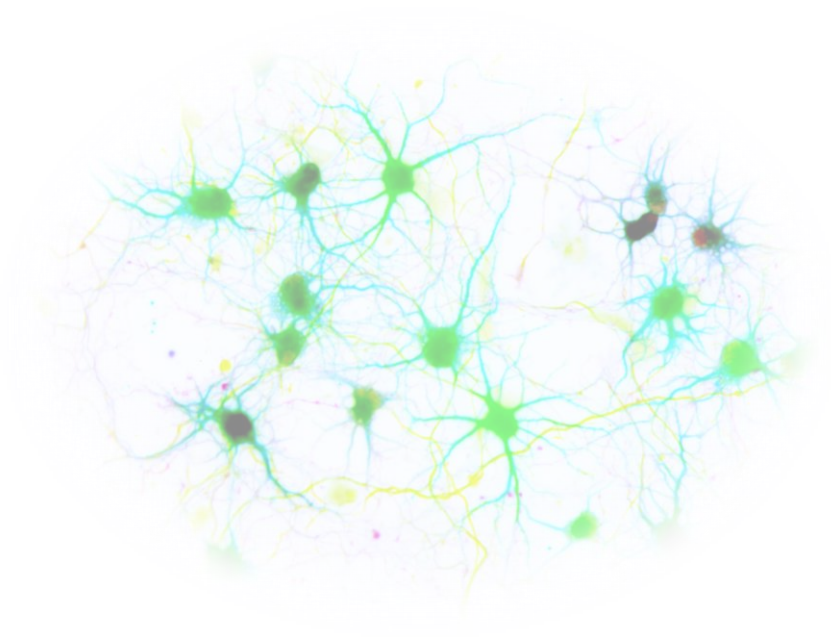
DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

BDNF-induced local protein synthesis at the synapse:

a regulatory role for hnRNPK



Luís Miguel Sousa Rodrigues

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Doutor Carlos Jorge A. M. Bandeira Duarte (Departamento de Ciências da Vida, Universidade de Coimbra)

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Abstract

The synaptic strength is known to respond to neuronal activity in a dynamic manner, and changes in neuronal connectivity are thought to underlie learning and memory formation. Long-term synaptic potentiation (LTP) involves two different types of mechanisms: the initial changes depend on posttranslational modifications of existing synaptic proteins whereas the delayed responses are mediated by local protein synthesis in dendrites and modifications in gene expression. The transport of newly synthesized mRNAs from the nucleus to dendrites, to allow local protein synthesis, is conducted by RNA granules that are responsible for the delivery and stabilization of transcripts. These granules are disassembled in response to synaptic stimulation and this is thought to release the mRNAs for subsequent local protein synthesis. Accordingly, local translation at the synapse is induced by neuronal activity, playing an important role in the maintenance of LTP. The coupling between neuronal activity and local protein synthesis suggests that the interaction of RNA binding proteins and the transcripts is regulated by intracellular signaling mechanisms.

The neurotrophin BDNF and the growth factor PDGF were both shown to enhance LTP. TrkB and PDGF-β receptors, activated by BDNF and PDGF respectively, share several parallel signaling pathways, including the PI3-K/Akt, the Ras/ERK and the PLCγ pathways. TrkB receptors have been shown to activate the translation machinery contributing to the protein synthesis-dependent phase of LTP (Santos et al., 2010).

In this work we aimed at characterizing the role of the RNA binding protein hnRNPK in the regulation of global protein synthesis in dendrites. Furthermore, given the evidence indicating that hnRNPK interacts with transcripts coding for proteins relevant for synaptic plasticity, we investigated the effects of BDNF and PDGF, which target distinct receptor tyrosine kinases, in the modulation of this interaction at the synapse. Protein synthesis was studied using the recently described surface sensing of translation (SUnSET) method (Schmidt et al., 2009). Overexpression of hnRNPK in COS-7 cells decreased total protein synthesis, but co-transfection with TrkB enhanced protein synthesis. Preliminary experiments indicated that hnRNPK knock-down in cultured hippocampal neurons with a specific shRNA downregulated the BDNF-induced increase in translation activity in dendrites. This suggests that hnRNPK plays an important role in the delivery and/or supply of mRNAs that are translated at the synapse in response to stimulation with BDNF.

The effect of BDNF in the regulation of the interaction of hnRNPK with transcripts relevant for synaptic plasticity was investigated in hippocampal synaptoneurosomes, and the results were compared with the effect of PDGF which activates a different receptor tyrosine kinase. The presence of functional receptors for both ligands in hippocampal synaptoneurosomes was confirmed by the activation of the ERK and Akt signaling pathways, as determined by western blot with phospho-specific antibodies. Quantitative reverse-transcription PCR experiments showed that GluA1, GluN1 and BDNF transcripts co-immunoprecipitated with hnRNPK from hippocampal synaptoneurosomes, suggesting that the protein is involved in the transport of those transcripts. However, while stimulation of synaptoneurosomes with BDNF decreased the interaction of the transcripts with hnRNPK, PDGF increased the interaction of GluA1 and GluN1 mRNA with the ribonucleoprotein, and was without effect on the binding of BDNF transcripts. These evidences support a role for BDNF in the late phase of LTP by promoting the release of the transported mRNAs associated with hnRNPK, which should allow them to be locally translated. If PDGF proves to induce local protein synthesis at the synapse, distinct mechanisms may be involved.

Keywords: hnRNPK; BDNF; synaptoneurosomes; local translation; synaptic plasticity.

Resumo

A força da comunicação sináptica pode ser alterada de forma dinâmica em resposta a alterações da actividade neuronal, e pensa-se que esta plasticidade seja responsável pela formação de memórias e pela aprendizagem. A potenciação sináptica de longa duração (LTP) envolve dois tipos diferentes de mecanismos: as alterações iniciais dependem de modificações pós-traducionais de proteínas pré- e pós-sinápticas, enquanto as respostas mais tardias são mediadas por síntese proteica local e modificações na expressão de genes. O transporte dos mRNA sintetizados *de novo* do núcleo para as dendrites, de modo a permitir a síntese local de proteínas, é realizado por grânulos de RNA que estabilizam os transcritos. Estes grânulos desagregam-se em resposta à estimulação sináptica e pensa-se que estas alterações estão associadas à libertação dos mRNA para subsequente síntese local de proteínas. Esta síntese de proteínas na sinapse é particularmente importante para a manutenção da LTP. O acoplamento entre a actividade neuronal e a estimulação da síntese proteica na sinapse sugere que a interacção entre as proteínas que ligam ao RNA e os transcritos é regulada por mecanismos de sinalização intracelular.

A neurotrofina BDNF e o factor de crescimento PDGF desempenham um papel importante na LTP. Os receptores TrkB e PDGF-β, que são activados por BDNF e PDGF, respectivamente. activam vias de sinalização comuns, incluindo a PI3-K/Akt, a Ras / ERK e a da PLCγ. Foi demonstrado que os receptores TrkB são capazes de activar a maquinaria de tradução, contribuindo para a fase dependente de síntese proteica da LTP (Santos et al., 2010).

Este trabalho teve como objectivo identificar o papel de uma proteína que liga RNA, a hnRNPK, na síntese proteica nas dendrites de neurónios do hipocampo em cultura. Tendo em conta as evidências indicando a interacção desta proteína com RNAm que codificam proteínas com importância ao nível da plasticidade sináptica, investigámos também os efeitos do BDNF e do PDGF, os quais interagem com receptores distintos, na modulação desta interacção ao nível da sinapse. A síntese de proteínas foi estudada utilizando a técnica de percepção de tradução superficial (SUnSET) recentemente descrita (Schmidt et al., 2009). Quando a hnRNPK foi sobre-expressa em células COS-7 observou-se uma diminuição da síntese proteica, enquanto a co-transfecção com TrkB aumentou a tradução. Resultados preliminares sugerem que a redução dos níveis da hnRNPK em neurónios do hipocampo em cultura, usando um shRNA específico, atenua o aumento da síntese proteica ao nível das dendrites induzido pela estimulação com BDNF. Estes resultados sugerem que a hnRNPK desempenha

um papel importante no transporte e/ou na entrega dos mRNA que são traduzidos nas dendrites em resposta à estimulação com BDNF.

Neste trabalho investigámos também o efeito do BDNF na regulação da interacção da hnRNPK com mRNA que codificam proteínas importantes para a plasticidade sináptica, em sinaptoneurossomas, e os resultados obtidos foram comparados com o efeito do PDGF que também activa receptores acoplados a cinases de resíduos de tirosina. A presença de receptores funcionais para BDNF e PDGF em sinaptoneurossomas isolados a partir do hipocampo de rato foi confirmada por western blot, usando anticorpos contra a forma fosforilada da Akt, ERK 1 e ERK2. Os dados provenientes do PCR de transcrição reversa quantitativa mostrou a presença dos transcritos de GluA1, GluN1 e BDNF em imunoprecipitados da hnRNPK preparados a partir de sinaptoneurossomas de hipocampo, sugerindo que a proteína está envolvida no transporte destes transcritos ao longo das dendrites. Porém, a estimulação dos sinaptoneurossomas com BDNF reduziu a interacção dos transcritos com a hnRNPK, enquanto o PDGF aumentou a interacção dos mRNA para o GluA1 e GluN1, e não teve qualquer efeito nos transcritos para o BDNF. Estes resultados apoiam uma função para o BDNF na fase tardia da LTP, como promotor da libertação de mRNA associados à hnRNPK, o que poderá permitir que estes sejam traduzidos localmente. No caso do PDGF também regular a tradução ao nível da sinapse os mecanismos envolvidos serão certamente distintos.

Palavras-chave: hnRNPK; BDNF; sinaptoneurossomas; tradução local; plasticidade sináptica.

Abbreviations

4EBP	eIF4E-binding protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
Arc	Activity-regulated cytoskeleton-associated protein
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CaMKII	Calcium/calmodulin-dependent protein kinase II
CHX	Cycloheximide
CNS	Central nervous system
CREB	cAMP response element-binding protein
CPEB	Cytoplasmic polyadenylation element binding protein
DAG	Diacylglycerol
DICE	Differentiation control element
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
DOC	Deoxycholic acid
DOI	Days of infection
DTT	Dithiothreitol
ECF	Enhanced chemifluorescence
eEF	Eucaryotic elongation factor
EGTA	Ethylene glycol tetraacetic acid
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FMRP	Fragile X mental retardation protein
FT	Flow-through
GAP	GTPase activating protein
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HBSS	Hank’s balanced salt solution

HEK	Human embryonic kidney cells
HEPES	N-(2-hydroxyethyl)-1-piperazine-N'-(2-ethanesulfonic acid)
HFS	High-frequency stimulation
hnRNP	Heterogeneous nuclear ribonucleoprotein
IEG	Immediate-early gene
IgG	Immunoglobulin G
IP	Immunoprecipitation
IP3	Inositol 1, 4, 5-trisphosphate
KH	K homology domain
KI	K-protein-interactive region
KNS	Nuclear shuttling domain
LTD	Long-term depression
LTP	Long-term potentiation
MAP2	Microtubule-associated protein 2
MBP	Myelin basic protein
mGluR	Metabotropic glutamate receptor
mRNP	mRNA-protein complex
mTOR	Mammalian target of rapamycin
NLS	Nuclear localization signal
NMDA	N-methyl-D-aspartate
P-bodies	Processing bodies
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4, 5-bisphosphate
PKA	Protein kinase A
PKCδ	Protein kinase C delta
PLC_{γ}	Phospholipase C gamma
PMSF	Phenylmethanesulfonylfluoride
PSD	Postsynaptic density
PVDF	Polyvinylidene fluoride
PDGF	Platelet-derived growth factor
RBP	RNA-binding protein

RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RRM	RNA recognition motif
SEM	Standard error of the mean
Shc	Src homology 2 domain-containing
SDS	Sodium dodecyl sulfate
SUnSET	Surface sensing of translation
TrkB	Tropomyosin-related kinase B
UTR	Untranslated region
VGAT	Vesicular GABA transporter
ZBP1	Zipcode-binding protein 1

CHAPTER 1

Introduction

1 - Glutamatergic synapse

In the early 1900s, the work of Santiago Ramón y Cajal defined neurons as the primary functional units of the CNS (central nervous system) that communicate with each other via specialized junctions, the synapses (Lopez-Munoz et al., 2006). There are billions of neurons in the mammalian brain that communicate with each other via synapses. These synapses mainly occur at contacts between presynaptic axons and postsynaptic dendrites, and in most cases use glutamate or γ-aminobutyric acid (GABA) as excitatory and inhibitory neurotransmitters, respectively. The glutamate released from the presynaptic terminal binds to synaptic glutamate receptors belonging to the ionotropic and metabotropic categories. Transmission of the signal to postsynaptic neurons depends mainly on the activation of two types of ionotropic receptors, which can be classified based on their molecular, pharmacological and electrophysiological properties in AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (AMPAR) and NMDA (N-methyl-D-aspartic acid) receptors (NMDAR). AMPAR are formed by assembly of GluA1-GluA4 subunits while NMDAR are tetrameric structures formed by oligomerization of GluN1 subunits together with GluN2A-GluN2D and/or GluN3A-GluN3B subunits (Greger et al., 2007; Paoletti and Neyton, 2007). Activation of AMPAR increases the influx of Na^+ while NMDAR are also permeable to Ca^{2+}. The influx of cations through the receptor channels allows propagating the depolarization to the postsynaptic neuron.

One of the most important features of the mammalian brain is its plasticity that enables the modification of neural circuits by neural activity, thereby generating experience and thus altering its pattern of response (e.g. thoughts, feelings and behaviour). This phenomenon is called synaptic plasticity and was first proposed in the late 1940s by Donald Hebb when he postulated that associative memories are formed in the brain by a process of synaptic modification that strengthens connections when presynaptic activity correlates with postsynaptic firing (Hebb, 1949). Only later did Timothy Bliss, Tony Gardner-Medwin and Terje Lomo reported this phenomenon, when they observed an enhancement of synaptic transmission between the stimulated axons and the dentate areas of the hippocampus after a high-frequency stimulation of the perforant path fibers (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973).

Synaptic plasticity specifically refers to the activity-dependent modification of the strength or efficacy of synaptic transmission at preexisting synapses, and plays a central role in the capacity of the brain to incorporate transient experiences into persistent memory traces.

Several observations have suggested synaptic plasticity impairment as a cause of many psychiatric and neurologic disorders, such as mental retardation (Pfeiffer and Huber, 2009), schizophrenia (Stephan et al., 2006), Parkinson's disease (Calabresi et al., 2006), autism (Sudhof, 2008), Alzheimer's disease (Selkoe, 2002), compulsive behavior (Welch et al., 2007), and addiction (Kauer and Malenka, 2007).

2 - LTP

The long-term potentiation (LTP) and the long-term depression (LTD) of synaptic transmission are the most widely studied physiological models of memory formation in the mammalian brain. LTP was first described in vivo by Bliss and Lomo, resulting from the coincident activity of pre- and post-synaptic components that leads to the facilitation of the chemical transmission (Abraham et al., 2002; Bliss and Lomo, 1973). Experimentally, LTP can be achieved using high-frequency trains of electrical stimuli (tetani) delivered to Schaffer collateral/commissural fibres projecting from CA3 to CA1 pyramidal neurons. This ensures sufficient synaptic input to strength synaptic communication (Cooke and Bliss, 2006).

Several lines of evidence point to a central role of GluA1-containing AMPAR in hippocampal LTP. Accordingly, mature GluA1-knockout mice lack LTP in the CA1 region of the hippocampus (Zamanillo et al., 1999) and this defect is rescued by genetically expressing GluA1 subunits (Mack et al., 2001). The translocation of GluA1-containing AMPAR to the synapse requires high-frequency stimulation of the synapse and is dependent on NMDAR activation (Shi et al., 1999).

The NMDA-type of glutamate receptors also play a key role in LTP. These receptors are present on the post-synaptic membrane where they bind glutamate released into the synaptic cleft following the arrival of an afferent action potential at the pre-synaptic terminal. Binding of glutamate to NMDAR is not sufficient to open the receptor intrinsic cation channel, because at near-resting membrane potentials the channel is blocked by magnesium ions (Nowak et al., 1984). Activation of NMDAR requires the costimulation of AMPAR to depolarize the plasma membrane, thereby expelling the magnesium ions from the NMDA receptor channel and allowing the influx of sodium and calcium ions. It is the calcium influx through NMDA receptors that is thought to initiate LTP induction (Lynch et al., 1983; Malenka et al., 1988) by activating the calcium/calmodulin dependent kinase II (CaMKII) or the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA; Figure 1). These molecules initiate LTP expression mechanisms, in short-term by phosphorylating

receptors and altering the intrinsic properties of their ligand-gated ion channels, and in long-term by activating local protein synthesis at the synapse and intracellular signaling to the nucleus via transcription factors, thereby altering gene expression (Alberini et al., 1995; Goelet et al., 1986). This is in accordance with the model proposing that LTP can be divided into at least two temporally distinct phases:

- Early phase-LTP (E-LTP) lasts for 1 – 2 hours and requires modification of existing proteins and their trafficking at synapses, but no *de novo* protein synthesis is required (Bliss and Collingridge, 1993; Malenka and Bear, 2004). This form of plasticity can be induced, for example, by a weak high frequency tetanus (e.g. a train of 100 pulses at 100 Hz).
- Late phase-LTP (L-LTP) follows an early translation-independent phase of synaptic potentiation and requires *de novo* mRNA transcription, new protein synthesis and structural changes at synapses (Frey et al., 1988; Harris et al., 2003). L-LTP results, for example, from repeated high frequency stimulations (e.g. multiple trains of 100 pulses at 100 Hz) which can induce an increase in synaptic efficacy lasting over hours or even days (Abraham, 2003).

The molecular mechanisms of LTP induction and maintenance vary somewhat from synapse to synapse. For instance, within the hippocampus, at the mossy fibre–CA3 pyramidal cell synapses, the NMDA receptor is not required for LTP induction (Harris and Cotman, 1986). In contrast, at both medial perforant path–dentate gyrus granule cell (Morris et al., 1986) and Schaffer collateral–CA1 pyramidal cell synapses (Collingridge et al., 1983), LTP induction is mediated by NMDAR (Cooke and Bliss, 2006). As such, there is no general molecular mechanism for LTP induction and expression at glutamatergic synapses. The available evidence indicate that the important properties of LTP, longevity, input specificity and associativity, can be implemented by a variety of receptors and signaling systems (Cooke and Bliss, 2006).

