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Regulation of heterogeneous nuclear RiboNucleoProtein K (hnRNP K) by BDNF:

changes in mRNA binding as determined by Microarray Analysis

DIOGO DE OLIVEIRA FERREIRA COMPRIDO

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professor Doutor Carlos Jorge A. M. Bandeira Duarte (Universidade de Coimbra) e co-orientação da Doutora Ana Rita A. Santos (Universidade de Coimbra)

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ABBREVIATIONS

AC	Adenylyl cyclase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
Arc	Activity-regulated cytoskeleton-associated protein
A2RE	A2-response element
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine 3',5'-monophosphate
CNS	Central nervous system
CREB	cAMP response element-binding protein
DAG	Diacylglycerol
DIV	Days *in vitro*
dlP-bodies	Dendritic-like P-bodies
DNA	Deoxyribonucleic acid
DOC	Deoxycholic acid
DTT	Dithiothreitol
ECF	Enhanced chemifluorescence
eEF	Eucaryotic elongation factor
EGTA	Ethylene glycol tetraacetic acid
eIF	Eukaryotic initiation factor
EMSA	Electrophoretic mobility shift assay
EPSP	Excitatory postsynaptic potential
ERK	Extracellular signal-regulated kinase
FMRP	Fragile X mental retardation protein

FXS	Fragile X Syndrome
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GO	Gene ontology
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
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
mRNP	mRNA-protein complex
mTOR	Mammalian target of rapamycin
NLS	Nuclear localization signal
NMDA	N-methyl-D-aspartate
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
RBP	RNA-binding protein
RIP	RNP immunoprecipitation
RIPA	Radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RRM	RNA recognition motif
Shc	Src homology 2 domain-containing
SDS	Sodium dodecyl sulfate
TLS	Translocated in liposarcoma
TrkB	Tropomyosin-related kinase B
ZBP1	Zipcode-binding protein 1

ABSTRACT

Neurons are highly polarized cells, and the local synthesis of proteins at the synapse requires mechanisms to deliver specific transcripts to dendrites. The transport of newly synthesized mRNAs from the nucleus to dendrites is conducted by RNA granules that allow the delivery and stabilize transcripts. These granules are disassembled in response to synaptic stimulation and this is thought to release the mRNAs for subsequent local protein synthesis. Local protein synthesis at the synapse is particularly important for the maintenance of LTP (long-term potentiation), a well-known form of synaptic plasticity that is thought to underlie learning and memory processes.

The interaction with different proteins is thought to provide specificity in the delivery and transport of mRNAs to dendrites. Furthermore, since local translation at the synapse is induced by synaptic activity, the interaction of RNA binding proteins and the transcripts is likely to be regulated by intracellular signaling mechanisms. In this work, we aimed at characterizing the mRNAs associated with the RNA binding protein hnRNP K in cultured hippocampal neurons, a protein present at the synapse, and the regulation by the neurotrophin BDNF (brain-derived neurotrophic factor). BDNF plays an important role in the late phase of LTP, which is dependent on protein synthesis. Furthermore, we investigated whether hnRNP K is phosphorylated in response to stimulation of hippocampal neurons with BDNF. hnRNP K was immunoprecipitated from cultured hippocampal neurons, stimulated or not with BDNF (10 min), and the transcripts present in the immunoprecipitates were identified by microarray analysis. A total of 11422 transcripts were identified in hnRNP K immunoprecipitates from cultured hippocampal neurons under resting conditions. Gene Ontology analysis showed that

these mRNAs code for proteins involved in cellular processes, metabolic processes, biological regulation and cellular signaling, among others. The interaction of about 50 % of these transcripts was sensitive to stimulation of hippocampal neurons with BDNF, and in most cases (99.9 %) the neurotrophin decreased the amount of mRNAs co-immunoprecipitated with hnRNP K. The results obtained for 4 transcripts coding for synaptic proteins (GluA1, GluA2, GluN1, and CaMKIIβ), as well as for BDNF and its TrkB receptor, were validated by real-time PCR. The decrease in transcript co-immunoprecipitation with hnRNP K was correlated with an increase in the phosphorylation of the protein on Ser302 in hippocampal neurons stimulated with BDNF

Taken together the results obtained allowed identifying a large group of mRNAs that are associated (directly or indirectly) with hnRNP K in cultured hippocampal neurons, and may be transported to dendrites by this RNA binding protein. Also, the observed BDNF-induced phosphorylation of hnRNP K may change the RNA-protein interaction since a significant fraction of the mRNAs were dissociated after stimulation of hippocampal neurons with the neurotrophin. These evidences support the role of BDNF on the late phase of LTP since we clearly showed that it promotes the release of the transported mRNAs associated with hnRNP K, which should allow them to be locally translated.

Key words: hnRNP K; BDNF; RNA Transport; Dendritic Translation; Synaptic Plasticity.

RESUMO

Os neurónios são células altamente polarizadas, e a síntese local de proteínas na sinapse requer mecanismos para o endereçamento de transcritos específicos para as dendrites. O transporte de mRNAs recém-sintetizados desde o núcleo até às dendrites é feito por grânulos de RNA que permitem a entrega e estabilização dos transcritos. Estes grânulos são desorganizados em resposta à estimulação sináptica, permitindo possivelmente a libertação dos mRNAs e subsequente síntese proteica local. A síntese de proteínas na sinapse é particularmente importante para a manutenção da LTP (potenciação sináptica de longa duração), uma forma bem conhecida de plasticidade sináptica que se pensa estar na base dos processos de aprendizagem e memória.

A interacção dos diferentes transcritos com proteínas distintas é provavelmente responsável pela especificidade no endereçamento e no transporte de mRNAs para as dendrites. Além disso, uma vez que a síntese proteica local na sinapse é induzida pela actividade sináptica, a interacção entre as proteínas que ligam RNA e os transcritos deverá sofrer regulação por mecanismos de sinalização intracelular. Neste trabalho, caracterizámos os mRNAs associados à hnRNP K, uma proteína presente na sinapse e com capacidade para ligar RNA, em culturas de neurónios de hipocampo. Comparou-se a população de transcritos que interage com a hnRNP K em condições de repouso e em células estimuladas com a neurotrofina BDNF (factor neurotrófico derivado do cérebro). O BDNF desempenha um papel importante na fase tardia do LTP, que é dependente de síntese proteica. Além disso, investigámos a fosforilação da hnRNP K em resposta à estimulação dos neurónios de hipocampo com BDNF. A hnRNP K foi imunoprecipitada a partir de culturas de neurónios de hipocampo, estimuladas ou não com BDNF (10 min), e os transcritos presentes nos imunoprecipitados foram

identificados por análise de *microarray*. Foram identificados 11422 transcritos nos imunoprecipitados de hnRNP K obtidos a partir de neurónios do hipocampo em cultura em condições de repouso. A análise através do *Gene Ontology* revelou que estes mRNAs codificam proteínas envolvidas em processos celulares, metabolismo, regulação celular, e sinalização celular, entre outras funções. A interacção de cerca de metade destes mRNAs foi afectada pela estimulação dos neurónios com BDNF, tendo-se observado uma redução na co-imunoprecipitação com a proteína hnRNP K em cerca de 99.9 % dos casos. Os resultados obtidos para 4 transcritos que codificam proteínas sinápticas (GluA1, GluA2, GluN1 e CaMKIIβ), bem como para o BDNF e o seu receptor TrkB, foram validados por PCR em tempo real. A diminuição na co-imunoprecipitação de transcritos com a hnRNP K foi correlacionada com um aumento na fosforilação da proteína na Ser 302 em neurónios de hipocampo estimulados com BDNF.

No conjunto, os resultados obtidos permitiram identificar um grande grupo de mRNAs que estão associados (directa ou indirectamente) com a hnRNP K em culturas de neurónios de hipocampo. Estudos adicionais são necessários para investigar o papel da hnRNP K no transporte destes mRNAs para as dendrites. Além disso, a fosforilação da hnRNP K induzida pelo BDNF poderá afectar a interacção proteína-RNA uma vez que uma fracção significativa dos transcritos deixou de co-imunoprecipitar com a hnRNP K após a estimulação dos neurónios de hipocampo com a neurotrofina. Estas evidências reforçam o papel do BDNF na fase tardia do LTP, uma vez que mostram claramente que este promove a libertação dos mRNAs transportados associados à hnRNP K, o que deverá contribuir para a síntese local de proteínas.

Palavras-chave: hnRNP K; BDNF; Transporte de RNA; Tradução nas Dendrites; Plasticidade Sináptica.

CHAPTER 1 - INTRODUCTION

Neurons were recognized as the primary functional unit of the CNS (central nervous system) in the beginning of the 20th century by the work of the Spanish anatomist Santiago Ramón y Cajal. He proposed that neurons were discrete cells that communicate with each other via specialized junctions, which are now called synapses (López-Muñoz et al., 2006). Following presynaptic stimulation, neurotransmitters are released from the presynaptic terminal into the synaptic cleft, where they can diffuse and reach the neurotransmitter receptors located on the postsynaptic membrane. In excitatory synapses the postsynaptic region is usually located in dendritic spines (Figure 1), which are highly dynamic structures that undergo changes in size and shape upon synaptic activity, and these alterations are very important for synaptic plasticity events (Hering and Sheng, 2001). In contrast, in inhibitory synapses the postsynaptic region is located on the dendritic shaft (Megías et al., 2001).

Figure 1 – Three-dimensional reconstruction of a rat pyramidal cell dendrite from stratum radiatum (CA1) in the hippocampus, illustrating the location of excitatory and inhibitory synapses. Excitatory synapses are colored in red and occur mainly in the dendritic spines. Inhibitory synapses are colored in blue and occur mainly in the dendritic shaft. Adapted from "Comparative Morphology of Dendritic Spines and Spine Synapses" by Josef Spacek.
(http://synapses.clm.utexas.edu/anatomy/compare/compare.stm).

Glutamate, the major excitatory neurotransmitter in the CNS, acts on synaptic transmission primarily through activation of ionotropic glutamate receptors present in the postsynaptic membrane, belonging to the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA (N-methyl-D-aspartate) categories (Traynelis et al., 2010). Activation of AMPA receptor channels by glutamate leads to sodium entry into the postsynaptic compartment, depolarizing the membrane towards the threshold required for activation of an action potential. The depolarization of the postsynaptic membrane removes the voltage-dependent magnesium block of the NDMA receptors resulting in a rapid increase of intracellular calcium levels in dendritic spines. Calcium can activate several enzymes and signaling cascades in the post-synaptic compartment, functioning as a second messenger for activity-dependent, synapse-specific changes. The efficient and specific activation of Ca^{2+}-dependent signaling mechanisms relies on the clustering of the receptors and target proteins on the post-synaptic region, mediated by protein-protein interactions (Scannevin and Huganir, 2000).

In 1973, Bliss, Gardner-Medwin and Lomo reported that a few seconds of HFS (high-frequency stimulation) of the perforant path fibers enhances synaptic transmission between the stimulated axons and the dentate areas of the hippocampus (Bliss and Gardner-Medwin 1973; Bliss and Lomo 1973). This phenomenon has been called LTP (long-term potentiation) and is the most studied form of synaptic plasticity (Figure 2). These changes in synaptic connectivity due to alterations in activity and following structural modifications are thought to be the basis of learning and memory formation.

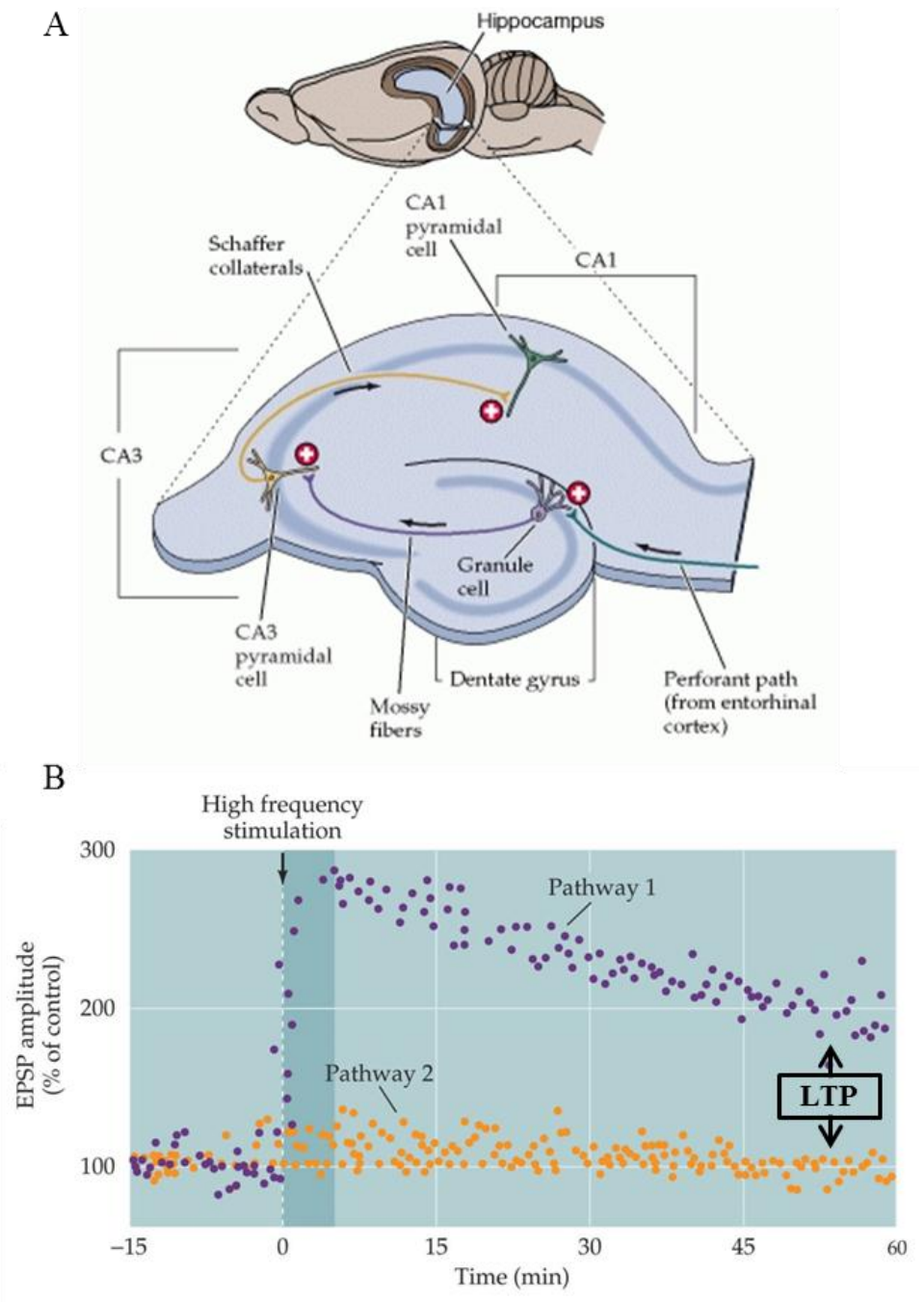

Figure 2 – (A) Illustration of a section through the rodent hippocampus showing the major regions and synaptic connections. (B) LTP induced at the Schaffer collateral-CA1 synapses in a hippocampal slice. The population responses were recorded in the CA1 region and where indicated a train of high-frequency stimulation was applied at the Schaffer collaterals in one of the tests (Pathway 1). High-frequency stimulation induces a long-term enhancement of the EPSCs in Pathway 1. In contrast, the synaptic pathway that did not receive a high frequency train of stimuli (Pathway 2) shows EPSCs with constant amplitude. Adapted from (Purves et al., 2004).

1. THE BDNF-TrkB RECEPTOR SYSTEM AND SYNAPTIC PLASTICITY

LTP in the hippocampus comprises three sequential phases: short-term potentiation, early-LTP and late-LTP. The first two phases are transcription- and translation -independent, lasting for 1-2 h, while late-LTP depends on transcription and *de novo* protein synthesis, lasting for hours and under certain conditions, even for days and weeks (Malenka and Bear, 2004; Costa-Mattioli et al., 2009).

LTP occurs after a cascade of events that is initiated by the glutamate release as described above. *In vitro*, LTP can be induced by HFS, or by pairing of presynaptic stimulation with postsynaptic depolarization, and is dependent on Ca^{2+} entry following activation of NDMA receptors (Lisman, 2007). The $[Ca^{2+}]_i$ increase has also the contribution from voltage-gated calcium channels and the release of calcium from intracellular stores, promoting the activation of CaMKII (calcium/calmodulin-dependent protein kinase II) and AC (calcium-stimulated adenylyl cyclase). The resulting increase in intracellular cAMP (cyclic adenosine 3',5'-monophosphate) concentration activates PKA (protein kinase A) which, together with CaMKII promote the insertion of AMPAR-containing vesicles into the postsynaptic membrane, thereby increasing the content of these receptors in the PSD (postsynaptic density) (Figure 3). The potentiation of synaptic transmission is also due to an increase in AMPAR conductance and CaMKII binding to NMDAR, which locks these receptors in a permanent activated state that cannot be reversed by phosphatases (Sheng and Kim, 2002; Xia and Storm, 2005).

