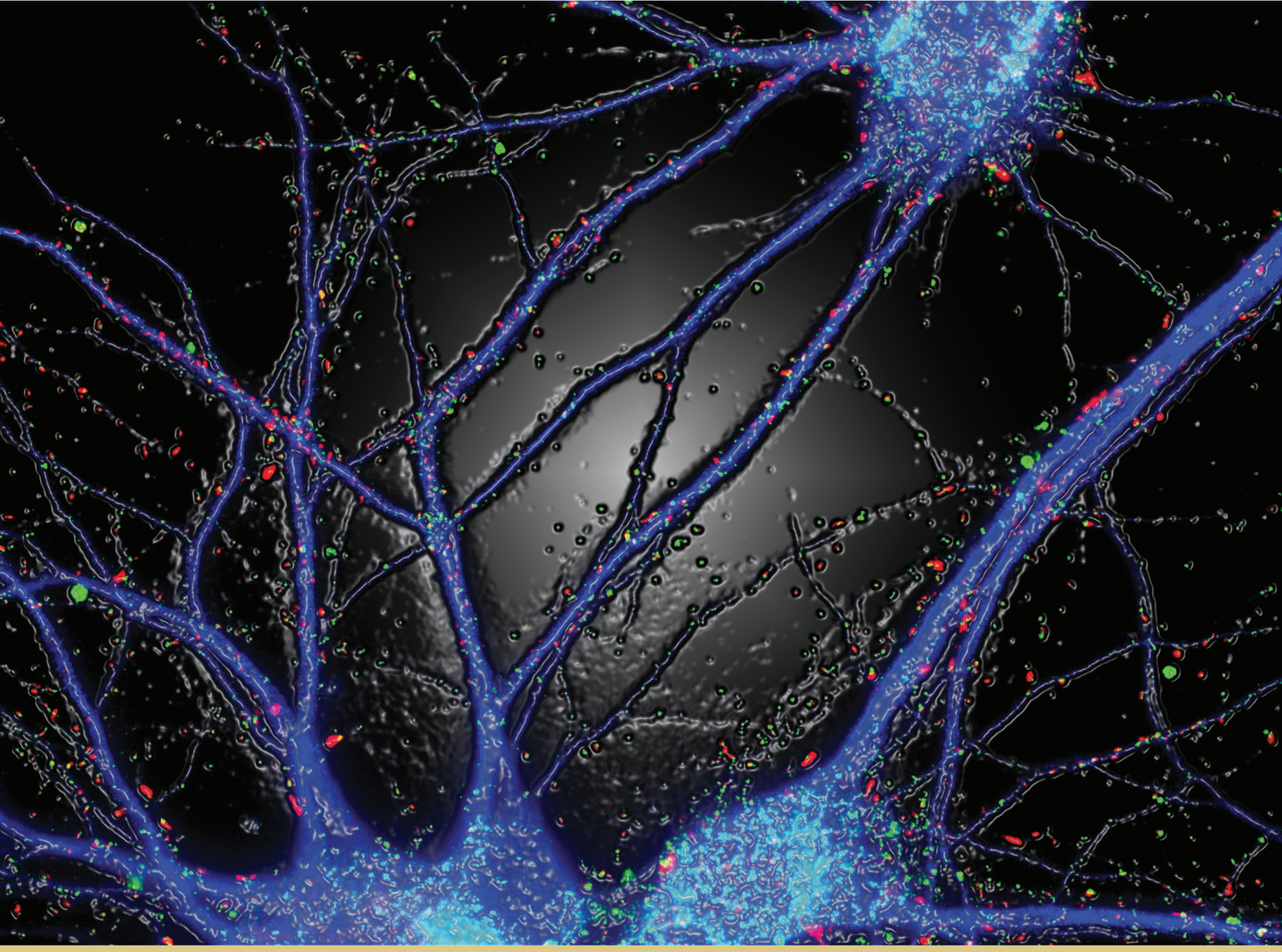

Pedro João Madeira Afonso

Regulation of Local Translation by BDNF

Effects on NMDA Receptor Trafficking

Doctoral Thesis in Pharmaceutical Sciences with specialization in Cellular and Molecular Biology, under supervision of Professor Carlos Jorge Alves Miranda Bandeira Duarte and Professor Armanda Emanuela Castro e Santos and presented to the Faculty of Farmacy of the University of Coimbra

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Universidade de Coimbra

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Regulação da Tradução Local pelo BDNF

Efeitos no Tráfego dos Receptores NMDA

Pedro João Madeira Afonso

Faculdade de Farmácia

Universidade de Coimbra

2015

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“The most beautiful thing we can experience is the mysterious. It is the source of all true art and science.”

-Albert Einstein

À minha mãe e irmã Vera…

Cover note: Deconstruction of microscopy image of rat hippocampal neuron co-labeled with dendritic marker, MAP2 (blue), GluN2B (green) and vGlut1 (red).

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Um sincero muito obrigado…

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LIST OF ABBREVIATIONS

4E-BP – eIEF4E-binding protein

5-HT – 5-hidroxitriptamine or serotonin

Abi1 – Abelson-interacting protein 1

AD – Alzheimer´s disease

ADP – actin-depolymerizing factor

Akt – Protein kinase B

AMPAR – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

Arc – Activity-regulated cytoskeleton-associated protein

Arg – Arginine

ATD – Amino-(N)-terminal domain

Aβ – Amyloid peptide

BDNF – Brain-derived neurotrophic factor

BSA – Bovine serum albumin

CAKβ – cell adhesion kinase β

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

CaRF – Ca^{2+}-response factor

CASK – Ca^{2+}/calmodulin-dependent serine protein kinase 3

CdK5 – Cyclin-dependent kinase 5

cDNA – Complementary DNA

CHX – Cycloheximide

CK2 – Casein kinase 2

cKO – Conditional knockout

cLTP – Chemical long-term potentiation

CNS – Central nervous system

CREB – cAMP-responsive element-binding protein

CTD – Carboxyl-(C)-terminal domain

CYFIP1 – Cytoplasmic Fmr-interacting protein 1

DAG – Diacylglycerol

DG – Dentate Gyrus

DICE – Differentiation control element

DIV – Days in vitro

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DREAM – Downstream regulatory element antagonist modulator

Dyrk1a – Dual specificity tyrosine-phosphorylation-regulated kinase 1a

ECM – Extracellular matrix

ECS – Extracellular solution

EEA1 – Early endosomal antigen 1

eEF – Eukaryotic elongation factor

eEF2K – Eukaryotic elongation factor 2 kinase

EFA6 – Arf6-specific guanine exchange factor

EGF – Epidermal growth factor

eIF – Eukaryotic initiation factor

E-LTP – Early-LTP

EphB – Ephrin receptor B

EPSC – Excitatory post-synaptic current

EPSP – Excitatory post-synaptic potential

ER – Endoplasmic reticulum

ERK – Extracellular-regulated protein kinase

FAK – Focal adhesion kinase

Fbxo2 – F-box only protein 2

FMRP – Fragile mental X retardation protein

FRS2 – Fibroblast growth factor receptor substrate 2

GAB-1 – GRB2-associated binder 1

GDT – Guanosine diphosphate

GEF – Guanine nucleotide exchange factor

GFP – Green fluorescent protein

GIPC1 – GAIP-interacting protein C-terminus

GKAP – Guanylate kinase-associated protein

GPCRs – G-protein coupled receptors

GRB2 – Receptor-bound protein 2

GSK-3β – Glycogen synthase kinase 3β

GST – Glutathione S-transferase

GTP – Guanosine triphosphate

HD – Huntington´s disease

HEK293T – Human embryonic kidney 293 cells

HFS – High-frequency stimulation

His – Histidine

hnRNPK – Heterogeneous nuclear ribonucleoprotein K

hnRNPU – Heterogeneous nuclear ribonucleoprotein U

IGF – Insulin-like growth factor

IgG – Immunoglobulin G

iGluRs – Ionotropic glutamate receptors

Ins(1,4,5)P_3 – Inositol-1,4,5-triphosphate

IPSP – Inhibitory post-synaptic potential

IRES – Internal ribosome entry site

IRS – Insulin-receptor substrate

KAR – Kainate receptors

KIF – Kinesin

KO – Knockout

LBD – Ligand-binding domain

L-LTP – Late-LTP

LOX – 15-lipoxigenase

LPA – Lysophosphatidic acid

LTD – Long-term depression

LTM – Long-term memory

LTP – Long-term potentiation

Lys – Lysine

MAGUK – Membrane-associated guanylate kinase

MAP1b – Microtubule-associated protein 1b

MAP2 – Microtubule-associated protein 2

MAPK – Ras-mitogen activated protein kinase

MEM – Minimal essential medium

mEPSC – Miniature excitatory post-synaptic currents

mGluRs – Metabotropic glutamate receptors

Mint1, LIN10 or X11 – Amyloid beta A4 precursor protein-binding family A member 1

miRNA – micro RNA

MLB – Mammalian Lysis Buffer

MOI – Multiplicity of infection

mRNA – messenger RNA

MS – Mass spectrometry

MSN – Medium spine neurons

mtHTT – Mutant huntington protein

mTOR – Mammalian target of rapamycin

MudPIT – Multidimensional protein identification technology

NF-M – Neurofilament medium

NGF – Nerve growth factor

NMDAR – N-methyl-D-aspartate receptor

NT – Neurotrophin

PAK – p21-activated kinase

PATs – Palmitoyltransferases

PDK1 – 3-phosphoinosiide-dependent protein kinase 1

PDZ – Post-synaptic disc large ZO1

PI3K – Phosphatidylinositol 3-kinase

PKA – Protein kinase A

PKC – Protein kinase C

PLCγ – Phospholipase Cγ

PP2B – Protein phosphatase 2B or Calcineurin

PSC – Postsynaptic current

PSD – Postsynaptic density

PSD-93 – Postsynaptic density protein 93

PSD-95 – Postsynaptic density protein 95

PSF – polypyrimidine tract binding protein-associated splicing factor

PSP – Postsynaptic potential

PtdIns(4,5)P_2 – Phosphatidylinositol-4,5-biphosphate

PTEN – Phosphatase and tensin homolog deleted on chromosome 10

PTM – Post-translational modifications

PTP – Protein tyrosine phosphatase

Pyk2 – Proline-rich tyrosine kinase 2

qRT-PCR – Quantitative real-time polymerase chain reaction

RAFTK – Related adhesion focal tyrosine kinase

RBP – RNA-binding protein

RNA – Ribonucleic acid

rpS6 – Ribosomal protein S6

RRP – Rapidly recycling pool

S6K – Ribosomal protein S6 kinase

SAP102 – Synapse-associated protein 102

SAP97 – Synapse-associated protein 97

Sch-CA1 – Schaffer collateral-CA1 synapse

SEM – Standard error of the mean

Ser – Serine

SER – Smooth endoplasmic reticulum

SH3 domain – SRC Homology 3 domain

SHP2 – Src homology phosphatase 2

shRNA – Small hairpin RNA

SOS – Sevenless

STEP – Striatal-enriched protein tyrosine phosphatase

TBS – Theta-burst stimulation

TBS – Tris-buffered saline

TCF – Ternary complex

Thr – Threonine

TMD – Transmembrane domain

tPA – Tissue plasminogen activator

Trk – Tyrosine receptor kinase

tRNA – Transfer RNA

TRPC – Transient receptor-potential cation channel subfamily C

TTX – Tetrodotoxin

Tyr – Tyrosine

UPS – Ubiquitin-proteasome system

UTR – Untranslated region

VGCC – Voltage-gated calcium channels

vGlut – Vesicular glutamate transporters

WT – Wild-type

RESUMO

As sinapses excitatórias são estruturas dinâmicas e a forma como neurónios vizinhos comunicam entre si é ajustada consoante a actividade neuronal. A esta propriedade chama-se plasticidade sináptica e a nível molecular está correlacionada com a aprendizagem e a memória. A potenciação sináptica de longa duração (LTP) é a forma de plasticidade sináptica mais estudada sendo definida como um fortalecimento duradouro na comunicação entre neurónios vizinhos desencadeado pela actividade neuronal. Pelo contrário, a depressão sináptica de longa duração (LTD) é caracterizada por uma diminuição duradoura da potência sináptica. Alterações moleculares nos mecanismos de plasticidade sináptica estão na base de muitas doenças neurológicas e psiquiátricas.

Algumas das modificações sinápticas ao nível estrutural, bioquímico e funcional associadas com a plasticidade sináptica requerem a tradução de mRNAs (RNA mensageiros) localizados nas dendrites, resultando em alterações no proteoma sináptico. Várias evidências mostram que a síntese proteica em dendrites desempenha um papel fundamental em várias formas de plasticidade sináptica, incluindo a LTP mediada pelo BDNF (factor neurotrófico derivado do cérebro). Contudo, pouco se sabe sobre a identidade dos mRNA que são traduzidos ao nível da sinapse em resposta ao BDNF e sobre os mecanismos de regulação envolvidos. Além disso, também está ainda por esclarecer de que modo muitas das alterações no proteoma sináptico contribuem para os fenómenos de plasticidade sináptica.

Neste trabalho investigámos o papel da Pyk2 (cinase de resíduos de tirosina rica em prolina do tipo 2) na mediação dos efeitos do BDNF na sinapse. A Pyk2 é uma cinase de resíduos de tirosina pertencente à família das FAK (cinases de adesão focal), que desempenha uma grande variedade de funções no sistema nervoso central, incluindo o control da LTP e da LTD por mecanismos que envolvem a regulação dos receptores NMDA (N-metil-D-aspartato). Além disso, pensa-se que esta cinase desempenha um papel importante na remodelação da arquitectura das espículas sinápticas e da arborização dendritica, induzidas pela actividade neuronal. Observámos que a Pyk2 é traduzida ao nível da sinapse e acumulada nas densidades pós-sinápticas de neurónios do hipocampo após a estimulação com BDNF. A acumulação dendritica da Pyk2 em resposta à estimulação com BDNF requer a participação da RBP (proteína que liga RNA), hnRNPK (ribonucleoproteína nuclear heterogénea do tipo K). Estas observações estão de acordo com os resultados anteriores do nosso laboratório mostrando que: (i) a hnRNPK é acumulada nas dendrites dos neurónios do hipocampo após o estimulação com BDNF; (ii) a ligação do mRNA da Pyk2 à hnRNPK é regulada por BDNF. Usando um protocolo químico para aumentar a actividade neuronal e induzir LTP também observámos que a Pyk2 se acumula na sinapse por um mecanismo dependente de BDNF.

A principal função da Pyk2 ao nível da densidade pós-sináptica tem sido associada à regulação das correntes mediadas pelos recetores NMDA através da interação direta com a Src, outra cinase de resíduos de tirosina. Neste estudo, observámos que o tratamento com BDNF aumenta a expressão superficial dos receptores NMDA que contêm a subunidade GluN2B, ao nível da sinapse, por um mecanismo dependente da síntese proteica. De acordo com estas observações,

observou-se que a estimulação com BDNF aumenta os níveis de Pyk2 fosforilada/activada de forma específica ao nível da sinapse, o que sugere uma regulação diferencial da atividade da cinase. O aumento dos níveis sinápticos dos recetores NMDA induzido pelo BDNF também depende da Pyk2 e da sua actividade de cinase. Por outro lado, também se observou que em condições de repouso a manutenção na membrana celular dos recetores NMDA contendo subunidades GluN2B depende da atividade de cinase da Pyk2. Finalmente, a sobreexpressão da Pyk2 em neurónios do hipocampo foi suficente para mimetizar os efeitos do BDNF na expressão superficial dos receptores NMDA que contêm a subunidade GluN2B.

No seu conjunto, os resultados mostram que o BDNF induz a activação/acumulação da Pyk2 por um mecanismo que envolve a hnRNPK e a síntese dendritica da Pyk2, resultando num aumento da expressão superfical dos receptores NMDA que contêm a subunidade GluN2B. Este mecanismo pode mediar os efeitos do BDNF nos défices cognitivos que são característicos de certas doenças do cérebro.

Palavras-chave: Potenciação sináptica de longa duração (LTP); Hipocampo; BDNF; hnRNPK; GluN2B; Pyk2.

ABSTRACT

Excitatory synapses are dynamic entities and adjust their strength depending on the activity. This property is named synaptic plasticity and is considered the cellular correlate of learning and memory. Long-term potentiation (LTP) is the best studied form of synaptic plasticity and by definition it is considered as an activity-induced sustained increase in synaptic strength. Long-term depression (LTD) is the opposite form of plasticity, and is characterized by an activity-induced sustained decrease in synaptic strength. Alterations in the molecular basis of synaptic plasticity events underlie several neurological and psychiatric disorders.

Some of the structural, biochemical and functional modifications of the synapse associated with synaptic plasticity require translation of dendritic-localized mRNAs, with concomitant alterations in the synaptic proteome. Multiple lines of evidence show that dendritic protein synthesis plays a key role in several forms of synaptic plasticity, including in brain-derived neurotrophic factor (BDNF)-mediated LTP. However, the identity of the mRNAs that are synaptically translated in response to BDNF and the regulatory mechanisms involved are poorly understood. Furthermore, how these changes in the proteome contribute to the plastic alterations of the synapse also remains to be uncovered.

In this work, we investigated a role for Pyk2 (proline-rich tyrosine kinase 2) as a mediator of the effects of BDNF at the synapse. Pyk2 is a non-receptor tyrosine kinase, belonging to the FAK (focal adhesion kinase) family of proteins, which plays a wide range of functions in the central nervous system (CNS), including the control of LTP and LTD by mechanisms involving the regulation of NMDA (N-methyl-D-aspartate) receptors. Furthermore, this kinase is thought to play an important role in the activity-induced remodeling of spine architecture and dendritic arborization. We found that the protein kinase Pyk2 is synaptically translated in hippocampal neurons and accumulates at post-synaptic density following BDNF treatment. The dendritic accumulation of Pyk2 upon stimulation with BDNF requires the participation of the RNA-binding protein hnRNPK (heterogeneous nuclear ribonucleoprotein K). This is in accordance with previous results from our laboratory showing that (i) hnRNPK is accumulated in dendrites of hippocampal neurons upon BDNF treatment and (ii) the binding of *Pyk2* mRNA to hnRNPK is regulated by BDNF. Using a chemical protocol to increase neuronal activity and to induce LTP, we also observed that Pyk2 accumulates at the synapse by a mechanism requiring BDNF.

The main function of Pyk2 at the post-synaptic compartment has been attributed to the regulation of NMDA receptor currents through a direct interaction with a different tyrosine kinase, Src. Herein we found that BDNF treatment increases the surface expression of GluN2B-containing NMDA receptors (NMDAR) at synapses, by a mechanism dependent on protein synthesis. In agreement with these observations, the levels of phosphorylated/activated Pyk2 were specifically enhanced at the synapse upon BDNF treatment, suggesting a compartment-specific regulation of Pyk2 activity by BDNF. The BDNF-induced increase on surface NMDARs also requires Pyk2 and its kinase activity. The maintenance of basal levels of GluN2B-containing NMDAR at the cell surface was also dependent on Pyk2 kinase activity. Finally, overexpression of Pyk2 in hippocampal neurons was sufficient, *per se*, to mimic the BDNF-induced increase in GluN2B-NMDAR surface expression.

Taken together, the results show that BDNF induces synaptic activation/accumulation of Pyk2 by a mechanism involving hnRNPK and dendritic Pyk2 synthesis, resulting in an enhancement in the surface levels of GluN2B-containing NMDAR. This mechanism may mediate the effects of BDNF on synaptic plasticity and may constitute a novel therapeutic target to restore the cognitive deficits characteristic of some brain disorders.

Keywords: Long-term potentiation (LTP); Hippocampus; BDNF; hnRNPK; GluN2B; Pyk2.

Chapter 1

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

The Excitatory Synapse

The brain is the most complex mammalian organ, and regulates processes that provide the ability to learn, remember and feel, as well as those involved in emotions. The human brain is comprised by at least 100 billion neurons connected with each other in specialized regions, the synapses. Each neuron is therefore able to influence the activity of other neurons, directly or indirectly, and the activity of many other different cells such, as glial cells.

Synapses are the basic units of information storage in the brain and they contain more than 300 types of proteins, from membrane receptors, to cytoskeleton components and signaling elements (Husi and Grant 2001, Peng *et al.* 2004). These specialized cell units are composed by pre- and postsynaptic terminals, which are involved in interneuron communication. Two categories of synapses, electrical and chemical, can be distinguished on the basis of their mechanisms of transmission. In electrical synapses, currents flow through gap junctions, which are specialized membrane channels that connect two cells, while in chemical synapses cell-cell communication is achieved via the secretion of neurotransmitters. These chemical agents released from the presynaptic neuron produce a secondary current flow in the postsynaptic neuron by activating specific receptor molecules. Other neurotransmitter receptors play a modulatory role, regulating synaptic transmission mediated by other receptors and/or molecules.

The release of neurotransmitters is triggered by Ca^{2+} influx through voltage-gated channels, which gives rise to a transient increase in the Ca^{2+} concentration within the pre-synaptic terminal. This increase in intracellular Ca^{2+} concentration induces the fusion of synaptic vesicles with the pre-synaptic plasma membrane allowing the release of their content into the synaptic cleft (the space between the pre- and postsynaptic terminals). The neurotransmitters remain in the synaptic cleft for only a brief period of time, but sufficient to activate receptors located on the postsynaptic neuron, thereby producing a postsynaptic conductance change. This conductance change typically generates an electric current, the postsynaptic current (PSC), which in turn changes the postsynaptic membrane potential to produce the postsynaptic potential (PSP). Depending on the nature of the neurotransmitter and the response of the postsynaptic neuron, depolarization or hyperpolarization, the synapse is classified as excitatory or inhibitory, respectively. Excitatory synapses induce an excitatory postsynaptic potential (EPSP) that depolarizes the membrane towards the threshold required for activation of an action potential. Conversely, inhibitory synapses induce an inhibitory postsynaptic potential (IPSP) that hyperpolarizes the membrane away from the threshold potential (Hausser *et al.* 2000). The excitatory synapses occur mainly on tiny protrusions called dendritic spines (Bourne and Harris 2008), while inhibitory synapses are formed on the shaft of dendrites, or on the cell bodies and axon initial segments (Sheng and Kim 2011).

Glutamatergic Synapse

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) (Hayashi 1954, Watkins and Jane 2006). In glutamatergic neurons, some of the glutamate is packaged within synaptic vesicles by specific vesicular glutamate transporters (vGluts) and is released into the synaptic cleft following presynaptic activation. The neurotransmitter diffuses within the synaptic cleft to reach the postsynaptic membrane, activating primarily postsynaptic receptors, which belong to two major classes: the ionotropic glutamate receptors (iGluRs), which are ligand-gated ion channels, and the metabotropic glutamate receptors (mGluRs), which belong to the class of G protein-coupled receptors (GPCRs). Some synapses also contain pre-synaptic glutamate receptors which control neurotransmitter release.

In excitatory synapses the postsynaptic region is highly specialized from the structural and functional points of view, and is usually located at the tip of a dendritic spine (Sheng and Kim 2011) **(Fig. 1.1)**. When observed in electron micrographs the postsynaptic region is electrodense and, therefore, it was named postsynaptic density (PSD). The existence of this structure was first reported in 1950s (Palade and Palay 1954, Palay 1958) using electron microscopy, but it was only in the 1970s that PSDs were purified upon treatment of synaptosomes with detergent (Davis and Bloom 1973, Cotman *et al.* 1974, Blomberg *et al.* 1977, Cohen *et al.* 1977). The PSD is essentially a proteinaceous organelle attached to the post synaptic plasma membrane and held by cytoplasmic actin filaments. More recently, with the development of mass spectrometry (MS), a large number of proteins (approximately 400) categorized in 13 functional groups were identified to reside in the post-synaptic density (Peng *et al.* 2004), including cytoskeleton proteins, plasma membrane proteins (mainly glutamate receptors and adhesion molecules), scaffold proteins (mainly PSD-95, GKAP/SAPAP, Shank/ProSAP and Homer families) and signaling proteins (such as CaMKII family members) (Cho *et al.* 1992, Hunt *et al.* 1996, Brakeman *et al.* 1997, Kim *et al.* 1997, Boeckers *et al.* 1999, Naisbitt *et al.* 1999, Walikonis *et al.* 2000, Cheng *et al.* 2006, Sheng and Kim 2011). More than 1000 proteins were identified up to now in the mammalian postsynaptic densities. However, the protein content of the PSD is not static, undergoing a continuous molecular turnover under basal conditions and showing large changes in response to neuronal activity (Inoue and Okabe 2003). These alterations are mediated by different mechanisms, including protein phosphorylation, ubiquitination, synthesis and proteasome-mediated degradation.

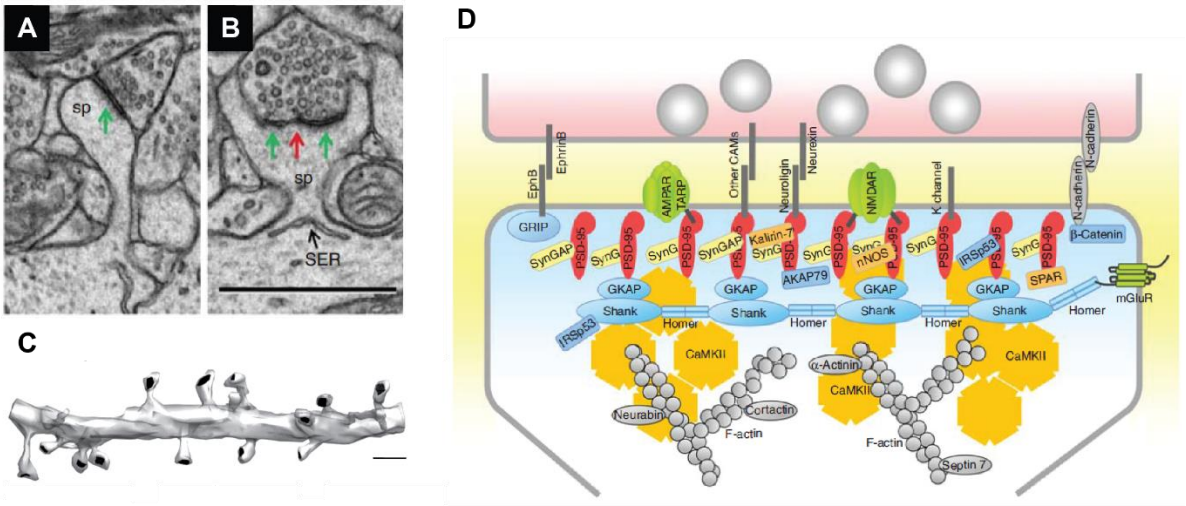


Figure 1.1. The glutamatergic synapse. A. Synapse with a PSD (green arrow) on a medium-size dendritic spine (sp) from CA1 hippocampal neurons **B.** PSD (green arrows) perforated by an electron-lucent region (red arrow) on a large dendritic spine (sp) with smooth endoplasmic reticulum (SER) at its base. Adapted from (Harris and Weinberg 2012). **C.** Three-dimensionally reconstructed dendritic segment of a hippocampal neuron. Scale = 1 µm. Adapted from (Nikonenko *et al.* 2008). **D.** Schematic diagram of the major proteins of the PSD, with protein interactions indicated by direct contacts or overlap between the proteins. Adapted from (Sheng and Kim 2011).