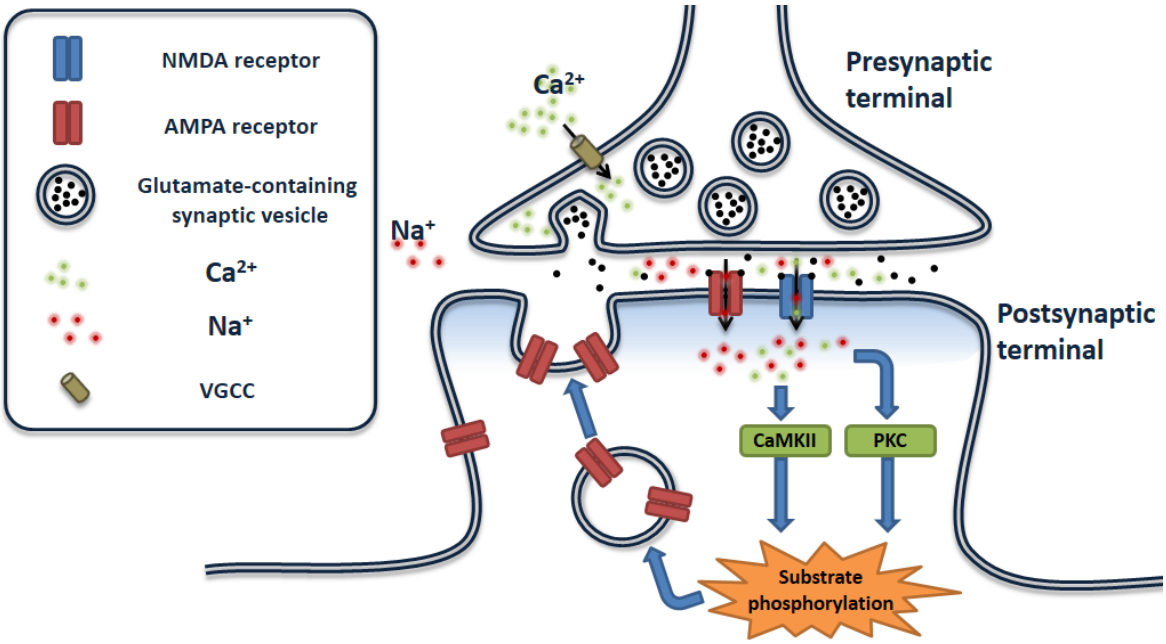

Figure 1- Mechanisms underlying LTP. Glutamate released from the presynaptic terminal into the synaptic cleft bind to AMPA and NMDA ionotropic receptors. Activation of AMPA receptor channels by glutamate leads to sodium entry into the postsynaptic compartment. The depolarization of the postsynaptic membrane induced by sodium-entry removes the voltage-dependent magnesium block of NDMA receptors resulting in a rapid increase of intracellular calcium levels in dendritic spines. Calcium activates several signaling cascades that lead to AMPAR synaptic insertion.

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In addition to the ionotropic glutamate receptors, other receptors play an important role in the regulation of excitatory synapses and in LTP in the hippocampus, including the metabotropic glutamate receptors and the TrkB receptors (tropomyosin-related kinase B) for BDNF (brain-derived neurotrophic factor), which have been genetically identified as required for the induction and strengthening of LTP, respectively (Minichiello et al., 1999; Tsien et al., 1996; Zamanillo et al., 1999). The role of BDNF in LTP will be discussed in the next section.

2.1 - BDNF in LTP

2.1.1 - BDNF and TrkB signaling

BDNF belongs to the neurotrophin family of trophic factors and controls several functions in the CNS, including neuronal survival and differentiation as well as synaptogenesis, in addition to an important role in activity-dependent forms of synaptic plasticity (Lewin and Barde, 1996; Santos et al., 2010). In hippocampal neurons BDNF is mainly present in dendrites, stored in vesicles of the regulated secretory pathway. These vesicles fuse with the membrane and release the neurotrophin to the synaptic cleft in response to signals that

increase the $[Ca^{2+}]_i$, by a mechanism that is also dependent on the activity of CaMKII and PKA (Hartmann et al., 2001; Kolarow et al., 2007).

At the synapse BDNF binds to TrkB receptors located at the pre- and postsynaptic membranes, resulting in receptor dimerization (Jing et al., 1992) and transphosphorylation on specific tyrosine residues located in the intracellular domain. The effects of BDNF are mediated by activation of various parallel signal transduction cascades, with distinct functions, which are initiated after docking of different adaptor proteins and signaling enzymes (Figure 2; Reichardt, 2006). There are several tyrosine residues on the intracellular domain of TrkB receptors that undergo phosphorylation after receptor dimerization. Phosphorylation of tyrosine 515 leads to the recruitment of Shc (Src homology 2 domain-containing) to the active TrkB receptors, and the adaptor protein is itself phosphorylated on tyrosine. This allows the interaction with additional adaptor proteins, thereby activating the Ras/ERK (extracellular signal-regulated kinase) signaling pathway (Minichiello, 2009). Activation of ERK regulates transcription events, including the activation of CREB (cAMP response element-binding protein). Shc binding to activated TrkB receptors also stimulates the PI3K/Akt (phosphatidylinositol 3-kinase) signaling pathway. In addition to the regulation of transcription activity, Akt may also induce rapid and local changes in the proteome by regulating the translation machinery (Takei et al., 2004). Phosphorylation of TrkB receptors on tyrosine 816 recruits and activates PLCγ (phospholipase C gamma) by tyrosine phosphorylation (Minichiello, 2009). PLCγ hydrolyses PIP2 (phosphatidylinositol 4, 5-bisphosphate), giving rise to DAG (diacylglycerol), which activates PKC (protein kinase C), and inositol 1,4,5-trisphosphate, which releases calcium from intracellular stores. This signaling pathway also contributes to ERK and CREB activation, thereby increasing cell survival, and plays a key role in synaptic plasticity (Minichiello et al., 2002).

2.1.2 - BDNF in E-LTP

BDNF plays an important role in LTP induced by high frequency stimulation in the hippocampus CA1 region (Chen et al., 1999; Kang et al., 1997), 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).

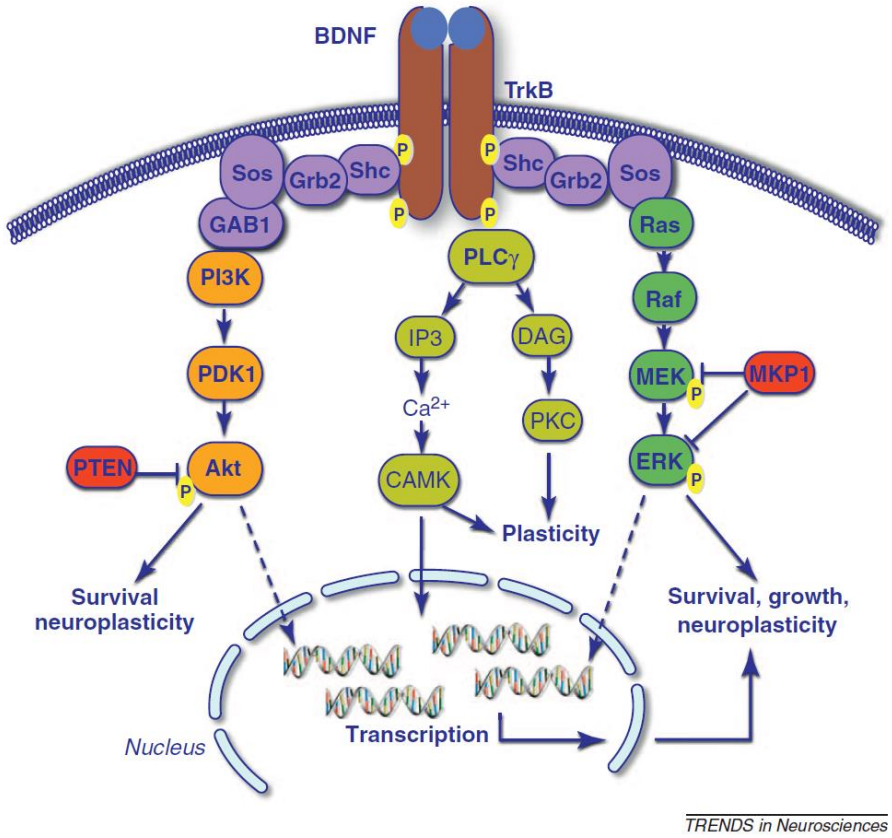

Figure 2- BDNF-TrkB intracellular signaling pathways (Duman and Voleti, 2012).

An interesting and yet unresolved issue is whether the endogenous BDNF is derived pre- or post-synaptically. Early work suggested that E-LTP at the Schaffer collateral- CA1 synapses requires BDNF derived from presynaptic CA3 neurons, but not from postsynaptic CA1 neurons (Zakharenko et al., 2003). The early increase in extracellular BDNF may be due to exocytosis of preexisting BDNF-containing vesicles from presynaptic terminals of CA3 neurons induced by high frequency stimulation (HFS). This may be important for the induction of E-LTP.

Protein phosphorylation accounts for some of the early post-synaptic effects of BDNF in the potentiation of glutamatergic synapses. BDNF was shown to induce tyrosine phosphorylation of GluN1 and GluN2B NMDAR subunits in cultured hippocampal neurons (Lin et al., 1998; Suen et al., 1997). The stimulation of cultured hippocampal neurons with BDNF increases NMDA receptor single channel open probability (Levine et al., 1998), presumably through phosphorylation of GluN2B subunits (Levine and Kolb, 2000). Furthermore, treatment with BDNF induced a rapid synaptic delivery of GluA1-containing AMPA receptors in cultured hippocampal neurons (Fortin et al., 2012) and in hippocampal organotypic cultures, by a

mechanism dependent on TrkB receptor activation (Figure 3; Caldeira et al., 2007; Fortin et al., 2012). Although the translocation of GluA1-containing AMPA receptors to the synapse was first included in the list of events contributing to E-LTP, recent studies showed that BDNF-induced increase in the surface expression of GluA1-containing AMPA receptors follows the opening of transient receptor potential canonical channels which allow the entry of Ca^{2+} with consequent stimulation of CaM-kinase kinase. This kinase induces the local translation of AMPA receptors which are rapidly incorporated at the synapse (Fortin et al., 2012). BDNF was also shown to induce synaptic delivery of GluA1-containing AMPA receptors from a local pool in cultured cerebrocortical neurons (Nakata and Nakamura, 2007).

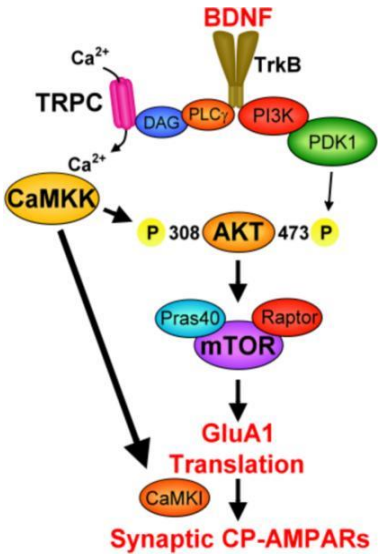

Figure 3- Signaling pathway involved in BDNF-induced synaptic delivery of GluA1-containing Ca^{2+}-permeable AMPAR (CP-AMPAR). GluA1 translation in response to BDNF depends on the activation of CaMKK (Fortin et al., 2012).

2.1.3 - BDNF and transcription in L-LTP

Experiments with an anti-BDNF antibody or with the TrkB-Fc peptide showed a role for the neurotrophin in the L-LTP induced by high frequency stimulation, which is also dependent on transcription and translation activity (Chen et al., 1999; Kang et al., 1997). These results were supported by the effect of exogenous addition of BDNF which induces synaptic potentiation (BDNF-LTP) in hippocampal CA1 slices (Ji et al., 2010; Kang and Schuman, 1996) and in the dentate gyrus (Messaoudi et al., 2002), in experiments performed in hippocampal slices and in vivo, respectively. Under the latter conditions the effect of BDNF in L-LTP was

abrogated by the transcription inhibitor actinomycin D (Messaoudi et al., 2002), showing that changes in gene expression underlie some of the effects of BDNF in LTP.

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 signal that upregulates the expression of the IEG (immediate-early gene) *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 the activity of TrkB receptors to induce gene expression is required mainly in the soma compartment. However, distinct mechanisms are involved in the regulation of the expression of the two genes 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. Whether these BDNF-induced transcripts coding for synaptic proteins are mainly translated in the soma before being transported to dendrites and/or delivered to the dendritic compartment in RNA granules also remains to be elucidated.

2.1.4 - BDNF turnover

Due to the low expression level of BDNF in neurons, the existing BDNF would eventually be exhausted in the event of repeated strong synaptic stimulation. Thus, for the long-term maintenance of L-LTP, a sustained supply of BDNF may come primarily from new protein synthesis triggered by the repeated strong synaptic stimulation. This hypothesis is supported by the findings that BDNF mRNA levels in the postsynaptic CA1 are significantly increased 1-3 hours after the L-LTP-inducing tetanic stimulation (Patterson et al., 1992). High K^+-induced depolarization was also shown to facilitate the translocation of BDNF mRNA into the dendrites of cultured hippocampal neurons (Tongiorgi et al., 1997). BDNF itself has been shown to induce dendritic targeting of BDNF mRNA (Righi et al., 2000). Additional studies are required to demonstrate that local and activity-dependent secretion of BDNF could recruit BDNF mRNA to the active synapses. An alternative mechanism could be that BDNF mRNA is nonselectively transported to dendritic spines, being trapped by synapses that undergo high-frequency transmission. This hypothesis resembles the “synaptic tagging” model for the synapse specificity of L-LTP (Frey and Morris, 1997; Redondo and Morris, 2011). Thus, local translation of BDNF mRNA is a key feature both in the dendritic targeting and dendritic trapping models.

Another important issue that attracted the attention of the investigators is how locally synthesized secretory or transmembrane proteins get processed. The BDNF protein undergoes a number of post-translational modifications, including glycosylation, proper folding, cleavage, and sorting to the constitutive or regulated secretion pathways. Folding and N-glycosylation are processed in ER (endoplasmic reticulum) whereas cleavage and sorting occur in the Golgi apparatus or subsequent organelles. Mike Ehlers and colleagues showed that at least in cultured hippocampal neurons, the Golgi apparatus is absent in the majority of dendrites (Horton et al., 2005). Using markers specific for Golgi, they showed that small, Golgi-like organelles (named Golgi outposts) are selectively localized to dendritic branch points and are typically present in only one of the dendrites. This study poses a number of important conceptual challenges to local synthesis of BDNF. First, most dendritically synthesized BDNF has to be transported back to the neuronal soma to be processed in Golgi apparatus. Second, even in the long (apical) dendrite, BDNF synthesized in the distal region still needs to be transported to the branch point to be sorted in the Golgi outposts. In both cases, a round-trip trafficking of BDNF is implicated, which would cause a loss of synapse specificity. Third, assuming that locally translated BDNF could be secreted after glycosylation and correct folding in the ER in distal dendrites, which lack Golgi apparatus, it would only be secreted in a constitutive manner in the form of proBDNF, because sorting to the regulated secretion pathway and intracellular cleavage can only happen in the Golgi. The requirement of round-trip transport for BDNF processing makes it difficult to ensure a selective modulation of the stimulated synapse without affecting other synapses (Lu et al., 2008).

2.1.5 - BDNF and local translation

2.1.5.1 - Local translation

Local translation is a requirement for synaptic potentiation induced by BDNF (Kang and Schuman, 1996), long-term depression elicited by mGluR (Huber et al., 2000), late-phase LTP induced by high frequency stimulation (Bradshaw et al., 2003) and dopamine-induced plasticity (Smith et al., 2005). However, in most cases the identity of the proteins that are locally synthesized during plasticity and the mechanisms controlling their translation have not been identified (Cajigas et al., 2012).

Although most mRNAs are translated in the neuronal cell body, local translation of specific mRNAs might be of particular importance for the regulation of protein expression within dendrites. The hypothesis that protein translation can take place in postsynaptic compartments came from a pioneer study reporting the presence of polysomes at the base of several spines in a rosette-like structure (Figure 4), which is the distinctive evidence that they are bound to mRNAs and actively engaged in protein synthesis (Steward and Levy, 1982).

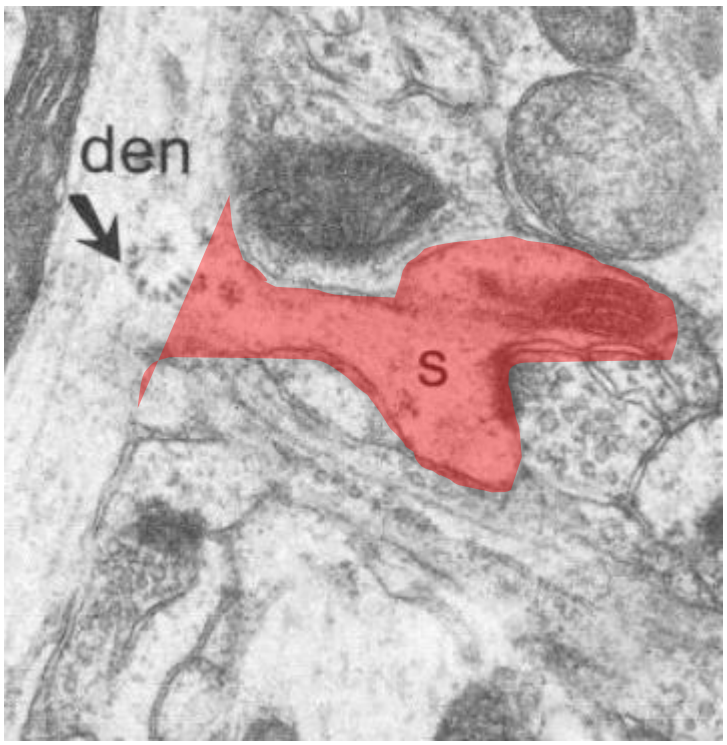

Figure 4- Distribution of polysomes in dentate granule cells. The electron micrograph shows highly dense structures, corresponding to ribosomes arranged into a rosette-like formation, at the base of dendritic spines (s). The dendritic spine is highlighted in red and the polysomes are indicated by the arrow at the spine-dendritic (den) shaft intersection. Adapted from Steward and Levy (1982).