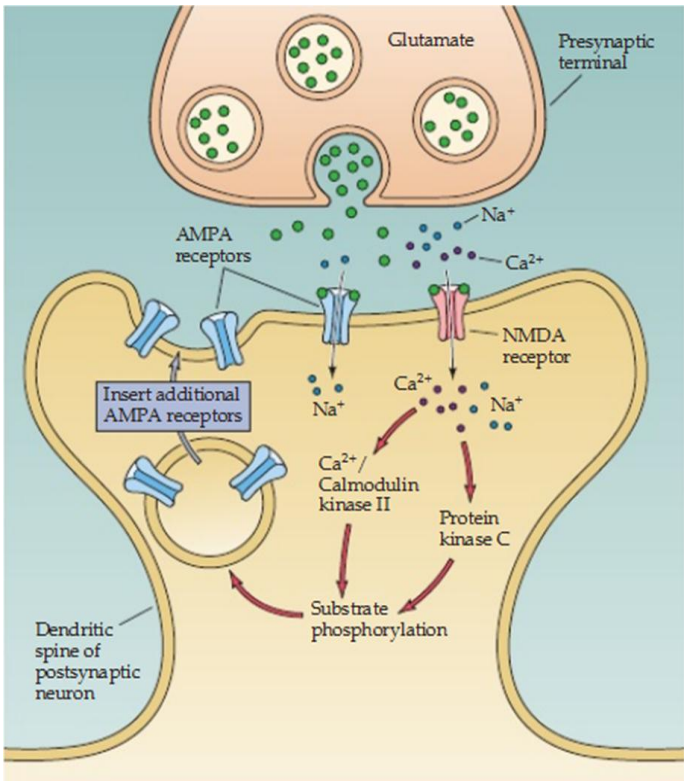

Figure 3 – Mechanisms underlying LTP. Glutamate released from the presynaptic terminal into the synaptic cleft bind to the AMPA and NMDA ionotropic receptors. Activation of AMPA receptor channels by glutamate leads to sodium entry into the postsynaptic compartment. The depolarization of the postsynaptic membrane, induced by sodium-entry, removes the voltage-dependent magnesium block of the NDMA receptors resulting in a rapid increase of intracellular calcium levels in dendritic spines. Calcium can activate several enzymes and signaling cascades in the post-synaptic compartment, functioning as a second messenger for activity-dependent, synapse-specific changes. From (Purves et al., 2004).

1.1. BDNF and the TrkB receptors

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 (Tsien et al., 1996; Zamanillo et al., 1999; Minichiello et al., 1999).

BDNF belongs to the neurotrophin family of trophic factors, which control 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 in both pre and postsynaptic membranes, resulting in receptor dimerization (Jing et al., 1992) and transphosphorylation on specific tyrosine residues located in the intracellular domains. The effects of BDNF are mediated by activation of various parallel signal transduction cascades, with distinct functions, that are initiated after the docking of different adaptor proteins and signaling enzymes (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 IP3 (inositol 1, 4, 5-trisphosphate), which releases calcium from intracellular stores. This also contributes to ERK and CREB activation, thereby increasing cell survival, and play a key role in synaptic plasticity (Figure 4) (Minichiello et al., 2002).

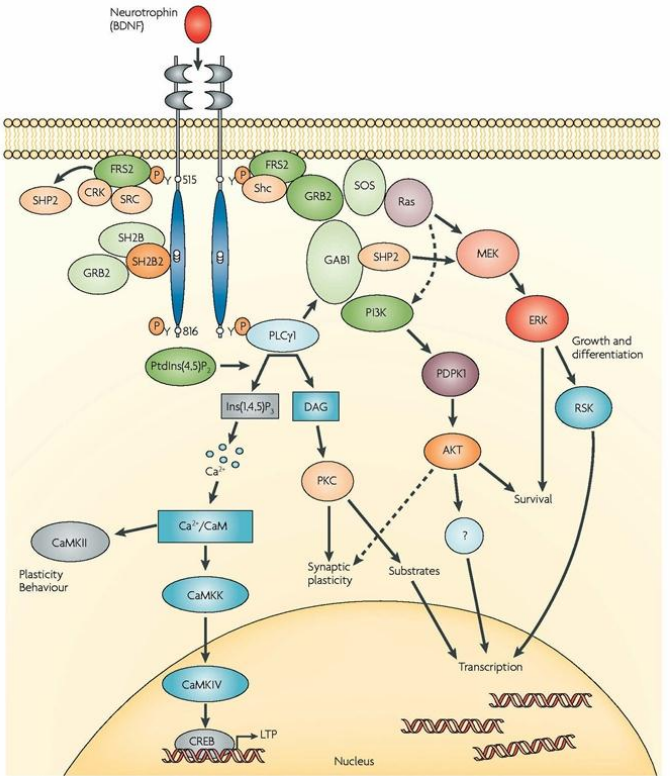

Figure 4 – The neurotrophin BDNF activates TrkB receptors that undergo dimerization and transphosphorylation on specific tyrosine residues of the intracellular domain. The phosphorylation of the receptors is followed by docking of several different adaptor proteins and signaling enzymes which set in motion three major parallel signal transduction cascades: PLC$_\gamma$, PI3K/Akt and Ras/ERK signaling pathways. The TrkB receptors play important roles in cell growth and differentiation, survival and synaptic plasticity. From (Minichiello, 2009).

1.2. The role of BDNF in LTP

The importance of BDNF in LTP in the hippocampus has been shown either by sequestering endogenous BDNF with TrkB-immunoglobulin G fusion protein, using BDNF- or TrkB-antiserum, and in experiments performed in TrkB- or BDNF-deficient mice where LTP was shown to be impaired (Korte et al., 1995, 1998; Figurov et al., 1996; Kang et al., 1997; Minichiello et al., 1999). On the other hand, BDNF application was shown to induce synaptic potentiation by a mechanism dependent on protein synthesis (Kang and Schuman, 1995, 1996; Ji et al., 2010). In fact, BDNF plays a very important role in the late-LTP, which is dependent on gene transcription and *de novo* protein synthesis, playing a key role in establishing long-term memories (Kandel, 2001; Kelleher et al., 2004). Though, this is the step lacking more information and in need of further clarification.

In addition to the delayed synaptic effects of BDNF requiring transcription and translation activity, the neurotrophin also induces rapid pre- and post-synaptic responses, most likely mediated by phosphorylation of the existing proteins. The presynaptic effects of BDNF include facilitation of synaptic vesicle docking to the plasma membrane (Tyler and Pozzo-Miller, 2001) and increase of neurotransmitters release (Lessmann et al. 1994; Pereira et al. 2006). This results in a sustained glutamate release during bursts of action potentials which facilitates LTP induction in response to high-frequency stimulation. Postsynaptically, BDNF may act by increasing NMDA receptor single channel open probability (Levine et al. 1998), presumably through phosphorylation of the receptor subunits. Accordingly, BDNF was shown to induce tyrosine phosphorylation of GluN1 and GluN2B NMDA receptor subunits in cultured hippocampal neurons (Suen et al., 1997; Lin et al., 1998), and the effects of the neurotrophin on the electrophysiological properties of the receptor depend on the

GluN2B subunits (Levine et al. 2000). Furthermore, studies in cultured organotypic hippocampal slices treated with BDNF also showed a rapid synaptic delivery of GluA1-containing AMPA receptors which was dependent on the activation of Trk (presumably TrkB) receptors (Caldeira et al., 2007). The synaptic delivery of GluA1 receptor subunits induced by BDNF was also correlated with an increase in receptor phosphorylation (Caldeira et al., 2007). Similarly, immunocytochemistry experiments in cultured cerebrocortical neurons showed that BDNF induces synaptic delivery of GluA1-containing AMPA receptors to the synapse from a local pool (Nakata and Nakamura, 2007). These rapid pre- and post- synaptic changes induced by BDNF are relevant in the first two phases of LTP, the short-term potentiation and early-LTP; both are protein synthesis-independent and rely only on proteins that were already available in the synapse.

2. LOCAL PROTEIN SYNTHESIS IN DENDRITES

Although the bulk of 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 5), which is the distinctive evidence that they are bound to mRNAs and actively engaged in protein synthesis (Steward and Levy, 1982).

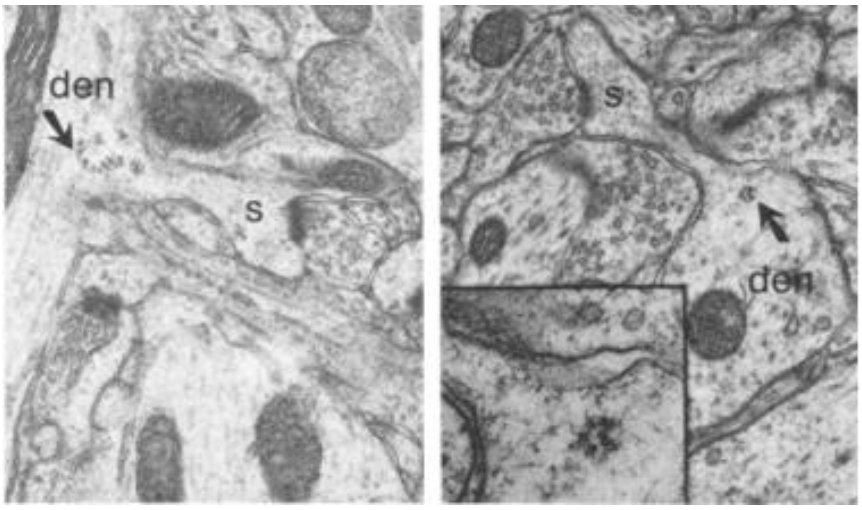

Figure 5 – Distribution of polysomes in dentate granule cells. The electron micrograph shows highly dense structures, corresponding to a series of ribosomes arranged into a rosette-like formation, in the base of dendritic spines (s). The arrows show polysomes clusters at the spine neck-dendritic (den) 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. Since the pioneer work of O Steward et al. 1982, many studies have addressed this issue, and it is now clear that local protein synthesis at synaptic sites occurs independently of the soma, and various stimuli, including BDNF, are known to induce local protein synthesis in dendrites. Furthermore, several neuronal processes have been related to local protein synthesis, such as synaptic plasticity (Steward and Schuman, 2001; Costa-Mattioli et al., 2009), neurite growth and development (Sebeo et al., 2009).

The translation of a given mRNA requires three steps: initiation, elongation and termination. The initiation and elongation steps are considered to be rate-limiting (Herbert and Proud, 2007) and, therefore, subjected to regulation. For initiation to begin the complex eIF4F (eukaryotic initiation factor 4F) has to be formed in order to recruit

both the ribosome and the mRNA molecule. The eIF4F complex is comprised by three subunits with specific functions: eIF4E binds to 5' capped mRNAs; eIF4A unwinds the secondary structure of the mRNA; eIF4G bridges the mRNA to the 43S pre-initiation complex. The assembly of eIF4F is modulated by 4EBPs (eIF4E-binding proteins). Non-phosphorylated 4EBPs bind to eIF4E suppressing translation, whereas phosphorylation of 4EBPs induces eIF4F complex formation and translation activation. Several studies have shown that synaptic plasticity is associated with an increase in phosphorylation of 4EBPs as well as eIF4E itself (Richter and Klann, 2007). BDNF promotes the phosphorylation of eIF4E and its binding protein-1 (4EBP1), by mechanisms sensitive to chemical inhibitors of the ERK and PI3K signaling pathways, respectively, and the phosphorylation of 4EBP1 is mediated by activation of the mTOR pathway (mammalian target of rapamycin) (Takei et al., 2004). This pathway is downstream of the PI3K/Akt signaling pathway and regulates both the 5'cap-dependent translation, by inducing eIF4F complex formation, and the 5' TOP containing mRNAs, by activating the S6 kinase pathway. Once initiation is completed, elongation factors are recruited including the eEF2, which promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome. The activity of 4EBPs, eIF4E and eEF2 is regulated by different signaling pathways, including the ERK, PI3K and mTOR pathways, highlighting the role of BDNF in local protein synthesis (Takei et al., 2001, 2009; Tang et al., 2002; Inamura et al., 2005).

The number of identified mRNAs that are localized to the synapse is increasing, many of them encoding synaptic proteins, including most components of the postsynaptic density, and components of the translation machinery (Zhong et al., 2006; Poon et al., 2006). Studies have been performed to clarify how local translation is regulated by extracellular signals, including neurotransmitters and trophic factors, but

the mechanisms that target specific mRNAs to dendrites and their selective docking at specific synapses remain largely unknown. Furthermore, although the synaptic effects of BDNF have been extensively characterized (see above), the mechanisms underlying BDNF-induced local changes in the synaptic proteome and their role in synaptic plasticity are not fully understood.

3. mRNA TRAFFICKING ALONG DENDRITES

Although mRNA translation in dendrites is presently quite established, the mechanisms contributing to the delivery of transcripts to synapses, at significant distance from the nucleus, where the transcription takes place, still remains largely unknown and is currently attracting the attention of many research groups in the field. Some of the questions that remain to be addressed are: 1) how are the mRNAs transported to dendrites; 2) what mRNAs are transported; 3) which stimuli promote translation of these mRNAs; 4) what is the physiological role of the newly synthesized proteins; and 5) how is regulated each step described above.

Dendritic protein synthesis depends on the coordinated transport, localization, and translation of mRNAs. All these mechanisms can be modulated by changes in synaptic activity, suggesting an extraordinary fine-tuning for controlling the time and place of protein synthesis (Steward and Schuman, 2003; Klann and Dever, 2004) (Figure 6). BDNF was shown to induce the trafficking of mRNAs from soma to dendrites (Righi et al., 2000) supporting the major role of the neurotrophin in the late-phase of LTP.

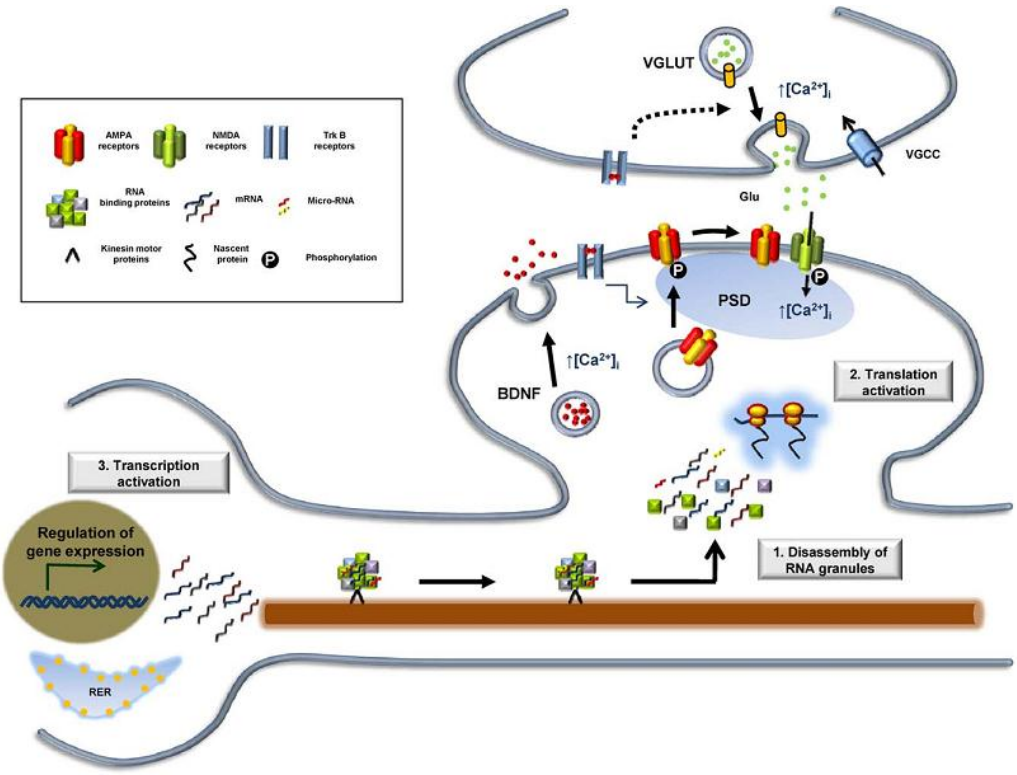

Figure 6 – Effects of BDNF on RNA granules. BDNF induces local protein synthesis at the synapse from mRNAs transported along dendrites in RNA granules, by promoting the disassembly of the granules (1) and activating the translation machinery (2). Additional effects of BDNF include the regulation of RNA transport along dendrites, which is mediated by kinesin motor proteins, and activation of gene expression (3). From (Santos et al., 2010).

