coated glass coverslips in neuronal plating medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1 mM pyruvic acid). After 2-4 h the coverslips were flipped over an astroglial feeder layer in Neurobasal medium (GIBCO - Life Technologies) supplemented with SM1 supplement (1:50 dilution, STEMCELL Technologies), 25 µM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin (GIBCO - Life Technologies). The neurons grew face down over the feeder layer but were kept separate from the glia by wax dots on the neuronal side of the coverslips. To prevent overgrowth of glial cells, neuron cultures were treated with 5 µM cytosine

arabinoside (Sigma-Aldrich) after 3 DIV. Cultures were maintained in a humidified incubator with 5% CO_2/95% air at 37°C for up to 2 weeks, feeding the cells once per week. At DIV 14-15 the neurons were stimulated for 30 min with 100 ng/ml BDNF (Peprotech) or with 50 µM bicuculline (Tocris), 2.5 mM 4-AP (Tocris) and 10 µM glycine (Sigma-Aldrich), to increase synaptic activity. Where indicated, cells were pre-treated for 30 min with the Trk receptor inhibitor SHN722 (1 µM) (Gomes et al., 2012; Martin et al., 2011) or with the scavenger of extracellular ligands of TrkB receptors TrkB-Fc (1 µg/ml) (R&D Systems) before stimulation with 100 ng/ml BDNF (Peprotech) or with the cocktail solution containing bicuculline (50 µM bicuculline, 2.5 mM 4-AP and 10 µM glycine), respectively. Alternatively, the cells were incubated with 100 ng/ml BDNF (Peprotech) for 3 h. The experiments were performed in a basal saline solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 2.5 mM $CaCl_2$, 6 mM glucose, and 10 mM HEPES at a final pH 7.4).

2.2. Compartmentalized system to culture neurons

High-density hippocampal cultures were prepared from the hippocampi of E18-E19 Wistar rat embryos, as described above. The cells were plated at a density of 80 000 cells/cm^2 in 3 µm pore 75 mm polyethylene terephthalate (PET) membrane filter inserts (Corning) coated with poly-D-lysine (0.1 mg/ml) as previously described (Manadas et al., 2009; Poon et al., 2006). The cultures were maintained in a humidified incubator of 5% CO_2/95% air at 37°C for 14-15 days and then stimulated with 100 ng/ml BDNF (Peprotech) for the indicated periods of time.

2.3. Preparation of hippocampal culture extracts

Hippocampal cultures were washed twice with ice-cold PBS, and once more with PBS buffer supplemented with 1 mM dithiothreitol (DTT) and a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF) and CLAP [1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml antipain, 1 µg/ml pepstatin; Sigma]). The cells were then lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5) supplemented with 50 mM sodium fluoride (NaF), 1.5 mM sodium ortovanadate (Na_3VO_4) and the cocktail of protease inhibitors. After sonication and centrifugation at 16,100 x g for 10 min at 4°C, protein in the supernatants was quantified using the Bicinchoninic acid (BCA) assay kit (Pierce),

and the samples were denaturated with 2x concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM Na_3VO_4, and 0.01% bromophenol blue) for 5 min at 95°C. The proteins of interest were then analysed by Western blot.

2.4. Adult rat total hippocampal extracts

Hippocampi were dissected from adult Wistar rats and the tissue was minced with scissors and homogenized with a glass homogenizer in 10 volumes of ice-cold supplemented RIPA buffer as indicated for the preparation of extracts from hippocampal cultures. The homogenate was sonicated and centrifuged at 16,100 x g for 10 min at 4°C, and the protein in the supernatants was quantified using the Bicinchoninic acid (BCA) assay kit (Pierce). The samples were aliquoted at -20°C until further use.

2.5. Synaptoneurosome preparation

Synaptoneurosomes were prepared as previously described with slight modifications (Yin et al., 2002). Briefly, 6-8 hippocampi were dissected from adult Wistar rats and the tissue was minced with scissors and homogenized with a glass homogenizer in a buffer containing 0.32 M sucrose, 10 mM HEPES-Tris pH 7.4 and 0.1 mM EGTA. After centrifugation for 3 min at 1,000 x g, the supernatant was collected and passed initially through nylon membranes (150 and 50 µm, VWR) and finally through an 8 µm pore size filter (Millipore, MA). The flow-through was centrifuged for 15 min at 10,000 x g, and the pellet was resuspended in incubation buffer (in mM: 8 KCl, 3 $CaCl_2$, 5 Na_2HPO_4, 2 $MgCl_2$, 33 Tris, 72 NaCl, 100 sucrose). All the procedure was done at 4°C. Synaptoneurosomes were incubated or not with 50 ng/ml BDNF (Peprotech) for 10 min at 30°C and were then centrifuged at maximum speed, in a Minispin microcentrifuge for 30 s. The pellet was resuspended in RIPA buffer supplemented as indicated for the extract preparation, followed by sonication and protein quantification using the BCA method. The samples were processed for RNA co-immunoprecipitation assays. For the analysis of polysomal profiles, synaptoneurosomes were prepared from adult rats (12-13 weeks) as described previously (Troca-

Marin et al., 2010), with minor alterations. The hippocampi of 6 adult rats were used in each preparation, and the final washing steps were performed by centrifugation at 33,000 x g.

2.6. Gel electrophoresis and Western blot

Proteins were resolved by SDS-PAGE in 10% polyacrylamide gels. For Western blot analysis, proteins were transferred onto a PVDF membrane (Millipore) by electroblotting (40V, overnight at 4ºC). The membranes were blocked for 1 h with skin milk and 0.1% Tween 20 in TBS [(20 mM Tris, 137 mM NaCl, pH 7.6 (TBS-T)], and probed with the primary antibody overnight at 4ºC. Following several washes with TBS-T, the membranes were incubated with an alkaline phosphatase-conjugated IgG secondary antibody (anti-mouse or anti-rabbit, depending on the primary antibody host-species) for 1 h at room temperature. The membranes were then washed again and immunostaining was visualized by the enhanced chemifluorescence method (ECF) on a Storm 860 Gel and Blot Imaging System (GE Healthcare, Carnaxide, Portugal). For the analysis of total extracts from dentate gyrus homogenates, horseradish peroxidase (HRP)-conjugated secondary antibodies were used and immunostaining was developed using chemiluminescence reagents (ECL, Amersham Biosciences). In this case the blots were scanned using Gel DOC EQ (Bio-Rad). Antibodies for Western blot were as follows: anti-hnRNP A2/B1 (sc-53531, 1:1000; Santa Cruz Biotechnology), anti-hnRNP K (sc-28380, 1:1000; Santa Cruz Biotechnology), anti-Staufen1 (AB5781, 1:1000; Millipore), anti-eEF2 (ab40812, 1:12.500; Abcam), anti-rpS6 (2217, 1:1000; Cell Signaling Technology), anti-RBMX (sc-48796, 1:1000; Santa Cruz Biotechnology). When indicated, anti-ß-tubulin (T7816, 1:30.000; Sigma-Aldrich), anti-ß-actin (A5441, 1:5000, Sigma-Aldrich) and anti-GAPDH (sc32233, 1:5000; Santa Cruz Biotechnology) antibodies were used as loading control.

2.7. Polysome preparation

Synaptoneurosomes prepared from adult rats (12-13 weeks) were lysed in 800 µl of lysis buffer [15 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 0.3 M NaCl, 0.5 mM DTT, 0.1 mg/ml Cycloheximide and 1% Triton X-100] supplemented with a cocktail of protease inhibitors (0.1 mM PMSF; CLAP: 1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml antipain, 1 µg/ml pepstatin; Sigma-Aldrich) and

50U/ml of RNase inhibitor (SUPERaseIn™, Ambion Applied Biosystems). Membranous structures were removed by spinning at 12,000 x g for 10 min. The resulting supernatant was loaded on a 10-50% linear sucrose gradient [prepared in 20 mM Tris-HCl, pH 8, 140 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mg/ml Cycloheximide and 5U/ml of RNase inhibitor (SUPERaseIn™, Ambion Applied Biosystems)] and spun at 35,000 rpm for 180 min (4°C) using a SW41 rotor (Beckman Coulter). Each gradient was separated into 10 fractions, with approximately 1.2 ml each. Equal volumes (30 µl) of the first nine fractions isolated from the 10-50% linear sucrose gradient were denatured and protein samples were separated by SDS-PAGE, transferred to PVDF membranes (Millipore, Madrid, Spain) and immunoblotted.

2.8. Immunoprecipitation assays

Antibody-immobilized beads were prepared by incubating 6 µg of hnRNP K or Mouse IgG antibodies with 100 µl of Protein G PLUS-Agarose beads (Santa Cruz Biotechnology), overnight at 4ºC in NT_2 2x buffer containing 100 mM Tris-HCl, 300 mM NaCl, 2 mM $MgCl_2$, 0.1% IGEPAL, pH 7.4, and supplemented with 1 mM dithiothreitol (DTT) and a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF), CLAP [1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml antipain, 1 µg/ml pepstatin; Sigma]). The immobilized antibodies were incubated with 1 mg of protein for 1 h at 4ºC, and the beads were washed twice (2 min centrifugations, 2,000 x g) with NT_2 1x buffer (at 4ºC) and once with NT_2 1x buffer supplemented with 1M Urea. After an additional wash using the NT_2 1x buffer, the supernatant was discarded and the final pellet, containing the immunoprecipitated hnRNP bound to the antibody-immobilized beads, was resolved by SDS-PAGE followed by in-gel protein detection using the Silver or colloidal Coomassie staining methods. Where indicated, precipitates were subjected to Western blot analysis. For RNA co-immuprecipitations, the NT_2 buffer was supplemented with 50U/ml of RNase inhibitor (SUPERaseIn™, Ambion Applied Biosystems) and the TRIzol Reagent (Invitrogen) was immediately added to the pellet. The RNA was extracted according to manufacturer's instructions. For the RNA co-immunoprecipitations performed using total extracts from dentate gyrus homogenates, the same procedure was performed with minor changes: 50 µl of Protein G Sepharose 4 Fast Flow (Ge Healthcare) beads and 500 µg of protein were used for

the immunoprecipitations and the NT_2 buffer was supplemented with c*O*mplete, Mini EDTA-free protease inhibitors cocktail (Roche) and 40U/ml RiboLockRNase inhibitor (Thermo Fischer).

2.9. Silver staining for in-gel protein detection

Silver staining was performed as previously described (O'Connell and Stults, 1997). Briefly, the proteins in the gel were fixed for 30 min using a fixative solution containing 25% methanol and 25% acetic acid. The gel was washed in 50% ethanol and 30% ethanol and then sensitized with sodium thiosulfate (0.2 g/l) for only 1 min to avoid peptide recovery from the gel. After washing twice with water, the gel was stained with silver nitrate (2 g/l) for 20 min and developed in a solution containing 37% formaldehyde (0.7 ml/l), 30 g/l sodium carbonate and 10 mg/l sodium thiosulfate until bands become visible (about 20 min). After stopping the staining with a solution containing Tris (50 g/l) and 2.5% acetic acid, the gel was stored in water at 4°C.

2.10. Colloidal Coomassie staining

Colloidal Comassie staining was performed as previously described (Candiano et al., 2004), with minor modifications. Gels were allowed to stain overnight in a solution containing 10% ammonium sulfate, 10% phosphoric acid, 20% methanol and 0.2% Coomassie Brilliant Blue G-250 dye (20279, Pierce). Once the desired staining was obtained, the gels were washed with water and the lanes were immediately cut with a scalpel. The resulting gel fragments were transferred to a 1.5 ml tube containing water and stored until further use.

2.11. Protein identification by liquid chromatography coupled to mass spectrometry analysis (LC-MS/MS)

Protein identification was performed as previously described (Chen et al., 2011). Briefly, immunoprecipitates were separated through SDS-PAGE and the gel was stained with colloidal Coomassie blue. The stained gel was cut into five slices for each gel lane and the bands were excised and subjected to tryptic-digestion. The resulting peptides were separated through capillary HPLC using a capillary reverse phase C18 column. The capillary HPLC system with

auto-sampler/auto-injector (Eksigent nano LC-ultra 1D) was equipped with a 5 mm Pepmap 100 C18 (Dionex) trapping column (300 µm ID, 5 µm particle size), a capillary C18 column and a 200 mm home-made Alltima C18 analytical column (100 µm ID, 3 µm particle size). Protein identification was carried out on the Electrospray LTQ-Orbitrap (Thermo Fisher Scientific) with a Stainless Steel Nano-bore emitter (Proxeon, 30 µm ID).

2.12. RNA isolation and RNA quantification

After immunoprecipitation of hnRNP K with a specific antibody, the co-immunoprecipitating RNAs were immediately isolated using TRizol reagent (Invitrogen) following the manufacturer's specifications. After the addition of chloroform and phase separation, the RNA was precipitated by the addition of isopropanol. The precipitated RNA was washed once with 75% etanol, centrifuged, air-dried and resuspended in 10 µl of RNase-free water (GIBCO - Invitrogen). In parallel experiments the non-specific binding of RNAs to Protein G PLUS-Agarose beads (or Protein G Sepharose 4 Fast Flow beads) was determined and the RNAs were resuspended in the same volume of RNase-free water. The RNA concentration was determined using NanoDrop (Thermo Scientific) and samples were stored at -80°C until further use.

2.13. Cell body and neurite total RNA

Total RNA was extracted from 14-15 DIV hippocampal neurons cultured in 3 µm pore 75 mm PET membrane filter inserts. To avoid contamination between the two cellular compartments, the neurites grown on the bottom of the filter were carefully scraped and immediately added to TRIzol Reagent (Invitrogen). The cell bodies and neuronal processes present in the inner compartment of the insert were directly lysed in TRIzol Reagent. The remaining steps were performed according to manufacturer's instructions and as described above. RNA quality and integrity was assessed using the Experion automated gel-electrophoresis system (Bio-Rad) and the RNA concentration was determined using NanoDrop (Thermo Scientific).

2.14. Reverse transcription

For first strand cDNA synthesis 500-1000 ng of isolated RNA was mixed with 4 µl of 5x iScript Reaction Mix, 1 µl of Reverse Transcriptase and Nuclease-free water, up to a total volume of 20 µl per experimental condition (iScript™cDNA Synthesis Kit, Bio-Rad). This kit utilizes a blend of oligo (dT) and random hexamer primers in the reaction mix. The reaction was performed at 25ºC for 5 min, followed by 30 min at 42ºC, for primer annealing to the template and cDNA synthesis, respectively. The reverse transcriptase was then denatured for 5 min at 85ºC, and the sample cooled to 4ºC, before storage at -20ºC until further use.

2.15. Primer design

Primers for qRT-PCR were designed by Beacon Designer 7 software (Premier Biosoft International, Palo Alto, CA). The following considerations were taken: 1) GC content about 50%; 2) annealing temperature (T_a) between 55 ± 5°C; 3) secondary structures and primer dimers were avoided; 4) primer length 18-24 bp; 5) final product length 100-200 bp. See Table 2.1 for the list of primers and corresponding sequences:

Table 2.1- Primer sequences

Genes	Primer Forward Sequence (5'- 3')	Primer Reverse Sequence (5'- 3')
Hnrnpa2	GCTACGGAGGTGGTTATG	AGTTAGAAGGTTGCTGGTTAT
Ppia	TTTGGGAAGGTGAAAGAAGGC	ACAGAAGGAATGGTTTGATGGG
Hnrnpk	AACACTCAGACAACAATCA	TCCTCCAATAAGAACAACTC
Grin1	CGGCTCTTGGAAGATACAG	GAGTGAAGTGGTCGTTGG
Gria1	ACTACATCCTCGCCAATCTG	AGTCACTTGTCCTCCATTGC
Bdnf	TAACCTCGCTCATTCATTA	TCAACTCTCACTCACCTT
Camk2b	GCTATACGACGATATTGG	TCTTGGTGTTAATGATCT
Hprt1	CCTTGACTATAATGACCACTTC	GCCACATCAACAGGACTC
Bdnf	TGGGACTCTGGAGAGCGTGAATGG	CGGGACTTTCTCCAGGACTGTGAC
Bdnf (cds)	GATGACCATCCTTTTCCTTAC	ATTCACGCTCTCCAGAGTCC

Hnrnpa2, heterogeneous nuclear ribonucleoprotein A2; *Ppia*, Peptidylprolylisomerase A; *Hnrnpk*, heterogeneous nuclear ribonucleoprotein K; *Grin1*, glutamate receptor ionotropic NMDA 1; *Gria1*, glutamate receptor ionotropic AMPA 1; *Bdnf*, brain-derived neurotrophic factor; *Camk2b*, calcium/calmodulin-dependent protein kinase II beta; *Hprt1*, hypoxanthine guanine phosphoribosyltransferase 1.

For mRNA measurements in hnRNP K immunoprecipitates prepared from dentate gyrus total homogenates, two additional sets of primers for *Bdnf* (two bottom primers in Table 2.1) were used. One of these primers was designed to target the *Bdnf* coding sequence [*Bdnf* (cds)].

2.16. Quantitative RT-PCR

Quantitative PCR was performed using the SsoFast™ EvaGreen Supermix (172-5201; Bio-Rad). 2 µl of 1:10 diluted cDNA was used and the final concentration of each primer was 250 nM in a final volume of 20 µl. The thermocycling reaction was initiated with activation of Taq DNA polymerase by heating at 95°C during 30 s, followed by 45 cycles of a 10 s denaturation step at 95°C, a 30 s annealing step at the optimal primer temperature of annealing, and a 30 s elongation step at 72°C. The fluorescence was measured after the extension step by the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). After the thermocycling reaction, the melting step was performed with slow heating, starting at 55°C and with a rate of 0.5°C per 10 s, up to 95°C, with continuous measurement of fluorescence to allow the detection of nonspecific products. To measure the mRNA co-immunoprecipitated with hnRNP K from dentate gyrus total homogenates, the qRT-PCR reaction was performed in a final volume of 8 µl with 2x SYBR Green Master Mix (Bio-Rad), and using a LightCycler 480 (Roche). The reaction was initiated with a pre-amplification step of 3 min at 95°C, followed by 45 cycles of a 10 s denaturation step at 95°C, a 10 s annealing step at the optimal primer temperature of annealing, a 10 s elongation step at 72°C, and warming from 65°C to 95°C for the melting curve

2.17. Processing and analysis of qRT-PCR data

The comparative Ct method ($2^{-(\Delta\Delta Ct)}$) was used to quantitate the relative gene expression across the experimental conditions. The threshold cycle (Ct) represents the detectable fluorescence signal above background resulting from the accumulation of amplified product, and is a proportional measure of the starting target sequence concentration. Ct was measured on the exponential phase and, for every run, Ct was set at the same fluorescence value. Data analysis of the log-transformed expression data was performed using the GenEx (MultiD Analysis, Sweden) software for Real-time PCR expression profiling.