The hypothesis of local translation at the synapse suggests that several key components can be specifically synthesized and regulated by signaling events initiated in a specific synapse.

2.1.5.2 - The translation machinery

BDNF was shown to promote local protein translation within dendrites through direct activation of the translation machinery (Aakalu et al., 2001). Thus, BDNF was shown to

activate the mammalian target of rapamycin (mTOR) and the extracellular signal regulated kinase (ERK) signaling pathways (Patterson et al., 2001; Takei et al., 2004), two critical regulators of translation during LTP formation (Kelleher et al., 2004). These pathways mediate phosphorylation of several translation initiation factors, including the eukaryotic initiation factor 4E (eIF4E), the eIF4E-binding protein (4EBP), and ribosomal protein S6, resulting in an increase in mRNA translation (Figure 5; Lujian Liao, 2007). Translation activation by BDNF was also shown by the upregulation of the polysome fraction in cultured hippocampal neurons stimulated with the neurotrophin (Schratt et al., 2004).

A great number of mRNAs have been identified in dendrites (Cajigas et al., 2012; Eberwine et al., 2001; Steward and Worley, 2001; Zhong et al., 2006), and different classes of proteins were shown to be synthesized at the synapse following stimulation with BDNF. Some of the proteins that are locally translated in response to BDNF are the activity-regulated cytoskeletal protein (Arc; Yin et al., 2002), the CaMKIIα (Takei et al., 2004), NMDA receptor subunit 1 (GluN1) and Homer 2 (Schratt et al., 2004), which are involved in synapse formation, maturation and plasticity. BDNF was also shown to upregulate the local translation of PSD95 and GluA1 subunit of AMPA receptors by activating the translation machinery through mTOR and its downstream target p70S6K (Figure 5; Schratt et al., 2004; Yang et al., 2008).

A high-throughput proteomic analysis showed that BDNF up-regulates 230 proteins in a synaptic fraction isolated from cultured cerebrocortical neurons. Many of the identified proteins have direct and indirect functions in the modulation of synaptic structure and function. Of particular interest, among the identified proteins up-regulated by BDNF were components of the translation machinery, such as ribosomal proteins from the 40S and 60S subunits, tRNA synthetases, and initiation and elongation factors. These components seem to be locally synthesized at or near synapses (Figure 6), because the mRNAs were detected in dendrites by in situ hybridization, and the increase in the expression of these proteins was sensitive to protein synthesis inhibitors and abrogated by the mTOR inhibitor rapamycin (Lujian Liao, 2007). These data strongly suggest that the increase in synaptic translation capacity and the widespread changes in the synaptic proteome induced by BDNF account for the synaptic changes associated with the consolidation phase of LTP (Lujian Liao, 2007).

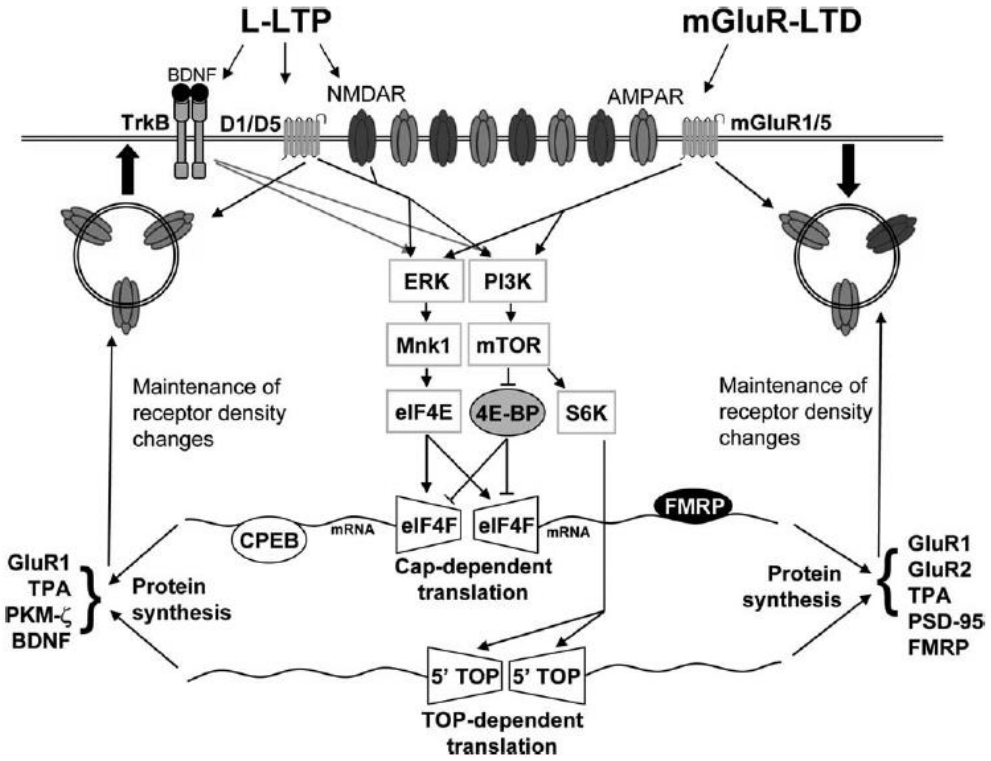

Figure 5- Convergence and divergence of mechanisms for protein synthesis-dependent LTP and LTD (Pfeiffer and Huber, 2006).

Arc, an immediate early gene, is translated locally at the synapse upon BDNF stimulation (Yin et al., 2002). Arc plays a role in the dephosphorylation of cofilin, a major regulator of F-actin dynamics in spines, thereby contributing for synaptic potentiation. Studies performed in synaptoneurosomes also showed that BDNF upregulates RhoA, a member of the Rho GTPase family that is involved in the remodeling of actin cytoskeleton (Troca-Marin et al., 2010). Arc and RhoA combine the induction of protein translation to F-actin expansion, which may ultimately lead to morphological changes (Matsuzaki et al., 2004; Tanaka et al., 2008) in BDNF-induced LTP (Messaoudi et al., 2007).

BDNF may also target RNPs which act as modulators of mRNA localization and/or translational repressors. Similarly to KCl depolarization, BDNF stimulation induces the translocation of dendritic-like P-bodies towards the distal region of the dendrites (Cougot et al., 2008).

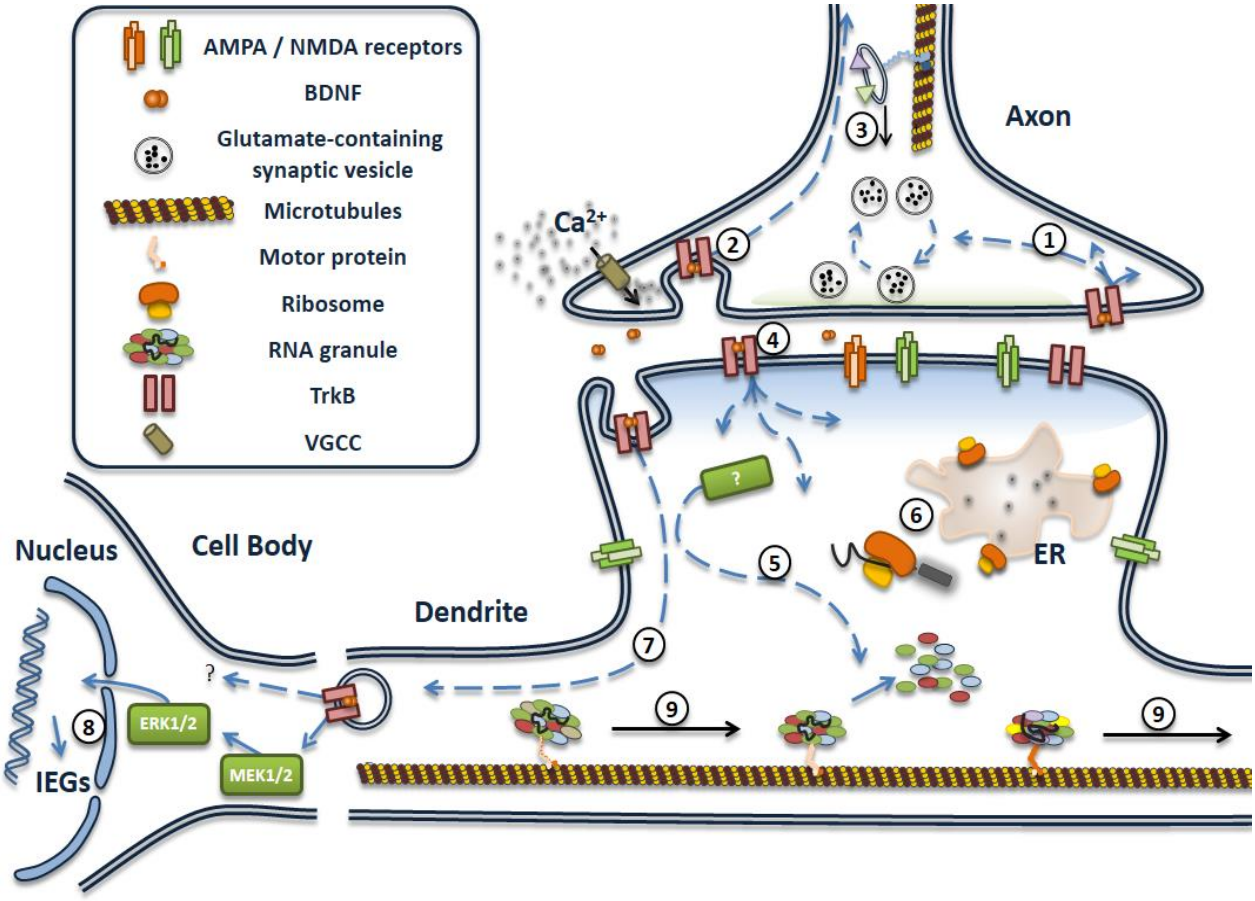

Figure 6- BDNF-induced local translation at the synapse and upregulation of gene expression. TrkB receptor activation upon BDNF binding increases the accumulation of synaptic vesicles at the active zone in the presynaptic region, thereby potentiating synaptic transmission (1). BDNF-TrkB complex can be 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 signalling 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). Adapted from Leal et al. (2013).

2.2 - PDGF in LTP

Platelet-derived growth factor (PDGF) was identified more than three decades ago as a serum growth factor for fibroblasts, smooth muscle cells, and glia cells (Kohler and Lipton, 1974; Ross et al., 1974; Westermark and Wasteson, 1976). Members of the PDGF family are disulfide-bonded polypeptides that have multifunctional roles ranging from embryonic

development to wound healing (Heldin and Westermark, 1999). PDGFs comprise four polypeptide chains (A-D) that can form homo- or heterodimers (PDGF-AA, -BB, -AB, -CC, and -DD) and can also bind two types of receptors (PDGF-α and -β receptor) (Fredriksson et al., 2004).

The early studies showed that PDGF-A is produced in neurons and glia cells in the mouse central nervous system (Yeh et al., 1991), while PDGF-B was only found in neurons throughout the brain of a nonhuman primate (Sasahara et al., 1991). In mice, the PDGF-α receptor is expressed in oligodendroglial O-2A precursors or glial cells but not in neurons (Yeh et al., 1993), while in rats PDGF-β receptor is mainly expressed in neurons (Smits et al., 1991).

PDGFR-β receptor undergoes dimerization and activation upon binding to PDGF-B homodimers (-BB) (Andrae et al., 2008). As described for TrkB receptors (Guiton et al., 1994), dimerization of PDGF receptors induces their autophosphorylation and increases the catalytic efficiencies of the kinases domain (Kazlauskas and Cooper, 1989). Phosphorylation of the receptor creates a docking site that enables the recruitment and activation of signal transduction molecules containing SH2 domains, such as PI3-kinase, phospholipase C (PLC)-γ (Meisenhelder et al., 1989), the Src family of tyrosine kinases (Erpel and Courtneidge, 1995), the tyrosine phosphatase SHP-2 (Pluskey et al., 1995), a GTPase activating protein (GAP) for Ras (Heidaran et al., 1993) and members of the Stat family (Patel et al., 1996).

PDGFR-β was shown to colocalize with synaptophysin and postsynaptic density 95 (PSD95) used as pre- and post-synaptic markers, respectively. Interference with PDGFR-β function in hippocampal neurons impairs LTP induction and hippocampus-dependent memory formation, and disrupts dendritic spine morphology (Shioda et al., 2012). In a different study, PDGF was found to enhance LTP in the rat hippocampal CA1 synapses and regulated the expression of Arc/Arg3.1 gene, which has been implicated in LTP (Peng et al., 2010). Although inhibition of PDGF receptors abrogated the effects on LTP, synaptic potentiation was maintained at levels similar to those observed in the absence of PDGF when the experiments were performed in the presence of the inhibitor. These results suggest that although PDGF upregulates LTP induced by high-frequency stimulation, the endogenous PDGF may not play an important role in long-term synaptic potentiation under certain experimental conditions.

3- RNA transport along dendrites

3.1 - mRNA targeting

The targeting of mRNAs to specific subcellular sites involves multiple steps. The cis-acting elements in mRNA enable the mRNA sorting to a given cellular compartment. These cis-acting elements are usually found in the 3'untranslated region (UTR), but may also be present in the 5' UTR or in the coding sequence, and be recognized by specific RNA-binding proteins. RNA-binding proteins are involved both in transcript transport and translational regulation. Several studies indicate that the processing of pre-mRNAs in the nucleus is required for the recruitment of RNA-binding proteins that determine the targeting of the RNAs to different locations in the cytoplasm (Giorgi and Moore, 2007). RNAs and RNA-binding proteins can form complexes, called ribonucleoproteins (RNPs) that may integrate a RNA transport granule, being transported by motor proteins along the cytoskeleton to their final destination (Figure 7). Finally, there are mechanisms to maintain the RNA in a translationally repressed state during delivery and to regulate its spatiotemporal translation (Martin and Ephrussi, 2009).

Splicing and alternative polyadenylation site selection are regulated nuclear events that can generate different RNA isoforms with different targeting specificities (Figure 8A). In rat hippocampal neurons, the differential use of polyadenylation sites in BDNF mRNA results in several different mRNA isoforms with different 3' UTR length. The short 3' UTR mRNAs are restricted to soma, whereas the long 3' UTR mRNAs are also localized in dendrites (Figure 8B; An et al., 2008).

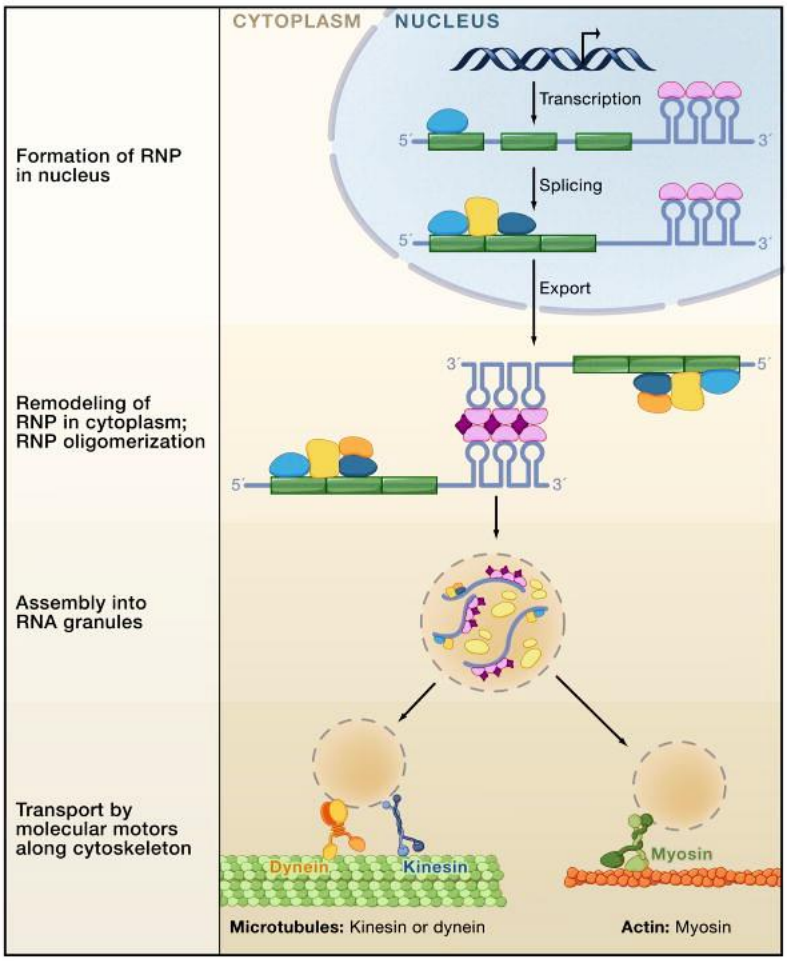

Figure 7- Targeting of mRNA as a multistep process. The pre-mRNA (exons in green; introns, 5' and 3'UTRs in grey) has *cis*-acting localization elements in its primary sequence. These are usually in the 3'UTR and often form stem-loop structures. RNA-binding proteins (blue and purple) bind the pre-mRNA. During splicing, additional RNA-binding proteins (golden and dark blue) are added to form a ribonucleoprotein (RNP) complex. Following export into the cytoplasm, the RNP is remodeled as additional proteins (orange, dark purple) are added. In some cases, the RNP can form oligomers with other RNPs through protein-protein interactions. In the cytoplasm, RNPs are assembled into RNA granules that are likely to be a heterogeneous population of structures containing diverse RNAs, ribosomal subunits (yellow), as well as many factors involved in translational regulation. Recent studies suggest a dynamic relationship between RNA transport granules, P-bodies, and stress granules. The RNA granules associate with motor proteins and are transported by cytoskeletal elements to their final destination (Martin and Ephrussi, 2009)**.**

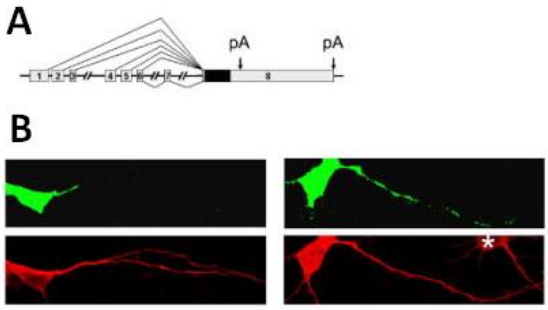

Figure 8- Role of the 3' UTR region in the targeting Bdnf mRNA to dendrites. (A) Diagram of the mouse *Bdnf* gene depicting two alternative polyadenylation (pA) sites in exon 8 (arrows). Curved lines linking boxes (exons) indicate alternative splicing from the first seven exons to exon 8. The filled box within exon 8 represents the coding sequence. (B) Localization of GFP mRNA in neurons transfected with GFP- short 3' UTR (left) and GFP- long 3' UTR (right) of the Bdnf mRNA. Top panels: FISH of cultured neurons with a GFP antisense riboprobe; Bottom panels: MAP2 immunocytochemistry; *untransfected neuron. Adapted from An et al. (2008).