Glutamate Receptors

Glutamate receptors are highly expressed in the mammalian CNS in accordance with the key role played by glutamate in excitatory neurotransmission (Watkins and Jane 2006). The effects of glutamate are mediated by activation of ionotropic receptors (iGluR), which are involved in rapid neurotransmission (msec range), and metabotropic (mGluRs) type receptors which mediate the delayed responses to glutamate, occurring over seconds or minutes.

Ionotropic Glutamate Receptors

Ionotropic glutamate receptors are ligand-gated cation channels that mediate excitatory neurotransmission crucial for brain development and function, including learning and memory formation. iGluR (Traynelis *et al.* 2010, Smart and Paoletti 2012) are encoded by a total of 16 genes belonging to four major families: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, Kainate (KA) receptors, N-methyl-D-aspartate (NMDA) receptors and delta (δ) receptors, named after their synthetic agonists (Watkins *et al.* 1981, Hollmann *et al.* 1989, Seeburg 1993, Nakanishi and Masu 1994, Dingledine *et al.* 1999) **(Table 1.1)**.

Table 1.1. The four families of iGluR subunits. Each subunit is encoded by a distinct gene. There is no known oligomerization of subunits belonging to different families. Adapted from (Smart and Paoletti 2012).

Ionotropic glutamate receptors (iGluRs)			
AMPARs	KainateRs	NMDARs	DeltaRs
GluA1	GluK1 GluK4	GluN1* GluN2A GluN3A*	GluD1
GluA2	GluK2 GluK5	GluN2B GluN3B*	GluD2*
GluA3	GluK3	GluN2C	
GluA4		GluN2D	

* These subunits bind glycine or D-serine.

Each of the iGluR is formed by oligomerization of four large subunits that form a central ion pore. Amino acid sequence similarity among all known glutamate receptor subunits, including the AMPA, Kainate (KA), NMDA, and δ receptors, suggested that they share a similar architecture. Accordingly, ionotropic glutamate receptor subunits are modular structures containing four discrete semiautonomous domains: (i) the extracellular amino-(N)-terminal domain (ATD); (ii) four (M1, M2, M3 and M4) transmembrane domains (TMD), the second of which loops in and out of the membrane, and it is thought to form the pore, as confirmed by resolving the crystallographic structure (Sobolevsky *et al.* 2009); (iii) the extracellular ligand-binding domain (LBD), which is formed by part of the N-terminal region (S1) adjacent to the first transmembrane domain (M1) and the extracellular domain (S2), between the third (M3) and the fourth (M4) transmembrane domains (Stern-Bach *et al.* 1994); and (iv) the intracellular carboxyl-(C)-terminal domain (CTD), which is the most variable domain of glutamate receptors (Traynelis *et al.* 2010) **(Fig. 1.2).** NMDAR form functional ion channels only as heterotetramers of at least two distinct subunits, while non-NMDAR are homo- or heterotetramers (Karakas *et al.* 2015).

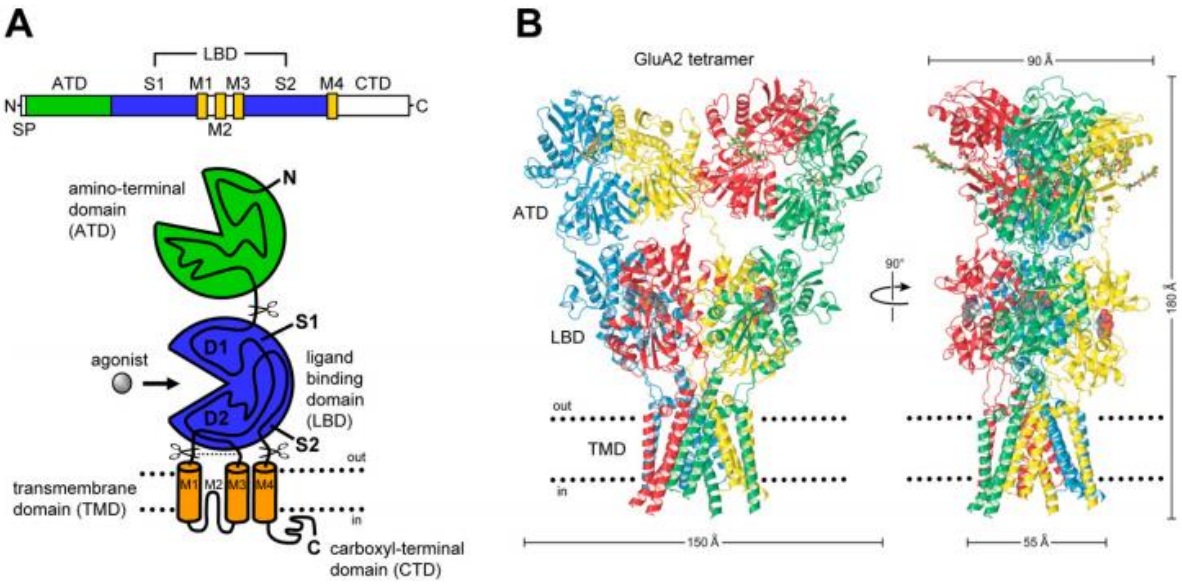


Figure 1.2. **Structure and domain organization of glutamate receptors**. **A.** Linear representation of the subunit polypeptide chain and schematic illustration of the subunit topology. Glutamate receptor subunits have a modular structure composed of two large extracellular domains [the ATD (green) and the LBD (blue)], a TMD (orange) that forms part of the ion channel pore, and an intracellular CTD. The LBD is defined by two segments of amino acids termed S1 and S2. The TMD contains three membrane-spanning helices (M1, M3, and M4) and a membrane re-entrant loop (M2). The isolated S1 and S2 segments have been constructed by deleting the ATD along with the TMD and joining S1 and S2 with a hydrophilic linker (dotted line). SP, signal peptide. **B.** Crystal structure at 3.6 Å of the membrane-spanning tetrameric GluA2 AMPA receptor. Adapted from (Traynelis *et al.* 2010).

AMPA Receptors

AMPA receptors (AMPAR) mediate the fast component of excitatory transmission, and are characterized by their high affinity for AMPA (Hollmann and Heinemann 1994). These receptors are tetrameric assemblies of high homologous subunits encoded by four different genes, GluA1-4 **(Table 1.1)**. Each of these subunits is alternatively spliced giving rise to a "flip" or a "flop" isoform (Sommer *et al.* 1990), which confers different desensitization properties to the receptor (Mosbacher *et al.* 1994). The expression of "flip" or "flop" isoforms of AMPAR subunits is regionally and developmentally regulated (Monyer *et al.* 1991). The lack of the flip flop exons originates AMPAR subunits characterized by the absence of the flip/flop cassette, the fourth transmembrane domain and the intracellular C-terminus. Truncated GluA1 subunits were found to associate with full-length GluA1 subunits, exerting a dominant negative effect that may constitute an intrinsic neuroprotective mechanism under conditions of high excitatory activity (Gomes *et al.* 2008).

AMPAR are voltage-independent ion channels considered to be sodium (Na^+) and potassium (K^+) permeable. Therefore, activation of AMPAR leads to strong influx of Na^+ and only a small efflux of K^+, which induces a net depolarizing effect in target neurons. Depending on their subunit composition, some of the AMPA receptors may also be Ca^{2+} permeable (Burnashev *et al.* 1992). Given their low Ca^{2+} permeability, the influx of current through GluA2-containing AMPAR (most AMPAR contain at least one GluA2 subunit) is largely carried by the influx of Na^+ from the extracellular compartment.

The low Ca^{2+}-permeability of GluA2-containing AMPA receptors results from the editing of the *GluA2* mRNA, which replaces the RNA sequence coding for a glutamine (Q) residue in the channel pore-forming region (into the short reentrant membrane M2 loop) by an arginine (R) coding sequence. Thus, this site is named "Q/R" site (Sommer *et al.* 1991). This editing site (Arg607) also provides an important endoplasmic reticulum (ER) quality control check point, reducing the formation of GluA2 homomeric channels (Greger *et al.* 2002). The Arg607 residue located in the pore region of the channels limits the flow of Na^+ and K^+, and prevents divalent ions from entering the cell. Thus, the majority of AMPAR carry inward currents at negative potentials and outward currents at positive potentials, and the reverse potential is 0 mV (i.e., the current-voltage relationship is linear) (Luscher and Malenka 2012) **(Fig. 1.3)**.

In contrast, GluA2-lacking AMPAR (e.g., GluA1 homomeric channels or GluA1/3 heteromeric channels) possess a glutamine residue in the pore (residue 607) instead of the arginine present in edited GluA2 subunits. These channels have a high conductance for Na^+ and are permeable to Ca^{2+}, and because endogenous polyamines, which are negatively charged, can also access a site close to

the cytoplasmic mouth of the pore, the channels are inhibited at positive potentials. In consequence, GluA2-lacking AMPARs have an inward-rectifying current voltage relationship (i.e. they conduct current more easily into the cell than out the cell) (Luscher and Malenka 2012) **(Fig. 1.3)**.

It is widely accepted that the regulation of AMPAR trafficking in and out of the synapses is a highly dynamic process that constitutes the basis for activity-induced synaptic plasticity. The traffic of AMPAR is regulated by several receptor interacting proteins, as well as by post-translational modifications at the C-terminal region of AMPAR subunits, such as phosphorylation (Shepherd and Huganir 2007, Kessels and Malinow 2009, Choquet 2010, Anggono and Huganir 2012, Bassani *et al.* 2013, Chater and Goda 2014).

Kainate Receptors and delta (δ) Receptors

Kainate receptor (KAR) subunits are classified based on their affinity for kainate into low- and high-affinity subunits. These receptors are tetrameric structures formed by oligomerization of five different subunits (GluK1-GluK5) **(Table 1.1)**. However, the number of possible subunit combinations is limited by the fact that only low-affinity subunits (GluK1 through GluK3) can form functional homomeric receptors, while it is mandatory for the high-affinity subunits (GluK4 and GluK5) to oligomerize together with low-affinity subunits to form functional KARs (Pahl *et al.* 2014). The diversity of KARs is further increased by post-transcriptional modifications such as RNA editing and alternative splicing (Pinheiro and Mulle 2006). The RNA editing of KAR is similar to that described above for the AMPAR. However, although editing also regulates the Ca^{2+} permeability of KAR (Kohler *et al.* 1993), they show a higher Ca^{2+} permeability than AMPAR. In addition to their role as ionotropic receptors, KAR also activate G-protein coupled second messenger-mediated signaling cascades (Rodriguez-Moreno and Lerma 1998). These iGluR show a strong developmental and spatial regulation (Huntley *et al.* 1993, Wisden and Seeburg 1993, Bahn *et al.* 1994, Roche and Huganir 1995) and play important roles in pre- and postsynaptic regulation, contributing to synaptic plasticity (Jane *et al.* 2009, Perrais *et al.* 2010, Contractor *et al.* 2011, Carta *et al.* 2014, Sihra *et al.* 2014).

Delta (δ) receptors are the least understood iGluR largely because, in contrast to other receptors of the same class, they are unable to gate an ion channel following ligand binding, which makes them electrically “silent” (Kohda *et al.* 2000, Schmid *et al.* 2009). Therefore, the excitatory postsynaptic currents (EPSC) are typically mediated by members of the AMPAR and NMDAR families of glutamate receptors **(Fig. 1.3)**.

NMDA Receptors

NMDA receptors (NMDAR) are highly permeable to Ca^{2+}, blocked by magnesium (Mg^{2+}) in a voltage-depended manner and exhibit slow kinetic **(Fig. 1.3)**. This subtype of iGluR will be described in detail below.

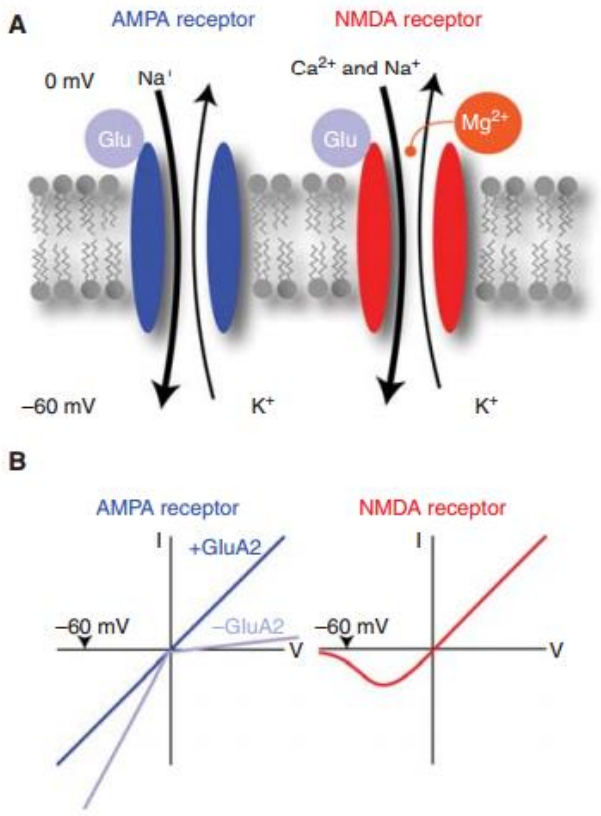


Figure 1.3. Schematic representation of AMPA and NMDA receptor channels and their biophysical properties. A. Glutamate binding to AMPA receptors induces a strong influx of sodium ions while a lower amount of potassium ions leave the neuron, causing a net depolarization of the membrane. NMDA receptors are also permeable to calcium but only if the magnesium is expelled by a slight depolarization of the neuron. **B.** The current–voltage (I–V) relationship provides a biophysical signature for the different receptors. AMPA receptors have a linear I–V relationship when they contain the GluA2 subunit, but are inward-rectifying (see text for definition) without GluA2. NMDA receptors have a complex I–V curve because Mg^{2+} blocks the pore at negative potentials. Adapted from (Luscher and Malenka 2012).

Metabotropic Receptors

mGluR belong to the super family of G-protein-coupled receptors (Masu *et al.* 1991). There are eight different subunits of mGluR (mGluR1-mGluR8) classified into three groups: group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8), according to their amino acid sequence homology, pharmacology and signal transduction mechanisms (Yin and Niswender 2014). Some of the mGluR isoforms may undergo alternative splicing generating further diversity (Chaudhari *et al.* 2009, Yin and Niswender 2014). mGluR are broadly distributed throughout the CNS and may be localized both presynaptically (Group II and III mGluR), where they regulate neurotransmitter release, and postsynaptically (Group I), regulating the depolarization of the membrane and neuronal excitability. The latter effects may be mediated by modulation of a variety of ion channels and other regulatory and signaling proteins (Niswender and Conn 2010, Yin and Niswender 2014).

NMDA Receptors

NMDAR play a key role in numerous physiological processes in the mammalian brain, such as synaptic transmission, synaptic plasticity, neuronal development, as well as in brain disorders such as epilepsy, depression, Alzheimer´s disease, Parkinson´s disease, Huntington's disease and schizophrenia [reviewed in (Lau and Zukin 2007, Sanz-Clemente *et al.* 2013, Zhou and Sheng 2013)].

NMDA receptor composition

Seven NMDA receptor subunits were identified and cloned: one GluN1 subunit, four GluN2 subunits (GluN2A-D) and two GluN3 subunits (GluN3A-B) **[Table 1.2 and Fig. 1.5 (*right*)]**. Functional NMDAR are heterotetrameric complexes formed by two glycine-binding GluN1 subunits assembled with two isoforms of the glutamate-binding GluN2 (A, B, C or D subtypes) subunit or the glycine-binding GluN3 (A or B subtypes) subunit (Dingledine *et al.* 1999, Cull-Candy and Leszkiewicz 2004).

Table 1.2. NMDAR subunits. NMDAR subunits identified and respective references.

NMDAR Subunit	Reference
GluN1	(Moriyoshi *et al.* 1991, Yamazaki *et al.* 1992)
GluN2A	(Meguro *et al.* 1992, Monyer *et al.* 1992, Ishii *et al.* 1993)
GluN2B	(Kutsuwada *et al.* 1992, Monyer *et al.* 1992, Ishii *et al.* 1993)
GluN2C	(Kutsuwada *et al.* 1992, Monyer *et al.* 1992, Ishii *et al.* 1993)
GluN2D	(Ikeda *et al.* 1992)
GluN3A	(Ciabarra *et al.* 1995)
GluN3B	(Nishi *et al.* 2001, Chatterton *et al.* 2002)

NMDAR are the longest subtype of iGluR, with a range of amino acids per subunit that goes from approximately 900 in GluN1 subunit, to approximately 1500 amino acids in the GluN2B subunit. This difference is mainly noted in the length of their C-terminal domain which may influence NMDAR localization (Paoletti 2011).

NMDA receptor assembly

NMDAR are assembled in the endoplasmic reticulum (ER) and exported after passing the quality control mechanisms that ensure that subunits are correctly folded and the receptor is active [reviewed in (Prybylowski and Wenthold 2004, Traynelis *et al.* 2010)]. The GluN1 subunit is produced in large excess relative to GluN2 subunits, ensuring that sufficient amounts of GluN1 subunits are available for newly synthesized GluN2 and GluN3 subunits (Huh and Wenthold 1999). Three different working models for the assembly of functional NMDAR in the ER have been proposed. First, several studies suggest that the GluN1-GluN1 and GluN2-GluN2 homodimers, which are initially formed, are required for the formation of functional heterotetramers (Meddows *et al.* 2001, Schorge and Colquhoun 2003, Papadakis *et al.* 2004, Qiu *et al.* 2005, Hansen *et al.* 2010). Second, other studies proposed that the GluN1-GluN2 and GluN1-GluN3 heterodimers are required for the formation of

heterotetrameric receptors (Schuler *et al.* 2008) or, alternatively, GluN1-GluN1 homomers may be the substrate for the oligomeric assembly of the heterotetramer (Atlason *et al.* 2007). The latter model has been extended by a recent study suggesting that the N-terminal domain of GluN1 subunits initially forms homodimers and the subsequent dimer dissociation was proposed to be essential for the formation of functional GluN1/GluN2 heteromers (Farina *et al.* 2011). In fact, the reported promiscuity between the GluN1 and GluN2 N-terminus may explain the different models proposed for NMDAR assembly.

Different regions of the GluN subunits have been proposed to regulate the assembly and/or retention of NMDAR in the ER. The C-terminus of some GluN1 splice variants contains ER retention motifs, like KKK and RRR in the C1 cassette (Standley *et al.* 2000, Scott *et al.* 2001, Mu *et al.* 2003). PKA and PKC can also modulate the ER retention by phosphorylation of serine residues nearby the RRR motif, as shown using chimeric proteins of the C-terminus and single transmembrane proteins (Scott *et al.* 2001). This contrasts with the results of a different study using a full-length GluN1 construct which did not confirm the previous results (Horak and Wenthold 2009). No specific signals were identified up to now for GluN2B retention in the ER. However, the C-terminus of GluN2B subunit attached to Tac is retained in the ER, suggesting the presence of an ER retention signal (Hawkins *et al.* 2004). The structure of the transmembrane domains is also likely to regulate the ER processing of the functional NMDAR. Critical structural determinants were identified within the M3 domains of GluN1 and GluN2A-B subunits that cause unassembled subunits to be retained it the ER (Horak *et al.* 2008, Kaniakova *et al.* 2012). In addition, the extracellular structure of NMDAR also plays a role in the ER processing of the receptor. For example, an ER retention signal was identified in the A2 segment of the amino-terminal domain of the GluN2A subunit that must be masked by interaction with GluN1 so that the functional NMDAR leaves the ER (Qiu *et al.* 2009).

NMDA receptor localization

NMDAR localization is tightly coupled to their function and, therefore, plays an important role in their aforementioned physiological roles. These receptors are mainly located at postsynaptic sites, although they can also be found at perisynaptic (Zhang and Diamond 2009), extrasynaptic (Papouin *et al.* 2012, Papouin and Oliet 2014, Parsons and Raymond 2014, Garcia-Munoz *et al.* 2015) and presynaptic sites (Bidoret *et al.* 2009, Larsen *et al.* 2014, Park *et al.* 2014, Urban-Ciecko *et al.* 2014). The later population of NMDAR regulates the release of neurotransmitters and other synaptic modulators such as neurotrophins (Park *et al.* 2014).

NMDAR are widely distributed throughout the CNS, but the expression of each individual subunit is highly dependent on the brain area and developmental stage. In fact, NMDAR subunits exhibit distinct, yet often overlapping expression patterns. Despite some differences reported in the expression pattern of GluN1, the heterogeneity in the expression of NMDAR subunits is more pronounced for GluN2 subunits. For example, GluN2B subunits are widely expressed during prenatal development but in the adult brain they are restricted to the forebrain. In contrast, the expression of GluN2A, which is ubiquitous in the CNS, is dramatically increased just after the second post-natal week. GluN2C expression is enriched in adult cerebellum, while GluN2D is present early in development, but its abundance increases in adulthood, particularly in the diencephalon,

mesencephalon and spinal cord (Monyer *et al.* 1994). The expression of GluN3 subunits also follows a specific pattern: the abundance of GluN3A peaks early in postnatal life (Wong *et al.* 2002) while GluN3B increases throughout development, especially in the motor neuron population (Nishi *et al.* 2001, Fukaya *et al.* 2005, Henson *et al.* 2010) **(Fig. 1.4).**

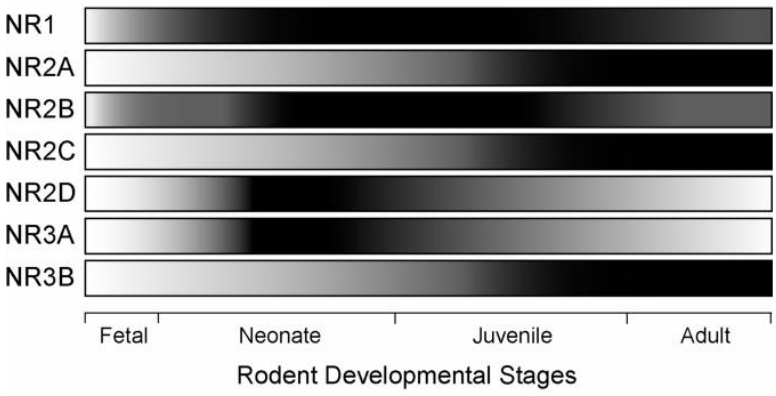


Figure 1.4. Schematic representation of NMDAR subunit expression in the developing rat brain. The gray scale gradient shows the differences in the expression of each subunit relative to the maximum, with the darkest regions reflecting the strongest expression. NR3A appears to be expressed in a similar temporal fashion as NR2D, with both subunits peaking between P7 and P14. This contrasts with NR3B, NR2A, and NR2C, which increase developmentally and peak in the third postnatal week. The old nomenclature was used in this figure, with NR1, NR2A, NR2B, NR2C, NR2D, NR3A and NR3B corresponding to GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A and GluN3B, respectively. Adapted from (Henson *et al.* 2010).

There are also important differences in what concerns the subcellular expression of NMDAR subunits. GluN1 exists in two pools, assembled with GluN2 or GluN3 subunits, and is predominantly found in the plasma membrane or retained in the ER with a short half-life (Huh and Wenthold 1999). In contrast, GluN2 subunits are mainly located in the plasma membrane, predominantly in the post-synaptic membrane, although their presynaptic expression was also reported (Scott *et al.* 2001). Currently, there is a simplified model according to which GluN2A subunits are predominantly synaptic, while GluN2B subunits are mainly found in extrasynaptic sites in the adult brain (Groc *et al.* 2009). However, there are many regulatory mechanisms that can affect the intracellular distribution of NMDAR, which will be discussed in detail in the next sections.

In addition to their role in neuron to neuron communication, NMDAR may also be expressed in glial cells, including macroglia, astrocytes and oligodendrocytes (Verkhratsky and Kirchhoff 2007, Cao and Yao 2013). In fact, all the seven subunits were found in human astrocytes (Lee *et al.* 2010).

NMDA receptor activation and modulation

NMDAR are cation channels permeable to sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}). These receptors are activated by simultaneous binding of glycine (or D-serine) and glutamate. Glycine binds to GluN1 (Furukawa and Gouaux 2003) and GluN3 subunits (Yao *et al.* 2008), whereas glutamate binds to GluN2 subunits (Furukawa *et al.* 2005) **(Fig. 1.5)**. These differences are explained by the presence of a tryptophan residue in position 731 at the binding domain of the GluN1 subunits, which makes the GluN1 binding cavity smaller than the one formed in the GluN2 subunits. Thus,

GluN1 binds to glycine because it is smaller than glutamate, allowing its access to the binding cavity on this NMDAR subunit (Furukawa and Gouaux 2003).

NMDAR activity is also characterized by the requirement of plasma membrane depolarization, which releases the Mg^{2+} blockade of the receptor channel (Mayer *et al.* 1984). This feature makes the activation of NMDAR dependent on the simultaneous release of glutamate and membrane depolarization. The membrane potential-sensitive blockade of NMDAR by Mg^{2+} makes the current-voltage relationship for these receptors more complex than that observed for AMPAR. At resting and hyperpolarized membrane potentials, the concentration of Mg^{2+} in the extracellular fluid is sufficient to virtually abolish the ion flux through NMDAR channels, even in the presence of glutamate and the co-agonist glycine. Thus, although the binding of glutamate and glycine to their respective sites and the virtual "activation" of the NMDAR, the presence of Mg^{2+} into the channel pore blocks the flux of ions. In the presence of this ion channel blocker, NMDAR channels exhibit the characteristic J-shaped current-voltage relationship **(Fig. 1.3)**. When the membrane potential becomes less negative or even positive (depolarization), the affinity of Mg^{2+} for its binding site decreases and the blockade is ineffective, allowing the entry of positive ions. Under physiological conditions, the activation of AMPA receptors that are found in the postsynaptic membrane contribute to the rapid depolarization of the membrane necessary for the activation of NMDAR. It is important to note that through this mechanism NMDAR play a role as molecular coincidence detectors, essential for several forms of synaptic transmission and in synaptic plasticity.