2.18. Immunocytochemistry

Hippocampal neurons (low-density) were fixed in 4% sucrose/paraformaldehyde (in PBS) for 15 min at room temperature and permeabilized with 0.3% Triton X-100 in PBS. The neurons were

then incubated with 10% BSA in PBS, for 30 min at 37°C, to block non-specific staining, and incubated overnight at 4°C with the primary antibodies diluted in 3% BSA in PBS. The following primary antibodies and dilutions were used: anti-hnRNP A2/B1 (sc-53531, 1:200; Santa Cruz Biotechnology), anti-hnRNP K (sc-28380, 1:200; Santa Cruz Biotechnology), anti-MAP2 (ab5392, 1:10.000, Abcam), anti-PSD95 (D27E11, 1:200; Cell Signaling) and anti-Staufen1 (AB5781, 1:250; Millipore). The cells were washed 6 times with PBS for 2 min and incubated with Alexa Fluor 568 (1:500, Invitrogen), Alexa Fluor 488 (1:500; Invitrogen) and AMCA (1:200; Jackson ImmunoResearch) conjugated secondary antibodies, for 45 min at 37°C. After washing the cells 6 times with PBS for 2 min, the coverslips were mounted with a fluorescence mounting medium (DAKO).

2.19. Microscopy and quantitative fluorescence analysis

Imaging was performed on a Zeiss Observer Z.1 microscope using a 63x 1.4 NA oil objective. Images were quantified using the ImageJ image analysis software. For quantitation, sets of cells were cultured and stained simultaneously, and imaged using identical settings. The protein signals were analysed after setting the thresholds, and the recognizable clusters under those conditions were included in the analysis. The number, area and the integrated intensity of hnRNP A2/B1 or hnRNP K particles in dendrites were determined and represented per dendritic area. For colocalization analysis, regions around thresholded puncta were overlaid as a mask in the PSD95 (or Staufen1) channel, and the integrated intensity, area and number of colocalized particles determined.

2.20. HFS-induced LTP in the dentate gyrus in live anesthetized rats

2.20.1. Animals and pre-surgical treatment

The animals used in this study were Sprague-Dawley outbreed strain (M&B A/S, Ry, Denmark) weighing a 250-300 g at the time of use. Animals were housed in a temperature- and light-controlled vivarium (21 ± 1°C; 12:12 hour artificial circadian rhythm) and supplied with a high protein diet type MR1 (Special Diet Services) and water for at least 1 week before surgery. Animals were retrieved from the animal facility into the lab in separate cages and were

anesthetized with Urethane (250 mg/ml; 1.4-1.8 mg/kg) according to their individual weight. Urethane was administered via intraperitoneal injection. The first injection contained 1/3 of the total dosage and after 5 min the animal was weighed for accurate measurements and the final 2/3 of the anesthetic was given.

2.20.2. Stereotaxic surgery and electrode positioning

Rats were positioned in a stereotaxic frame (David Kopfs Instruments, USA) with the upper incisor bar 2 mm below the interaural line (skull flat position), the ear bars placed at the side of the head in the natural jaw sockets and a nose-and-tooth bar supported by the upper jaw of the animal (Fig. 2.1A). If required, supplemental doses of urethane were given to maintain a surgical level of anesthesia. Rectal temperature was maintained at 36°C with a thermostatically controlled electric heating pad. A scalpel was used to make a 1.5 cm longitudinal cut on the top of animals head. Four bulldog clamps (FST, Germany) were used to reflect the skin giving open access to the scalp. The surface of the scalp was kept dry and free from blood. Burr holes were drilled in the appropriate location for the insertion of the stimulating and recording electrodes (Fig. 2.1A). A sharp needle was used to incise the dura to facilitate the penetration of the electrodes. Two holes were drilled anterior to bregma in the frontal bone to attach the ground and reference electrodes (Fig. 2.1A). The stimulation electrodes were bipolar, concentric, stainless steel, and with a vertical tip separation of 500 µm (SNEX 100; Rhodes Medical Instruments, Woodland Hills, CA) (Fig. 2.1B-left). The recording electrodes were Teflon coated 7-strand stainless steel (or tungsten) wires (#7955/7960, A-M System Inc, USA) of approximately 8-10 cm length. The ground and reference electrodes were Teflon coated 7-strand stainless steel wires (#7925/7935, A-M System Inc, USA). Stereotaxic coordinates for the unilateral stimulation of the medial perforant path fibers in the angular bundle were as follows (in mm, relative to bregma): 7.9 posterior and 4.2 lateral. Stereotaxic coordinates for recording in the hilar region of the dentate gyrus were as follows (in mm, relative to bregma): 3.9 posterior and 2.3 lateral (Fig. 2.1A). When positioned on the accurate coordinates, both stimulation and recording electrodes were gradually lowered down to their final position. The final depth for the stimulation and recording electrodes was 2.2-2.8 and 3.3 mm below dura, respectively.

2.20.3. *in vivo* electrophysiology and intrahippocampal infusion

After the correct positioning of the electrodes, the medial perforant path fibers in the angular bundle were unilaterally stimulated and the evoked field potentials (fEPSP) were recorded in the hilar region of the dentate gyrus. After stabilization, baseline was recorded for 20 min. Test pulses were applied at 0.033 Hz throughout the experiment except during the period of high-frequency stimulation (HFS). The intensity of the stimulus for test pulses and for HFS was set to the intensity that evoked 1/3 of maximum population spike. The HFS paradigm to induce long-term potentiation (LTP) consisted of eight pulses of 400 Hz, repeated four times at 10 s intervals. Three sessions of HFS were given at intervals of five minutes. Intrahippocampal infusions were made using a stainless steel cannula system (Plastics One, Roanoke, VA) consisting of an outer guide tube (24 gauge) and an inner infusion tube (31 gauge). The guide cannula was beveled sharp at the tip to facilitate brain insertion. The recording electrode was attached to the guide cannula and cut so that the distance between the electrode and the tip of the inner infusion cannula was 0.8-0.9 mm (Fig. 2.1B-right). The guide cannula-electrode assembly was slowly lowered until a positive-going field EPSP (fEPSP) of maximum slope was obtained in the dentate hilus. The infusion cannula was then inserted so that the tip protruded 300 µm below the end of the guide cannula. The infusion site was located 700 µm above the hilar recording site (corresponding to deep CA1 *stratum lacunosum-moleculare*), and 300-400 µm above the medial perforant path-granule cell synapses. The response was allowed to stabilize for 1 h. After baseline recordings (20 min) the infusions were performed with a pump that ensures the gradual infusion of 1 µl TrkB-Fc (100 µg/µl, 688-TK) or IgG-Fc (100 µg/µl,110-HG) (R&D Systems) over a 12.5 minute time period. HFS was performed 18 min after the infusion.

2.20.4. Dissection and preparation of dentate gyrus homogenates

At the end of the electrophysiological recording, the rats were decapitated and the brain rapidly removed and transferred to a glass plate covered with ice-cold saline-soaked filter paper. The dentate gyrus and the hippocampal CA1 and CA3 regions were rapidly dissected and stored in eppendorf tubes instantly frozen in a mixture of 96% methanol and dry ice. Samples were kept at -80°C until further use. The dentate gyri were homogenized in 400 µl ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final

pH 7.5) containing 50 mM sodium fluoride (NaF) and supplemented with c*O*mplete, Mini EDTA-free protease inhibitors cocktail (Roche), and 40U/ml RiboLock RNase inhibitor (Thermo Fischer). A fraction of the homogenate sample was set aside for Western blot analysis and the remaining homogenate used for the RNA co-immunoprecipitation experiments.

2.20.5. Field potential analysis

The fEPSP was analysed using the software Datawave Experimental Workbench (Data Wave Systems, USA). Between two points on the fEPSP, five points were randomly selected to calculate the steepness of the slope (Fig. 2.1C). Data files were coverted to ASCII format and further analysed in the Microsoft Office Excel 2010 (Microsoft Corporation, USA). fEPSP is presented as percentage change from baseline.

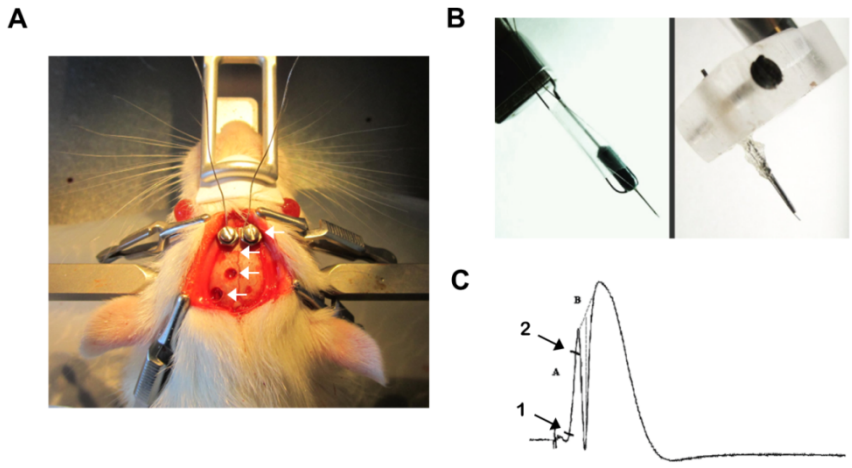

Figure 2.1 - (A) Image of a rat positioned in a stereotaxic frame during the surgery process. White arrows point to the ground and reference electrodes, bregma, the hole drilled to insert the recording electrode and the hole drilled to insert the stimulation electrode, from the top to the bottom, respectively. (B) Stimulation electrode (left) and a recording electrode with an attached guide cannula (right). (C) Between the two points indicated in the fEPSP slope (1 and 2), five points were randomly selected to calculate the steepness. B and C were adapted from (Berentsen, 2009).

2.21. Statistical analysis

Statistical analysis was performed using Student's *t* test and one-way ANOVA followed by the Dunnett's or Bonferroni's test, as indicated in the figure captions.

Chapter 3

Neuronal Activity Induces Synaptic Delivery of hnRNP A2/B1 by a BNDF-dependent Mechanism in Hippocampal Neurons

Chapter submitted for publication

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The experiments shown on panel 3.1C were performed by Pedro M. Afonso

3.1. Summary

Dendritic protein synthesis plays a critical role in several forms of synaptic plasticity, including BDNF (brain-derived neurotrophic factor)-mediated long-term synaptic potentiation. Dendritic transcripts are typically transported in a repressed state as components of large ribonucleoprotein complexes, and then translated upon stimulation at, or near, activated synapses. hnRNP A2/B1 is one of the best described *trans*-acting factors involved in dendritic mRNA trafficking, but how the transport of the protein in dendrites is regulated has not been characterized. Here we found that a fraction of hnRNP A2/B1 colocalizes with Staufen1 in dendrites of cultured hippocampal neurons, suggesting that the two proteins are present in the same granules. Accordingly, both hnRNP A2/B1 and Staufen1 were identified in synaptic mRNP-fractions, and Staufen1 was also associated with synaptic polysomes. Neuronal activity and BDNF treatment increased hnRNP A2/B1 protein levels in dendrites and induced the delivery of this protein to synaptic sites. Although the activity-dependent upregulation in dendritic hnRNP A2/B1 was independent of TrkB receptor activation, the accumulation of the ribonucleoprotein in synaptic sites required, at least in part, the contribution of endogenous released BDNF. The neurotrophin BDNF also upregulated the hnRNP A2/B1 mRNA in the soma but was without effect on the abundance of neuritic-localized hnRNP A2/B1 transcripts. These results show that the distribution of hnRNP A2/B1 is regulated by BDNF and by neuronal activity, and this effect may play a role in BDNF-induced plasticity events.

3.2. Introduction

Experience-dependent changes in synapse structure and function are thought to underlie learning and memory formation (Kandel, 2001). Some of these modifications require activity-dependent transport and translation of dendritic-localized mRNAs, with concomitant alterations in the proteome (Sutton and Schuman, 2006). These biochemical, structural and functional alterations are required for several forms of synaptic plasticity, including brain-derived neurotrophic factor (BDNF)-mediated long term potentiation (LTP) (Leal et al., 2013).

Dendritic mRNAs are usually packaged into large messenger ribonucleoprotein complexes (mRNPs) in the cell body and transported along the microtubule cytoskeleton until they reach their

destination. During this process, the transcripts are usually kept in a dormant state and then translated upon synaptic activation (Bramham and Wells, 2007). One of the best described RNA-binding protein involved in mRNA trafficking in both oligodendrocytes and neurons, is the heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) (see section 1.8.1). Given the nature of some of the hnRNP A2/B1-associated transcripts, the protein may play a role in long-term synaptic potentiation. Staufen1 is another RNA-binding protein present in neuronal RNA granules that migrate along dendrites of hippocampal neurons (Kiebler et al., 1999; Kohrmann et al., 1999), regulating the transport of mRNA (Kanai et al., 2004; Tang et al., 2001). Neuronal mRNPs are heterogeneous structures that may contain Staufen1 and hnRNP A2/B1 (Elvira et al., 2006; Kanai et al., 2004).

Several transcripts are transported to dendrites upon synaptic activity, including mRNAs containing A2RE-like elements in their sequences, such as the mRNAs encoding for the activity-regulated cytoskeleton-associated protein (Arc), CaMKIIα and BDNF (Gao et al., 2008; Havik et al., 2003; Link et al., 1995; Lyford et al., 1995; Raju et al., 2011; Rook et al., 2000; Steward et al., 1998; Steward and Worley, 2001; Thomas et al., 1994; Tongiorgi et al., 1997). In addition, the accumulation of Arc and BDNF transcripts in dendrites is also induced by the neurotrophin BDNF (Messaoudi et al., 2007; Rao et al., 2006; Righi et al., 2000; Ying et al., 2002). Although the BDNF mRNA may be transported along dendrites through interaction with other RNA-binding proteins (Chiaruttini et al., 2009; Oe and Yoneda, 2010), these findings suggest that the transport of hnRNP A2/B1 in dendrites may be regulated by neuronal activity.

Here we show that hnRNP A2/B1 protein colocalizes in a subset of Staufen1-containing granules in dendrites of hippocampal neurons and is in part present at the synapse under resting conditions. hnRNP A2/B1 and Staufen1 were both identified in mRNP-fractions isolated from adult hippocampal synaptoneurosomes, being Staufen1 associated with synaptic polysomes. We also show that synaptic activity and the neurotrophin BDNF increase the levels of hnRNP A2/B1 in dendrites and induce the accumulation of the protein in synaptic sites. Importantly, BDNF mediates the synaptic accumulation of hnRNP A2/B1 induced by neuronal activity.

3.3. Results

hnRNP A2/B1 is required for the dendritic localization of mRNAs encoding proteins that are relevant for synaptic plasticity, such as CaMKIIα and Arc (Gao et al., 2008). Staufen1 is another well described *trans*-acting factor localized in dendritic RNA granules (Kiebler et al., 1999; Kohrmann et al., 1999) that regulates the transport of mRNA (Kanai et al., 2004; Tang et al., 2001), and this protein was shown to play a role in protein synthesis-dependent long-term synaptic potentiation in hippocampal pyramidal neurons (Lebeau et al., 2008). Both Staufen1 and hnRNP A2/B1 are present in neuronal mRNPs (Elvira et al., 2006; Kanai et al., 2004) but whether these proteins localize in the same set of granules is unknown. We stained cultured hippocampal neurons (14-15 DIV) using antibodies against hnRNP A2/B1, Staufen1, and the dendritic marker MAP2, and the results show that hnRNP A2/B1 exhibits a punctate distribution along dendrites and often localizes in particles that also contain Staufen1 (Fig. 3.1A-white arrows). Colocalization analysis demonstrate that a significant fraction (22.42 ± 1.89%, n=37 cells from three independent preparations) of dendritic hnRNP A2/B1 signal colocalizes with Staufen1 (Fig. 3.1A'). These results suggest that hnRNP A2/B1 and Staufen1 can assemble in the same mRNPs. However, a significant fraction of the puncta containing hnRNP A2/B1 and Staufen1 did not colocalize.

Dendritic-localized mRNAs are typically transported in a dormant state until the translational-block is relieved upon activity at the synapse (Bramham and Wells, 2007). To test if hnRNP A2/B1 is present at the synapse, we analysed the colocalization with the postsynaptic marker PSD95, in cultured hippocampal neurons (14-15 DIV) (Fig. 3.1B; Fig. 3.1B'). A considerable fraction of dendritic hnRNP A2/B1 (11.19 ± 0.85%; n=71 cells from six independent preparations) localizes at PSD95-positive clusters (Fig. 3.1B-white arrows; Fig. 3.1B'). Similarly, a significant percentage of total PSD95-positive synapses contain hnRNP A2/B1 (7.68 ± 0.47%; n=48 cells from four independent prepararations) (data not shown). Taken together, these observations suggest that hnRNP A2/B1 is present in synaptic sites under resting conditions. In addition, we detected hnRNP A2/B1 in the mRNPs, but not in the polysomal-fractions, obtained from synaptoneurosomes isolated from rat hippocampus (Fig. 3.1C). In contrast with hnRNP A2/B1, which was excluded from polysomal fractions isolated from hippocampal synaptoneurosomes, Staufen1 was present in both mRNPs- and polysomal-associated fractions (Fig. 3.1C).