3.2 - mRNA transport in RNP

The first characterization of intracellular movement of mRNA in living cells and description of the role of RNA granules in transport and localization of mRNA in cells was done by Carson and colleagues. They observed the myelin basic protein (MBP) mRNA transport in oligodendrocytes and concluded that a population of large RNA granules, containing multiple localized transcripts, served as the vehicle for mRNA transport (Ainger et al., 1993). Later studies in neurons showed that these RNA granules were composed by a heterogeneous population (Kiebler et al., 1999). Purification of RNA granules from neurons has revealed some of the components of these large RNPs. Krichevsky and Kosik isolated complexes larger than polysomes from cultured neurons and showed that they contained ribosomes and the RNA-binding protein Staufen. They further showed that depolarization of neurons disrupted the structure of the RNA granule, releasing the mRNAs to be translated (Krichevsky and Kosik, 2001).

Hirokawa and colleagues purified large RNA granules from mouse brain that associate with the tail of the kinesin motor protein KIF5. These RNA granules contained CamKIIα and Arc mRNAs. Proteomic analysis led to the identification of proteins previously known to be involved in mRNA localization in neurons, including Staufen and FMRP (fragile X mental retardation protein), as well as new trans-acting factors involved in mRNA localization, including Pur-α, hnRNP U, and polypyrimidine tract binding protein-associated splicing

factor (PSF). Using RNAi knockdown the authors further showed that Pur-α, hnRNP U, PSF, and Staufen were all required for the dendritic localization of CamKIIα mRNA (Kanai et al., 2004).

A proteomic analysis provided information about the components of RNA granules isolated from developing rodent cortex (Elvira et al., 2006). The composition of these RNA granules differed somewhat from those identified by Kanai and colleagues (Kanai et al., 2004). They were found to be enriched in β-actin mRNA but not CamKIIα mRNA, and contained ribosomes, RNA-binding proteins, such as Staufen and hnRNP A2, as well as the DEAD-box 3 helicase which had previously been implicated in RNP assembly (Elvira et al., 2006). Taken together, these two studies suggest that there are multiple species of RNA granules, each containing distinct populations of mRNAs and RNA-binding proteins.

3.3 - RNA transport granules, stress granules and processing bodies

Recent studies have focused on neuronal RNA transport granules, stress granules, and RNA processing bodies (P-bodies; Kiebler and Bassell, 2006). Stress granules formed following environmental stress consist of stalled ribosomal initiation complexes, mRNAs that encode most cellular proteins other than heat-shock proteins, translation initiation factors, as well as a number of RNA-binding proteins involved in mRNA localization, such as Staufen, FMRP, and cytoplasmic polyadenylation element binding protein (CPEB). Stress granules have been postulated to serve as triage centers that sort, remodel, and export specific mRNA transcripts for reinitiation, decay, or storage (Anderson and Kedersha, 2006). P-bodies contain components of the 5'-3' mRNA decay machinery, nonsense-mediated decay pathway, and RNA induced silencing complex. Similarly to stress granules, P-bodies have been shown to contain RNA-binding proteins that are components of RNA transport granules, including Staufen and FMRP. This finding, together with recent indications that the translation of transcripts localized to dendrites may be regulated by miRNAs, raises the possibility that mRNAs may undergo dynamic trafficking between RNA transport granules, P-bodies, and stress granules (Kiebler and Bassell, 2006).

3.4 – Motor proteins for mRNAs transport

Microtubules and actin filament networks provide a railway for trafficking of mRNAs within the cytoplasm, with the microtubule motor proteins kinesin, dynein and myosin providing the vehicle for transport along these pathways (Figures 6 and 7). In neurons, where the distances

travelled by mRNAs are especially great, microtubules have been demonstrated to play a critical role. Kiebler studies demonstrated a fundamental role for microtubules in the staufen-dependent dendritic mRNA transport (Kiebler et al., 1999). Hirokawa and colleagues demonstrated a role for the microtubule anterograde motor KIF5 in transporting many dendritically localized transcripts and further showed that alterations in the concentrations of KIF5 modulate the dendritic localization of RNA granules in neurons (Kanai et al., 2004). Genetic, pharmacological and siRNA-mediated inhibition of kinesins have been shown to inhibit FMRP transport into dendrites and have further indicated that FMRP interacts with at least two distinct kinesin isoforms, KLC (the light chain component of KIF5; Dictenberg et al., 2008) and KIF3C (Davidovic et al., 2007). The finding that FMRP can use two kinesin motors indicates that molecular motors may play redundant roles in mRNA transport.

Several studies have indicated that neuronal activity modulates the transport of mRNAs into dendrites (Sossin and DesGroseillers, 2006). It will be interesting to determine whether this modulation occurs as a result of posttranslational changes in the RNA-binding proteins, in the composition of RNA granules, or perhaps as modifications of microtubules or motor proteins. Studies performed in Aplysia neurons showed that kinesin heavy chain isoforms are upregulated during learning-related synaptic plasticity and showed that this upregulation results in an increase in transport of essential components from the soma to the synapse (Puthanveettil et al., 2008).

3.5 - hnRNPK

The hnRNPs are a different class of RNA-binding proteins (RBPs), consisting in a large group of primarily nuclear proteins that bind to nascent transcripts. They are composed of multiple domains connected by linker regions of varying length. The predominant domain shared by these proteins is called RRM (RNA recognition motif) which allows RNA binding through hydrophobic interactions. However, the RRM domain is not present in all hnRNPs being replaced in some cases by other domains that are responsible for the RNA binding. Both hnRNP E and K possess K homology domains (KH) that are known to participate in a range of biological processes through interactions with RNA or ssDNA (Han et al., 2010).

hnRNPK is a 65 kDa protein composed by 463 amino acids containing different regions with distinct functions (Figure 9). The protein contains three KH domains, responsible for the RNA and ssDNA binding with a high affinity towards polycytosine tracts, as well as a NLS (nuclear localization signal), a KNS (nuclear shuttling domain) and a KI region (K-protein-

interactive region), responsible for numerous protein-protein interactions (Bomsztyk et al., 2004; Bomsztyk et al., 1997). A lot of KI-binding partners were already identified via mass spectrometry, including kinases and proteins controlling mRNA splicing, transcription and translation processes (Mikula et al., 2006).

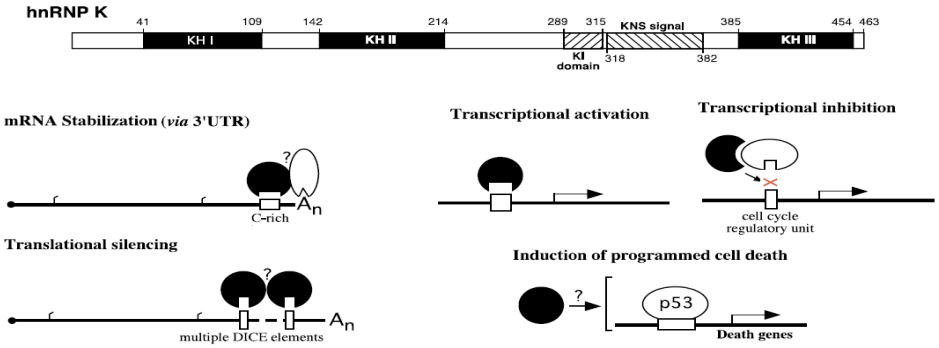

Figure 9- hnRNPK structure and functions.(Makeyev and Liebhaber, 2002)

3.5.1 - hnRNPK role in transcription and cell proliferation

hnRNPK has a specific binding site on the SV40 early promoter (Gaillard et al., 1994) and in the pyrimidine-rich strand of the CT element in the promoter of human c-myc gene (Tomonaga and Levens, 1996). In both cases this interaction activates transcription (Figure 9) in *in vitro* systems, apparently by an hnRNPK-dependent assembly of the transcription factor II D complex at target promoters (Michelotti et al., 1996). In the case of the thymidine kinase promoter, hnRNPK itself cannot physically interact with promoter but may repress transcription (Figure 9) by inhibiting the binding of other *trans*-factors to the cell cycle regulatory determinant of the promoter (Lau et al., 2000).

In breast cancer cells hnRNPK was found to increase the activity of the target c-myc promoter and to upregulate c-Myc and hnRNPK protein levels, together with an increase in cell proliferation and growth (Mandal et al., 2001). In a loss-of-function screening system based on intracellular expression of single domain antibodies, hnRNPK was found as a potential target for cell migration and metastasis of human fibrosarcoma cells (Gao et al., 2013).

3.5.2 - Modulation of hnRNPK's RBP function

hnRNP A2, hnRNP E1 and hnRNPK were shown to prevent translation initiation of DICE (differentiation control element, an evolutionarily conserved CU-rich repeated motif within the 3' UTR)-containing 15-lipoxygenase mRNA (Ostareck et al., 2001). The RNA binding protein ZBP1 also inhibits translation of β-actin mRNA (Huttelmaier et al., 2005) by preventing the association of the 60S ribosomal subunit with the 48S pre-initiation complex. These translationally silenced states can be halted following the phosphorylation of hnRNP A2 by Fyn (White et al., 2008) and Src-mediated phosphorylation of ZBP1 (Sasaki et al., 2010).

The KI region of hnRNPK contains proline-rich docking sites that interact with SH3 domains, characteristic of the Src-family kinases (Vanseuningen et al., 1995). In fact, along the entire amino acid sequence, several serine, threonine and tyrosine residues are known to be phosphorylated (Ostareck-Lederer et al., 2002; Ostrowski et al., 2000; Schullery et al., 1999) and the regulation of the phosphorylation state of some of these residues was shown to be important for the translational silencing relieve (Ostareck-Lederer et al., 2002)

The available evidence also suggest that phosphorylation may change the mRNA binding ability of hnRNPK (Feliers et al., 2007; Habelhah et al., 2001; Ostareck-Lederer et al., 2002; Ostrowski et al., 2000), and this may contribute to the release of specific transcripts in response to stimuli that induce the appropriate signaling activity. Additional studies have shown that hnRNPK is a substrate of Src-family of tyrosine kinases and PKCδ. Thus, the Src-family kinase Lck binds to the KI region of hnRNPK through the SH3 docking sites and phosphorylates the protein causing the mRNA-RBP dissociation (Ostareck-Lederer et al., 2002; Ostrowski et al., 2000). This phosphorylation on tyrosine creates SH2 docking sites that allow the recruitment of PKCδ, since this kinase only binds to hnRNPK when it is dissociated from the mRNA (Schullery et al., 1999). Lck activates PKCδ by phosphorylation and the active PKC phosphorylates hnRNPK on Ser302 in addition to other effector proteins that may either be bound to hnRNPK or in the surrounding microenvironment (Bomsztyk et al., 1997; Schullery et al., 1999). Furthermore, hnRNPK is a target of other enzymes and signaling cascades that may play an important regulatory role. Phosphorylation of hnRNPK on Ser284 by ERK leads to its cytoplasmic accumulation and inhibition of mRNA translation (Habelhah et al., 2001), suggesting a pleiotropic effect for the hnRNPK phosphorylation.

3.5.3 - hnRNPK in neurons

hnRNPK was identified as a partner of Abi-1 at postsynaptic sites. The interaction with the Abi-1 SH3 domain is mediated by the hnRNPK-interaction (KI) domain (Proepper et al., 2011). At the postsynaptic density Abi-1 is in a complex with several other proteins including WASP/WAVE or cortactin, thereby regulating the actin cytoskeleton via interaction with the Arp 2/3 complex. In the same study, the downregulation of hnRNPK in cultured hippocampal neurons using RNAi enlarged the dendritic tree and significantly increased filopodia formation, and a decrease in the number of mature synapses was also observed (Proepper et al., 2011). hnRNPK has also been identified as a direct interaction partner of N-WASP via KI/WH1-domain-interaction (Yoo et al., 2006).

Results from a proteomic study performed in our laboratory suggested that hnRNPK may be phosphorylated upon stimulation of cultured hippocampal neurons with BDNF (Manadas et al., 2009). However, this was not further investigated using other methodologies and, therefore, the functional consequences of the regulation of hnRNPK by BDNF still remain to be determined.

CHAPTER 2

Objectives

In a gel-based proteome profiling of the long-term effects of BDNF in cultured hippocampal neurons conducted in our laboratory, it was observed that BDNF upregulated several protein spots identified as hnRNPK. These results suggest that hnRNPK undergoes posttranslational modifications in neurons exposed to the neurotrophin. In particular, one of the up-regulated spots had a more acidic pI, suggesting that hnRNPK may be phosphorylated upon stimulation with BDNF (Manadas et al., 2009). More recently, hnRNPK phosphorylation on Ser302 was observed in cultured hippocampal neurons stimulated with BDNF (Comprido, 2011). A microarray screening identified 11422 transcripts that co-immunoprecipitated with hnRNPK in cultured hippocampal neurons, and the interaction of about 50% of these transcripts (5833) decreased upon stimulation of the cells with BDNF (Figure 10). The decrease in the interaction of hnRNPK with the mRNAs for GluA1, GluA2, GluN1, CaMKIIβ, BDNF and TrkB following BDNF stimulation, observed in the microarray screening, was validated by quantitative PCR (Comprido, 2011).

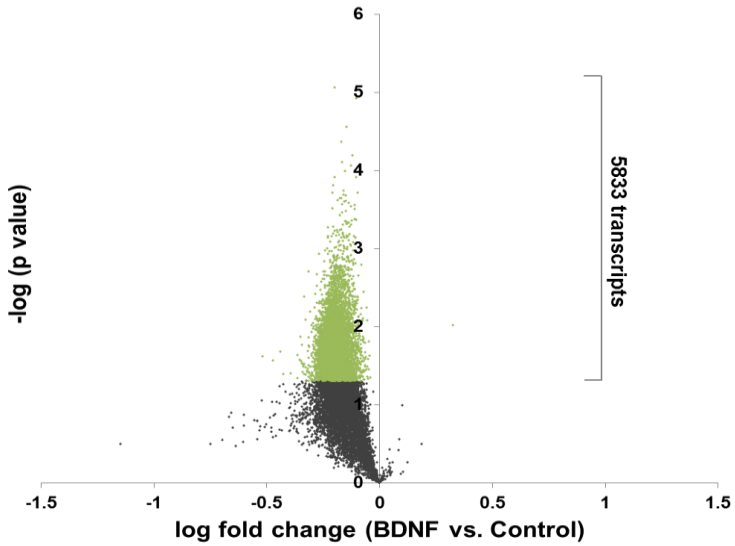

Figure 10- Effect of BDNF on the co-immunoprecipitation of mRNAs with hnRNPK in cultured hippocampal neurons: characterization of the percentage of transcripts regulated by BDNF. The results were obtained from the quantitation of four different experiments performed in independent preparations, and are expressed as -log (p value) and log fold change (BDNF vs. Control). From the list of 11422 mRNAs that were co-immunoprecipitated with hnRNPK, only 5833 were significantly regulated by BDNF; $p<0.05$ (green dots) as determined by the paired Student's *t*-test. (Comprido, 2011)

Since hnRNPK is present at the synapse (Lujian Liao, 2007), its regulation by BDNF may be relevant to understand the effects of the neurotrophin on local protein synthesis. Given the large number of transcripts that interact with hnRNPK in cultured hippocampal neurons, we hypothesized that this RNP could play an important role in the regulation of protein synthesis at the synapse. Therefore, the main goals of this work were the following:

1) The large number of transcripts that bind to hnRNPK suggest that the protein may act as a global repressor of translation. This hypothesis was addressed in COS-7 cultures overexpressing hnRNPK and the effects on protein synthesis were investigated using SUnSET followed by western blot.

2) A large number of the transcripts that interact with hnRNPK were found to be released following stimulation of hippocampal neurons with BDNF (Comprido, 2011). Although we hypothesized that under resting conditions hnRNPK may act as a suppressor of translation, signals that release the transcripts should have a great impact in total translation activity. This question was first addressed using COS-7 cells transfected with hnRNPK and with the TrkB receptors for BDNF, and total protein synthesis was measured as indicated above. Additional experiments were performed in cultured hippocampal neurons infected with short-hairpin RNA against hnRNPK, and the effect of BDNF on protein synthesis was investigated by SUnSET followed by immunocytochemistry.

3) The results of the microarray experiments showing the interaction of hnRNPK with several transcripts coding for proteins relevant in synaptic plasticity in the hippocampus suggest that this protein may play an important role in the transport of those mRNAs to the synapse and in making them available for translation. This hypothesis was addressed by analysing the presence of the mRNA for the synaptic proteins GluA1, GluN1 and BDNF in immunoprecipitates of hnRNPK prepared from hippocampal synaptoneurosomes. Furthermore, we compared the effect of BDNF- or PDGF-stimulation on the interaction of the transcripts with hnRNPK immunoprecipitated from the synaptic preparation. The two ligands activate distinct receptor tyrosine kinases, and these studies allowed determining i) whether BDNF dissociates the transcripts from hnRNPK at the synapse, possibly making them available to be translated, and ii) if the effects are specific for BDNF or can also be induced by other receptors that mediate long-term synaptic potentiation.