Small molecules and ions may modulate NMDAR mediated activity [reviewed in (Traynelis *et al.* 2010)]. In particular, it was shown that extracellular protons inhibit NMDAR by trapping them into a closed state. This mechanism is voltage-independent and does not change the sensitivity to the agonists (Tang *et al.* 1990, Traynelis and Cull-Candy 1990, Vyklicky *et al.* 1990), but it was shown that protons alter the receptor open probability (Banke *et al.* 2005). One particular feature of this type of receptors is that they are inhibited by zinc (Zn^{2+}), which acts like Mg^{2+}, blocking the channel pore (Peters *et al.* 1987, Westbrook and Mayer 1987). NMDAR activity may be also modulated by polyamines (Rock and Macdonald 1995, Igarashi and Kashiwagi 2010). For example, spermine regulates NMDAR through a voltage-dependent mechanism, either by the blocking the channel pore or by promoting glycine sensitivity, thereby increasing receptor activity (Benveniste and Mayer 1993). It is important to underline that endogenous polyamines are released in the brain by an activity-dependent manner (Li *et al.* 2007).

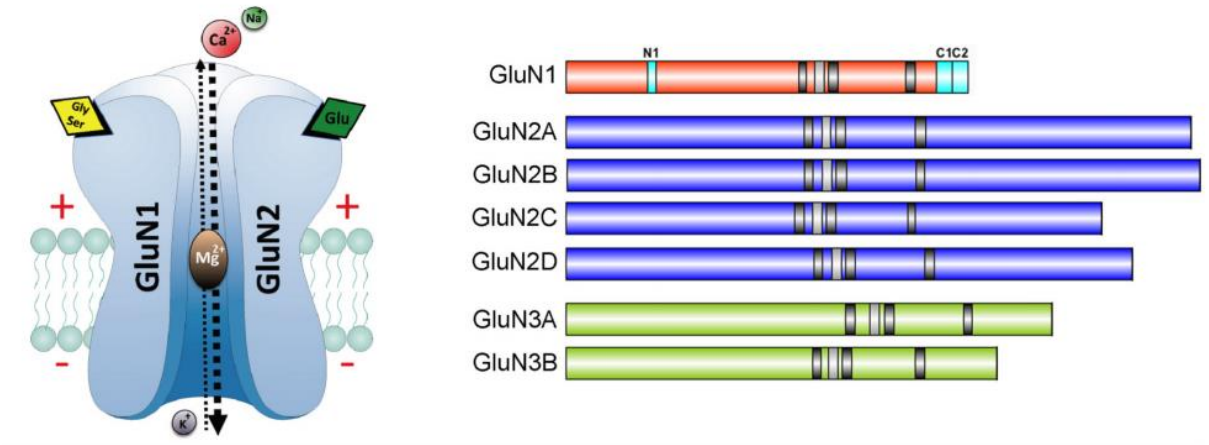


Figure 1.5. NMDA receptor structure, subunits, and topology. NMDA receptors are ionotropic glutamate receptors composed of two GluN1 subunits and two GluN2 or GluN3 subunits. NMDAR are permeable to Ca^{2+}, Na^{+} and K^{+}. To be activated, NMDAR need to bind glutamate (via GluN2 subunits) and glycine (via GluN1), and the Mg^{2+} blockade has to be relieved by membrane depolarization (left). Seven different NMDAR subunits have been identified: GluN1, GluN2A-D and GluN3A-B (right). Adapted from (Sanz-Clemente *et al.* 2013).

GluN1 subunit

GluN1 is the obligatory subunit of NMDAR and it is encoded by a single gene that load three alternatively spliced exons: exon 5 encoding the N1 cassette on the N-terminal domain and exons 21 and 22 encoding the C1 and C2 cassettes respectively, on the C-terminus domain. Alternative splicing of these exons gives rise to eight splicing isoforms of GluN1 (Moriyoshi *et al.* 1991), which confers different properties and localization to NMDAR (Rumbaugh *et al.* 2000, Zhang and Diamond 2009).

The essential role of the GluN1 subunit was confirmed by Forrest and colleagues, who showed that disruption of the expression of this subunit abolishes NMDAR response and that GluN1 (-/-) mice die shortly after birth due to respiratory failure (Forrest *et al.* 1994). This specific subunit is more abundant than GluN2 and it is present in two populations, one that is unassembled and rapidly degraded, and another population that is oligomerized with GluN2 or GluN3 subunits, lasting for hours (Huh and Wenthold 1999). On the other hand, the intracellular pool of GluN1 corresponds to about 60% of the total GluN1 expression (Huh and Wenthold 1999). This subunit possesses two membrane-proximal endocytic motifs (YKRH and VWRK) which guide the internalized receptors into late endosomes (Scott *et al.* 2004). In addition, GluN1 determines several properties of NMDAR, including their inhibition by protons, zinc and the potentiation by polyamines (Cull-Candy and Leszkiewicz 2004).

The surface expression of NMDAR is regulated by direct phosphorylation of the receptor subunits, preferentially GluN1, which is a substrate of protein kinase C (PKC) and protein kinase A (PKA) (Tingley *et al.* 1997). In agreement with these findings, GluN1 conditional knockout (cKO) mice showed an absence of functional NMDAR as the GluN2 subunits were retained in the ER (Fukaya *et al.* 2003). GluN1 phosphorylation regulates the surface expression of NMDAR by affecting the export of newly assembled receptors from ER to the plasma membrane. Phosphorylation of GluN1 by PKC- and PKA, on S896 and S897, respectively, promotes the release of NMDAR from the ER, most likely

by masking the adjacent ER retention motif RXR (893-895) (Scott *et al.* 2001). Moreover, NMDAR phosphorylation by PKC was shown to increase the traffic of NMDAR to the plasma membrane (Lan *et al.* 2001), thereby increasing the amplitude of NMDAR currents and their open probability (Xiong *et al.* 1998). In addition, it was also proposed that PKA can indirectly associate with the GluN1 subunit through PSD-95 (Colledge *et al.* 2000), and the three isoforms of PKC (β, γ and ε) were also found to be a part of an NMDAR complex (Husi *et al.* 2000). PKA-induced phosphorylation of NMDAR further promotes their synaptic targeting under activity-dependent blockage of the receptors (Crump *et al.* 2001) and enhances their Ca^{2+} permeability (Skeberdis *et al.* 2006). Finally, the GluN1 C-terminus contains different motifs that regulate receptor trafficking and interaction with several proteins, including calmodulin, CaMKII, yotiao, alpha-actinin, tubulin, neurofilaments and the downstream regulatory element antagonist modulator (DREAM) (Cull-Candy and Leszkiewicz 2004, Zhang *et al.* 2010).

GluN2 subunit

The majority of native NMDAR are tetrameric assemblies of two GluN1 subunits and two GluN2 subunits (Traynelis *et al.* 2010). These receptors may form diheteromeric structures, but a compelling body of evidence shows that a large proportion of native NMDAR are triheteromers assembled from two GluN1 and two different GluN2 subunits. In particular, it has been demonstrated that GluN1/GluN2A/GluN2B triheteromers account for >50% of the total NMDA receptors in the hippocampus and cortex of the adult rodent brain (Sheng *et al.* 1994, Luo *et al.* 1997, Al-Hallaq *et al.* 2007, Rauner and Kohr 2011, Tovar *et al.* 2013). The functional and pharmacological properties of these triheteromers were assessed recently using engineered C-terminus peptide tags (Hansen *et al.* 2014). These studies showed that in the rat hippocampus, GluN2A and GluN2B subunits exist primarily in di-heteromeric complexes that interact similarly with PSD-95-related proteins but are associated with different protein complexes (Al-Hallaq *et al.* 2007). In addition, it was also proposed that the combination of GluN1, GluN2B and GluN2D (Dunah *et al.* 1998, Brickley *et al.* 2003), as well as combinations of GluN1, GluN2A and GluN2C (Sundstrom *et al.* 1997, Cathala *et al.* 2000), are also possible.

Taking advantage of animal genetic engineering, several mice strains were generated to study the specific role of GluN2 subunits. GluN2B (-/-) [knockout (KO)] mice die shortly after birth because they miss suckling response (Kutsuwada *et al.* 1996), indicating a preponderant role of this subunit during neuronal development. Mice overexpressing GluN2B subunits (GluN2B-Tg), the highly publicized “smart animal” (*doogie mouse*), show enhanced memory and learning ability (Tang *et al.* 1999). In contrast, GluN2A (-/-) mice, although viable, show impairment in synaptic plasticity mechanisms, which translates in a deficiency on spatial learning (Sakimura *et al.* 1995). Moreover, animals lacking both GluN2A and GluN2C subunits do not express NMDAR in the cerebellum or any spontaneous or evoked EPSPs in cerebellar granule cells, and they exhibit deficits in motor coordination. Finally, GluN2D (-/-) mice show less sensitivity to stress and altered monoaminergic neuronal function (Miyamoto *et al.* 2002).

NMDAR transport and endocytosis

The transport and endocytosis of NMDAR are very important mechanisms in the mammalian brain, because they directly regulate the synaptic content of NMDAR **(Fig. 1.6)**. Dendritic transport and synaptic recruitment of NMDAR occur via distinct mechanisms at different developmental stages *in vitro* (Lau and Zukin 2007). Experiments using time-lapse imaging of fluorescently tagged receptor subunits in rat cortical neurons showed that NMDAR and AMPAR subunits are present in distinct mobile transport packets at young ages, which are recruited rapidly and independently to sites of axodendritic contact (Washbourne *et al.* 2002, Washbourne *et al.* 2004). During synaptogenesis NMDAR are transported along microtubules faster than AMPAR. NMDAR packets are vesicle-associated protein complexes containing the scaffold protein SAP-102 and the early endosomal antigen 1 (EEA1) (Washbourne *et al.* 2004). In contrast, at older ages, when the majority of synapses are already formed, NMDAR are recruited more gradually to nascent synapses in the form of clusters containing a small number of receptors (Bresler *et al.* 2004).

NMDAR are transported in large protein complexes to and from the synapses. One MAGUK (membrane-associated guanylate kinase) protein, SAP102, was found to be associated with NMDAR during transport to dendritic compartments, as well as Sec8, a component of exocytosis complex, which is involved in the intracellular trafficking and membrane delivery of the receptor (Matern *et al.* 2001, Yeaman *et al.* 2001, Inoue *et al.* 2003), and mPins (mammalian homologue of Drosophila melanogaster partner of inscuteable) (Sans *et al.* 2003, Sans *et al.* 2005). Moreover, disruption of the interaction between SAP-102 and Sec8 reduces the expression of NMDAR both in heterologous system and in neurons (Sans *et al.* 2003). In addition, the NMDAR trafficking pathway requires SAP97 and CASK, which appear to be essential for the efficient synaptic delivery of the receptors (Jeyifous *et al.* 2009). This trafficking pathway involves a dendritic endoplasmic reticulum subcompartment and Golgi outposts, and is used by NMDAR, but not AMPAR (Jeyifous *et al.* 2009). Similarly, NMDAR subunits form complexes with CASK, Velis/MALS, Mint and KIF17 on vesicles that move rapidly along dendritic microtubules (Setou *et al.* 2000, Setou *et al.* 2002).

Kinesin KIF17, a plus-end-directed motor, transports NMDAR-containing vesicles along microtubules in dendrites, together with the adaptor protein LIN10 (also known as Mint1 or X11), towards nascent synapses (Hirokawa *et al.* 2010). KIF17 interacts through its N-terminal domain with the PDZ domain of LIN10, which binds to intermediate adaptor proteins (LIN2 and LIN7) that ultimately bind to the GluN2B subunit **(Fig. 1.7)**. Interestingly, the genes encoding KIF17 and GluN2B are coregulated (Setou *et al.* 2000, Guillaud *et al.* 2003). In neurons, KIF17 colocalizes with GluN2B and moves along dendrites in anterograde and retrograde directions (Setou *et al.* 2000, Guillaud *et al.* 2003). Similarly to phenotypic alterations characteristic of GluN2B-Tg mice (see above), transgenic mice overexpressing KIF17 also showed enhanced working memory and spatial learning (Wong *et al.* 2002), suggesting a role for KIF17-dependent transport (and possibly GluN2B-NMDAR) in synaptic plasticity.

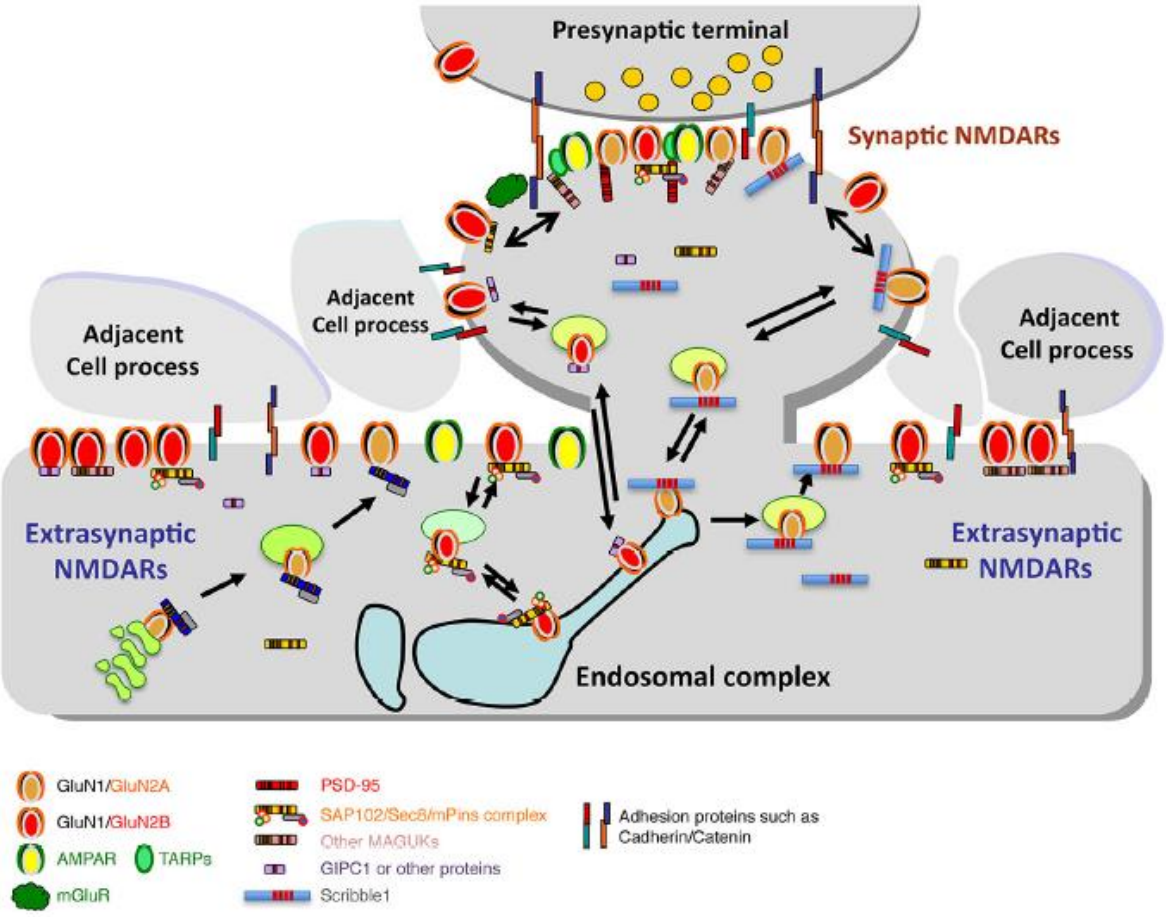


Figure 1.6. Diagram illustrating the synaptic and extra-synaptic distributions of NMDAR and associated scaffolding and adhesion proteins, and especially the association of extra-synaptic NMDAR with adjacent cell processes. Adapted from (Horak *et al.* 2014).

In contrast to GluN2B, GluN2A transport seems to be preferentially mediated by interaction with SAP-97 and regulated by CaMKII (Bassand *et al.* 1999, Gardoni *et al.* 2003, Mauceri *et al.* 2007). SAP-97 phosphorylation by CaMKII on Ser39 (N-terminus) promotes the release of the complex GluN2A-SAP97 from ER to the postsynaptic compartment in hippocampal neurons (Mauceri *et al.* 2007). In contrast, CaMKII-dependent phosphorylation of Ser232 located in the postsynaptic disc-large ZO1 (PDZ) domain of SAP97 disrupts its interaction with GluN2A subunit and thus promotes the insertion of GluN2A-containing receptors in the membrane (Gardoni *et al.* 2003).

Another level of regulation of NMDAR is based on the control of their endocytosis, which utilizes clathrin-coated vesicles via association of the receptors with the AP-2 adaptor complex (Roche *et al.* 2001, Petralia *et al.* 2003, Lavezzari *et al.* 2004, Prybylowski *et al.* 2005). However, internalization of NMDAR may occur by an alternative, non-clathrin-mediated endocytosis mechanism (Swanwick *et al.* 2009). Both GluN2A and GluN2B subunits undergo clathrin-mediated internalization by interaction with AP-2. Interaction with GluN2A occurs via C-terminus di-leucine (LL) motif (Lavezzari *et al.* 2004) although an additional AP-2 binding motif may also be involved (Vissel *et al.* 2001). GluN2B has an AP-2 binding motif close to the C-terminus (YEKL) (Roche *et al.* 2001) but a MAGUK-dependent Src kinase-mediated phosphorylation of the receptor subunit on Tyr1472 (Y1472) prevents internalization and therefore increases NMDAR currents (Prybylowski *et al.* 2005). These findings suggest that GluN2B endocytosis may be regulated by binding of PSD-95 and Src-mediated

phosphorylation of the Y1472 residue, which prevent AP-2 binding and clathrin-mediated GluN2B endocytosis. NMDAR internalization may also occur via a mechanism mediated by calpains, which cleave GluN2A and GluN2B upon prolonged activity of the NMDAR, leading to their degradation (Wu *et al.* 2005).

After internalization, NMDAR can follow different pathways before they are incorporated into endosomes. GluN2A-containing NMDAR are preferentially incorporated into late endosomes for degradation, while GluN2B-containg NMDAR tend to move to recycling endosomes from where they can return to the surface and to the synapse (Lavezzari *et al.* 2004). However, recent studies showed that Scribble1 prevents GluN2A subunits from undergoing lysosomal trafficking and degradation by increasing their recycling to the plasma membrane following NMDAR activation (Piguel *et al.* 2014). Furthermore, Arf6 and the Arf6-specific guanine exchange factor (EFA6) were shown to be involved in that process (Fukaya *et al.* 2014). GluN2A and GluN2B subunits also contain membrane-proximal endocytic motifs, YWKL and YWQF, respectively, which are responsible for targeting receptors to the late endosomes (Scott *et al.* 2004).

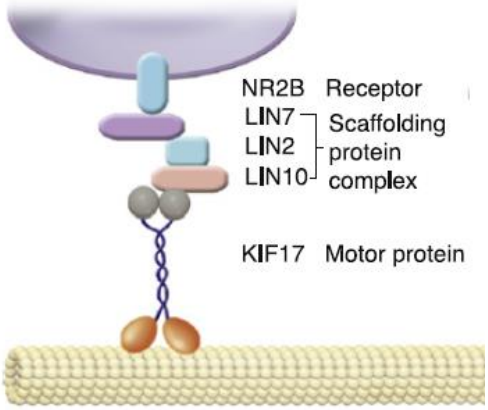


Figure 1.7. KIFs transport receptors in dendrites via scaffolding proteins. KIF17 transports NMDA-type glutamate receptor containing GluN2 subunits by associating with a scaffolding complex consisting of LIN10 (Mint1), LIN2, and LIN7. Old nomenclature was used in the figure: NR2B corresponds to GluN2B. Adapted from (Hirokawa *et al.* 2010).

GluN2 subunit: Role in NMDA receptors properties

The presence of different GluN2 subunits confers distinct features to NMDAR (Gielen *et al.* 2009). For example, receptors composed by GluN2A or GluN2B show larger conductance and higher sensitivity to Mg^{2+} (Stern *et al.* 1992) and Zn^{2+} inhibition (Paoletti *et al.* 1997) than receptors containing GluN2C or GluN2D subunits. Additionally, GluN2A or GluN2B-containing NMDAR are more permeable to Ca^{2+} than GluN2C- or GluN2D-NMDAR (Burnashev *et al.* 1995, Schneggenburger 1996) and exhibit higher open probability (Chen *et al.* 1999). The interaction of glycine with NMDAR also depends on their subunit composition: GluN2A-NMDAR exhibit lower affinity for glycine, due to their N-terminus (Yuan *et al.* 2009), but higher sensitivity to glutamate and faster deactivation kinetics (Vicini *et al.* 1998). In contrast, GluN2D-containing receptors show the lowest sensitivity to glutamate and the

slowest deactivation kinetics, whereas NMDAR containing GluN2B or GluN2C show intermediate properties when compared with receptors containing the other two GluN subunits. Only GluN2B containing NMDAR are also characterized by their sensitivity to polyamines, which potentiate receptor activity, most likely by relieving the proton inhibition (Williams *et al.* 1994, Traynelis *et al.* 1995).

The triheteromeric structure of NMDAR was first described in heterologous systems, by overexpressing GluN1 and two GluN2 (A and B) subunits. Under these conditions, NMDAR containing both GluN2A and GluN2B subunits were formed, which showed intermediate characteristics between pure GluN2A- or GluN2B-containing NMDAR (Vicini *et al.* 1998, Hatton and Paoletti 2005). Taking advantage of the $GABA_B$ receptor leucine zipper motifs and engineered C-terminal peptide tags that were used to selectively express recombinant triheteromeric receptors at the cell-surface without confounding coexpression of diheteromeric receptors, recent studies characterized the pharmacological properties of the triheteromeric GluN1/GluN2A/GluN2B-NMDAR (Hansen *et al.* 2014). This approach showed that the rate of deactivation of triheteromers following stimulation with glutamate is distinct from that recorded in GluN1/GluN2A and GluN1/GluN2B NMDAR. Furthermore, triheteromeric NMDAR channels were found to be modulated by subunit-specific antagonists (ifenprodil, CP-101,606, TCN-201) as well as by extracellular Zn^{2+} (Hansen *et al.* 2014).

GluN2A versus GluN2B subunits

Over the past few decades GluN2A and GluN2B have been the subject of intense investigation. Both subunits are highly expressed in the cerebral cortex as well as in the hippocampus, and play a central role in synaptic function by controlling different forms of synaptic plasticity (discussed in detail below).

As aforementioned, the GluN2 subunits confer different functional and spatial properties to the NMDAR, which are summarized in **Table 1.3**. Briefly, GluN2B-containing receptors have lower open probability (Chen *et al.* 1999) and peak current (Erreger *et al.* 2005), as well as slower deactivation (Erreger *et al.* 2005), rise (Monyer *et al.* 1994, Chen *et al.* 1999) and decay times (Vicini *et al.* 1998) than GluN2A-containing receptors. Furthermore, the GluN2B subunit confers higher Ca^{2+} permeability (Sobczyk *et al.* 2005) and charge transfer (Erreger *et al.* 2005) than GluN2A.

Table 1.3. Comparison of GluN2A and GluN2B-containing NMDA receptors. Adapted from (Yashiro and Philpot 2008).

	GluN2A	*GluN2B*
Developmental Expression		
Expression Start	After birth	Embryonically
Expression Peak	Adulthood	P7 to P10
Channel Properties		
Open probability	High	Low
Deactivation	Fast	Slow
Rise and Decay time	Fast	Slow
Peak Current	High	Low
Charge Transfer	Low	High
Ca^{2+} influx	Low	High
Transport		
Preferential binding	SAP97	KIF17
Endocytosis		
Preferential association	Late endosomes	Recycling Endosomes
Knockout mice		
	Viable	Non-viable

Studies with conditional KO mice in which GluN2B was ablated exclusively in hippocampal CA3 pyramidal cells showed that this subunit is important for the maintenance of the number of NMDAR at the synapse, as well as the normal spine density (Akashi *et al.* 2009), and similar observations were made in other brain regions (Abe *et al.* 2004, Brigman *et al.* 2010). It was also shown that GluN2B-containing receptors regulate the AMPA receptor content at the postsynaptic membrane. In particular, it was found that this type of NMDAR negatively regulates the insertion of AMPAR in developing cortical synapses (Hall *et al.* 2007) and additional studies suggested that this process requires the GluA2 subunit (Lu *et al.* 2011).

Deletion of the GluN2A or GluN2B subunit also provided evidence for their role early in development. Thus, deleting either GluN2A or GluN2B subunits in neurons of the hippocampal CA1 region increases AMPA-mediated currents, although different mechanisms are involved (Gray *et al.* 2011). However, while deleting GluN2A in the CA1 region early in development strengthens pre-existing synapses, deletion of GluN2B has an effect similar to that described upon GluN1 deletion, i.e. an increase in the number of functional synapses, as previously reported (Adesnik *et al.* 2008). The authors proposed a model according to which not only the presence but also the activity of GluN2B-NMDAR early in development acts to limit the constitutive traffic of AMPAR (Gray *et al.* 2011), possibly through long term depression (LTD)-like mechanisms (Xiao *et al.* 2004) or via homeostatic mechanisms (Lu *et al.* 2011). To further understand the regulation of AMPAR by GluN2B-containing receptors early in development, a mouse was generated in which GluN2B was genetically replaced for GluN2A (2B→2A) (Wang *et al.* 2011). Although this manipulation restore NMDAR-mediated currents at glutamatergic synapses, the effects on AMPAR-mediated currents are not. Moreover, it resulted in an occlusion of protein translation-dependent homeostatic synaptic plasticity by a mechanism mediated by the specific binding of GluN2B-containing receptors to CaMKII and the regulation of mammalian target of rapamycin (mTOR) (Wang *et al.* 2011). These findings suggest that GluN2B-containing NMDAR activate unique cellular processes that cannot be rescued by replacement with GluN2A.

Recently, a novel mechanism was proposed according to which GluN2B-mediated anchoring of the synaptic proteasome is responsible for fine tuning the synaptic levels of AMPAR under basal conditions (Ferreira *et al.* 2015). In addition, the ubiquitin ligase substrate adaptor protein Fbxo2 (F-box only protein 2), previously reported to facilitate the degradation of the NMDAR subunit GluN1 *in vitro (Kato et al. 2005)*, also functions to regulate GluN1 and GluN2A subunit levels in the adult mouse brain (Atkin *et al.* 2015). The loss of Fbxo2 results in greater surface localization of GluN1 and GluN2A, together with an increase in the synaptic markers PSD-95 and vGlut1 (vesicular glutamate transporter type 1) (Atkin *et al.* 2015). Although the E3 ligase Fbxo2 does not affect GluN2B levels (Atkin *et al.* 2015), this NMDAR subunit is ubiquitinated by a distinct E3 ligase, Mib2, by a mechanism requiring the Fyn kinase (Jurd *et al.* 2008). Accordingly, Mib2 overexpression also decreases NMDAR-mediated currents (Jurd *et al.* 2008). Taken together, these evidence suggest an important role for the ubiquitin-proteasome system (UPS) in the regulation of NMDAR activity.