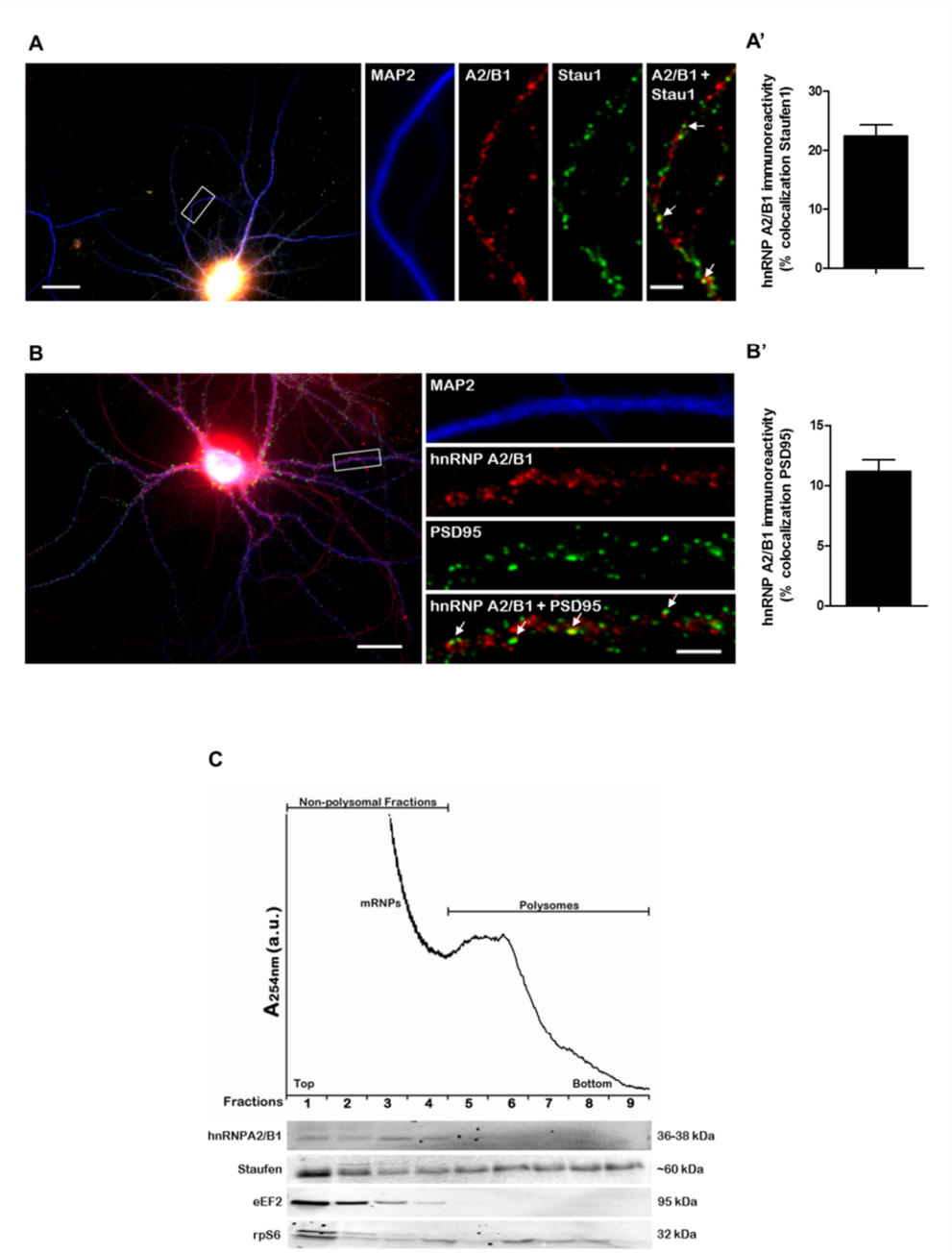


Figure 3.1 - hnRNP A2/B1 is present in synaptic mRNPs and colocalizes with Staufen1 in dendrites of hippocampal neurons. (A) Cultured hippocampal neurons (14-15 DIV) immunostained for hnRNP A2/B1 (red), Staufen1 (green) and the dendritic marker MAP2 (blue) show a punctate distribution of hnRNP A2/B1 along dendrites which is partly colocalized in particles that also contain Staufen1 (white arrows). Scale bars = 25 µm and 3 µm for low and high magnification images, respectively. The images are representative of three different experiments performed in independent preparations, with a total of 37 cells analysed. (A') The fraction of dendritic hnRNP A2/B1 signal that colocalizes with Staufen1 was analysed using ImageJ software (mean ± SEM). (B) Cultured hippocampal neurons (14-15 DIV) immunostained for MAP2 (blue), hnRNP A2/B1 (red) and PSD95 (green) show that hnRNP A2/B1 is present in synaptic sites as indicated by the colocalization with the postsynaptic marker PSD95 (white arrows). Scale bars = 25 µm and 4 µm for low and high magnification images, respectively. The images are representative of six different experiments performed in independent preparations, with a total of 71 cells analysed. (B') The percentage of dendritic hnRNP A2/B1 signal that colocalizes with PSD95 was analysed using ImageJ software (mean ± SEM). (C)

Co-sedimentation of synaptoneurosomal proteins using a 10-50% linear sucrose gradient. The polysomes and mRNPs (non-polysomal fractions) were detected by UV absorbance at 254 nm and the gradient was collected in 9 fractions. Equal volumes from each fraction were analysed by SDS-PAGE and Western blot using antibodies that recognize hnRNP A2/B1, Staufen1, elongation factor eEF2 and ribosomal protein S6 (rpS6). The results are representative of three (hnRNP A2/B1), two (staufen1), four (eEF2) and three (rpS6) different experiments performed in independent synaptoneurosomal preparations.

Altogether, these findings strongly suggest that hnRNP A2/B1 and Staufen1 can assemble to some extent in the same granules in dendrites of hippocampal neurons and localize in synaptic mRNPs under basal conditions

Several transcripts are transported to dendrites and dendritic spines upon neuronal activation, including the mRNAs for Arc (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998; Steward and Worley, 2001), β-actin (Tiruchinapalli et al., 2003), CaMKIIα (Havik et al., 2003; Rook et al., 2000; Thomas et al., 1994), TrkB and BDNF (Tongiorgi et al., 1997). The mRNAs encoding for Arc, CaMKIIα and BDNF have A2RE sequences in their sequence (Gao et al., 2008; Raju et al., 2011) and the Arc and CaMKIIα transcripts appear to be transported along dendrites in an hnRNP A2/B1-dependent manner (Gao et al., 2008). However, it remains to be determined whether the hnRNP A2/B1-dependent mRNA transport is a constitutive or regulated process. Therefore, we investigated if synaptic activity changes the levels of hnRNP A2/B1 in dendrites and regulates the delivery of this protein into synapses. For that purpose, we stimulated cultured hippocampal neurons (14-15 DIV) for 30 min with a cocktail solution with bicuculline to increase the excitatory activity of the neuronal network (Hardingham et al., 2002). Bicuculline treatment significantly increased the integrated intensity, as well as the number and area of hnRNP A2/B1 puncta in dendrites (Fig. 3.2A, B, C, D) and in synaptic sites (Fig. 3.2A-white arrows, E, F, G). Since the increase in synaptic levels of hnRNP A2/B1 could be a result of the general increase observed in dendrites, we also analysed the percentage of dendritic hnRNP A2/B1 signal that colocalizes with the synaptic marker PSD95. We found that synaptic activity also increased the percentage of dendritic hnRNP A2/B1 that is present at the synapse (Fig. 3.2H), suggesting that there is a preferential increase in the delivery of hnRNP A2/B1 into synaptic sites, which is not only due to the overall change that occurs in dendrites.

The neurotrophin BDNF plays a key role in several forms of synaptic plasticity (Waterhouse and Xu, 2009). Some of the actions of BDNF in the CNS rely, in part, on the ability of this neurotrophin to change synaptic proteome through the regulation of the delivery of dendritic-

localized transcripts and by regulating local protein synthesis at the synapse (Leal et al., 2013; Santos et al., 2010). Furthermore, several activity-inducing paradigms were shown to induce the release of endogenous BDNF (Kuczewski et al., 2009; Lessmann and Brigadski, 2009). Therefore, we investigated whether the endogenous released BDNF was involved in the bicuculline-induced regulation of hnRNP A2/B1. For that purpose we took advantage of the TrkB-Fc chimera, an effective extracellular scavenger of TrkB ligands which has been widely used to access BDNF functions in the CNS. TrkB-Fc application did not block bicuculline-induced increase in dendritic hnRNP A2/B1 puncta intensity and area (Fig. 3.2A, B, and C) but partially prevented the increase on synaptic hnRNP A2/B1 observed upon bicuculline treatment (Fig. 3.2A-white arrows, E, F, and G). Altogether, our data indicate that the activity-dependent delivery of hnRNP A2/B1 into synaptic sites depends on the release of BDNF and extracellular activation of TrkB receptors. In contrast, the accumulation of hnRNP A2/B1 in dendrites upon synaptic activity does not require the actions of endogenous released BDNF.

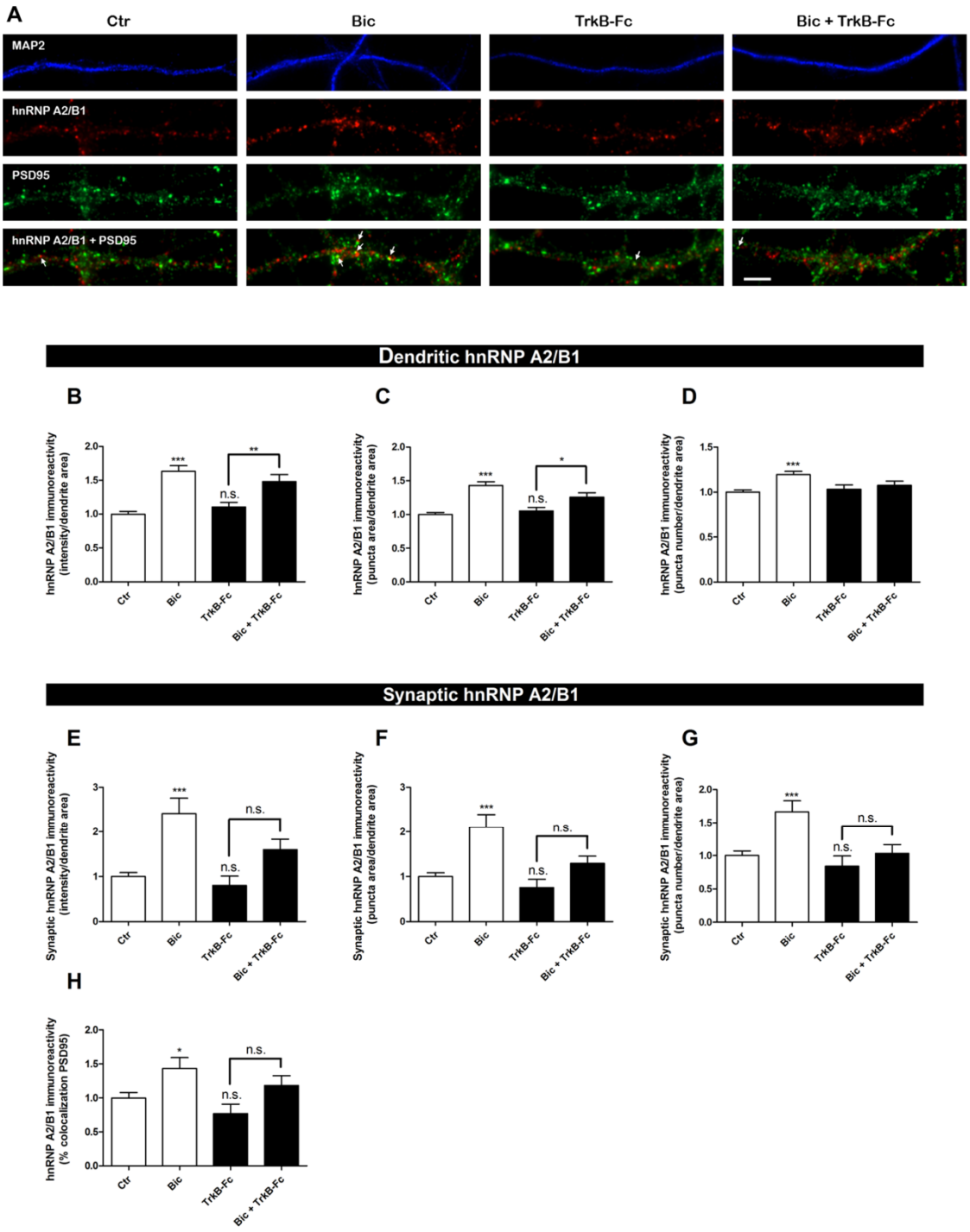


Figure 3.2 - Synaptic activity increases hnRNP A2/B1 in dendrites and induces the delivery of hnRNP A2/B1 to the synapse by a BDNF-dependent mechanism. Cultured hippocampal neurons (14-15 DIV) were stimulated or not with bicuculline (50 µM), 4-AP (2.5 mM) and glycine (10 µM), for 30 min. Where indicated, neurons were treated with the extracellular scavenger of TrkB ligands TrkB-Fc (1 µg/ml) for 30 min and then stimulated or not with bicuculline in the presence of the BDNF scavenger. The cells were immunostained for hnRNP A2/B1 (red), MAP2 (blue), and PSD95 (green) (A). White arrows indicate PSD95-positive clusters that also contain hnRNP A2/B1 (A). The integrated fluorescence intensity, area and number of hnRNP A2/B1 puncta in dendrites (B, C, and D) and at the synapse (as defined by the signal of hnRNP A2/B1 that overlaps with PSD95) (E, F, and G) was analysed using ImageJ software and represented per dendritic area. The percentage of dendritic hnRNP A2/B1 signal that colocalizes with PSD95 was also analysed (H). Results are normalized to control and are averaged of 3-7 different experiments performed in independent preparations. For the analysis of dendritic-localized hnRNP A2/B1 puncta, Ctr (n=87 cells); Bic

(n=64 cells); TrkB-Fc (n=47 cells); TrkB-Fc + Bic (n=48 cells). For the analysis of synaptic-localized hnRNP A2/B1 puncta, Ctr (n=76 cells); Bic (n=54 cells); TrkB-Fc (n=35 cells); TrkB-Fc + Bic (n=35 cells). Error bars, mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni's test. n.s. Not significant, * P<0.05; ** P<0.01; *** P<0.001. Scale bar = 4 µm.

Thus, we next investigated whether the exogenous application of BDNF alters the dendritic and synaptic distribution of hnRNP A2/B1 in hippocampal neurons. Cultured hippocampal neurons (14-15 DIV) were stimulated with 100 ng/ml BDNF for 30 min in the presence or in the absence of the Trk receptor inhibitor SHN722 (1 µM) (Gomes et al., 2012; Martin et al., 2011). BDNF treatment resulted in a significant increase of hnRNP A2/B1 integrated intensity, area and puncta number in dendrites (Fig. 3.3A, B, C, and D) and in synaptic sites (Fig. 3.3A-white arrows, E, F, and G). Inhibition of Trk receptor activity with SHN722 induced a modest increase in hnRNP A2/B1 puncta area and integrated intensity in dendrites (Fig. 3.3A, B, and C). Nevertheless, further stimulation with BDNF had no effect in every parameter evaluated in both dendritic and synaptic hnRNP A2/B1 particles, showing that the effects of BDNF were mediated by Trk receptors. Taken together, our findings indicate that the neurotrophin BDNF induces a robust accumulation of hnRNP A2/B1 in dendrites and at the synapse in hippocampal neurons by activation of Trk (presumably TrkB) receptors.

Control experiments showed no significant changes in total hnRNP A2/B1 protein levels in cultured hippocampal neurons stimulated with BDNF or following synaptic activity, as determined by Western blot (supplementary Fig. 3.1), suggesting that effects observed on hnRNP A2/B1 distribution upon neuronal activation, or in response to BDNF treatment, are not due to a global upregulation of hnRNP A2/B1 protein in the cells

The dendritic transcriptome is not yet fully characterized but a recent study using deep RNA sequencing in microdissected synaptic neuropil (*stratum radiatum* and *lacunosum moleculare*) segments from the CA1 region of the adult rat hippocampus, allowed the identification of approximately 2550 mRNAs in dendrites and/or axons (Cajigas et al., 2012), including the hnRNP A2/B1 mRNA (Cajigas et al., 2012). Using a neuronal culture system that allows the mechanical separation of neurites from cell bodies (Manadas et al., 2009; Poon et al., 2006) we investigated the effect of BDNF on the levels of hnRNP A2/B1 transcripts in the two compartments. qRT-PCR experiments showed that BDNF treatment for 2 h significantly increased the hnRNP A2/B1 mRNA

in the soma but had no effect on the abundance of the transcript in the neurite compartment (Fig. 3.4).

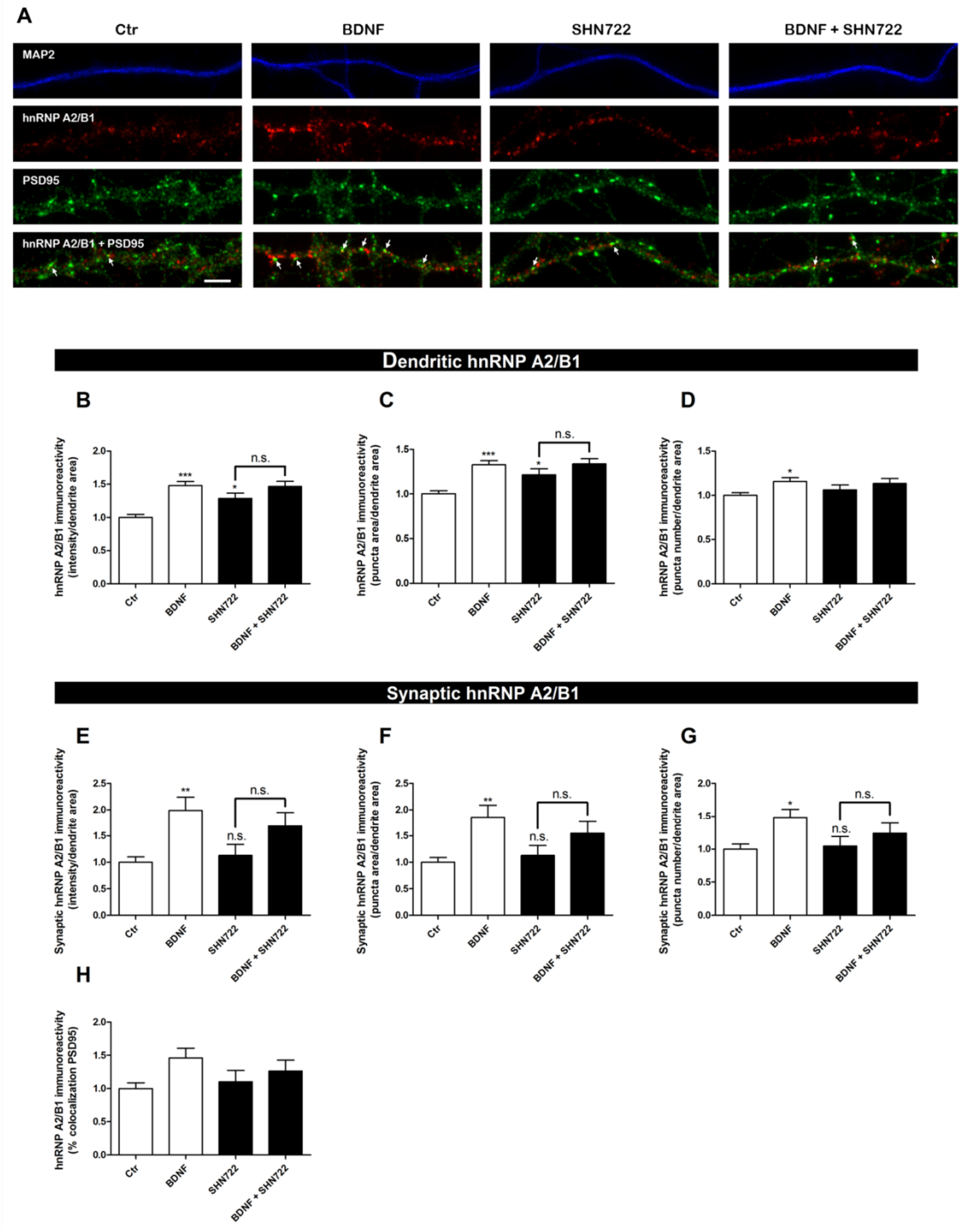


Figure 3.3 - BDNF induces the accumulation of hnRNP A2/B1 in dendrites and at the synapse in hippocampal neurons. Cultured hippocampal neurons (14-15 DIV) were stimulated or not with 100 ng/ml BDNF for 30 min. The role of Trk receptor activity in BDNF-induced regulation of hnRNP A2/B1 distribution was tested using the inhibitor SHN722. Where indicated, neurons were treated for 30 min with 1 µM SHN722 and then stimulated or not with 100 ng/ml BDNF in the presence of the inhibitor. The cells were immunostained for hnRNP A2/B1 (red), MAP2 (blue), and PSD95 (green) (A). White arrows indicate PSD95-positive clusters that also contain hnRNP A2/B1 (A). The integrated fluorescence intensity, area and number of hnRNP A2/B1 puncta in dendrites (B, C, and D) and at the synapse (as defined by the signal of hnRNP

A2/B1 that overlaps with PSD95) (E, F, and G) was analysed using ImageJ software and represented per dendritic area. The percentage of dendritic hnRNP A2/B1 signal that colocalizes with PSD95 was also analysed (H). Results are normalized to control and are the average of 3-6 different experiments performed in independent preparations. For the analysis of dendritic-localized hnRNP A2/B1 puncta, Ctr (n=73 cells); Bic (n=64 cells); TrkB-Fc (n=47 cells); TrkB-Fc + Bic (n=48 cells). For the analysis of synaptic-localized hnRNP A2/B1 puncta, Ctr (n=62 cells); Bic (n=67 cells); TrkB-Fc (n=37 cells); TrkB-Fc + Bic (n=35 cells). Error bars, mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni's test. n.s. Not significant, * P<0.05; ** P<0.01; *** P<0.001. Scale bar = 4 µm.