CHAPTER 3

Methods

COS-7 cell culture and transfection

COS-7 cells, a fibroblast-like cell line derived from immortalized kidney cells of the African green monkey, were maintained at 37°C in a humidified incubator with 5% CO_2/ 95% air, in Dulbecco's modified Eagle's medium (Sigma-Aldrich), supplemented with 10% foetal bovine serum (Gibco, Invitrogen), 1% penicillin/streptomycin (Gibco, Invitrogen) and 44mM $NaHCO_3$ at pH 7.2, up to a subconfluence of 60-80%, and were diluted 1:5 every three days. The day before transfection the cells were plated in 6 well plates (Corning; 9.5cm^2/well).
COS-7 cells were transfected with Lipofectamine LTX and PLUS reagent (Invitrogen) following the protocol of the manufacturer as follows: 2.5µg of plasmid DNA (GFP, hnRNPK or TrkB construct) and 2.5µL of PLUS reagent were diluted in 500µL OptiMEM (Gibco, Invitrogen) per well and incubated at room temperature for 10min. Then 12.5µL of Lipofectamine LTX were added to the mixture and incubated for 25 min to allow the formation of complexes. The complexes were then added to the cells and incubated for 24 h at 37°C before the experiment.

COS-7 cell stimulation and analysis of protein synthesis

To study protein synthesis in transfected COS-7 cells, a surface sensing of translation (SUnSET) method was used (Lin et al., 2009; Schmidt et al., 2009). This method is based on the ability of puromycin, a chain-terminating tRNA analogue, to tag the carboxyl terminus of nascent proteins. The cells were incubated with 0.5 µM puromycin (Sigma) for 15 min in culture conditioned medium, and where indicated were pre-incubated with 40 µg/mL cycloheximide (Sigma) for 45 min and during the puromycin incubation. Stimulation with 50 ng/mL BDNF was performed during the period of incubation with puromycin. Cell extracts were prepared after incubation with puromycin, as indicated below.

Preparation of extracts

The cultures were washed with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4 and 10mM $Na_2HPO_4.2H_2O$, pH 7.4) before lysis with RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS pH 7.5) supplemented with a cocktail of protease inhibitors (0.1 mM PMSF; CLAP: 1 µg/mL chymostatin, 1 µg/mL leupeptin, 1 µg/mL antipain and 1 µg/mL pepstatin; Sigma-Aldrich) and phosphatase inhibitors (50 mM NaF and 1.5 mM Na_3VO_4). The extracts were then sonicated and

centrifuged at 16,100 x g for 10 min at 4ºC. Protein concentration in the supernatants was quantified using the BCA method (Pierce) and the extracts were then diluted with 2× concentrated denaturating buffer (125 mM Tris pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue) before incubation at 95ºC for 5 min. The proteins of interest were then analyzed by Western Blot.

Western Blotting

Protein samples were separated by SDS-PAGE, in 10% polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) in 10 mM CAPS buffer pH 11 with 10% of methanol (overnight, 4ºC, 40V), and immunoblotted. The blocking of the membranes was performed with 5% milk or 5% bovine serum albumin (BSA; in the case of detection of phosphorylated proteins) prepared in TBS supplemented with 0.1% Tween 20. Blots were incubated with primary antibodies (overnight at 4°C), washed, and exposed to alkaline phosphatase-conjugated secondary antibodies (1h at room temperature). Alkaline phosphatase activity was visualized by enhanced chemifluorescence (ECF) on the Molecular Dynamics Storm 860, and quantified using ImageQuant program (GE Healthcare). Anti-β-actin and anti-β-tubulin were used as loading controls and the results were expressed after normalization. Statistical analysis of the results was performed using one-way ANOVA followed by the Dunnett's or Bonferroni Multiple Comparison Test. See Table 1 for the antibody list.

Table 1- Primary and secondary antibodies used for Western Blotting experiments.

Primary Antibodies	Dilution	Host	Supplier
β-actin	1:5000	Rabbit	Sigma-Aldrich
β-tubulin	1:300000	Mouse	Sigma-Aldrich
hnRNPK	1:1000	Mouse	Santa Cruz Biotechnology
p-Erk1/2 Thr202/Tyr204	1:500	Rabbit	Promega
p-Akt Ser473	1:1000	Rabbit	Cell Signaling
Puromycin	1:1000	Mouse	KeraFAST
TrkB	1:1000	Mouse	BD Biosciences
GFP	1:2000	Rabbit	MBL International

Secondary Antibodies	Dilution	Supplier
Anti-rabbit[1]	1:20000	GE Healthcare
Anti-mouse[1]	1:20000	GE Healthcare

[1]Alkaline phosphatase

Hippocampal Cultures

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18–E19 Wistar rat embryos after treatment with trypsin (0.06%) for 15 min at 37ºC (Gibco, Invitrogen), in Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution (HBSS; 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). The hippocampi were then washed with HBSS containing 10% foetal bovine serum (Gibco, Invitrogen) to stop trypsin activity, further washed in HBSS to avoid the development of glial cells and finally transferred to Neurobasal medium (Gibco, Invitrogen) supplemented with NeuroCult® SM1 supplement (1:50 dilution; StemCell Technologies), 25 μM glutamate, 0.5 mM glutamine, and 120 μg/mL gentamycin. The cells were dissociated in this solution and were plated in poly-D-lysine (0.1 mg/mL) coated plates at a density of 90,000 cells/cm^2, for high density cultures, or at a density of 15,000 cells/cm^2, for low density cultures.

The cultures were maintained in a humidified incubator of 5% CO_2/95% air at 37ºC for 15 days. After one week in culture, half of the medium was exchanged for fresh supplemented Neurobasal medium without glutamate. The extracts were then prepared from high density cultures, as previously described, or the preparations were processed for immunocytochemistry (for low density cultures).

Viral infection

pTRIP vectors were used to generate lentiviral shRNA vectors (See Table 2) for hnRNPK knockdown following methods previously described (Janas et al., 2006). Lentiviruses were generated by triple transfection of pTRIP-shRNA, pCMV-dR8.2, and pMD2.G (which provide structural viral proteins) into HEK 293T cells.

After generating the virus and determining the viral titers, neuronal cultures were transduced at different time points with a multiplicity of infection equal to 5, which represents 80% of neuronal infection. At 15 days *in vitro* (DIV) the neurons were processed for either immunocytochemistry, after the stimulation protocol, or western blot.

Table 2 - shRNAs sequences targeting the rat coding sequences of hnRNPK (SH5 and SH6) or none (SH1). The shRNA sequences were inserted in the pTRIP vector.

Name	Target Sequence	Sense oligo	Anti-sense oligo
SH1	None	GATCCCC **GATGAACGCTCTGGATGCG** TTCAAGAGA **CGCATCCAGAGCGTTCATC** TTTTTGGAAA	AGCTTTTCCAAAAA **GATGAACGCTCTGGATGCG** TCTCTTGAA **CGCATCCAGAGCGTTCATC** GGG
SH5	980 - 998 GAGAUCUCAUGGCUUACGA	GATCCCC **GAGATCTCATGGCTTACGA** TTCAAGAGA **TCGTAAGCCATGAGATCTC** TTTTTGGAAA	AGCTTTTCCAAAAA **GAGATCTCATGGCTTACGA** TCTCTTGAA **TCGTAAGCCATGAGATCTC** GGG
SH6	1201 - 1219 GUAACUAUUCCCAAAGAUU	GATCCCC **GTAACTATTCCCAAAGATT** TTCAAGAGA **AATCTTTGGGAATAGTTAC** TTTTTGGAAA	AGCTTTTCCAAAAA **GTAACTATTCCCAAAGATT** TCTCTTGAA **AATCTTTGGGAATAGTTAC** GGG

Hippocampal neurons stimulation and analysis of protein synthesis

The infected and non-infected hippocampal neurons were incubated with 0.5 µM puromycin (Sigma) for 15 min in culture conditioned medium, and where indicated were pre-incubated with 40 µg/mL cycloheximide (Sigma) for 45 min and during the puromycin incubation. Stimulation with 50 ng/mL BDNF was performed during the period of incubation with puromycin. After the stimulation the neurons were processed for immunocytochemistry as indicated below.

Immunocytochemistry

Low-density hippocampal cultures with 15 DIV were used and the coverslips fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. The cells were permeabilized with 0.25% Triton X-100/PBS for 5 min at 4ºC and blocked with 10% BSA/PBS for 30 min at 37ºC. Primary antibodies were diluted in 3% BSA/PBS and incubated overnight at 4ºC. The coverslips were then washed five times with ice-cold PBS and incubated for 2 h at 37ºC with the secondary antibodies (in PBS with 3% BSA; see Table 3). The preparations were washed five times with ice-cold PBS before being mounted in fluorescence mounting medium (Dako).

Microscopy and quantitative fluorescence analysis

Fluorescence images of neurons were obtained with a Zeiss Axiovert 200 fluorescence microscope with a 63x and 1.4 numerical aperture oil objective coupled to an AxioCam HRm camera and using AxioVision 4.8 and ZEN 2011 software with customized filter sets (see Table 3 for antibodies list). Images were quantified using ImageJ image analysis software. For quantification, sets of cells were cultured and stained simultaneously, and imaged using identical settings. The protein signals were analysed after thresholds were set to avoid unspecific tagging. Measurements of the quantified area, length, mean intensity and integrated intensity were taken for soma and dendrites.

Table 3- Primary and secondary antibodies used in the immunocytochemistry experiments.

Primary Antibodies	Dilution	Host	Supplier
MAP2	1:100	Chicken	Abcam
hnRNPK	1:200	Mouse	Santa Cruz Biotechnology
GFP	1:400	Rabbit	MBL International
Puromycin	1:1000	Mouse	KeraFAST

Secondary Antibodies	Dilution	Fluorophores	Supplier
Anti-chicken	1:200	AMCA	Jackson Immunoresearch
Anti-mouse	1:500	Alexa568	Invitrogen
Anti-rabbit	1:500	Alexa488	Invitrogen

Synaptoneurosome preparation

Synaptoneurosomes were prepared as previously described with slight modifications (Hollingsworth et al., 1985). Briefly, 4-6 hippocampi were dissected from adult Sprague–Dawley rats (Figure 11) and the tissue was minced with scissors and homogenized with a Kontes® Dounce Tissue Grinder, using first a pestle with large clearance, 0.889–0.165 mm (8-10 strokes), followed by a small clearance pestle, 0.025–0.076 mm (8-10 strokes), in a buffer containing 0.32 M sucrose, 10mM 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). The flow-through was centrifuged for 15 min at 10,000 x g and the resulting pellet was resuspended in incubation buffer (8 mM KCl, 3 mM $CaCl_2$, 5 mM Na_2HPO_4, 2 mM $MgCl_2$, 33 mM Tris, 72 mM NaCl, and 100 mM sucrose). All the procedure was done at 4°C. Incubations were made at 30°C and the effect of BDNF (Peprotech) and PDGF (Peprotech)

was tested at a concentration of 50 ng/mL and 20 ng/mL, respectively. For each time point considered a control experiment was also performed in the absence of the neurotrophic factors. Synaptoneurosomes were then centrifuged for 30 s at maximum speed, using a MiniSpin microcentrifuge (Eppendorf). The pellet was resuspended in RIPA supplemented with 50 U/mL of the RNase inhibitor SUPERase-In (Ambion), in addition to the protease inhibitors indicated for the preparation of extracts, before sonication. Protein quantification was performed using the BCA method.

hnRNPK immunoprecipitation

Antibody-immobilized beads were prepared by incubating 6 μg of anti-hnRNPK or mouse IgG antibodies with 100 μL of Protein G PLUS-Agarose beads (Santa Cruz Biotechnology), overnight at 4ºC. The immobilized antibodies were incubated with 1 mg of synaptoneurosomal protein during 1 h at 4ºC, and the beads were washed four times (2 min centrifugations, 2000g) at 4ºC with wash buffer (100 mM Tris-HCl pH 7.4, 300 mM NaCl, 2 mM $MgCl_2$, 0.1% IGEPAL), supplemented as the described for the RIPA buffer in addition to the RNase inhibitor SUPERase-In (Ambion). The final pellet, containing the immunoprecipitated hnRNPK bound to the antibody-immobilized beads, was used for Western Blot analysis or RNA isolation.

mRNA isolation, quality and concentration analysis

After immunoprecipitation of hnRNPK with a specific antibody, the co-immunoprecipitating RNAs were immediately isolated using a TRIzol extraction protocol. Briefly, 500μL of TRIzol (Ambion) were used per experimental condition, and incubated for 5 min before addition of 100 μL of chloroform. The samples were then centrifuged at 12,000 x g for 15 min and after separation of the aqueous phase the RNA was precipitated in 250 μL of isopropanol and 1 μL GlycoBlue (Ambion) for 10min. After centrifugation at 12,000 x g for 10 min, the pellets were washed in 500 μL ethanol (75% solution). The samples were then centrifuged at 7,500 x g for 5 min and the resulting pellets were resuspended in 10 μL of RNase-free water (Gibco, Invitrogen). RNA quality and integrity was assessed using the Experion automated gel electrophoresis system (Bio-Rad). A virtual gel was also created for the total RNA isolated from synaptoneurosomes, allowing the detection of degradation of the reference markers 18S and 28S rRNA. RNA concentration was determined using the NanoDrop 2000 spectrophotometer (Thermo). The RNA samples were stored at -80ºC until further use.

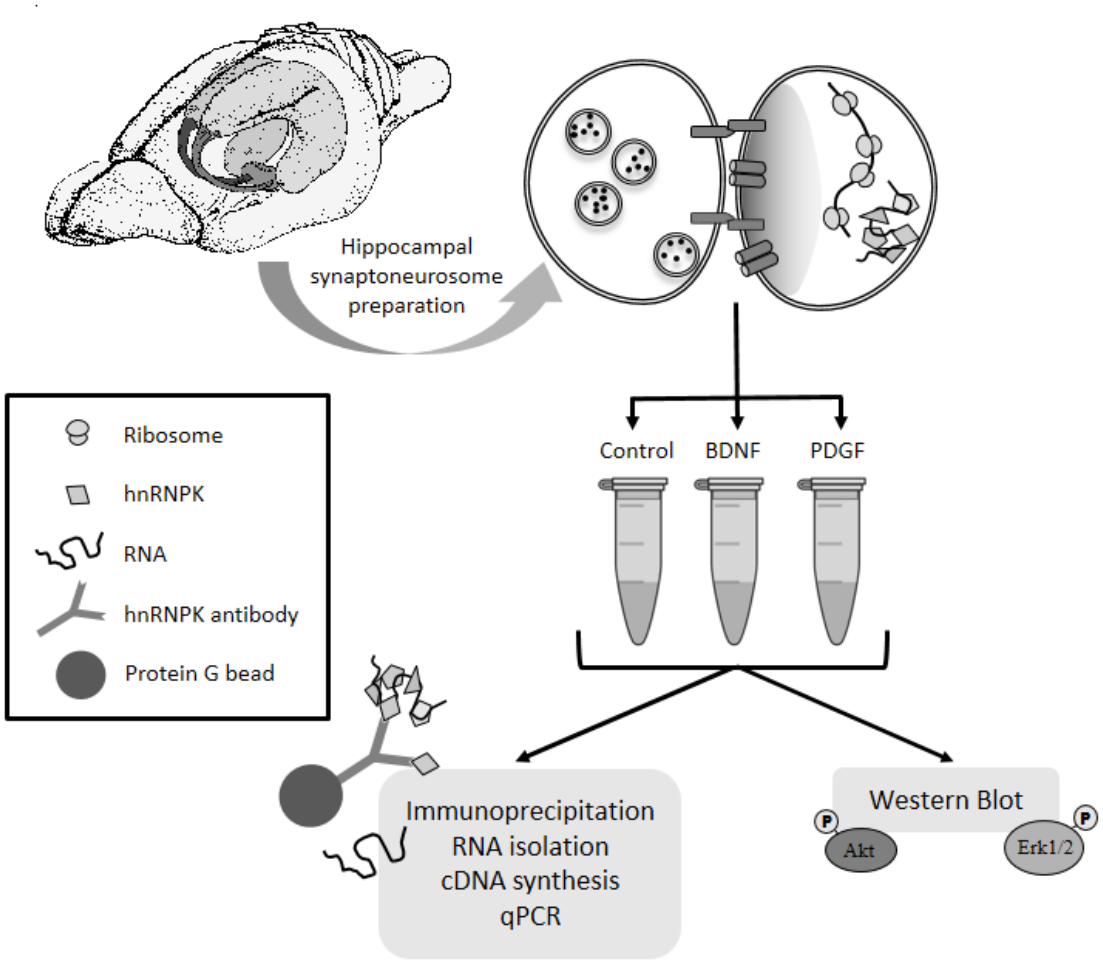

Figure 11- Workflow used to determine the effect of trophic factors on Akt and ERK activity, as well as on interaction of mRNAs with hnRNPK. Hippocampal synaptoneurosomes were stimulated either with BDNF (50 ng/mL) or PDGF (20 ng/mL) for 10 min before preparation of the extracts. The extracts were subjected to Western blot to detect the pAkt and pErk1/2 protein levels. For isolation of the transcripts that interact with hnRNPK, antibodies specific for the ribonucleoproteins were incubated overnight with Protein G beads before addition of the extracts and incubation for 1 h. RNA was isolated from the immunoprecipitates, converted to cDNA and subjected to qPCR.

Reverse Transcription

For first strand cDNA synthesis 500 ng of isolated RNA were mixed with 4 µL of 5x iScript Reaction Mix, 1 µL of Nuclease-free Reverse Transcriptase and water, in 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 samples were cooled to 4ºC before storage at -20ºC until further use.

Primer Design

Primers for real-time PCR were designed using the Beacon Designer 7 software (Premier Biosoft International), according to the following criteria: 1) GC content about 50%; 2) annealing temperature (Ta) between 55-58 ºC; 3) secondary structures and primer–dimers were avoided; 4) primer length 18–24 bp; 5) final product length 100–200 bp (See Table 4).

Table 4- Primer sequences used in this study.

Gene Name	Primer Forward Sequence (5'→3')	Primer Reverse Sequence (5'→3')
Bdnf	TAA CCT CGC TCA TTC ATT A	TCA ACT CTC ATC CAC CTT
Gria1	ACT ACA TCC TCG CCA ATC TG	AGT CAC TTG TCC TCC ATT GC
Grin1	CGG CTC TTG GAA GAT ACA G	GAG TGA AGT GGT CGT TGG

Bdnf, brain-derived neurotrophic factor; Gria1, glutamate receptor ionotropic AMPA 1; Grin1, glutamate receptor ionotropic NMDA 1.