3.1. mRNP assembly

Gene expression occurs in the nucleus and results in synthesis of pre-mRNAs by transcription, and these pre-mRNAs are assembled into pre-mRNPs (pre-mRNA-protein complexes). Before the mRNAs can be exported from the nucleus they undergo a series of modifications that include 5' capping, splicing, 3' cleavage and polyadenylation, and for most mRNAs this is carried out co-transcriptionally by a host of proteins that bind the C-terminal domain of RNA polymerase II (Stewart, 2010). The resulting mRNP complexes are then exported first into the interchromatin space, and at a later point to the cytoplasm (Björk and Wieslander, 2011).

The RNA containing particles can be classified as processing bodies, stress granules, RISC complexes (RNA-induced silencing complexes), transport particles and RNA granules according to their molecular composition and biological function (Sossin and DesGroseillers, 2006). Studies regarding the composition of mRNP complexes in different systems have identified the following components: hnRNPs (heterogeneous nuclear ribonucleoproteins), nuclear cap binding proteins, splicing machinery, SR proteins, 3' end processing machinery, transcription-export complexes, mRNA export adaptors and receptors, and gene-specific components, among other elements (Björk and Wieslander, 2011). A proteomics study performed using RNA granules isolated from the developing brain identified ribosomal proteins, RNA-binding proteins, microtubule-associated proteins, and several proteins described only as potential open reading frames (Elvira et al., 2006). Some of the components of the mRNP complexes may leave these structures soon after leaving the nucleus, while others may accompany the mRNPs all the way to the dendrites. The latter group includes hnRNPs, which are found at the synapse and play a role in the regulation of gene expression (Kiesler et al., 2005; Björk and Wieslander, 2011).

3.2. mRNP transport and localization

The delivery of new mRNAs to the dendrites occurs in motile structures called RNA granules. These granules are constituted by different proteins, including RBPs (RNA-binding proteins), which control the stability, localization and translation of mRNAs, ribosomes and translational factors (Martin and Zukin, 2006) in a rapid (average speed is 0.1 μm/s), bidirectional and microtubule-dependent manner (Knowles et al., 1996). Interestingly, stimulation of cultured hippocampal neurons with BDNF was found to increase the proportion of moving RNA granules labeled with YFP-tagged

DEAD box 3, one of the proteins identified in a proteomics screening to determine the composition of the granules (Elvira et al., 2006). More recently, dlP-bodies (dendritic-like P-bodies structures) were proposed as the structures responsible for transporting mRNAs to dendrites. dlP-bodies were found both in the soma and dendrites of hippocampal neurons and contain many P-body components involved in RNA processes, such as Dcp1a, GW182, RCK/p54 and Ago2 proteins (Cougot et al., 2008). Furthermore, dlP-bodies were also shown to contain RBPs, including FMRP (fragile X mental retardation protein), ZBP1 (zipcode-binding protein 1) and Staufen (Barbee et al., 2006; Cougot et al., 2008), in addition to translational repressor proteins. Noticeably, depolarization of hippocampal neurons, or stimulation with NMDA or with BDNF, relocates dlP-bodies to the distal part of the dendrites (Cougot et al., 2008), in agreement with the numerous studies showing an activity-dependent translocation of mRNAs to dendrites (Tongiorgi et al., 1997).

One of the most studied RBPs is the Staufen 2, a protein that is mainly expressed in the brain. Almost two thousand mRNAs coding for proteins involved in cellular processes such as RNA metabolism, post-translational modifications, intracellular transport and translation were co-immunoprecipitated with Staufen 2-containing mRNPs (Maher-Laporte and DesGroseillers, 2010). Down-regulation of Staufen 2 induces morphological defects in cultured hippocampal neurons, including loss of dendritic spines and a change of shape from mushroom to filopodia, reorganizaton of the actin cytoskeleton in dendrites, a significant decrease in the β-actin mRNA levels, both in cell body and in dendrites, and attenuation of EPSPs (Excitatory postsynaptic potentials) (Goetze et al., 2006). Furthermore, Staufen 2 is necessary for DHPG-induced protein synthesis-dependent long-term depression (mGluR-LTD), but is not required for the protein synthesis-dependent long-term potentiation (L-LTP) in hippocampal

pyramidal cells (Lebeau et al., 2011). In contrast, Staufen 1 is important and necessary for the protein synthesis-dependent late-LTP and for the maintenance of mature dendritic spines and synaptic activity in hippocampal CA1 pyramidal cells, strongly suggesting a role of mRNA regulation by Stau1 in these processes (Lebeau et al., 2008). In neurites the two paralogues of Staufen are found in distinct RNA granules (Duchaine et al., 2002; Thomas et al., 2005).

FMRP is a RBP, the absence of which causes the Fragile X Syndrome (FXS), the most common inherited cause of mental retardation, characterized by deficits in learning and memory (Bagni and Greenough, 2005). FMRP contains multiple RNA-binding domains and is widely thought to function as a translational suppressor of specific mRNAs, including MAP1b, CaMKIIa, and Arc (Bassell et al. 2008). The protein is present at synapses, where it associates with mRNA and polyribosomes, and accumulating evidences show a role for FMRP in synapse development, elimination, and plasticity (Pfeiffer and Huber, 2009). Although the importance of RBPs is not yet completely elucidated, it is undeniable that they play an essential role in synaptic plasticity events as well as in normal brain development.

The hnRNPs are a different class of 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. Glycine-rich domains can also be found in others

hnRNPs (Figure 7) (Han et al., 2010). The modularity of these proteins results in a combination of domains that can form different hnRNPs which accounts for the variety of functions attributed to them.

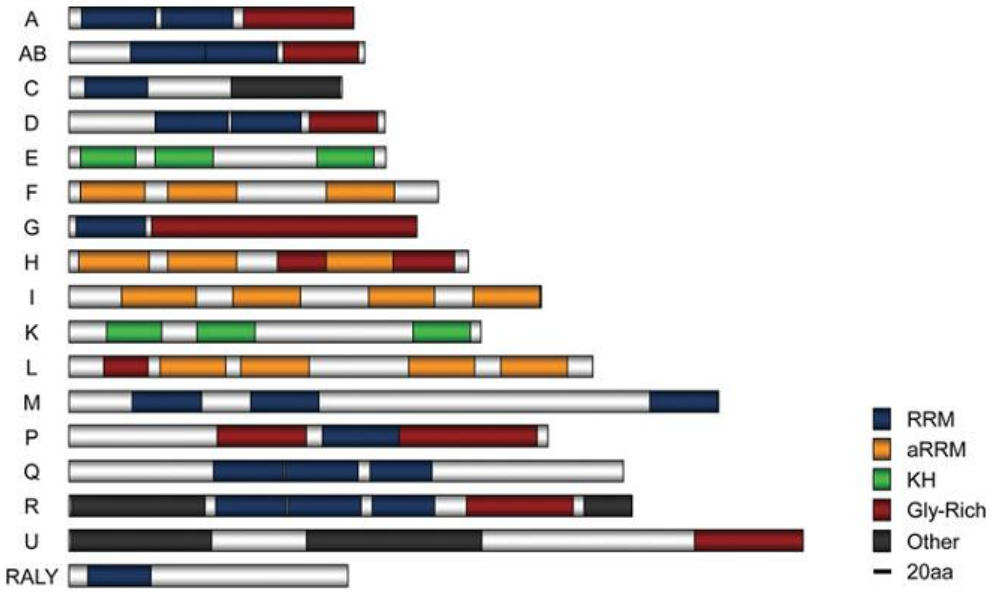

Figure 7 – Domain composition and assembly of different hnRNP proteins. RRM, RNA recognition motif; aRRM, atypical RNA recognition motif; KH, K homology domain; Gly-Rich, Glycine-Rich domain. From (Han et al., 2010).

hnRNP K is a 65 kDa protein composed by 463 amino acids containing different regions with distinct functions (Figure 8). Three KH domains, responsible for the RNA and dsDNA binding are found, 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., 1997, 2004). A total of 114 KI-binding partners were already identified via mass spectrometry, including kinases and proteins controlling mRNA splicing, transcription and translation processes (Mikula et al., 2006). The KI region contains, for example, proline-rich docking sites that interact with SH3 domains, characteristic of the Src-family kinases (Van Seuningen et

al., 1995). In fact, along the entire amino acid sequence, several serine, threonine and tyrosine residues are known to be phosphorylated (Schullery et al., 1999; Ostrowski et al., 2000; Ostareck-Lederer et al., 2002) 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).

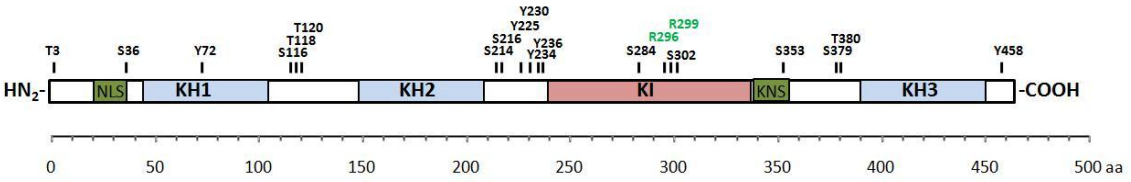

Figure 8 – hnRNP K structure and covalent modification sites. The residues subjected to phosphorylation (black) and methylation (green) are represented above the structure. NLS, nuclear localization signal; KH, K homology domains; KI, K interactive region; KNS, nuclear shuttling domain. From (Mikula and Ostrowski, 2010).

hnRNP A2/B1 is another RBP present in RNA granules and is known to play a role in mRNA trafficking in both oligodendrocytes and neurons (Hoek et al., 1998; Shan et al., 2003). It recognizes a 21-nucleotide *cis*-acting element, the A2RE (A2-response element), and is necessary and sufficient for the localization of mRNAs containing the A2RE-like sequences such as those encoding MBP (myelin basic protein), CaMKII, neurogranin and Arc (activity-regulated cytoskeleton-associated protein) (Ainger et al., 1997; Munro et al., 1999; Gao et al., 2008). Interestingly, this hnRNP recruits hnRNP E1 to RNA granules, a protein structurally related to hnRNP K, and this was shown to inhibit translation of trafficked mRNAs until they reach their intended destination (Kosturko et al., 2006).

3.3. mRNP disassembly and mRNA release

Once they are incorporated into RNA granules, mRNAs are translationally silenced either by hnRNPs (Krichevsky and Kosik, 2001) and/or microRNAs (Schratt et al., 2006). The microRNAs are emerging as key synaptic modulators, acting by targeting partially complementary mRNAs leading to translation repression or mRNA degradation (Konecna et al., 2009).

Depolarization of cerebrocortical neurons was shown to induce a reorganization of RNA granules, as observed by electron microscopy, and induces a less compact organization of their ribosomes. These structural changes were accompanied by a shift of many mRNA from the granule fraction into polysomes, including transcripts coding for proteins involved in synaptic plasticity (Krichevsky and Kosik, 2001). However, the mechanisms by which transcripts become available for translation and the trigger that promotes the disassembly of the RNP structures responsible for the transport of mRNAs to dendrites is not fully understood. This is partially due to the diversity of proteins present in RNA granules and the complexity of interactions established among them. The available evidences suggest that phosphorylation may change the mRNA binding ability of hnRNP K (Ostrowski et al., 2000; Habelhah et al., 2001; Ostareck-Lederer et al., 2002; Feliers et al., 2007), 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 hnRNP K is a substrate of Src-family of tyrosine kinases and PKCδ. Thus, the Src-family kinase Lck binds to the KI region of hnRNP K through the SH3 docking sites and phosphorylate the protein causing the mRNA-RBP dissociation (Ostrowski et al., 2000; Ostareck-Lederer et al., 2002). This phosphorylation on tyrosine creates SH2 docking sites that allow the recruitment of PKCδ, since this kinase only binds to hnRNP K when it is dissociated from the mRNA

(Schullery et al., 1999). Lck is able to phosphorylate PKCδ which becomes activated and phosphorylates hnRNPK on Ser302 in addition to other effector proteins that may either be bound to hnRNP K or in the surrounding microenvironment (Bomsztyk et al., 1997; Schullery et al., 1999). One of the PKCδ targets is EF1α, a protein belonging to the translation machinery (Kielbassa et al., 1995). Furthermore, hnRNP K is a target of other enzymes and signaling cascades that may play an important regulatory role. Phosphorylation of hnRNP K on Ser284 by ERK leads to its cytoplasmic accumulation and inhibition of mRNA translation (Habelhah et al., 2001) suggesting a pleiotropic effect for the hnRNP K phosphorylation. Taken together, these evidences show a dynamic regulation of the mRNA transport particles and indicate that phosphorylation of RBP may control their stability, translational state and fate.

4. DENDRITICALLY TRANSPORTED mRNAS

Despite the difficulties in the identification of dendritic transcripts, associated with contamination with somatic material and the low sensitivity of the methods available, in addition to the problems resulting from the use of different cell types and distinct neuronal development stages, which also makes difficult the comparison between the results available in the literature, some dendritic transported mRNAs are already known as their synaptic functions have been identified.

Hundreds of dendritic transcripts were already identified, coding for proteins belonging to the translation machinery, like initiation and elongation factors (Poon et al., 2006), signaling pathways, [e.g. CaMKII (Burgin et al., 1990)], neurotrophins, [e.g. BDNF (Tongiorgi et al., 1997)], cytoskeleton, [e.g. Arc (Ying et al., 2002)], receptors,

[e.g. NDMA (Schratt et al., 2004)], among others. The diversity of dendritic mRNAs identified reinforces the importance that mRNA trafficking to dendrites has on the late-LTP where new protein synthesis is essential. However, many other questions remain unsolved in this field: which mRNAs are transported together in the same granule?; is the mRNA binding different when distinct proteins clusters are together?; is there a different packaging of mRNAs upon BDNF stimulation?

CHAPTER 2 – OBJECTIVES

In a gel-based proteome profiling of the long-term effects of BDNF in cultured hippocampal neurons recently conducted in our laboratory, changes were found in the abundance of proteins involved in a series of processes: 1) nucleobase, nucleoside, nucleotide and nucleic acid metabolism; 2) protein metabolism; 3) carbohydrate metabolism; 4) regulators of apoptosis; and 5) regulators of cell proliferation (Manadas et al. 2009). It was observed that BDNF up-regulated several protein spots identified as hnRNP K, suggesting that this RBP undergoes posttranslational modifications in neurons exposed to the neurotrophin. In particular one of the up-regulated spots (spot 5704) had a more acidic pI (Figure 9), suggesting that hnRNP K may be phosphorylated upon stimulation with BDNF. Since hnRNP K is present at the synapse (Liao et al., 2007), its regulation by BDNF may be relevant to understand the effects of the neurotrophin on local protein synthesis.

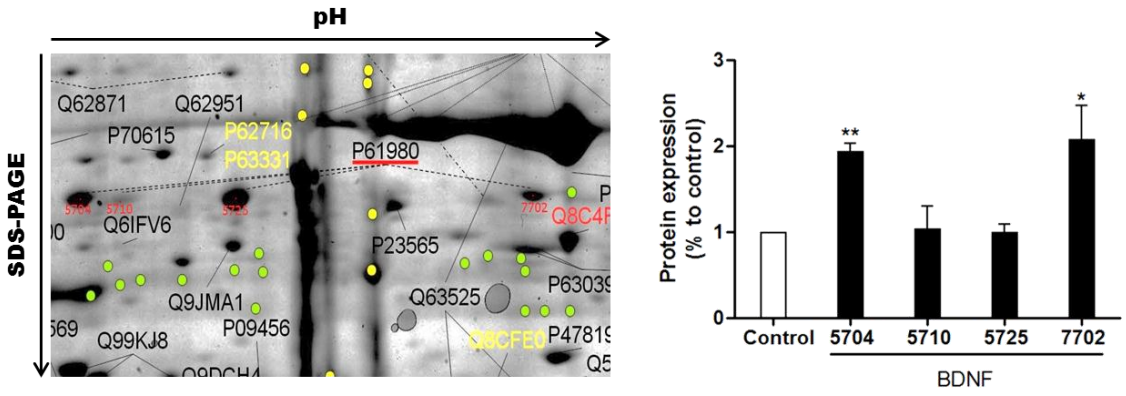

Figure 9 – Section of a 2D SDS-PAGE containing four spots corresponding to hnRNP K protein that were up-regulated by BDNF. In particular, one of the up-regulated spots (spot 5704) has a more acidic pI suggesting that hnRNP K may be phosphorylated upon BDNF stimulation. From (Manadas et al. 2009).