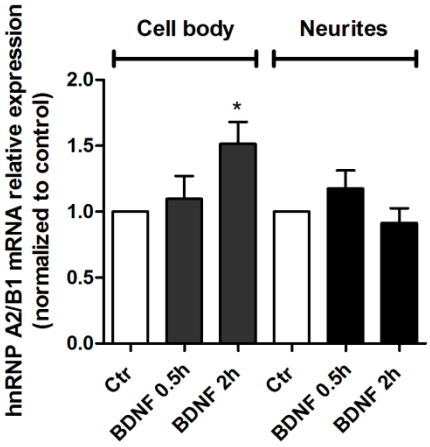


Figure 3.4 - BDNF upregulates hnRNP A2/B1 mRNA in the cell body compartment of hippocampal neurons. Cultured hippocampal neurons (14-15 DIV) were stimulated or not with 100 ng/ml BDNF, for 30 min or 2 h. The cell body mRNA was mechanically separated from the transcripts of neurites and 500-1000 ng of RNA from each compartment was used in the reverse transcription reaction. The analysis of hnRNP A2/B1 mRNA levels was performed by qRT-PCR using *Ppia* as internal control gene. The results are the average ± SEM of five (cell body compartment) or seven (neurite compartment) independent transcription reactions, performed in different preparations. Statistical analysis of logtransformed expression data was performed by one-way ANOVA, followed by Dunnet's test. * P< 0.05.

3.4. Discussion

In this work we show that hnRNP A2/B1 is partly colocalized with the RNA granule protein Staufen1 in dendrites of cultured hippocampal neurons and is also found at synapses. Furthermore, we found that hnRNP A2/B1 protein levels are rapidly increased in dendrites and synapses of hippocampal neurons upon neuronal activation or BDNF stimulation. Together, these results point to a tight regulation of the dendritic distribution of hnRNP A2/B1, and do not favor a model of constitutive delivery of the protein. This is particularly relevant since hnRNP A2/B1 is a *trans*-acting factor involved in the transport of several mRNAs along dendrites (Gao et al., 2008; Muslimov et al., 2006; Muslimov et al., 2011; Shan et al., 2003). BDNF plays an important role in the protein synthesis-dependent late phase of LTP induced by high-frequency stimulation in the hippocampus CA1 region (Chen et al., 1999; Kang and Schuman, 1996) and BDNF-induced

synaptic potentiation has also been reported, both *in vitro* (Kang and Schuman, 1996) and *in vivo* (Messaoudi et al., 2002; Ying et al., 2002).

The observed increase in the dendritic distribution and synaptic clustering of hnRNP A2/B1 in hippocampal neurons stimulated with BDNF, suggest that the neurotrophin may act, at least in part, by regulating the transport of mRNAs during plasticity-related events. In particular, the BDNF-induced clustering of hnRNP A2/B1 at the synapse may bring the Arc and CaMKII mRNAs (among others) that are locally translated and may contribute to the protein synthesis-dependent late phase of LTP. Accordingly, intrahippocampal infusion of BDNF resulted in the accumulation of Arc transcripts in dendrites and triggered long-term potentiation (BDNF-LTP) at medial perforant path-granule cell synapses *in vivo* (Messaoudi et al., 2007; Ying et al., 2002). Furthermore, exogenous application of BDNF is sufficient to induce the transport of A2RE-containing mRNAs, such as BDNF (Righi et al., 2000) and Arc (Rao et al., 2006) transcripts into dendrites. Although synaptic activity and BDNF stimulation increased hnRNP A2/B1 protein levels and clustering in dendrites, the effect of neuronal activity was insensitive to the presence of TrkB-Fc, indicating that it is not mediated by the release of endogenous BDNF. This contrasts with the synaptic delivery of the protein upon neuronal activation, which is likely to require the activation of TrkB receptors by BDNF. Further research is needed to clarify the differential mechanisms induced by synaptic activity and BDNF that promote the accumulation of hnRNP A2/B1 in dendrites.

Processing-bodies (P-bodies), which are typically involved in mRNA degradation or storage (Anderson and Kedersha, 2006; Kiebler and Bassell, 2006), are transported to synapses and release mRNAs upon synaptic activation (Oh et al., 2013). Several components of RNA transport granules, such as ZBP1 and FMRP, were localized in P-bodies in mammalian neurons (Cougot et al., 2008), supporting the hypothesis that these two types of granules may be structurally related. In contrast with previous findings (Zeitelhofer et al., 2008), a recent study raised the possibility that some of the P-bodies might contain Staufen1 in mammalian neurons (Oh et al., 2013). Since Staufen1 and hnRNP A2/B1 are likely to assemble in the same granules to some extent (Fig. 3.1A-white arrows, A') it will be important to investigate the nature and functional significance of these structures. Given the evidence reported here, it is tempting to speculate that Staufen1 and hnRNP A2/B1 might also constitute a pool of storage silent-foci that respond to neuronal

activation and BDNF, in addition to the role in dendritic mRNA transport. Interestingly, we found that Staufen1 is present in synaptic polysomal fractions (Fig. 3.1C) suggesting a role for this protein not only in the delivery but also in the translational control of synaptic-localized mRNAs. To our knowledge this is the first indication that Staufen1 may play a role in the translational control at the synapse in mammalian neurons. This is in agreement with the results showing the presence of Staufen proteins in polysomal fractions isolated from COS7 and HeLa cell lines (Luo et al., 2002; Marion et al., 1999), and with the role of Staufen1 in the translational control of mRNAs (Dugre-Brisson et al., 2005).

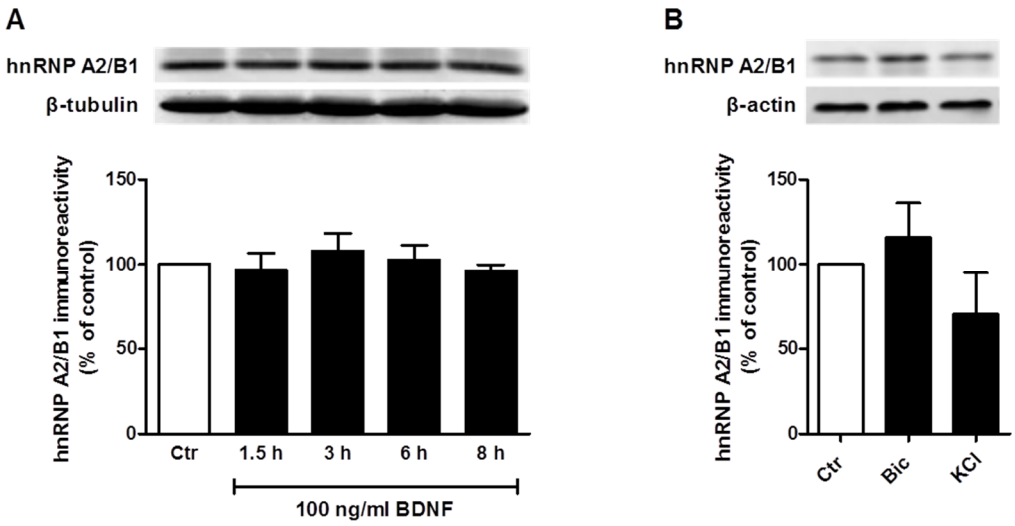
The fractionation studies showed that hnRNP A2/B1 is present in the mRNPs but not in the polysomal-fractions obtained from synaptoneurosomes isolated from rat hippocampus. Because polysomes are sites of active translation (Bagni et al., 2000; Weiler et al., 1997), these results suggest that although hnRNP A2/B1 plays a role in the delivery of mRNAs into synaptic sites it is unlikely to be present during active translation of the target mRNAs at the synapse. This contrasts with the role of hnRNP A2/B1 in oligodendrocytes, where the protein enhances the translation of A2RE-carrying mRNAs following phosphorylation by the Fyn kinase (Kosturko et al., 2006; White et al., 2008). On the other hand, the direct binding of hnRNPE1 to hnRNP A2/B1 apparently represses the translation of its target mRNAs (Kosturko et al., 2006).

Using a culture system that allows a physical separation of the soma and neurite compartment, we found that stimulation of cultured hippocampal neurons with BDNF increased the hnRNP A2/B1 mRNA only in the former compartment. The BDNF-induced upregulation of hnRNP A2/B1 mRNA in cell bodies may provide a layer to support the effect of the neurotrophin on the hnRNP A2/B1 protein localization in dendrites or even to contribute to the wide variety of functions that hnRNP A2/B1 plays in the nucleus. The lack of effect of BDNF on the dendritic levels of hnRNP A2/B1 mRNA contrasts with the effects observed for transcripts encoding several translation-related proteins (Manadas et al., 2009). Although the abundance of hnRNP A2/B1 mRNA in neurites remained unchanged following BDNF treatment, it is not possible to rule out the existence of subtle changes with a magnitude under the limit of sensitivity of the technique.

3.5. Conclusions

Overall, our study provides strong evidence supporting the dendritic accumulation of hnRNP A2/B1 in response to synaptic activity and upon BDNF treatment, most likely through independent and distinct mechanisms. Similarly, the activity-dependent BDNF-mediated synaptic delivery of hnRNP A2/B1 further suggests a role for hnRNP A2/B1 in local mRNA metabolism and is likely to play a role in plasticity-related events.

3.6. Supplementary Figures



Supplementary Figure 3.1 - hnRNP A2/B1 total protein levels are not significantly altered following synaptic activity or BDNF treatment in hippocampal neurons. (A) High-density primary hippocampal neurons cultured for 14-15 DIV were incubated with or without 100 ng/ml BDNF (1.5 h, 3 h, 6 h and 8 h) as indicated. Total hnRNP A2/B1 protein levels were detemined by Western blot and ß-tubulin was used as loading control. The results are the average ± SEM of 4-5 different experiments, performed in independent preparations. (B) High-density primary hippocampal neurons cultured for 14-15 DIV were stimulated or not with bicuculline (50 µM), 4-AP (2.5 mM) and glycine (10 µM), or depolarized with KCl (30 mM) for 30 min. Total hnRNP A2/B1 protein levels were determined by Western blot and ß-actin was used as loading control. The results are the average ± SEM of 3 independent experiments, performed in distinct preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnet's test.

Chapter 4

Regulation of hnRNP K and hnRNP K-associated mRNAs by BDNF and Synaptic Activity: *in vitro* and *in vivo* Evidence

The experiments shown on panel 4.5A were performed by Diogo Comprido

4.1. Summary

Dendritic protein synthesis plays a critical role in several forms of synaptic plasticity, including BDNF (brain-derived neurotrophic factor)-mediated long-term synaptic potentiation. Dendritic transcripts are typically transported in a repressed state as components of large ribonucleoprotein complexes, and then translated upon stimulation at, or near, activated synapses. hnRNP K is present in neuronal mRNPs but whether it is involved in the regulation of local mRNA metabolism at the synapse is unknown. Here we found that a fraction of hnRNP K colocalizes with Staufen1 in dendrites of cultured hippocampal neurons, suggesting that the two proteins may be found in the same granules. Neuronal activity and BDNF treatment increased hnRNP K levels in dendrites and induced the delivery of this protein to synaptic sites. Studies with hippocampal synaptoneurosomes showed that several hnRNP K-bound mRNAs, including the transcripts coding for GluA1, GluN1 and BDNF, are released from hnRNP K-containing mRNPs locally at the synapse in response to BDNF stimulation. Similarly, these mRNAs are differentially regulated during high-frequency (HFS)-induced long term potentiation (LTP) in the dentate gyrus in live anesthetized rats, an *in vivo* model of synaptic potentiation requiring the activation of TrkB receptors, presumably by BDNF. In additional studies we found that hnRNP K may interact with several RNA-binding proteins, which may also contribute to the alterations in hnRNP K interaction with mRNAs reported here. Taken together, these results show that hnRNP K and hnRNP K-associated mRNAs are regulated by synaptic activity and BDNF, both *in vitro* and *in vivo*, and suggest hnRNP K as an important regulator of neuronal function, particularly in BDNF-induced plasticity events.

4.2. Introduction

Experience-dependent changes in synapse structure and function are thought to underlie learning and memory formation (Kandel, 2001). The activity-dependent transport and translation of dendritic-localized mRNAs, with a concomitant change in the synaptic proteome, contribute to some of these modifications (Sutton and Schuman, 2006). The biochemical, structural and functional alterations resulting from local translation are required for several forms of synaptic

plasticity, including brain-derived neurotrophic factor (BDNF)-mediated long-term potentiation (LTP) (Leal et al., 2013).

Several transcripts are transported to dendrites and dendritic spines upon neuronal activation, including the mRNAs for Arc (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998; Steward and Worley, 2001), β-actin (Tiruchinapalli et al., 2003), CaMKIIα (Havik et al., 2003; Rook et al., 2000; Thomas et al., 1994), TrkB and BDNF (Tongiorgi et al., 1997). In addition, the accumulation of Arc and BDNF transcripts in dendrites is also induced by the neurotrophin BDNF (Messaoudi et al., 2007; Rao et al., 2006; Righi et al., 2000; Ying et al., 2002). Dendritic mRNAs are typically transported along dendrites in a repressed state, as components of large messenger ribonucleoprotein complexes (mRNPs). Upon docking at their final destination, the mRNA content present in these granules is released and translated in response to synaptic activation (Bramham and Wells, 2007). The neuronal mRNPs are heterogeneous structures composed by a wide variety of proteins, including the RNA-binding proteins hnRNP K and Staufen1 (Elvira et al., 2006; Kanai et al., 2004). Staufen1 is present in neuronal RNA granules that migrate along dendrites of hippocampal neurons (Kiebler et al., 1999; Kohrmann et al., 1999), regulating the transport of mRNA (Kanai et al., 2004; Tang et al., 2001). Although the role of hnRNP K in the regulation of several aspects of nucleic acids metabolism, such as transcription, splicing or translation (Bomsztyk et al., 2004) is well established, the function of the protein in the CNS is poorly understood. hnRNP K was shown to interact with Abelson-interacting protein 1 (Abi1) at postsynaptic sites and to modulate dendritic spine morphology in hippocampal neurons (Proepper et al., 2011). Furthermore, BDNF stimulation increases hnRNP K protein levels in synaptoneurosomes isolated from cortical neurons (Liao et al., 2007). Despite these reports, the possible role of hnRNP K in the transport and/or translation of dendritic-localized mRNAs remains to be investigated.

BDNF is released at the synapse upon high-frequency stimulation (Aicardi et al., 2004), and this neurotrophin is required for the maintenance of LTP (Figurov et al., 1996; Korte et al., 1998; Minichiello et al., 2002). Furthermore, BDNF application can induce protein synthesis-dependent synaptic potentiation *in vitro* (Kang and Schuman, 1996) and *in vivo* (Messaoudi et al., 2002). Here we show that BDNF stimulation and neuronal activity induce the rapid delivery of hnRNP K into synapses in cultured hippocampal neurons. Synaptic activity rapidly increases hnRNP K

protein levels in dendrites whereas prolonged treatment with BDNF is required to produce similar effects. We also found that several hnRNP K-associated mRNAs, such as the transcripts for GluN1, GluA1 and BDNF, are released from hnRNP K-containing mRNPs in synaptoneurosomes stimulated with BDNF. A similar effect was observed for some of these transcripts 30 min upon HFS-induced LTP in the medial perforant path-granule cell synapses in the dentate gyrus of live anesthetized rats. Importantly, we showed that activation of TrkB, presumably by BDNF, is required for synaptic potentiation in this *in vivo* model.

4.3. Results

4.3.1. Neuronal activity and BDNF stimulation upregulate the dendritic levels of hnRNP K

The available evidence suggests that hnRNP K is a component of neuronal mRNPs (Elvira et al., 2006) but the distribution of this protein in neurons remains to be investigated. Staufen1 is a well described *trans*-acting factor localized in dendritic RNA granules (Kiebler et al., 1999; Kohrmann et al., 1999) and is known to regulate the transport of mRNA (Kanai et al., 2004; Tang et al., 2001). This protein was shown to play a role in protein synthesis-dependent long-term synaptic potentiation in hippocampal pyramidal neurons (Lebeau et al., 2008). To investigate whether hnRNP K is present in dendritic-localized granules we stained cultured hippocampal neurons (14-15 DIV) using antibodies against hnRNP K, Staufen1, and the dendritic marker MAP2. The results show that hnRNP K exhibits a punctate distribution along dendrites (Fig. 4.1A) which is in agreement with the hypothesis of hnRNP K being a component of neuronal mRNPs involved in dendritic mRNA transport. Furthermore, hnRNP K often localizes in Staufen1-containing particles (Fig. 4.1A-white arrows). Colocalization analysis demonstrate that a significant fraction (19.77 ± 1.58%, n=30 cells from three independent preparations) of the dendritic hnRNP K signal colocalizes with Staufen1 (Fig. 4.1A') suggesting that hnRNP K and Staufen1 can assemble, to some extent, in the same mRNPs. However, a significant fraction of the puncta containing hnRNP K and Staufen1 did not colocalize.

