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Activity-dependent changes in the dendritic distribution of hnRNP K:

functional implications

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Doutor Professor Carlos Duarte (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra) e do Doutor Graciano Leal (Centro de Neurociências e Biologia Celular)

Eduardo Manuel Firmo Morais

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"Acima de tudo, não temas os momentos difíceis. O melhor resulta deles."

Rita-Levi Montalcini

(tradução livre)

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Abbreviations

Abi-1 - Abelson interactor 1

ADF – Actin-depolymerizing factor

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Arbp – Attachment region binding protein

Arp2 – Actin-related protein 2

ATM - Ataxia telangiectasia-mutated gene

ATR - ATM- and rad3-related

BDNF - Brain-derived neurotrophic factor

c/EBP - CCAAT/enhancer-binding protein

CacyBP - Calcyclin-binding protein

CaMKII - Ca^{2+}/calmodulin-dependent kinase II

CNS – Central nervous system

CT element - Consensus sequence CCCTCCCC

DICE - Differentiation control element

DIV – Days *in vitro*

DNA – Desoxyribonucleic acid

EF-1α – Elongation factor-1α

EGR-1 - Early growth response protein-1

eIF4E - Eukaryotic translation initiation factor 4E

EPSC – Excitatory post synaptic current

ERK1/2 - Extracellular-signal-regulated kinases

FMRP – Fragile X mental retardation protein

Grb2 - Growth factor receptor-bound protein 2

HDM2 – Human double minute 2

hnRNP – Heterogeneous nuclear Ribonucleoprotein

Hsc70 - Heat shock cognate 70

IL-1 – Interleukin 1

JNK - c-Jun N-terminal kinase

Kd – Dissociation constant

KH – K homology domain

KI – K interactive region

KNS – K nuclear shuttling

Lck - Leukocyte C-terminal Src kinase

LTP – Long-term potentiation

MAP2 - Microtubule-associated protein 2

MAPK - Mitogen-activated protein kinase

mRNA – Messenger RNA

NGF – Nerve growth factor

NLS – Nuclear localization signal

NMDA - N-methyl-D-aspartate

NT-4 – Neurotrophin-4

PCBP - Poly(C)-binding protein

PI3K - Phosphatidylinositol 3-kinase

PKC - Protein kinase C

PLC – Phospholipase C

Pol. II – DNA polymerase II

PSD – Postsynaptic density

RBP – RNA-binding protein

RhoA – Ras homolog family member A

RISC – RNA-induced silencing complex

RNA – Ribonucleic acid

RNP – Ribonucleo particle

Rplp1/2 - Ribosomal protein large P1/2

RRM – RNA recognition motif

SCA-10 – Spino-cerebellar ataxia 10

SH2/3 – Src homology domain

shRNA – Small hairpin RNA

ssDNA - Single-stranded DNA

SIC – Spreading initiation centre

siRNA – Small interfering RNA

SPAR - Spine-associated Rap-specific GTPase-activating protein

Src - Cytoplasmic protein tyrosine kinase

ssDNA – Single stranded DNA

SUnSET – Surface sensing of translation

TBP – TATA-box binding protein

TrkB – Tropomyosin receptor kinase B

UTR – Untranslated region

ZBP1 – Z-DNA binding protein 1

Zik-1 - Zinc finger protein interacting with K protein 1

Key words

- Heterogeneous nuclear Ribonucleoprotein K (hnRNP K)
- Brain-derived neurotrophic factor (BDNF)
- Synaptic plasticity
- Local protein synthesis
- Surface GluA1 expression

Palavras-Chave

- *Heterogeneous nuclear Ribonucleoprotein K* (hnRNP K)
- Factor neurotrófico derivado do cérebro (BDNF)
- Plasticidade sináptica
- Síntese local de proteínas
- Expressão superficial de GluA1

Abstract

Neurons communicate with each other through highly specialized structures, the synapses, and the synaptic strength can be bidirectionally modulated. Long-term potentiation (LTP) refers to a long-lasting enhancement of the excitatory synapse strength which may be induced by high-frequency presynaptic stimulation or by pairing low-frequency presynaptic stimulation with postsynaptic depolarization. Experimental evidence relates LTP to memory acquisition and learning, especially at the hippocampus. The late stages of LTP require local synthesis of specific proteins and, accordingly, the essential cellular machinery required for translation activity was found at the vicinities of synapses. Furthermore, LTP is associated with structural changes of dendritic spines, namely their enlargement. Brain-derived neurotrophic factor (BDNF) is a neurotrophin capable of modulating synaptic transmission and is a mediator of late-phase LTP at hippocampal synapses. BDNF has been implicated, among other functions of the neurotrophin, in the control of mRNA localization in dendrites, in the regulation of translation machinery activity, as well as in spine plasticity. Heterogeneous nuclear Ribonucleoprotein K (hnRNP K) belongs to the heterogeneous nuclear ribonucleoprotein family of proteins and binds nascent transcripts. This ribonucleoprotein virtually regulates every step of mRNA biology and a recent study showed a role for hnRNP K in synaptic plasticity in the hippocampus. In this work we aimed at further characterizing the activity-dependent changes on hnRNP K levels in dendrites of cultured hippocampal neurons, as well as its possible functions in the regulation of BDNF-induced dendritic protein synthesis and surface expression of GluA1-containing AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors. We developed lentiviral vectors to deliver small hairpin RNAs (shRNA) targeting hnRNP K mRNA in cultured hippocampal neurons. With this approach we validated two shRNA constructs (SH5 and SH6) with full complementarity to the rat hnRNP K mRNA which induced a strong knockdown of hnRNP K protein levels, both in the cell soma and in dendrites.

Immunocytochemistry experiments showed an increase in hnRNP K protein levels in dendrites of cultured hippocampal neurons following activation of the BDNF-TrkB (tropomyosin receptor kinase B) signalling cascade. Increasing synaptic activity with bicuculline also uprgulated the dendritic levels of hnRNP K through a mechanism

dependent on endogenous released BDNF. These results show an important role of BDNF in the accumulation of hnRNP K in dendrites upon neuronal activation. Preliminary results obtained using a SUnSET (surface sensing of translation) methodology in both control and hnRNP K-silenced neurons also suggest an important role for this ribonucleoprotein in the regulation of dendritic protein synthesis. Finally, our preliminary findings indicate that hnRNP K may also be involved in the regulation of the surface expression of GluA1-containing AMPA receptors.

Altogether, our data support the available evidence suggesting hnRNP K as a crucial player in the regulation of synapse function. Importantly, hnRNP K is likely involved in the modulation of local mRNA metabolism in dendrites which may have a role in BDNF-dependent forms of synaptic plasticity.

Resumo

Os neurónios são capazes de comunicar entre eles através de estruturas altamente especializadas, as sinapses, cuja potência pode ser modulada bidireccionalmente. A potenciação sináptica de longa duração (LTP) refere-se a um aumento prolongado da força sináptica e pode ser induzida por estimulação de alta-frequência do terminal pré-sináptico ou por emparelhamento de uma estimulação pré-sináptica de baixa-frequência com despolarização da região pós-sináptica. As evidências experimentais disponíveis relacionam a LTP com a aquisição de memória e com a aprendizagem, sobretudo ao nível do hipocampo. As fases mais tardias da LTP requerem síntese local de proteínas específicas e a maquinaria celular essencial para a tradução foi encontrada nas regiões adjacentes às sinapses. A LTP está também associada a alterações nas espículas dendríticas, nomeadamente ao aumento do seu tamanho.

O BDNF (factor neurotrófico derivado do cérebro) é uma neurotrofina capaz de modular a transmissão sináptica e mediar a fase-tardia do LTP em sinapses do hipocampo. Entre outros processos, o BDNF está envolvido na regulação da localização de mRNAs nas dendrites, assim como na regulação da actividade da maquinaria envolvida na tradução, e da plasticidade sináptica. A hnRNP K é uma proteína pertencente à família das *heterogeneous nuclear ribonucleoproteins*. Esta proteína liga-se a sequências de RNA após a transcrição e assim tem o potencial de regular todos as etapas do seu metabolismo. Estudos recentes mostraram também que esta proteína desempenha um papel importante na plasticidade sináptica ao nível do hipocampo. Com este trabalho pretendemos caracterizar as alterações na distribuição dendrítica da hnRNP K em neurónios do hipocampo resultantes da actividade sináptica, e estudar a possível função da proteína na regulação da síntese proteica induzida por BDNF e na inserção superficial de receptores AMPA (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) contendo a subunidade GluA1. Para tal, desenvolvemos vectores lentivirais para entregar "*small hairpin RNAs*" (shRNAs) que reconhecem o mRNA que codifica a proteína hnRNP K de rato, em neurónios de hipocampo em cultura. Foi possível validar duas construções de shRNA (SH5 e SH6), com completa complementaridade pelo mRNA que codifica a proteína hnRNP K de rato, as quais

induziram uma redução significativa dos níveis desta proteína no corpo celular e nas dendrites.

Através de experiências de immunocitoquímica observámos um aumento dos níveis proteicos de hnRNP K em dendrites de neurónios de hipocampo em cultura devido à activação da cascata de sinalização induzida pela interacção entre o BDNF e o receptor TrkB (*tropomyosin receptor kinase B*). O aumento de actividade sináptica induzido pela bicuculina fez também aumentar os níveis dendríticos da proteína hnRNP K através de um mecanismo dependente da libertação de BDNF endógeno. Estes resultados sugerem que a neurotrofina é necessária para a acumulação de hnRNP K nas dendrites em resposta à actividade neuronal. Resultados preliminares usando a técnica SUnSET (*"surface sensing of translation"*) em neurónios controlo ou em neurónios com níveis proteicos de hnRNP K reduzidos, sugerem igualmente um importante papel para esta ribonucleoproteina no controlo da síntese proteica ao nível das dendrites. Por fim, os nossos resultados preliminares também sugerem que a proteína hnRNP K pode estar envolvida na regulação dos níveis membranares de receptores AMPA que contêm a subunidade GluA1.

Em conclusão, os nossos resultados apoiam as evidências já existentes que sugerem um papel para a proteína hnRNP K na regulação da actividade sináptica. De notar que a proteína hnRNP K poderá estar envolvida na modulação do metabolismo do mRNA nas dendrites, o que poderá ter implicações nos eventos de plasticidade sináptica dependentes do BDNF.

1. Introduction

1.1. Neurons and chemical synapses

If nowadays the concept of neuron individuality is widely accepted, it is mainly due to the remarkable work of Jamón y Cajal during the 19th and 20th centuries. With the consolidation of the neuron doctrine several disciplines could emerge and rapidly improve. This theory postulates the contiguity in disregard of continuity of neuronal cells, and the communication of these cells through specialized contacts which were named synapses by Sherrington in 1906 (Lopez-Munoz et al., 2006). Neurons are now recognized as the basic components of the nervous tissue and they are highly diverse and dynamic cells. Synapses are viewed as the subcellular region responsible for the specific and highly regulated flow of information between nerve cells. Additional cells are found in the nerve tissue, the glia cells, including microglia, astrocytes and oligodendrocytes. They provide neurotrophic support essential for the maintenance of neurons, confer the principal immunological defenses in the brain, and are capable of modulating the activity of neurons, among other important functions (Hering and Sheng, 2001; Purves, 2008).

Although neuronal cells are highly diverse in shape, size and functions, there are several structural features that allow their identification. Neurons are polar cells, with several ramifications: one longer, the axon, and many shorter, the dendrites. Dendrites may be simple short cellular membrane projections, or long and complex arborizations composed by several order dendrites (second, third…), forming tree-like structures. By rule, the information is transmitted unidirectionally from the dendrites to the postsynaptic region, and then to the cell soma. From this region of the cell, the information travels along the axon, arriving at the presynaptic region and finally the message is sent to the next neuron (Purves, 2008).

The typical chemical synapse is composed by a presynaptic region that secretes the neurotransmitter to the synaptic cleft, which will activate receptors located on the postsynaptic region of an adjacent neuron. The synaptic cleft should not be seen as an empty space to be crossed, but instead should be regarded as an extracecullar space with many proteins responsible for the modulation of diffusion, binding and clearance of neurotransmitters (Purves, 2008). Numerous studies have shown the structural distinction between excitatory and inhibitory synapses (Bourne and Harris, 2008). The inhibitory synapses release the neurotransmitters directly to the synaptic shaft, while the

excitatory synapses generally release the neurotransmitters to a specialized postsynaptic structure called dendritic spine (Bourne and Harris, 2008; Purves, 2008).

Dendritic spines also show a great variability of morphology in hippocampal neurons (Figure 1). The majority have narrow necks and bigger heads resembling mushrooms. These spines are enriched in postsynaptic density (PSD) proteins, glutamate receptors and regularly contain protein synthesis machinery, namely polyribosomes and endosomal compartments. This morphology is therefore believed to correspond to mature and stronger synapses. In contrast, spines with no head or with heads slightly larger than the neck (filopodia shaped) are believed to be immature and weaker synapses. However, weaker synapses seem to be more dynamic and mutable depending on the arriving stimuli (Bosch and Hayashi, 2012; Bourne and Harris, 2008; Purves, 2008). It was hypothesized that the spine neck could provide electric and biochemical isolation to the spine but several studies did not find a significant effect of the spine neck on electric conductance, while others have suggested a possible restriction on molecular diffusion in this region (Bosch and Hayashi, 2012; Lopez-Munoz et al., 2006; Purves, 2008).

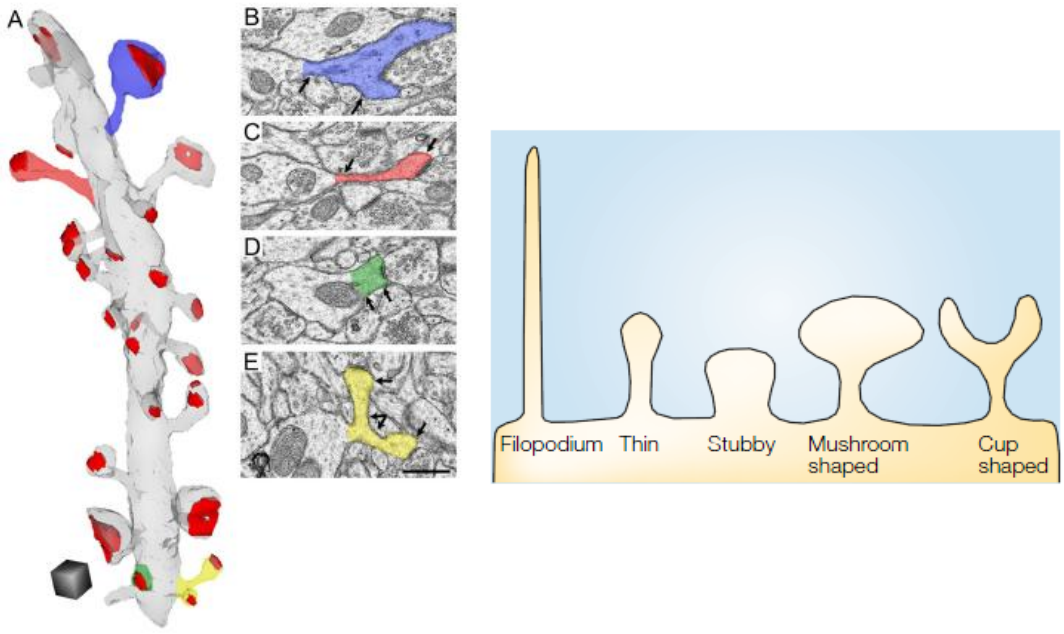

Figure 1 - Representation of the morphological variability of dendritic spines. A. Model of the tridimensional structure of a dendrite. (B-E) Images from electronic microscopy illustrating the diversity of morphologies of dendritic spines. B. Mushroom-shaped spine; C. Filopodia-like spine; D. Stubby spine, with the same head diameter and neck length; E. Cup-shaped spine. The right panel illustrates the morphological variability of dendritic spines. From (Bourne and Harris, 2008; Hering and Sheng, 2001).