GluN2A and GluN2B are also thought to regulate the synaptic localization of NMDAR through their interaction with different partners and to trigger different signaling cascades, as described below.

GluN2A- and GluN2B-containing NMDA receptors subcellular localization

There has been much speculation in recent years about the preferential localization NMDAR depending on their subunit composition. A compelling body of evidence have emerged suggesting that GluN2A-containing receptors are mainly synaptic, while GluN2B are predominantly extrasynaptic (Stocca and Vicini 1998, Rumbaugh and Vicini 1999, Tovar and Westbrook 1999, Shinohara *et al.* 2008, Zhang and Diamond 2009). However, this model has been questioned based on the results of several studies. For example, experiments using uncaged glutamate suggest a similar sensitivity to ifenprodil (selective GluN2B-containing receptors antagonist) between NMDAR expressed in synaptic and extrasynaptic sites (Harris and Pettit 2007). In any case, the segregated subcellular localization is not absolute, as the presence of GluN2A in the extrasynaptic membrane of cultured neurons has been reported (Thomas *et al.* 2006) and GluN2B is also present at the postsynaptic density (PSD) (Shinohara *et al.* 2008). Taken together these evidence suggest that both types of receptors can be located in synaptic and extrasynaptic compartments. The differential localization of NMDAR, favoring synaptic localization of GluN2A and extrasynaptic localization of GluN2B may occur only in specific subtype of synapses, such as the Schaffer Collateral-CA1 (Sch-CA1) pyramidal cells (Shinohara *et al.* 2008) and retinal ganglion cells (Zhang and Diamond 2009). However, it is well accepted that GluN2-containing NMDAR diffuse in and out of the synapse (Tovar and Westbrook 2002, Groc *et al.* 2004, Ladepeche *et al.* 2014), and this mechanism may regulate the synaptic content in addition to their distribution (Triller and Choquet 2008, Choquet and Triller 2013). Their mobility is dependent on the subunit composition, since GluN2B-NMDAR show a higher mobility when compared with GluN2A-NMDAR, being the latter more retained within the synapse (Groc *et al.* 2006). Finally, about 30-40% of the NMDAR at the surface are thought to be mobile (Groc and Choquet 2008).

Nevertheless, the question that arises is how is the subcellular localization of GluN2 subunits regulated? It is believed that protein-protein interaction within the cytoplasmic C-terminus and the extracellular N-terminus of the receptors determine the precise localization of GluN2 subunits. For

example, the PDZ binding motif at the C-terminus end of both GluN2A and GluN2B subunits binds to the second PDZ domain of MAGUK proteins, which act as scaffold proteins.

GluN2A and GluN2B binding partners regulating NMDAR subcellular localization

Synaptic NMDAR are localized at postsynaptic densities, where they are structurally organized and spatially restricted in a large macromolecular complex of synaptic scaffolding and adaptor proteins, which physically links the receptors to kinases, phosphatases and other downstream signaling proteins.

PDZ domain-containing proteins, such as the MAGUK proteins, PSD-93, PSD-95, SAP102, and SAP97, were first identified as the major synaptic scaffolding proteins anchoring NMDAR at glutamatergic synapses (Kornau *et al.* 1997, Kim and Sheng 2004, Gardoni *et al.* 2009, Zheng *et al.* 2011), but many studies have also implicated these proteins in the trafficking of receptors to and/or from synapses (Wenthold *et al.* 2003, Elias and Nicoll 2007). The MAGUK family members directly interact with the four GluN2 subunits (Kornau *et al.* 1995, Brenman *et al.* 1996, Kim *et al.* 1996, Lau *et al.* 1996, Muller *et al.* 1996, Niethammer *et al.* 1996). Members of this family of proteins show differential subcellular localization, with PSD-95 being predominantly expressed at the postsynaptic density and SAP102 being more evenly distributed between synaptic and extrasynaptic sites. In addition, a preferential association GluN2A/PSD-95 and GluN2B/SAP102 has been reported (Sans *et al.* 2000, Townsend *et al.* 2003), although PSD-95, PSD-93 and SAP102 interact with both GluN2A and GluN2B subunits (Lau *et al.* 1996, Muller *et al.* 1996, Sans *et al.* 2000, Al-Hallaq *et al.* 2007). Therefore, it was proposed that binding of GluN2 subunits to different MAGUK proteins controls NMDAR localization. This hypothesis is based on experimental evidence showing that SAP102 is abundantly expressed early in development, while PSD-95 expression increases with age, in accordance with the GluN2B and GluN2A expression patterns, respectively (Sans *et al.* 2000, Petralia *et al.* 2005). Although this model is not supported by the results of biochemical studies (Al-Hallaq *et al.* 2007), other evidence support this scenario for GluN2B, since disruption of GluN2B PDZ binding domain results in a loss of synaptic GluN2B as demonstrated by electrophysiological and confocal imaging (Chung *et al.* 2004, Prybylowski *et al.* 2005). In contrast, the literature for GluN2A is less consistent. GluN2A subunits expressing a point mutation disrupting the PDZ binding domain mediate NMDAR-mEPSCs in a way that is similar to those resulting from the activity of wild-type (WT) GluN2A in transfected cerebellar granule cells (Prybylowski *et al.* 2005). However, studies using a genetically-modified mouse line expressing GluN2A lacking the C-terminus ($GluN2A^{\Delta C/\Delta C}$) showed a reduced synaptic GluN2A expression and slower NMDAR kinetics (Steigerwald *et al.* 2000). A possible explanation for these results is the existence of additional protein binding domains in the GluN2A C-terminus, other than the PDZ binding, that may act to stabilize the receptors at synaptic sites. Recently, it was identified a PDZ-independent binding site between GluN2 and MAGUKs, supporting this model (Cousins *et al.* 2009, Chen *et al.* 2011). Furthermore, two new interaction motifs on SAP102, resulting from two alternatively spliced regions named I1 (located in N-terminus) and I2 (located within a hinge region between the SH3 and GK domains), were also described (Chen *et al.* 2011, Chen *et al.* 2012, Wei *et al.* 2015). Interestingly, SAP102 I1 specifically binds to the GluN2B subunit, regulating its surface expression and dendritic spine morphology (Chen *et al.* 2011), while

SAP102 I2 knockdown differentially affects the surface expression of GluN2A and GluN2B (Wei *et al.* 2015). In addition, PSD-95 binding also seems to regulate both GluN2A and GluN2B subunits. For example, PSD-95 interaction with GluN2A- and GluN2B-containing receptors promotes their clustering (El-Husseini *et al.* 2000) and surface expression (Lin *et al.* 2004, Cousins *et al.* 2008), inducing the synaptic targeting of GluN2A- over GluN2B-receptors in cerebellar granule cells (Losi *et al.* 2003).

Another intriguing question is the possible compensatory mechanism between different MAGUKs. This is suggested based on the results showing no significant losses on NMDAR currents in the PSD-95 and PSD-93 single KOs mice show (McGee *et al.* 2001, Beique *et al.* 2006), which contrast with the significant reduction on NMDAR-mediated currents in PSD-95 and PSD-93 double KO mice (Elias *et al.* 2006). PSD-95 and PSD-93 are responsible for the localization of different NMDAR-signaling complexes within lipid rafts or PSD domains (Delint-Ramirez *et al.* 2010). Almost half of NMDAR were shown to be associated with lipid rafts (Fernandez *et al.* 2009) and their recruitment to synaptic lipid rafts, as well as PSD-95, is induced by spatial memory formation (Delint-Ramirez *et al.* 2010). In fact, both GluN2A and GluN2B subunits interact with the lipid raft associated protein flotilin-1, while interaction with flotilin-2 is GluN2B-specific (Swanwick *et al.* 2009). Moreover, comparing PSD-95 complexes isolated from lipid fractions with PSD-95 complexes isolated from PSD fractions, the former fraction exhibits a lower expression of GluN2B-containing receptors, SynGAP and CaMKIIα, while the latter preparation exhibits an increased expression of Arc (activity-regulated cytoskeleton-associated protein) and Src kinase (Delint-Ramirez *et al.* 2010). Cytoskeleton-binding proteins may also regulate the subcellular localization of NMDAR. For example, α-actinin, which belongs to the spectrin gene superfamily, directly binds to the GluN2B subunit but not to GluN2A (Wyszynski *et al.* 1997), and plays an important role in the localization of NMDAR (Leonard *et al.* 2002, Jalan-Sakrikar *et al.* 2012).

The extracellular domain of NMDAR also plays a role in controlling their subcellular localization via interaction with other postsynaptic proteins. For example, EphB, a receptor tyrosine kinase, is required for the enhanced localization of GluN2B-containing NMDAR at synapses of mature neurons (Dalva *et al.* 2000, Nolt *et al.* 2011). Recently, a mechanism was proposed according to which EphB regulates PSD-95 anchoring and synaptic stability, which may then contribute to anchor NMDAR at the synapse (Hruska *et al.* 2015). In addition, Reelin, a component of extracellular matrix (ECM), also regulates the synaptic localization and function of GluN2B-containing NMDAR in postnatal hippocampal neurons (Groc *et al.* 2007). Other proteins are likely to modulate NMDAR localization via indirect processes, including neuroligins (Jung *et al.* 2010, Budreck *et al.* 2013).

Dynamic regulation of GluN2A and GluN2B subunits by phosphorylation

Phosphorylation is defined as the reversible addition of a phosphate group (PO_4^{3-}) to a protein, typically to serine (Ser), threonine (Thr) or tyrosine (Tyr) residues, although phosphorylation of histidine (His), arginine (Arg) and lysine (Lys) has also been reported (Ciesla *et al.* 2011). This phenomena is regulated by several kinases and phosphatases that rapidly and efficiently regulate the addition and removal of phosphate groups, respectively.

GluN2 phosphorylation is a key regulatory mechanism controlling the trafficking of NMDAR and it occurs predominantly in the C-terminus of GluN2 subunits (Sanz-Clemente *et al.* 2013, Lussier *et al.* 2015) **(Fig. 1.8)**. In addition to the effects on NMDAR localization, the phosphorylation of GluN2 also regulates the function of these receptors. PKA and PKC phosphorylate the C-terminus of the GluN2B subunit in distinct residues (Leonard and Hell 1997). It was recently shown that GluN2B phosphorylation by PKA (S1166) plays an important role in the regulation of NMDAR-dependent Ca^{2+} permeability (Aman *et al.* 2014). PKC phosphorylates GluN2B subunit in two distinct residues, S1303 and 1323, resulting in enhanced currents through NMDAR (Liao *et al.* 2001).

Phosphorylation also regulates the surface and synaptic expression of NMDAR in a subunit-specific manner, providing a highly plastic and precise mechanism to accurately control different subunits in response to distinct stimuli. For example, GluN2B internalization is mediated by clathrin and is tightly controlled by Src/Fyn-mediated phosphorylation of Y1472 (Lavezzari *et al.* 2003). This residue belongs to the endocytic motif of the GluN2B subunit, YEKL, which is recognized by the clathrin protein adaptor AP-2 as a required step to induce receptor internalization. GluN2B phosphorylation of Y1472 blocks the binding of AP-2, thus preventing its endocytosis and thereby increasing its surface expression (Lavezzari *et al.* 2003, Prybylowski *et al.* 2005, Sanz-Clemente *et al.* 2010). Src/Fyn kinases directly bind to MAGUKs, such as PSD-95 and SAP-102, and GluN2B phosphorylation on Y1472 is promoted by the interaction of the receptor with these scaffold proteins. Therefore, synaptic GluN2B exhibits elevated levels of Y1472 phosphorylation. Src/Fyn also phosphorylates GluN2B C-terminus in two additional tyrosines, Y1252 and Y1336 (Nakazawa *et al.* 2001), and the phosphorylation of the latter tyrosine residue (Y1336) decreases calpain-mediated proteolysis of GluN2B (Wu *et al.* 2007) which may regulate NMDAR properties during synaptic plasticity (Wu *et al.* 2007).

GluN2B is also phosphorylated on S1480 by casein kinase 2 (CK2), which inversely controls the phosphorylation of GluN2B Y1472. The CK2-mediated serine (S1480) phosphorylation occurs within the PDZ binding domain, thereby disrupting the interaction of the receptor with MAGUK proteins (Chung *et al.* 2004). The decrease in the interaction between NMDAR and the anchoring proteins at the postsynaptic density allows the receptors to diffuse laterally to extrasynaptic sites, similarly to the effects of Y1472 dephosphorylation by the action of STEP (striatal-enriched protein tyrosine phosphatase) (Venkitaramani *et al.* 2011, Chen *et al.* 2012). Additionally, the disruption of the PDZ interaction “dissociates” the receptor and Src/Fyn kinases, decreasing the phosphorylation of Y1472 (Chen *et al.* 2012). Thus, S1480 phosphorylation of the GluN2B C-terminal results in a decrease of Y1472 phosphorylation, thereby promoting the internalization of the receptor (Sanz-Clemente *et al.* 2010).

An additional mechanism involved in the regulation of NMDAR synaptic expression is by PKC/CaMKII-mediated phosphorylated of GluN2B on S1303 (Omkumar *et al.* 1996, Liao *et al.* 2001). Synaptic activity enhances the physical interaction of CaMKII with GluN2B (residues 1290-1310) but the phosphorylation of S1303 disrupts the interaction of the NMDAR subunit with CaMKII (O'Leary *et al.* 2011). Furthermore, the active form of CaMKII interacts with CK2, which phosphorylates the PDZ binding domain of GluN2B (Sanz-Clemente *et al.* 2013). In this process, CaMKII acts as a “scaffold” protein to couple GluN2B and CK2, thereby promoting the phosphorylation of GluN2B on S1480

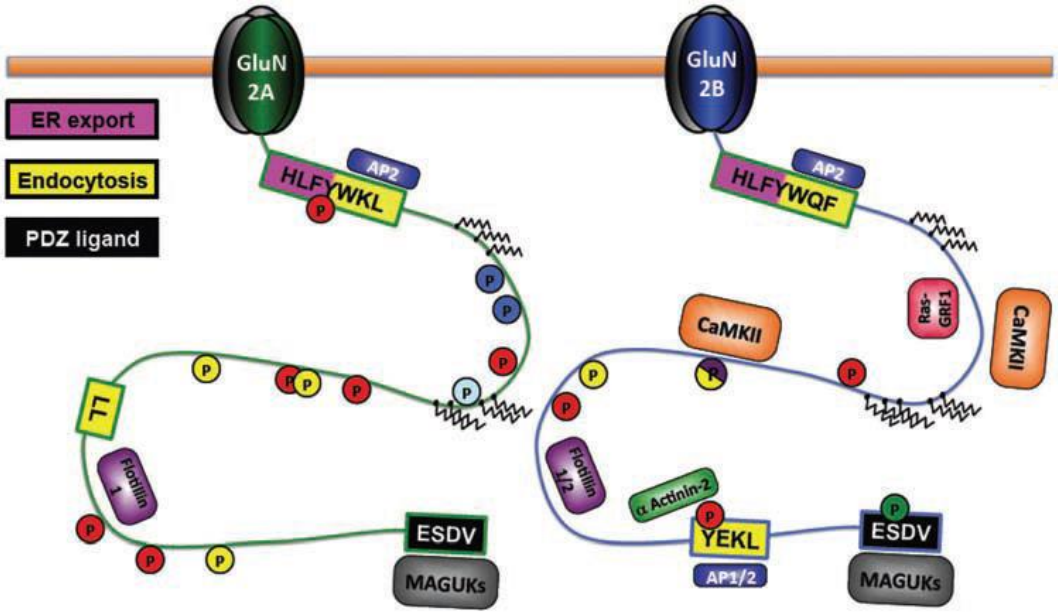
(Sanz-Clemente *et al.* 2013). Since phosphorylation of S1303 by PKC reduces GluN2B/CaMKII association, it has a secondary effect on the CK2-mediated phosphorylation of S1480 and ultimately on Y1472 phosphorylation by Src/Fyn. To summarize, the phosphorylation of three distinct residues on the C-tail of GluN2B subunit (S1303, Y1472, S1480) by four different kinases regulates in a coordinated manner the synaptic expression of GluN2B-containing receptors. An additional level of complexity in the mechanisms of GluN2B phosphorylation results from the association of MAGUK proteins with Src kinase, which is modulated by Cdk5-mediated PSD-95 phosphorylation (Morabito *et al.* 2004). In addition, Cdk5 also regulates the GluN2B phosphorylation on Y1472 (Zhang *et al.* 2008), likely by a mechanism involving the disruption of the interaction between the Src and PSD-95 or by regulation of PSD-95 ubiquitination (Bianchetta *et al.* 2011). Recent studies showed that Cdk5 binds directly and phosphorylates the C-tail of GluN2B on S1116 to decrease receptor surface expression in an activity-dependent manner (Plattner *et al.* 2014). A new phosphorylation site on GluN2B C-tail was recently identified, Y1070, which is phosphorylated by Fyn and is required to couple the Fyn kinase and the GluN2B subunit, resulting in the upregulation of Y1472 phosphorylation and GluN2B-NMDAR surface expression (Lu *et al.* 2015). Together, these multiple lines of evidence suggest a tight and complex mechanism for the synaptic regulation of GluN2B-containing NMDAR, with direct implications in the functionality of the receptors.

The high degree of sequence homology between GluN2A and GluN2B and the close proximity of the aforementioned kinases, would suggest that synaptic expression of GluN2A-containing NMDAR is also tightly regulated by phosphorylation. Indeed, several putative phosphorylation sites were identified in the C-terminus of GluN2A (Lau and Huganir 1995, Yang and Leonard 2001, Ghafari *et al.* 2012). However, the lack of GluN2A phosphorylation is striking and may explain the dynamic differences between the two NMDAR subunits highly expressed in adult cortex and hippocampus. While GluN2B is highly mobile and after endocytosis is incorporated into recycling vesicles, GluN2A is more static and after endocytosis it is mainly incorporated into late endosomes targeted for degradation (Lavezzari *et al.* 2004). In fact, GluN2A and GluN2B are subjected to a differential regulation despite their homology in the C-terminal tail. For example, the PDZ binding domain of GluN2A is not required for the maintenance of GluN2A synaptic localization (Sanz-Clemente *et al.* 2013). Furthermore, although there are examples of GluN2A regulation by phosphorylation, it is upstream of the C-terminal end. For example, GluN2A is phosphorylated in its C-terminal domain, specifically on S1048, by the Dyrk1a kinase, which increases surface expression by impairing internalization (Grau *et al.* 2014), but the molecular mechanisms regulating this process and the physiological relevance of these findings remain unknown.

The complexity of the mechanisms that regulate in a coordinated manner the GluN2A and GluN2B properties and tracking is further increased by the presence of triheteromeric structures composed by GluN1/GluN2A/GluN2B. These structures represent more than one-third of the total NMDAR (Al-Hallaq *et al.* 2007) and their electrophysiological/functional contribution to NMDA-mediated currents in adult CA1 pyramidal neurons is estimated in over than 50% (Rauner and Kohr 2011). Furthermore, it has been shown that GluN2B plays a dominant role in NMDAR trafficking, with triheteromers showing a similar ratio of internalization and recycling compared to GluN1/GluN2B receptors (Tang *et al.* 2010).

Other post-translational modifications of GluN2A and GluN2B subunits

In addition to phosphorylation, other post-translational modifications (PTM) of GluN2 subunits, such as palmitoylation and ubiquitination, are gaining attention as well. Palmitoylation is defined by the covalent and reversible addition of a palmitic acid molecule (saturated 16-carbon lipid) to a cysteine residue in a given protein through the action of palmitoyltransferases (PATs). Palmitoylation is a reversible process, since the palmitoyl group can be removed from target proteins through activity of acyl-protein-thioesterases. Palmitoylation is important for the trafficking of NMDAR, and GluN2A and GluN2B palmitoylation occurs in "two clusters" **(Fig. 1.8)**. The function of these clusters is distinct: While Cluster I, close to the last transmembrane domain of GluN2A and GluN2B, is associated with an increase in surface expression of the receptor, Cluster II, located in the middle of intracellular C-terminus, plays the opposite role being associated with receptor accumulation in the Golgi apparatus (Hayashi *et al.* 2009, Thomas and Huganir 2013).



	GluN2A	GluN2B
PKA	S900 ; S929	
PKC	S1291 ; S1312 ; S1416	S1323
CK2		S1480
Cdk5	S1232	
PKC/CaMKII	S1303	
Src/Fyn	Y842 ; Y1105 ; Y1267 Y1292 ; Y1325 ; Y1387	Y1252 ; Y1336 ; Y1472
Palmitoylation	C848 ; C853 ; C870 C1214 ; C1217 ; C1236 ; C1239	C849 ; C854 ; C871 C1215 ; C1218 ;C1239 ; C1242 ; C1245

Figure 1.8. Sites of intracellular modulation of NMDAR. Schematic representation of the distribution of selected posttranslational regulatory sites on the intracellular C-terminal domains of GluN2A and GluN2B NMDAR subunits. Adapted from (Sanz-Clemente *et al.* 2013).

In addition to palmitoylation, GluN2A and GluN2B may also be regulated by ubiquitination. Ubiquitination is a highly regulated ATP-dependent process that requires the conjugated and sequential action of three enzymes, an E1 activating enzyme, an E2 conjugating enzyme and finally an E3 ubiquitin ligase. Many studies have characterized the ubiquitination of mammalian iGluRs. For example, the ubiquitin E3 ligases Fbxo2 (Atkin *et al.* 2015) and Mind Bomb-2 (Jurd *et al.* 2008) ubiquitinate the NMDAR subunits GluN1 and GluN2B in an activity-dependent manner.

GluN3 subunit

GluN3 subunits were the last NMDAR subunits to be cloned **(Table 1.2)**. Despite the lack of information about its role in the brain, it is known that GluN3 subunits can assemble with GluN1 and GluN2, regulating NMDAR currents. However, the stoichiometry of GluN3-containing receptors remains to be identified. These subunits bind glycine and cannot form homomeric functional receptors (Chatterton *et al.* 2002, Yao and Mayer 2006). Heteromeric GluN3-containing receptors are characterized by a lower Ca^{2+} permeability, reduced sensitivity to voltage-dependent block by extracellular Mg^{2+} and reduced single-channel conductance (Matsuda *et al.* 2002, Sasaki *et al.* 2002).

The GluN3 family comprises two different types of NMDAR subunits, GluN3A and GluN3B, which are developmentally regulated as aforementioned. Hippocampal neurons from GluN3A transgenic (GluN3A-Tg) mice show a decreased sensitivity to Mg^{2+} and a reduced Ca^{2+} permeability (Tong *et al.* 2008), while GluN2A KO mice exhibit increased NMDAR/AMPA EPSC ratio (Tong *et al.* 2008). GluN3B (-/-) mice show some impairment on motor learning and coordination skills, and exhibit a mild anxiety-like behavior (Niemann *et al.* 2007).

Recent advances in the understanding of glutamatergic synapse function and structure showed that astrocytes play an active role through the release of glutamate and D-serine, in a Ca^{2+}-dependent manner, which modulates synaptic transmission (Fellin *et al.* 2004, Volterra and Meldolesi 2005, Panatier *et al.* 2006, Reichenbach *et al.* 2010). These gliotransmitters can act on neuronal GluN3-containing NMDAR located at extrasynaptic sites (Fellin *et al.* 2004, Perez-Otano *et al.* 2006). Moreover, astrocytes also express GluN3-NMDAR (Lee *et al.* 2010) which can influence their intracellular Ca^{2+} homeostasis.

NMDA receptors and disease

NMDAR play an important role in neuronal development, synaptic transmission and in synaptic plasticity. Therefore, it is not surprising that mislocalization and abnormal trafficking of NMDAR have been associated with several brain disorders and pathological conditions such as Huntington´s or Alzheimer´s disease **(Table 1.4)**.

Huntington´s disease (HD) is a genetic neurodegenerative disorder, characterized by an expanded CAG repeat mutation in the *huntington* gene which leads to a mutant polyglutamine repeat in the huntington protein (mtHTT). Patients with HD are characterized by defects in muscle coordination and dementia. The disease is also characterized by degeneration of striatal medium spine neurons (MSN), which are enriched in NMDAR. Interestingly, activation of synaptic NMDAR enhances the formation of non-toxic mtHTT inclusions and reduces mtHTT-induced toxicity (Okamoto

et al. 2009). The extrasynaptic NMDAR are mainly GluN2B-NMDAR and crossing GluN2B-overexpressing mice with HD model mice exacerbates the loss of MSN (Heng *et al.* 2009). Thus, extrasynaptic GluN2B-NMDAR may play an important role in neuronal cell death in HD.

Alzheimer´s disease (AD) is a dementia characterized by the increased generation of beta-amyloid peptide (Aβ) and the formation of intracellular tangles mainly composed by protein tau in a hyperphosphorylated state. Given the role of NMDAR in cognitive processes, it has long been thought that NMDAR play a critical role in the effects of Aβ [reviewed in (Malinow 2012). Aβ oligomers bind to the synapse and decrease the surface expression of GluN2B, but not GluN2A (Snyder *et al.* 2005). Aβ also increases the activity of $STEP_{61}$, promoting the dephosphorylation of GluN2B Y1472 and thus reducing its surface expression (Snyder *et al.* 2005), suggesting a critical role of NMDAR in this neurodegenerative disease.

Several additional lines of evidence are available pointing to alterations in NMDAR in many other pathological conditions such as Parkinson disease (PD), ischemia and stroke and schizophrenia **(**summarized in **Table 1.4).**

Table 1.4. NMDAR alteration in pathological conditions. Adapted from (Sanz-Clemente *et al.* 2013).