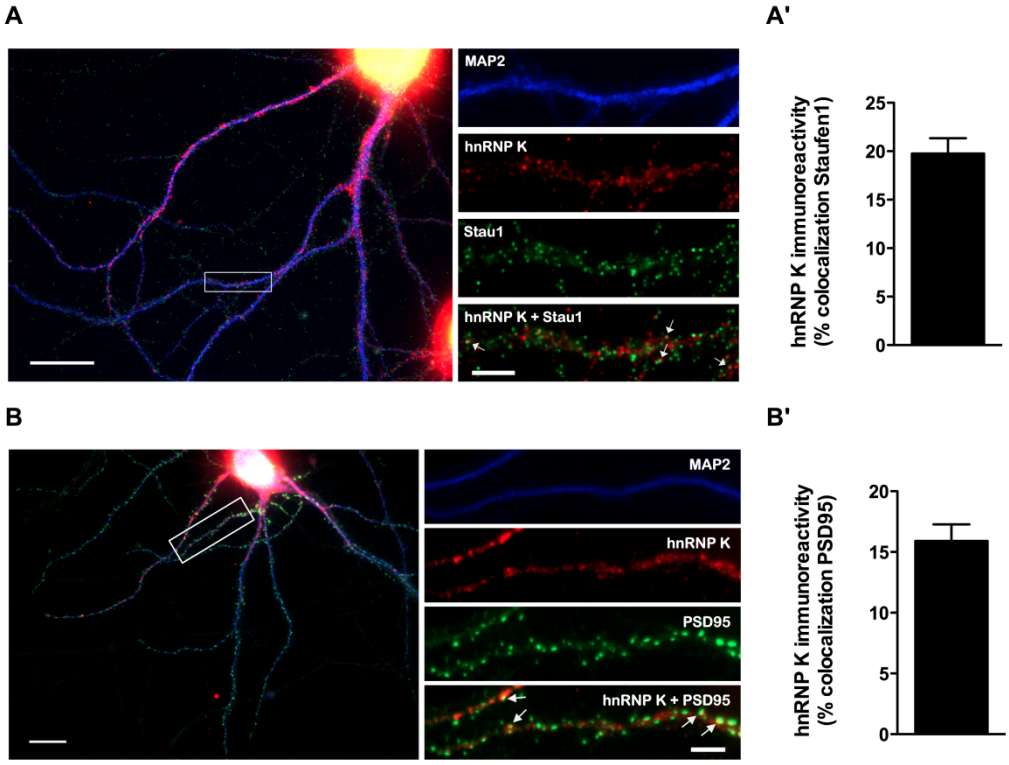


Figure 4.1 - hnRNP K is present in synaptic sites under resting conditions and colocalizes with Staufen1 in dendrites of hippocampal neurons. (A) Cultured hippocampal neurons (14-15 DIV) immunostained for hnRNP K (red), Staufen1 (green) and the dendritic marker MAP2 (blue) show that hnRNP K exhibits a punctate distribution along dendrites and may localize in particles that also contain Staufen1 (white arrows). Scale bars = 20 µm and 4 µm for low and high magnification images, respectively. The image is representative of 3 different experiments performed in independent preparations with a total of 30 cells analysed. (A') The fraction of dendritic hnRNP K signal that colocalizes with Staufen1 was analysed using ImageJ software. Error bars, mean ± SEM; (19.77 ± 1.58% n=30). (B) Cultured hippocampal neurons (14-15 DIV) immunostained for MAP2 (blue), hnRNP K (red) and PSD95 (green) show that hnRNP K is present in synaptic sites as indicated by the colocalization with the postsynaptic marker PSD95 (white arrows). Scale bars = 20 µm and 5 µm for low and high magnification images, respectively. The image is representative of 8 different experiments performed in independent preparations with a total of 80 cells analysed. (B') The percentage of dendritic hnRNP K signal that colocalizes with PSD95 was analysed using ImageJ software. Error bars, mean ± SEM (15.91 ± 1.35% n=80).

Dendritic-localized mRNAs are typically transported in a dormant state until the translational-block is relieved by synaptic activity (Bramham and Wells, 2007). To test if hnRNP K is present at the synapse, we analysed the colocalization with the postsynaptic marker PSD95, in cultured hippocampal neurons (14-15 DIV) (Fig. 4.1B; Fig. 4.1B'). A considerable fraction of dendritic hnRNP K (15.91 ± 1.35%; n=80 cells from eight independent preparations) localizes at PSD95-positive clusters (Fig. 4.1B-white arrows; Fig. 4.1B'). Taken together, these observations suggest that hnRNP K may localize in dendritic particles that also contain Staufen1 and is present in synaptic sites under resting conditions.

Several transcripts are transported to dendrites and dendritic spines upon neuronal activation, including the mRNAs encoding for Arc (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998; Steward and Worley, 2001), β-actin (Tiruchinapalli et al., 2003), CaMKIIα (Havik et al., 2003; Rook et al., 2000; Thomas et al., 1994), TrkB and BDNF (Tongiorgi et al., 1997). Therefore, we investigated if synaptic activity changes the levels of hnRNP K in dendrites and regulates the delivery of the protein into synaptic sites. For that purpose, we stimulated cultured hippocampal neurons (14-15 DIV) for 30 min with a cocktail solution with bicuculline to increase the excitatory activity of the neuronal network (Hardingham et al., 2002). Bicuculline treatment significantly increased the integrated intensity, as well as the number and area of hnRNP K puncta in dendrites (Fig. 4.2A, B, C, D), and the intensity of hnRNP K clusters in synaptic sites (Fig. 4.2A-white arrows, I).

We next questioned whether the effects observed on hnRNP K distribution upon neuronal activation require changes in total hnRNP K protein levels. For this purpose, cultured hippocampal neurons (14-15 DIV) were stimulated with the cocktail solution that contain bicuculline to increase synaptic activity, or with KCl (30 mM), and the hnRNP K protein levels were determined by Western blot. Interestingly, bicuculline stimulation did not change hnRNP K total protein levels (Fig. 4.3), in contrast with the effect of KCl depolarization which decreased total hnRNP K protein levels (Fig. 4.3). These results indicate that effects of bicuculline stimulation of hnRNP K protein levels in dendrites are not due to a global upregulation of the ribonucleoprotein in the cells.

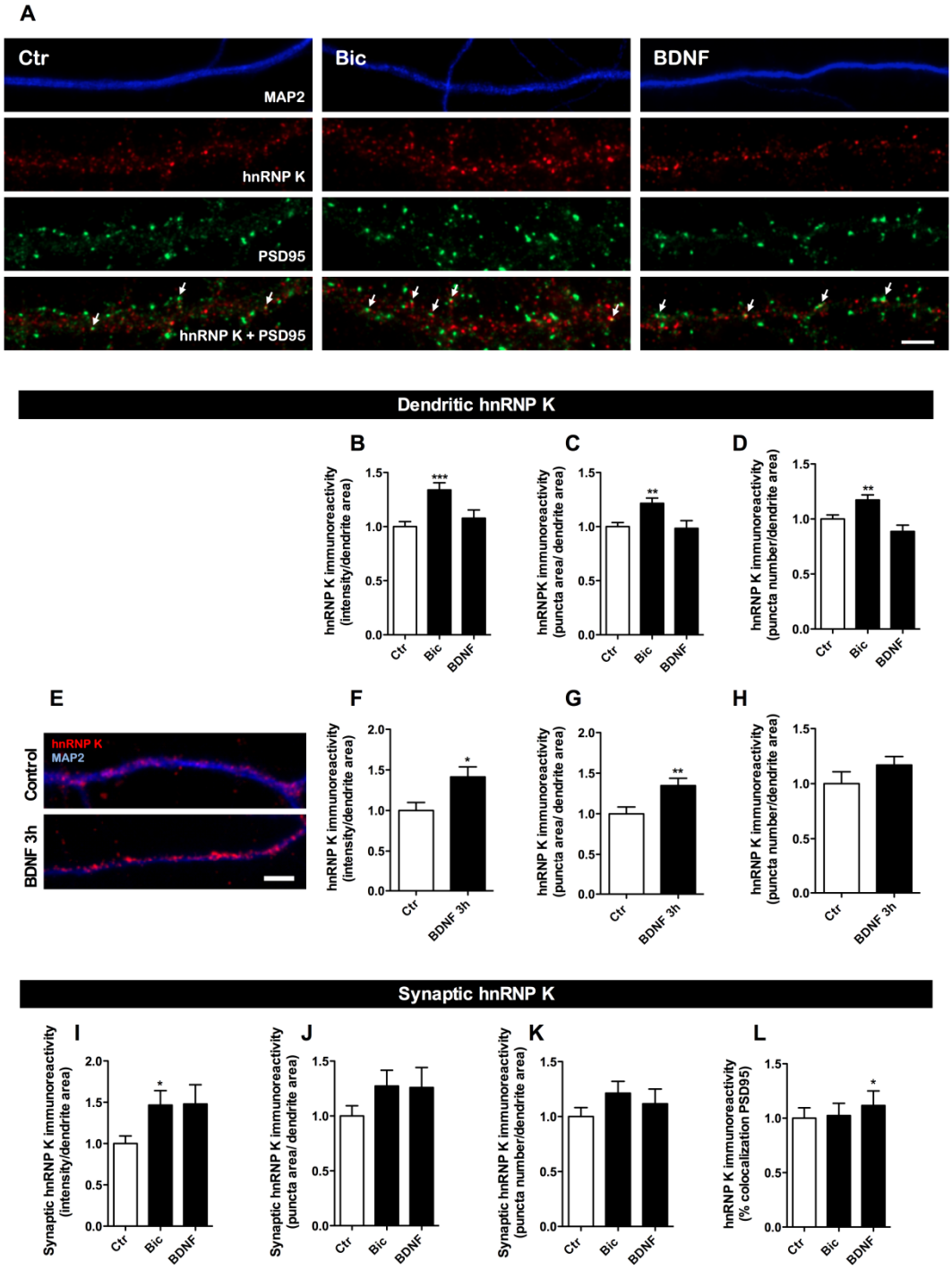


Figure 4.2 - Synaptic activity induces the accumulation of hnRNP K in dendrites and the delivery of the protein into synaptic sites. Cultured hippocampal neurons (14-15 DIV) were stimulated or not with 100 ng/ml BDNF or with bicuculline (50 µM), 4-AP (2.5 mM) and glycine (10 µM), for 30 min. The cells were immunostained for hnRNP K (red), MAP2 (blue), and PSD95 (green) (A). White arrows indicate PSD95-positive clusters that also contain hnRNP K (A). The fluorescence intensity, area and the number of hnRNP K puncta in dendrites (B, C and D) and at the synapse (as defined by the signal of hnRNP K that overlaps with PSD95) (I, J and K) was analysed using ImageJ software and represented per dendritic area. The percentage of dendritic hnRNP K signal that colocalizes with PSD95 was also analysed (L). Results are normalized to control and are the average of five (Ctr and Bic) or three (BDNF) independent experiments performed in different preparations. Ctr (n=47 cells); Bic (n=50 cells); BDNF (n=22 cells). Error bars, mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by Dunnet's test.* P<0.05; ** P<0.01;

*** P<0.001. Scale bar = 4 µm. (E) Cultured hippocampal neurons (14-15 DIV) were stimulated or not with 100 ng/ml BDNF for 3 h, and immunostained for hnRNP K (red) and MAP2 (blue). The images are representative of two different experiments performed in independent preparations with a total of 18 (Ctr) and 23 (BDNF 3 h) cells analysed. Scale bar = 4 µm. (F, G and H) The integrated intensity, area and number of hnRNP K puncta in dendrites was analysed using ImageJ software. Error bars, mean ± SEM (Ctr n=18; BDNF 3 h n=23). Statistical analysis was performed using unpaired Student's *t* test. * P<0.05; ** P<0.01.

The neurotrophin BDNF plays a key role in several forms of synaptic plasticity (Waterhouse and Xu, 2009). Some of the actions of BDNF in the CNS rely, in part, on the ability of this neurotrophin to change the synaptic proteome by inducing the delivery of dendritic-localized transcripts and by promoting local protein synthesis at the synapse (Leal et al., 2013; Santos et al., 2010). BDNF is stored in vesicles of the regulated secretory pathway, being released in response to synaptic activity (Kuczewski et al., 2009; Lessmann and Brigadski, 2009). Thus, we next investigated whether BDNF regulates the dendritic and synaptic distribution of hnRNP K in hippocampal neurons. To do so, we stimulated cultured hippocampal neurons (14-15 DIV) with BDNF (100 ng/ml) for 30 min. We found that BDNF treatment under these conditions has no effect on the levels of hnRNP K in dendrites (Fig. 4.2A, B, C, D). However, incubation of the cells with BDNF for 30 min slightly increased the hnRNP K integrated intensity at the synapse (although it did not reach statistical significance), the number of synaptic puncta and the percentage of hnRNP K in synaptic sites (Fig. 4.2A-white arrows, I, L), although statistical significance was obtained only under the latter conditions. These results suggest that short-term stimulation with BDNF specifically modulates hnRNP K at the synapse. Interestingly, longer periods of stimulation with BDNF (3 h) resulted in a significant increase in the integrated intensity and area of hnRNP K-containing particles in dendrites (Fig. 4.2E, F, G), showing a differential regulation of hnRNP K distribution by transient or sustained activation of TrkB receptors.

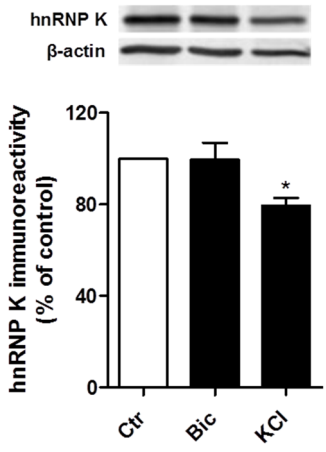


Figure 4.3 - hnRNP K total protein levels are downregulated upon KCl depolarization in hippocampal neurons. High-density primary hippocampal neurons cultured for 14-15 DIV were stimulated or not with bicuculline (50 µM), 4-AP (2.5 mM) and glycine (10 µM), or depolarized with KCl (30 mM) for 30 min. Total hnRNP K protein levels were assessed by Western blot and β-actin was used as loading control. The results are the average ± SEM of 3 independent experiments, performed in different preparations. Statistical analysis was performed by one-way ANOVA, followed by Dunnet's test. * P< 0.05

The dendritic transcriptome is not yet fully characterized but a recent study using deep RNA sequencing in microdissected synaptic neuropil (*stratum radiatum* and *lacunosum moleculare*) segments from the CA1 region of the adult rat hippocampus allowed the identification of approximately 2550 mRNAs in dendrites and/or axons (Cajigas et al., 2012), including the hnRNP K mRNA (Cajigas et al., 2012). Using a neuronal culture system that allows the mechanical separation of neurites from cell bodies (Manadas et al., 2009; Poon et al., 2006) we investigated the effect of BDNF on the levels of hnRNP K transcripts in the two compartments. qRT-PCR experiments showed that BDNF treatment (30 min and 2 h) had no significant effect on the abundance of hnRNP K mRNA in both cell body- and neurite-compartments (Fig. 4.4). Therefore, the delayed effects of BDNF on the hnRNP K protein levels in dendrites cannot be attributed to the transport of new transcripts to this compartment.

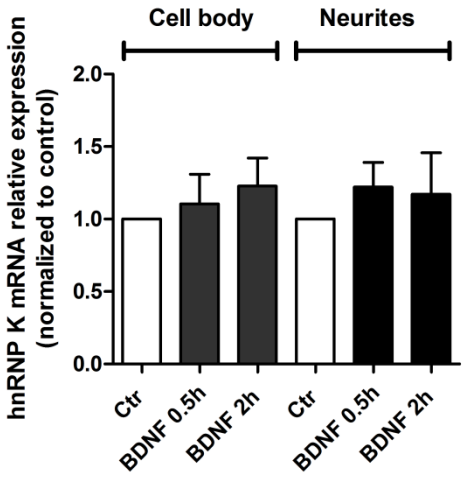


Figure 4.4 - BDNF stimulation does not change hnRNP K mRNA levels in the cell body and neurite compartments. Cultured hippocampal neurons (14-15 DIV) were stimulated or not with 100 ng/ml BDNF, for 30 min or 2 h. The cell body and neurite mRNA was isolated after mechanical separation of the two compartments, and 500-1000 ng of RNA was used in each case for the reverse transcription reaction. The analysis of hnRNP K mRNA levels was performed by qRT-PCR using *Ppia* as internal control gene. The results are the average ± SEM of five (cell body compartment) or seven (neurite compartment) independent transcription reactions, performed in different preparations. Statistical analysis of logtransformed expression data was performed by one-way ANOVA, followed by Dunnet's test.

4.3.2. BDNF stimulation downregulates the interaction of hnRNP K with different mRNAs in hippocampal synaptoneurosomes

Previous studies from our laboratory used RNP immunoprecipitation assays (RIP) followed by DNA microarray to identify the transcripts associated with hnRNP K in hippocampal neurons. This study identified thousands of hnRNP K-associated transcripts, including mRNAs encoding proteins with well established roles in synaptic plasticity such as GluN1, GluA1, CamKIIβ or BDNF (Comprido, 2011). A similar approach was used to evaluate the effect of BDNF stimulation on the hnRNP K-bound transcripts in hippocampal neurons and the results showed that 51% of hnRNP K-associated transcripts were regulated by BDNF. The large majority (99.9%) of these mRNAs were partially released within 10 min of stimulation with the neurotrophin. This clearly shows a massive effect of BDNF on the dissociation of mRNAs from hnRNP K and/or hnRNP K-associated proteins (Comprido, 2011).

Since hnRNP K may be localized at synaptic sites under resting conditions (Fig. 4.1B) (Proepper et al., 2011), we next tested if BDNF regulates the association of hnRNP K with target mRNAs locally at the synapse. For that purpose, synaptoneurosomes isolated from adult rat hippocampus were stimulated with 50 ng/ml BDNF for 10 min and the levels of transcripts co-

immunoprecipitated with hnRNP K were evaluated through qRT-PCR. We found that BDNF decreases the amount of GluA1, GluN1, CamKIIβ, hnRNP K and BDNF mRNAs co-immunoprecipitated with hnRNP K (Fig. 4.5A) suggesting that, as previously observed at the whole cell level, BDNF also induces the dissociation of mRNAs from hnRNP K-containing complexes at synaptic sites. These findings strongly suggest a role for hnRNP K in the regulation of mRNA metabolism locally at the synapse.