The main goal of this work was to better understand the role of BDNF on the regulation of hnRNP K, with two main objectives:

1) To study the BDNF-induced phosphorylation on hnRNP K

hnRNP K possesses multiple phosphorylation sites (Bomsztyk et al. 2004) and the activation of insulin receptors in hepatocytes was shown to induce phosphorylation of this RNP on tyrosine and to affect its interaction with RNA (Ostrowski et al., 2001). Furthermore, hnRNP K was shown to be phosphorylated on serine upon up-regulation of the ERK pathway (Habelhah et al., 2001). Given the similarities between the signaling cascades activated by the TrkB receptors for BDNF and insulin receptors (Reichardt, 2006; Chiu and Cline, 2010), and taking into account the role of the ERK pathway in the cellular responses induced by BDNF, we hypothesized that this neurotrophin could regulate the function of hnRNP K and its phosphorylation. The hypothesis that hnRNP K may became phosphorylated upon BDNF stimulation was addressed in cultured hippocampal neurons, and the neurotrophin-induced changes in the phosphorylation state of this RNP were investigated using Western Blot.

2) To identify the RNA transcripts associated with selected hnRNPs and the regulation by BDNF

Some hnRNPs are described as being present in RNA granules, being responsible for the transport of mRNAs from nucleus to dendrites (Hoek et al., 1998; Shan et al., 2003; Han et al., 2010). The transcripts that are associated with hnRNPs-containing granules were unknown and their identification was one of the objectives of this work. Given the results obtained in the first part of the work showing that BDNF stimulation changes the phosphorylation state of hnRNP K we investigated how the

neurotrophin affects the binding of transcripts to this RNP. In additional studies we aimed at characterizing the population of mRNAs associated with hnRNP A2/B1. This particular hnRNP is necessary and sufficient for the localization of mRNAs containing an A2RE-like sequence such as those encoding CaMKII, neurogranin and Arc mRNAs (Gao et al., 2008). The role of CaMKII in synaptic plasticity was briefly described in the first section of the introduction, and neurogranin is a CaMKII regulator which binds to the kinase in the absence of calcium. Neurogranin phosphorylation by PKC lowers its binding affinity to CaMKII (Martínez de Arrieta et al., 1999). Arc is induced as an immediate early gene, being activated transiently and rapidly in response to a wide variety of cellular stimuli, such as BDNF (Bramham et al., 2008). Arc protein is related to the cytoskeleton reorganization in the dendritic spines and synapse rearrangements associated with the LTP (Bramham, 2008). Disturbance in the expression of the Arc mRNA results in deficits in the late-phase LTP and impaired learning and memory tasks (Plath et al., 2006; Messaoudi et al., 2007). Moreover, hnRNP A2/B1recruits hnRNP E1 to RNA granules, a protein structurally related to hnRNP K, and this was shown to inhibit translation of trafficked mRNAs until they reach their intended destination (Kosturko et al., 2006). In order to accomplish these objectives, we used DNA Microarrays to identify the mRNAs associated with the hnRNPs and the results obtained with a selected group of transcripts were validated using real-time PCR.

CHAPTER 3 – METHODS

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 % fetal 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 50 µg/mL gentamycin. The cells were dissociated in this solution and were plated in six-well microplates (MW6) coated with poly-D-lysine (0.1 mg/mL) at a density of 85 500 cells/cm^2. The cultures were maintained in a humidified incubator of 5 % CO_2/95 % air at 37 ºC for 15 days. After one week in culture, half of the medium was exchanged for supplemented Neurobasal medium without glutamate. Cultures were stimulated with 50 ng/mL BDNF (Peprotech) for the indicated periods of time.

Low density hippocampal cultures were prepared as previously described (Goslin et al., 1998). Briefly, hippocampi were dissected from E18 rat embryos and dissociated using trypsin (0.02 %). Neurons were plated at a final density of 1.5 x 10^4 cells/cm^2 (60 mm culture dishes) on poly-D-lysine-coated coverslips and cultured in the presence of an astroglial feeder layer. Cultures were maintained in Neurobasal medium

supplemented with B27 supplement (1:50, Gibco Invitrogen), 25 µM glutamate, 0.5 mM glutamine and 0.12 mg/mL gentamycin. To prevent the overgrowth of glia, neuron cultures were treated with 5 µM cytosine arabinoside after 3 DIV and maintained in a humidified incubator with 5 % CO_2/95 % air, at 37 °C for 2 weeks, feeding the cells once per week by replacing one-third of the medium.

Preparation of extracts

Cultures of hippocampal neurons with 15 DIV (85 500 cells/cm^2), stimulated or not with BDNF, were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM $Na_2HPO_4.2H_2O$, pH 7.4). The cells were then lysed with RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EGTA, 1 % Triton, 0.5 % DOC and 0.1 % SDS, 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, 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 4 ºC. Protein in the supernatants was quantified using the BCA method and then denaturated 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), at 95 ºC for 5 min. The proteins of interest were then analyzed by Western Blot.

Western Blotting

Protein samples were separated by SDS-PAGE, in 7.5 % or 12 % polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) in 10 mM CAPS buffer with 10 % of Methanol (overnight, 4 ºC, 40 V), and immunoblotted. The blocking of the membranes was made with 5 % milk or 5 % bovine serum albumin (BSA) in the case of detection of phosphorylated proteins, prepared in TBS supplemented with 0.1 % Tween20. Blots were incubated with primary antibodies (overnight at 4 °C), washed, and exposed to alkaline phosphatase-conjugated secondary antibodies (1 h at room temperature). Alkaline phosphatase activity was visualized by enhanced chemifluorescence (ECF) on the Bio-Rad model 3000 VersaDoc Gel Imaging System, and quantified using ImageQuant program (GE Healthcare). Anti-GAPDH, anti-β-Actin and anti-β-Tubulin were used as loading controls and the results expressed after normalization. Statistical analysis of the results was performed using one-way ANOVA followed by the Dunnett's 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	1:5 000	Mouse	Sigma-Aldrich
β-Tubulin	1:300 000	Mouse	Sigma-Aldrich
GAPDH	1:5 000	Mouse	Sigma-Aldrich
hnRNP A2/B1	1:250	Mouse	Santa Cruz Biotechnology
hnRNP K	1:250	Mouse	Santa Cruz Biotechnology
p-hnRNP K Ser284	1:250	Rabbit	Abcam
p-hnRNP K Ser302	1:100	Goat	Santa Cruz Biotechnology

Secondary Antibodies	Dilution	Supplier
Anti-Goat (Alkaline phosphatase)	1:7 500	Santa Cruz Biotechnology
Anti-Mouse (Alkaline phosphatase)	1:20 000	GE Healthcare
Anti-Rabbit (Alkaline phosphatase)	1:20 000	GE Healthcare

Synaptoneurosomes Preparation

Synaptoneurosomes were prepared as previously described with slight modifications (Yin et al., 2002). Briefly, 4-6 hippocampi were dissected from adult Sprague–Dawley rats and the tissue was minced with scissors and homogenized with 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, 10 mM HEPES-Tris pH 7.4 and 0.1 mM EGTA. After centrifugation for 3 min at 1 000 x g, the supernatant was collected and passed initially through nylon membranes (150 and 50 μm, VWR) and finally through an 8 μm pore size filter (Millipore). The flow-through was centrifuged for 15 min at 10 000 x g, and the resulting pellet was resuspended in incubation buffer (in mM: 8 KCl, 3 CaCl2, 5 Na_2HPO_4, 2 $MgCl_2$, 33 Tris, 72 NaCl, and 100 sucrose). All the procedure was done at 4 °C. Incubations were made at 30 °C and the effect of BDNF was tested at a concentration of 50 ng/mL. For each time point considered a control experiment was also performed. Synaptoneurosomes were then centrifuged at maximum speed, in a Minispin microcentrifuge for 30 s, and the pellet was resuspended in RIPA supplemented as indicated for the extract preparation, followed by sonication and protein quantification using the BCA method.

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 4 ºC and blocked with 10 % BSA/PBS for 30 min at 37 ºC. Primary antibodies were diluted in 3 % BSA/PBS and

incubated overnight at 4 ºC. The coverslips were then washed twice with ice-cold PBS and incubated for 2 h at 37 ºC with the secondary antibodies (in PBS with 3 % BSA). The preparations were washed twice with ice-cold PBS before being mounted in fluorescence mounting medium (Dako). Fluorescence images of neurons were obtained with a Zeiss Axioplan fluorescence microscope with a 63x and 1.4 numerical aperture oil objective, and a Photometrics Sensys cooled CCD camera, using MetaVue imaging software (Molecular Devices) with customized filter sets. See Table 2 for the antibody list.

Table 2 - Primary and secondary antibodies used for Immunocytochemistry experiments.

Primary Antibodies	Dilution	Host	Supplier
MAP2	1:10 000	Chicken	Abcam
p-hnRNP K Ser284	1:400	Rabbit	Abcam
PSD95	1:200	Mouse	Pierce Antibodies (Thermo Scientific)

Secondary Antibodies	Dilution	Fluorophores	Supplier
Anti-Chicken	1:200	AMCA	Jackson Immunoresearch
Anti-Rabbit	1:500	Alexa568	Invitrogen
Anti-Mouse	1:500	Alexa488	Invitrogen

hnRNP Immunoprecipitation

Primary cultures of hippocampal neurons (85 500 cells/cm^2, 15 DIV) were prepared as described above, and incubated for 10 min in the presence or in the absence of 50 ng/mL BDNF in a humidified incubator with 5 % CO_2/95 % air at 37 ºC. Cellular

extracts were prepared using the RiboCluster Profiler RIP-Assay Kit (MBL International Corporation) and supplemented with 50 mM NaF, 1:200 Protease Inhibitor Cocktail Set III (Calbiochem®, Merck), 1 mM DTT and 80 U of RNase inhibitor (SUPERaseIn™, Ambion Applied Biosystems). Antibody-immobilized beads were prepared by incubating 6 µg of hnRNP or Mouse IgG antibodies with 100 µL of Protein G PLUS-Agarose beads (Santa Cruz Biotechnology), overnight at 4 ºC. The immobilized antibodies were incubated with 1 mg protein during 1 h at 4 ºC, and the beads were washed four times (2 min centrifugations, 2 000 x g) at 4 ºC with wash buffer, supplemented as the described for the lysis buffer. The final pellet, containing the immunoprecipitated hnRNP bound to the antibody-immobilized beads, was used for Western Blot analysis and RNA isolation (see Figure 10).

mRNA Isolation, Quality and Concentration analysis

After immunoprecipitation of hnRNPs with a specific antibody, the co-immunoprecipitating RNAs were immediately isolated using the RiboCluster Profiler RIP-Assay Kit and resuspended in 4 µL of RNase-free water (Gibco Invitrogen) (see Figure 10). In parallel experiments the non-specific binding of RNAs to Protein G PLUS-Agarose beads was determined (see above) and the RNAs were resuspended in the same volume of RNase-free water. 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 the same cultures, allowing the detection of degradation of the reference markers 18S and 28S rRNA. RNA concentration was determined using the NanoDrop 2 000 spectrophotometer (Thermo). The RNA samples were stored at -80ºC until further use.

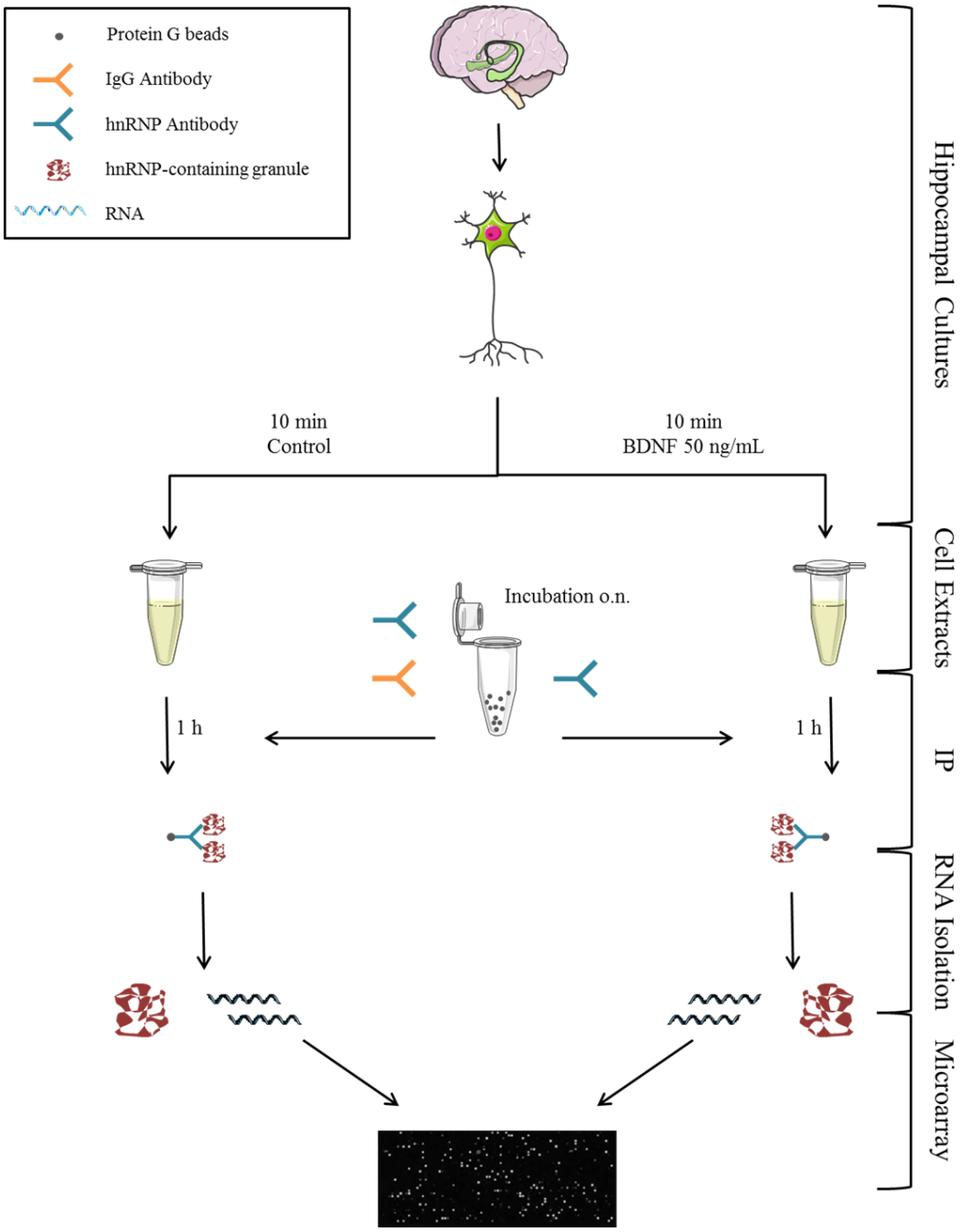

Figure 10 – Gene Expression Microarray workflow. Primary hippocampal neurons were stimulated or not with BDNF (50 ng/mL) for 10 min before preparation of cell extracts. Incubation of the anti-hnRNP antibody or IgG antibody with Protein G beads was performed overnight (o.n.). Homogenates were incubated for 1 h with the immobilized antibody-Protein G beads and the RNA was isolated from the resulting immunoprecipitates. RNA was subjected to microarray analysis. IP, immunoprecipitation.

Gene Expression Microarray

The RNA isolated from the hnRNP/IgG immunoprecipitates was subjected to microarray analysis (Figure 10) using the One-Color Microarray-Based Gene Expression Protocol v6.0. Low Input Quick Amp Labeling (Agilent Technologies) protocol was used for the preparation and labeling of the biological targets, hybridization, washing, scanning, and data analysis, as recommended by the manufacturer. The Whole Rat Genome Microarray Kit (4 x 44K, Agilent Technologies) was used and analyzed with a high-resolution microarray scanner (G2565AA, Agilent Technologies). Two different bioinformatics tools were used to analyze the results obtained: the GoMiner, which allowed the classification and assessment of the genes into biologically coherent categories (Zeeberg et al., 2003) and the MultiExperiment Viewer to compare the results from different samples (Saeed et al., 2003). Where indicated, the statistical analysis of the log-transformed expression data was performed by paired Student's *t*-test.

Reverse Transcription

For first strand cDNA synthesis 5 ng of isolated RNA was used mixed with 4 µL of 5x iScript Reaction Mix, 1 µL of Reverse Transcriptase and Nuclease-free water for 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 25 ºC for 5 min, followed by 30 min at 42 ºC for primer annealing to the template and cDNA synthesis, respectively. The reverse transcriptase was then denatured for 5 min at 85 ºC, and the sample cooled to 4 ºC, before storage at -20 ºC until further use.