Disorder	NMDAR alteration	References
Huntington's disease (AD)	Enhanced extrasynaptic NMDAR activity	(Gladding and Raymond 2011)
Alzheimer´s disease (AD)	Reduction in GluN2B surface expression Pathological activation of extra-synaptic NMDAR	(Snyder *et al.* 2005, Ronicke *et al.* 2011)
Parkinson´s disease (AD	GluN2B redistribution from synaptic to extra-synaptic sites Increased synaptic GluN2A	(Dunah *et al.* 2000)
Ischemia and stroke	Enhanced extrasynaptic NMDAR activity	(Hardingham and Bading 2010)
Schizophrenia	Decreased NMDAR function Altered GluN2A trafficking via neuregulin/ErbB4 receptor	(Gaspar *et al.* 2009)

Synaptic Plasticity

The influx of calcium through NMDAR is a trigger for plasticity of excitatory synapses. This concept was firstly postulated by Hebb in 1949, when he suggested that experience can modify synapses favoring some neuronal pathways within a circuit and weakening others (Hebb 1949). The best studied forms of Hebbian plasticity, extensively associated with learning and memory mechanisms, are long-term potentiation (LTP) and long-term depression (LTD). In both forms of synaptic plasticity, the NMDAR composition, particularly the GluN2 subunit content, seems to play a crucial role [reviewed in (Yashiro and Philpot 2008, Fetterolf and Foster 2011, Paoletti *et al.* 2013)].

The first full description of LTP was published more than 40 years ago (Bliss and Lomo 1973) when it was reported that trains of high-frequency stimulation of the rabbit perforant path induce a sustained increase in the efficiency of synaptic transmission in the granule cells of the dentate gyrus. This form of synaptic plasticity is characterized by three important properties: i) the cooperativity,

which describes the existence of an intensity threshold for induction; ii) LTP is associative, in the sense that a weak input can be potentiated by other separated but convergent input; iii) LTP is input-specific indicating that potentiation is not shared with other pathways distinct from the potentiated synapse (Bliss and Collingridge 1993).

LTP can occur in several brain regions and much is known about the molecular mechanisms underlying this form of plasticity. However, LTP in the hippocampus is the most studied form of synaptic plasticity and it is typically divided into at least three distinct and sequential phases: Short-term potentiation, early-LTP (E-LTP), and late LTP (L-LTP). Short-term potentiation and E-LTP are transient and involve the modification of pre-existing proteins, lasting minutes, whereas L-LTP requires gene expression and *de novo* protein synthesis, lasting hours and under certain conditions even days (Sweatt 1999, Kandel 2001).

NMDAR activity allows the postsynaptic influx of Ca^{2+} which is necessary and sufficient for the induction of E-LTP (Bliss and Collingridge 1993). The postsynaptic increase of the $[Ca^{2+}]_i$ activates the intracellular signaling machinery that mediates E-LTP induction, including CaMKII and PKC (Sweatt 1999). The sustained activation of these enzymes also plays an important role in the maintenance of E-LTP (Bliss and Collingridge 1993, Lynch 2004). PKC and CaMKII become autonomously active and phosphorylate downstream targets that underlie E-LTP maintenance, such as AMPAR, resulting in an increase in their trafficking and insertion in the postsynaptic membrane, with a consequent upregulation of the receptor activity (Malenka 2003, Malenka 2003, Derkach *et al.* 2007, Shepherd and Huganir 2007, Lin *et al.* 2009). Additionally, a surface increase of NMDAR was also observed upon E-LTP induction (Grosshans *et al.* 2002).

Sequentially, E-LTP is followed by L-LTP which is dependent on protein synthesis (Sutton and Schuman 2006) and gene transcription activity (Lynch 2004, Adams and Dudek 2005) **(Fig. 1.9)**. Several signaling proteins such as PKA, CaMKIV and extracellular-regulated protein kinase (ERK) are involved in the activation of transcription factors, including cAMP-responsive element-binding (CREB) protein and the ternary complex (TCF) Elk-1 (Platenik *et al.* 2000, Lynch 2004, Adams and Dudek 2005). The increase in gene expression is followed by an upregulation in the synthesis of proteins that mediate structural and/or functional changes in the synapses, which is thought to be required for the maintenance of L-LTP. The maintenance of this form of synaptic plasticity also requires structural changes in the architecture of dendritic spines. The actin-mediated enlargement of the spine head, together with an increase in the spine number, is believed to support the long-term changes in synaptic transmission (Krucker *et al.* 2000, Fukazawa *et al.* 2003).

LTD was described in 1977 as a long-lasting decrease of the synaptic strength following low-frequency synaptic stimulation (Lynch *et al.* 1977). The prevailing view is that NMDAR activation is responsible for triggering both LTP and LTD depending on the type of stimulus and the levels of intracellular Ca^{2+} reached. In a classical model, the difference between NMDAR-dependent LTP and LTD has been explained by distinct levels and/or kinetics of NMDAR-mediated calcium influx, that ultimately triggers different signaling pathways (Malenka and Bear 2004). Increased levels of calcium during LTP induces activation of CaMKII and AMPAR insertion into the postsynaptic membrane

(Malenka and Bear 2004, Lisman *et al.* 2012, Lisman and Raghavachari 2015), whereas calcium influx during LTD increases the rate of AMPAR endocytosis by a mechanism involving the activation of phosphatases, such as calcineurin (PP2B) (Malenka and Bear 2004, Xia and Storm 2005, Collingridge *et al.* 2010).

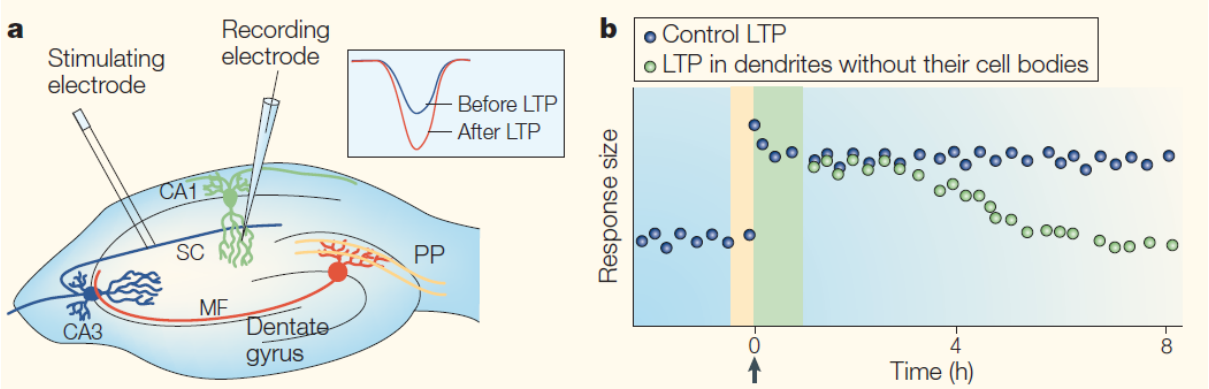


Figure 1.9. LTP in the Shaffer collateral-CA1 synapses and the dependence on transcription of its late phase. A. Illustration of a coronal slice of the rodent hippocampus. A stimulating electrode placed in the *stratum radiatum* (pyramidal cell dendrites) activates the fibers of the Schaffer collateral-commissural pathway that forms synapses on CA1 pyramidal cells. Synaptic responses can be recorded extracellularly with a recording electrode, also placed in the *stratum radiatum*. Inset: extracellular responses before and after induction of long-term potentiation (LTP). CA3 and CA1, CA3 and CA1 hippocampal region; MF, Mossy fibers; PP, Perforant path; SC Shaffer collaterals. **B.** LTP induced by high-frequency stimulation (arrow) often lasts more than 8 h in a slice preparation (blue dots). If dendrites are severed from their cell bodies, LTP decays to baseline level within 4 h (green dots). In the old nomenclature NR2A and NR2B corresponds to GluN2A and GluN2B, respectively. Adapted from (Adams and Dudek 2005).

It was also proposed that the content on GluN2A and GluN2B subunits may define the threshold for the induction of LTP or LTD (Yashiro and Philpot 2008) **(Fig 1.10)**. This hypothesis predicts that the same frequency of stimulation produces different outcomes in synaptic plasticity depending on the ratio of GluN2A/GluN2B content of NMDAR at the synapse. In synapses with a higher levels of GluN2B (low GluN2A/GluN2B ratio), large amounts of Ca^{2+} enter through NMDAR in response to synaptic stimulation and/or calcium is more likely to activate CaMKII, even with a modest synaptic activity, and therefore activates LTP pathways. In this kind of synapses, only a very weak stimulation activates calcineurin (PP2B) without sufficiently activating CaMKII, allowing LTD to be induced. Conversely, when GluN2A-containing NMDAR are predominant in the postsynaptic membrane, Ca^{2+} entry is limited and/or there is less CaMKII brought to the site of calcium entry via GluN2B. This increases the stimulation requirements necessary to activate CaMKII rather than PP2B, favoring the LTD induction in this kind of synapses (Yashiro and Philpot 2008).

Consistently, GluN2B-containing receptors are preferentially expressed at smaller dendritic spines (Sobczyk *et al.* 2005, Shinohara *et al.* 2008) which are more likely to undergo LTP than larger spines (Matsuzaki *et al.* 2004). Furthermore, experience-dependent upregulation of the GluN2A/GluN2B ratio increases the threshold needed for LTP induction in the visual cortex (Philpot *et al.* 2007). Interestingly, GluN2B subunit was shown to mediate the metaplasticity at the level of a single synapse (Lee *et al.* 2010). This work demonstrates that silenced synapses exhibit an enhanced expression of GluN2B-containing receptors, which favors LTP mechanisms, lowering the threshold for LTP induction (Lee *et al.* 2010).

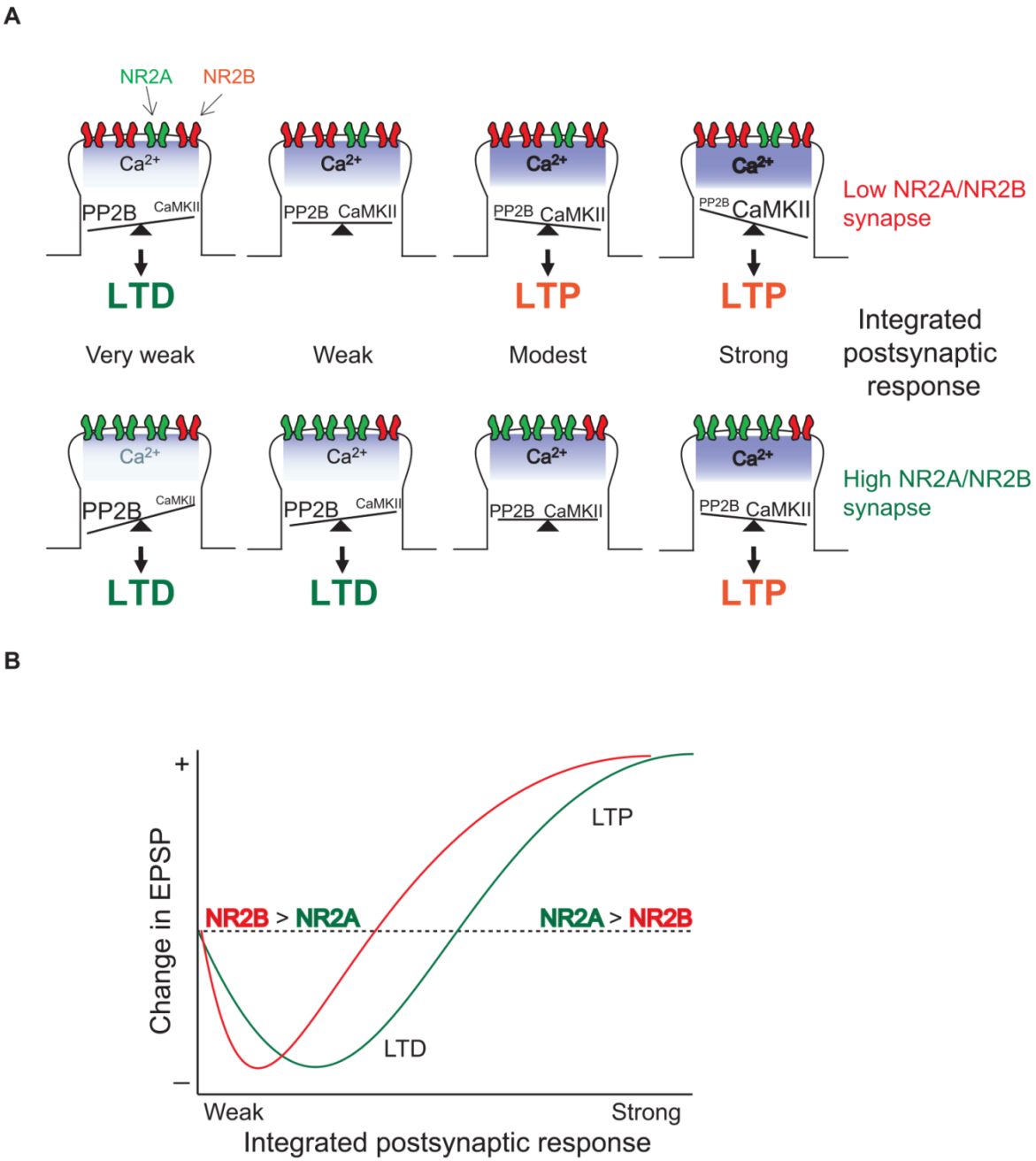


Figure 1.10. **Hypothetical model of synaptic plasticity regulation by NMDAR subunits. A**. A model to explain why the LTD/LTP induction threshold may differ between synapses with low (upper) and high (lower) NR2A/NR2B ratios. In these two types of synapses, the same frequency of stimulation will produce different outcomes in synaptic plasticity, because of the difference in the relative levels of activated PP2B and CaMKII, which are coupled to LTD and LTP pathways, respectively. **B.** Schematic representation of how NMDAR subunits are coupled to different forms of synaptic plasticity. The x-axis represents the level of the integrated postsynaptic response (which is related to the frequency of synaptic activation), while the y-axis represents the lasting change in synaptic strength. Adapted from (Yashiro and Philpot 2008).

Multiple lines of evidence have emerged indicating that neurotrophins, in particular brain-derived neurotrophic factor (BDNF), are potent mediators of hippocampal LTP [reviewed in (Bramham and Messaoudi 2005, Lu *et al.* 2008, Minichiello 2009, Leal *et al.* 2015)]. BDNF is a downstream gene induced by LTP and this protein has been shown to be crucial player in both induction and maintenance of LTP.

Regulation of hippocampal LTP by BDNF

BDNF belongs to the neurotrophin family of trophic factors. Nerve growth factor (NGF) was the first member of this family to be discovered as a target-derived protein that promotes the growth and survival of sympathetic and sensory neurons during development (Cohen *et al.* 1954). BDNF was firstly purified from pig brain in the early 80´s and showed similar neurotrophic actions in sensory neurons (Barde *et al.* 1982). In addition, two more neurotrophins were discovered since then, neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5) (Lewin and Barde 1996). It is now well established that neurotrophins can be released, at least to some extent, in response to neuronal activity (Thoenen 1991), and that they have pleiotropic function in the nervous system, including roles in synaptogenesis, neuronal survival and differentiation, and as mediators of activity-dependent synaptic plasticity (Park and Poo 2013).

Activation of two distinct classes of membrane-bound receptors mediate the physiological responses to the neurotrophins, namely $p75^{NTR}$ receptors and tropomyosin-related kinase (Trk) receptors (TrkA-TrkC). NGF binds to TrkA receptors, BDNF and NT4/5 bind to TrkB receptors, while NT3 binds to TrkC (Chao 2003, Reichardt 2006). In contrast with Trk receptors, activation of $p75^{NTR}$ does not display the same specificity since this receptor binds both the mature and the precursor forms (pro-neurotrophins) of neurotrophins (Lee *et al.* 2001, Teng *et al.* 2005).

Activity-dependent synthesis and secretion of BDNF

Transcriptional and post transcriptional regulation of BDNF

The *Bdnf* gene is very complex, containing at least nine promoters (Aid *et al.* 2007), each driving the transcription of BDNF mRNAs carrying one of the alternative 5' non coding exons spliced to the common 3' coding exon (Aid *et al.* 2007). Among these, promoter IV is highly responsive to neuronal activity, which is coupled to transient increases in the $[Ca^{2+}]_i$. Ca^{2+} influx through voltage-gated calcium channels (VGCC) and NMDAR triggers *bdnf* transcription in cultured hippocampal neurons by promoting the binding of transcription factors such as cyclic AMP (cAMP) response element (CRE)-binding protein (CREB) and Ca^{2+}-response factor (CaRF) to the correspondent regulatory regions (Tao *et al.* 1998, Tao *et al.* 2002). Several activity-inducing paradigms, such as kainic acid-induced seizures (Zafra *et al.* 1990) and high-frequency stimulation (HFS) (Patterson *et al.* 1992) strongly induce the expression of the BDNF mRNA. The observation that HFS, a stimulation paradigm known to induce LTP, upregulates BDNF mRNA in CA1 neurons (Patterson *et al.* 1992) provided the first hint suggesting that the neurotrophin could be involved in synaptic plasticity. Recent studies performed with mutant mice in which promoter IV-dependent BDNF expression is selectively disrupted showed that activity-induced BDNF expression is required for BDNF-dependent L-LTP but not E-LTP at CA1 synapses (Sakata *et al.* 2013). Surprisingly, hippocampal-dependent spatial and contextual memories were normal in these mice (Sakata *et al.* 2013). Given the role of BDNF in cognition, and since basal levels of BDNF were not dramatically changed in these mice, it may be hypothesized that those cognitive processes are more sensitive to changes in basal levels of BDNF. Taking into account the complexity of *bdnf* gene structure, it is possible that activation of specific promoters may confer specificity to the actions of BDNF in multiple brain processes and regions.

Although BDNF transcripts contain the same encoding region, multiple splicing sites on the 5'UTR and alternative polyadenylation site selection on the 3'UTR result in the generation of multiple variants of the BDNF mRNA (Aid *et al.* 2007). The presence of two polyadenylation sites in the BDNF transcript results in two distinct pools of mRNAs in the brain containing either a short or a long 3'UTR (Timmusk *et al.* 1993). The short 3'UTR-carrying mRNAs are restricted to somatic regions whereas the long 3'UTR-containing mRNAs are also found in neuronal dendrites (An *et al.* 2008). Several lines of evidence also suggest the alternative 5'UTR regions of BDNF transcript as important determinants for the localization of the mRNA in the soma versus dendrites (Tongiorgi 2008, Baj *et al.* 2011). Importantly, blocking the dendritic localization of *BDNF* mRNA, *in vivo*, reduced BDNF protein levels in dendrites and resulted in a selective impairment of LTP in dendrites (An *et al.* 2008). Together, these results suggest a critical role for dendritically synthesized BDNF in synaptic plasticity. However, in contrast with these observations, a recent study using high resolution imaging and deep sequencing techniques, to evaluate the distribution of endogenous *BDNF* mRNA in hippocampal neurons (Will *et al.* 2013), showed that *BDNF* mRNA is present in the somatic compartment but rarely detected (or even absent) in dendrites (Will *et al.* 2013). In agreement with these observations, super resolution fluorescence microscopy studies showed that endogenous BDNF protein is preferentially located at presynaptic structures in cultured hippocampal neurons (Andreska *et al.* 2014). This is also in accordance with the detection of endogenous BDNF in presynaptic large dense core vesicles in the Schaffer collaterals but not in the CA1 pyramidal cell dendrites (Dieni *et al.* 2012). These results raise questions about the ability of endogenous BDNF mRNA to localize in dendrites and the potential of being translated locally under basal conditions. Further research should address this question and investigate whether LTP-inducing stimuli is capable of inducing local synthesis of BDNF *in vivo*. Interestingly, activity-driven local synthesis of BDNF controls plasticity of dendritic spines in hippocampal neurons (Verpelli *et al.* 2010).

Secretion of BDNF

Pro-BDNF versus mBDNF

Similarly to many other secreted proteins, BDNF is initially synthesized in the endoplasmic reticulum as a precursor protein, pre-pro-BDNF, which is then converted into pro-BDNF by removal of the signal peptide. Pro-BDNF is then cleaved to generate the mature form of the neurotrophin, but whether this cleavage occurs intracellularly or after its secretion remains controversial. Unlike other neurotrophins (e.g. NGF), BDNF is released through constitutive and regulated pathways. Several lines of evidence suggest that BDNF is released in the precursor form (pro-BDNF) (Pang *et al.* 2004, Woo *et al.* 2005, Nagappan *et al.* 2009, Yang *et al.* 2009, Mizoguchi *et al.* 2011, Yang *et al.* 2014), and the molecule is further processed into the mature form extracellularly, via the action of metalloproteinases and the extracellular plasmin (Pang *et al.* 2004, Nagappan *et al.* 2009, Mizoguchi *et al.* 2011). Studies performed with cultured hippocampal neurons using epitope tagged BDNF suggested that BDNF is mainly released in the precursor form and the formation of mature BDNF was proposed to depend on the availability of the enzymes that cleave proBDNF (Nagappan *et al.* 2009). Using electrical stimulation at different frequencies the authors found that the extracellular accumulation of the mature form of BDNF following high-frequency stimulation depends on the co-

release of tissue plasminogen activator (tPA), which may not occur under conditions of low frequency stimulation (Nagappan *et al.* 2009). These findings suggest that secreted pro-BDNF may undergo activity-dependent conversion into the mature form of the neurotrophin that is required for LTP. A study using a BDNF-hemagglutinin knock-in mice indeed reported the presence of a significant amount of endogenous pro-BDNF during the perinatal period in mice, and both BDNF and pro-BDNF were found to be secreted in hippocampal cultures (Yang *et al.* 2009). (Nagappan *et al.* 2009).

In contrast with the evidence described above, studies performed using adult brain tissue indicated that pro-BDNF is rapidly converted intracellularly into mature BDNF for later storage and release by excitatory input (Matsumoto *et al.* 2008). In agreement with the later findings, a recent study reported the presence of BDNF and its cleaved pro-peptide in large dense core vesicles located at the presynaptic terminals of excitatory neurons in the adult brain hippocampus, suggesting that the cleavage may occur inside the secretory granule (Dieni *et al.* 2012). Understanding whether BDNF is secreted in the precursor or in the mature form in adult brain versus cultured neurons is an important issue since the pro- and mature forms of BDNF have different functions in the mammalian nervous system [for a review see (Teng *et al.* 2010)]. For example, activation of the complex of $p75^{NTR}$-sortilin by pro-BDNF induces neuronal apoptosis (Teng *et al.* 2005) in contrast with the neuroprotective effects of BDNF acting on Trk receptors [e.g. (Almeida *et al.* 2005)]. Several studies demonstrated that the binding of pro-BDNF to $p75^{NTR}$ is required for long-term depression (LTD) induction at CA3 - CA1 synapses (Woo *et al.* 2005, Yang *et al.* 2014) in contrast with the effects of BDNF in LTP (Figurov *et al.* 1996, Kang *et al.* 1997, Korte *et al.* 1998, Chen *et al.* 1999, Minichiello *et al.* 1999).

Activity-dependent release of BDNF

BDNF produced in the cell body is targeted to vesicles of the regulated secretory pathway before being transported to postsynaptic dendrites in secretory granules (Hartmann *et al.* 2001, Kohara *et al.* 2001, Adachi *et al.* 2005, Matsuda *et al.* 2009). Alternatively, the neurotrophin may be delivered to presynaptic terminals in large dense core vesicles through anterograde transport (Kohara *et al.* 2001, Adachi *et al.* 2005, Matsuda *et al.* 2009, Dieni *et al.* 2012). BDNF stands out among all neurotrophins in the activity-dependent regulation of its expression and secretion [reviewed in (Lessmann and Brigadski 2009)]. Upon HFS, BDNF is secreted in response to the influx of Ca^{2+} through NMDA receptors and/or voltage-gated Ca^{2+} channels (Hartmann *et al.* 2001, Balkowiec and Katz 2002, Gartner and Staiger 2002, Aicardi *et al.* 2004). Synaptic stimulation may also induce the release of the dendritic pool of BDNF by activating N-type Ca^{2+} channels and the release of Ca^{2+} from intracellular stores (Balkowiec and Katz 2002). Back propagation potentials were also shown to provide enough excitation to trigger the dendritic secretion of BDNF in cultured hippocampal neurons (Kuczewski *et al.* 2008). Using a pH-sensitive fluorescent protein-tagged BDNF it was recently shown that axonal secretion requires much higher levels of neuronal spiking than dendritic secretion (Matsuda *et al.* 2009), suggesting that normal neuronal activity is more likely to induce the fusion of BDNF-containing vesicles at postsynaptic sites in hippocampal neurons.

From the studies above mentioned, it is clear that cultured neurons develop the machinery necessary to release BDNF from dendrites. The current knowledge about the maturation patterns and secretion of BDNF from pre- and postsynaptic sites in the adult hippocampus is however, very limited.

Convincing is the fact that neuronal release of BDNF is largely induced by protocols also known to induce LTP in hippocampal slices, such as HFS and theta-burst stimulation (TBS) [for a review see (Lessmann and Brigadski 2009)], further supporting the role of the neurotrophin in this form of synaptic plasticity.

BDNF actions in synaptic plasticity in the hippocampus

BDNF and long-term potentiation (LTP)

The first evidence linking BDNF to hippocampal LTP was provided in studies showing that mice with a target disruption on *BDNF* exhibit a significant impairment on LTP at Schaffer collateral - CA1 synapses (Korte *et al.* 1995, Patterson *et al.* 1996). Importantly, the deficits observed in LTP in *BDNF* knockout mice were rescued upon acute treatment with recombinant BDNF (Patterson *et al.* 1996) or viral-mediated re-expression of *BDNF* gene (Korte *et al.* 1996). There are several lines of evidence pointing to a role for TrkB receptors as the prime candidates mediating BDNF actions in LTP (Minichiello 2009): i) LTP is significantly inhibited when HFS of Schaffer collateral - CA1 synapses is performed in the presence of the BDNF scavenger TrkB-IgG or with anti-TrkB antibodies (Figurov *et al.* 1996, Kang *et al.* 1997, Korte *et al.* 1998, Chen *et al.* 1999); ii) the use of TrkB-IgG fusion protein and BDNF antibodies similarly impair LTP suggesting that only BDNF and no other TrkB ligands are involved in LTP induction (Chen *et al.* 1999); iii) deletion of *TrkB* in mice impairs LTP (Minichiello *et al.* 1999). Using a knockin mice carrying a point mutation either in the Shc- or phospholipase Cγ (PLCγ)-docking sites on TrkB allowed dissecting the pathways involved in the BDNF-TrkB-mediated actions on LTP (Minichiello *et al.* 2002). These studies showed that TrkB mediates hippocampal LTP via recruitment of PLCγ and subsequent induction of CREB and CaMKIV phosphorylation (Minichiello *et al.* 2002). Confirming the important role of BDNF in hippocampal synaptic plasticity, it was reported that exogenous BDNF facilitates LTP in young hippocampal slices receiving tetanic stimulation that would normally result in short-term potentiation (Figurov *et al.* 1996). Facilitation effects of BDNF in the induction of LTP have also been reported in the hippocampal dentate granule cells but in this case were attributed to postsynaptic effects of the neurotrophin, requiring activation of NMDA receptors and voltage-gated Ca^{2+} channels (Kovalchuk *et al.* 2002). Importantly, deficits in BDNF-TrkB-mediated hippocampal synaptic plasticity correlate with a reduction in the performance in certain learning tasks (Linnarsson *et al.* 1997, Minichiello *et al.* 1999, Tyler *et al.* 2002, Gruart *et al.* 2007). Furthermore, the Val66Met polymorphism in the *BDNF* gene which results in a defect in regulated release of BDNF and affects episodic memory (Egan *et al.* 2003) also leads to an impairment of LTP (Ninan *et al.* 2010).