The hnRNP K protein interactome is vast and has been studied in several biological contexts (Bomsztyk et al., 2004; Mikula et al., 2006), but so far no proteomic study was performed in the CNS. Some of the hnRNP K-associated transcripts identified in cultured hippocampal neurons (Comprido, 2011) may in fact interact with hnRNP K binding proteins, and the effects of BDNF may reflect alterations in the interaction of those mRNAs with hnRNP K binding partners. To characterize the hnRNP K binding partners in the nervous system we used a proteomic approach, and the RNP was immunoprecipitated from the adult rat hippocampus. Figure 4.5B shows hnRNP K immunoprecipitation from total extracts of adult rat hippocampus. The immunoprecipitates were separated through SDS-PAGE and the proteins in the gel were stained using Silver (Fig. 4.5C) or colloidal Coomassie (data not shown) staining methods. We found that hnRNP K immunoprecipitation pulled-down several proteins that were absent in the control immunoprecipitation performed with IgG (Fig. 4.5C-blue arrows). The lanes corresponding to the hnRNP K or IgG immunoprecipitations were excised from the gel and further analysed by LC-MS/MS analysis. Only proteins that were not present in the control immunoprecipitation performed with IgG and with a minimum of two peptides (with eight or more amino acids) were considered hnRNP K binding partners and used for further analysis.

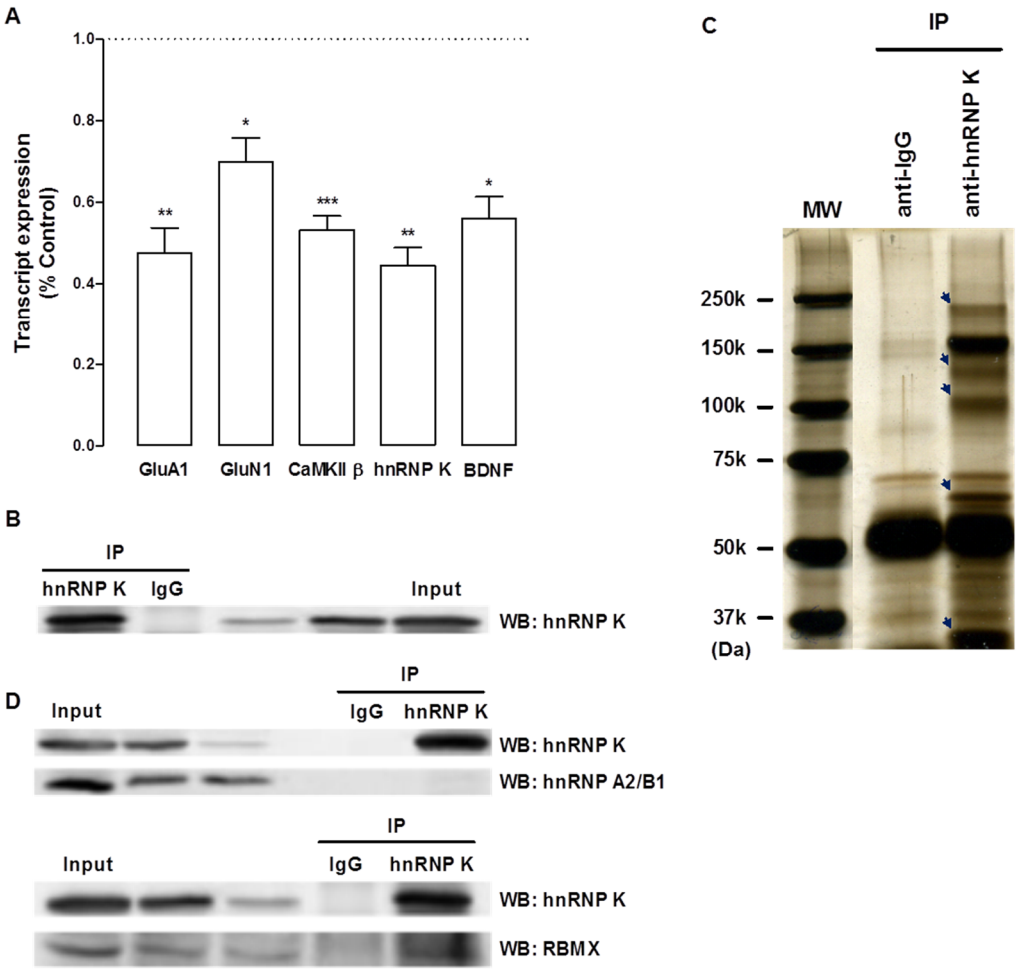


Figure 4.5 - BDNF induces the release of hnRNP K-associated mRNAs at the synapse. (A) Synaptoneurosomes isolated from rat hippocampus were stimulated or not with BDNF for 10 min and hnRNP K was immunoprecipitated using equal amounts of synaptoneurosomal extracts. The levels of GluA1, GluN1, CaMKIIβ, hnRNP K and BDNF transcripts co-immunoprecitated with hnRNP K were assayed by qRT-PCR. The results are presented as mean ± SEM normalized to non-stimulated synaptoneurosomes and are the average ± SEM of 4 independent experiments performed in 4 different transcription reactions. ** P<0.01; * P<0.05; *** P<0.001; as determined by the Student's *t*-test. (B) hnRNP K immunoprecipitation from total rat hippocampus homogenates (1 mg). Western blot analysis of the immunoprecipitates shows that hnRNP K is detected in the corresponding immunoprecipitation (IP) but not in the control IP performed with IgG. (C) Several proteins are specifically pulled-down by hnRNP K in co-immunoprecipitation experiments. The immunoprecipitates were subjected to a gel electrophoresis followed by in-gel protein detection by Silver staining. Immunorecipitation of hnRNP K pulled-down several proteins (blue arrows) that were absent in the control IP performed with IgG. In parallel experiments, the gels were stained with colloidal Coomassie and the bands were excised and processed for LC-MS/MS analysis. (D) hnRNP K interacts with RBMX (hnRNP G) in rat hippocampus. hnRNP K was immunoprecipitated from hippocampal homogenates. The immunoprecipitates were separated through SDS-PAGE and immunoblotted using antibodies against hnRNP K, hnRNP A2/B1, and RBMX. Precipitation of hnRNP K also pulled-down RBMX (bottom). The interaction between hnRNP K and hnRNP A2/B1 (suggested by LC-MS/MS data) was not confirmed (top).

The proteins co-immunoprecipitated with hnRNP K and identified by LC-MS/MS were analysed with the GoMiner tool that uses the GO (Gene Ontology) algorithm. This approach allows grouping the proteins according to their function and/or localization. We identified a vast number

of hnRNPs as interactors of hnRNP K (see Table 4.1). Interestingly, the majority of the proteins identified are also RNA-binding proteins involved in several aspects of RNA metabolism (Table 4.1), and some of those were previously described as hnRNP K interactors in rat hepatoma cells (Mikula et al., 2006). We did not detect any of the described kinases that associate with hnRNP K (Bomsztyk et al., 2004), similarly to the results previously reported in rat hepatoma cells (Mikula et al., 2006). A wide variety of ribosomal proteins were found to interact with hnRNP K (Table 4.1) (Mikula et al., 2006). Our results show new putative binding partners for hnRNP K, such as the GTPase-activating protein Asap1 (ArfGAP with SH3 domain, ankyrin repeat and PH domain 1), Camk2a (calcium/calmodulin-dependent protein kinase II alpha) and Syngr1 (Synaptogyrin-1), which were grouped into cytoskeleton and/or synaptic regulation proteins (Table 4.1), suggesting that indeed hnRNP K is very likely to have a role in synaptic RNA metabolism and/or cytoskeleton regulation.

Table 4.1 - hnRNP K-interacting proteins identified by LC-MS/MS.

hnRNP K interacting proteins							
hnRNPs	**RNA binding**	**RNA splicing**	**RNA stability**	**Other RNA regulators**	**Ribosome structure**	**Cytoskeleton regulation**	**Synapse**
Hnrnpa2b1	Elavl2	Dhx9	Hnrnpu	Spen	Mrpl15	Fhdc1	Camk2a
Hnrnpab	Ilf2	Hnrnpa2b1	Dhx9	Xrn2	Mrpl23	Macf1	Slc1a2
Hnrnpc	Ilf3	Hnrnpc			Mrpl28	Syne1	Slc1a3
Hnrnpd	Matr3	Hnrnpl			Mrpl37	Actn3	Syngr1
Hnrnph1	Dhx9	Hnrnpr			Mrpl38	Asap1	Nsf
Hnrnpl	Pabpc1	Hnrnpu			Mrpl39	Calm3	Syne1
Hnrnpr	Tert	Pabpc1			Mrps2		
Hnrnpu		Pcf11			Mrps22		
Hnrnpul2		Sart1			Mrps30		
Hnrpdl		Srrm1			Mrps9		
Rbmxrt							

Proteins are named with their gene symbols and grouped according to their function and/or localization, as determined with the GoMiner tool that uses the GO (Gene Ontology) algorithm.

To validate this proteomic study we performed immunoprecipitation experiments with the anti-hnRNP K antibody followed by Western blot analysis of the immunoprecipitates. This approach allowed the detection of RBMX (hnRNP G) in the hnRNP K co-immunoprecipitates (Fig. 4.5D-bottom). However, we could not confirm the interaction between hnRNP K and hnRNP A2/B1 (Fig. 4.5D-top).

4.3.3. HFS-induced LTP in the dentate gyrus differentially modulates the interaction of hRNP K with the transcripts

Given the evidence here reported, we next questioned whether hnRNP K and hnRNP K-associated mRNAs are regulated by synaptic activity *in vivo*. To address this question we used a model of HFS-induced LTP in the dentate gyrus of live anesthetized rats. The LTP at medial perforant path-granule cell synapses induced by HFS prompt the transport of Arc (Messaoudi et al., 2007; Panja et al., 2009; Steward et al., 1998) and Matrix Metalloproteinase 9 (MMP9) (Dziembowska et al., 2012) mRNAs into dendrites. Intrahippocampal infusion of BDNF also resulted in the accumulation of Arc transcripts in dendrites and triggered long-term potentiation (BDNF-LTP) at medial perforant path-granule cell synapses *in vivo* (Messaoudi et al., 2002; Ying et al., 2002).

The medial perforant path fibers in the angular bundle were unilaterally stimulated and the evoked field excitatory postsynaptic potential (fEPSP) was recorded in the hilar region of the dentate gyrus (Fig. 4.6A, B). LTP was induced by spaced stimulation consisting of three sessions of HFS (4 trains of 400 Hz, eight pulses) with 5 min between sessions. This paradigm induces a robust and sustained increase in the fEPSP (Fig. 4.6C). The fEPSP is presented in percentage change from the baseline (Fig. 4.6C) and represents the slope measured between two points in the fEPSP (see methods for more details). To test whether TrkB signaling mediates the HFS-induced LTP *in vivo* in the dentate gyrus, we took advantage of TrkB-Fc, an effective extracellular scavenger of TrkB ligands which has been widely used to access BDNF function in the CNS. Intrahippocampal infusion of TrkB-Fc (1 µl, 100 µg, 12.5 min) prior to HFS induces a selective impairment of HFS-induced increase in the evoked fEPSP measured in the hilar region of the dentate gyrus (Fig. 4.6D), indicating that TrkB signaling is required for the induction of this form of synaptic potentiation. Conversely, infusion of IgG-Fc (1 µl, 100 µg, 12.5 min) did not change significantly the non-decremental enhancement of the fEPSP induced by HFS (Fig. 4.6D).

To investigate the effect of LTP induction in the interaction of hnRNP K with mRNAs the dentate gyrus was dissected 30 min after HFS as described above (at the end of experiments shown in Fig. 4.6C), and the tissue was processed for Western blot and co-immunoprecipitation assays. We evaluated putative changes in the interaction of hnRNP K with the mRNAs for GluA1, GluN1, hnRNP K and BDNF (two distinct sets of primers), and the effect of HFS on the total

content of the same transcripts in dentate gyrus homogenates. The effect of HFS-induced LTP in both total (Fig. 4.7A) and co-immunoprecipitated (Fig. 4.7B) RNAs was determined by qRT-PCR. HFS resulted in a massive increase in the total abundance of BDNF mRNA (Fig. 4.7A) and significantly increased total GluN1 mRNA levels (Fig. 4.7A), but no effect was observed for the total GluA1 and hnRNP K mRNA content ($p>0.05$). HFS decreased the hnRNP K mRNA levels associated with hnRNP K protein (Fig. 4.7B) suggesting that, as previously demonstrated in response to BDNF stimulation, synaptic activity induces the dissociation of hnRNP K-containing complexes. Interestingly, BDNF mRNA levels are increased in the co-immunoprecipitates 30 min post-HFS (Fig. 4.7B), but this is likely to be related with the massive increase in the total number of transcripts induced by synaptic potentiation (Fig. 4.7A). HFS also induced a small decrease in the interaction of hnRNP K with GluN1 mRNA, and slightly upregulated the interaction with GluA1 transcripts, but these effects were not statistically significant. Control experiments showed no changes in total hnRNP K protein levels 30 min post-HFS (Fig. 4.7C) as determined by Western blot, indicating that the differences observed in the interaction with mRNAs are not due to alterations in the total amount of protein expressed.

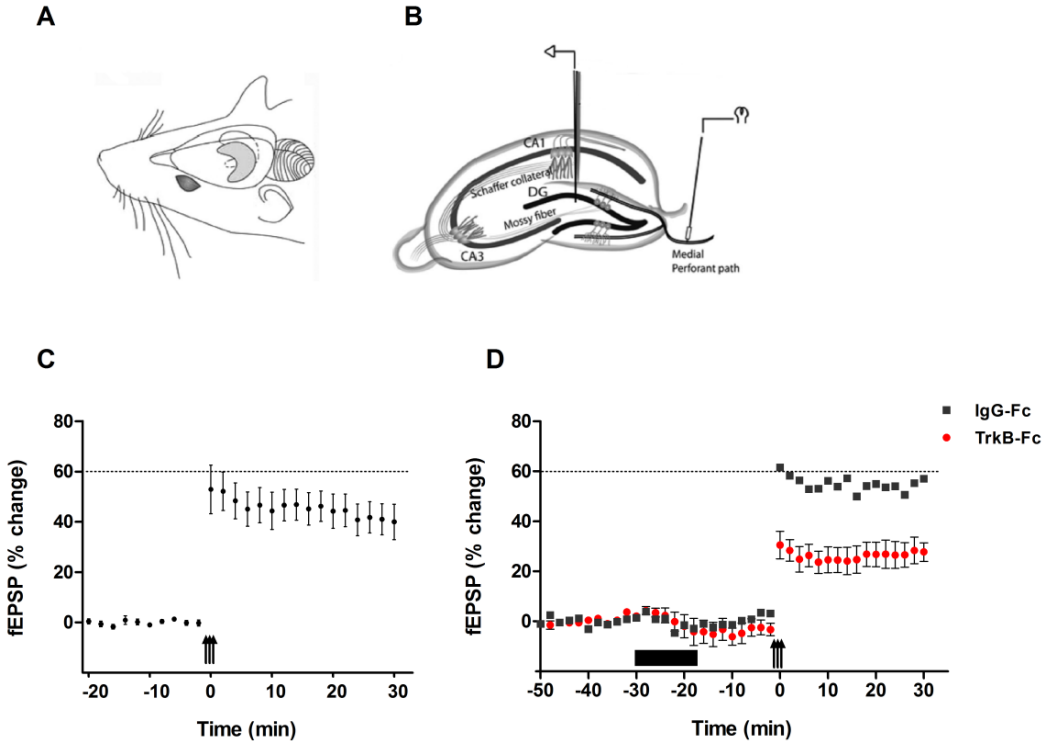


Figure 4.6 - TrkB activation is required for the induction of HFS-induced LTP in the dentate gyrus *in vivo*. Experiments were performed in live anesthetized rats. (A) The schematic represents the hippocampus on the left and right side of the forebrain. (B) The electrodes were positioned for selective unilateral stimulation of the medial perforant path fibers in the angular bundle and for recording the evoked field potentials in the hilar region of the dentate gyrus. (C) Time course plots showing changes in the medial perforant path-evoked fEPSP slope expressed in percentage of the baseline. Values are means ± SEM. Test pulses were applied at 0.033 Hz. The HFS paradigm (indicated by arrows) consisted of eight pulses of 400 Hz, repeated four times at 10 s intervals. Three sessions of HFS were given at intervals of five min. n=6 for each time point. (D) Time course plots showing changes in the medial perforant path-evoked fEPSP in rats receiving TrkB-Fc (1µl, 100 µg, 12.5 min) or IgG-Fc (1µl, 100 µg, 12.5 min) infusion before HFS (arrows). Values are means ± SEM and are expressed in percentage of the baseline. Test pulses were applied at 0.033 Hz. HFS was applied in three series of 400 Hz bursts separated by 5 min. TrkB-Fc (1µl, 100 µg) or IgG-Fc (1µl, 100 µg) were infused in the dorsal dentate gyrus at 0.08 µl/min during the period indicated by the black bar. Test pulses were not given during the HFS period. n=5 (TrkB-Fc infusion); n=1 (IgG-Fc infusion). A and B were adapted from (Panja et al., 2009).