Real-Time PCR

For gene expression analysis 2 µL of cDNA were added to 10 µL of SsoFast EvaGreen Supermix (Bio-Rad), and the final concentration of each primer was 125 nM in 20 µL (total volume). The thermocycling reaction was initiated with activation of Taq DNA polymerase by heating at 95ºC during 3 min, followed by 45 cycles of a 10 s denaturation step at 95ºC, a 30 s annealing step at 55ºC, and a 30 s elongation step at 72ºC. The fluorescence was measured after the extension step using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). After the thermocycling reaction, the melting step was performed with slow heating, starting at 55ºC and with a rate of 0.5ºC per 10 s, up to 95ºC, with continuous measurement of fluorescence, allowing detection of possible non-specific products. The assay included a non-template control and a standard curve (in 10-fold steps) of cDNA for assessing the efficiency of each set of primers. All reactions were run in duplicate to reduce confounding variance.

Data Processing

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 in the exponential phase and therefore was not affected by possible limiting components in the reaction. For every run performed Ct was set at the same fluorescence value. Data analysis was performed by GenEx (MultiD Analyses) software for real-time PCR expression profiling with several adjustments. The adjustments performed take into account primer efficiency for each set of primers, and provide a normalization to the amount of sample, to the technical repeat average and against a reference sample. Where indicated, statistical analysis of the log-transformed expression data was performed by the one-way ANOVA followed by the Dunnett's Multiple Comparison Test.

CHAPTER 4

Results & Discussion

1 - Effect of hnRNPK overexpression in protein synthesis

To test the effect of hnRNPK on the global protein synthesis, translation activity was compared in COS-7 cells transfected with hnRNPK-GFP or with GFP. Protein synthesis was analysed using the SUnSET method (Lin et al., 2009; Schmidt et al., 2009), a methodology based on the ability of puromycin, a chain-terminating tRNA analogue, to tag the carboxyl terminus of nascent proteins. The puromycin labelled proteins were then detected by western blot, using an anti-puromycin antibody.

COS-7 cells were transfected with a plasmid expressing hnRNPK-GFP or GFP for 24h, and the transfection efficiency was then confirmed under the fluorescence microscope using GFP as reporter (not shown). The expression of hnRNPK-GFP was also confirmed by western blot with an anti-hnRNPK antibody, which detected a protein of about 90 kDa, in addition to the endogenous hnRNPK protein of about 65 kDa (Figure 12A). Labelling of proteins synthesized *de novo* was performed by exposing the cells to 0.5 µM puromycin for 15 min in culture conditioned medium before preparation of the extracts. The resulting smear obtained for each experimental condition was quantified and the results were normalized to actin (Figure 12A). The normalized results were expressed as a percentage of the total immunoreactivity detected in cells transfected with GFP (control condition), which showed maximal accumulation of puromycin (Figure 12B).

Transfection with hnRNPK reduced puromycin immunoreactivity to 77% ± 5% of the control, while pre-incubation of the cells with cycloheximide (40µg/mL), a protein synthesis inhibitor, decreased the accumulation of puromycin in proteins to 64% ± 4%. The results obtained under these conditions were not significantly different ($p>0.05$) from those obtained in cells transfected with hnRNPK and pre-incubated with cycloheximide (57% ± 6% of the control) (Figure 12B). This effect of cycloheximide is similar to that reported in B3Z cells treated with the translation inhibitor at concentrations ranging from 1-100 µM (Schmidt et al., 2009). In additional control experiments we tested the immunoreactivity of protein extracts prepared from non-transfected cells that were not incubated with puromycin. Under these conditions, which represent the unspecific binding of the anti-puromycin antibody, the total immunoreactivity was 22% ± 4% of the control (Figure 12B), determined in cells transfected with GFP and incubated with puromycin. These results were also within the range reported in B3Z cells under similar conditions (Schmidt et al., 2009).

Taken together, these results support a role for hnRNPK in the translation repression under basal conditions as previously reported for other RBPs (Huttelmaier et al., 2005; Ostareck et al., 2001).

2 - Effect of hnRNPK in BDNF-induced protein synthesis

Previous results from our laboratory showed that numerous transcripts bound to hnRNPK are released following stimulation of hippocampal neurons with BDNF (unpublished observations). This suggests that although hnRNPK may act as a buffer of mRNAs, thereby decreasing global translation activity, the release of a high number of transcripts following appropriate stimulation of the cells may increase total protein synthesis. According to this hypothesis, the total translation activity induced by the BDNF-TrkB complexes should be dependent on the hnRNPK protein levels. This was tested by comparing the TrkB receptor-induced translation activity in COS-7 transiently transfected with TrkB-GFP, together with GFP or hnRNPK-GFP. The transfection efficiency was confirmed under the fluorescence microscope, using GFP as the reporter (not shown). Translation activity was measured using the SUnSET method, as described in the previous section, and the results were normalized to the actin protein levels, used as a loading control, and expressed as fold change in comparison with the total puromycin immunoreactivity detected in cells transfected with GFP.

The results of Figure 13B show a slight reduction in puromycin immunoreactivity in COS-7 cells transfected with TrkB-GFP for 24 h, to 77 ± 3%, although the effect was not statistically significant ($p>0.05$). Similar effects were observed when the TrkB-GFP transfected cells were stimulated with BDNF (50 ng/ml) for 15 min, during the period of incubation with puromycin (83% ± 12%, when compared with the puromycin immunoreactivity in GFP transfected cells). The similarity in the translation activity in COS-7 cells transfected with TrkB-GFP, in the presence and in the absence of BDNF, suggest that receptor levels expressed in the cells allows spontaneous oligomerization and transactivation, even in the absence of BDNF. Accordingly, western blot experiments using an anti-pTrk antibody showed similar phosphorylation of the receptor under the two experimental conditions (Figure 13C). The lack of effect of TrkB receptor expression in protein synthesis in COS-7 cells is not in agreement with the numerous reports showing activation of the translation machinery in neurons stimulated with BDNF (Takei et al., 2004; Takei et al., 2001). This discrepancy may be due to the incubation period with puromycin (15 min) which may not be long enough to observe the effects of TrkB receptors on translation activity in transfected COS-7 cells. Additional

experiments testing longer incubation periods with puromycin may allow addressing this hypothesis.

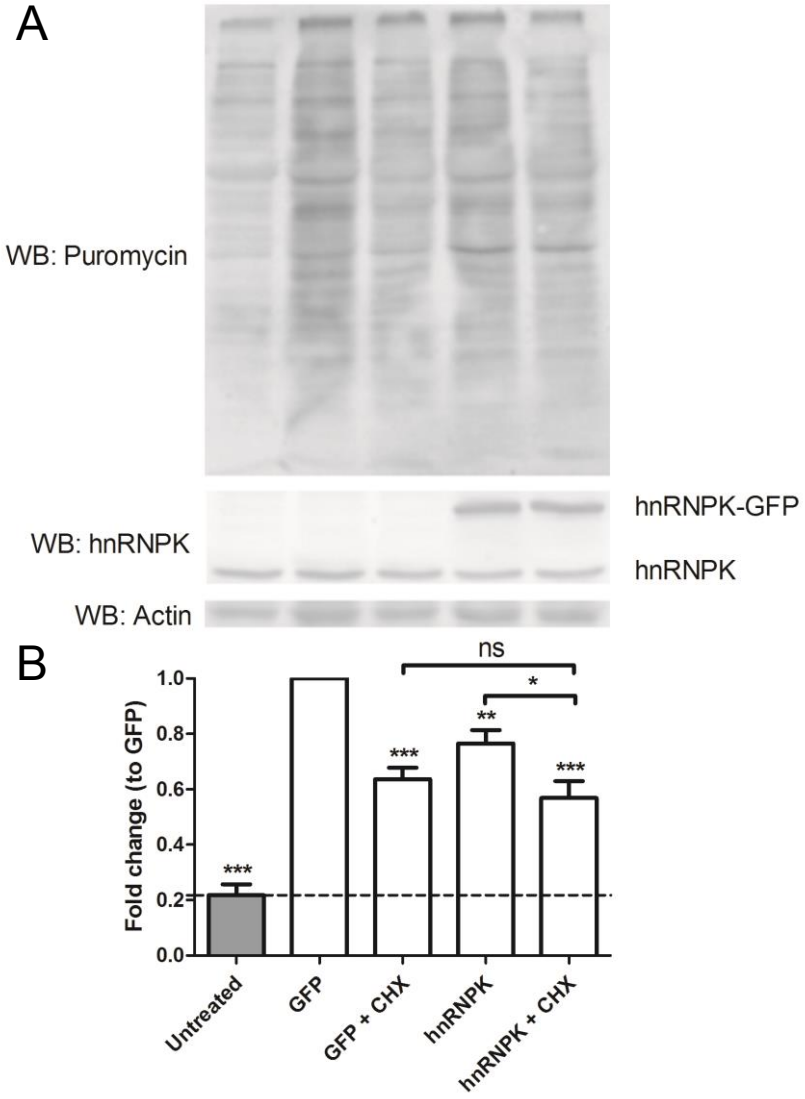

Figure 12- Effect of hnRNPK overexpression in protein synthesis in COS-7 cells, as determined using SUnSET. (A) COS-7 cells transfected with GFP or with hnRNPK-GFP were incubated with 0.5µM puromycin for 15 min in culture conditioned medium before preparation of the extracts. Where indicated the cells were also pre-incubated with cycloheximide (40µg/mL) for 45 min before incubation with puromycin, and the translation inhibitor was also present during the incubation with puromycin. In control conditions to test for the specificity of the antibody, the non-transfected cells were not treated with puromycin (untreated). After striping, the membranes were also incubated with anti-actin and anti-hnRNPK antibodies. Quantification of the results obtained in four independent experiments is shown in panel (B). The results were normalized to actin protein levels and expressed as fold change (mean ± SEM) in comparison with the total immunoreactivity detected in cells transfected with GFP. Statistical analysis was performed by the One-way ANOVA followed by the Bonferroni Multiple Comparison Test.*$p<0.05$, **$p<0.01$, ***$p<0.001$, ns (non-significant) when compared with GFP transfected cells, or for the indicated comparisons.

A decrease in puromycin accumulation in *de novo* synthesized proteins was also observed when COS-7 cells transfected with TrkB-GFP were incubated with the translation inhibitor cycloheximide, in the presence or in the absence of BDNF (Figure 13B). However, more experiments should be performed in order to conclude about the effects of the protein synthesis inhibitor.

To determine the effect of hnRNPK on the translation activity induced by TrkB receptors, protein synthesis was measured in COS-7 cells co-transfected with TrkB receptors and hnRNPK. Interestingly, when both proteins were expressed there was an increase in translation activity when compared with COS-7 cells transfected with GFP (to 146 ± 17% of the control). Similar results were obtained when COS-7 cells co-transfected with TrkB-GFP and hnRNPK-GFP were stimulated with BDNF (50 ng/ml) during the period of incubation with puromycin (142 ± 5% of the control). These results contrast with the slight dowregulation of protein synthesis when COS-7 cells were transfected with hnRNPK (Figures 12B and 13B). Given the previous results from our laboratory showing that TrkB signaling leads to the dissociation of a large number of transcripts bound to hnRNPK (Comprido, 2011), the present results may suggest that the increased number of mRNAs bound to hnRNPK when this protein is overexpressed are released by the TrkB-induced signaling contributing to a significant increase in translation activity. As discussed above, the lack of effect of BDNF in total translation activity measured in cells transfected with TrkB-GFP and hnRNP-GFP ($p>0.05$) may be due to spontaneous oligomerization and activation of TrkB receptors.

Control experiments showed that co-transfection with TrkB-GFP did not affect significantly the hnRNPK-GFP protein levels expressed in COS-7 cells (Figure 13A). However, when the two proteins were expressed together there was a significant reduction in TrkB-GFP protein levels when compared with the expression levels detected in cells that were not co-transfected with hnRNPK-GFP (Figure 13A). Additional studies should be performed to determine whether the lower expression of TrkB-GFP in cells co-transfected with hnRNPK account for the increase in translation activity, and to ensure that higher TrkB protein levels do not prevent the upregulation of protein synthesis.

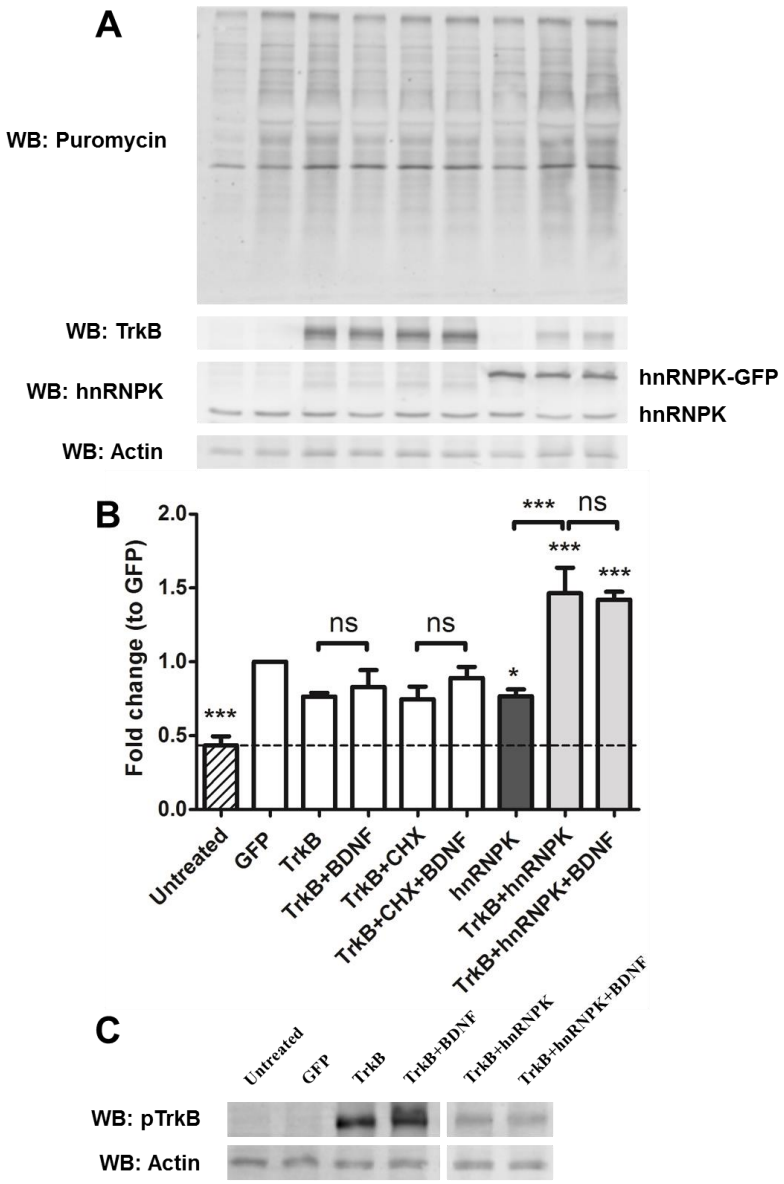

Figure 13- Effect of hnRNPK in BDNF-induced protein synthesis in COS-7 cells, as determined with the SUnSET method. (A) COS-7 cells transfected with GFP, hnRNPK-GFP and/or hnRNPK (as indicated) were incubated with 0.5µM puromycin for 15 min in culture conditioned medium before preparation of the extracts. Where indicated the cells were also pre-incubated with cycloheximide (40µg/mL) for 45 min before incubation with puromycin, and the translation inhibitor was also present during the incubation with puromycin. In control conditions to test for the specificity of the antibody, the non-transfected cells were not treated with puromycin (untreated). After striping, the membranes were also incubated with anti-actin, anti-hnRNPK amd anti-TrkB antibodies. Quantification of the results obtained in three independent experiments is shown in panel (B). The results were normalized to actin protein levels and expressed as fold change (mean ± SEM) in comparison with the total immunoreactivity detected in cells transfected with GFP. (C) Membranes were incubated with anti-pTrk and anti-actin for the given conditions. Statistical analysis was performed by the One-way ANOVA followed by the Bonferroni Multiple Comparison Test. *$p<0.05$, ***$p<0.001$, ns (non-significant) when compared with GFP transfected cells or for the indicated comparisons.

It was previously shown that hnRNPK interacts with different promoters (Gaillard et al., 1994; Tomonaga and Levens, 1996). As such, the overexpression of hnRNPK may increase the transcription of different genes and the resulting transcripts are likely to be maintained translationally silenced in the cytoplasm by hnRNPK. The activity of TrkB receptors in transfected COS-7 cells may be sufficient to trigger the release of the mRNAs from hnRNPK granules and thus increase their translation. The main limitation of these experiments is the use of an heterologous system to express TrkB receptors and to investigate their coupling to the regulation of hnRNPK. Some of the questions that may arise from the use of an heterologous system are: i) Are TrkB receptors indeed expressed on the surface of transfected COS-7 cells? ii) Are TrkB receptors properly coupled to the downstream signaling machinery in transfected COS-7 cells? iii) Are TrkB receptors expressed in transfected COS-7 cells coupled to the phosphorylation of hnRNPK?

3 - Effect of hnRNPK downregulation in neuronal protein synthesis

Previous results from our laboratory showed the presence of hnRNPK in synaptoneurosomes isolated from the rat hippocampus (not shown), indicating that the ribonucleoprotein is expressed at the synapse. A nuclear and post-synaptic distribution of hnRNPK in cultured hippocampal neurons was also found using immunocytochemistry (Proepper et al., 2011). In this part of the work we aimed at determining the role of dendritic hnRNPK in the regulation of local translation in dendrites.

We first performed immunocytochemistry experiments to confirm the presence of hnRNPK in dendrites of cultured hippocampal neurons. Co-localization with MAP2 was used to assess the dendritic localization of the protein. The results of Figure 14 show the presence of hnRNPK in neurites that are also labeled with an anti-MAP2 antibody, confirming the dendritic distribution of the ribonucleoprotein.