Spines are composed by a large number of different proteins, some with overlapping functions, which allows a tight regulation and modulation of the signals triggered in the postsynaptic neuron. In addition to the crowded protein agglomeration near the membrane, spines also contain highly organized and stimuli-sensitive actin filaments, the main cytoskeleton elements in spines, and may possess recycling endosomes, polysomes, proteasomes, smooth endoplasmic reticulum, mitochondria, among other cellular components (Bosch and Hayashi, 2012; Bourne and Harris, 2008; Purves, 2008).

In the hippocampus, as well as in other brain regions, dendritic spines are formed during development and in adulthood as a plasticity response. Early postnatal neurons have many filopodia spines that are subsequently selected: only spines that form synapses are able to mature and stabilize. Several elements are essential for spine stabilization such as PSD95 assembly, insertion of AMPA receptors on the postsynaptic membrane and their activation (Bosch and Hayashi, 2012; Bourne and Harris, 2008). Dendritic spines are highly dynamic structures and the changes in their morphology and number may correspond to the conversion of short-term changes in synaptic transmission into long-lasting alterations. Indeed, long-term potentiation in the hippocampus, a long-lasting form of synaptic plasticity, has been shown to induce enlargement of spine head along with shortening of the neck, as well as the appearance of a new subpopulation of small spines dependent on the activation of N-methyl-D-aspartate (NMDA) receptors (Bosch and Hayashi, 2012). Most experimental findings appear to fit in a bimodal relationship between intracellular calcium concentrations and spine growth. Thus, moderate levels of calcium support spine stability and maturation, whereas increased levels of calcium may cause shrinkage and even collapse of spines (Hering and Sheng, 2001). Several studies have also characterized the molecular pathways responsible for the regulation of dendritic spine dynamics, and identified several partners of actin filament as important mediators: Rac, RhoA (members of Ras homolog family), Ras/MAP (mitogen-activated protein) kinase pathway, SPAR (spine-associated Rap-specific GTPase-activating protein), PSD95, Shank and syndecan2, among others (Bourne and Harris, 2008). However, the complete dissection of the signalling interconnections is still missing.

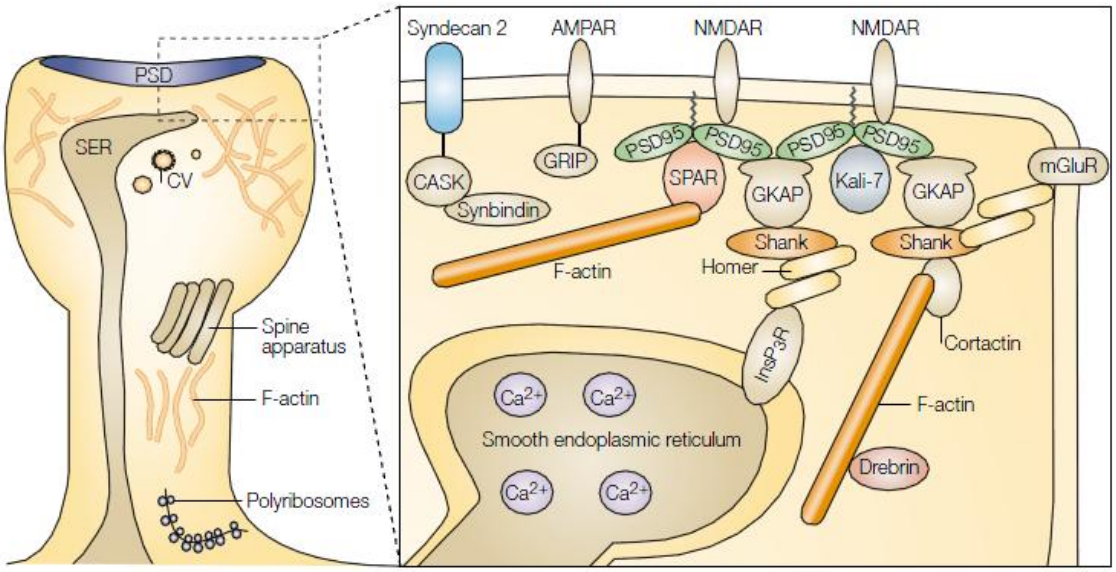

Figure 2 - Cellular components of a dendritic spine. A typical mushroom-shaped spine is represented. In the right, a detail of the synaptic region is shown, with molecular elements present in the postsynaptic density and the intracellular Ca^{2+} stores. From (Hering and Sheng, 2001).

1.2. Glutamatergic synapses and long-term potentiation

As described above, changes in the strength of synaptic transmission play an essential role in the information coding and integration in the neuronal circuits. Such claim is supported by many experimental studies finding causal relations between changes in the synaptic transmission and cognitive impairments (e.g. in memory and learning) and *vice-versa*. Moreover, several diseases of the nervous system, such as Autism spectrum disorders, have been directly related to structural changes in spines and to changes in synaptic transmission (Bliss et al., 2014; Penzes et al., 2011).

Glutamatergic synapses are the most abundant excitatory synapses in the central nervous system (CNS). At these synapses glutamate is released from the presynaptic terminal and the neurotransmitter acts through activation of ionotropic and metabotropic receptors on the postsynaptic membrane. There are three types of ionotropic receptors for glutamate, named AMPA, NMDA and kainate receptors, which can be distinguished based on their molecular, electrophysiological and pharmacological properties. All ionotropic receptors for glutamate form nonselective cation channels permeable to Na^+

and K^+, and NMDA receptors also allow the influx of Ca^{2+} (Niswender and Conn, 2010; Traynelis et al., 2010).

The plasticity of glutamatergic synapses has been extensively studied in the hippocampus, particularly in the CA1 area, but similar activity-dependent alterations in synaptic strength are observed in other brain regions. Long-term potentiation (LTP) of glutamatergic synapses in the hippocampus is believed to underlie memory acquisition and learning. This form of synaptic plasticity refers to a long-lasting strengthening of synaptic transmission as measured by an increased magnitude of the excitatory postsynaptic potentials (EPSP). It is also characterized by two important properties, the synapse specificity and associative stimulation. Thus, only the stimulated synapses are able to develop LTP, but a single input (~500 μV) is not able to induce LTP by itself. The induction of LTP may require a significant membrane depolarization which is only achieved by simultaneous activation of several adjacent excitatory synapses (Lisman, 2007; Purves, 2008).

The molecular mechanisms responsible for LTP have been extensively investigated. The calcium influx induced by glutamate receptor activation is the major trigger of LTP in the hippocampus. Glutamate binds to AMPA and NMDA receptors, but at resting membrane potential the NMDA cationic channel is blocked by physiological concentrations of Mg^{2+}. However, if the membrane is already depolarized, magnesium is expelled from the NMDA receptor channel allowing an additional influx of calcium (Bliss and Collingridge, 1993; Lisman, 2007; Purves, 2008). Indeed, studies knocking out or using blockers of NMDA receptors, or using calcium buffers, showed LTP impairments and weaker performances in memory-tasks (Bliss and Collingridge, 1993; Lynch et al., 1983; Tsien et al., 1996). Nonetheless, the early influx of calcium does not account for the long-term changes in synaptic strength. It has been shown that calcium exerts its effect by reversible activation of kinases, mainly Ca^{2+}/calmodulin-dependent kinase II (CaMKII), activating several signalling pathways that will at last potentiate the synapse. One mechanism is through phosphorylation of GluA1 on Ser831 which enhances the activity of AMPA receptors (Lee et al., 2000).

LTP as well as memory, have distinct temporal phases – short-term potentiation, early LTP (E-LTP) and late LTP (L-LTP). Short-term potentiation and E-LTP are transient and depend mainly on the changes in the activity of ion channels and on post-translational modifications of the proteins present at the synapse (Minichiello, 2009).

However, translation and transcription play an important role in the late phase of LTP as assessed by interdisciplinary studies. Blocking protein synthesis impairs LTP formation and long-term memory acquisition, in a time frame earlier than the impairments caused by transcription inhibitors (Costa-Mattioli et al., 2009). Although the early protein synthesis burst is dependent on previously transcribed mRNAs, at later time points it may also require new transcripts coming from the nucleus (Lisman, 2007).

1.3. Dendritic protein synthesis

Dendritic protein synthesis, or local protein synthesis, refers to the translation of new proteins directly in dendrites and in the vicinity of spines, which also requires the presence of the correspondent cellular machinery. Evidence for the presence of polyribosomes near the spines was firstly described by Steward and Levy in 1982 using electron microscopy. Additional studies have shown an important role for local protein synthesis in the late phase of LTP and in neurite growth and development, and lower performances in memory tasks were observed when protein synthesis was impaired (Santos et al., 2010; Sutton and Schuman, 2006). Producing proteins locally has several advantages, including the spatial accuracy in the location of the protein, the capacity to produce specific proteins when necessary, and avoids changes of protein structure and function that could otherwise occur during transport, among others (Jung et al., 2014). Several attempts have been made to characterize the pool of mRNAs present in dendrites, and a recent microarray study suggested the presence of about 2550 different transcripts in dendrites (Cajigas et al., 2012; Sutton and Schuman, 2006). The transport of mRNA-containing granules along dendrites was also observed using fluorescent dyes (Knowles et al., 1996).

For their correct function, eukaryotic mRNAs usually require information for their nuclear export, subcellular localization, translation and stability. Some of this information is coded in the sequence of ribonucleotides itself, but the specificity in the transport of transcripts along dendrites is determined, to a large extent, by the interaction with specific RNA binding-proteins (RBP). Accordingly, the pre-mRNA and mRNA molecules are usually found in ribonucleoprotein complexes (RNPs) in the cell, bound to RNA-binding proteins, and their structure and composition may encode the fate of the RNA. About 270 RBP were identified up to now based on the known RNA-binding domains (Holt and Schuman, 2013). As described later in this section,

heterogeneous ribonucleoproteins (hnRNPs) are a family of proteins capable of binding to and participating in every step of mRNA metabolism in eukaryotic cells, from alternative splicing, to translation and degradation (Bjork and Wieslander, 2011; Dreyfuss et al., 2002; Sinnamon and Czaplinski, 2011). However, several questions still remain to be answered, including how many transcripts are transported in each complex, and if there is more than one mRNA molecule per complex it will be important to find out whether they code for the same or distinct proteins. It is noteworthy mentioning that there are several different RNA-protein complexes in the cells: translating polysomes, processing bodies (P-bodies), stress granules, microRNA particles (miRNPs) or the RNA interfering silencing complex (RISC), transport particles and RNA granules, which participate in different cellular functions (Leal et al., 2014; Sossin and DesGroseillers, 2006).

Initiation of translation is the rate-limiting step of protein synthesis in eukaryotic cells, conferring to translation a great means of controlling the synthesis of new proteins. Translation initiation is composed by three main steps: formation of the 43S ribosomal pre-initiation complex, followed by binding of the mRNA to the 43S ribosomal complex, and finally 80S ribosomal complex formation. Thus, protein synthesis is a highly regulated process, even more when activated as response to specific stimuli such as in the case of LTP-dependent protein synthesis (Costa-Mattioli et al., 2009). Local protein synthesis was also shown to be inhibited by mini EPSCs, leading to the stabilization of dendritic spines, and pharmacological inhibition of mini EPSCs was found to activate local translation. Local protein synthesis is therefore important not only for structural and synaptic plasticity, but also in the stabilization of the synaptic function (Sutton and Schuman, 2006).

Although the requirement for local translation has been experimentally observed in different contexts, the identity, the wide range of functions, and the specific changes induced by the newly synthesized proteins remains largely elusive (Costa-Mattioli et al., 2009; Sutton and Schuman, 2006). Are the newly synthesized proteins just increased copies of the locally available proteins, or are there proteins that are exclusively expressed under LTP? The proteins synthesized during LTP are intermediate factors triggering activation and/or potentiation of signalling pathways already present at synapses, or are they bringing new functions not present in the synapse before LTP?

1.4. BDNF signalling and synaptic plasticity

Brain-derived neurotrophic factor was first described in 1980s by Yves Barde, Hans Thoenen and colleagues, and it is a close relative to nerve growth factor (NGF) (Chao, 2003). BDNF binds with high affinity to the TrkB receptor, which can also be activated by NT-4. The binding of both neurotrophins to TrkB induces its dimerization and autophosphorylation on tyrosine residues located in the intracellular kinase domain, followed by phosphorylation of tyrosine residues at the C-terminus which allow the docking of adaptor proteins. TrkB receptors activate three major signalling pathways (Figure 3): the Ras-MAPK pathway, the phosphatidylinositol 3-kinase (PI3K)–Akt pathway and the PLCγ–Ca^{2+} pathway (Chao, 2003; Leal et al., 2014; Minichiello, 2009). Additionally, but still controversial, TrkB receptors may activate Na^{+} channels thereby depolarizing the cell by a mechanism independent of the above mentioned classic signalling pathways (Minichiello, 2009).

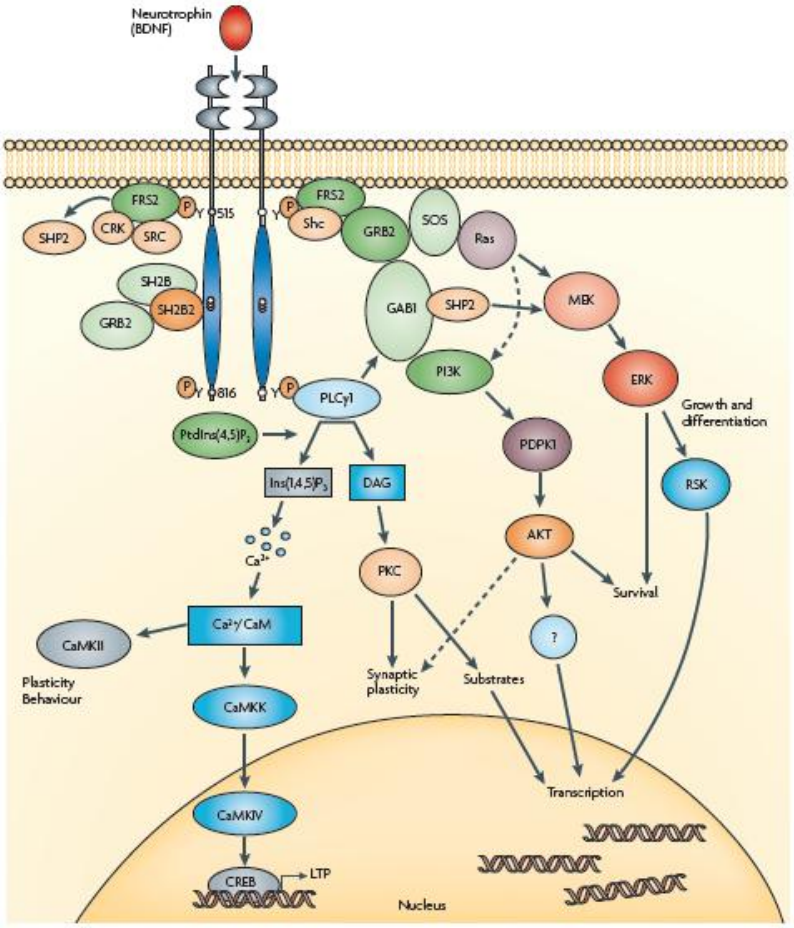

Figure 3 – Representation of the main TrkB receptor-activated signalling pathways (Minichiello, 2009).

The role of BDNF and TrkB activation in synaptic plasticity was first suggested based on the evidence showing the release of BDNF upon synaptic activation and the localization of TrkB receptors in glutamatergic synapses. Additional studies showed that stimuli strong enough to induce LTP also sharply increase BDNF release in a Ca^{2+} and CaMKII-activation dependent mechanism in the hippocampus (Hartmann et al., 2001; Kolarow et al., 2007; Minichiello, 2009). Furthermore, depletion of BDNF disrupts early and late phases of LTP, similar to the results obtained when TrkB receptors were blocked. Addition of BDNF also induces LTP in hippocampal slices (Ji et al., 2010; Kang and Schuman, 1996), and this effect is age dependent (Minichiello, 2009).