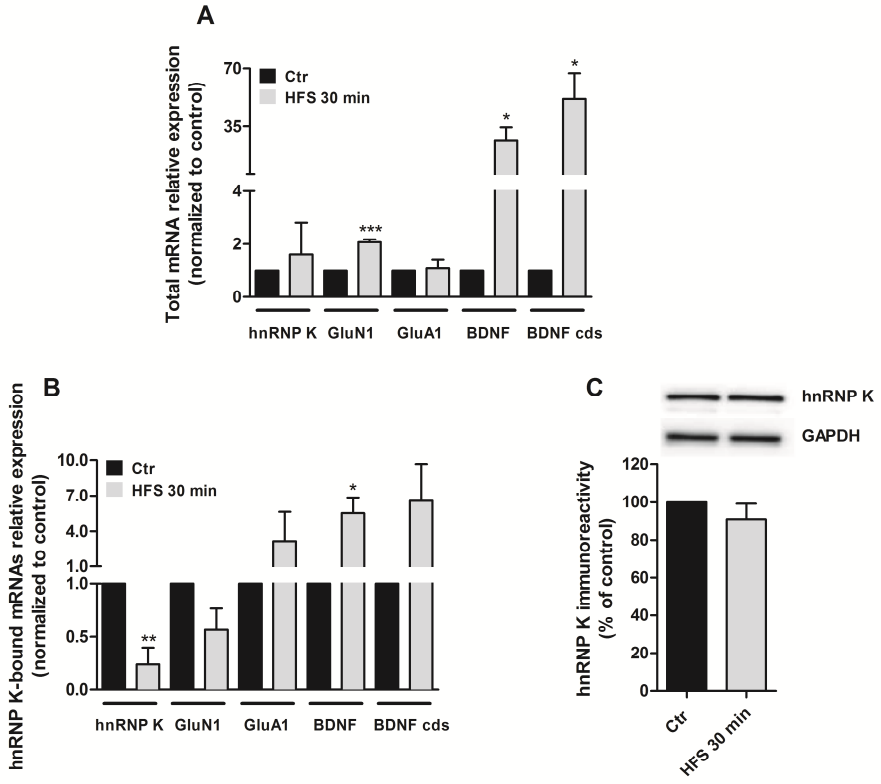


Figure 4.7 - hnRNP K-associated mRNAs are differentially regulated during HFS-induced LTP in the dentate gyrus *in vivo*. (A) The variation of hnRNP K, GluA1, GluN1 and BDNF mRNA levels was assayed by qRT-PCR using total RNA samples obtained from dentate gyrus homogenates collected 30 min post-HFS (at the end of the experiments shown in Fig. 4.6C) and the non-stimulated contralateral control tissue. For the reverse transcription, 500 ng of total RNA was used. The results are presented as mean ± SEM normalized to the contralateral non-stimulated dentate gyrus and *Hprt1* (hypoxanthine guanine phosphoribosyl transferase 1) was used as internal control gene. Results are the average ± SEM of 6 experiments (n=6 DG) analysed in 3 independent preparations (2 DG were analysed together in each preparation). Two distinct sets of primers were used for BDNF (BDNF and BDNF cds). *** P<0.001; * P<0.05; as determined by the Student's *t*-test. (B) The levels of hnRNP K, GluA1, GluN1 and BDNF transcripts co-immunoprecitated with hnRNP K were assayed by qRT-PCR. hnRNP K protein was immunoprecipitated from equal amounts (500 µg) of total extracts from homogenized dentate gyrus collected 30 min post-HFS (at the end of the experiments shown in Fig. 4.6 C) and the contralateral tissue. 500 ng of co-immunoprecipitated RNA was used in the reverse transcription reaction. The results are presented as mean ± SEM normalized to the contralateral dentate gyrus and are the average ± SEM of 6 experiments (n=6 DG), as described above. Two distinct sets of primers were used for BDNF (BDNF and BDNF cds). ** P<0.01; * P<0.05; as determined by the Student's *t*-test. (C) hnRNP K protein levels were measured by Western blot using dentate gyrus homogenate samples collected 30 min post-HFS (at the end of the experiments shown in Fig. 4.6C) and the contralateral non-stimulated tissue. The results are the average ± SEM of 6 experiments (n=6 DG), as described above, and are presented as the percentage change in hnRNP K protein levels in the treated dentate gyrus relative to the non-stimulated contralateral tissue. GAPDH was used as loading control.

Using a similar approach we evaluated how hnRNP K and hnRNP K-bound mRNAs are regulated 2 h upon LTP induction in the medial perforant path-granule cell synapses. We observed that LTP induced by HFS was sustained for two hours (Fig. 4.8A). The levels of total (Fig. 4.8B) and co-immunoprecipitated (Fig. 4.8C) mRNA for hnRNP K, GluA1, GluN1, and BDNF

2h post-HFS were accessed by qRT-PCR. We observed a significant decrease in total GluA1 mRNA levels and a striking increase in BDNF mRNA abundance (Fig. 4.8B), although in this case it did not reach statistical significance. All the co-immunoprecipitated mRNAs were apparently increased 2 h post-HFS (Fig. 4.8C), although the effects were not statistically significant, suggesting that the hnRNP K-bound mRNAs are differentially regulated during different stages of HFS-LTP. Western blot analysis of total extracts from dentate gyrus total homogenates showed that hnRNP K total protein levels did not change 2 h post-HFS (Fig. 4.8D), indicating that the differences observed in the interaction with mRNAs are not due to alterations in the total amount of protein expressed.

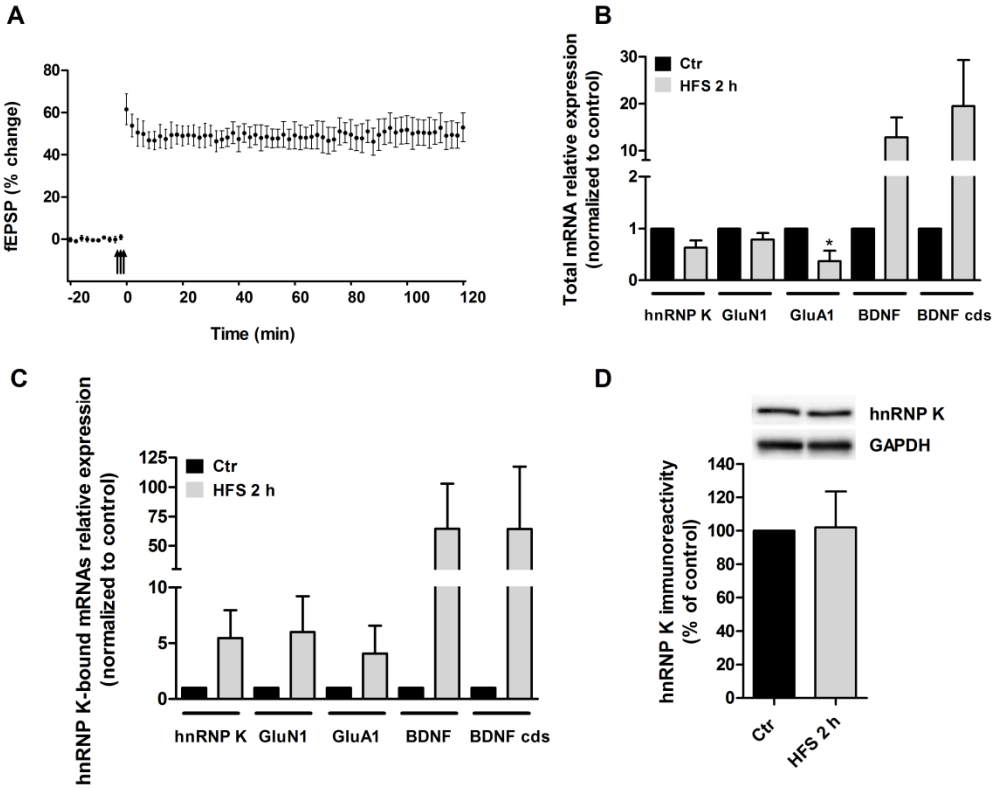


Figure 4.8 - Regulation of hnRNP K-bound mRNAs at 2 h after induction of LTP in the dentate gyrus synapses *in vivo*. (A) Time course plots showing changes in the medial perforant path-evoked fEPSP slope before and after HFS. Values are means ± SEM and are represented as percentage from baseline. Test pulses were applied at 0.033 Hz. HFS (indicated by arrows) was applied in three series of 400 Hz bursts separated by 5 min. n=6 for each time point. (B) The variation of hnRNP K, GluA1, GluN1 and BDNF mRNA levels was assayed by qRT-PCR of total RNA samples obtained from dentate gyrus homogenates collected 2 h post-HFS (at the end of experiments shown in A) and from the contralateral non-stimulated tissue. For the reverse transcription, 500 ng of total RNA was used. The results are presented as mean ± SEM normalized to the contralateral non-stimulated dentate gyrus and *Hprt1* (hypoxanthine guanine phosphoribosyl transferase 1) was used as internal control gene. Results are the average ± SEM of 6 experiments (n=6 DG) analysed in 3 independent preparations (2 DG were analysed together in each preparation). Two distinct sets of primers were used for BDNF (BDNF and BDNF cds). * P<0.05 as

determined by the Student's *t*-test. (F) The levels of hnRNP K, GluA1, GluN1 and BDNF transcripts co-immunoprecitated with hnRNP K were assayed by qRT-PCR. hnRNP K protein was immunoprecipitated from equal amounts (500 µg) of total extracts from homogenized dentate gyrus collected 2 h post-HFS (at the end of the experiments shown in A) and from the non-stimulated contralateral tissue. 500 ng of co-immunoprecipitated RNA was used in the reverse transcription reaction. The results are presented as mean ± SEM normalized to the contralateral dentate gyrus and are the average ± SEM of 6 experiments (n=6 DG), as described above. Two distinct sets of primers were used for BDNF (BDNF and BDNF cds). (D) hnRNP K protein levels were measured by Western blot using dentate gyrus homogenate samples collected 2 h post-HFS (at the end of the experiments shown in A) and from the contralateral non-stimulated tissue. The results are the average ± SEM of 6 independent experiments (n=6 DG), as described above, and are presented as the percentage of change in hnRNP K expression in the treated dentate gyrus relative to the non-stimulated contralateral tissue. GAPDH was used as loading control.

4.4. Discussion

Herein, we showed a punctate distribution of hnRNP K in dendrites of hippocampal neurons consistent with the idea that the protein is present in neuronal mRNPs (Elvira et al., 2006). hnRNP K was partially localized in dendritic particles that also contain Staufen1 (Fig. 4.1A-white arrows, A') further supporting the hypothesis that hnRNP K has a role in the dendritic transport of mRNAs. We further showed that hnRNP K is present at excitatory synapses under resting conditions in primary hippocampal neurons (Fig. 4.1B, B') as previously demonstrated (Proepper et al., 2011). Whether the delivery of hnRNP K into synaptic sites was a constitutive or regulated process was unknown. Here we found that synaptic activity and BDNF induce the accumulation of hnRNP K in dendrites and in synaptic sites, as observed for hnRNP A2/B1 (Chapter 3) and other hnRNPs (Zhang et al., 2012). Studies performed in hippocampal synaptoneurosomes stimulated with BDNF showed a decrease in the interaction of hnRNP K with transcripts relevant in synaptic plasticity (GluA1, GluN1, BDNF and CaMKIIβ), suggesting a role for this ribonucleoprotein in the regulation of local protein synthesis required for the late phase of LTP. However, the interaction of hnRNP K with the transcripts investigated showed a more complex pattern of response when the protein was immunoprecipitated from the dentate gyrus after induction of LTP, possibly due to the increased complexity of the system.

The results of the immunocytochemistry experiments showed that the hnRNP K puncta is only partly colocalized with Staufen 1, indicating that it is distributed in different types of structures along dendrites. Interestingly, although some proteins that associate with Staufen1 were not detected in hnRNP K-mRNPs in HEK293 cells (Brendel et al., 2004), Staufen1-mRNPs contain other hnRNPs (e.g. hnRNP U) that were found to interact with hnRNP K in adult rat hippocampus in the proteomic study that we performed (Table 4.1). These results, and the observation that not

all Staufen1 puncta present in dendrites colocalize with hnRNP K, suggest that Staufen1 is present in different types of granules.

Quantitative immunocytochemistry experiments showed a rapid effect (30 min) of neuronal activity on the dendritic accumulation of hnRNP K, in contrast with a delayed response (3 h) resulting from the stimulation with BDNF. The rapid accumulation of hnRNP K in dendrites induced by neuronal activity was correlated with an increased accumulation at the synapse. Interestingly, treatment of hippocampal neurons with 100 ng/ml BDNF for 30 min increased the accumulation of hnRNP K at the synapse, although the results were not statistically significant, without affecting the total protein levels in dendrites. These results suggest that BDNF specifically regulates the synaptic-localized hnRNP K, but whether this effect is mediated by local protein synthesis remains to be determined. Considering the low percentage of hnRNP K that is expressed at the synapse, this mechanism may allow a significant upregulation in local hnRNP K protein levels without affecting the total protein content in dendrites. In fact, local translation was proposed to mediate the effects of BDNF on hnRNP K protein levels in synaptoneurosomes isolated from cultured cerebrocortical neurons (Liao et al., 2007). Alternatively, the BDNF-induced upregulation of hnRNP K protein levels at the synapse may be due a redistribution of the protein within the dendrites, so that the total protein content is not affected. When hippocampal neurons were incubated with BDNF for a longer period of time (3 h) there was a significant increase in hnRNP K puncta intensity and area in dendrites (Fig. 4.2E, F, G), suggesting the activation of distinct physiological responses. Additional studies are required to determine whether long incubations with BDNF also have an impact on hnRNP K protein accumulation at the synapse. Consistent with the idea that hnRNP K is specifically and differentially regulated in different compartments of the cell, synaptic activity did not change total hnRNP K protein levels, both *in vitro* and *in vivo*.

Using a culture system that allows a physical separation of the soma and neurite compartment, we found that stimulation of cultured hippocampal neurons with BDNF did not change hnRNP K mRNA in both cell bodies and neurites. These findings indicate that most likely this neurotrophin does not regulate hnRNP K total mRNA levels in these subcellular compartments. Furthermore, no changes were observed in the total mRNA for hnRNP K in the dentate gyrus after induction of LTP *in vivo*. Taken together, these results suggest that the

expression of hnrnpk is not affected significantly by neuronal activity. The lack of effect of BDNF on the dendritic levels of hnRNP K mRNA contrasts with the effects observed for transcripts encoding several translation-related proteins (Manadas et al., 2009).

Interestingly, KCl depolarization decreased hnRNP K total protein levels in primary hippocampal neurons (Fig. 4.3). Although the physiological significance of this observation may be questionable considering that KCl induces a permanent depolarization of the membrane, this result *per se* is a good indicator that hnRNP K is regulated in an activity-dependent manner.

Previous results from our laboratory identified several transcripts associated with hnRNP K in cultured hippocampal neurons, such as the mRNAs encoding for hnRNP K, GluA1, GluN1 and BDNF (Comprido, 2011). Furthermore, BDNF was shown to induce the dissociation of these mRNAs from hnRNP K-containing ribonucleoprotein complexes in cultured hippocampal neurons (Comprido, 2011). Interestingly, stimulation with BDNF upregulates GluA1 (Schratt et al., 2004), hnRNP K and CaMKIIβ (Liao et al., 2007) protein levels in synaptoneurosomes. GluN1 mRNA is also among the RNAs that are present in both soma and dendrites (Benson, 1997; Gazzaley et al., 1997; Schratt et al., 2004) and is associated with RNA granules (Krichevsky and Kosik, 2001). The BDNF transcript is one of the best studied dendritic-localized mRNAs (see section 1.6.2). A recent study also identified the transcripts for CamKIIβ, GluN1, GluA1 and BDNF in dendrites and/or axons in hippocampal neuropil (Cajigas et al., 2012). Given these observations, and since hnRNP K is present at the synapse under resting conditions (Fig. 4.1B-white arrows, B'), we have also evaluated whether BDNF induces the release of these mRNAs from the hnRNP K-containing complexes in synaptoneurosomal fractions isolated from adult rat hippocampus. The results obtained show that BDNF prompts the dissociation of the GluA1, hnRNP K, GluN1, BDNF and CaMKIIß transcripts from the hnRNP K-carrying mRNPs locally at the synapse.

In addition to the effects of BDNF on hnRNP K observed in this work, a previous study reported the effects of the neurotrophin in the dissociation of granules containing RNG105 (Shiina et al., 2005). Interestingly, as we observed for hnRNP K, RNG105 also colocalizes with Staufen proteins in dendrites of hippocampal neurons. BDNF induces the release of RNG105 from RNA granules and this effect is coincident with the translation of an mRNA reporter near the granules (Shiina et al., 2005). However, the translation of RNG105-associated mRNAs was still suppressed in the absence of RNG105 (Shiina et al., 2010), suggesting that translation

repression is not the primary role of RNG105. In fact, the RNG105-deficient mice showed reduced levels of RNG105-associated mRNAs in dendrites indicating a role for RNG105 in the dendritic localization of these mRNAs.

hnRNP K is a well known regulator of translation (Bomsztyk et al., 2004). The protein was shown to activate the translation of silenced mRNAs upon c-Src-mediated phosphorylation (Ostareck-Lederer et al., 2002). Accordingly, the hnRNP K-mRNA complexes were disrupted by tyrosine phosphorylation of hnRNP K suggesting that the binding of hnRNP K to RNA may be responsive to extracellular stimuli that activate tyrosine kinases (Ostrowski et al., 2000). Considering that the signaling activity induced by BDNF in hippocampal neurons induces the phosphorylation of hnRNP K at Serine 302 (Comprido, 2011), it is tempting to propose a model in which the activation of TrkB receptors would induce the phosphorylation of hnRNP K with the concomitant release of hnRNP K-bound mRNAs from the hnRNP K-carrying mRNPs, allowing the local translation of these transcripts at the synapse. Future research is required to determine whether hnRNP K is involved in the dendritic delivery of these transcripts and/or in the translational control at the synapse.

The landscape of hnRNP K protein-protein interactions is extensive and well described in several biological contexts (Bomsztyk et al., 2004; Mikula et al., 2006). In neurons though, with the exception of target oriented studies, it remains largely unknown. Herein, we showed that a large number of RNA-binding proteins and regulators may interact with hnRNP K in adult rat hippocampus (Table 4.1). It is therefore difficult to determine whether each of the mRNAs that co-immunoprecipitated with hnRNP K bind directly or indirectly to the protein**.** Future studies should address this question, for instance by performing EMSA (electrophoretic mobility shift assay) experiments. Importantly, as previously demonstrated in rat hepatoma cells (Mikula et al., 2006), other hnRNPs were found to interact with hnRNP K in adult rat hippocampus (Table 4.1). Several of those hnRNPs and hnRNP K were identified in two of the largest proteomic studies performed until now that tried to unravel the protein composition of neuronal mRNPs (Elvira et al., 2006; Kanai et al., 2004), suggesting that hnRNP K, together with other hnRNP K-interacting hnRNPs, may constitute a core machinery for the transport of mRNA granules in neurons.

A wide variety of ribosomal proteins were also found to interact with hnRNP K (Table 4.1) (Mikula et al., 2006) demonstrating the prominent role of this protein in the translational control.