Primer Design

Primers for real-time PCR were designed by 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 3 for the list of primers and corresponding sequences.

Table 3 - Primer sequences.

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
Camk2b	GCT ATA CGA GGA TAT TGG	TCT TGG TGT TAA TGA TCT
Clrn2	TTC TCT TCC TTC CTT CTG A	ACT CCA GTC TGA CAC AAG
Gria1	ACT ACA TCC TCG CCA ATC TG	AGT CAC TTG TCC TCC ATT GC
Gria2	TCT CTT CTA ACA GCA TAC A	AAA CTG AAC CAT CCC TAC
Grin1	CGG CTC TTG GAA GAT ACA G	GAG TGA AGT GGT CGT TGG
Hnrnpk	AAC ACT CAG ACA ACA ATC A	TCC TCC AAT AAG AAC AAC TC
Npas4	AAT GGA GAT ATT CAG GCT	TAG TTA TTG GCA GTA ATA GG
Ntrk2	GAT CTT CAC CTA CGG CAA GC	TCG CCA AGT TCT GAA GGA GT

Bdnf, brain-derived neurotrophic factor; Camk2b, calcium/calmodulin-dependent protein kinase II beta; Clrn2, clarin 2; Gria1, glutamate receptor ionotropic AMPA 1; Gria2, glutamate receptor ionotropic AMPA 2; Grin1, glutamate receptor ionotropic NMDA 1; Hnrnpk, heterogeneous nuclear ribonucleoprotein K; Npas4, neuronal PAS domain protein 4; Ntrk2, neurotrophic tyrosine kinase receptor type 2 (TrkB).

Real-Time PCR

For gene expression analysis, 2 µL of 1:4 diluted cDNA was 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 95 ºC during 3 min, followed by 45 cycles of a 10 s denaturation step at 95 ºC, a 30 s annealing step at 53 ºC, and a 30 s elongation step at 72 ºC. 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 55 ºC and with a rate of 0.5 ºC per 10 s, up to 95 ºC, 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 in account primer efficiency for each set of primers, provide a normalization to the amount of sample, to the technical repeats average and normalization 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

BDNF-induced phosphorylation of hnRNP K

The effect of BDNF on the phosphorylation of hnRNP K was investigated by western blot, using antibodies that interact specifically with the phosphorylated form of the protein. hnRNP K phosphorylation was assessed with antibodies against two different residues, which are targeted by kinases typically activated downstream of TrkB receptors: serine 302, a target of PKCδ, and serine 284, which is phosphorylated by ERK (Schullery et al., 1999; Habelhah et al., 2001; Sataranatarajan et al., 2008). The experiments were performed using cultured hippocampal neurons and synaptoneurosomes isolated from the adult rat hippocampus. The latter preparation is enriched in resealed presynaptic structures (equivalent to synaptosomes) with attached sealed postsynaptic entities (Troca-Marín et al., 2010) making it suitable for the investigation of postsynaptic mechanisms. In particular this preparation has been used to investigate local translation at the synapse in the hippocampus and cerebral cortex (Bagni et al., 2000; Schratt et al., 2004; Matsumoto et al., 2007; Di Nardo et al., 2007)), which plays an important role in synaptic plasticity (Steward and Schuman, 2001; Costa-Mattioli et al., 2009).

Hippocampal neurons were maintained in culture for 15 DIV in order to allow neuronal differentiation, and the cells were stimulated with 50 ng/mL of BDNF for 10, 20 and 30 min. This incubation period allows maximal activation of TrkB signaling mechanisms, as previously described in studies where the activity of ERK and Akt were investigated (Almeida et al., 2005). Stimulation of hippocampal neurons with BDNF for 10 min increased the phosphorylation of hnRNP K on Ser302 to 137.2 % ± 16.4 % of

the control, and similar results were obtained in cells incubated with the neurotrophin for 20 min or 30min (Figure 11). Since the hnRNP K total protein levels were unaltered during the exposure to BDNF, the observed increase in the phosphorylation status of hnRNP K arises from the changes in phosphorylation of the existing proteins by kinases and is not due to new protein synthesis.

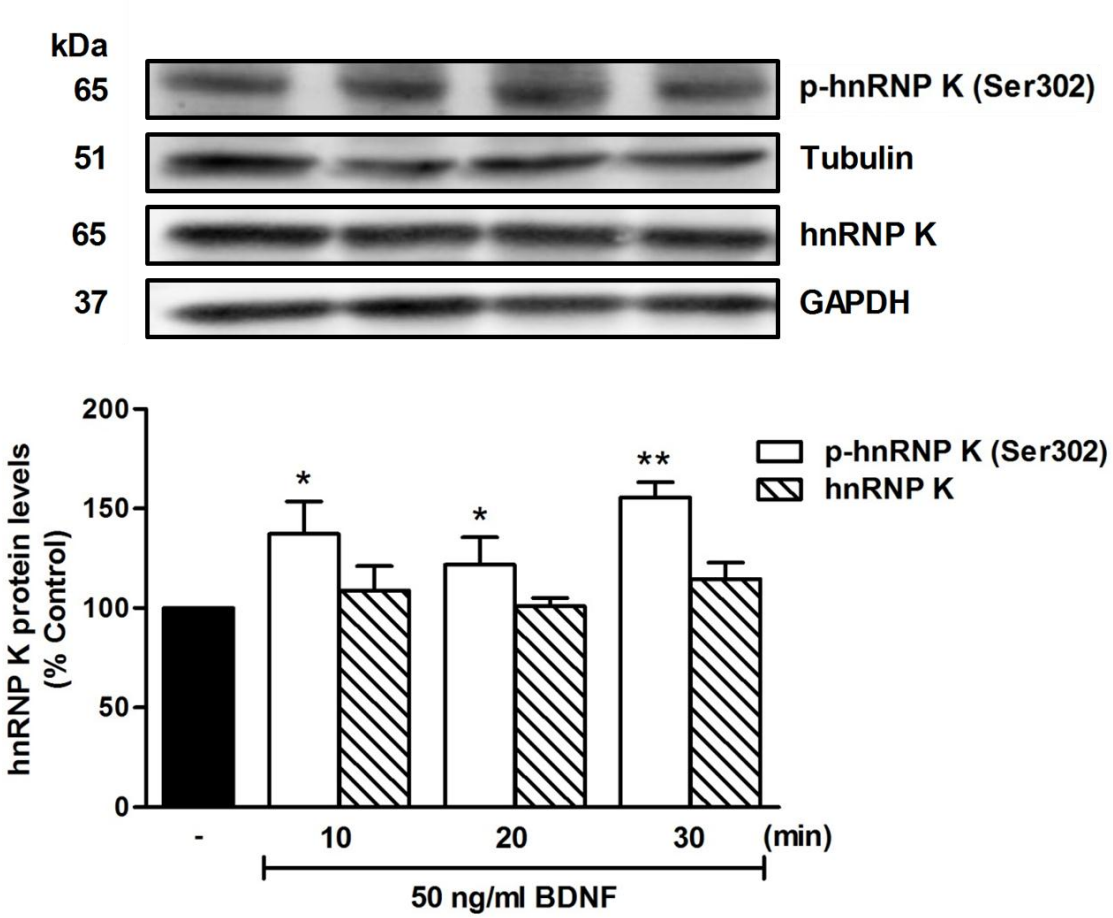

Figure 11 – Effect of BDNF on p-hnRNP K (Ser302) and hnRNP K protein levels. Primary hippocampal neurons were stimulated with 50 ng/mL of BDNF for 10, 20 and 30 min and the cellular extracts were analyzed by Western Blot. Tubulin and GAPDH were used as loading controls for p-hnRNP K (Ser302) and hnRNP K, respectively. The results represent quantification of three independent experiments, and are expressed as percentage (mean ± SEM) of control. * $p<0.05$, ** $p<0.01$ by ANOVA followed by Dunnett's Multiple Comparison Test.

To confirm that BDNF-induced phosphorylation on Ser302 was mediated by activation of TrkB receptors we tested the effect of K252a, a commonly used inhibitor of the Trk receptor tyrosine kinase activity. However, incubation of cultured hippocampal neurons with K252a (200 nM) for 15 min, in the absence of BDNF, increased p-hnRNP K (Ser302) immunoreactivity (Figure 12). This may be due to unspecific effects of K252a that have been reported, such as activation of both Akt and ERK (Roux et al., 2002) among other targets (Chin et al., 1999; Pan et al., 2007).

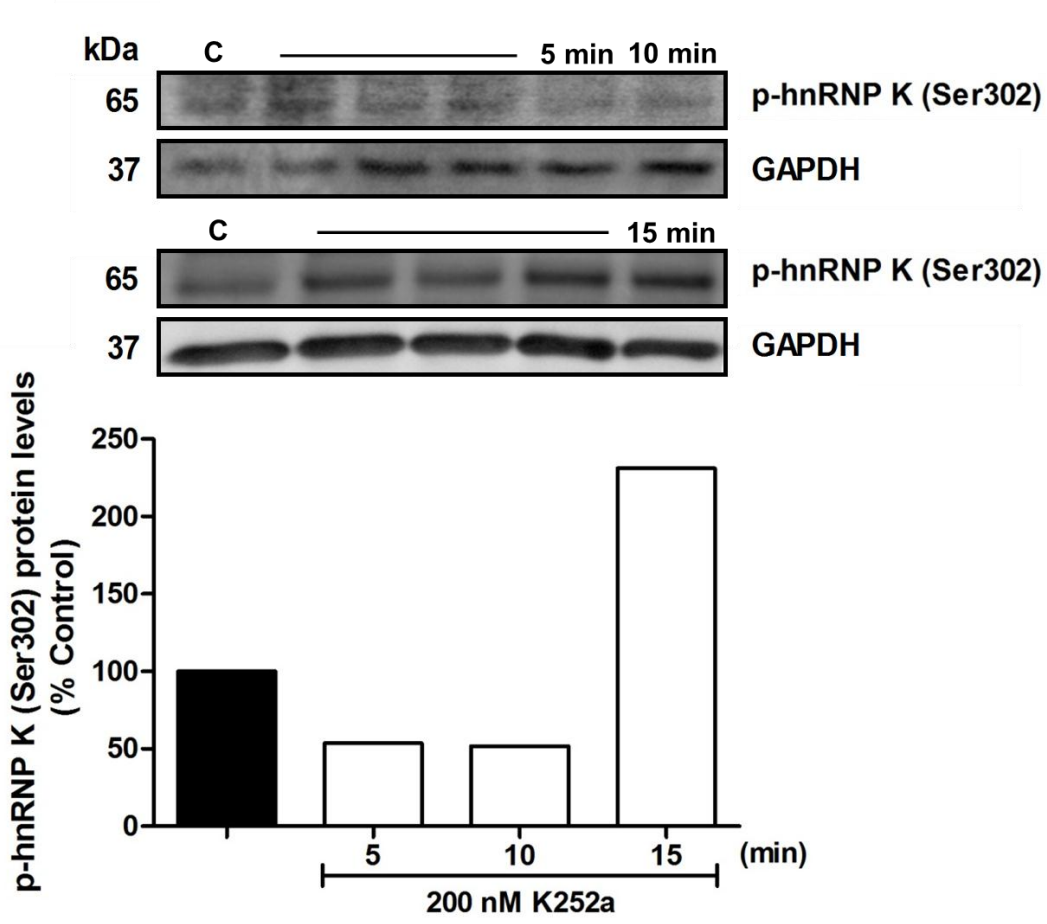

Figure 12 – Effect of K252a on p-hnRNP K Ser302 protein levels. Cultured hippocampal neurons were incubated with 200 nM K252a for 5, 10 or 15 min and the cellular extracts analyzed by Western Blot. GAPDH was used as loading control for p-hnRNP K Ser302. The results represent quantification of one experiment, expressed as percentage of control. C, control; 5, 10, 15 min of incubation with K252a. The remaining bands represent correspond to other experimental conditions not relevant for this study.

To determine the effect of BDNF on the phosphorylation of hnRNP K at the synapse, similar experiments were performed using synaptoneurosomes. The presence of hnRNP K in synaptoneurosomes isolated from cultured cerebrocortical neurons was previously reported (Liao et al., 2007). Surprisingly, although the western blot experiments with an anti-hnRNP K antibody showed a band with an apparent molecular weight of about 65 kDa (Figure 13A), this was not observed when the anti-p-hnRNP K (Ser302) antibody was used (Figure 13B). The phosphospecific antibody also reacted with a protein with an apparent molecular weight of 50 kDa, similarly to the results obtained with the antibody against total hnRNP K, suggesting that this may correspond to an isoform of the protein that is also phosphorylated on the same site (see above). In contrast, the band corresponding to an apparent molecular weight of 70 kDa, observed in the western blot experiments with the p-hnRNP K antibody may be non-specific since no equivalent labeling was observed with the antibody that binds to the total hnRNP K protein.

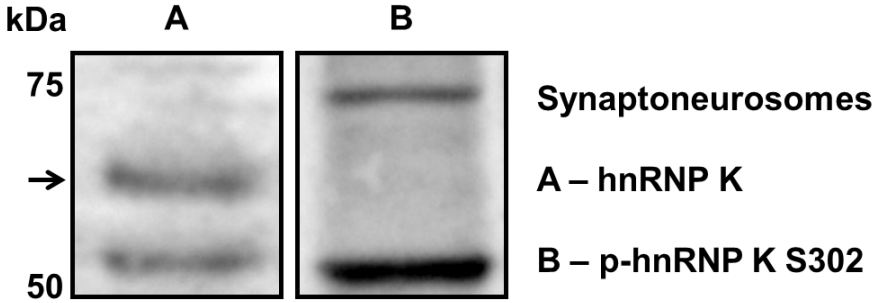

Figure 13 – Western Blot profile for hnRNP K (A) and p-hnRNP K Ser302 (B) proteins in extracts prepared from rat hippocampal synaptoneurosomes. The arrow points to a 65 kDa band corresponding to hnRNP K.

In contrast with the results obtained with the p-hnRNP K (Ser302) antibody, which reacted with a protein with an apparent molecular weight of 65 kDa in extracts

from cultured hippocampal neurons (similarly to the results obtained with the antibody against the total protein), the antibody raised against p-hnRNP K (Ser284) is reported by the manufacturer (Abcam) to react with a protein with a molecular weight of 51 kDa. However, no immunoreactivity was observed in western blot experiments using cultured rat hippocampal neurons stimulated with 50 ng/mL BDNF (Figure 14B). As a positive control we confirmed that the p-hnRNP K (Ser284) antibody reacted with a protein of about 51kDa in extracts from the human embryonic kidney HEK293 cells (Figure 14A).

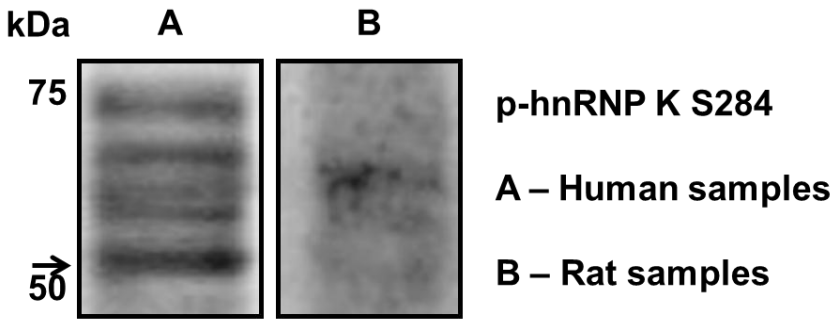

Figure 14 – Western Blot profile for p-hnRNP K (Ser284) protein with cellular extracts from the human embryonic kidney HEK293 cells (A) and cultured rat hippocampal neurons (B). The arrow points to a band with approximately 51 kDa corresponding to p-hnRNP K (Ser284).

According to the information provided by the manufacturer, the antibody reacts with p-hnRNP K (Ser284) from *Homo sapiens* (human) and *Mus musculus* (mouse) samples. As rat and mouse are genetically related, we compared the amino acid sequences in the three species and no differences were found (not shown). Therefore, the lack of immunoreactivity observed in extracts from cultured hippocampal neurons may be due to differences in the expression of hnRNP K between HEK293 cells and cultured hippocampal neurons.

Immunocytochemistry experiments were performed to determine the localization of p-hnRNP K (Ser284) in cultured hippocampal neurons. Co-localization with MAP2 was used to assess the dendritic localization of the protein and PSD95 was used as a marker of excitatory synapses (Figure 15A).