The effects of BDNF on LTP are likely to be mediated by activation of pre- and postsynaptic receptors (Gartner et al., 2006) and distinct mechanisms account for the early and delayed responses to the neurotrophin (Leal et al., 2014). The fast effects of BDNF involve post-translational modifications of locally available proteins, which change their activity and/or distribution. For example, BDNF induces GluN2B phosphorylation which increases the opening probability of the ionic channel (Leal et al., 2014). Some of the delayed effects of BDNF, which may contribute to the late-phase of LTP, include the upregulation in the synthesis and trafficking of AMPA and NMDA receptors to the membrane (Caldeira et al., 2007a; Caldeira et al., 2007b; Santos et al., 2010).

The late phase of LTP, also known as synaptic consolidation, is dependent on protein synthesis. BDNF induces protein synthesis by regulating the initiation and elongation phases of translation, and the effect on the transport and recruitment of mRNAs to the dendrites also contributes to local translation activity (Leal et al., 2014; Minichiello, 2009). In fact, BDNF may induce translation through phosphorylation and activation of the proteins involved in this process. However, the identity of the proteins synthesized locally in response to the increase in BDNF at the synapse remains largely to be determined.

Hundreds of mRNAs have been identified in the dendrites (Cajigas et al., 2012; Leal et al., 2014; Santos et al., 2010), and one of the challenges in the field is to determine how they are specifically delivered to dendrites and transported to synaptic sites, and how their translation is regulated. The interaction between the mRNA *cis*-acting elements with *trans*-acting elements of RBPs plays a key role in the regulation of the transport of mRNAs along dendrites. The most studied RBPs include ZBP1 (Z-DNA

Binding Protein 1), Staufen, FMRP (Fragile X Mental Retardation Protein) and hnRNP A2. These proteins are able not only to transport mRNA but also to repress their translation. BDNF was shown to induce the dendritic accumulation of activity-regulated cytoskeleton-associated protein (Arc) transcripts and the phosphorylation of ZBP1 which allows the local synthesis of β-actin (Costa-Mattioli et al., 2009; Leal et al., 2014).

BDNF also contributes to structural modifications associated to LTP, such as the increase in the number and volume of dendritic spines (Cowansage et al., 2010; Ji et al., 2010). The spine density increase induced by BDNF is dependent on the activation of Ras/ERK pathway and the transient receptor-potential cation channel subfamily C (TRPC) type 3. BDNF also increases actin polymerization by a mechanism dependent on p21-activated-kinase and ADF/cofilin (Leal et al., 2014). Other forms of plasticity are also modulated by BDNF and changes in the levels of this neurotrophin have been associated to several diseases or to developmental benefits, such as in maternal deprivation and environmental enrichment. The expression of BDNF itself was demonstrated to be regulated by an experience-dependent epigenetic mechanism (Cowansage et al., 2010).

1.5. Heterogeneous nuclear Ribonucleoproteins (hnRNPs)

The heterogeneous nuclear ribonucleoproteins are a set of 20 RNA-binding proteins (hnRNP A-U) with multiple functions in nucleic acid metabolism, from nascent transcript stabilization to the regulation of translation (Bjork and Wieslander, 2011; Han et al., 2010; Swanson and Dreyfuss, 1988). They are modular proteins composed mainly by multiple domains connected by linker regions. The variability of domain composition in number, combination and arrangements, contributes to their functional diversity (Han et al., 2010). As shown in Figure 4, almost every hnRNP share structural characteristics such as RNA-binding domains (RBDs), frequently in tandem, RGG boxes (repeats of Arg-Gly-Gly tripeptides) and auxiliary domains of distinctive amino acid composition. Additionally, many of the proteins belonging to this family are expressed in alternative spliced variants and undergo post-translational modifications, namely phosphorylation of serine and threonine residues, and methylation of arginine residues (Dreyfuss et al., 2002; Han et al., 2010).

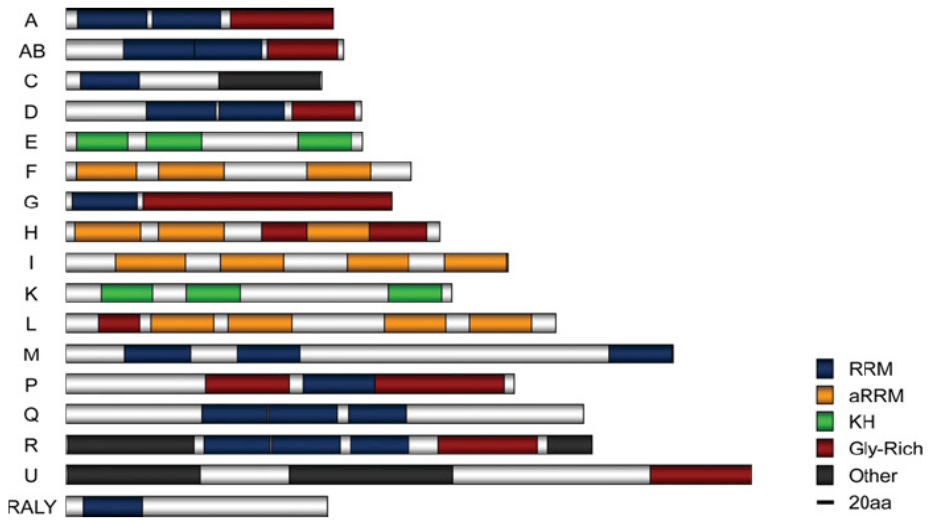

Figure 4 - Domain composition of the proteins belonging to the family of the heterogeneous nuclear ribonucleoproteins; from (Han et al., 2010).

hnRNPs are typically found disperse in nucleoplasm, but excluded from the nucleoli, and may undergo nucleocytoplasmic shuttling, as visualized by immunostaining (Dreyfuss et al., 2002; Han et al., 2010; Matunis et al., 1992; Pinol-Roma et al., 1988). This process is important for the transport of mRNAs across the nuclear membrane, and is essential for the hnRNPs cytoplasmic functions. However, while paralogues A1 and I do not contain classical nuclear localization signals (NLSs), other paralogues such as hnRNP K contain NLS and nuclear shuttling domains (KNS) (Bomsztyk et al., 2004). Therefore, their movements are respectively coupled to transcription and transcription-independent (Han et al., 2010). Nevertheless, it is important to note that these studies have been largely performed in HeLa cells (human carcinoma epithelial cells) and diverse localization patterns may be found in different cell types or under distinct physiological conditions (Han et al., 2010; Mikula et al., 2006; Moumen et al., 2005).

Although different hnRNPs (especially paralogues) share the same functions, they are not functionally redundant (Han et al., 2010). In fact, their functions can be divided in generalised roles, for example as RNA-packaging proteins, and specialised roles that are dependent on specific RNA-protein or protein-protein interactions. More specifically, hnRNPs regulate every step of mRNA metabolism - composed by transcription, splicing, 5' capping, polyadenylation, transport, translation and

degradation - and other processes of nucleic acid dynamics - for example telomere maintenance, chromatin remodelling and DNA repair (Dreyfuss et al., 2002; Han et al., 2010). Remarkably, hnRNPs bind to nascent transcripts produced by RNA polymerase II but fail to associate with other RNA-protein complexes (Han et al., 2010).

The interaction of hnRNPs with nucleic acids relies mainly in the presence of a RNA recognition motif (RRM). This motif is composed by two highly degenerate RNP consensus sequences (RNP-1 and RNP-2) that establish hydrophobic interactions with RNA or single-stranded DNA (ssDNA) and therefore account for the non-sequence-specific binding (Han et al., 2010; Pinol-Roma et al., 1988). The external β_2 and β_4 strands, linker regions and C- and N-termini increase the affinity for specific sequences or types of nucleic acids. Exceptionally, hnRNP K/E contain a K homology (KH) domain that establishes hydrogen bonds and electrostatic interactions with nucleic acids (Han et al., 2010; Swanson and Dreyfuss, 1988; Valverde et al., 2008).

Abnormal expression of hnRNPs has been related to several diseases. For example, hnRNP E/K control the levels of c-Myc, the androgen receptors, eIF4E, p53 and C/EBPα, and dysregulation of hnRNP E and K proteins may lead to excessive cell proliferation (Han et al., 2010).

1.5.1. hnRNP K

Characterization: the hnRNP K protein is a member of the hnRNP set of proteins, thus sharing many of the characteristics above mentioned. hnRNP K is one of the most abundant proteins of the heterogeneous nuclear ribonucleoprotein group and ubiquitous in the mammalian cells, with levels similar to histones (Matunis et al., 1992). In humans, it is encoded by the gene *HNRNPK* localized in chromosome 9 (Dejgaard et al., 1994). Four splice variants of hnRNP K were described (there are only three entries in UniProt database), which are distinguished by the presence or absence of exon A (15bp) after position 1316, and by the presence or absence of exon B (60bp) after position 1569 (Dejgaard et al., 1994; Kimura et al., 2010). hnRNP K is composed by 463 amino acids (Figure 5) with a correspondent molecular weight of 50976.25 Da as estimated by matrix-assisted laser desorption/ionization, or an apparent molecular weight of 65kDa as determined in SDS-PAGE (Bomsztyk et al., 1997) (UniProt). Its

isoelectric point was estimated as pH 6.1 – 6.4 (Pinol-Roma et al., 1988). The subcellular distribution of hnRNP K was studied by immunocytochemistry and electron microscopy and showed that the protein is expressed with higher levels in the nucleus, being also present in the cytoplasm, mitochondria, and in the vicinity of the plasma membrane (Bomsztyk et al., 2004; Mikula et al., 2006).

```
        10         20         30         40         50         60
METEQPEETF PNTETNGEFG KRPAEDMEEE QAFKRSRNTD EMVELRILLQ SKNAGAVIGK

        70         80         90        100        110        120
GGKNIKALRT DYNASVSVPD SSGPERILSI SADIETIGEI LKKIIPTLEE GLQLPSPTAT

       130        140        150        160        170        180
SQLPLESDAV ECLNYQHYKG SDFDCELRLL IHQSLAGGII GVKGAKIKEL RENTQTTIKL

       190        200        210        220        230        240
FQECCPHSTD RVVLIGGKPD RVVECIKIIL DLISESPIKG RAQPYDPNFY DETYDYGGFT

       250        260        270        280        290        300
MMFDDRRGRP VGFPMRGRGG FDRMPPGRGG RPMPPSRRDY DDMSPRRGPP PPPPGRGGRG

       310        320        330        340        350        360
GSRARNLPLP PPPPPRGGDL MAYDRRGRPG DRYDGMVGFS ADETWDSAID TWSPSEWQMA

       370        380        390        400        410        420
YEPQGGSGYD YSYAGGRGSY GDLGGPIITT QVTIPKDLAG SIIGKGGQRI KQIRHESGAS

       430        440        450        460
IKIDEPLEGS EDRIITITGT QDQIQNAQYL LQNSVKQYSG KFF
```

Figure 5 - Sequence of amino acids that compose the hnRNP K protein; from Uniprot http://www.uniprot.org/uniprot/P61978.

Structure: hnRNP K shares common characteristics with the other members of the hnRNP family but has distinctive features that allow its recognition. Similar to the other paralogues, hnRNP K contains several modules that allow the simultaneous binding of kinases or nucleic acids, and recruitment of a multitude of protein partners (Figure 6) (Bomsztyk et al., 2004). Like hnRNP J, hnRNP K binds strongly both to poly(rC) and to oligo(dC) sequences (Matunis et al., 1992), and its structure is similar to four other poly(C)-binding proteins (PCBP), hnRNP E1, hnRNP E2, aCP-3 and aCP-4 (Bomsztyk et al., 2004). As aforesaid, hnRNP K contains three distinctive KH domains similar to hnRNP E, instead of the typical RRMs or RRM-like domains found in the other hnRNPs (Han et al., 2010). These three KH domains mediate RNA and single- and double-stranded DNA binding (Bomsztyk et al., 2004). Additionally, hnRNP K has one

K protein interactive region (KI) with SH2 and SH3 docking sites that mediate specific protein-protein interactions (Mikula et al., 2006). Worth to note, hnRNP K has multiple phosphorylation sites capable of regulating the affinity of the protein with their nucleic acid and protein partners in response to signalling cascades (Bomsztyk et al., 2004). Phosphorylation of specific amino acids of hnRNP K has also been effectively related with alterations in the cellular distribution and functional regulation (Kimura et al., 2010).

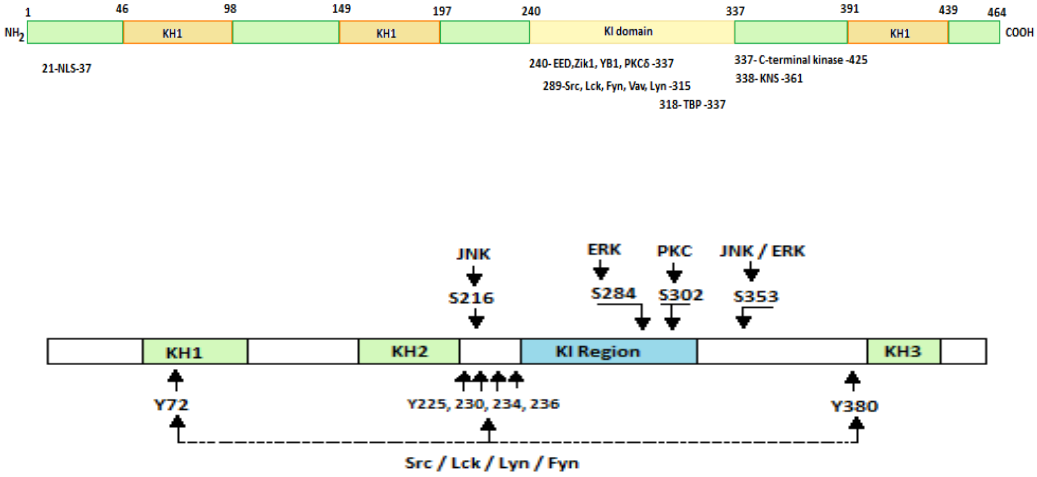

Figure 6 - Domain composition of hnRNP K. Some identified partners (top) and their binding sites. The identified phosphorylation sites and the respective kinases are indicated below. Adapted from (Bomsztyk et al., 2004).

Protein partners: hnRNP K is a component of highly dynamic complexes composed by proteins and nucleic acids, regulated by cellular signalling mechanisms (Mikula et al., 2006; Ostrowski et al., 2000). A great effort has been made in the study of the hnRNP K partners with identification of long lists of proteins. Recurrent described partners include tyrosine kinases such as Src and Lck, serine/threonine kinases namely ERK1/2, JNK, PKC_{α} PKC_{β} and PKC_{ε}, the nucleotide exchange factor Vav, the transcription factor TATA-box binding protein (TBP), the zink finger protein interacting with K protein 1 (Zik-1) and the elongation factor 1 alpha (EF-1α) (Bomsztyk et al., 1997; Mikula et al., 2006). Immunoprecipitation assays suggest the formation *in vivo* of complexes of hnRNP K and several kinases (Src, Fyn and Lyn) (Bomsztyk et al., 1997). Additionally, hnRNP K is phosphorylated in the C-terminus to

form a complex with an interleukin 1 (IL-1)-responsive kinase (Van Seuningen et al., 1995). Interestingly, the interaction of hnRNP K with some of its molecular partners is regulated by RNA (Bomsztyk et al., 1997), and six proteins were found to interact with hnRNP K *via* RNA: three acidic ribosomal proteins (Arbp, Rplp2, Rplp1), CacyBP, Ccct5, and Hnrph (Mikula et al., 2006). Furthermore, the latter study showed 22 proteins belonging to the Grb2-SH2 complex, and 29 proteins of the spreading initiation centre (SIC) that co-immunoprecipitate with hnRNP K, as well as several proteins involved in RNA metabolism, namely RNA helicases Ddx1, Ddx5, and Ddx17 (Mikula et al., 2006). The distribution of hnRNP K-binding partners identified in this work according to their function is represented in Figure 7.