Accordingly, it was demonstrated that hnRNP K together with hnRNP E1/2 regulates the recruitment of 60S ribosomal subunits to form a competent 80S ribosome to initiate the translation of 15-lipoxygenase (LOX) mRNA in reticulocytes (Ostareck et al., 2001; Ostareck et al., 1997)

The proteomic study that we performed to analyse the hnRNP K interactome showed several new binding partners, which remain to be validated. Some of those are synaptic-localized proteins with well established functions in plasticity-related events such as CaMKIIα [for a review see (Coultrap and Bayer, 2012)] and cytoskeleton regulators. These observations contribute to the growing evidence suggesting a role for hnRNP K in the regulation of cytoskeleton composition. hnRNP K directly interacts with the regulator of actin dynamics, N-WASP via its KI domain (Yoo et al., 2006). This study showed that hnRNP K negatively regulates N-WASP-induced cell spreading and filopodia formation in mouse fibroblasts (Yoo et al., 2006). Furthermore, it was recently demonstrated that hnRNP K interacts with Abelson-interacting protein 1 (Abi1) at postsynaptic sites in hippocampal neurons (Proepper et al., 2011). Abi1 is localized in dendritic spines and postsynaptic densities (PSDs) where it plays an important role in regulating cytoskeleton reorganization and synaptic maturation (Courtney et al., 2000). Accordingly, hnRNP K knockdown results in a significant increase in filopodia formation and decrease in the number of mature synapses, an effect that mimics the neuronal morphology in the absence of Abi1 (Proepper et al., 2011). Furthermore, hnRNP K was shown to post-transcriptionally regulate multiple transcripts that collectively organize microfilaments, microtubules, and neuronal intermediate filaments to make the axon (Liu and Szaro, 2011). Altogether, our results highlight a possible major role of hnRNP K in the regulation of local mRNA metabolism and/or cytoskeleton regulation at the synapse.

In the final set of experiments we provide direct evidence that synaptic activity regulates the mRNA content of hnRNP K-containing mRNPs *in vivo*. We found that the LTP paradigm that we used induces a massive increase in the total abundance of BDNF mRNA in total extracts of dentate gyrus homogenates and chelation of extracellular BDNF with TrkB-Fc reduced synaptic potentiation induced by high-frequency stimulation. These observations are in agreement with the key role that this neurotrophin plays in this form of synaptic plasticity (Minichiello, 2009). Several activity-inducing paradigms were shown to promote the release of endogenous BDNF (Kuczewski et al., 2009; Lessmann and Brigadski, 2009), including the response to high-frequency stimulation

(Aicardi et al., 2004). Furthermore, BDNF was shown to play an important role in the protein synthesis-dependent late phase of LTP induced by high-frequency stimulation in the hippocampus CA1 region (Chen et al., 1999; Kang and Schuman, 1996) and BDNF-induced synaptic potentiation has also been reported, both *in vitro* (Kang and Schuman, 1996) and *in vivo* (Messaoudi et al., 2002; Ying et al., 2002). Accordingly, BDNF is required for the maintenance of LTP in the CA1 region of the hippocampus (Figurov et al., 1996; Korte et al., 1998; Minichiello et al., 2002).

Induction of LTP in the dentate gyrus *in vivo* also resulted in a significant increase in the total mRNA levels of GluN1, when determined 30 min after high-frequency stimulation. Importantly, under the same conditions there was a decrease in the levels of hnRNP K and GluN1 transcripts associated with hnRNP K, although the effect was not statistically significant in the latter case. These results are in agreement with the observations in hippocampal synaptoneuronsomes stimulated with BDNF. In contrast with these results, 2 h upon HFS all the hnRNP K-associated mRNAs are increased in the hnRNP K-containing complexes, although the effect is not statistically significant for the number of experiments performed. Given the role of TrkB signaling in the induction of this form of synaptic potentiation, and although our analysis was performed in total homogenates, it is tempting to speculate that BDNF may contribute to the regulation of hnRNP K and hnRNP K-bound mRNAs at the synapse at the early phase after high-frequency stimulation. The effect of BDNF may be mediated by increasing the delivery of hnRNP K into synapses, as we observed in cultured hippocampal neurons, and by inducing the release of hnRNP K-bound mRNAs from the mRNPs, as we observed in synaptoneurosomes. The observed increase in all hnRNP K-bound mRNAs 2 h post-HFS may be secondary to the initial increase in transcription activity and/or due to the desensitization of the signaling mechanisms that account for the initial decrease in the interaction of hnRNP K with the transcripts. Since stimulation of cultured hippocampal neurons provides a simple system to investigate the regulation of the interaction between hnRNP K and the associated transcripts, it will be interesting to determine whether long incubations (2-3 h) with BDNF also change the pattern of interaction of hnRNP K with the transcripts. Further analysis of dentate gyrus homogenates obtained after the TrkB-Fc infusion experiments should also elucidate about the role of BDNF in the HFS-induced regulation of hnRNP K-associated transcripts.

4.5. Conclusions

In this work we demonstrate that synaptic activity and the neurotrophin BDNF regulate hnRNP K and hnRNP K-bound mRNAs *in vitro* and *in vivo*. We showed that hnRNP K partly colocalizes with Staufen1 in dendrites of hippocampal neurons further demonstrating a role for hnRNP K in the transport of dendritic-localized transcripts. In addition, hnRNP K is rapidly delivered into synapses in response to neuronal activation. Moreover, studies performed in hippocampal synaptoneurosomes showed that several hnRNP K-bound mRNAs, such as the transcripts for GluN1, GluA1 and BDNF, are released from hnRNP K-containing mRNPs in response to stimulation with BDNF. These results suggest a key role for hnRNP K in the local translation of several proteins that contribute to the late phase of LTP. Accordingly, we found that the hnRNP K and possibly the GluN1 mRNAs are dissociated from the hnRNP K-carrying mRNPs in the early phase of HFS-induced LTP in the medial perforant path-granule cells synapses, a phenomenon that is dependent on extracellular BDNF. Altogether, the evidence here reported suggests a fundamental role for hnRNP K in the regulation of neuronal function and is likely to play a role in BDNF-mediated plasticity events.

Chapter 5

General Conclusions and Future Perspectives

The present work has focused on two members of the hnRNP family of proteins and overall, we provide evidence indicating that hnRNPs, besides the well established functions in the nucleus, may also play a prominent role in the regulation of dendritic mRNA metabolism in the central nervous system.

5.1. General conclusions and open questions

5.1.1. Regulation of the activity-dependent accumulation of hnRNP A2/B1 in dendrites and synapses

We found that the delivery of hnRNP A2/B1 into dendrites and synaptic sites is a regulated process since both synaptic activity and BDNF treatment increased hnRNP A2/B1 levels in dendrites and at the synapse in cultured hippocampal neurons. Importantly, we showed that synaptic activity induces the delivery of hnRNP A2/B1 into synaptic sites by a mechanism that depends on endogenous released BDNF. In contrast, the activity-dependent increase in dendritic hnRNP A2/B1 was not affected by the TrkB-Fc-mediated blocking of extracellular ligands of TrkB receptors, indicating that this effect does not require the actions of BDNF. Whether these effects depend on the redistribution of preexisting hnRNP A2/B1 or require new protein synthesis is unknown. To answer this question, additional experiments should be performed in the presence of inhibitors of translation to evaluate the relative contribution of protein synthesis to the observed effects. Furthermore, it will be of interest to dissect the mechanisms involved in the activity-dependent changes in the distribution of this ribonucleoprotein. The use of pharmacological inhibitors of AMPA and NMDA receptors would allow evaluating the relative contribution of these receptors in the dendritic and synaptic accumulation of hnRNP A2/B1 following neuronal activation. Since BDNF mediates the activity-dependent increase of hnRNP A2/B1 in synaptic sites, it would be also important to dissect the signaling pathways (e.g. using pharmacological inhibitors) required for the BDNF-induced accumulation of hnRNP A2/B1 in dendrites and, in particular, at the synapse.

The immunocytochemistry experiments also showed that hnRNP A2/B1 and Staufen1 are likely to be present, to some extent, in the same subset of dendritic mRNPs in cultured hippocampal neurons. Interestingly, we also found that Staufen1, in contrast with hnRNP A2/B1,

was present in synaptic polysomal fractions which, to our knowledge, provides the first evidence suggesting a role for Staufen1 in the translational control of synaptic-localized mRNAs.

5.1.2. Regulation of hnRNP K distribution in neurons

We observed that hnRNP K is present in dendritic processes and in synaptic sites under resting conditions in cultured hippocampal neurons. Furthermore, the hnRNP K distribution was found to be regulated by synaptic activity and by the neurotrophin BDNF. The results show that, as observed for hnRNP A2/B1, synaptic activity upregulates hnRNP K protein levels in dendrites and in synaptic sites. Interestingly, short-term stimulation with BDNF did not change dendritic levels of hnRNP K but induced the delivery of the protein to synapses. A sustained activation of TrkB receptors was required to increase hnRNP K in dendrites, suggesting that short or long incubations with BDNF result in distinct physiological responses. As previously mentioned for hnRNP A2/B1, it is currently unknown whether these changes in hnRNP K levels in dendrites and at the synapse result from the redistribution of pre-existing hnRNP K or instead require new hnRNP K synthesis. To test this hypothesis, experiments should be performed in the presence of protein synthesis inhibitors. Moreover, it will be of interest to investigate the mechanisms by which synaptic activity and BDNF differentially regulate hnRNP K distribution. This can be addressed using pharmacological inhibitors of AMPA and NMDA receptors to unravel if the activation of these receptors contributes to the delivery of hnRNP K into dendrites and synapses following neuronal activation. Furthermore, as previously shown for hnRNP A2/B1, we are currently investigating the role of endogenous released BDNF in the activity-dependent synaptic delivery of hnRNP K, using the scavenger of TrkB ligands TrkB-Fc.

5.1.3. The nature of hnRNP A2/B1 and hnRNP K ribonucleoprotein complexes

One of the major questions in the field is related to the nature and composition of the mRNPs involved in the transport of mRNA to distal dendritic domains. It is thought that the transport mRNPs may be in a close interplay with other types of RNA granules. This hypothesis was raised after the findings suggesting that neuronal mRNPs and P-bodies may share some structural features. For instance, some *trans*-acting proteins usually involved in mRNA transport, such as

FMRP and ZBP, were identified in P-bodies (Cougot et al., 2008). Furthermore, a recent study reported the activity-dependent accumulation of neuronal P-bodies (usually involved in mRNA degradation or storage) in synaptic sites with a concomitant release of mRNA (Oh et al., 2013). More interestingly, in contrast with previous findings (Zeitelhofer et al., 2008), it was suggested that Staufen1 may also belong to the group of proteins with roles in mRNA transport present in mammalian neuronal P-bodies (Oh et al., 2013).

In this work we observed that both hnRNP A2/B1 and hnRNP K can, to some extent, localize in dendritic particles that also contain Staufen1. Since Staufen1 is a well known player in the dendritic-transport of mRNA, these findings *per se* indicate that both hnRNPs play a role in this process. This is well established for hnRNP A2/B1 but little evidence is available for hnRNP K.

We have performed a proteomic study to identify hnRNP K-interacting proteins in adult rat hippocampus. The results suggested an interaction between hnRNP K and hnRNP A2/B1. However, we could not confirm this interaction by Western blot analysis of the hnRNP K immunoprecipitates. In the future it will be important to investigate whether these two hnRNPs are found in the same structures in dendrites of hippocampal neurons, and if hnRNP K and/or hnRNP A2/B1 are present in silent-foci that respond to synaptic stimulation. Experiments should be performed to analyse the colocalization between hnRNP A2/B1 and hnRNP K, or between both proteins and markers of P-bodies, in dendrites of cultured hippocampal neurons. In addition, the transfection of hnRNP K and/or hnRNP A2/B1 constructs into hippocampal neurons will allow performing live-cell imaging to evaluate whether synaptic activation or BDNF stimulation increase the motility of exogenous hnRNP K and/or hnRNP A2/B1. This approach will also provide a valuable tool to visualize and evaluate the kinetics of the activity-dependent delivery of these proteins into the synapse. Since the putative role of hnRNP K in the delivery of mRNAs into dendritic domains in neurons remains to be proved, it will be of interest to generate contructs lacking the KHs (KH1, KH2, and KH3) or the KI domains to investigate if the results obtained depend on the binding to mRNA or other proteins, respectively.

5.1.4. Regulation of dendritic and synaptic mRNA by hnRNP K

The present work showed that hnRNP K associates with several transcripts with important roles in synaptic plasticity. More importantly, we showed that BDNF stimulation induces the release of these transcripts at the synapse. Since hnRNP K accumulates in dendrites and at the synapse upon synaptic activation, and likely also following BDNF stimulation, it will be of interest to perform *in situ* hybridization experiments targeting some of the hnRNP K-bound transcripts (for instance BDNF, GluA1 or GluN1), together with immunocytochemistry using an antibody against hnRNP K, to investigate the colocalization of the mRNAs with hnRNP K in dendrites of hippocampal neurons. This approach will allow evaluating if BDNF treatment or synaptic activity preferentially disrupts hnRNP K-mRNA interactions in synaptic processes. To evaluate whether hnRNP K is required for the dendritic transport and/or synaptic delivery of these transcripts experiments can be performed knocking down hnRNP K (e.g. using siRNAs or shRNAs) in hippocampal neurons followed by *in situ* hybridization to detect the selected hnRNP K-bound mRNAs. Quantification of the amount of mRNA in dendrites and at the synapse (e.g. colocalization with PSD95) would provide valuable information about the role of the protein in the dendritic transport and synaptic delivery of these transcripts. To test our working hypothesis, hnRNP K may also be overexpressed to determine if the expected increase in the dendritic- and/or synaptic-localized mRNAs occurs.

It is important to note that, at the moment, it remains to be determined whether hnRNP K binds directly the hnRNP K-bound transcripts that we have identified. EMSA (electrophoretic mobility shift assay) experiments will further elucidate the nature of the interaction between hnRNP K and mRNAs.

5.1.5. Does hnRNP K regulate the translation of the selected hnRNP K-bound mRNAs?

hnRNPs are involved in several aspects of mRNA metabolism, including the translational control of transcripts (Han et al., 2010). We did not detect hnRNP A2/B1 in polysomal fractions obtained from synaptoneurosomes isolated from the rat brain, suggesting that hnRNP A2/B1 is not present in the functional units of protein synthesis at the synapse. These results suggest that hnRNP A2/B1 does not play a role in the translation control of synaptic-localized mRNAs.

Since hnRNP K has been shown to play a role in the regulation of translation (Bomsztyk et al., 2004) and we observed that hnRNP K-bound mRNAs are released from hnRNP K-carrying complexes locally at the synapse in response to BDNF, it is tempting to speculate that hnRNP K may be controlling the translation of its associated mRNAs. First, it will be important to determine whether hnRNP K is present in synaptic polysomes. Additional studies can be performed to further evaluate the possible role of hnRNP K in translation regulation. For instance, hnRNP K knockdown experiments will allow investigating the role of the protein in the regulation of the total rate of translation in neurons (e.g. using a general luciferase reporter). This question may be addressed using a recently developed methodology with a metabolic labeling approach based on incorporation of noncanonical amino acids into proteins followed by chemoselective fluorescence tagging by means of 'click chemistry' (Dieterich et al., 2010). This method enables the observation of new proteins in both soma and dendrites and has already been used to demonstrate that BDNF induces the synthesis of new proteins in dendrites (Dieterich et al., 2010). This method would allow comparing translation in dendrites in the presence or in the absence of hnRNP K, and determining the effect of BDNF stimulation. However, this type of strategy does not allow distinguishing between hnRNP K specific targets. For that purpose it would be necessary to generate luciferase reporter constructs containing the sequences of specific hnRNP K-bound mRNAs and perform luciferase reporter assays. Since one of hnRNP K-bound mRNAs is BDNF, experiments may be performed to knockdown hnRNP K and transfect the d2EGFP-long 3'UTR BDNF construct into hippocampal neurons. This reporter harbors a membrane insertion tag for limiting diffusion of newly synthesized d2EGFP from the site of translation, and was recently used to evaluate the activity-dependent translation of BDNF in dendrites (Lau et al., 2010). Although this is very challenging from an experimental point of view, it may provide an insight into the role of hnRNP K in the activity-dependent dendritic translation of BDNF. It is important to note that, as previously mentioned, it is still not known if hnRNP K binds directly the BDNF mRNA and, despite the majority of translational control elements being localized in the 3'UTR, other regulatory elements may also be present in other regions. This may be tested using other BDNF constructs, containing distinct sequences (e.g. coding sequence).

5.1.6. Do the HFS-induced changes in hnRNP K-bound mRNAs require BDNF?

In the last set of experiments we showed that hnRNP K-associated mRNAs are differentially regulated upon HFS-induced LTP in the dentate gyrus of live anesthetized rats, a model of synaptic potentiation that requires TrkB signaling. Given the almost lack of *in vivo* studies in dendritic mRNA metabolism research, this represents an important contribution to the field.

We found that hnRNP K mRNA is released from hnRNP K-carrying ribonucleoprotein complexes in dentate gyrus homogenates 30 min after LTP-induction *in vivo*, but whether this dissociation is prompt by BDNF is at the moment unknown. Analysis of the dentate gyrus homogenates obtained from the animals injected with the TrkB-Fc chimera should allow addressing this question

Interestingly, although the changes were not statistically significant, no clear differences were observed in the regulation pattern of hnRNP K-associated mRNAs 2 h post-HFS, where apparently all the tested hnRNP K-bound mRNAs were increased.

An important question is related to the localization in the cell of the alterations in hnRNP K binding to mRNAs after induction of LTP. The use of synaptic fractions may provide an insight about the compartment where these changes occur, increasing the functional relevance of these findings, but the amount of tissue that can be obtained may constitute a limitation.

5.1.7. hnRNP K-mediated regulation of synaptic function

A recent study demonstrated that hnRNP K interacts with Abi1 in postsynaptic sites in hippocampal neurons (Proepper et al., 2011). Abi1 is an actin-cytoskeleton regulator protein enriched in dendritic spines. Accordingly, hnRNP K and Abi1 are required for normal spine morphology (Proepper et al., 2011). Our proteomic study suggested several new binding partners for hnRNP K in adult hippocampus. Among those were proteins with well established roles at the synapse and/or in the regulation of cytoskeleton, supporting the idea that hnRNP K may have other functions in neurons that might not dependent on its ability to bind mRNA. This is also in agreement with the versatile structure of hnRNP K. Additional immunoprecipitation studies are required to confirm the interaction between hnRNP K and other proteins suggested by our proteomic study. The functional relevance of these findings also deserves further investigation.

Knockdown of hnRNP K was shown to reduce the number of mature synapses in hippocampal neurons (Proepper et al., 2011) but whether this affects the number of functional synapses remains to be determined. This possibility may be evaluated by knocking down hnRNP K and evaluating the surface levels of GluA1 in hippocampal neurons. Complementary experiments may be performed using whole-cell patch clamp recordings of AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs), in the CA1 region of organotypic cultures subjected to lentiviral-mediated siRNA knockdown of hnRNP K. Similarly, it would be also possible and challenging to perform the lentiviral-mediated siRNA knockdown of hnRNP K *in vivo* in the dentate gyrus of rats. This would allow to test whether hnRNP K is required for the induction, persistence and/or maintenance of the HFS-induced form of synaptic potentiation in the perforant path-dentate gyrus synapse.

Altogether, the findings reported in this thesis strongly support a scenario where both hnRNP A2/B1 and hnRNP K regulate mRNA metabolism in dendrites, which is likely to play a role in BDNF-mediated synaptic plasticity.

Chapter 6

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