To test for the role of hnRNPK in the regulation of local protein synthesis in dendrites we developed two shRNAs to knock-down hnRNPK (SH5 or SH6) and a scramble shRNA (SH1) as a control. To test for the optimal period of infection to achieve an efficient knock-down of hnRNPK, cultured hippocampal neurons were infected for 4, 5 or 6 days with SH1, SH5 or SH6, and processed for immunocytochemistry at 15 DIV. Images of the soma and dendrites were taken from GFP-positive cells (except for the experimental condition corresponding to non-infected cells [0 DOI]) and the levels of hnRNPK were quantified relative to the control (0 DOI) in the different experimental conditions conditions (Figure 15A). In hippocampal

neurons infected with the SH5 or SH6 shRNA, which target two different hnRNPK mRNA sequences (Table 2), there was a downregulation of hnRNPK protein levels both in the soma and in dendrites, although the effects were more significant in the latter compartment. Maximal effects were observed with SH6 and SH5 shRNAs at 5 and 6 DOI, and similar results were obtained with the SH6 at 4 DOI. However, this infection period was less efficient when the SH5 shRNA was used. Infection with the scramble shRNA (SH1) for 4 days did not affect significantly hnRNPK protein levels in both dendrites and soma, as expected. However an increase in the levels of hnRNPK was observed when longer infection periods were tested (5–6 DOI), possibly as a non-specific response to infection.

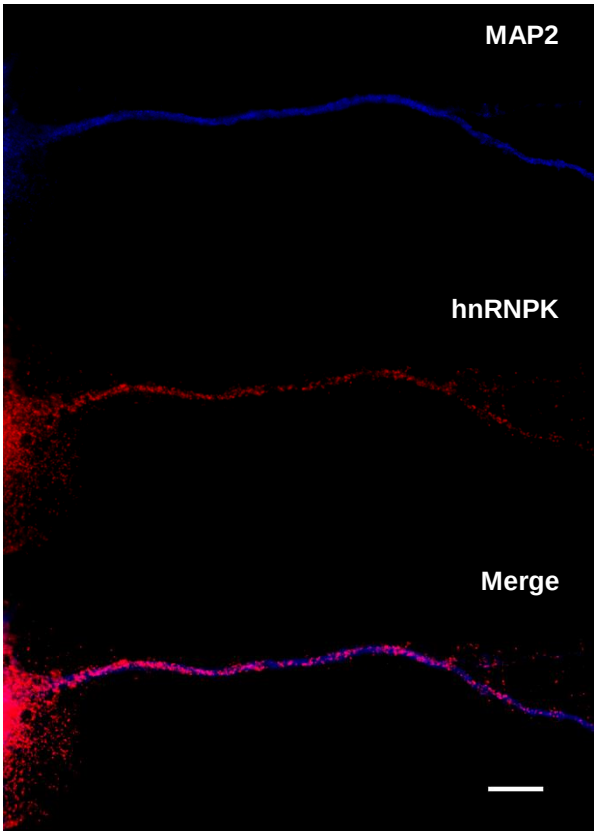

Figure 14- Dendritic distribution of hnRNPK in a cultured hippocampal neuron. Top panel shows MAP2 in blue, the middle panel shows hnRNPK in red and the bottom panel presents the merge image. White bar corresponds to10 µm.

Given the results obtained in the preliminary immunocytochemistry experiments with the SH5 and SH6 shRNAs, we used the SH6 shRNA to further evaluate the effects of infection for 2, 3 or 4 days on hnRNPK protein levels. In this set of experiments hnRNPK protein levels were

determined by western blot at DIV 14. Infection of the neurons was confirmed by fluorescence microscopy (not shown) and by analysing the expression GFP with western blot (Figure 15B). The results for the hnRNPK knock-down showed a similar decrease in the levels of hnRNPK for the three different infection times tested, in comparison with non-infected cells. Total hnRNPK protein levels were decreased by about 40% at 3 and 4 DOI (Figure 15B), which is similar to the results obtained in the soma compartment using the immunocytochemistry experiments. In fact, considering the relative volume of the soma and dendritic compartments, the hnRNPK knock-down determined by western blot analysis is likely to be influenced mainly by the alteration in the former compartment.

Considering the effects of the SH6 shRNA in hippocampal neurons observed in the immunocytochemistry and western blot experiments, 3 DOI was considered the most suitable incubation period to know-down hnRNPK.

To determine the role of the hnRNPK in the regulation of local translation in dendrites we have adapted the SUnSET method (Lin et al., 2009; Schmidt et al., 2009). A major limitation of this method as originally described, with puromycin detection by western blot, is the lack of spatial resolution in the cell. This is particularly relevant in the study of protein synthesis in neurons, which are highly polarized cells expressing distinct mechanisms of translation regulation in different compartments. Therefore, we performed preliminary experiments to set up the SUnSET with immunocytochemistry detection of puromycin. Cultured hippocampal neurons were infected with the SH6 shRNA for 3 days, and dendritic protein synthesis was determined at DIV 14 by exposing the neurons to 0.5 µM puromycin for 15 min. The preparations were then processed for immunocytochemistry with an anti-puromycin antibody, and the dendrites were identified by co-staining with an anti-MAP2 antibody. In parallel immunocytochemistry experiments we subjected hippocampal neurons not incubated with puromycin to the same staining procedures. The immunoreactivity determined under these conditions was used to set a baseline threshold for puromycin specific signal when acquiring the fluorescence images.

Under control conditions puromycin immunoreactivity was stronger in the soma when compared with the dendrites, indicating a higher rate of translation activity (Figure 16B). Incubation of cultured hippocampal neurons with BDNF (50 ng/mL) for 15 min increased puromycin staining in the dendritic compartment 1.76 times, and this effect was significantly inhibited when the cells were incubated with the protein synthesis inhibitor cycloheximide (40µg/mL). The effect of BDNF in translation activity in dendrites determined here with the SUnSET method is similar to the effect of BDNF in dendritic protein synthesis determined

with FUNCAT (fluorescent noncanonical amino acid tagging; Dieterich et al., 2010). In the soma compartment there was also a small upregulation of translation activity in the presence of BDNF, which was sensitive to the presence of the translation inhibitor (Figure 16), but additional experiments should be performed to confirm these results.

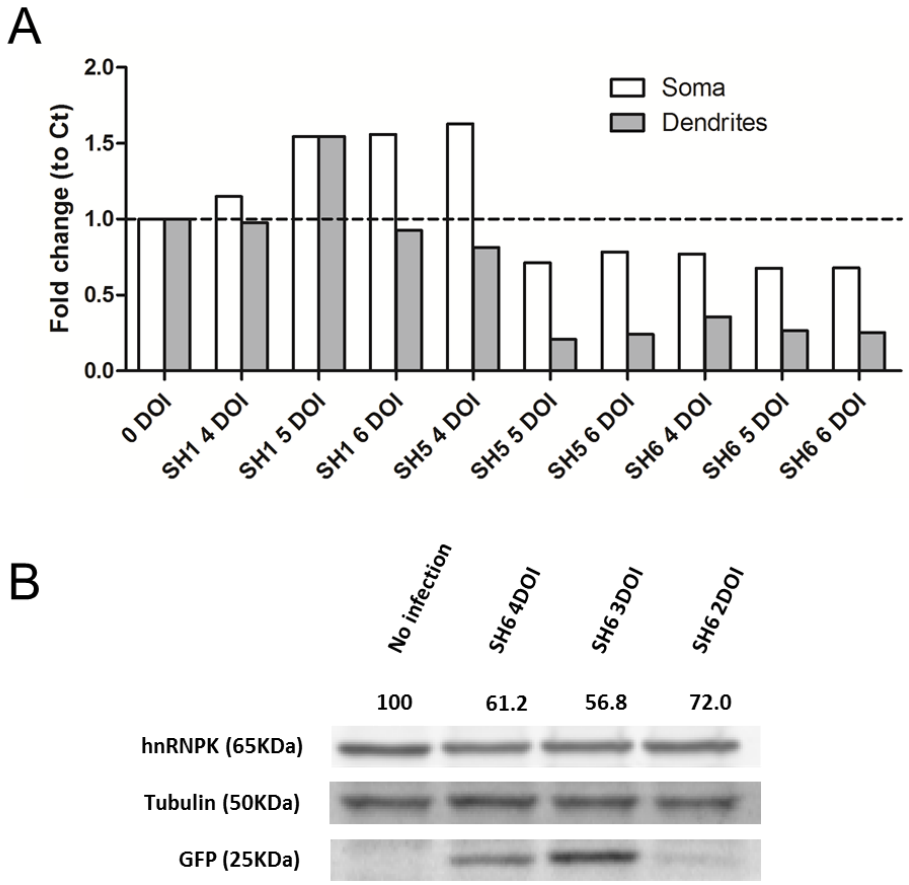

Figure 15- Optimization of the infection time to downregulate hnRNPK with specific shRNA. (A) Hippocampal neurons were infected for 4, 5 or 6 days with SH1 (scramble shRNA), SH5 or SH6, and hnRNPK protein levels were determined at DIV14 by immunocytochemistry with a specific antibody. The dendritic localization of hnRNPK was determined by colocalization with the dendritic marker MAP2. The quantification of hnRNPK was performed with the Image J software in 12 randomly selected dendrites per condition and compared to control. (B) Western blot of hippocampal neuron extracts infected with the SH6 shRNA for 2, 3 or 4 days, and total hnRNPK protein levels was determined at DIV 14 by western blot. The results represent quantification of one experiment.

To determine whether hnRNPK regulates the effects of BDNF on translation activity in dendrites, we performed experiments in cultured hippocampal neurons infected with the SH6 shRNA to decrease hnRNPK protein levels. The results show an inhibition of the BDNF-

induced translation activity in dendrites of hippocampal neurons expressing reduced levels of hnRNPK. These results suggest that hnRNPK is a major player in the transport of mRNAs used in dendritic protein synthesis in response to BDNF stimulation, in agreement with the large number of transcripts that interact with this RNA binding protein (Comprido, 2011). Additional experiments should be performed to confirm these findings and to determine whether similar effects are observed in the soma compartment. In these experiments it will be important to test the effect of the scramble SH1 shRNA, which does not target hnRNPK, and rescue experiments should also be performed.

4 - Modulation of synaptic hnRNPK association to specific mRNAs by BDNF and PDGF

The results described above suggest that hnRNPK plays an important role in the regulation of protein synthesis in dendrites. Using a DNA microarray screening we identified in our laboratory a total of 11422 transcripts that co-immunoprecipitated with hnRNPK from cultured hippocampal neuron homogenates. Furthermore, the results showed that 50% of those transcripts were dissociated from hnRNPK following BDNF stimulation. Additional experiments using qPCR showed a decrease in the amount of transcripts for GluA1, GluA2, GluN1, BDNF, TrkB, hnRNPK and CaMKII β that co-immunoprecipitated with hnRNPK in extracts from hippocampal neurons stimulated with BDNF. Therefore, we hypothesized that this ribonucleoprotein could play a role in the delivery of some of those transcripts to dendrites, which would be released for translation following stimulation with BDNF. This hypothesis is addressed in this section and we compared the effect of BDNF with PDGF, which activates a different type of receptor tyrosine kinase. These studies were performed using hippocampal synaptoneurosomes isolated from the adult rat hippocampus. Synaptoneurosomes are resealed presynaptic structures (synaptosomes) with attached sealed postsynaptic entities (neurosomes), making this preparation suitable for the investigation of synaptic mechanisms (Troca-Marin et al., 2010). Synaptoneurosomes have been used to investigate local translation at the synapse in the hippocampus and cerebral cortex (Bagni et al., 2000; Schratt et al., 2004).

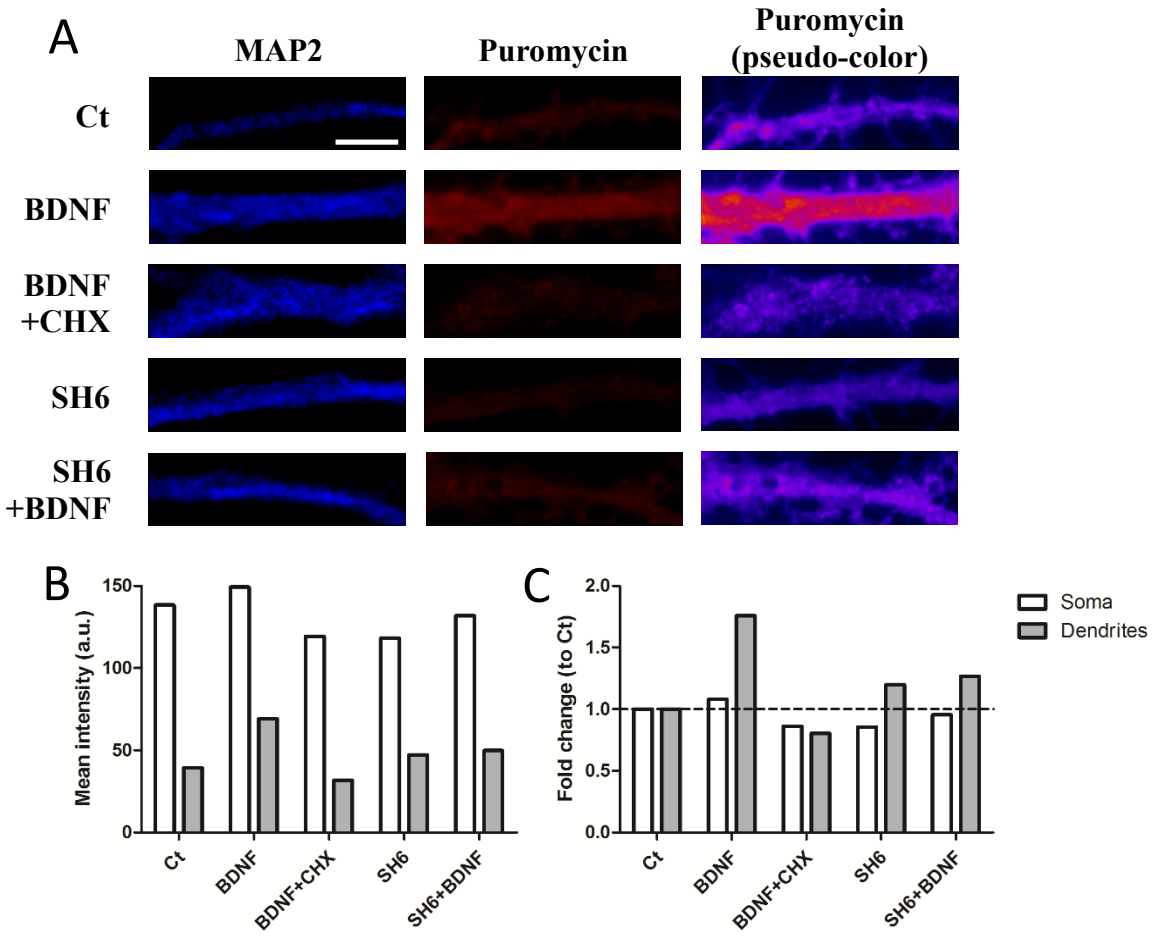

Figure 16- Immunocytochemical analysis of protein synthesis in hippocampal neurons. Cultured hippocampal neurons (DIV14) were incubated with 0.5µM puromycin for 15 min in culture conditioned medium, in the presence or in the absence of BDNF (50 ng/ml), before preparation of the extracts (DIV14). Where indicated the cells were also pre-incubated with cycloheximide (40µg/mL) for 45 min before incubation with puromycin, and the translation inhibitor was also present during the incubation with puromycin. Downregulation of hnRNPK was performed by infection of the cells with SH6 for 3 days. (A) Representative images of dendrites for the different conditions. The first row of panels represents MAP2 immunoreactivity in blue, the middle row shows puromycin immunoreactivity in red and last row represents puromycin immunoreactivity pseudo-color image to increase the contrast. (B) Quantification of the mean intensity for puromycin immunoreactivity for the different conditions in the soma and dendrites. (C) Quantification of the fold change in translation activity when compared with the control condition for the different conditions. White bar: 5 µm.

The protocol used for synaptoneurosomal preparation was validated for enrichment in synaptic proteins, such as postsynaptic density protein 95 (PSD 95), synaptophysin and vesicular GABA Transporter (VGAT), and decrease in the somatic and astrocytic proteins histone 3 and glial fibrillary acidic protein (GFAP), respectively (Santos, 2010). The results showed that synaptoneurosomes are enriched in the presynaptic markers VGAT and

synaptophysin, and in PSD95, a postsynaptic marker of excitatory synapses (Figure 17), when compared with hippocampal homogenates. In contrast, synaptoneuromes contain less tubulin than total homogenates and are slightly enriched in actin, as expected based on the distribution of cytoskeleton proteins at the synapse (Matus, 2000). The presence of low amounts of GFAP in the synaptoneurosome preparation indicates a minor contamination with glial components. Furthermore, the lack of histone 3 shows that there is no contamination with nuclei.

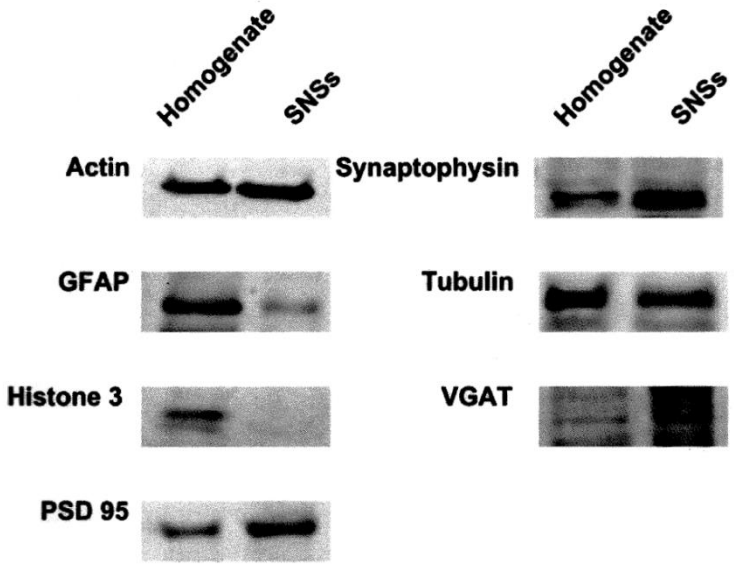

Figure 17- Characterization of purified synaptoneurosomes obtained from the adult rat hippocampus. The panel shows the enrichment in synaptic proteins in the synaptoneurosomal (SNS) preparation when compared to the total homogenates (Santos, 2010).