Reinforcing the idea of specificity of the interaction of hnRNP K with other proteins, three SH3-binding sites localized between the amino acids 265-278, 285-297, and 303-318, all present in the KI domain, were found to selectively engage c-Src and Vav SH3 domains, but failed to bind SH3 domains of Abl, p85 phosphatidylinositol 3-kinase, Grb-2, and Csk (Van Seuningen et al., 1995). In this context, the three hnRNP K proline-rich sites may bind independently the Src SH3 domain or the Vav COOH terminus SH3 domain. Moreover, each sequence binds specifically the c-Src SH3 domain, with different affinities but with Kd (dissociation constant) values in the micromolar range. The SH3 domain of Src is important not only for the binding but also for hnRNP K phosphorylation (Ostareck-Lederer et al., 2002). Recent mass spectrometry studies showed the interaction of H1 (histone 1) mRNA with hnRNP A1, hnRNP K and Hsc70 (heat shock cognate 70). In this study the hnRNP K protein was found to co-immunoprecipitate both with hnRNP A1 and Hsc70 (Di Liegro et al., 2013).

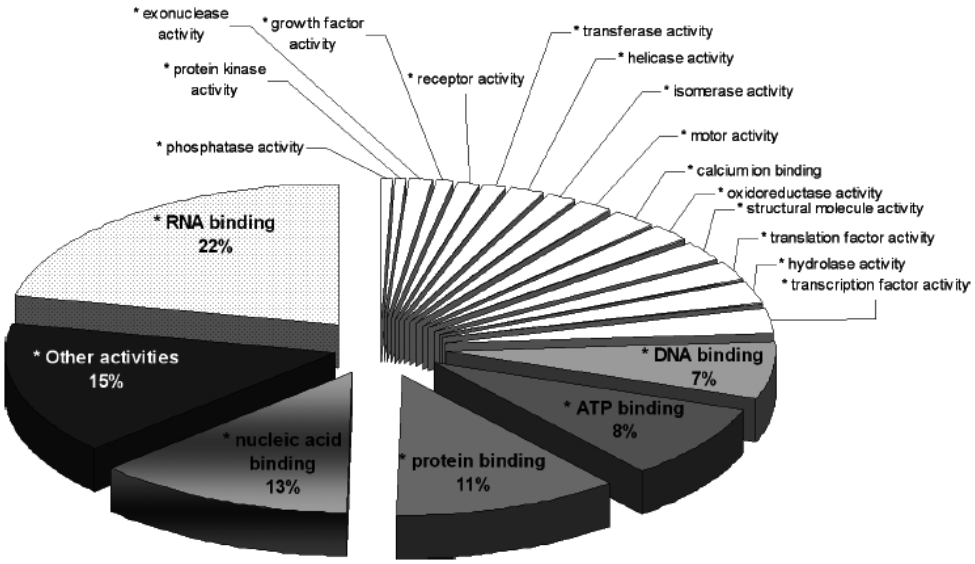

Figure 7 - Distribution of the cellular functions of hnRNP K protein partners identified by (Mikula et al., 2006).

Cellular functions: The modular structure and the large number of partners are the basis for the wide range of nuclear and cytoplasmic functions of hnRNP K (Han et al., 2010). In fact, hnRNP K has been implicated in many steps of RNA biology, including mRNA transcription, splicing, nuclear shuttling, stabilization, silencing and translation (Bomsztyk et al., 2004; Han et al., 2010). Furthermore, the protein has been related to chromatin remodelling, histone interaction, cell cycle regulation and with some cases of disease such as spinocerebellar ataxia type 10 (SCA10) and cancer (Bomsztyk et al., 2004; Moumen et al., 2005; White et al., 2012). hnRNP K has also been considered a docking platform allowing the integration of several signalling cascades (PKCM, MAPK, Lck) and the crosstalk between many cellular factors (Bomsztyk et al., 1997; Bomsztyk et al., 2004)

hnRNP K is involved in the regulation of gene transcription and a role for hnRNP K as a transcriptional factor was also proposed (Michelotti et al., 1996). Expression of hnRNP K mRNA was found to stimulate the transcription of a CT-dependent reporter, which was even increased with the simultaneous overexpression of TBP (Michelotti et al., 1996). However, more recently hnRNP K was identified as a gene transcription repressor, in particular for the osteocalcin gene. The repression appears to be achieved by competitive removal of a *trans*-activator from the CT element, and is not mediated by protein-DNA interactions (Stains et al., 2005).

Furthermore, in the amino acid sequence 206-327 of hnRNP K there is one GRGG box and one SH3-binding domain, responsible for the interaction with the transcriptional repressor Zik1, presenting an alternative mechanism of transcription regulation (Bomsztyk et al., 1997).

There is also evidence that hnRNP K regulates the rate of mRNA elongation and splicing (Mikula and Bomsztyk, 2011). In this context, the EGR-1 transcription by Pol II after stimulation of HCT116WT colon carcinoma cells with serum seems to be dependent of hnRNP K and requires the activity of ERK. Indeed, siRNA-induced knockdown of hnRNP K was found to decrease active MEK and ERK levels, and these alterations were associated with decreased levels of elongating pre-mRNA and less efficient splicing (Mikula and Bomsztyk, 2011). ERK1/2 activation with consequent phosphorylation of hnRNP K on Ser284 and Ser353 was also reported to induce the cytoplasmic accumulation of the protein, presumably due to increased nuclear export and nuclear shuttling regulation (Bomsztyk et al., 2004).

The role of hnRNP K in translation regulation is probably the best described function of the protein. Here, hnRNP K validates its classification as a docking platform for signalling cascades. hnRNP K is a substrate of the MEK/ERK signalling pathway, both *in vitro* and *in vivo*, being phosphorylated on Ser 284 and Ser 353. As abovementioned, MEK/ERK activity is required and sufficient to induce the accumulation of phosphorylated hnRNP K in the cytoplasm (Habelhah et al., 2001). In this compartment, hnRNP K together with hnRNP E1/2 (aCP1/2, PCBP1/2) blocks the recruitment of the 60S ribosomal subunit necessary to form the competent 80S ribosome, thereby inhibiting translation initiation (Mikula et al., 2006). Moreover, expression of a phosphomutant hnRNP K protein was suggested to attenuate the translational inhibition caused by ERK-dependent phosphorylation (Habelhah et al., 2001).

The Src kinase is another very important signalling mediator in the regulation of translation through hnRNP K. The differentiation control element (DICE) in the 3' UTR of mRNAs was proposed to interact with the KH domain of hnRNP K. It was shown that Src and Lck are able to tyrosine-phosphorylate the hnRNP K protein, both *in vitro* and *in vivo* (Ostareck-Lederer et al., 2002; Ostrowski et al., 2000). The c-Src mediated phosphorylation of hnRNP K causes a reversible loss of the binding with DICE in the mRNA 3'UTR, and specifically de-represses the translation of DICE-containing

mRNAs *in vivo* (Ostareck-Lederer et al., 2002). In addition, the same authors suggested that hnRNP K is unlikely to be a substrate of Src kinase, but rather functions as its activator. Together, the ERK and c-Src are two examples of different mechanisms that control the role of hnRNP K in the regulation of translation in two opposite directions – repression and stimulation. Other mechanisms have also been recognized, as the enhancing of translation initiation through binding to eIF4E (Lynch et al., 2005), and many are probably yet to be identified.

DNA damage: DNA damage causes rapid upregulation of hnRNP K, as assessed through differential proteomics. After DNA damage hnRNP K is stabilized by inhibition of its HDM2-mediated ubiquitin-dependent degradation. In fact, hnRNP K proteins from non-irradiated cells were found to co-immunoprecipitate with HDM2. This upregulation of hnRNP K also depended on ATM or ATR DNA-damage signalling kinases (Moumen et al., 2005). In the same work, hnRNP K was reported to cooperate with p53 to activate p53 target genes, thereby triggering cell-cycle checkpoint events. This cooperation seems to be done at the level of transcription instead of downstream steps (Moumen et al., 2005).

Oxidative stress: Oxidative stress has been reported to stimulate tyrosine phosphorylation of hnRNP K in cultured cells and intact livers, but the responsible kinase(s) was not identified. This phosphorylation enhances the binding of Lck and Vav to the hnRNP K protein *(in vivo)*, with a consequent decrease in the binding to poly(C) RNA (*in vitro)*. Also, phosphorylation of hnRNP K on tyrosine activates PKCδ with consequent phosphorylation of the RNP on Ser302. Therefore, under oxidative stress there is a chain of phosphorylation reactions and the hnRNP K protein may release a large repertoire of RNAs (Ostrowski et al., 2000).

Disease: Dysregulation of hnRNP K levels has been correlated to several diseases. The role of hnRNP K protein in several types of cancer has been gradually dissected. In a recent study, upregulation of hnRNP K levels was associated to development of malignancy and metastasis, mainly by regulation of the extracellular matrix, cell motility and angiogenesis pathways. Exciting results were observed using hnRNP K antibodies, with reduction of chemotaxis, cell viability, and colony-forming efficiency, with a global reduction of tumoral size (Gao et al., 2013).

In addition, a model of toxic RNA-mediated gain of function, dependent on hnRNP K, was proposed in SCA10. SCA10 is an autosomal dominant

neurodegenerative disorder characterized by ataxia and seizures. In SCA10 cells, hnRNP K is sequestered by the spliced intron-9 containing an expanded AUUCU repeat. hnRNP K protein sequestration may trigger the recruitment of PKCδ to mitochondria thereby activating caspase-3 and initiating apoptosis (White et al., 2012). Interestingly, a decrease in the number of neurons at the hippocampal CA3 region was observed in transgenic mouse model of the disease, starting at 6 months of age. They also presented morphological defects, such as small perikarya, corkscrew-like dendrites, and densely basophilic nuclei (White et al., 2012).

Neurons: Despite its abundance and important roles, very few studies have addressed the role of hnRNP K in neurons. A study to visualize the distribution of hnRNP K in the rat brain showed an enrichment of the protein in the cerebellum, cortex and hippocampus during development. An SH3-mediated interaction of hnRNP K with the Abi-1 protein was also observed at postsynaptic densities. Double knockdown for these proteins resulted in enlarged dendritic trees, with increased filopodia shape, and a decrease of the number of mature synapses (Proepper et al., 2011).

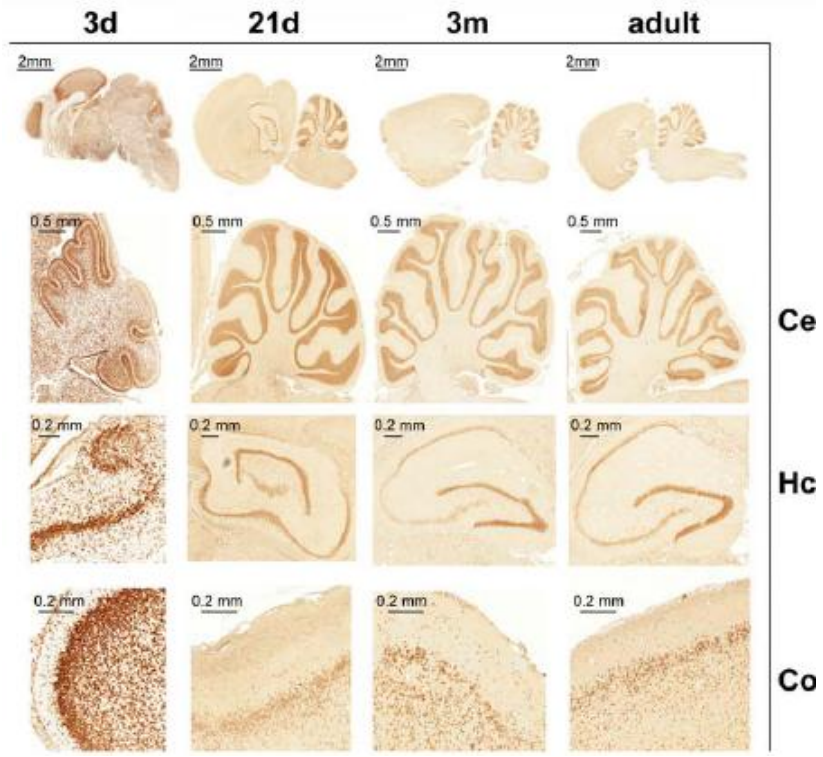

Figure 8 - Expression of hnRNP K mRNA in the CNS during development assessed by *in situ* hybridization; 3d – 3 days, 21d – 21 days, 3m – 3 months. Ce- cerebellum, Hc- hippocampus and Co- cortex. From (Proepper et al., 2011).

Forskolin induces neuronal differentiation through splicing regulation at the 3' polypirimidine tract. hnRNP K was shown to be essential for the neuritic growth in cells stimulated with forskolin. Interestingly, neither the location nor the abundance of hnRNP K changed after forskolin application, suggesting an important role for post translational modifications of the protein (Cao et al., 2012).

The role of hnRNP K in axonal development and repair has been investigated in *Xenopus laevis* neurons. hnRNP K protein knockdown disrupted the cytoskeleton polymer structure during the initial events of neuronal polarity generation and induced the loss of axons in embryos and cell cultures. These results are in accordance with the observed assembly of hnRNP K in messenger RNP complexes linking together multiple RNAs coding for proteins essential for axonal outgrowth, allowing their post-transcriptional regulation (Liu and Szaro, 2011). Axotomized optic axons of *Xenopus laevis*, which normally retain the ability to regenerate in adulthood, were unable to regrow after hnRNP K knockdown, pointing to another role of the protein (Liu et al., 2012).

Recent studies also addressed the role of hnRNP K in synaptic plasticity in hippocampal neurons (Folci et al., 2014). The protein was shown to play a role in the regulation of dendritic outgrowth, spine density and long-term potentiation, in cultured hippocampal neurons. Knockdown of hnRNP K reduced the length and branching of dendrites, and reduced the spine density. Moreover, a chemical LTP paradigm failed to increase the amplitude of mEPSCs in hnRNP K-silenced neurons. The deficits in LTP were attributed to a reduction on GluA1 S845-phosphorylation and impairments on GluA1 surface expression, attributed to inhibition of the ERK signalling pathway.

Future outlook: Several research groups contributed in the nineties to the characterization of hnRNP K protein. This was followed by many studies looking for its partners and its cellular functions. However these studies have been very limited in the cell types used, namely little work has been done in neurons. Considering the functions here described, its cellular distribution and abundance, hnRNP K is expected to be a very important player in signalling integration, through the transport of mRNAs and translation regulation at the level of neuronal dendrites where many of external cues arrive. The possible roles of hnRNP K in synaptic plasticity only recently have been addressed (Folci et al., 2014). However, previous results from our laboratory identified upregulated levels of hnRNP K in dendrites of cultured hippocampal neurons after

BDNF stimulation, suggesting that the effects of the neurotrophin in the regulation of translation in dendrites may be mediated, at least in part, by this RNP. Also, microarray analysis of the mRNAs co-immunoprecipitated together with hnRNP K showed a significant downregulation of several hnRNP K-bound transcripts following BDNF treatment (Comprido, 2011). The relevance of the effects of BDNF in the upregulation of hnRNP K protein levels along dendrites, and presumably in the dendritic release of mRNAs bound to the RNP, remains to be addressed. In particular, the results of the microarray experiments suggest that hnRNP K may act as a repressor of translation during the transport of mRNAs along dendrites, and these transcripts may become available to be translated following activation of TrkB receptors at the synapse.