In addition to the facilitatory effects described above, whether BDNF is itself able to induce acute potentiation of glutamatergic synapses has been a highly controversial issue. Several lines of evidence now show that BDNF can elicit a sustained increase in the synaptic efficacy at the Schaffer collateral - CA1 synapses (Kang and Schuman 1995, Diogenes *et al.* 2007, Ji *et al.* 2010), an effect that is highly dependent on the delivery rate of the neurotrophin (Ji *et al.* 2010) and may also be age-specific (Diogenes *et al.* 2007). Indeed, whether BDNF potentiates or facilitates the potentiation of Schaffer collateral - CA1 synapses depends on the kinetics of TrkB activation. Fast delivery of BDNF (transient activation of TrkB) enhances basal synaptic transmission, whereas slow delivery (sustained activation of TrkB) facilitates LTP induced by weak TBS (Ji *et al.* 2010). Intrahippocampal infusion of

BDNF was also shown to induce a long-lasting form of synaptic potentiation (BDNF-LTP) at the medial performant path – granule cell synapses in vivo (Messaoudi *et al.* 1998, Ying *et al.* 2002) by a mechanism that requires translation and transcription activities (Messaoudi *et al.* 2002, Messaoudi *et al.* 2007).

BDNF is released during or shortly after LTP induction (Aicardi *et al.* 2004) and experiments using TrkB-IgG fusion protein suggest that endogenous BDNF may indeed be an active instructor of this form of synaptic plasticity (Figurov *et al.* 1996, Kang *et al.* 1997, Chen *et al.* 1999). This hypothesis was later supported by studies performed in synaptotagmin-IV knockout mice which attributed the upregulation in LTP observed in these animals to an increase in the release of endogenous BDNF (Dean *et al.* 2009). BDNF was also shown to exert long-lasting effects to maintain LTP. Initial evidence for BDNF-mediated effects on L-LTP maintenance came from studies using *bdnf* knockout mice (Korte *et al.* 1998), TrkB antibodies and TrkB-IgG fusion proteins (Kang *et al.* 1997, Korte *et al.* 1998), and conditional *trkB* knockout mice (Minichiello *et al.* 1999). The effects observed in these experimental models were largely attributed to the role of the neurotrophin in the upregulation of protein synthesis-dependent functional and structural changes at synapses [for a review see (Panja and Bramham 2014)]. Exogenous application of BDNF was capable of restoring L-LTP deficits even when protein synthesis was inhibited (Pang *et al.* 2004), suggesting BDNF as the key (if not the only) plasticity-related product (PRP) necessary for L-LTP maintenance. More recently, TrkB receptors were also proposed to act as a synaptic tag induced in a protein synthesis-independent manner upon E-LTP, capturing L-LTP induced in a distinct synaptic pathway, being BDNF the obvious PRP in the paradigm (Lu *et al.* 2011). Moreover, BDNF-TrkB-mediated tagging and capture was suggested to play a role in the consolidation of long-term memory (LTM) (Lu *et al.* 2011).

Taken together, the available experimental evidence points to a key role for BDNF in the regulation (modulation and/or mediation) of both early and late phases of LTP, but the sites of BDNF secretion and action during LTP are still unclear.

Pre- and/or postsynaptic source of BDNF in LTP

Despite the unequivocal role of BDNF in the regulation of hippocampal LTP, the source of the neurotrophin and whether it acts on pre- or postsynaptic sites remains unclear. Using a genetic deletion to selectively disrupt *BDNF* in the entire forebrain (CA3 and CA1) or only in the CA1 neurons, it was shown that the source of BDNF required for LTP (with 200 Hz TBS or HFS) is located at the presynaptic CA3 neurons (Zakharenko *et al.* 2003). These findings are in agreement with the presynaptic (and not postsynaptic) detection of endogenous BDNF at these synapses (Dieni *et al.* 2012). More recently, the use of super resolution imaging techniques also indicated that endogenous BDNF is preferentially (90%) found within glutamatergic presynaptic structures in cultured hippocampal neurons (Andreska *et al.* 2014). These results suggest that the major source of BDNF regulating LTP in the hippocampus may be presynaptic.

In contrast with the evidence described above, the early findings showed that LTP-inducing stimuli results in an upregulation of *BDNF* mRNA in the CA1 postsynaptic cells (Patterson *et al.* 1992) and the dendritic localization of the transcript was also shown to be crucial for CA1 LTP (An *et al.* 2008). Moreover, the deficits in LTP observed in *bdnf* knockout mice were restored upon re-

expression of the *bdnf* gene in the CA1 postsynaptic cells (Korte *et al.* 1996), pointing to a role for dendritic release of BDNF. This is in accordance with recent data showing that axonal secretion of BDNF from cultured hippocampal neurons requires much higher levels of neuronal spiking than dendritic secretion (Matsuda *et al.* 2009), within the range typically found under pathological conditions, i.e., status epilepticus. Pairing glutamate uncaging at single spines together with repetitive postsynaptic spikes at single CA1 pyramidal cells was also shown to trigger the release of endogenous BDNF (Tanaka *et al.* 2008). This spike-timing protocol induces a protein synthesis- and BDNF-dependent spine enlargement at these neurons and, although the source of endogenous BDNF was not directly addressed in this study, it is likely to be postsynaptic (Tanaka *et al.* 2008). Studies using TrkB-IgG fusion proteins showed that there is a critical time window (up to one hour after the synaptic stimulation) at which TrkB signaling is required for L-LTP (Kang *et al.* 1997). It is therefore possible that E-LTP relies on the presynaptic BDNF whereas L-LTP may also depend on the newly transcribed and translated BDNF which is then secreted by the postsynaptic cell. Furthermore, endocytosed BDNF at the pre and/or postsynaptic level can undergo activity-dependent secretion to maintain L-LTP, even when protein synthesis is inhibited (Santi *et al.* 2006). If this is the case, dendritic release of BDNF can result, at least in part, from the secretion of endocytosed BDNF.

Pre- and/or postsynaptic actions of BDNF in LTP

It is now widely accepted that BDNF-mediated actions on LTP require the activation of TrkB receptors [see (Minichiello 2009)]. The binding of neurotrophins to TrkB receptors induces ligand-receptor dimerization and autophosphorylation of tyrosine residues in the intracellular kinase domain of the receptor. This leads in turn to the phosphorylation of tyrosine residues in the intracellular juxtamembrane domain as well as in the carboxyl terminus of the receptor, which act as docking sites for adaptor molecules **(Fig. 1.11)**. Phosphorylation of these two tyrosine residues, located outside the kinase domain of the TrkB receptors, mediates the interaction with Shc (Src homology 2-containing protein) and phospholipase Cγ (PLCγ), respectively (Reichardt 2006). Moreover, another adaptor molecule, fibroblast growth factor receptor substrate 2 (FRS2), competes with Shc adaptor molecule for the binding to TrkB receptor (Kouhara *et al.* 1997, Meakin *et al.* 1999). In addition, adaptor proteins containing pleckstrin homology and SH2 domain, such as SH2B and SH2B2, bind the phosphotyrosine residues on the catalytic domain of Trk receptors and can activate Trk signaling (Qian *et al.* 1998). Due to the high homology of the intracellular domains, the signaling cascades are highly conserved among Trk receptors (Atwal *et al.* 2000). The three main intracellular signaling cascades activated by TrkB receptors are the Ras-mitogen activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3-K)-Akt pathway and the PLCγ-Ca^{2+} pathway (Kaplan and Miller 2000).

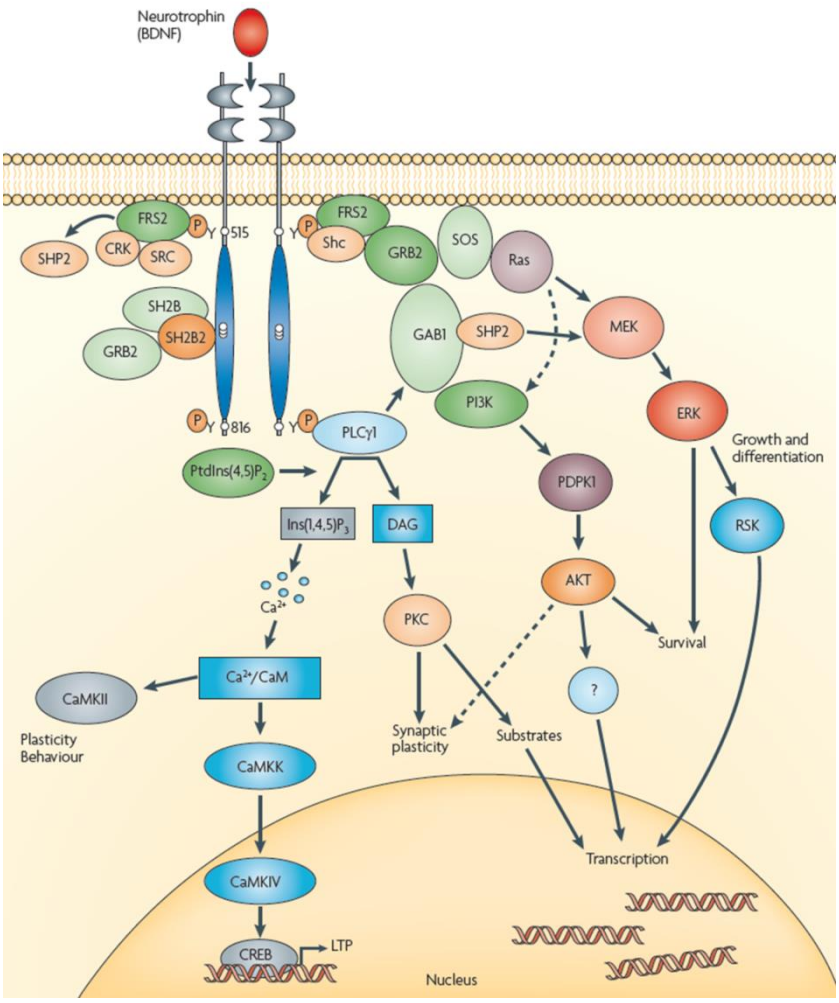


Figure 1.11. - Major signaling cascades activated by TrkB receptors. Binding of BDNF to TrkB receptors induces the dimerization and transphosphorylation of the receptors on tyrosine residues localized in the intracellular kinase domain. This increases the tyrosine kinase activity of the receptors and leads to the phosphorylation of other tyrosine residues that recruit several adaptor and signaling molecules such as Shc, FRS2 and PLCγ (more details in the text). TrkB signaling activates three major signaling pathways: the Ras-MAPK, the PI3-kinase and the PLCγ pathways. The activation of these pathways is coupled to several physiological actions that control neuronal survival, differentiation and synaptic plasticity. Adapted from (Minichiello 2009).

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

There are several adaptor protein that mediate the association and activation of PI3-K by TrkB receptors. Insulin-receptor substrate 1 (IRS1), ISR2 and GRB2-associated binder-1 (GAB1) bind to the activated TrkB receptors through GRB2 to stimulate the PI3-K pathway (Holgado-Madruga *et al.* 1997,

Yamada *et al.* 1997). Activation of PI3-K generates 3-phosphoinositides recruits to the plasma membrane and stimulate 3-phosphoinositide-dependent protein kinase 1 (PDK1) which in turn, together with the 3-phosphoinositides, activate the Akt protein kinase (Crowder and Freeman 1998). Among the targets of Akt is SHP2, which enhances MAPK signaling (Liu and Rohrschneider 2002). The PI3-kinase pathway has several targets that promote axon growth and pathfinding as well as neuronal differentiation [(Wagner-Golbs and Luhmann 2012) reviewed in (Reichardt 2006).

Phosphorylation of the TrkB receptor on tyrosine 816 recruits PLCγ1 which is phosphorylated by the active receptor (Kaplan and Miller 2000). Activated PLCγ1 hydrolyses phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) to generate inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG) which activates PKC. Ins(1,4,5)P_3 promotes the release of Ca^{2+} from internal stores, which results in the activation of Ca^{2+}-dependent enzymes such as Ca^{2+}/calmodulin-dependent protein kinases. Signaling through this pathway controls the expression and/or activity of many proteins, including ion channels and transcription factors, and plays an important role in TrkB-mediated long-term synaptic potentiation (Minichiello *et al.* 2002).

TrkB receptors are expressed in axons, nerve terminals and dendritic spines of the hippocampal granule and pyramidal neurons of the adult rat hippocampus (Drake *et al.* 1999, Pereira *et al.* 2006), raising the question of whether the neurotrophin acts at the pre- and/or postsynaptic level in LTP, and the signaling mechanisms involved. Thus, modulation of TrkB expression in the brain has been used to address this question. Using two different lines of TrkB mutant mice with reduced expression of the receptor throughout the brain (including the CA3 and CA1 regions) and mice lacking TrkB in the hippocampus CA1 region, it was found that LTP was only affected in the former mice (Xu *et al.* 2000) suggesting that BDNF acts preferentially presynaptically to modulate LTP.

As previously mentioned, studies performed in mice with targeted mutations in either the Shc or the PLCγ binding sites of TrkB showed that recruitment of PLCγ is preferentially coupled to potentiation of the CA3-CA1 synapse and associative learning (Minichiello *et al.* 2002, Gruart *et al.* 2007). Inhibition of the PLCγ signaling by virus-mediated overexpression of the PLCγ plekstrin homology (PH) domain at the hippocampal CA3 and CA1 regions suggested a role for pre- and postsynaptic TrkB receptors on LTP (Gartner *et al.* 2006). In this study, concomitant pre- and postsynaptic inhibition of PLCγ signaling was necessary to significantly reduce LTP to levels similar to those observed in the *Bdnf* and *trkB* knockout mice (Gartner *et al.* 2006). Conversely, blocking pre- or postsynaptic PLCγ recruitment alone did not significantly affect LTP (Gartner *et al.* 2006).

In contrast with the reports suggesting a concurrent role of pre- and postsynaptic TrkB receptors in LTP in the CA1 region of the hippocampus, a postsynaptic effect was proposed to account for the robust induction of LTP at the medial perforant path-granule cells synapses when BDNF was applied together with a weak synaptic stimulation that would not normally induce synaptic potentiation (Kovalchuk *et al.* 2002). Repetitive pairing of postsynaptic spikes together with two-photon glutamate uncaging at single spines (spike-timing protocol) elicits the release of endogenous BDNF and results in a neurotrophin- and protein synthesis-dependent spine enlargement (Tanaka *et al.* 2008), suggesting a postsynaptic action of BDNF.

The sites of BDNF actions on LTP are ultimately dictated by the characteristics inherent to the LTP form, meaning that depending on the synapse studied and on the pattern of stimulation, BDNF may elicit effects in pre- and/or postsynaptic sites [for a complete review of the literature please see (Edelmann *et al.* 2014)].

Molecular mechanisms underlying BDNF effects in LTP

Modulation of excitatory synapses

The molecular mechanisms mediating the effects of BDNF on LTP are not fully understood. However, the effects of the neurotrophin are thought to be mediated, at least in part, by modulation of glutamate release and glutamate receptors at excitatory synapses **(Fig. 1.12)**. Incubation of cultured hippocampal neurons with BDNF upregulates the vesicular glutamate transporter 1 and 2 (vGlut1 and 2) protein levels and increases their clustering along neurites (Melo *et al.* 2013). Furthermore, BDNF was found to increase the number of docked vesicles at the active zone of excitatory synapses in the CA1 region and increases the frequency of miniature excitatory postsynaptic currents (Tyler and Pozzo-Miller 2001), suggesting a presynaptic action of the neurotrophin. [For a review of the effects of BDNF on the regulation of glutamate release please see (Tyler *et al.* 2002, Carvalho *et al.* 2008)]. It was also shown that BDNF induces a long-lasting enhancement of glutamate release by acting on the rapidly recycling pool (RRP) of vesicles at the CA3–CA1 synapses (Tyler *et al.* 2006). The molecular mechanisms underlying the presynaptic effects of BDNF are still not fully understood, but the available evidence points to a role for synapsin (Jovanovic *et al.* 2000, Valente *et al.* 2012) and the Myosin 6-GIPC1 (GAIP-interacting protein, C terminus) motor complex (Yano *et al.* 2006). The major limitation in the current understanding of the presynaptic effects of BDNF is the fact that no attempt was made to investigate the effect of direct axonal stimulation. Additional studies are required to determine to what extent the TrkB-BDNF complexes formed at the presynaptic membrane may travel to the soma as signaling endosomes (Watson *et al.* 2001, Ye *et al.* 2003, Ha *et al.* 2008, Xie *et al.* 2012), which may then regulate the transcription of genes coding for presynaptic proteins. This type of mechanism may upregulate the synthesis of synaptic proteins (e.g. vesicular glutamate transporters) in the soma compartment, which may contribute to a delayed upregulation of neurotransmitter release.

BDNF actions on LTP may also depend on the ability of the neurotrophin to modulate glutamate receptors at excitatory synapses. BDNF was shown increase the phosphorylation of the NMDAR GluN2A and GluN2B subunits (Suen *et al.* 1997, Lin *et al.* 1998) which may increase the open probability of the ion channel (Levine *et al.* 1998). More recently, BDNF was also shown to modulate NMDAR and AMPAR traffic and expression in cultured hippocampal and cortical neurons (Caldeira *et al.* 2007, Caldeira *et al.* 2007, Fortin *et al.* 2012, Jourdi and Kabbaj 2013). Regarding NMDAR, it was shown that BDNF induces the phosphorylation of GluN2B subunit on Y1472 (Nakai *et al.* 2014, Xu *et al.* 2015) located in the endocytic motif which regulates the endocytosis of NMDAR mediated by AP-2/clathrin complex. It may explain the enhanced NMDAR-currents elicited in hippocampal slices acutely stimulated with BDNF (Ji *et al.* 2010). The effects on AMPAR are also particularly relevant since alterations in the trafficking of these receptors to the synaptic plasma membrane has an essential role in LTP (Malenka 2003). Stimulation with BDNF also induces the phosphorylation of GluA1-containing AMPAR and elicits the rapid incorporation of these receptors at

the synapse in hippocampal slices (Caldeira *et al.* 2007), and similar effects were reported in cultured cortical (Nakata and Nakamura 2007) and hippocampal neurons (Fortin *et al.* 2012). However, direct evidence that endogenous BDNF regulates the surface expression of AMPA and NMDA receptors during LTP is still lacking.

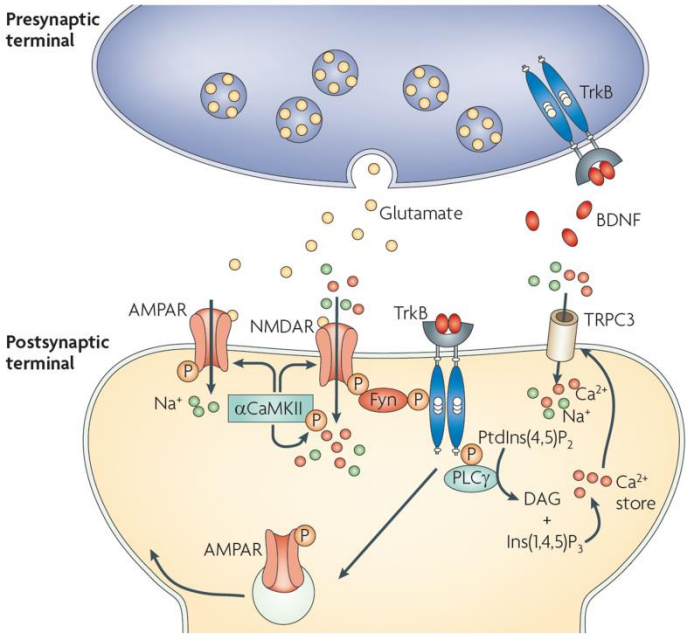


Figure 1.12. Modulation of glutamatergic synapses by BDNF. BDNF activates pre- or postsynaptic TrkB receptors with distinct effects on the glutamatergic synaptic transmission. At the presynaptic level, BDNF upregulates glutamate release. Activation of postsynaptic TrkB receptors also upregulates NMDA and AMPA receptor activity by different mechanisms. In particular, the Fyn kinase activated by TrkB receptors interacts with NMDAR and increases the open probability of the ion channel. BDNF-TrkB can also induce cation influx through TRPC3 channels facilitating the entry of Ca^{2+} through voltage-gated calcium channels and the activity of NMDA receptors. Ca^{2+} influx through NMDAR activates CaMKIIα which in turn contributes to the positive regulation of AMPAR and NMDAR with a consequent induction and expression of LTP. BDNF also modulates the trafficking and membrane insertion of AMPAR. Adapted from (Minichiello 2009).

BDNF-mediated regulation of spine plasticity

Activity-dependent LTP is also accompanied by structural changes at synapses including an increase in spine number and volume (Bosch and Hayashi 2012). Recent studies shed light on the dynamics of LTP-induced structural plasticity at the single spine level (Bosch *et al.* 2014, Meyer *et al.* 2014). The reorganization of postsynaptic structures and spine enlargement during LTP was proposed to occur in three sequential and distinct phases: (i) reorganization of actin cytoskeleton, (ii) stabilization of new remodeled actin through the persistent association of cofilin with F-actin and (iii) protein synthesis-dependent accumulation of postsynaptic density (PSD) scaffold proteins (Bosch *et al.* 2014). The initial two phases were proposed to be the structural equivalent of E-LTP whereas L-LTP would correspond to the third and protein synthesis-dependent phase.

BDNF has emerged as an essential factor necessary for spine remodeling during LTP and evidence suggests that it may be involved in the regulation of both early and late activity-dependent structural changes. Thus, incubation with BDNF enhanced TBS (threshold level)-induced increase of actin polymerization in dendritic spines, through regulation of p21-activated kinase (PAK) and ADF

(actin-depolymerising factor)/cofilin pathway (Rex *et al.* 2007). Furthermore, the BDNF scavenger TrkB-Fc abrogated the increases in spine F-actin produced by suprathreshold levels of theta stimulation (Rex *et al.* 2007), further showing a role for endogenous BDNF in spine remodeling under those conditions. F-actin remodeling is essential for hippocampal LTP maintenance in vivo (Fukazawa *et al.* 2003). The BDNF-TrkB signaling may also regulate F-actin remodeling by acting at Vav GEFs (guanine nucleotide exchange factor) to stimulate Rac-GTP production (Hale *et al.* 2011). BDNF-dependent activation of Rac was also shown to occur via the interaction of phosphorylated TrkB (Ser478) with the GEF TIAM1 (Lai *et al.* 2012), an event necessary for the activity-dependent spine enlargement.

The endogenous BDNF released in response to LTP-inducing stimuli (spike-timing protocol) is also required for the protein synthesis-dependent increase in spine volume at the CA3 – CA1 synapse (Tanaka *et al.* 2008). Accordingly, BDNF was shown to facilitate the activity-dependent spine enlargement (Bosch *et al.* 2014) with a concomitant but delayed protein synthesis-dependent accumulation of Homer1b at the PSD (Bosch *et al.* 2014). In a distinct study, the PSD-associated protein PSD-95 was also found to undergo a delayed activity-dependent accumulation in enlarged spines but only when the enlargement was persistent (Meyer *et al.* 2014). The localization of newly synthesized PSD-95 to stimulated synapses is also induced by BDNF (Butko *et al.* 2012), by a mechanism that is likely to be dependent on the dynamic invasion of dendritic spines by microtubules (Hu *et al.* 2011). In fact, a role for microtubules in HFS-induced LTP at CA3 – CA1 synapses (Jaworski *et al.* 2009) and in BDNF-induced changes in spine morphology (Gu *et al.* 2008) was also suggested.

Studies performed with exogenous application of BDNF to cultured hippocampal neurons and hippocampal slice cultures further support the role of the neurotrophin in the regulation of dendritic spine plasticity (Tyler and Pozzo-Miller 2001, Tyler and Pozzo-Miller 2003, Alonso *et al.* 2004, Amaral and Pozzo-Miller 2007, Ji *et al.* 2010). BDNF stimulation increases synaptic spine density by a mechanism dependent on the Ras/ERK pathway (Alonso *et al.* 2004) and the transient receptor-potential cation channel subfamily C (TRPC) type 3 (Amaral and Pozzo-Miller 2007).

Altogether, the available evidence suggests that BDNF actions on LTP may also result from the activity-dependent and BDNF-mediated structural changes in dendritic spines. Of particular interest is the fact that BDNF may be involved in the regulation of distinct processes that differentially determine spine and synapse plasticity depending on the period of stimulation of TrkB receptors. Future studies are required to evaluate whether endogenous BDNF released in response to stimuli that induce LTP mediates structural changes at dendritic spines in vivo, which are required for the persistence of synaptic potentiation.

BDNF and long-term depression (LTD)

In contrast with the role of the mature form of BDNF in LTP, its precursor form has been implicated in NMDA receptor-dependent LTD in the hippocampal CA1 region (Woo *et al.* 2005, Yang *et al.* 2014). The effects of pro-BDNF on LTD are mediated by the $p75^{NTR}$ which were localized in dendritic spines of CA1 neurons, as well as in the afferent terminals (Woo *et al.* 2005). Although this initial study reported the effect of exogenous pro-BDNF, a recent report using a cleavage-resistant

proBDNF knockin mice demonstrated that the endogenous overexpressed pro-BDNF facilitates the Schaffer collateral LTD (Yang *et al.* 2014). In accordance with these findings, pro-BDNF is the major form of BDNF secreted in response to LTD-inducing low-frequency stimulation (Nagappan *et al.* 2009). However, a distinct study using conditional *BDNF*-knockout mice showed that LTD induction was normal in these synapses, suggesting that neither the mature BDNF nor the pro-BDNF is involved in LTD (Matsumoto *et al.* 2008).