The presence of functional receptors for BDNF and PDGF in hippocampal synaptoneurosomes was investigated by analysing the activation of the Akt and ERK signaling pathways, using western blot and antibodies against pAkt and pERK1/2. PDGFR-β and TrkB receptors activate similar signalling mechanisms, and PDGFR-β receptors were shown to localize in pre- and post-synaptic sites in the hippocampus where they mediate LTP induction (Shioda et al., 2012). Both BDNF and PDGF induced a transient activation of Akt and ERK1/2, and a similar increase in the phosphorylation of the three kinases was observed after 10 min of stimulation with the trophic factors. However, after 20 min of stimulation ERK1 was the only kinase that remained active, and this was observed in synaptosomes

incubated with BDNF but not with PDGF (Figure 18). These results indicate that BDNF and PDGF stimulation lead to fast but transient activation of the signalling machinery. This contributes to spine specific responses and reduces the signal spreading to adjacent spines (Yasuda and Murakoshi, 2011). The kinetics of activation of the ERK and Akt signaling pathways by BDNF in hippocampal synaptoneurosomes is similar to the kinetics described in cultured hippocampal neurons (Almeida et al., 2005).

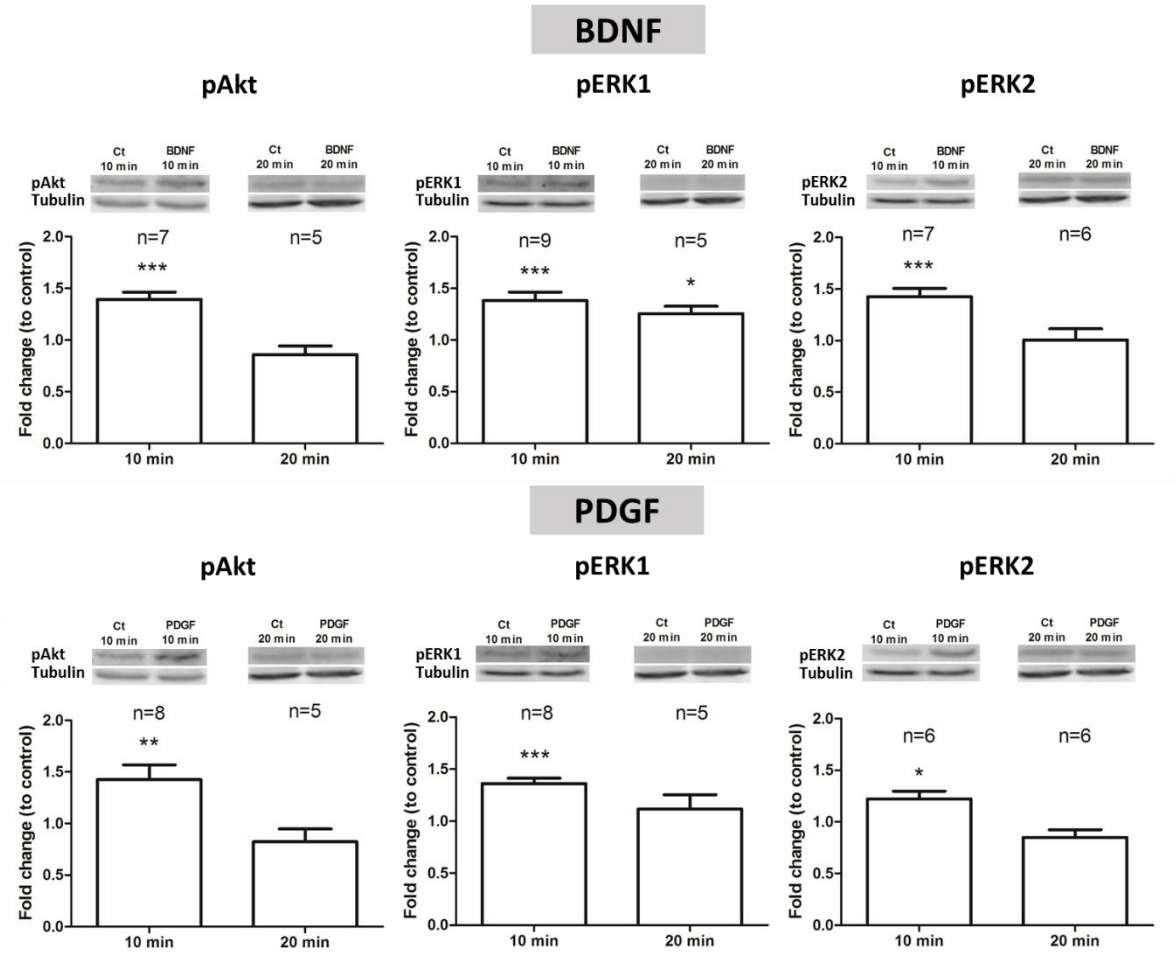

Figure 18- Activation of Akt and ERK1/2 by BDNF and PDGF in hippocampal synaptoneurosomes. The graphs represent the fold change in the levels of pAkt, pERK1 or pERK2 in hippocampal synaptoneurosomes stimulated with BDNF or PDGF for 10 min and 20 min, as indicated. Representative blots show the results obtained for the phosphorylated proteins and tubulin under control conditions (Ct) and following stimulation with BDNF or PDGF. The results represent the quantification of the indicated number of experiments performed in independent preparations, and are expressed as fold change (mean ± SEM) to control. *p<0.05, **p<0.01, ***p<0.001 as determined by ANOVA followed by Dunnett's Multiple Comparison test.

To study the effect of BDNF and PDGF on the association of specific mRNAs to hnRNPK at the synapse, synaptoneurosomes were stimulated for 10 min with either 50 ng/mL of BDNF

or 20 ng/mL of PDGF prior to the hnRNPK immunoprecipitation and mRNA extraction. This stimulation period allows maximal activation of the signaling pathways induced by both ligands (Figure 18).

In preliminary experiments we compared the stability of the RNAs present in total homogenates of hippocampal synaptoneurosomes incubated 1 h at 4ºC against the ones incubated 3 h at 4ºC. The degradation of the reference markers rRNA 18S and 28S was used to determine the RNA integrity by automated electrophoresis (Experion). At 1 h of incubation there was no significant RNA degradation, while at 3 h of incubation there was a significant degradation of the RNA that could be observed by the loss of the reference markers rRNA 18S and 28S and increase in the quantity of low size RNA in the electropherogram. Based on these results, the hnRNPK immunoprecipitation protocol used in the following experiments consisted in the incubation of the antibody overnight with Protein G beads before incubation with the homogenates for 1 h. This protocol allowed an efficient immunoprecipitation of hnRNPK as confirmed by the 65 kDa band in the immunoblot from the immunoprecipitation (IP) and the absence of the band in the flow-through (FT) (Figure 19). The hnRNPK immunoprecipitation also confirmed the presence of hnRNPK in hippocampal synaptoneurosomes (Figure 19) as observed in synaptoneurosomes isolated from cultured cerebrocortical neurons (Lujian Liao, 2007). Another control experiments using a mouse IgG antibody showed no hnRNPK immunoprecipitation (data not shown) confirming the specificity of the method.

After extraction of the mRNA and synthesis of the cDNA, a qPCR was run for GluA1 (AMPA receptor subunit), GluN1 (NMDA receptor subunit) and BDNF transcripts. These genes, coding for proteins important for the synaptic function and synaptic plasticity, were previously found to be associated with hnRNPK in cultured hippocampal neurons, as determined by qPCR after co-immunoprecipitation with the ribonucleoprotein. These studies also showed that GluA1, GluN1 and BDNF transcripts are dissociated to some extent from hnRNPK following stimulation of cultured hippocampal neurons with BDNF. Similar results were obtained in synaptoneurosomes where stimulation with BDNF reduced the interaction of hnRNPK with the mRNA for GluA1, GluN1 and BDNF to 47, 65, 55 % of the control, respectively (Figure 20). These results showing a partial reduction of the interaction of hnRNPK with the transcripts following stimulation with BDNF suggest that the neurotrophin may only target a subset of the RNP structures present in synaptoneurosomes. CLIP (cross-linking and immunoprecipitation) studies are necessary to determine whether the mRNAs investigated bind directly to hnRNPK or if they interact with a binding partner of this RBP.

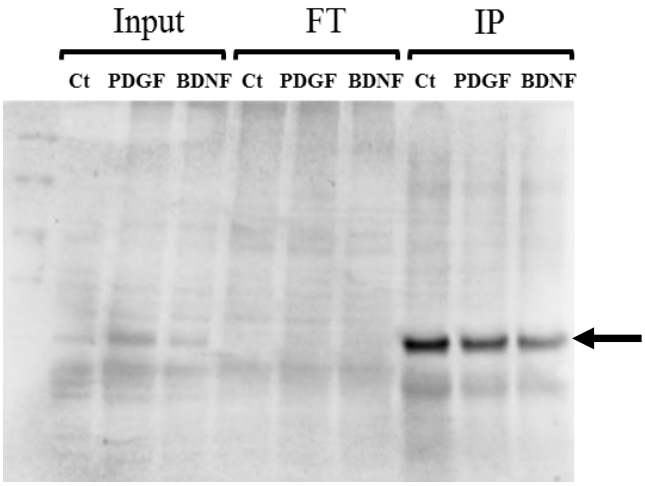

Figure 19- Optimization of the protocol for hnRNPK IP immunoprecipitation from hippocampal synaptoneurosome homogenates. Immunoblot for hnRNPK of the samples from input, flow-through (FT) and immunoprecipitate (IP) for the different conditions (Ct, PDGF and BDNF). The band with an apparent molecular weight of about 65 kDa (black arrow) corresponds to hnRNPK.

In contrast with the results obtained in synaptoneurosomes stimulated with BDNF, PDGF induced a non-significant increase in the interaction of the GluA1 mRNA with hnRNPK and a significant increase in GluN1 mRNA interaction. Interestingly, no effect was observed for the interaction of hnRNPK with the BDNF mRNA (Figure 20). These results show clear differences in the effects of BDNF and PDGF in the mobilization of the transcripts bound to hnRNPK, which are likely to affect protein synthesis. This PDGF-induced increase in the amount of transcripts associated with hnRNPK is surprising considering that synaptoneurosomes do not possess transcriptional activity. However, the results suggest that PDGF stimulation causes a redistribution of some transcripts between two distinct populations of RNA binding proteins. Whether this is the case, and the physiological relevance of these alterations remain to be investigated.

The BDNF-induced dissociation of the GluA1 mRNA from hnRNPK is in agreement with the effect of the neurotrophin in upregulating GluA1 protein levels in total forebrain synaptoneurosomes (Schratt et al., 2004). Therefore, the BDNF-mediated activation of ERK and PI3K-mTOR signalling activates the translation machinery (Takei et al., 2004; Takei et al., 2001) and halts the translation repressor activity of hnRNPK (or its interacting partners in RNA granules), thereby increasing local protein synthesis. Furthermore, BDNF was shown to

promote the trafficking and synaptic incorporation of *de novo* synthesized GluA1-containing AMPA receptors, resulting in increased synaptic strength (Fortin et al., 2012). Additional studies should be performed to determine whether a downregulation of hnRNPK protein levels affects the BDNF-induced synaptic accumulation of GluA1-containing AMPA receptors. Given the observed dissociation of GluN1 transcripts from the hnRNPK protein in synaptoneurosomes stimulated with BDNF, it will be of interest to determine whether the ribonucleoprotein modulates the effects of BDNF on the synaptic level and distribution of GluN1 at the synapse. Although BDNF was shown to upregulate GluN1 protein levels in cultured hippocampal neurons (Caldeira et al., 2007), it remains to be determined if NMDA receptors are locally synthesized at the synapse in response to stimulation with the neurotrophin.

BDNF also induced the dissociation of BDNF mRNA from hnRNPK in synaptoneurosomes. This evidence supports the hypothesis of local BDNF translation at the synapse that may account for the fast replenishing of the BDNF pool at dendrites after repeated strong stimulation. The local synthesis of BDNF at dendrites induces the local activation of TrkB receptors, and may therefore provide trophic support at the synapse (Baj et al., 2011; Righi et al., 2000; Tongiorgi et al., 1997).

In contrast with the results obtained regarding the effects of BDNF on the interaction of GluA1, GluN1 and BDNF mRNA with hnRNPK, PDGF stimulation either has no effect or increases the interaction of the mRNAs with hnRNPK. This difference may be due to a differential distribution of the BDNF and PDGF receptors at the synapse, which may prevent the PDGF receptors from acting as regulators of hnRNPK. Alternatively, the receptors may be located in distinct synapses. At this point, there are no studies with a comparative analysis of the distribution of the two types of receptors and further studies are required to explain the differences observed between the responses to BDNF and PDGF. The results showing no dissociation of GluA1 mRNA from hnRNPK in synaptoneurosomes stimulated with PDGF, suggest that this ligand does not upregulate GluA1 protein levels at the synapse. Alternatively, PDGF may release transcripts from a different ribonucleoprotein that also transports GluA1 mRNAs along dendrites. Interestingly, insulin was also shown to increase the interaction of hnRNPK with RNA in a rat hepatoma cell line (Ostrowski et al., 2001). To the best of our knowledge there are no studies concerning the effect of PDGF in GluA1 protein levels in synaptoneurosome preparations. PDGF was reported to upregulate GluA1 protein levels in cultured cerebrocortical neurons, but this study did not assess the relative role of transcription

and whether the alterations in GluA1 levels occurred at the synaptic level (Narisawa-Saito et al., 1999).

Although PDGF did not release the GluA1 or GluN1 transcripts from hnRNPK, this trophic factor was shown to induce LTP and PDGF receptors play a role in memory formation (Peng et al., 2010; Shioda et al., 2012). This may be mediated by inducing the local translation of a distinct set of proteins and/or recruitment of transcripts for translation from distinct ribonucloproteins.

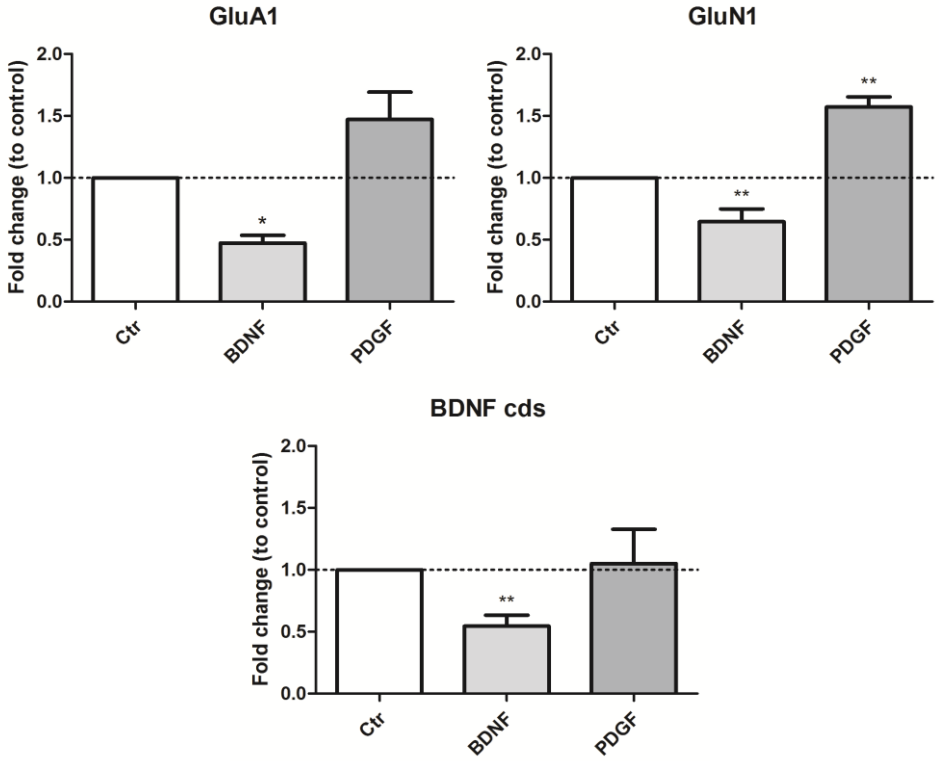

Figure 20- Effect of BDNF and PDGF on the interaction of the GluA1; GluN1 and BDNF transcripts with hnRNPK in synaptoneurosomes. The results are the mean ± SEM of four different experiments performed in independent preparations, and are expressed as fold change to control. *p<0.05, **p<0.01, ***p<0.001 as determined by ANOVA followed by Dunnett's Multiple Comparison test.

CHAPTER 5

Conclusions

This work shows a role for hnRNPK, a protein that interacts with a large number of transcripts, in the regulation of translation activity in COS-7 cells and in cultured hippocampal neurons. Transfection of COS-7 cells with hnRNPK significantly reduced translation activity, suggesting that the protein may act as a repressor of translation.

Previous studies showed that the interaction of hnRNPK with several transcripts coding for proteins relevant in synaptic plasticity is reduced in cultured hippocampal neurons stimulated with BDNF. The present work performed in an heterologous system and in cultured hippocampal neurons also suggest that the role of hnRNPK as a translation repressor may be relieved following stimulation of hippocampal neurons with BDNF.

We also found that the mRNA for GluA1, GluN1 and BDNF co-immunoprecipitate with hnRNPK in synaptoneurosomes isolated from the rat hippocampus. These results and the presence of hnRNPK in dendrites shown by immunocytochemistry, suggest an important role for this RNP in the transport of several mRNAs along dendrites. Given the nature of the transcripts analysed, it is tempting to suggest that hnRNPK plays a role in the transport of mRNAs necessary for local protein synthesis in long-term synaptic potentiation. In contrast with the effect of BDNF, PDGF increased (GluA1, GluN1) or was without effect (BDNF) in the interaction of hnRNPK with the mRNAs investigated. The results suggest that PDGF-mediated long-term synaptic potentiation may require the participation of distinct mechanisms to control translation activity.

The trigger that promotes the release of the mRNA cargo from the RNA granules following synaptic stimulation is not completely understood. However, BDNF was previously shown to increase the phosphorylation status of the hnRNPK, which might be related with the alteration in the affinity for the transcripts and/or with the disassembly of the RNA granules through a change in protein-protein interaction. The release of transcripts in the dendritic spines makes the mRNAs available for local protein synthesis, a critical step in late-phase LTP. The direct or indirect association of the mRNAs with hnRNPK and its binding partners in the RNA granules remain to be investigated.

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