1.6. Aims

Previous results from our laboratory have shown an active role of the neurotrophin BDNF in the regulation of hnRNP K levels in dendrites of hippocampal neurons. It was found that stimulation of cultured hippocampal neurons with BDNF increases the total levels of hnRNP K in dendrites and induces the phosphorylation of the protein, at Ser302. Furthermore, from the high number of transcripts shown to be associated with hnRNP K in cultured hippocampal neurons (more than eleven thousand), about 51% were partially released upon brief stimulation with BDNF, as assessed by RNA-coimmunoprecipitation assays and microarray analysis (Comprido, 2011). More recently, overexpression of hnRNP K in COS-7 cells was found to reduce total protein synthesis, an effect that was relieved by co-transfecting TrkB receptors (Rodrigues, 2013).

In the present work we aimed at further understanding the role of hnRNP K in the regulation of BDNF-induced synaptic plasticity, by addressing three main questions:

1) Is the dendritic enrichment in hnRNP K during chemical LTP dependent on BDNF actions and TrkB receptor activation?

Similarly to the increase of hnRNP K levels in dendrites of hippocampal neurons following chemical LTP (Folci et al., 2014), unpublished observations from our laboratory showed a dendritic enrichement of the protein in hippocampal neurons treated with BDNF. As at the time of the beginning of this project there was no evidence in the literature regarding the role of hnRNP K in synaptic plasticity, we

questioned whether the activity-dependent increase in dendritic hnRNP K levels requires BDNF-mediated activation of TrkB receptors (the major extracellular target of BDNF in hippocampal neurons), and whether this effect could be observed in other LTP paradigms.

2) Does hnRNP K regulate dendritic protein synthesis during BDNF-induced LTP?

Since hnRNP K regulates several aspects of mRNA metabolism and belongs to a family of proteins which includes several RNA-binding proteins involved in the regulation of mRNA transport and translation in dendrites, we hypothesized that this protein could have similar functions. As a first approach, we used the SUnSET technique to evaluate the effect of hnRNP K knockdown on the levels of protein synthesis in dendrites of cultured hippocampal neurons.

3) Does hnRNP K regulates the surface expression of GluA1-containing AMPA receptors during BDNF-induced LTP?

hnRNP K interacts with several proteins through the K protein interactive region (KI), and its three K-homology (KH) domains account for the binding of the protein to transcripts rich in cytosine. Due to its large set of partners, it is possible to hypothesize that the activity-induced increase of hnRNP K would be a by-stand consequence of the complex mechanisms occurring after BDNF stimulation. To access the functional relevance of hnRNP K regulation by BDNF, we investigated the impact of hnRNP K knockdown on the BDNF-induced increase in surface expression of GluA1-containing calcium-permeable AMPA receptors. BDNF is a crucial player involved in the regulation of AMPA receptor trafficking (Caldeira et al., 2007a; Carvalho et al., 2008), being the synaptic incorporation of AMPA receptors widely described to be necessary for the enhancement of synaptic efficacy in different LTP paradigms (Malenka, 2003). hnRNP K was hypothesized as a player in this regulatory mechanism since the protein interacts with the GluA1 mRNA (Comprido, 2011).

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2. Methods

2.1. Hippocampal cultures

Low-density hippocampal cultures were prepared as previously described (Kaech and Banker, 2006). Briefly, hippocampi were dissected from E18 - E19 Wistar rat embryos and the cells were dissociated using trypsin (0.06%, 15 min at 37°C). Neurons were plated at a final density of 1-5 x 10^4 cells/dish, on poly-D-lysine-coated glass coverslips, in neuronal plating medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1 mM pyruvic acid). After 2-4 h the coverslips were flipped over an astroglial feeder layer in Neurobasal medium (GIBCO - Life Technologies) supplemented with SM1 supplement (1:50 dilution, STEMCELL Technologies), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin (GIBCO - Life Technologies). The neurons grew face down over the feeder layer but were kept separate from the glia by wax dots on the neuronal side of the coverslips. To prevent overgrowth of glial cells, neuron cultures were treated with 5 μM cytosine arabinoside (Sigma-Aldrich) after 2 days *in vitro* (DIV). Cultures were maintained in a humidified incubator with 5% CO_2/95% air at 37°C for up to 2 weeks, feeding the cells once per week.

2.2. Cortical cultures

High-density cultures of rat cortical neurons were prepared from the cortices of E18 - E19 Wistar rat embryos. Briefly, cortices where washed with cold HBSS three and five times, prior and after trypsin (0.06%, 15 min at 37^0C) treatment, respectively. Cells were mechanically dissociated, no more than 10-15 times with HBSS. After counting, the cells were plated with neuronal plating medium (MEM supplemented with 10% horse serum, 0.6% glucose and 1mM pyruvic acid) for 2-4 h in 6- or 24-well plates ($94.7x10^3$cells/cm^2) coated with poly-D-lysine (0.1 mg/mL). After this period, the plating medium was removed and replaced by Neurobasal supplemented with SM1 supplement (1:50 dilution, STEMCELL Technologies), without glutamate, but containing 0.5 mM glutamine and 0.12 mg/mL gentamycin (GIBCO - Life Technologies). After 2 days in culture, division of glial cells was halted by addition of 10 μM 5-FdU-NOAC (5-FDU) to the medium. The culture was maintained in a humidified incubator with 5% CO_2/95% air, at 37°C.

2.3. Viral production and neuronal infection

pTRIP vectors were used to generate lentiviral shRNA vectors (Table I) for hnRNP K knockdown following methods previously described (Janas et al., 2006). Briefly, lentiviruses were generated by triple calcium-phosphate transfection of pTRIP-shRNA (coding also GFP), pCMV-ΔR8.91, and pMD.(VSVG) (which provide structural viral proteins) into HEK293T cells. The design of the viral vectors was performed in collaboration with Doctor Ramiro Almeida (CNC, Coimbra).

HEK293T cells grown for 2 days in 10 cm petri dishes until they reached about 60% confluence. A solution of $CaCl_2$ and DNA (Helper plasmids: 10 μg pCMV-ΔR8.91, 6 μg pMD.G(VSVG); plasmid with the specific constructs: 5 μg pTrip-shRNA) was added drop-wise to a solution of 2x HEPES buffered saline (HBS) (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.0). The solution of calcium-DNA was drop-wise added to 2xHBS and rested for 10 min to form the precipitates. The precipitates were then distributed evenly over the HEK293T cultures. The cells were allowed to incorporate the precipitates for 6 hours and were further incubated for about 60 hours to express the plasmid content. During these periods cells were maintained at 37ºC, with saturating humidity and 5% CO_2/95% air. After expression, the cellular media containing the lentivirus was collected, filtered using a cellulose acetate 0.45 μL syringe filter (Frilabo) and centrifuged at 22,000 rpm for 120 min at 22ºC (Beckman Coulter, Optima^{TM} L-100 XP ultracentrifuge). The supernatant was discarded, and 200 μL of 1% BSA (prepared in PBS) was added. The solution was kept vortexing for 40 min to allow the resuspension of lentivirus. The final solution was stored at -80ºC.

After preparation of the virus, the viral titers and the of multiplicity of infection (MOI) were determined using high-density cultures of cortical neurons, and using the following formulas:

$$\text{Titer (TU/ml)} = \frac{\text{Target cell number x \% of GFP} - \text{positive cells}}{\text{Volume of viral supernatant (ml)}/100}$$

$$\text{MOI} = \frac{\text{Volume of viral supernatant (ml) x titer (TU/ml)}}{\text{Target cell number}}$$

Neuronal cultures were transduced at different time points with a MOI equal to 5, which represents about 80% of neuronal infection. High-density cortical cultures

were transduced in 3 ml of conditioned media for 6 h, which was replaced by new neuronal media with glutamate after that period. Coverslips with low-density hippocampal neuronal cultures growing over a layer of astroglia cells were transferred to sterile 12 multiwell plates where the cells were transduced for 6 h in 500 µl of conditioned media. After that period, the coverslips were gently washed in sterile PBS and then transferred to the wells containing the astroglia cell layer. Neurons were allowed to express the shRNA for two, three, or four days, as indicated in the figure captions. At 14 DIV the neurons were treated with the appropriate stimulation paradigm (described below) and processed for immunocytochemistry. When indicated, cells were processed for Western blot.

Table I: shRNAs sequences targeting the rat mRNA of hnRNPK (SH5 and SH6) or scrambled sequence (SH1). The shRNA sequences were then inserted in the pTRIP vector.

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

2.4. Neuronal stimulation

Activity-dependent changes on hnRNP K-distribution: At DIV 14 low-density cultures of hippocampal neurons were stimulated for 30 min with 100 ng/ml BDNF (Peprotech) or with 50 µM bicuculline (Tocris), 2.5 mM 4-AP (Tocris) and 10 µM glycine (Sigma-Aldrich), to increase synaptic activity. Where indicated, cells were pre-treated for 30 min with the Trk receptor inhibitor SHN722 (1 µM) (Gomes et al., 2012; Martin et al., 2011) or with the scavenger of extracellular ligands of TrkB receptors, TrkB-Fc (1 µg/ml) (R&D Systems), before stimulation with 100 ng/ml BDNF

(Peprotech) or with the cocktail solution containing bicuculline (50 μM bicuculline, 2.5 mM 4-AP and 10 μM glycine), respectively. The experiments were performed in a basal saline solution (132 mM NaCl, 4 mM KCl, 1.4 mM MgCl2 , 2.5 mM CaCl2 , 6 mM glucose, and 10 mM HEPES at a final pH 7.4).

Protein synthesis: When indicated, low-density cultures of hippocampal neurons were infected at DIV 10 with SH1, SH5 or SH6. At DIV 14 the cells were stimulated for 15 min with 50 ng/ml BDNF (Peprotech) while they were incubated with 0.5 μM puromycin (Sigma-Aldrich). Puromycin is a tRNA analogue that blocks the elongation phase of translation. Thus, newly synthesized proteins incorporate puromycin and could be identified using antibodies against puromycin. The experiments were performed in conditioned media.

Surface GluA1 expression: When indicated, low-density cultures of hippocampal neurons were infected at DIV 10 with SH1, SH5 or SH6. At DIV 14 the cells were stimulated for 15 min with 50 ng/ml BDNF (Peprotech). The experiments were performed in conditioned media.

2.5. Cortical culture extracts and western blot

The cultures were washed with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4 and 10 mM $Na_2HPO_4.2H_2O$, pH 7.4) before lysis with RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS, pH 7.5) supplemented with a cocktail of protease inhibitors (0.1 mM PMSF; CLAP: 1 μg/mL chymostatin, 1 μg/mL leupeptin, 1 μg/mL antipain and 1 μg/mL pepstatin; Sigma-Aldrich) and phosphatase inhibitors (50 mM NaF and 1.5 mM Na3VO4). The extracts were then sonicated and centrifuged at 16,100 x g for 10 min at 4ºC. Protein concentration in the supernatants was quantified using the BCA method (Pierce) and the extracts were then diluted with 2× concentrated denaturating buffer (125 mM Tris pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue) before incubation at 95ºC for 5 min. Protein samples were separated by SDS-PAGE, in 10% polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) in 10 mM CAPS buffer pH 11 with 10% of methanol (overnight, 4ºC, 40V), and immunoblotted. The blocking of the membranes was performed with 5% milk prepared in TBS supplemented with 0.1% Tween-20. Blots were incubated with primary antibodies

(overnight at 4°C), washed, and exposed to alkaline phosphatase-conjugated secondary antibodies (1 h at room temperature). Alkaline phosphatase activity was visualized by enhanced chemifluorescence (ECF) on the Molecular Dynamics Storm 860, and quantified using ImageJ analysis software. Anti-β-actin was used as loading control and the results were expressed after normalization.

2.6. Immunocytochemistry

Hippocampal neurons (low-density) were fixed in 4% paraformaldehyde/ sucrose (in PBS) for 15 min at room temperature and permeabilized with 0.3% Triton X-100 in PBS. The neurons were then incubated with 10% BSA in PBS, for 30 min at 37°C, to block non-specific staining, and incubated overnight at 4°C with the primary antibodies diluted in 3% BSA in PBS (Table II). The following primary antibodies and dilutions were used: anti-hnRNP K (sc-28380, 1:200; Santa Cruz Biotechnology), anti-MAP2 (ab5392, 1:10.000, Abcam), anti-PSD95 (D27E11, 1:200; Cell Signaling), anti-GFP (598, 1:200; MBL) and anti-Puromycin (3RH11, 1:1000, KeraFAST). The cells were washed 6 times with PBS for 2 min and incubated with Alexa Fluor 568 (1:500, Invitrogen), Alexa Fluor 488 (1:500; Invitrogen) and AMCA (1:200; Jackson ImmunoResearch) conjugated secondary antibodies, for 45 min at 37°C. After washing the cells 6 times with PBS for 2 min, the coverslips were mounted with a fluorescence mounting medium (DAKO). For the detection of surface GluA1 levels, live hippocampal neurons were incubated for 10 min at room temperature with the primary antibody anti-GluA1 (kind gift from Dr. Andrew Irving) diluted in conditioned medium. The cells were fixed, permeabilized and incubated overnight with the Alexa Fluor 568 (1:500, Molecular Probes) conjugated secondary antibody. The neurons were then processed for immunocytochemistry as described above.

T**able II: List of antibodies and dilutions used in each immunocytochemistry experiment**.

Experiment	Primary antibodies	Secondary antibodies
Activity-dependent changes on hnRNP K distribution	Anti-hnRNP K (1:200 ; mouse) Anti-MAP2 (1:1000 ; chicken) Anti-PSD95 (1:200 ; rabbit)	Anti-mouse Alexa Fluor 568 (1:500) Anti-chicken AMCA (1:200) Anti-rabbit Alexa Fluor 488 (1:500)
shRNA validation	Anti-hnRNP K (1:200 ; mouse) Anti-MAP2 (1:1000 ; chicken) Anti-GFP (1:500 ; rabbit)	
Protein synthesis	Puromycin (1:1000 ; mouse) Anti-MAP2 (1:1000 ; chicken) Anti-GFP (1:500 ; rabbit)	
Surface GluA1 expression	Anti-GluA 1 (1:300 ; sheep) Anti-MAP2 (1:1000 ; chicken) Anti-GFP (1:500 ; rabbit)	Anti-sheep Alexa Fluor 568 (1:500) Anti-chicken AMCA (1:200) Anti-rabbit Alexa Fluor 488 (1:500)

2.7. Fluorescence microscopy and quantitative fluorescence analysis

Imaging was performed on a Zeiss Observer Z.1 microscope using a 63x 1.4 NA oil objective. Images were quantified using the ImageJ image analysis software. For quantification, sets of cells were cultured and stained simultaneously, and imaged using identical settings. The protein signals were analysed after setting the thresholds, and the recognizable clusters under those conditions were included in the analysis. The number, area and the integrated intensity of hnRNP K particles and surface GluA1 clusters in secondary dendrites were determined and represented per dendritic area. For colocalization analysis, regions around thresholded puncta were overlaid as a mask in the PSD95 channel, and the integrated intensity, area and number of colocalized particles determined. The intensity of Puromycin staining was determined and also represented per dendritic area. When indicated, the GFP signal was used to identify neurons successfully infected. Statistical analysis of the results was performed using one-way ANOVA followed by Bonferroni Multiple Comparison test.