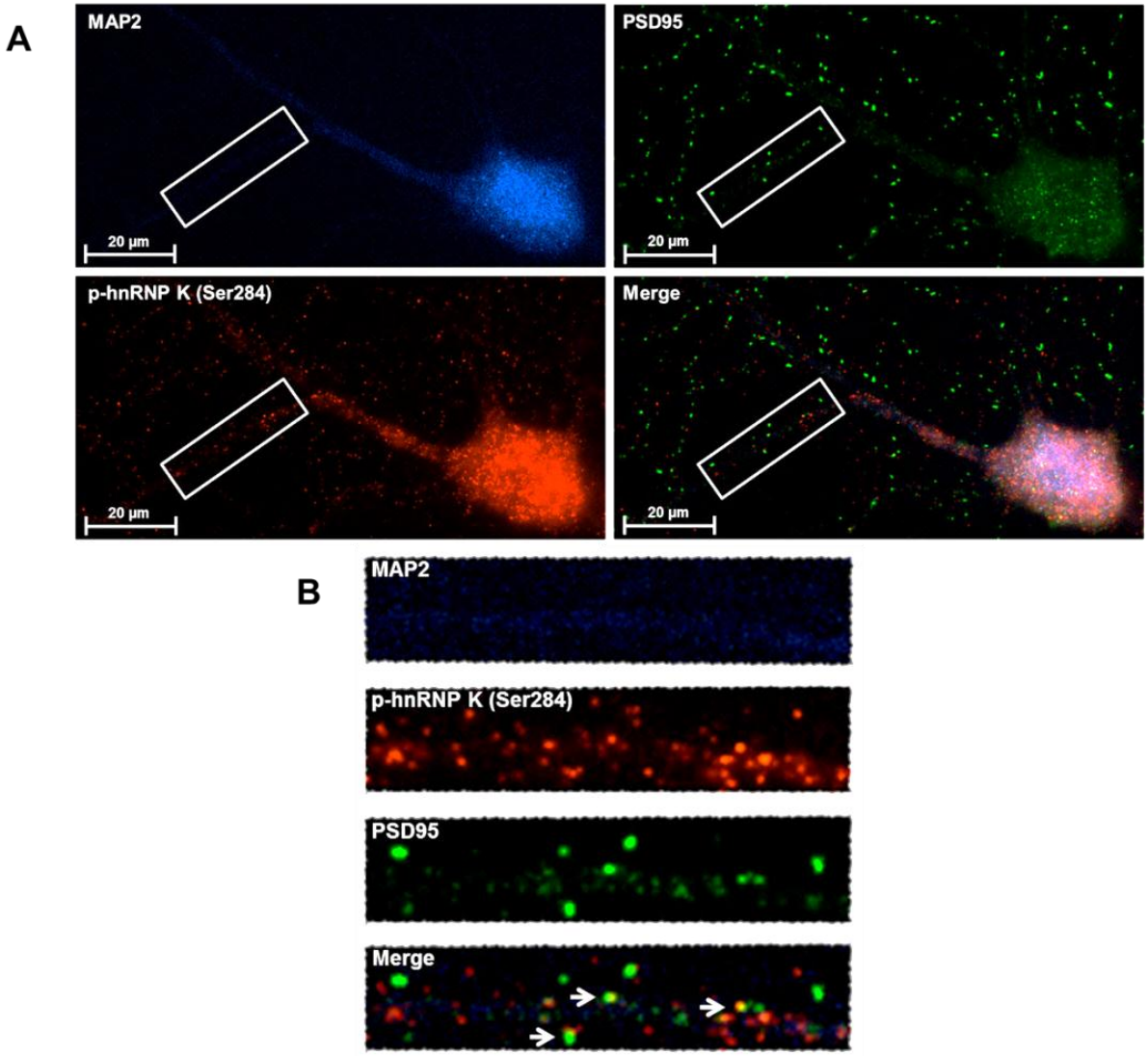

Figure 15 – Distribution of p-hnRNP K (Ser284) in cultured hippocampal neurons. Immunostaining for MAP2 (blue), PSD95 (green) and p-hnRNP K (Ser284) (red) was performed in 15 DIV neurons. (A) Cellular distribution of p-hnRNP K (Ser284) in rat hippocampal neurons. (B) The inset shows a high magnification of the boxed area shown in (A), and the arrows on the merged picture indicate co-localization of p-hnRNP K (Ser284) and PSD95.

The results show that in addition to the soma, p-hnRNP K (Ser284) is also present in dendrites. Furthermore, the partial co-localization with PSD95 shows the presence of p-hnRNP K (Ser284) at excitatory synapses (arrows in Figure 15B, bottom panel). In contrast, although the antibody against the p-hnRNP K (Ser302) was reported to be suitable for detection of the protein by immunofluorescence, we could not obtain reliable results with this antibody (not shown).

Identification of the RNA transcripts associated with selected hnRNPs

The RNA transcripts that interact with hnRNPs can be identified using the RIP (RNP immunoprecipitation) assay.

The first step consists in an IP (immunoprecipitation) of the targeted hnRNPs in order to isolate this protein from the starting sample. In this study we immunoprecipitated the hnRNP K and A2/B1 proteins. The latter is present in RNA granules and is responsible for mRNA trafficking in dendrites (Shan et al., 2003). hnRNP K is present at the synapse (Liao et al., 2007), but its subcellular localization has not been investigated in detail.

From the technical point of view the main difficulties in the identification of the transcripts associated with hnRNPs lie in the low amount of RNA co-immunoprecipitated with the proteins and the stability of the transcripts during the immunoprecipitation protocol. In preliminary experiments we compared the stability of the RNAs present in total homogenates of hippocampal neurons incubated at room temperature for 1 h or 3 h, to evaluate two different hnRNP immunoprecipitation protocols. After RNA isolation, an automated electrophoresis was performed to obtain information regarding the nucleic acid sizing and quantification, and to conclude about

degradation of the reference markers rRNA 18S and 28S. Observation of two sharp bands, corresponding to the large and small subunit rRNAs, with the intensity of the larger band being about twice that of the smaller band, is indicative of intact RNA. The results of Figure 16B show that 1 h incubation of hippocampal homogenates at 4 ºC does not induce a significant RNA degradation, since a two clear peaks corresponding to the rRNA 18S and 28S were observed. In contrast, homogenates incubated for 3 h at 4 ºC show a significant degradation of the RNA as observed by the loss of the reference markers rRNA 18S and 28S and increase in the quantity of low size RNA as observed by the predominant signal near the beginning of the electropherogram (Figure 16A).

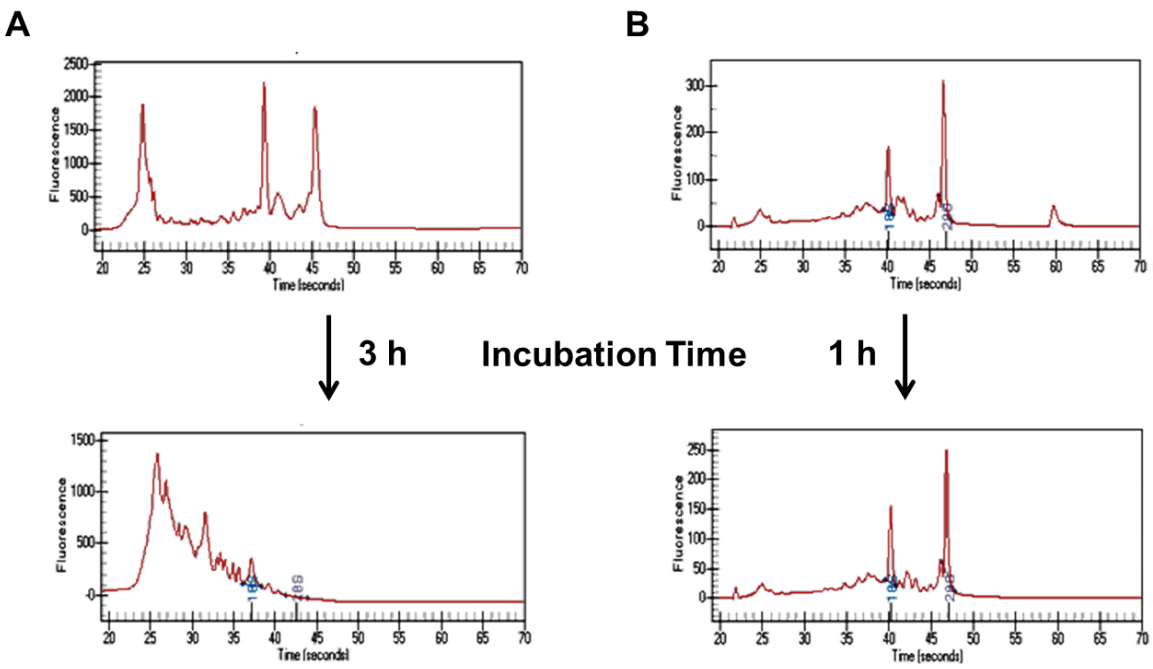

Figure 16 – Electropherogram of RNA samples isolated from cultured hippocampal neuron homogenates using two different incubation times. (A) 3 h of incubation caused a shift in the RNA electropherogram towards the left and loss of the reference markers 18S and 28S rRNA, corresponding to a higher degradation profile. (B) 1 h of incubation did not induce a significant change in the RNA profile of the homogenate samples.

Based on the results obtained in the preliminary experiments concerning the RNA stability, the RIP 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 the immunoprecipitation of hnRNP K and hnRNP A2/B1 as shown in Figure 17A, although the efficiency of protein precipitation was higher for hnRNP K since no protein was detected in the flow-through in this case. Control experiments using a mouse IgG antibody showed no hnRNP immunoprecipitation confirming the specificity of the method. Significant amounts of RNA were co-immunoprecipitated with hnRNP K but much lower amounts were present in the hnRNP A2/B1 immunoprecipitates Figure 17B. The latter results may be partially due to the lower efficacy in the precipitation of hnRNP A2/B1 with the antibody used. Due to the low amount of RNA isolated from hnRNP A2/B1 immunoprecipitation, the following studies to identify RNA transcripts associated with hnRNPs targeted the hnRNP K protein.

Identification of the transcripts associated with hnRNP K was performed using DNA microarray, in collaboration with the RNA Biology laboratory from the Aveiro University. The One-Color Microarray-Based Gene Expression Analysis Protocol was used, based on a Low Input Quick Amplification Labeling from Agilent Technologies. Briefly, the RNA samples are converted in cDNA which in turn is converted and amplified in cRNA that is labeled with the Cyanide 3-CTP fluorophore. A purification step was performed 17 h before hybridization with the microarray slides. The slides were washed to remove the unbound cRNA, scanned and the data extracted. Each microarray slide used is representative of the whole rat genome and contains over 41000 rat genes and transcripts that are highly represented in leading public databases, including RefSeq, Ensembl, UCSC Goldenpath, and Unigene. We performed a total of

10 microarrays: 8 with the RNA isolated from the hnRNP K immunoprecipitates (4 control samples and 4 BDNF-stimulated samples) and 2 with the RNA isolated from the IgG immunoprecipitates.

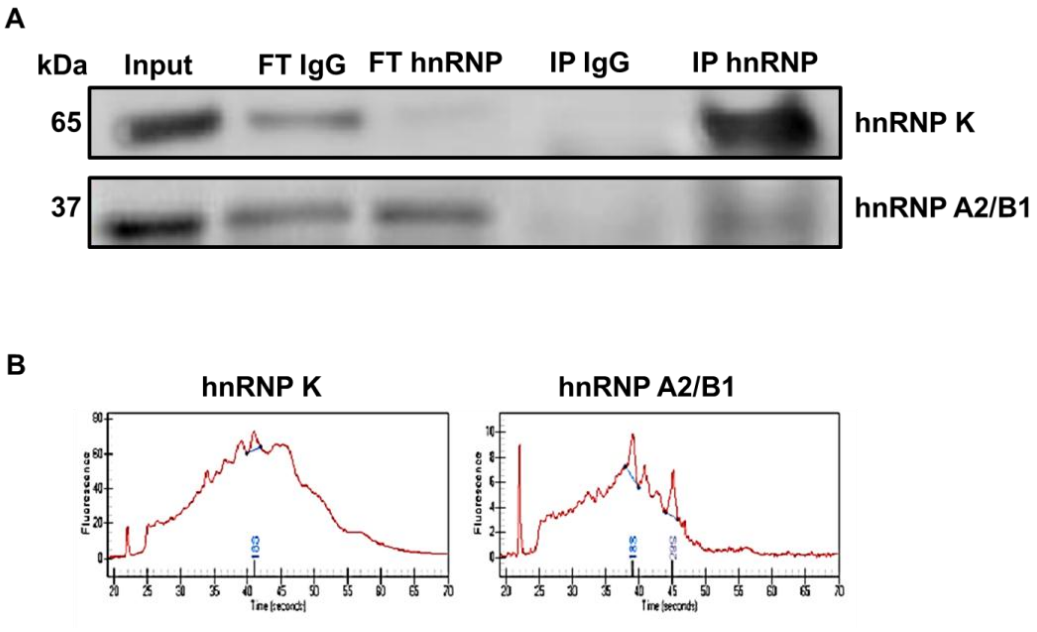

Figure 17 – hnRNPs immunoprecipitation and RNA profile of the immunoprecipitates. (A) Western Blot profile for hnRNP K and hnRNP A2/B1 after immunoprecipitation with the corresponding antibodies. Where indicated the homogenates were incubated with protein G beads coupled to mouse an IgG antibody (IgG). FT, flow-through; IP, immunoprecipitate. (B) Electropherogram obtained with the RNA isolated from hnRNPs immunoprecipitates.

Analysis of the RNA transcripts specifically associated with hnRNP K was performed by subtracting the results obtained with the mouse IgG antibody from those obtained following immunoprecipitation of hnRNP K using non-stimulated hippocampal neurons. The results obtained with the beads coupled to mouse IgG allow controlling for the non-specific binding of RNA to the antibody and/or to the Protein G beads during the IP. In fact, since the IgG antibody is structurally similar to the hnRNP K antibody, except for the antigen-binding site, the transcripts that bind non-specifically

to one of the antibodies should also bind to the other. The identification of the mRNAs that co-IP with hnRNP K may be performed using two different strategies: i) by subtracting the values from the non-specific binding (negative controls); or ii) by setting a cutoff value of the fold variation between the control and the IgG samples. In the analysis of the results obtained the latter strategy was used since it provides a more stringent method for identification of transcripts co-immunoprecipitating with hnRNP K. With a cutoff value of 8, i.e. those transcripts showing at least 8 fold variation when their abundance in the hnRNP K IP was compared with the IgG controls, a total of 11422 transcripts were identified. From this massive list, the transcript that was found to be more enriched in the hnRNP K IP (LOC290876 gene) exhibited a fold variation of 256, indicating that the transcripts found fall between 8 and 256 fold change values (Supplementary Table 1, Appendix).

The transcripts co-immunoprecipitated with hnRNP K found with the Microarray experiments were analyzed using the GoMiner tool that uses the GO (Gene Ontology) algorithm. Rather than analyzing the microarray results with a gene-by-gene approach, this strategy provides a rigorous and exhaustive organization of the genes according to three different categories: biological processes, molecular function and cellular component. Analysis of the 11422 transcripts that co-immunoprecipitated with hnRNP K according to their role in biological processes (Figure 18A), shows that 52.8% are related to cellular (about 23 %), metabolic /about 17 %) and biological regulation (about 13 %), suggesting a key role of hnRNP K in the functional regulation of the cells.

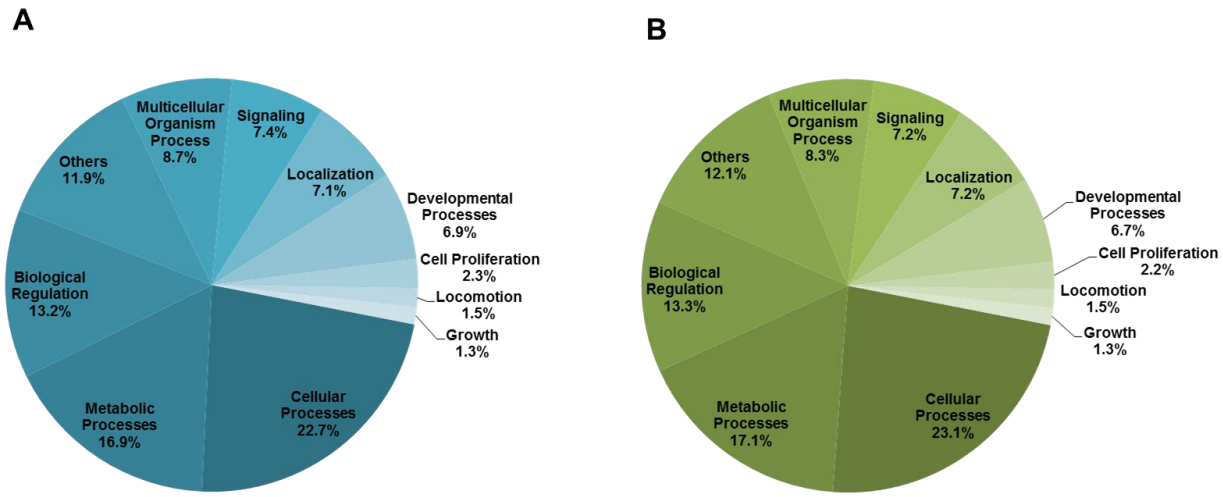

Figure 18 – Diagrams presenting the biological processes associated with the mRNAs co-immunoprecipitated with hnRNP K and the effect of BDNF on transcript binding, as determined by Gene Ontology. (A) Biological processes attributed to the 11422 transcripts that co-immunoprecipitate with hnRNP K under resting conditions and showed at least 8 fold variation when their abundance in the hnRNP K IP was compared with the IgG. (B) Biological processes attributed to the 10782 transcripts that co-immunoprecipitate with hnRNP K and were regulated by BDNF.