Although the effects of $p75^{NTR}$ on LTD were proposed to be mediated by an upregulation in the expression of GluN2B-NMDA receptor subunit (Woo *et al.* 2005), the signaling mechanisms involved in these alterations remain to be characterized. In fact, the $p75^{NTR}$ have been shown to interact with several different intracellular proteins making difficult the analysis of the pathways involved in the downstream responses (Schecterson and Bothwell 2010, Ibanez and Simi 2012).

Effect of BDNF on protein synthesis

It is well established that protein synthesis is required for BDNF-dependent LTP in the hippocampus [(Kang and Schuman 1996); for a review see (Santos *et al.* 2010, Leal *et al.* 2014)]. In fact, the effects of BDNF on L-LTP have been largely assigned to the effects of the neurotrophin on local protein synthesis at the synapse based on the transcripts locally available. The initial translation-dependent effects of BDNF are mediated by local activation of the translation machinery, while at later time-points protein synthesis may also follow the effects of BDNF on transcription activity.

Translation control occurs via regulation of a large number of translation mediators, including ribosomes, translation factors and tRNAs (Sonenberg and Hinnebusch 2009). Eukaryotic nuclear-transcribed mRNAs have a 5´-end cap structure and there are two major macromolecular complexes involved in cap-dependent translation initiation which are considered the main targets of translation regulation: the eukaryote initiation factor 4F (eIF4F) and the 43S pre-initiation complex (Jung *et al.* 2014). eIF4F is an heteromeric complex comprised by the eIF4E (cap-binding protein), which binds the 5´-capped mRNA, the eIF4A (RNA helicase), responsible for unwinding the secondary structure of mRNAs, and the eIF4G (scaffolding protein), that binds both eIF4E and eIF4A, bridging the transcripts to the 43S pre-initiation complex (Jung *et al.* 2014). The formation of eIF4F is promoted by the phosphorylation of eIF4E-binding proteins (4E-BPs), which dissociate from the complex and allow the translation activity; hypophosphorylated 4E-BPs binds to eIF4E thereby preventing it from associating with eIF4G (Gingras *et al.* 1999, Gingras *et al.* 2001). BDNF has been reported to induce translation at different levels, by altering the phosphorylation of specific proteins involved in initiation and elongation steps of protein synthesis as indicated below **(Fig. 1.13)**.

BDNF effects at the translation initiation

BDNF was first identified as a translation enhancer based on studies performed in primary cultures of cortical neurons, which showed that the effects on the protein synthesis machinery are mediated by activation of the PI3-K and ERK pathways. Activation of ERK by BDNF induces the phosphorylation of eIF4E and increases m^7 GTP binding (Takei *et al.* 2001). Stimulation of this signaling pathway by BDNF also activates the MAP-kinase interacting kinase1 (Mnk1) (Takei *et al.*

2001), which is known to bind directly to eIF4G and to phosphorylate eIF4E on Ser209 (Buxade *et al.* 2008, Shveygert *et al.* 2010). BDNF-induced increased in the phosphorylation of eIF4E was also observed in the dentate gyrus (DG) following infusion of BDNF to induce LTP (BDNF-LTP) as well as in DG synaptoneurosomes stimulated with BDNF (Kanhema *et al.* 2006), and is correlated with an increase in the rate of translation activity (Gingras *et al.* 2004). Studies performed in cultured hippocampal neurons also showed that BDNF induces the translocation of eIF4E into dendritic spines and increases the association of the protein with mRNA granules (Smart *et al.* 2003), but how this change in the distribution of eIF4E is related with the induction of translation activity remains to be investigated. The upregulation of protein synthesis by BDNF was also associated with the dissociation of eIF4E and CYFIP1 (FMRP interacting protein1), which acts like a 4E-binding protein (Napoli *et al.* 2008).

In addition to the effects mediated by ERK, BDNF also regulates translation initiation by the PI3-K/Akt pathway, which is coupled to the activation of mTOR and phosphorylation of 4E-BP1 (Takei *et al.* 2004) **(Fig. 1.13)**. A recent study uncovered a role for calpains in the stimulation of this pathway, by two distinct mechanisms: i) activation of calpain-2 by ERK leads to the degradation of PTEN (phosphatase and tensin homolog deleted on chromosome 10), a phosphatase that inactivates Akt (Zadran *et al.* 2010, Briz *et al.* 2013); ii) BDNF-induced activation of calpain leads to the degradation of hamartin/tuberin (TSC1/TSC2), two negative regulators of mTOR (Briz *et al.* 2013) **(Fig. 1.13)**. This contributes to the BDNF-induced upregulation of Akt and mTOR, two kinases involved in the BDNF-induced activation of the translation machinery as described below. The BDNF-induced activation of the PI3-K/Akt pathway was shown to increase 4E-BP1 phosphorylation by a mechanism sensitive to the mTOR inhibitor rapamycin, and the phosphorylated protein dissociates from eIF4E (Takei *et al.* 2001). The BDNF-induced phosphorylation of tuberin further contributes to activate mTOR in dendrites of cultured hippocampal neurons (Takei *et al.* 2004). The active mTOR promotes the initiation of translation by phosphorylating 4E-BP1, which enhances cap-dependent translation, while the phosphorylation and activation of the p70S6 kinase induces the translation of 5'-oligopyrimidine tract-containing mRNAs (referred to as TOP mRNAs) (e.g. transcripts coding for ribosomal proteins and elongation factors) (Fumagalli 2002, Thompson 2012). Similar results were obtained in synaptoneurosomes, a subcellular fraction containing the pre- and postsynaptic regions, suggesting that this pathway also plays a role in the BDNF-induced translation activity at the synapse (Takei *et al.* 2004). A functional coupling between the mTOR and ERK-dependent signaling pathways was also proposed based on studies performed in mice with a conditional forebrain expression of a dominant negative form of MEK1, which prevents ERK activation. Downregulation of MEK1 blocked BDNF-induced translation as well as BDNF-induced phosphorylation of the translation factors, eIF4E, 4E-BP1 and ribosomal protein S6 (Kelleher *et al.* 2004). Furthermore, these mice show selective deficits in hippocampal memory retention and the translation-dependent phase of hippocampal L-LTP (Kelleher *et al.* 2004).

Taken together, the available evidence indicates that multiple mechanisms are involved in the effects of BDNF in the initiation of translation. The analysis of BDNF-induced translation has identified seven mRNAs with increased polysome association in a rapamycin-sensitive manner (Schratt *et al.* 2004), but additional studies are required to identify additional mRNAs that are translated following

stimulation with BDNF, which may depend on the cellular compartment where TrkB receptors are located (e.g. synapse vs cell body).

Another component of the translation initiation machinery that is subjected to regulation by BDNF is the guanine nucleotide exchange factor eIF2B (Takei *et al.* 2001). This initiation factor catalyzes the exchange of eIF2.GDP to eIF2.GTP, which is required for the assembly of the ternary complex eIF2.GTP.Met.tRNAi. eIF2B binds to the 40S ribosomal subunit to form the 43S pre-initiation complex (Rhoads 1999). Incubation of cultured cortical neurons with BDNF increases the phosphorylation of the eIF2B by glycogen synthase kinase-3β (GSK-3β), thereby enhancing the activity of this initiation factor (Takei *et al.* 2001).

BDNF effects at the elongation step

Although initiation is usually the rate-limiting step of translation, the elongation phase is also a regulated process. The most studied mechanism involved in the regulation of elongation is the phosphorylation of the eukaryote elongation factor 2 (eEF2) by the eEF2 kinase (eEF2K; also known as Ca^{2+}- and calmodulin-dependent protein kinase III) (Costa-Mattioli *et al.* 2009). This kinase is also regulated by S6K and p90RSK mediated phosphorylation, which inactivate the enzyme (Wang *et al.* 2001).

Incubation of cultured cortical neurons with BDNF was shown to increase the elongation rate by approximately two-fold in a rapamycin-sensitive manner (Inamura *et al.* 2005). This increase in translation activity was correlated with an mTOR-dependent increase in eEF2K phosphorylation (Ser366) with a consequent downregulation in eEF2 phosphorylation (Inamura *et al.* 2005). In agreement with these results, increased levels of the dephosphorylated (active) form of eEF2 were detected in the cerebral cortex of *bdnf* transgenic mice, and the opposite was found in *bdnf* knockout mice (Takei *et al.* 2009). Furthermore, these changes in eEF2 phosphorylation were correlated with a decrease and an increase in the rate of protein synthesis measured in extracts from the cerebral cortex, respectively (Takei *et al.* 2009). In contrast with the results described above, BDNF-LTP led to rapid and transient phosphorylation of eEF2 in hippocampal dentate gyrus (Kanhema *et al.* 2006). Infusion of a MEK inhibitor blocked BDNF-LTP and decreased eEF2 phosphorylation showing a rapid ERK-dependent regulation of the elongation step during BDNF-LTP in vivo (Kanhema *et al.* 2006). However, this effect of BDNF on eEF2 phosphorylation was not observed in dentate gyrus synaptoneurosomes, indicating that the effects of the neurotrophin on the elongation step of translation are compartment-specific and the arresting of elongation may be limited to non-synaptic sites (Kanhema *et al.* 2006).

Taken together, these results show that distinct mechanisms may be involved in the regulation of the eEF2/eEF2K phosphorylation state by BDNF, depending on the specific cell compartment, the cell type and the upstream signaling cascades. However, an additional layer of complexity arises from the observations pointing to the specificity in the regulation of the translation of certain mRNAs. For example, stimulation of NMDA receptors in the superior colliculi of young rats was shown to increase the synthesis of Ca^{2+}- and calmodulin-dependent kinase IIα (CaMKIIα) (Scheetz *et al.* 2000) despite the increase in eEF2 phosphorylation and the reduction in overall protein synthesis. LTD induced by

agonists of group 1 mGluR is also associated with an increase in eEF2 phosphorylation with a reduction in overall translation activity, but an increase in activity-regulated cytoskeleton-associated protein (Arc) translation is also observed under these conditions (Park *et al.* 2008). Studies performed in Aplysia neurons showed a differential regulation of eEF2 by 5-HT (5-hydroxytryptamine, serotonin) in the dendritic and soma compartments (Weatherill *et al.* 2011), further suggesting a complex pattern of regulation. In the latter report, an increase in the expression of eEF2K was found to inhibit translation of a marker for translation dependent on an internal ribosome entry site (IRES), and the opposite effect was observed on the translation of a marker for cap-dependent translation (Weatherill *et al.* 2011). Additional studies are therefore required to fully elucidate the regulation of the eEF2, and the impact in translation activity.

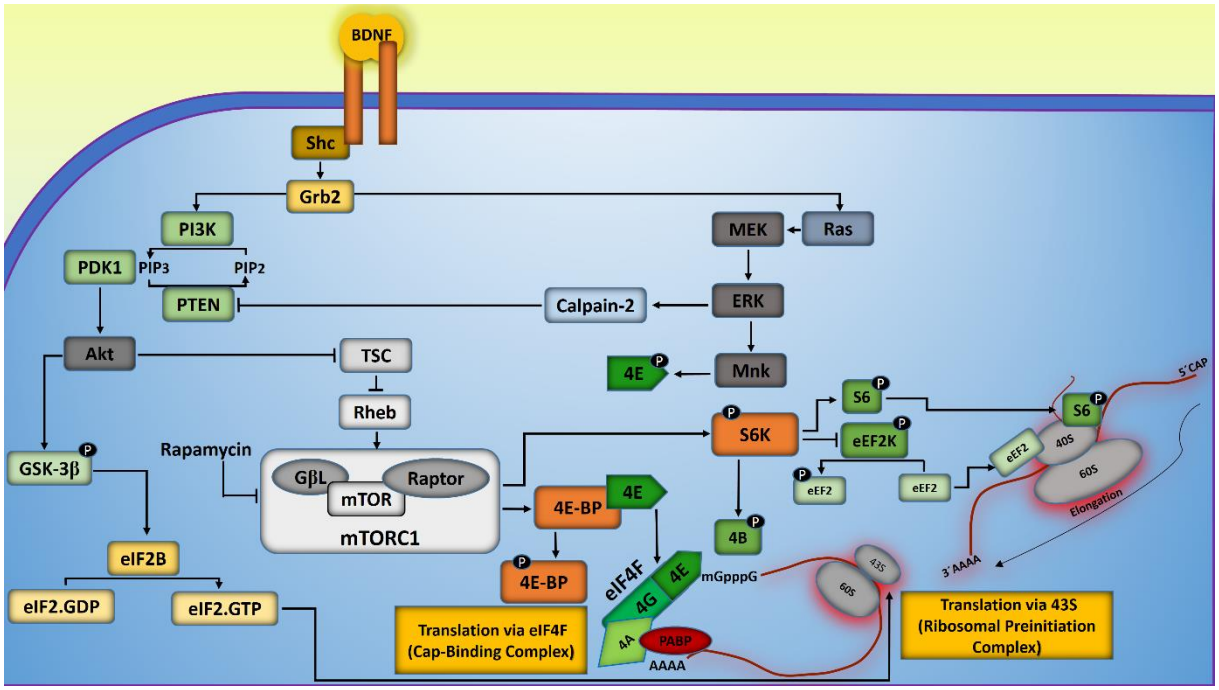


Figure 1.13. BDNF/TrkB signaling involved in the control of protein synthesis. The effects of BDNF on translation activity are mediated by activation of the PI3-K/mTOR and Ras/ERK pathways to induce translation via the eIF4F cap-binding complex and the 43S pre-initiation complex (See text for more details). Adapted from (Leal *et al.* 2015).

Studies performed in cultured cerebrocortical neurons showed an additional point of regulation of elongation by BDNF, through phosphorylation of the eukaryotic elongation factor 1A (eEF1A) (Inamura *et al.* 2005). The BDNF-induced phosphorylation (activation) of eEF1A was insensitive to inhibitors of MEK and mTOR (Inamura *et al.* 2005). In the active form eEF1 recruits aminoacyl-transfer RNA (tRNA) to the A-site of ribosomes in a GTP-dependent manner. The recognition of the codon by the tRNA induces the hydrolysis of GTP by the eEF1A, which allows accommodating the aminoacyl-tRNA into the ribosomal A-site (Dever and Green 2012).

BDNF-induced changes in local protein synthesis

All components required for translation have been found in dendrites, including polysomes (Steward and Levy 1982), mRNAs (Eberwine *et al.* 2001, Cajigas *et al.* 2012) and translation factors (Gardiol *et al.* 1999, Inamura *et al.* 2003). Accordingly, protein synthesis has been reported to occur in dendrites with a significant impact in synaptic plasticity (Sutton and Schuman 2006, Bramham and Wells 2007, Doyle and Kiebler 2011). In order to be translated at the synapse mRNAs are transported along dendrites in a dormant state, packaged into large messenger ribonucleoprotein complexes (mRNPs), and then protein synthesis takes place upon appropriate stimulation at or near activated synapses (Bramham and Wells 2007) **(Fig. 1.14)**.

Staufen proteins are among the best characterized proteins regulating mRNA localization in many species, and they are also involved in the binding and targeting of mRNAs into dendrites (Kiebler *et al.* 1999, Tang *et al.* 2001). The Staufen family of proteins comprises two members, Staufen1 and Staufen2, which are components of different RNA granules in neurons (Duchaine *et al.* 2002). Staufen2 is mainly expressed in the brain and is necessary for the microtubule-dependent delivery of *CaMKIIα* mRNA to dendrites (Jeong *et al.* 2007). Staufen1 is required for the late phase of long-term potentiation (L-LTP) in the hippocampus (Lebeau *et al.* 2008) whereas Staufen2 regulates mGluR-dependent long-term depression (mGluR-LTD) (Lebeau *et al.* 2011), suggesting distinct roles for the two Staufen proteins at the synapse. Another important regulator of mRNA localization in neurons is FMRP (Fragile mental X retardation protein). This RNA-binding protein (RBP) associates with several well described dendritic mRNAs, such as *CaMKIIα*, *Arc*, *MAP1b*, *PSD-95*, as well as its own mRNA (Bassell and Warren 2008). Moreover, FMRP interacts directly with the motor protein kinesin to promote the transport of FMRP and cognate mRNAs along dendrites (Dictenberg *et al.* 2008). FMRP is also one of the best described factors that regulate the translation of mRNAs at the synapse. For example, FMRP can recruit 4E-BP-like cytoplasmic Fmr-interacting protein-1 (CYFIP1) to repress the translation of target mRNAs (Napoli *et al.* 2008). Recent findings indicate that FMRP can also repress the translation of cognate mRNAs through the association with miRNAs (Edbauer *et al.* 2010, Muddashetty *et al.* 2011) and through the reversible stalling of ribosomes on polyribosomes containing FMRP-target mRNAs (Darnell *et al.* 2011).

Different approaches have been used to characterize the effects of BDNF on dendritic protein synthesis. BDNF treatment of hippocampal neurons was shown to induce translation of a protein synthesis reporter consisting in a green fluorescent protein (GFP) coding sequence flanked by 3´UTR and 5´UTR of CaMKIIα in intact dendrites isolated from cultured hippocampal neurons (Aakalu *et al.* 2001). More recently, live cell imaging studies using a fluorescent TimeSTAMP tag, an approach that allows detection of specific new proteins synthesized at defined times, showed that copies of PSD-95 are synthesized in response to local application of BDNF, and preferentially localize to stimulated synapses in rat hippocampal neurons (Butko *et al.* 2012).

Synaptoneurosomal preparations have also been instrumental in the characterization of the effects of BDNF in local translation at the synapse since this model comprises the pre- and postsynaptic regions and, therefore, avoid the interference from the effects of the neurotrophin on transcription which also lead to protein synthesis. Studies performed in rat hippocampal

synaptoneurosomes showed that BDNF induces the local translation of Arc in a Trk- and NMDA-dependent manner (Yin *et al.* 2002). Arc is a postsynaptic protein known to play an important role in LTP in the hippocampus [reviewed in (Bramham *et al.* 2010)]. Additional studies performed with total forebrain (Schratt *et al.* 2004) or cortical (Takei *et al.* 2004) synaptoneurosomes showed that BDNF stimulation increases translation of Homer2 (a PSD scaffold protein), GluA1, Arc and CaMKII in a rapamycin-sensitive manner (Schratt *et al.* 2004, Takei *et al.* 2004). The GluA1-AMPA receptor subunits synthesized in response to stimulation with BDNF are rapidly delivered to the synapse (Fortin *et al.* 2012) and this may play an important role in LTP. The local synthesis of CaMKII may also play a role in BDNF-induced synaptic potentiation given the role played by the kinase in enlarging and strengthening the synapse (Lisman *et al.* 2012).

Using a multidimensional protein identification technology (MudPIT), 214 proteins were found to be upregulated in synaptoneurosomes derived from cortical cultured neurons that were treated with BDNF (Liao *et al.* 2007). Since synaptoneurosomes were prepared after 30 min of incubation with BDNF the observed alterations in the proteome were attributed to local protein synthesis. These included proteins involved in synaptic vesicle formation and movement, maintenance or remodeling of synaptic structure, mRNA processing, transcription, and translation. This upregulation of translation components was sensitive to protein synthesis inhibitors and dependent on the activation of mTOR (Liao *et al.* 2007), and may constitute a mechanism to amplify locally the effects of BDNF on translation activity.

At the present time we have a very limited understanding of the diversity of mRNAs that are translated at the synapse in response to BDNF, and their role in synaptic plasticity. Given the diversity of transcripts present in neuronal dendrites (Cajigas *et al.* 2012) it is likely that additional proteins will be found to be translated locally in response to stimulation with BDNF. How exactly these locally translated mRNAs reach the synapse and how the transport is controlled are other questions that remain to be addressed.

A few studies have contributed to the characterization of RNA granules, the structures responsible for the transport of mRNAs along neurites. Large RNA granules were purified from mouse brain which were associated with the tail of conventional KIF5 (Kanai *et al.* 2004). These granules contained *CaMKIIα* and *Arc* mRNAs, as well as 42 different proteins including several well established *trans*-acting factors involved in mRNA localization such as Staufen, FMRP and Pur-α. In addition, this proteomic study led to the identification of new players in mRNA transport such as hnRNP (heterogeneous nuclear ribonucleoprotein) U and PSF (polypyrimidine tract binding protein-associated splicing factor) (Kanai *et al.* 2004). Interestingly, several members of the hnRNP family of proteins that were detected in this RNA granule preparation were found to accumulate at postsynaptic sites upon neuronal activation (Zhang *et al.* 2012), further supporting their role in local mRNA regulation.

In a different study, subcellular fractionation was used to obtain fractions enriched in ribonucleoprotein complexes from developing rodent cortex (Elvira *et al.* 2006). The composition of these granules was to some extent different from the KIF5-associated granules described by Hirokawa and colleagues (Kanai *et al.* 2004). They were enriched in *β-actin* and did not contain *CaMKIIα* or *Arc* transcripts, and a proteomic analysis of these fractions showed several RNA-binding proteins that

were not detected in the KIF5-associated granules (Elvira *et al.* 2006). Nevertheless, the two granule preparations had many common components, including several hnRNPs, Staufen2, and DEAD-box3 helicase. Interestingly, it was further demonstrated that BDNF treatment increased the number of motile DEAD-box3-carrying granules in hippocampal neurons (Elvira *et al.* 2006). This study identified yet a novel member of hnRNP family, hnRNPK to be contained in RNA granules from developing brain.

hnRNPK has a wide variety of nuclear and cytoplasmic functions, including in transcription (Ostrowski *et al.* 2003, Lynch *et al.* 2005, Stains *et al.* 2005), splicing (Expert-Bezancon *et al.* 2002, Cao *et al.* 2012) and regulation of mRNA stability (Skalweit *et al.* 2003, Fukuda *et al.* 2009), as well as in translation (Ostareck *et al.* 1997, Habelhah *et al.* 2001, Ostareck-Lederer *et al.* 2002, Yano *et al.* 2005, Feliers *et al.* 2007, Yoon *et al.* 2013). hnRNPK binds single or double stranded nucleic acids, especially in CU/CT rich regions via its three K homology (KH) domains (Grishin 2001, Bomsztyk *et al.* 2004, Fenn *et al.* 2007). In the central nervous system (CNS) this protein was shown to regulate spine morphology through interaction with Abi1 (Abelson-interacting protein 1) (Proepper *et al.* 2011). More recently, it was found that LTP in cultured hippocampal neurons requires ERK1/2-mediated hnRNPK phosphorylation on S284, and the cytoplasmic accumulation of the protein (Folci *et al.* 2014). Unpublished observations from our laboratory also showed that this RBP accumulates in dendrites of cultured hippocampal neurons upon BDNF treatment and high frequency stimulation of the perforant path - gentate gyrus synapses *in vivo* regulated the interaction of hnRNPK with several transcripts (Graciano Leal unpublished observations). In *Xenopus laevis*, hnRNP K is also required for the efficient nuclear export and translation of multiple cytoskeletal-related mRNAs that are essential for axonal-development (Liu *et al.* 2008, Liu and Szaro 2011) and optic axon regeneration (Liu *et al.* 2012). In addition, an ERK1 phosphorylation site was identified on *Xenopus laevis* hnRNPK (S257; homologous with S284 of human hnRNPK) which regulates NF-M (neurofilament-medium) protein expression and axon outgrowth (Hutchins *et al.* 2015).

BDNF-induced changes in transcription

In addition to the rapid effects of BDNF on translation activity at different subcellular compartments, including at the synapse (described above), the neurotrophin also affects transcription. Multiple lines of evidence suggest that the effects of BDNF on L-LTP are dependent on its activity in the regulation of gene expression. Accordingly, intrahippocampal injection of BDNF was shown to induce LTP at the medial perforant path-granule cell synapses in vivo (Messaoudi *et al.* 1998) by a mechanism sensitive to the transcription inhibitor actinomycin D (Messaoudi *et al.* 2002).

The effects of BDNF on transcription activity have been characterized mainly in response to the incubation of cultured neurons with the neurotrophin, an experimental paradigm that does not allow identifying the effects of synaptically active BDNF at the transcriptional level. In these studies BDNF was shown to induce the expression of genes coding for synaptic activity regulators, such as Arc (Ying *et al.* 2002, Zheng *et al.* 2009), several synaptic vesicles proteins, including vesicular glutamate transporters (Melo *et al.* 2013), translation machinery proteins, including eIF4E (Kanhema *et al.* 2006), eEF2 (Manadas *et al.* 2009) among others (Santos and Duarte 2008), and glutamate receptors subunits, such as GluN1, GluN2A, GluN2B (Caldeira *et al.* 2007), GluA1 and GluA2

(Caldeira *et al.* 2007). The BDNF-induced *Arc* expression was dependent on ERK-mediated activation of CREB (Ying *et al.* 2002, Zheng *et al.* 2009).

The role of synaptic BDNF in the regulation of gene expression was recently addressed using a microfluidic culture device to separate the soma and dendritic compartments. Addition of BDNF to the latter compartment elicited an anterograde signal that induces transcription of the immediate early genes, *c-fos* and *Arc*. *Arc* induction was dependent on the $[Ca^{2+}]_i$ in cell body- and dendritic compartments, whereas *c-Fos* was independent of the Ca^{2+} concentration (Cohen *et al.* 2011). The authors also showed that BDNF-mediated anterograde dendrite-to-nucleus signaling was dependent on MEK1/2 (Cohen *et al.* 2011) and activity of TrkB receptors in the soma compartment was required for the *Arc* and *c-Fos* induction by dendritically applied BDNF (Cohen *et al.* 2011). Additional studies are required to identify all genes regulated by BDNF when the neurotrophin acts on dendritic-localized TrkB receptors. It will also be of interest to understand the mechanisms underlying this anterograde signal which is consistent with a signaling endosome-like pathway observed in axons (Schmieg *et al.* 2014), as well as to unravel the role of this mechanism in L-LTP.