3. Results

3.1. Activity-dependent regulation of hnRNP K levels in dendrites

To understand the mechanisms that locally regulate the levels of hnRNP K in dendrites, we questioned whether the distribution of the protein in this compartment could be regulated by BDNF-mediated activation of Trk receptors. Low-density cultures of hippocampal neurons were stimulated at DIV14-15 with BDNF (100ng/ml, 30 min), in the presence or in the absence of SHN722 (1µM, 30 min), an inhibitor of the Trk family of receptors, with high affinity for TrkB. Cells were then fixed and immunostained for hnRNP K (red), the postsynaptic marker PSD95 (green), and the dendritic marker MAP2 (blue). Images were analyzed using ImageJ software and statistical analysis was performed by one-way ANOVA, followed by Bonferroni's multiple comparison test.

hnRNP K showed a punctate distribution in dendrites and BDNF treatment induced a robust ($P<0.001$) increase of about 34.6% on the integrated intensity of hnRNP K puncta in dendrites. A 24.6% increase in dendritic hnRNP K puncta area was also observed. As shown in figure 9, the number of hnRNP K puncta along dendrites remained unaltered following BDNF stimulation. Therefore, this data suggests that BDNF may increase the size of the granules containing hnRNP K and possibly the number of copies of the protein present in each granule, but it is unlikely to affect the number of the hnRNP K-containing granules in dendrites. Preincubation of hippocampal neurons with SHN722, thus inhibiting the activity of TrkB receptors, abolished the BDNF-induced effects on hnRNP K puncta intensity and area along dendrites. Although SHN722 seems to have a residual effect *per se* on the levels of hnRNP K, no changes were observed following stimulation with BDNF. Together, these results show that the BDNF-TrkB signalling plays a key role in the regulation of the dendritic distribution of hnRNP K during synaptic plasticity in the hippocampus.

In additional analysis of the immunocytochemistry experiments we evaluated the putative changes in the distribution of hnRNP K at the synapse following stimulation with BDNF, as determined by the colocalization with the postsynaptic marker PSD95. No significant changes were observed in the hnRNP K integrated fluorescence intensity at the synapse, the number of synaptic puncta, the area of the puncta at the synapse and the percentage of hnRNP K in synaptic sites (Figure 9E-H). The lack of effect of BDNF in the synaptic distribution of hnRNP K may be justified by the great variability observed between dendrites, with many measurements showing no colocalization

between PSD95 and hnRNP K, with a consequent increase in the magnitude of the error bars. hnRNP K is believed to be present mainly in granules bound to other proteins and mRNAs, which may not be present exactly at the synapses but on their vicinities causing an underestimation of the true levels of the RNP that is recruited to the synapses upon stimulation with BDNF.

Given the effect of BDNF on the dendritic distribution of hnRNP K, and the evidence showing that the neurotrophin is released in an activity-dependent manner, we characterized the alterations in the dendritic distribution of the RNP in low density cultures of hippocampal neurons stimulated with bicuculline (50 µM, 2.5 mM 4-AP, 10 µM glycine, 30 min) which upregulates the activity of the neuronal network. Furthermore, to investigate whether the activity-dependent changes in hnRNP K distribution require the action of endogenous released BDNF, hippocampal neurons were stimulated with bicuculline in the presence or in the absence of the extracellular scavenger TrkB-Fc (1 µg/ml, 30 min), a truncated and soluble form of the TrkB receptor that binds and sequesters BDNF.

Stimulation with bicuculline significantly increased the integrated intensity ($P<0.001\%$) and area of hnRNP K puncta in dendrites ($P<0.01\%$) (Figure 10A, B and C), by about 30.0% and 19.8%, respectively. However, bicuculline was without effect on the number of puncta along dendrites. Preincubation of the cells with TrkB-Fc prevented the bicuculline-induced increase in the intensity and area of the dendritic hnRNP K puncta (Figure 10 A, B and C). These results strongly suggest that the release of endogenous BDNF upon bicuculline treatment accounts for the activity-induced upregulation of dendritic hnRNP K levels.

Incubation of neurons with TrkB-Fc alone induced a slight decrease on the levels of hnRNP K at synapses (Figure 10A, E-H), although the effects were not statistically significant ($P>0.05\%$). These results stress the role of constitutively-released BDNF as an important regulator of neurons under resting conditions. Interestingly, subsequent incubation of neurons with bicuculline significantly increased the number of hnRNP K puncta that colocalizes with PSD95 ($P<0.05\%$) (Figure 10A and G). Thus, it is reasonable to suggest that neuronal activity regulates the recruitment of hnRNP K to the vicinities of synapses in a BDNF-independent manner.

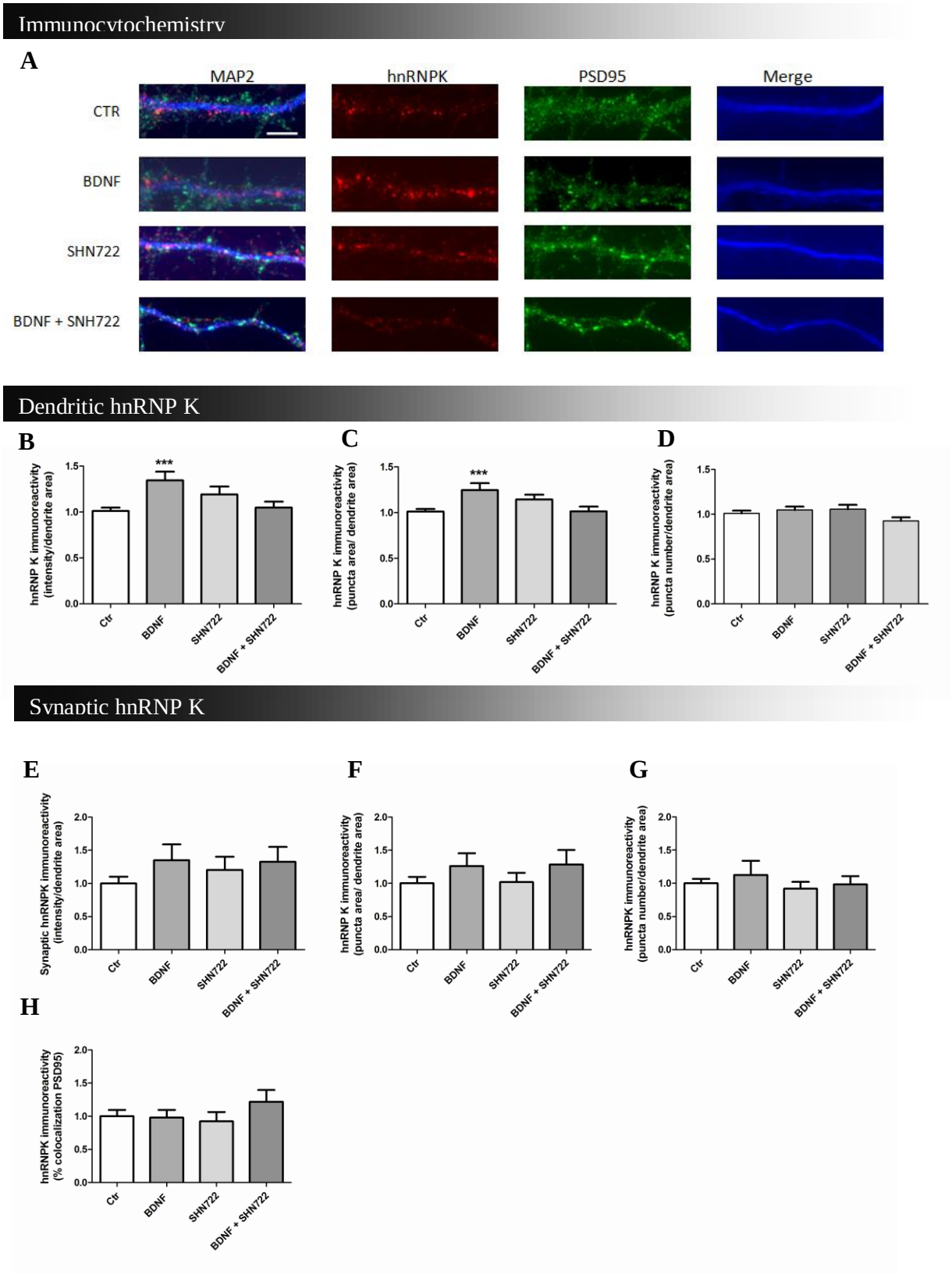

Figure 9 - BDNF increases hnRNP K in dendrites but does not induce a significant recruitment of this protein to synapses. Cultured hippocampal neurons (14-15 DIV) were stimulated or not with BDNF (100ng/ml) for 30 min. The role of Trk receptor activation in BDNF-induced regulation of hnRNP K levels along dendrites was tested using the inhibitor SHN722. In these experiments the cells were preincubated with the Trk receptor inhibitor for 30

min, and the inhibitor was also present during the period of incubation with the neurotrophin. (A) The cells were immunostained for hnRNP K (red), PSD95 (green), and MAP 2 (blue). The integrated fluorescence intensity, area and number of hnRNP K puncta in dendrites (B, C, and D) and at the synapse (as defined by the signal of hnRNP K that overlaps with PSD95) (E, F, and G) was analysed using ImageJ software and represented per dendritic area. The percentage of dendritic hnRNP K signal that colocalizes with PSD95 was also analysed (H). Results are normalized to control and are averaged of 4-10 different experiments performed in independent preparations. For the analysis of dendritic-localized hnRNP K puncta, Ctr (n=108 cells); BDNF (n=53 cells); SHN722 (n=41 cells); BDNF + SHN722 (n=51 cells). For the analysis of synaptic-localized hnRNP K puncta, Ctr (n=100 cells); BDNF (n=58 cells); SHN722 (n=43 cells); BDNF + SHN722 (n=54 cells). Error bars, mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni's multiple comparison test. *** $P<0.001$. Scale bar = 5 μm.

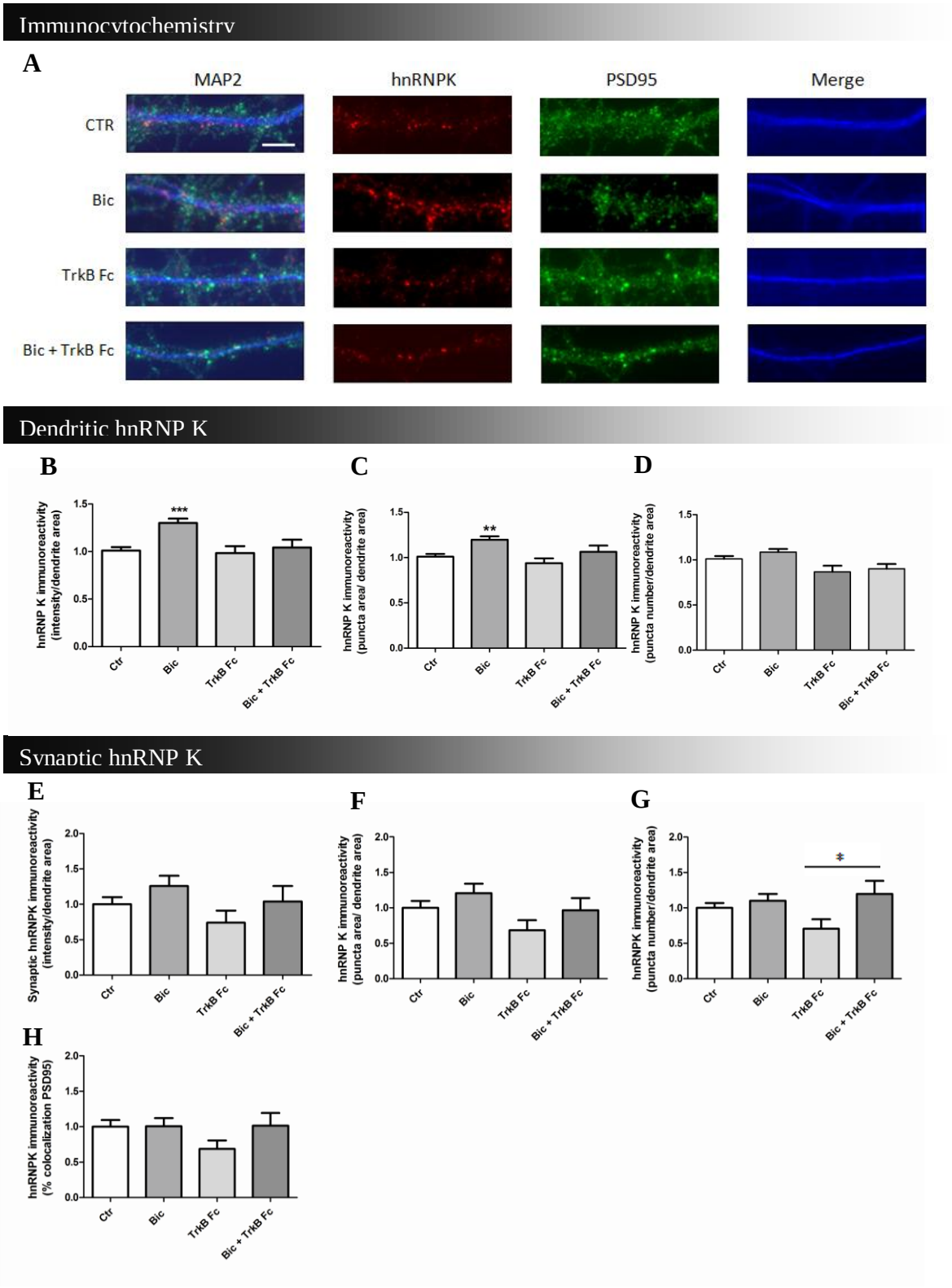

Figure 10 – Synaptic activity increases hnRNP K in dendrites but does not induce the delivery of the protein to the synapse. Cultured hippocampal neurons (14-15DIV) were stimulated or not with bicuculline (50 μM), 4-AP (2.5 mM) and glycine (10 μM), for 30 min. Where indicated, neurons were treated with the extracellular scavenger of TrkB ligands TrkB-Fc (1 μg/ml) for 30 min and were then stimulated or not with bicuculline in the presence of the BDNF scavenger. The cells were immunostained for hnRNP K (red), PSD95 (green), and MAP

2 (A). The integrated fluorescence intensity, area and number of hnRNP K puncta in dendrites (B, C, and D) and at the synapse (as defined by the signal of hnRNP K that overlaps with PSD95) (E, F, and G) was analysed using the ImageJ software and represented per dendritic area. The percentage of dendritic hnRNP K signal that colocalizes with PSD95 was also analysed (H). Results are normalized to control and are averaged of 4-10 different experiments performed in independent preparations. For the analysis of dendritic-localized hnRNP K puncta, Ctr (n=108 cells); Bic (n=97 cells); TrkB-Fc (n=49 cells); TrkB-Fc + Bic (n=52 cells). For the analysis of synaptic-localized hnRNP K puncta, Ctr (n=100 cells); Bic (n=91 cells); TrkB-Fc (n=40 cells); TrkB-Fc + Bic (n=43 cells). Error bars, mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni's multiple comparison test. * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Scale bar = 5 μm.

3.2. Validation of the small hairpin RNA (shRNA) constructs

To study the possible functions of hnRNP K in synaptic plasticity, two different small hairpin RNAs with complete specificity for rat hnRNP K mRNA were designed (SH5 and SH6), as well as a shRNA carrying a scrambled sequence (SH1) that was used as a control (Table II). Before starting the lack-of-function experiments, the three constructs were tested to determine their validity and efficiency.

To test the validity of each construct we used western blot to quantify the total levels of hnRNP K protein in dense cultures of cortical neurons infected with a MOI of 5. Neurons were allowed to express the shRNAs for two, three, or four days to track their efficiency throughout time. Figure 11 shows that after expressing the shRNA constructs for two, three, or four days, both SH5 and SH6 shRNAs showed no effect on total hnRNP K protein expression when compared to control or SH1-infected neurons. However, at four days of infection it was possible to identify a trend suggesting that SH5 and SH6 shRNAs may decrease the total levels of hnRNP K.

The effect of SH5 and SH6 shRNA constructs in knocking-down hnRNP K is likely to be underestimated in the western blot experiments performed in dense cultures (Figure 11) since there are neurons that are not infected and, therefore, will not show alterations in hnRNP K protein levels. Furthermore, since hnRNP K is mainly present in the soma compartment, with a comparatively small pool of the protein being present in dendrites (Figure 12), the estimation of the efficiency of the anti-hnRNP K shRNAs by western blot is likely to reflect mainly the downregulation of the protein in the soma. Since the half-life of hnRNP K in neurons is not known, and the kinetics of downregulation of the protein in the soma and in dendrites may be distinct, we assessed the effects of the three shRNAs on the hnRNP K protein levels in the soma and in

dendrites of cultured hippocampal neurons, using immunocytochemistry. In these experiments, the effect of shRNA delivery on hnRNP K expression was evaluated by staining the neurons for hnRNP K, the dendritic marker MAP2, and against GFP, which is an indicator of successful infections.