When the hnRNP K immunoprecipitated transcripts were analyzed according to molecular function, from the 13 categories considered, 70.6 % of the mRNAs were grouped in 2 classes, binding (about 46 %) and catalytic (about 25 %) activity functions (Figure 19A). A significant number of transcripts identified (about 8 %) code for proteins related with signaling activity.

Finally, the transcripts identified in hnRNP K immunoprecipitates were classified based in 'Cellular Component', as determined by Gene Ontology. The mRNAs identified code for proteins belonging to different cellular compartments, including cytoplasm (38 %), nucleus (22 %), organelles (21 %), plasma membrane (14 %) and cytoskeleton (5 %) (Figure 20A, upper diagram).

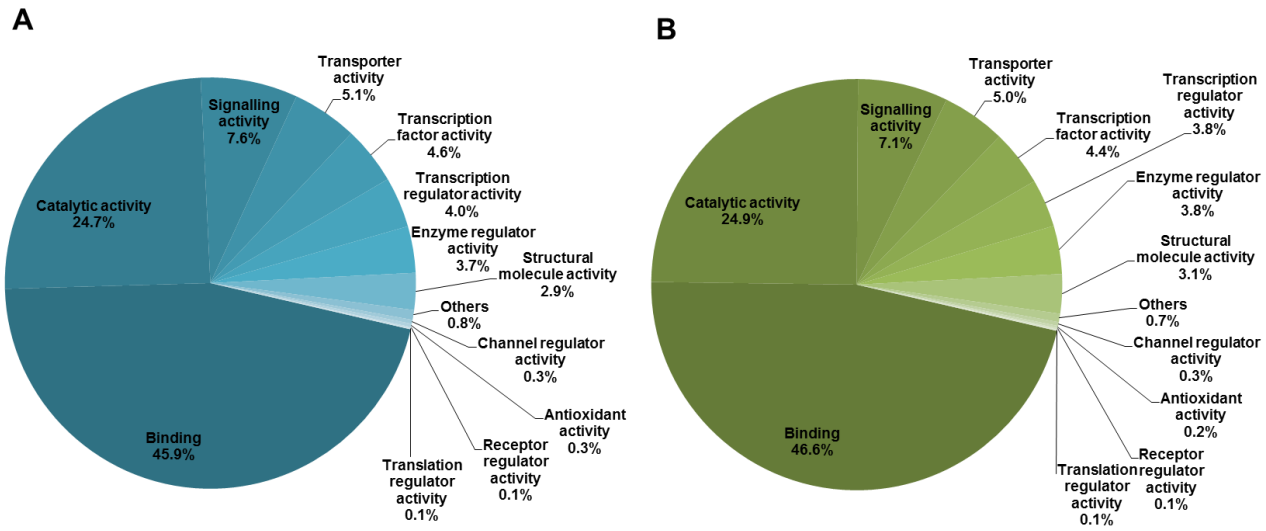

Figure 19 – Diagrams presenting molecular functions associated with the mRNAs co-immunoprecipitated with hnRNP K and the effect of BDNF on transcript binding, as determined by Gene Ontology. (A) Molecular functions attributed to the 11422 transcripts that co-immunoprecipitated with hnRNP K and showed at least 8 fold variation when their abundance in the hnRNP K IP was compared with the IgG. (B) Molecular functions attributed to the 10782 transcripts that co-immunoprecipitated with hnRNP K and were regulated by BDNF

Given the putative role of hnRNP K in synaptic regulation we also analyzed the co-immunoprecipitated transcripts related with the synapse environment. A total of 445 entries were found to be related with the synapse but the number of transcripts related to this structure is lower since some of the transcripts are associated with more than one sub-category (e.g. GluA1 mRNA, coding for subunit 1 of AMPA receptors, is present both in postsynaptic membrane and the postsynaptic density; Cadps2 mRNA, coding Ca^{2+}-dependent secretion activator 2 protein, is present both in postsynaptic membrane and in synaptic vesicles). Based on this classification, 67 % of the transcripts identified are associated with postsynaptic membrane/postsynaptic density/dendritic spines, suggesting that hnRNP K plays an important regulatory role at the postsynaptic level

(Figure 20A, lower diagram). Interestingly, a significant fraction of the transcripts identified (21 %) code for proteins of the synaptic vesicles.

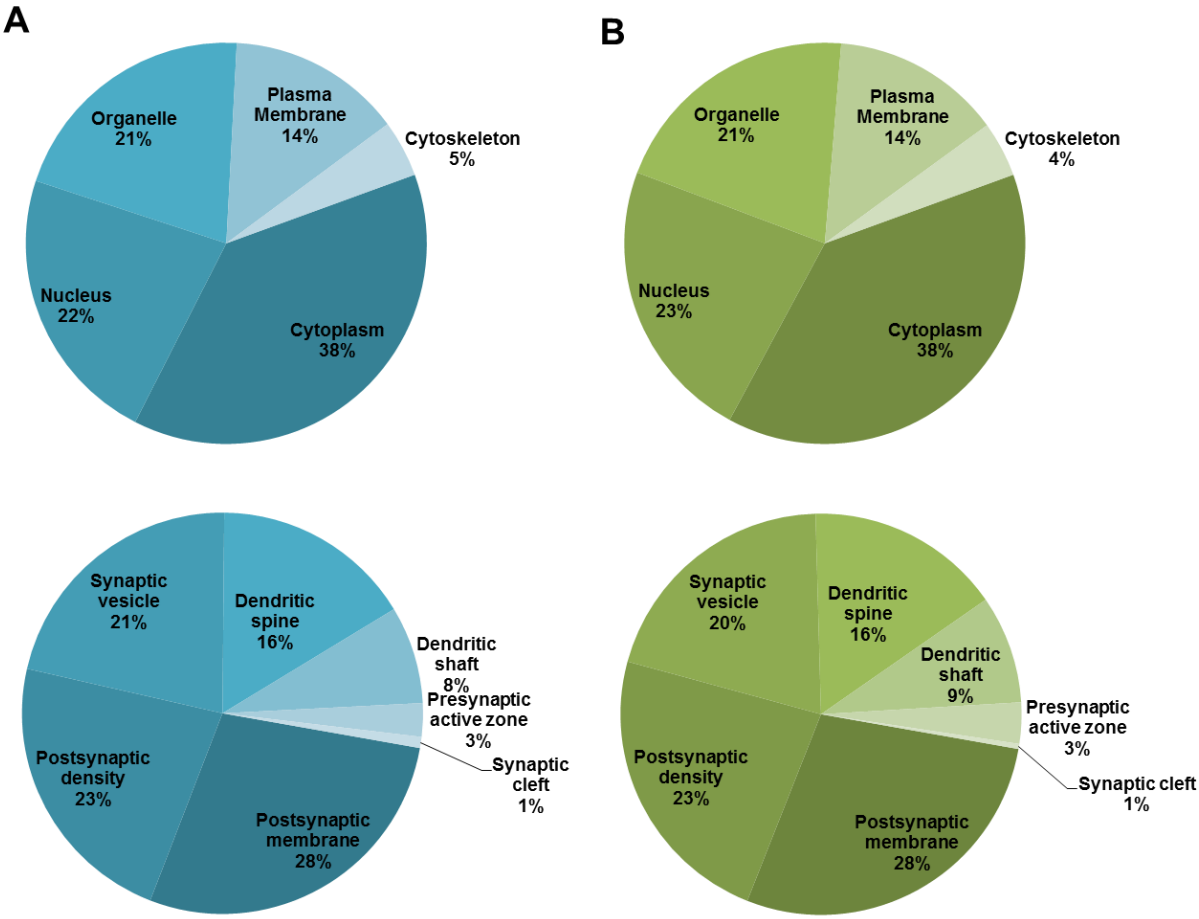

Figure 20 – Diagrams presenting the distribution between cellular components of the proteins coded by the transcripts that co-immunoprecipitated with hnRNP K and the effect of BDNF on transcript binding, as determined by Gene Ontology. In the upper diagrams the transcripts are grouped based on cell part distribution, and the lower panels show the distribution among synaptic components. (A) Distribution between cellular components of the 11422 transcripts that co-immunoprecipitate with hnRNP K under resting conditions and show at least 8 fold variation when their abundance in the hnRNP K IP was compared with the IgG. (B) Distribution between cellular components of the 10782 transcripts that co-immunoprecipitated with hnRNP K and are regulated by BDNF.

Effect of BDNF on the mRNAs co-immunoprecipitated with hnRNP K

A similar microarray analysis was performed to determine the effect of BDNF stimulation on the mRNAs that co-IP with hnRNP K, using homogenates from control cells and hippocampal neurons incubated with the neurotrophin (50 ng/mL) for 10 min. In this analysis we subtracted in both cases the results obtained in extracts incubated with mouse IgG, assuming that the non-specific binding of transcripts to the antibody and the Protein G beads is similar in extracts prepared from control hippocampal neurons and in cells stimulated with BDNF. From the list of 11422 mRNAs that were specifically pulled-down together with hnRNP K, 51 % (5833 transcripts) showed a significant change in the interaction with the RNP ($p<0.05$) (Figure 21). This shows that BDNF stimulation targets specifically a subpopulation of transcripts, rather than having a global effect.

In a separate analysis we calculated the ratio between the abundance of each given transcript in hnRNP K IPs from control and BDNF-stimulated hippocampal neurons, for the entire dataset, to calculate the fold variation in RNA-RBP binding induced by the neurotrophin. A paired Student's *t*-test analysis was performed, using a p value of 0.05, and the results showed that 10782 transcripts were affected by BDNF stimulation (Figure 22). Remarkably, 10773 (99.92 %) transcripts were negatively regulated by BDNF and only 9 (0.08 %) of the mRNAs showed an increase in binding (Supplementary Table 2, Appendix). This clearly shows a massive effect of BDNF on the dissociation of mRNAs from hnRNP K and/or hnRNP K-associated proteins. The total number of transcripts found to be regulated by BDNF was higher in this case than in the analysis shown in Figure 21 because of the stringent criteria used in the latter case, which may have reduced the dataset of transcripts considered to specifically co-IP with hnRNP K.

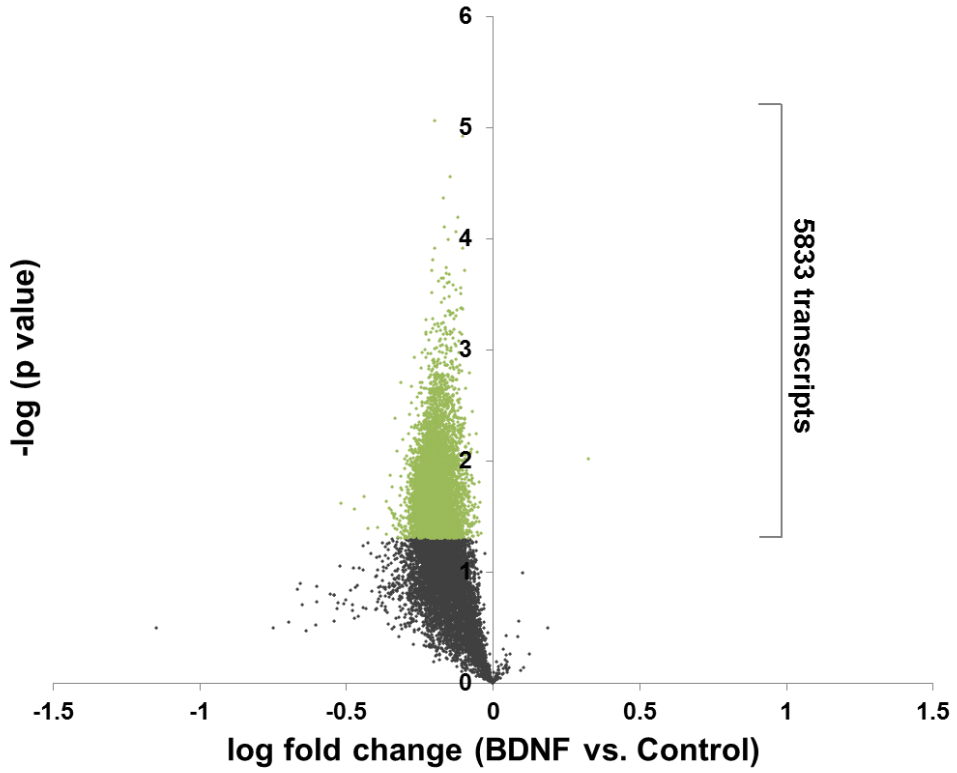

Figure 21 – Effect of BDNF on the co-immunoprecipitation of mRNAs with hnRNP K in cultured hippocampal neurons: characterization of the percentage of transcripts regulated by BDNF. The results were obtained from the quantitation of four different experiments performed in independent preparations, and are expressed as -log (p value) and log fold change (BDNF vs. Control). From the list of 11422 mRNAs that were co-immunoprecipitated with hnRNP K, only 5833 were significantly regulated by BDNF; $p<0.05$ (green dots) as determined by the paired Student's *t*-test.

In order to validate some of the results obtained we conducted several qPCRs (quantitative PCRs) with 9 selected genes, including 3 genes coding for proteins relevant for the present study (BDNF, TrkB and hnRNP K), 4 additional genes coding for proteins with synaptic functions (GluA1 and GluA2 [AMPA receptor subunits]; GluN1 [NMDA receptor subunit]; and CaMKII β [protein kinase highly expressed at the synapse playing an important role in synaptic plasticity]), one transcript showing

minor changes in response to BDNF stimulation (Clarin2), and a mRNA that showed a very robust increase (over 100 %) (NPAS4).

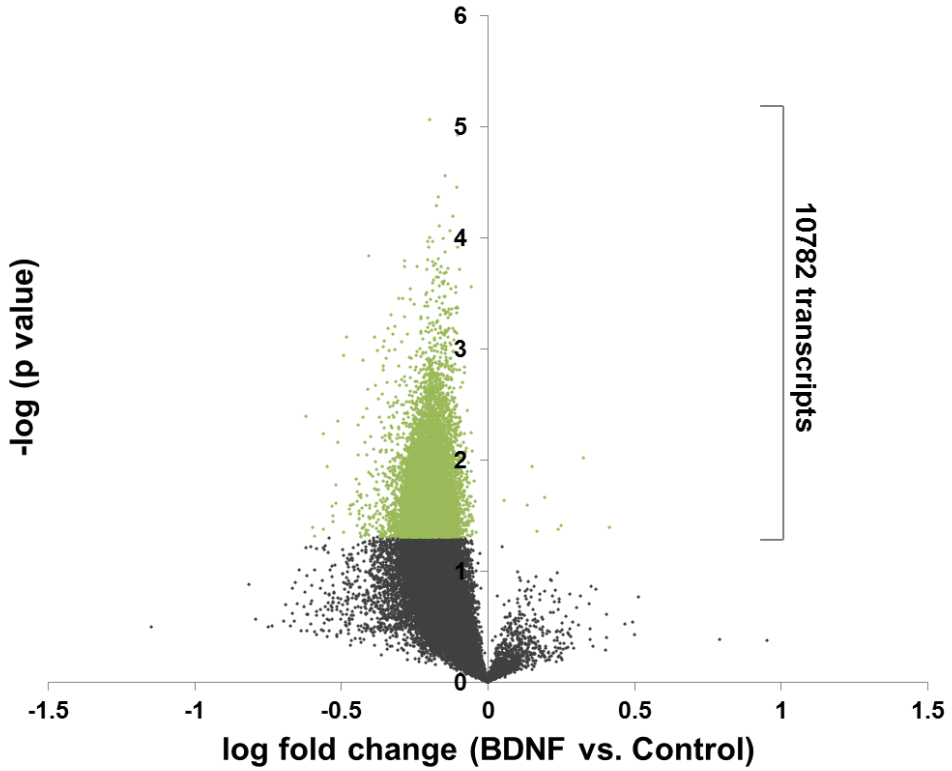

Figure 22 – Effect of BDNF on the co-immunoprecipitation of mRNAs with hnRNP K in cultured hippocampal neurons: characterization of transcripts binding vs. dissociation. The results were obtained from the quantitation of four different experiments performed in independent preparations, and are expressed as -log (p value) and log fold change (BDNF vs. Control). From the total list of mRNAs analyzed in the microarray, 10782 transcripts were found to be significantly regulated by BDNF; $p<0.05$ (green dots) as determined by the paired Student's *t*-test.