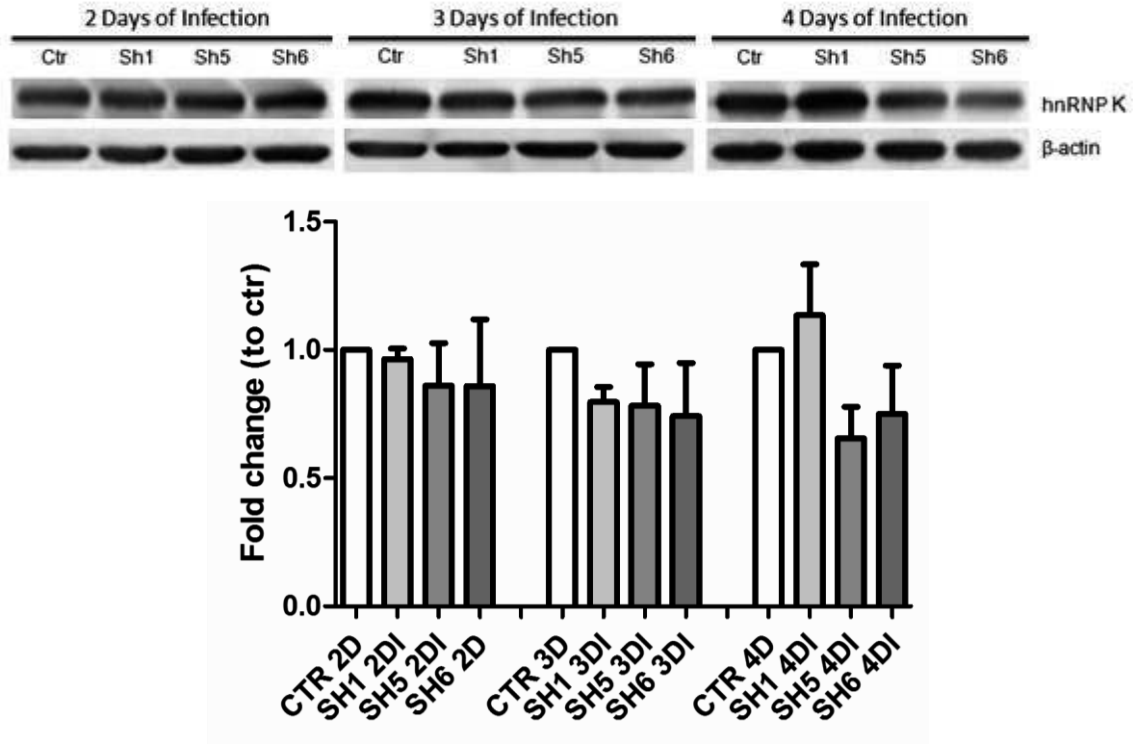

Figure 11 - Validation and measurement of the kinetics of hnRNP K protein downregulation following expression of the scrambled (SH1) and specific shRNA constructs targeting hnRNP K (SH5 and SH6). High density cultures of cortical neurons were infected from DIV10 to DIV12 with a MOI of 5. Protein extracts were prepared at DIV14, and the levels of hnRNP K protein were assessed by western blot. hnRNP K immunoreactivity was normalized for the signal of β-Actin, and the values were then normalized for the specific control condition according to the duration of viral expression. The results plotted correspond to two different experiments performed in independent preparations.

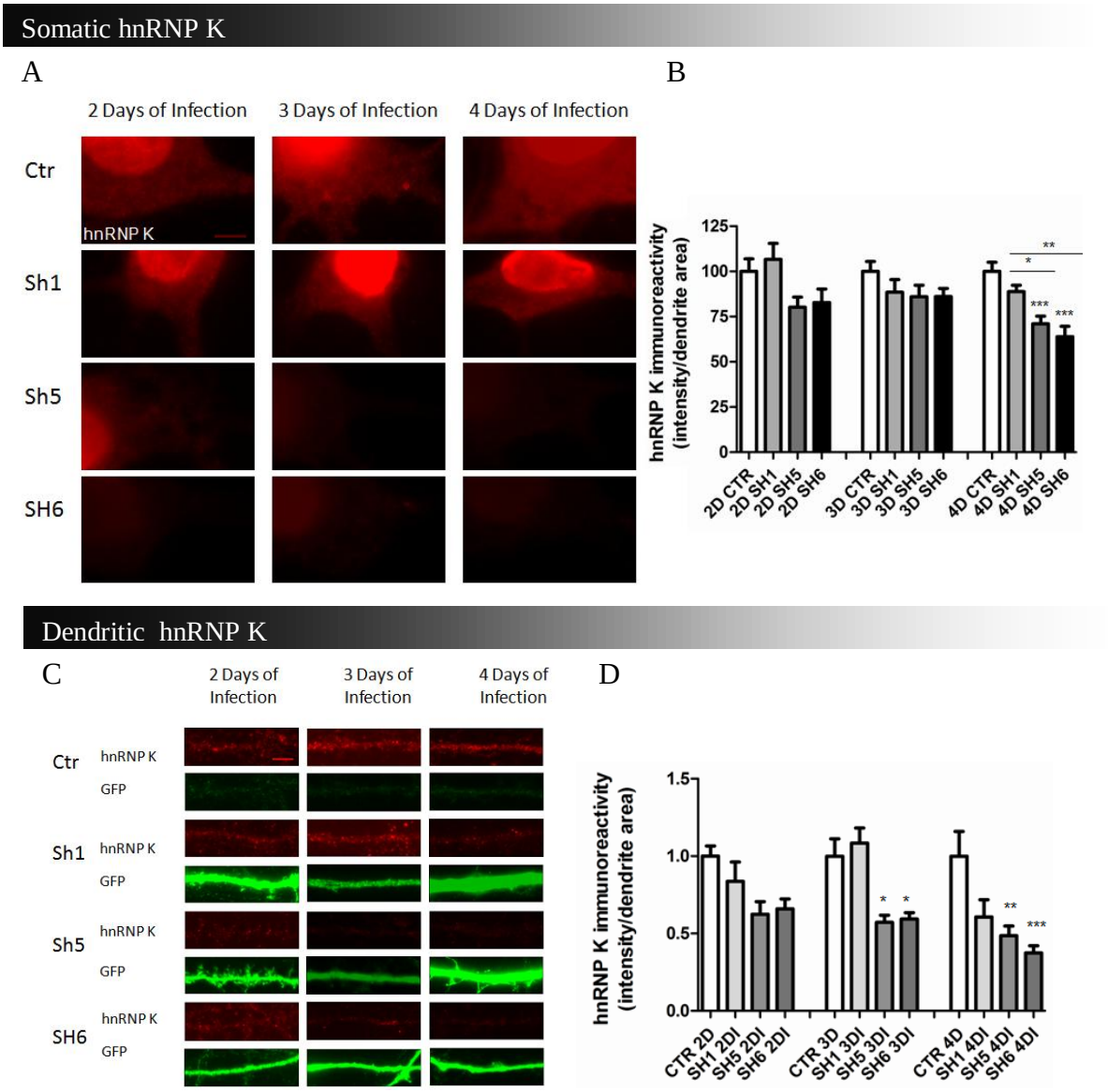

Figure 12 - Validation and measurement of the kinetics of hnRNP K protein downregulation for the scrambled (SH1) and specific designed shRNA constructs targeting hnRNP K (SH5 and SH6) by immunocitochemistry. Low density cultured hippocampal neurons were infected from DIV10 to DIV12 with a MOI of 5. The cells were fixed at DIV14 and then immunostained for hnRNP K (red), GFP (green), and MAP2 (not shown). The hnRNP K signal intensity was measured at the cell soma, outside the nucleus (A and B), and in dendrites (C and D), using the ImageJ software. Each condition represents two experiments performed in independent preparations, with the exception of the experiments performed in neurons expressing the shRNA constructs for 2 days, which represent only one experiment. Error bars, mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni's multiple comparison test. * P<0.05; ** P<0.01; *** P<0.001. Scale bar = 5 μm.

Measurement of the hnRNP K immunoreactivity in the soma, avoiding the nuclear region, showed a significant reduction on hnRNP K protein levels after four days of expression of SH5 and SH6 shRNAs when compared to control (P<0.001%) and SH1-infected neurons (P<0.05% for SH5; P<0.01% for SH6) (Figure 12A and B). Two and three days of shRNAs expression did not affect significantly the intensity of hnRNP K signal (Figure 12A and B).

At secondary dendrites, three days of expression of SH5 and SH6 was sufficient to induce a significant reduction of about 40% on hnRNP K levels (P<0.05%) (Figure 12C, D), and four days of expression induced an even more dramatic effect on hnRNP K expression, with a knockdown of about 50% with SH5 (P<0.01%) and 60% with SH6 (P<0.001%) (Figure 12C, D). With two days of shRNAs expression, the rate of infection is much lower and we observed no effects on hnRNP K expression both in the cell soma and dendrites (n=1). As expected, the scrambled shRNA (SH1) did not affect significantly the dendritic hnRNP K protein levels at 2 and 3 days of expression but, surprisingly, a reduction in the abundance of the protein in the dendritic compartment was detected at day 4. If this effect is confirmed by additional experiments, suppression of dendritic hnRNP K with SH5 and SH6 shRNAs should be performed using 3 days of infection.

3.3. Regulation of dendritic protein synthesis by hnRNP K

The heterogeneous nuclear ribonuclear family of proteins comprises several members involved in virtually all stages of mRNA metabolism (Han et al., 2010). Due to the capacity of hnRNP K to bind nascent transcripts and its multiple protein partners, and since this ribonucleoprotein is thought to be present in dendritic RNA granules, we hypothesized a role for hnRNP K in the regulation of local protein synthesis. A similar role for hnRNP K was observed in other cell types such as in oligodendrocytes (Torvund-Jensen et al., 2014).

A role for hnRNP K in the regulation of translation activity in dendrites was investigated in low density cultures of hippocampal neurons using the SUnSET method (Schmidt et al., 2009). Hippocampal neurons were infected at DIV10 with a MOI of 5 and allowed to express the small hairpin RNAs for four days. To investigate whether hnRNP K is required for BDNF-induced protein synthesis in dendrites, neurons were treated at DIV14 with puromycin (0.5 µM) for 15 min in the presence or in the absence

of BDNF (50 ng/ml). Puromycin is a chain-terminating analogue of tyrosil tRNA which tags carboxyl terminus of nascent proteins, and can be detected by immunocytochemistry to analyze protein synthesis in the dendritic and soma compartments. Expression of GFP was used as an infection reporter.

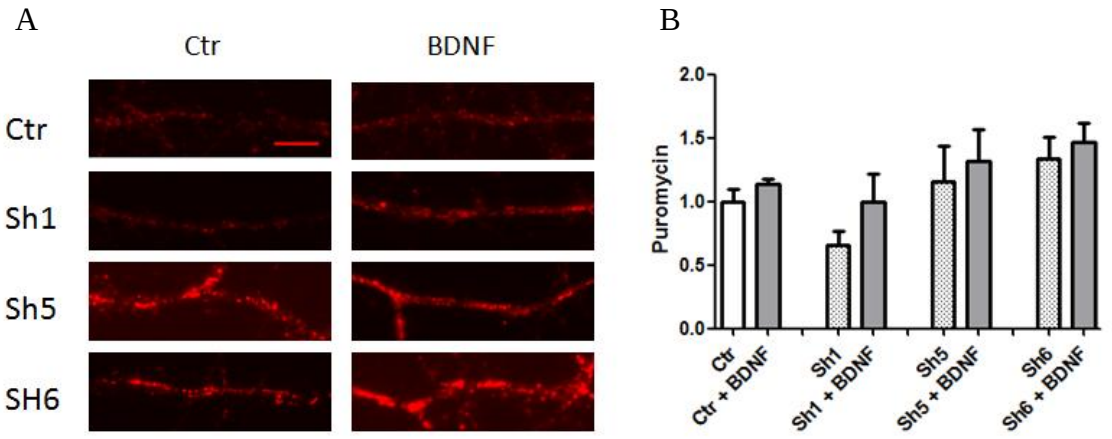

Figure 13 - Analysis of protein synthesis in neuronal dendrites by the SUnSET method. Cultured hippocampal neurons were incubated at DIV14 with 0.5 µM puromycin for 15 min, in the presence or in the absence of BDNF (50 ng/ml). Where indicated, neurons were allowed to express the shRNA constructs SH1, SH5 or SH6 for four days. Cells were immunostained for Puromycin (red), GFP (not shown), and MAP2 (not shown) (A). Puromycin immunoreactivity was normalized for the area of MAP2, and each condition was normalized for the control (B). The bar chart includes the results of one experiment. Error bars, mean ± SEM. Scale bar = 5µm.

Comparison of the total levels of dendritic protein synthesis under control conditions and in hnRNP K-silenced neurons, shows a trend to increase protein synthesis when hnRNP K protein levels are decreased. This is particularly notorious when comparing the results obtained with the SH5 and SH6 shRNAs with the scrambled shRNA (Figure 13A and B). These results suggest that indeed hnRNP K may modulate dendritic protein synthesis in hippocampal neurons under resting conditions.

We also tested the effect of 15 min incubation with BDNF on the translation activity in dendrites, but no significant changes in protein synthesis were detected using the SUnSET method (Figure 13). This may be due to the short incubation period tested in these experiments.

3.4. Regulation of surface GluA1 expression by hnRNP K

BDNF-induced LTP requires synaptic and extrasynaptic changes at different time scales, from post-translational modifications of specific proteins, to the novo synthesis of proteins at the cell soma and in dendrites (Leal et al., 2014). Recently, it was reported that treatment of hippocampal neurons with BDNF (50ng/ml) induces the synthesis, trafficking and membrane insertion of GluA1-containing calcium-permeable AMPA receptors (Fortin et al., 2012). hnRNP K is associated with the mRNAs coding for GluA1 in cultured hippocampal neurons and BDNF induces the dissociation of the transcripts from hnRNP K-containing complexes locally at the synapse (Comprido, 2011). Thus, we next questioned whether hnRNP K plays a role in BDNF-induced GluA1 membrane insertion. For this purpose, and to investigate the possible functional relevance of hnRNP K during BDNF-induced LTP, we measured the surface levels of GluA1 containing AMPA receptors using a live-cell labelling technique. The neurons were infected at DIV 10 and allowed to express the shRNA constructs for four days to ensure the successful knockdown of hnRNP K.

The knockdown of hnRNP K induced a slight increase in the surface expression of GluA1, although no statistically significant differences were observed (Figure 14A-D). In addition, BDNF showed a slight trend to further increase the surface levels of GluA1-containing receptors, with the exception of SH5-infected cells. Interestingly, normalization of the results obtained in hippocampal neurons stimulated with BDNF for the same condition without BDNF, showed a robust effect of BDNF under control conditions which was not observed in infected cells (Figure 14A and E). However, it is surprising that the scrambled shRNA also decreases the effect of BDNF in GluA1 surface levels, which may be attributed to the unexpected effect of this construct when expressed for 4 days (Figure 12). These preliminary results suggest that knockdown of hnRNP K affects the mechanisms regulating the surface expression of GluA1-containing receptors activated upon BDNF stimulation.

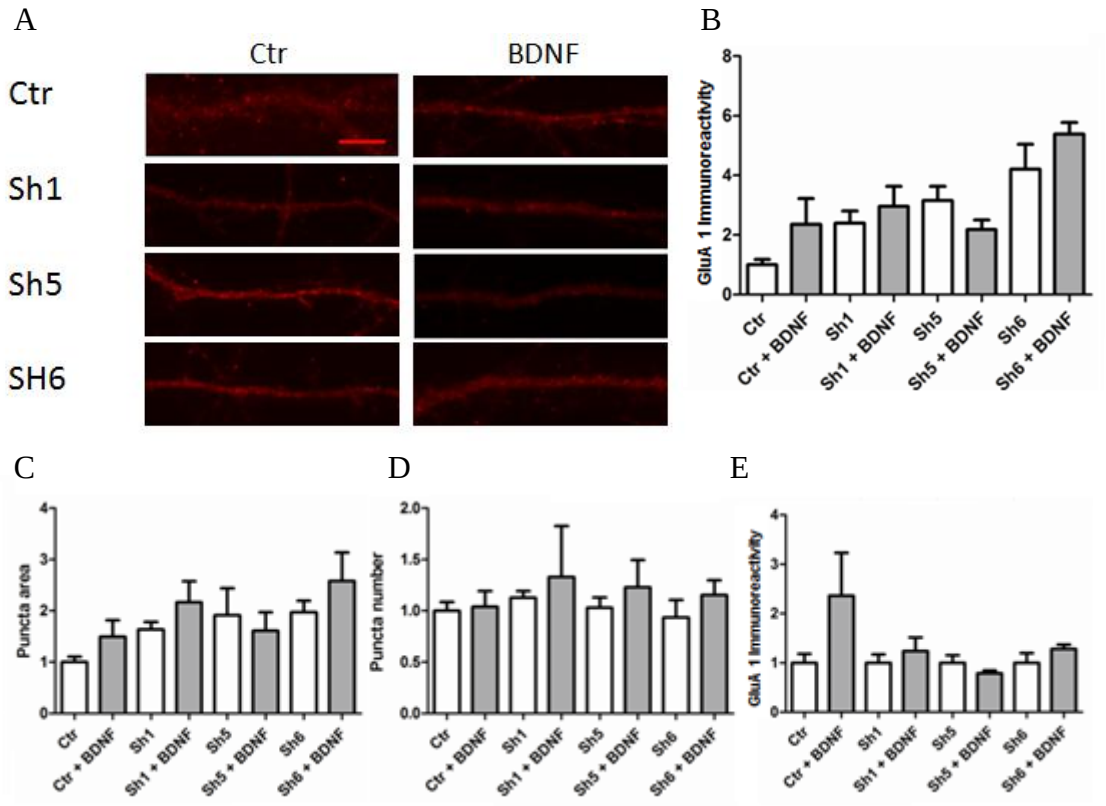

Figure 14 - Effect of hnRNP K knockdown on BDNF-induced GluA1 surface expression. (A) Culture hippocampal neurons (DIV14) were live-stained for GluA1 (red), and immunostained for GFP (not shown) and MAP2 (not shown). Where indicated, neurons expressing the shRNA constructs SH1, SH5 or SH6 for four days, were stimulated with BDNF (50 ng/ml) for 15 min or kept in neuronal conditioned medium. The intensity of GluA1 puncta (B), puncta area (C), and the number of puncta (D) were measured using the ImageJ software; the results were calculated per area of MAP2, and normalized for the control. The immunoreactivity of GluA1 measured following BDNF stimulation was normalized for the MAP2 area, and then normalized for the respective condition without BDNF (E). Error bars, mean ± SEM. Scale bar = 5 µm.

4. Discussion

In the first part of this work, we showed that TrkB signalling mediates the enrichment of hnRNP K in dendrites of cultured hippocampal neurons after BDNF treatment. Additionally, increasing the neuronal excitability with bicuculline also raised the levels of hnRNP K in dendrites by a mechanism dependent on endogenous BDNF. The synaptic levels of hnRNP K, as assessed by the colocalization of the ribonucleoprotein with the postsynaptic marker PSD95, did not change significantly with the treatments applied. Only the number of synaptic hnRNP K puncta increased after bicuculine treatment, but this effect was not sufficient to raise significantly the percentage of colocalization between hnRNP K and PSD95.

It is known that BDNF regulates synaptic transmission and modulates synaptic plasticity (such as LTP) in the hippocampus as well as in other brain regions. In the hippocampus BDNF acts at pre- and postsynaptic sites, through activation of TrkB receptors. The postsynaptic effects include the regulation of the transport and translation of dendritic-localized mRNAs. Furthermore, deficiencies on BDNF release and TrkB signalling in the hippocampus have been associated to lower performances in certain learning tasks (Leal et al., 2014). The finding that dendritic hnRNP K levels are regulated by BDNF-mediated TrkB signalling suggests a correlation between these biochemical postsynaptic effects of BDNF and the docking properties of hnRNP K. The activity-dependent and BDNF-mediated upregulation of dendritic hnRNP K is likely to be accompanied by an increase in its phosphorylation state and the release of a large number of mRNAs, including transcripts coding for proteins relevant in synaptic plasticity (Comprido, 2011; Leal, 2014). Further experiments should address how important is hnRNP K for BDNF-induced synaptic alterations and identify the steps at which hnRNP K may act.

Studies describing the activity-dependent synaptic functions of other hnRNP proteins further support a possible role of hnRNP K in synaptic plasticity. An increase in synaptic activity was found to change about 2% of the postsynaptic proteome of primary neuronal cultures (Zhang et al., 2012). These modifications included the synaptic accumulation of several members of the hnRNP family of proteins, including hnRNP A2/B1, G, M and D. In cultured hippocampal neurons hnRNP A2 is also recruited to dendrites alongside with transcripts coding for proteins with well established roles in synaptic plasticity, such as the Arc, CaMKIIα, and neurogranin (Gao et al., 2008). In oligodendrocytes, a model was proposed to explain the transport

and regulation of translation of the myelin basic protein (MBP) mRNA at the cellular projections. According to this model, hnRNP A2 may be responsible for transport, while hnRNP E1 and hnRNP K have an inhibitory and stimulatory effect on translation, respectively (Torvund-Jensen et al., 2014). In addition, hnRNP K was shown to bind Abelson interactor protein 1 (Abi-1) and to regulate the dendritic sprouting, the spine density and maturation in hippocampal neurons (Proepper et al., 2011).

In two studies using cell lines and *in vivo* models, hnRNP K was shown to accumulate at the cytoplasm after ERK activation and ERK-dependent phosphorylation of the serine residues 284 and 353 (Habelhah et al., 2001; Mikula et al., 2006). Accordingly, a recent study addressed the possible role of ERK in the modulation of hnRNP K and the impact in synapse function in cultured hippocampal neurons (Folci et al., 2014). The authors demonstrated that hnRNP K regulates dendritic spine density, basal synaptic transmission and LTP. Moreover, they found that LTP requires the expression and phosphorylation of hnRNP K, which is essential for ERK pathway activation, GluA1-Ser845 phosphorylation and membrane insertion of GluA1-containing AMPA receptors. These findings are in accordance with our results since ERK is also a downstream element of TrkB receptor signalling and the authors also reported an increase in the cytoplasmic levels of hnRNP K following LTP. It is tempting to speculate that BDNF-TrkB signalling may also be involved in the ERK-mediated phosphorylation and cytoplasmic accumulation of hnRNP K in response to the chemical LTP paradigm. Here we show that endogenous released BDNF is essential for the cytoplasmic enrichment of hnRNP K induced by bicuculline treatment. Moreover, BDNF was shown to increase hnRNP K phosphorylation at Ser302 (Comprido, 2011), which is believed to be mediated by PKC (Schullery et al., 1999), other downstream kinase of TrkB receptor signalling. Nonetheless, as PKC and ERK phosphorylate hnRNP K at different sites, they may regulate distinct processes and/or the affinity for partners with different cellular functions.

It is important to note, that unlike hnRNP A2/B1 (Leal, 2014), increased neuronal activity did not significantly change the synaptic levels of hnRNP K. Although it is possible that the true values of hnRNP K at the synapse could have been underestimated, this different outcome comparing to hnRNP A2/B1 may indicate a functional distinction between both proteins. hnRNP K may regulate elements not

located at synapses but in the dendritic shaft or in the spine neck, such as specific cytoskeleton or translation machinery components, among others.

Altogether, previous results from our laboratory and the evidence collected in this work strongly suggest that hnRNP K levels are tightly regulated, which is functionally relevant given the important functions of the protein in synaptic plasticity (Folci et al., 2014). To study the possible dendritic functions of hnRNP K we conducted a lack-of-function set of experiments as first approach. Two specific shRNA constructs were designed to completely match rat hnRNP K mRNA, induce its degradation and at last to reduce the hnRNP K protein levels. Although the majority of studies in the literature ignore the existence of four splice variants of hnRNP K, it is believed that only the splice variants 1 and 2 are found in the cytoplasm (Dejgaard et al., 1994). Accordingly, our shRNA construct SH5 targets the hnRNP K ribonucleotides 980-998, and the construct SH6 binds ribonucleotides 1201-1219, being both sequences found outside exon A (15bp after position 1316) and exon B (60bp after position 1569). Virtually our constructs have affinity to all splice variants of mature hnRNP K mRNA and are therefore able to induce their degradation, guaranteeing the successful knockdown of hnRNP K protein expression. Additionally, a construct (SH1) able to form a hairpin structure but with no target sequence in hnRNP K mRNA was used as control. These constructs were incorporated into pTRIP vectors to generate lentiviral shRNA vectors.

Cultured hippocampal neurons were infected with lentiviral shRNA vectors and allowed to express the shRNAs for two, three or four days (Figure 12). As hnRNP K has many described nuclear functions (Bomsztyk et al., 2004), we sought to significantly reduce dendritic hnRNP K levels, while keeping the nuclear levels of the protein as stable as possible. Four days of SH5 and SH6 expression resulted in a dramatic downregulation of hnRNP K levels in dendrites (50% and 60%, respectively) when compared to control neurons, while the soma-localized hnRNP K protein was only reduced in about 25%. Therefore, all other experiments were performed using hippocampal neurons expressing the shRNAs constructs for four days. Additional experiments will be required to rule out an effect of the scrambled shRNA used (SH1) in hnRNP K protein levels.

hnRNP K binds many mRNAs through its KH domains and may therefore act as a buffer of transcripts. In fact, a reduction of total protein synthesis in COS-7 cells was observed when hnRNP K was overexpressed (Rodrigues, 2013), presumably due to a reduction of free transcripts to be translated. In addition, the role of hnRNP K in repressing the translation of the target mRNAs has been widely described in several biological contexts (Bomsztyk et al., 2004). Previous studies from our laboratory demonstrated that hnRNP K specifically released a subpopulation of bound transcripts after a brief treatment of cultured hippocampal neurons or hippocampal synaptoneurosomes with the neurotrophin BDNF (Comprido, 2011). As a result we hypothesized that hnRNP K may assemble a pool of stable transcripts ready to be translated on demand in dendrites. To test this hypothesis, control (non-infected) or shRNA-expressing (for 4 days) cultured hippocampal neurons were incubated with puromycin to label nascent proteins. Although we did not observe statistically significant differences on the levels of protein synthesis in dendrites between the experimental conditions tested, which is likely due to the small number of experiments performed, hnRNP knockdown induced a slight increase on puromycin immunoreactivity. The increase in total dendritic protein synthesis observed in hnRNP K-silenced cells may be explained based on a decreased buffering capacity of transcripts by the remaining hnRNP K copies. However, hnRNP K may also be important for the transport and stabilization of mRNAs in dendrites. If this is the case, the reduction of hnRNP K in dendrites will also be accompanied by a reduction in the population of transcripts able to be translated in this subcellular compartment.

Our preliminary results also point for a lack of effect of a brief treatment with BDNF (50ng/ml for 15min) on dendritic protein synthesis. A longer period of TrkB activation may be necessary to induce visible effects of BDNF on dendritic protein synthesis in cultured hippocampal neurons. This particular experiment will provide further insights into the activity-dependent functions of hnRNP K in dendrites. Since BDNF induces protein synthesis in dendrites of hippocampal neurons (Dieterich et al., 2010), this experimental approach will allow evaluating whether hnRNP K is required for BDNF-induced dendritic protein synthesis.

Increased GluA1 surface expression is a hallmark of LTP and brief incubations with the neurotrophin BDNF also induce the surface delivery and synaptic incorporation of GluA1-containing AMPA receptors in cultured hippocampal neurons and in hippocampal slices (Caldeira et al., 2007a; Fortin et al., 2012). hnRNP K binds GluA1 mRNAs, being the transcripts released from the ribonucleoprotein complexes following treatment of hippocampal neurons or synaptoneurosomes with BDNF (Comprido, 2011; Leal, 2014). Our preliminary results suggest a slight increase on the surface GluA1 expression following hnRNP K knockdown in hippocampal neurons. Given the results suggesting a similar trend to increase total dendritic protein synthesis in neurons lacking hnRNP K, it is possible that hnRNP K silencing contributes to increase GluA1 local synthesis. Alternatively, hnRNP K may regulate AMPA receptor trafficking through other mechanisms that may not depend on the ability of the protein to modulate protein synthesis. In fact, recent findings suggest that hnRNP K silencing inhibited ERK-dependent GluA1-S845 phosphorylation which is essential for its surface delivery (Folci et al., 2014).

Brief treatment with BDNF induced a non-statistically significant trend to increase the surface expression of GluA1 in all conditions, with the exception of SH5-expressing neurons. However, when normalizing the surface levels of GluA1 measured after BDNF stimulation to the corresponding resting condition, it is clear that the BDNF-induced increase in GluA1 surface expression is more prominent in non-infected neurons, indicating that hnRNP K silencing somehow prevents the enrichment of GluA1-containing AMPA receptors in the plasma membrane following neurotrophin treatment. Using longer periods of incubation with BDNF, it was shown that the neurotrophin-induced surface enrichment in GluA1 is transcriptional and translational-dependent (Fortin et al., 2012). However, the slight increase in surface GluA1 expression observed in our experimental conditions may also reflect alterations in the trafficking of already existing subunits, especially considering the shorter incubation period with BDNF used. Indeed, as abovementioned, a recent study suggest that hnRNP K has a role in the activity-induced phosphorylation and membrane delivery of AMPA receptors in hippocampal neurons (Folci et al., 2014).

In the experiments performed in this work to assess BDNF-induced dendritic protein synthesis and surface GluA1 expression in hnRNP K-silenced neurons, the effects of the neurotrophin seem to be abolished. hnRNP K may therefore be an

important downstream element of the BDNF-TrkB signalling activated by synaptic activity. Although the surface GluA1 expression may give functional insights into the importance of hnRNP K in synaptic plasticity, particularly in LTP, electrophysiological and behavioural experiments would surely complement our understanding of the possible role of hnRNP K in learning and memory.

In addition to the alterations in the adult brain, hnRNP K distribution is refined during mammalian brain maturation, mainly in the hippocampus and cerebellum. hnRNP K loss was also associated to structural impairments at the levels of dendritic arborisation, spine maturation and spine density (Folci et al., 2014; Proepper et al., 2011). These observations suggest that the cellular functions regulated by hnRNP K in each given neuron may change according to the development stages.

5. Conclusion

In this work we show that BDNF-TrkB signalling regulates the levels of hnRNP K protein in dendrites but not at synapses of hippocampal neurons. Synaptic activity induced by bicuculline also increases the dendritic pool of hnRNP K by a mechanism dependent on endogenous BDNF release.

Two small hairpins RNAs were validated to successfully silence hnRNP K in cultured hippocampal neurons, both in dendrites and in the cell soma. These constructs could be therefore used in future experiments addressing the importance of hnRNP K in neurons.

Preliminary results point to an important role of hnRNP K as downstream element of BDNF-activated signalling cascades in dendrites. hnRNP K silencing may alter the basal levels of protein synthesis in dendrites as well as the surface expression of GluA1-containing AMPA receptors. Moreover, the neuronal responses to BDNF treatment appear to be affected by hnRNP K knockdown.

Altogether, our results suggest hnRNP K as an important regulator of mRNA metabolism in dendrites, an effect that may have a role in BDNF-induced forms of synaptic plasticity.

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