The results of the qPCR experiments were, in general, consistent with those obtained using the microarrays, showing a decrease in the amount of transcripts for GluA1, GluA2, GluN1, BDNF, TrkB, hnRNP K and CaMKII β that co-immunoprecipitated with hnRNP K in extracts from hippocampal neurons stimulated with BDNF. Furthermore, no significant effect was observed on the amount of Clarin2

mRNA immunoprecipitated with hnRNP K, in agreement with the minor effect observed in the microarray experiments. The only result obtained in the microarray experiments that could not be validated by qPCR was the increase in the NPAS4 transcripts immunoprecipitated with hnRNP K in cells stimulated with BDNF. This suggests that the increased interactions with hnRNP K observed for 0.08 % of the genes analyzed in cells stimulated observed by BDNF may not be real (Figure 23).

The 10782 transcripts that were found to co-immunoprecipitate with hnRNP K by a mechanism sensitive to BDNF-stimulation were also analyzed and distributed in three categories as performed for the total population of transcripts (see previous section): biological processes, molecular function and cellular component (Figures 18B, 19B and 20B). The comparison of the total population of transcripts that co-immunoprecipitate with hnRNP K and those that are affected by stimulation of hippocampal neurons with BDNF is relevant since it indicates whether stimulation with the neurotrophin acts on a particular group of mRNAs with a predominant molecular function, biological processes or cellular localization. However, comparison of the results obtained for the two different analyses (Figures 18, 19 and 20) shows no significant differences indicating that the effects of BDNF on transcript binding are wide-ranging, acting globally on the mRNAs that co-IP with hnRNP K.

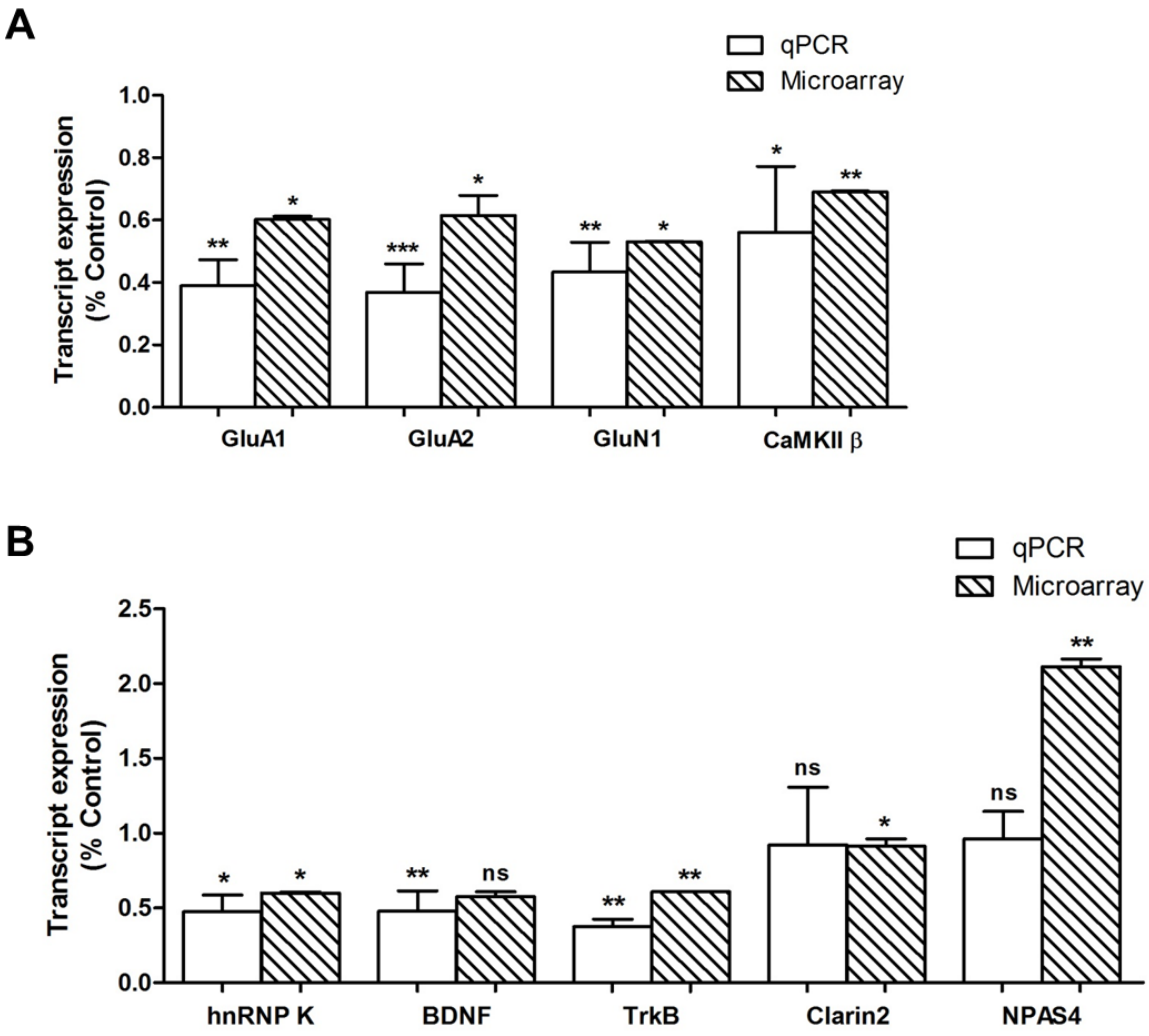

Figure 23 – Effect of BDNF stimulation on transcript co-immunoprecipitation with hnRNP K in cultured hippocampal neurons. (A) Genes coding for synaptic proteins: GluA1, AMPA receptor subunit 1; GluA2, AMPA receptor subunit 2; GluN1, NMDA receptor subunit 1; CaMKII β, calcium/calmodulin-dependent protein kinase II β. (B) Other genes: hnRNP K, heterogeneous nuclear RiboNucleoProtein K; BDNF, brain-derived neurotrophic factor; TrkB, Tropomyosin-related kinase B receptor; Clarin2, clarin 2; NPAS4, Neuronal PAS Domain Protein 4. The results represent quantitation of four different experiments performed in independent preparations, and are expressed as percent (mean ± SEM (qPCR) or SD (Microarray)) of control. *$p<0.05$, **$p<0.01$, ***$p<0.001$ as determined by ANOVA followed by Dunnett's Multiple Comparison Test (qPCR) or using the paired Student's *t*-test (Microarray).

CHAPTER 5 – DISCUSSION

In this work with identified a large number of mRNAs, belonging to different categories, including transcripts coding for synaptic proteins, in hnRNP K immunoprecipitates from cultured hippocampal neurons. Furthermore, stimulation with BDNF induced a massive dissociation of a subpopulation of transcripts that co-IP with hnRNP K, and this effect was correlated with the phosphorylation of the protein on Ser302. These results point to a key role of BDNF in hnRNP K regulation.

Under resting conditions we identified 11442 transcripts present in the hnRNP K immunoprecipitates, coding for proteins involved in different biological processes, and with distinct molecular functions and cellular localization, as described with the Gene Ontology. In a recent study a lower amount of transcripts (753) were identified after immunoprecipitation of hnRNP K from the juvenile froglet brain, although the similar functional categories were represented (Liu et al. 2011). In addition to the KH domains responsible for binding mRNAs, hnRNP K also possesses a KI region that is responsible for the interaction and binding to other proteins (Bomsztyk et al., 2004). Since some of these binding partners may also interact with specific mRNAs, it is not possible to determine whether each of the mRNAs that co-IP with hnRNP K bind directly or indirectly to the RNP. EMSA (electrophoretic mobility shift assay) studies are necessary in order to evaluate which mRNAs bind directly to hnRNP K, since this procedure can determine if a protein is capable of binding to a given DNA or RNA sequence.

hnRNP K has numerous putative phosphorylation sites, some of them located in the KH domains responsible for RNA and DNA binding (Bomsztyk et al., 2004; Mikula and Ostrowski, 2010). Since it was also described that hnRNP K phosphorylation influence the binding to mRNA (Ostrowski et al., 2000, 2001; Habelhah et al., 2001; Ostareck-Lederer et al., 2002; Feliers et al., 2007), we conducted microarray experiments to identify the transcripts associated with hnRNP K in cultured hippocampal neurons and to evaluate how stimulation with BDNF might affect the interactions of the RNP with the mRNAs. From the list of mRNAs that were specifically pulled-down together with hnRNP K in extracts prepared from hippocampal neurons under resting conditions, only 51 % showed a significant change in the interaction with the RNP following stimulation with BDNF. This shows that the signaling mechanisms activated by the neurotrophin target a specific subpopulation of transcripts, rather than having a global effect.

In a separate analysis we focused on the population of transcripts that co-immunoprecipitated with hnRNP K and were sensitive to BDNF, and we found that the large majority (99.9 %) of these mRNAs were partially released within 10 min of stimulation with the neurotrophin. Moreover, since we could not validate the observed increase in the binding of NPAS4 mRNA in immunoprecipitates from BDNF-stimulated hippocampal neurons, the neurotrophin may specifically induce the dissociation of transcripts from hnRNP K containing particles. Our results also showed that the effect of BDNF on mRNA interaction with hnRNP K is only partial, and the maximal reduction observed was by 76 % (Aldob mRNA, fructose-bisphosphate aldolase B protein). This suggests that the effects of BDNF on hnRNP K may target various RNP structures, with different compositions and/or distinct locations in the cell. Additional studies should be performed in synaptoneurosomes to determine whether

BDNF has similar effects at the synapse. Furthermore, studies aimed at characterizing the RNA granules containing hnRNP K may help to better understand the interaction partners that might bind to mRNA and their regulation.

Stimulation of hippocampal neurons with BDNF increased phosphorylation of hnRNP K on Ser302, an amino acid residue located in the KI region responsible for the interaction and binding to other proteins (Bomsztyk et al., 2004). Phosphorylation of this or other residues may therefore affect the interaction of hnRNP K with other ribonucleoproteins thereby contributing to the observed decrease in the number of transcripts co-immunoprecipitated. In vitro experiments showed that Ser302 is a major hnRNP K phosphorylation site by PKCδ (Schullery et al., 1999), and this is in line with the fact that the kinase is activated in the signaling cascade triggered by TrkB receptors (Reichardt, 2006).

BDNF activates Fyn (Narisawa-Saito et al., 1999), a Src-family protein tyrosine kinase that phosphorylates hnRNP K in both the KI region and the KH1 domain (Bomsztyk et al. 2004), and this may also contribute to decrease mRNA binding. Furthermore, stimulation of TrkB receptors by BDNF is coupled to the activation of ERK, a protein kinase that phosphorylates hnRNP K Ser284 and induces a cytoplasmic accumulation of the protein. Phosphorylation of hnRNP K on Ser284 also inhibits mRNA translation (Habelhah et al., 2001), which suggests a pleiotropic effect for the phosphorylation of hnRNP K. Whether ERK activation contributes to the trafficking of hnRNP K from the nucleus to the cytoplasm in hippocampal neurons stimulated with BDNF remains to be investigated.

Similarly to the intracellular signaling induced by BDNF and TrkB receptors, insulin receptors are coupled to stimulation of the ERK and PI3K pathways (Kim et al.

1997; Saltiel 1996) and to the activation of transcription and translation (Messina, 1991; Proud and Denton, 1997; Takei et al., 2001). Insulin was shown to induce tyrosine phosphorylation of hnRNP K, but this was accompanied by an increased interaction with RNA in a rat hepatoma cell line (Ostrowski et al., 2001). Since hnRNP K has several phosphorylation sites, the net effect of phosphorylation on the RNA binding affinity might be determined by the sum of serine, threonine, and tyrosine phosphorylated residues, rather than being determined by phosphorylation of a single residue.

A previous report have shown that hnRNP K is present at the synapse (Liao et al., 2007), and this is supported by our results showing the expression of the protein in rat hippocampal synaptoneurosomes (Figure 13A). Furthermore, the co-localization of hnRNP K (Ser284) with the synaptic marker PSD95 in cultured hippocampal neurons suggests that the protein is present at excitatory synapses (Figure 15B), where it may play a role in the regulation of the transcripts locally available for translation. In particular, the observed release of mRNAs for AMPA (GluA1 and GluA2) and NMDA (GluN1) receptor subunits, in addition to transcripts for CaMKII β, may be very important to allow local synthesis of these proteins after synaptic stimulation with BDNF, which may contribute to synaptic potentiation. Accordingly, stimulation of BDNF was shown to up-regulate GluA1 (Schratt et al., 2004) and CaMKII β (Liao et al., 2007) protein levels in synaptoneurosomes. GluN1 mRNA is also among the RNAs that are present in both soma and dendrites (Benson, 1997; Gazzaley et al., 1997; Schratt et al., 2004), and is associated with RNA granules (Krichevsky and Kosik, 2001), but the effects of BDNF on the expression levels of this receptor subunit at the synapse has not been yet reported. A similar role for hnRNP K (or its binding partners) in the release of transcripts used for local translation may be hypothesized in the BDNF-

induced dendritic synthesis of BDNF and TrkB receptors (Shiina et al., 2005), and in the translation of the hnRNP K mRNAs at the synapse (Liao et al., 2007). The local synthesis of RNA binding proteins at the synapse, including hnRNP K, following stimulation with BDNF is an intriguing observation, but it may contribute to increase the local buffering capacity of the transcripts released after the disassembly of the RNA granules, thereby contributing to the stabilization of the mRNAs.

In addition to the effects of BDNF on hnRNP K observed in this work, a previous study reported the effects of the neurotrophin in the dissociation of granules containing RNG105. In this report, the release of RNG105 was coincident with the induction of local translation near the granules (Shiina et al., 2005), suggesting that the protein could act in the inhibition of translation. However, more recent studies indicated that although RNG105 affect translation when expressed at non-physiological levels in cells, under physiological conditions the protein may not play the primary role in translational inhibition (Shiina et al., 2010).

Analysis of the proteome of synaptoneurosomes isolated from cultured cerebrocortical neurons showed the presence of several members of the hnRNP protein family, including the hnRNP K, H2, A1, M and Q (Liao et al., 2007), and a role of hnRNP A2 in the delivery of specific transcripts to dendrites was also reported (Gao et al., 2008). All these proteins are likely to contribute to the transport of mRNAs to the synapse, but the identity of the transcripts that interact with each of the proteins in hippocampal neurons remains largely unknown. Furthermore, additional studies are required to determine whether the interaction of hnRNP H2, A1, A2, M and Q with mRNAs is also regulated by BDNF stimulation, as observed in this work for hnRNP K. Although we have attempted to characterize the mRNAs associated with hnRNP A2/B1 the amount of RNA isolated was not enough to perform the microarray analysis.

CHAPTER 6 – CONCLUSION

This work presents a major contribution to the knowledge of the transcripts associated with the RNA-binding protein, hnRNP K, and to the regulation by the neurotrophin BDNF in hippocampal neurons. At least eleven thousand transcripts were identified, which are associated with RBP either through a direct interaction or by indirect binding through other RBPs present in the same transport particles.

The trigger that promotes the release of the mRNA cargo from the RNA granules following synaptic stimulation is not completely understood. We showed that BDNF changes the RBP-RNA binding since we have identified over ten thousand transcripts down-regulated by BDNF in hnRNP K immunoprecipitates. Also, BDNF increased the phosphorylation status of the hnRNP K, which might be related to the disassembly of the RNA granules through a change in protein-protein interactions, and resulting in the release of the mRNAs transported. Whenever the release of transcripts occurs near the dendritic spines, the mRNAs may be made available for local protein synthesis, critical for the late-phase LTP.

The precise mechanism initiated by BDNF and the resulting signaling events that ultimately lead to the phosphorylation of hnRNP K remains unclear. However, the results of this work support the hypothesis that the BDNF regulates the phosphorylation status of hnRNP K and that this event may be a key point in the fate of the transported mRNAs.

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APPENDIX

The supplementary tables are available for examination in the CD supplied in portable document format (.pdf).

Supplementary Table 1 – the corresponding file is entitled “hnRNP K co-immunoprecipitated transcripts”.

Supplementary Table 2 - the corresponding file is entitled “hnRNP K co-immunoprecipitated transcripts regulated by BDNF”.