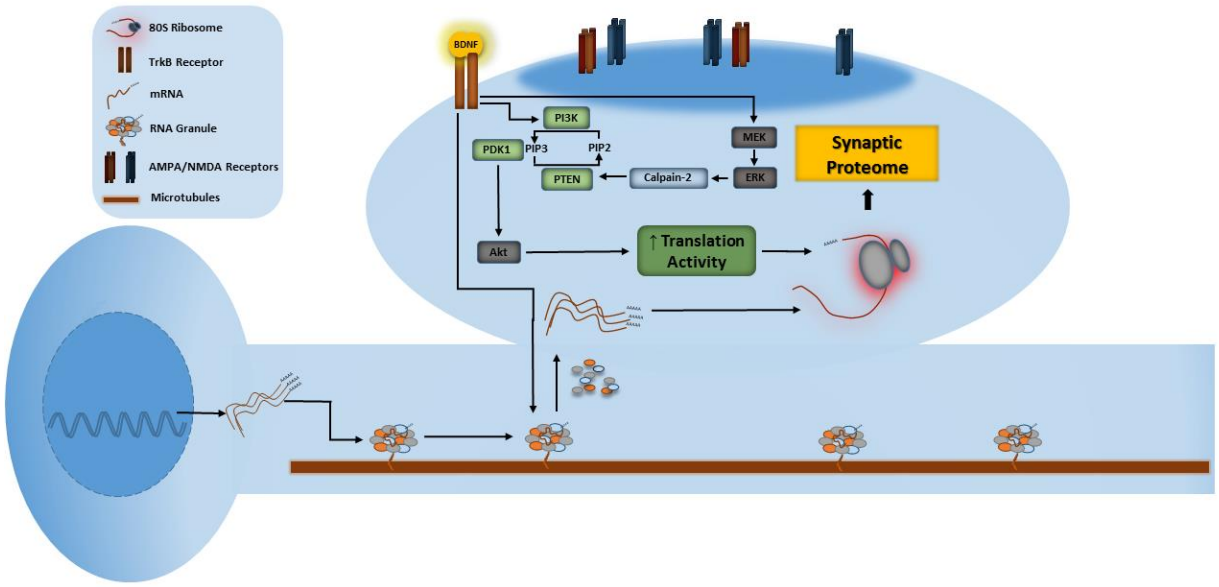


Figure 1.14. BDNF-induced activation of TrkB receptors promotes synaptic changes in the proteome by modulating protein synthesis pathways. TrkB activation results in PI3K activation and downstream phosphorylation of Akt, leading to hamartin/tuberin complex dissociation and degradation by calpain-1 and calpain-2. This relieves mTOR inhibition thereby stimulating protein synthesis. In parallel, BDNF induces PTEN degradation by ERK-mediated calpain-2 activation/phosphorylation further stimulating the PI3K/Akt/mTOR pathway. BDNF can regulate dendritic protein synthesis by acting at three different levels: i) controlling the expression and dendritic transport of specific mRNAs involved in LTP, in a dormant state, packaged into large RNA granules; ii) once mRNAs reach the synapse, by inducing the release of transcripts from RNA granules, allowing their local translation; iii) by activating the protein synthesis machinery locally**.** Adapted from (Leal *et al.* 2015).

Focal adhesion kinases (FAK) family of proteins

The PSD proteins are responsible for the regulation of signaling pathways that couple rapid events, such as the action potential and neurotransmitter release, to long-lasting changes in synaptic strengh and neuronal survival. These adaptations involve the interaction of neurons with other cells, as well as with the extracellular matrix, and for this purpose neurons use, in part, the same molecular

machinery that controls adhesion, motility and survival in non-neuronal cells. Two homologous non-receptor tyrosine kinases, FAK and Pyk2 (proline-rich tyrosine kinase 2), and the associated Src-family tyrosine kinases, play important roles in these mechanisms as described below.

Proline-rich tyrosine kinase 2 (Pyk2)

Pyk2 was identified near-contemporaneously by several groups and designated Pyk2 (Proline-rich tyrosine kinase), CAKβ (cell adhesion kinase beta), RAFTK (related adhesion focal tyrosine kinase), or FAK2 (Avraham *et al.* 1995, Lev *et al.* 1995, Sasaki *et al.* 1995, Herzog *et al.* 1996, Yu *et al.* 1996). The human Pyk2 gene is located on chromosome 8 (8p21.1; gene reference: PTK2B) (Herzog *et al.* 1996, Inazawa *et al.* 1996), and the early studies showed that the kinase shares a similar domain structure with FAK and exhibits approximately 45% sequence identity, particularly in the central kinase domain (approximately 60%). Despite their structural similarity, Pyk2 and FAK display a number of significant differences. While FAK is ubiquitously expressed, Pyk2 exhibits a more limited tissue distribution. Pyk2 expression is highest in cells of hematopoietic lineage and in the CNS (Avraham *et al.* 1995, Lev *et al.* 1995, Avraham *et al.* 2000). Interestingly, in certain cell types the knockdown of FAK expression is accompanied by a compensatory increase of Pyk2 expression (Sieg *et al.* 1998, Lim *et al.* 2008, Weis *et al.* 2008). However, Pyk2 and FAK are differentially regulated: FAK is primarily activated following integrin mediated adhesion to the extracellular matrix (ECM) whereas Pyk2 is mainly activated in response to a variety of stimuli that increase intracellular calcium (Lev *et al.* 1995). Nevertheless, Pyk2 can be also activated following integrin mediated adhesion.

Pyk2 structure and functional domains

Although a high resolution structure of full length Pyk2 has not been reported, a number of functional domains have been identified and characterized **(Fig. 1.15)**. These domains include: a N-terminal FERM domain, a central catalytic domain, a number of proline rich sequences, and a C-terminal focal adhesion targeting domain.

FERM domain

FERM domains are compact cloverleaf shaped and composed of three structural modules (designated A, B and C, or F1, F2 and F3 respectively) which mediates both protein-membrane targeting as well as protein-protein interaction. The functional activity of the prototypical FERM domain proteins ezrin, radixin and moesin is regulated by FERM domain mediated intramolecular associations (Edwards and Keep 2001). Protein membrane targeting is mediated by basic residues in a cleft between subdomains F1 and F3 that interact with PIP2 (Hirao *et al.* 1996, Hamada *et al.* 2000). It was also proposed that this domain regulates Pyk2 activity by mediating Ca^{2+}/ calmodulin dependent homodimer formation with consequent transphosphorylation (Kohno *et al.* 2008). In addition, the Nir family of proteins was found to interact specifically with the N-terminal of Pyk2 (Lev *et al.* 1999). The latter group comprises calcium binding proteins that possess phosphatidylinositol transfer activity and are Pyk2 substrates.

Kinase domain

Pyk2 contains a centrally located kinase domain that is connected to the FERM domain by a short linker segment which contains the autophosphorylation site at Tyr402. Phosphorylation at Tyr402 provides a binding site for SH2 containing proteins including Src and p85. Binding of Src leads to phosphorylation of Pyk2 residues Tyr579 and Tyr580 within the kinase domain and maximal Pyk2 kinase activity. FIP200 (FAK family kinase-interacting protein of 200 kDa) was identified as a protein that binds to the Pyk2 kinase domain and may function as a potential endogenous inhibitor of Pyk2 (Ueda *et al.* 2000).

C-terminal domains

The kinase domain of Pyk2 is followed by two proline-rich sequences that mediate the interaction of Pyk2 with a number of SH3 domain-containing proteins such as p130Cas, ASAP1 and Graf (Astier *et al.* 1997, Ren *et al.* 2001, Kruljac-Letunic *et al.* 2003, Takahashi *et al.* 2003). Interestingly, the mutation of the second proline rich sequences in Pyk2 leads to an exclusive nuclear localization of the protein (Aoto *et al.* 2002), suggesting that proline-rich domains may contribute to the intracellular distribution of Pyk2. The C-terminal domain of Pyk2 also includes a focal adhesion targeting (FAT) domain. This region of Pyk2 is well conserved when compared with the corresponding FAT domain of FAK, the latter being necessary and sufficient for efficient localization of FAK at focal adhesions (Hildebrand *et al.* 1993, Shen and Schaller 1999). The FAT domain of Pyk2 also interacts with the LD motif in the C-terminus of gelsolin and regulates the activity of Pyk2 in the control of cytoskeleton organization (Wang *et al.* 2003). The FAT domain includes Tyr881 which when phosphorylated by Src serves as a binding site for the adaptor protein Grb2 and is important for the interaction with the MAP kinase signaling pathway (Blaukat *et al.* 1999).

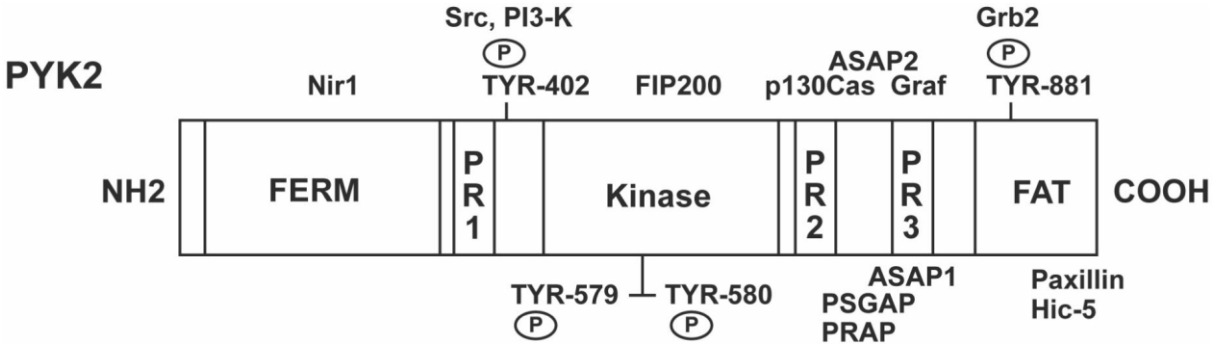


Figure 1.15. Schematic representation of Pyk2 functional domains and interacting proteins. Pyk2 contains an N-terminal FERM domain, a central kinase domain, three proline rich motifs (PR1, Pr2, PR3), and a C-terminal focal adhesion targeting domain (FAT). Phosphorylation of Tyr-402 serves as a binding site for Src, a kinase that phosphorylates the activation loop residues Tyr-579 and Tyr-580, as well as Tyr-881 in the FAT domain. Phosphorylation of the latter residue allows the interaction with the adapter protein Grb2. Proline rich motifs mediate interaction with a variety of SH3 domain containing proteins. Other indicated interactions are Nir1 with the FERM domain, FIP200 interaction with the Pyk2 kinase domain and paxillin and Hic-5 interaction with the FAT domain. Adapted from (Lipinski and Loftus 2010).

At least two isoforms of Pyk2 were identified, Pyk2-H or Pyk2s 66, lacking a frame of 126 base pairs coding for the C-terminal portion, in addition to PRNK (Pyk2 related nonkinase) which lacks both the N-terminal FERM domain and the kinase domain. However, unspliced full length Pyk2 is the predominant form of the kinase in the brain, especially in the hippocampus.

Pyk2 and hippocampal synaptic plasticity

Pyk2 has been shown to play an important role in synaptic plasticity in brain, particularly in the hippocampus. The kinase is physically associated with the NMDAR complex, together with Src (Lu *et al.* 2000, Huang *et al.* 2001), and studies performed in hippocampal slices showed that Pyk2 is activated by PKC following an increase of the intracellular Ca^{2+} concentration, downstream of NMDAR activation (Siciliano *et al.* 1996, Alier and Morris 2005); the active Pyk2 potentiates NMDAR currents (Lu *et al.* 2000, Huang *et al.* 2001). A similar mechanism was proposed involving the Pyk2/Src-family kinase pathway, which upregulates the NMDAR-currents through mGluR1 (Heidinger *et al.* 2002).

Pyk2 and Src have also been described to play a critical role in the induction of LTP in CA3-CA1 synapses (Lu *et al.* 1998, Xiong *et al.* 1999, Huang *et al.* 2001, Salter and Kalia 2004). Accordingly, Pyk2 tyrosine phosphorylation and its association with Src are enhanced by stimulation paradigms that produce LTP, and Pyk2-mediated enhancement of synaptic AMPAR responses was found to be prevented by blocking NMDAR, chelating intracellular Ca^{2+}, or blocking Src (Huang *et al.* 2001). These results show that Pyk2 activation is required for LTP induction and may depend upon downstream activation of Src to upregulate NMDAR activity. A recent study proposed a model for Pyk2-mediated activation of Src, according to which the Src-mediated phosphorylation of PSD-95 at Y523 facilitates the interaction between the PSD-95 SH3 domain and Pyk2, thereby activating Pyk2 that further phosphorylates Src, ultimately upregulating the function of NMDAR (Zhao *et al.* 2015).

The neuronal distribution of Pyk2 is also regulated by intracellular calcium and NMDAR activity. Ca^{2+} influx through NMDAR causes postsynaptic clustering and autophosphorylation of Pyk2 via Ca^{2+}- and calmodulin stimulated binding to PSD-95 (Bartos *et al.* 2010). The association between Pyk2 and PSD-95 is required for LTP induction, because injection of the peptide GST-tagged SH3 of PSD-95 or GST-tagged Pyk2 residues 671-875, which mediate Pyk2 binding to PSD-95, blocked the induction of LTP (Seabold *et al.* 2003). This mechanism of synaptic recruitment and activation of Pyk2 upon NMDAR activity and downstream Ca^{2+} influx is an economical and effective mechanism to elevate Pyk2 activity specifically at those synapses experiencing LTP.

The differential distribution of Pyk2 depending upon synaptic activity may explain the opposing roles reported for Pyk2 in hippocampal synaptic plasticity. It was shown that Pyk2 knockdown blocks LTD but not LTP in the CA1 region of hippocampal slice cultures, and in this model the NMDAR-mediated chemical LTD activates Pyk2 (Hsin *et al.* 2010). Furthermore, Pyk2 knockdown enhances NMDAR-dependent ERK phosphorylation and delays NMDAR-dependent GluA1 S845 dephosphorylation, further suggesting a role of Pyk2 in LTD (Hsin *et al.* 2010). However previous reports showed that Pyk2 activation facilitates ERK1/2 activation in cell lines (Nicodemo *et al.* 2010) and Pyk2 and ERK were found to be independently activated, in distinct cellular compartments, in hippocampal slices (Corvol *et al.* 2005). In addition to the role of Pyk2 in LTD, it was shown that

depolarization and tetanic stimulation induces nuclear translocation of Pyk2 in hippocampal slices, independently of its autophosphorylation and kinase activity, by a mechanism dependent on calcineurin and independent on Src-family of kinases (Faure *et al.* 2007). Therefore, it can be hypothesized that Pyk2 may be involved in at least two different mechanisms of hippocampal synaptic plasticity: (i) Pyk2 can be recruited and accumulated in synaptic compartments upon NMDAR activation favoring the LTP induction and/or (ii) Pyk2 may be translocated to the nucleus by a calcineurin-dependent mechanism upon synaptic activity, by a mechanism independent of Pyk2 activation, which may favor LTD. However, additional studies are required to full understand the role of Pyk2 in both forms of synaptic plasticity.

There are also evidence available suggesting a role for Pyk2 in the regulation of the neuronal structural complexity. It was shown that ArgBP2, an SH3 and SoHo-domain containing adaptor protein, links Pyk2 and Cbl in a signaling complex, which upon growth factor stimulation is translocated to lipid rafts, favoring the formation of lamellipodia in growing neurites of PC12 cells (Haglund *et al.* 2004). Pyk2 association with the membrane in brain synaptosomes relies predominantly on its phosphorylation state (Bongiorno-Borbone *et al.* 2002). Recently, it was also shown that Pyk2 inhibits Rac1, thereby regulating dendritic spine arborization in hippocampal neurons (Suo *et al.* 2012). In this study it was observed that overexpression of Pyk2 reduced the number of spines, the spine length and the spine width, while the Pyk2 knockdown had the opposite effect (Suo *et al.* 2012). However, a similar approach did not find a significant difference in the steady-state spine morphology with Pyk2-ShRNA or WT-Pyk2 expression in dissociated hippocampal neurons (Hsin *et al.* 2010). Thus, more studies are required to understand the role of Pyk2 in the regulation of spine morphology, in particular to determine whether Pyk2 plays a role in activity-dependent spine shrinkage or elimination. In neuronal cell lines, however, it was observed that glycogen synthase kinase-3 (GSK-3) is activated by Pyk2 during lipid mediator lysophosphatidic acid (LPA)-mediated neurite retraction (Sayas *et al.* 2006). In contrast, inhibition of Pyk2 in PC12 cells blocks EGF (epidermal growth factor) and IGF (insulin-like growth factor)-1-induced neurite outgrowth by a mechanism involving paxillin (Ivankovic-Dikic *et al.* 2000).

Pyk2 activity is negatively regulated by the phosphatase STEP (striatal-enriched protein-tyrosine phosphatase) (Xu *et al.* 2012), which binds to the kinase and dephosphorylates Tyr402. STEP KO mice also exhibit enhanced phosphorylation of Pyk2 at Y402, as well an upregulation in the phosphorylation of the Pyk2 substrates paxillin and ASAP1 (Xu *et al.* 2012). In addition, functional studies indicated that STEP opposes Pyk2 activation after KCl depolarization of cortical slices and blocks Pyk2 translocation to postsynaptic densities, a key step required for Pyk2 activation (Xu *et al.* 2012). Interestingly, STEP KO mice displayed enhanced tyrosine phosphorylation of ERK1/2, as well as an upregulation in the phosphorylation of the GluN2B subunit of NMDAR, which was accompanied by a significant increase in the performance in hippocampal-dependent learning and memory tasks (Venkitaramani *et al.* 2011). This enhancement in performance was also associated with an increase in the synaptosomal expression of GluN1/GluN2B NMDAR and in GluA1/GluA2-containing AMPAR, providing a potential molecular mechanism for the improved cognitive functions (Venkitaramani *et al.* 2011). A recent study showed that BDNF increases the phosphorylation of $STEP_{61}$ targets, such as Pyk2, ERK1/2 and GluN2B, by a mechanism involving the degradation of $STEP_{61}$ through the ubiquitin

proteasome system (UPS) (Saavedra *et al.* 2015, Xu *et al.* 2015). Furthermore, inhibition of $STEP_{61}$ reversed the hyperlocomotor activity in $BDNF^{+/-}$ mice (Xu *et al.* 2015), confirming the role of BDNF in the regulation $STEP_{61}$ activity.

In addition to the effect of STEP, Pyk2 activity is also regulated by the receptor protein tyrosine phosphatase alpha (PTPα) (Le *et al.* 2006). This receptor is a ubiquitously expressed transmembrane protein that is enriched in the brain. Studies performed in synaptosomes isolated from PTPα-deficient mice showed a reduced Pyk2 tyrosine phosphorylation, enhanced inhibitory tyrosine phosphorylation of four Src-family kinases (Src, Fyn, Yes and Lck), and reduced GluN2B and GluN2A phosphorylation (Le *et al.* 2006), consistent with the phenotype of this animal model, displaying deficits in learning and memory and in CA1 hippocampal LTP (Petrone *et al.* 2003, Skelton *et al.* 2003).

Taken together, these findings show a key role of Pyk2 in hippocampal synaptic plasticity, deeply related with the regulation of NMDAR. However, how the activity of Pyk2 is synaptically regulated remains to be uncovered.

OBJECTIVES

As discussed in the previous sections, new protein synthesis in dendrites is important not only for the induction, but also for the maintenance of BDNF-dependent forms of LTP. However, the identity of the proteins that are locally translated is not fully understood. Identification of these proteins will allow to further elucidate the molecular mechanisms underlying the late phase of LTP. The main objective of the present work was to further investigate the mechanisms underlying the effect of BDNF on synaptic plasticity, focusing on the alterations in the synaptic proteome.

In the work described in chapter 3, we further investigated the role of BDNF in synaptic protein synthesis. In 2004, Schratt and colleagues identified 48 mRNAs whose polysome-association was induced by BDNF by a mechanism sensitive to rapamycin (Schratt *et al.* 2004). Here we focused our work in one of the transcripts shown to be regulated by the neurotrophin, the *Pyk2* mRNA. In particular, we investigated whether Pyk2 is synaptically translated in response to BDNF. In addition, and because previous evidence have suggested that Pyk2 can accumulate at synapses upon NMDAR activation by a mechanism involving Ca^{2+} and calmodulin, we characterized the role of BDNF in the regulation of the subcellular distribution of Pyk2 and the putative role of translation activity in the alterations observed. This question is relevant given the opposite effects of Pyk2 in synaptic plasticity, which may depend on the intracellular localization of the protein. Since BDNF is released upon neuronal activity, we also explored the effect of the neurotrophin on the distribution of endogenous Pyk2 in a chemical-LTP protocol. The alterations in the dendritic and synaptic distribution of Pyk2 were extensively characterized by quantitative immunocytochemistry. Since Pyk2 directly binds to PSD-95, we also investigated the dendritic distribution of PSD-95 upon BDNF treatment and whether the effects of the neurotrophin require protein synthesis.

Given our evidence for local translation of Pyk2 in the dendritic compartment in response to stimulation with BDNF, we further investigated the mechanisms underlying the effects of the neurotrophin. Local translation of Pyk2 in dendrites requires the transport of the corresponding mRNA, which is typically mediated by the interaction with RNA binding proteins. Based on previous evidence of our laboratory suggesting that hnRNPK associates with *Pyk2* mRNA, and considering that the protein is accumulated in the dendritic compartment under conditions of neuronal activity, we investigated the putative role of this RNA binding protein in the regulation of Pyk2 protein levels in dendrites.

The results obtained in the work presented in Chapter 3 show that BDNF upregulates Pyk2 protein levels at the synapse. Therefore, we hypothezised that these alterations could regulate NMDAR trafficking because several reports have linked Pyk2 and NMDAR function. Furthermore, multiple lines of evidence have suggested that BDNF treatment of hippocampal slices increases the NMDAR-currents although the molecular mechanisms involved had not been elucidated. In the work described in Chapter 4 we evaluated the role of BDNF in regulating GluN2B-containg NMDAR surface expression as well as the role of Pyk2 phosphorylation/activation in these alterations. Additionally, we studied the putative role of protein synthesis on the effects BDNF on NMDAR surface expression. Since GluN2B-NMDAR localization may affect the specific function of the receptor, we characterized

the effects of BDNF on the surface expression of total synaptic GluN2B-containing NMDAR, as well as on the synaptic pool of the receptor, in cultured hippocampal neurons.

Chapter 2

MATERIAL AND METHODS

MATERIAL

Plasmids and Constructs

The pSHCMV 3XFLAG Pyk2 (Pyk2-WT) and p3XFLAG Pyk2 Y402F (Pyk2-Y402F) plasmids were a kind gift from Dr. Joseph C. Loftus (Mayo Clinic Arizona). Lentiviral plasmids Sh1-Scramble, sh5-hnRNPK and sh6-hnRNPK were designed and cloned as indicated in section viral production and neuron infection. To knockdown Pyk2 we used the sequences shA2 (shA2-Pyk2) and shA4 (shA4-Pyk2), published by Zang and colleagues (Zhang *et al.* 2014), and we cloned them as described in section cloning of small hairpins RNAs. In parallel we also designed and cloned the construct Sh1-Scramble. The GFP-hnRNPK (GFP-hnRNPK) plasmid was kindly provided by Dr. Antje Ostareck-Lederer (Institute of Biochemistry, Martin-Luther-University Halle-Wittenberg, Kurt-Mothes-Strasse). GFP-Empty and Flag-Empty plasmids were gently given by Dr. Ana Luísa Carvalho (Center for Neuroscience and Cell Biology and Department of Life Sciences, University of Coimbra, Portugal). All plasmid sequences were confirmed by DNA sequencing.

Antibodies

Table 2.1. List of primary antibodies used in immunocytochemistry and Western Blot assays.

Primary Antibody	Host Species	Dilution (application)	Source (reference No.)
Anti-eEF2	Rabbit	1:12 500 (WB)	Abcam (ab40812)
Anti-Flag	Mouse	1:500 (ICC)	Sigma-Aldrich (F3165)
Anti-GFP	Rabbit	1:500 (ICC)	MBL (598)
Anti-GFP	Mouse	1:500 (ICC)	Roche Life Science (11814460001)
Anti-GluN2B	Rabbit	1:100 (ICC)	Alomone Labs (AGC-003)
Anti-hnRNPK	Mouse	1:100 (ICC); 1:1000 (WB)	Santa Cruz Biotechnology (sc-28380)
Anti-hnRNPA2/B1	Mouse	1:1000 (WB)	Santa Cruz Biotechnology (sc-53531)
Anti-MAP2	Chicken	1:10 000 (ICC)	Abcam (ab5392)
Anti-pPyk2(Y402)	Rabbit	1:1000 (WB)	Invitrogen (44-618G)
Anti-PSD-95	Mouse	1:200 (ICC)	Thermo Scientific 7E3-1B8)
Anti-Pyk2	Goat	1:500 (WB)	Santa Cruz Biotechnology (sc-1514)
Anti-Pyk2	Rabbit	1:100 (ICC)	Santa Cruz Biotechnology (sc-9019)
Anti-rpS6	Rabbit	1:1000 (WB)	Cell Signaling Technology (5G10)
Anti-Staufen	Rabbit	1:1000 (WB)	Millipore (AB5781)
Anti-vGlut1	Guinea Pig	1:5000 (ICC)	Millipore (AB5905)
Anti-β-Actin	Mouse	1:50 000 (WB)	Sigma-Aldrich (A5441)
Anti-β-Tubulin	Mouse	1:400 000 (WB)	Sigma-Aldrich (T7816)

eEF2: eukaryotic elongation factor 2; GFP: green fluorescent protein; GluN2B: N-methyl D-aspartate receptor subtype 2B; hnRNPK: heterogeneous nuclear ribonucleoprotein K; hnRNPA2/B1: heterogeneous nuclear ribonucleoprotein A2/B1; MAP2: microtubule-associated protein 2; PSD-95: postsynaptic density protein 95; Pyk2: proline-rich tyrosine Kinase 2; rpS6: ribosomal protein S6; vGlut1: vesicular glutamate transporter 1; ICC: Immunocytochemistry; WB: Western Blot

Table 2.2. List of secondary antibodies used in immunocytochemistry and Western Blot assays.

Secondary Antibody	Dilution (application)	Source (reference No.)
Alexa 488-conjugated anti-Mouse	1:1000 (ICC)	ThermoFisher Scientific (A-11001)
Alexa 488-conjugated anti-Rabbit	1:1000 (ICC)	ThermoFisher Scientific (A-11034)
Alexa 568-conjugated anti-Mouse	1:1000 (ICC)	ThermoFisher Scientific (A-11004)
Alexa 568-conjugated anti-Rabbit	1:1000 (ICC)	ThermoFisher Scientific (A-11036)
Alexa 647-conjugated anti-Guinea Pig	1:500 (ICC)	ThermoFisher Scientific (A-21450)
Alkaline phosphatase-conjugated anti-Goat	1:10 000 (WB)	Jackson Immunoresearch (305-055-003)
Alkaline phosphatase-conjugated anti-Mouse	1:20 000 (WB)	Jackson Immunoresearch (115-055-003)
Alkaline phosphatase-conjugated anti-rabbit	1:20 000 (WB)	Jackson Immunoresearch (211-055-109)
AMCA-conjugated Anti-Chicken	1:200 (ICC)	Jackson Immunoresearch (103-155-155)

ICC: Immunocytochemistry; WB: Western Blot

METHODS

Cell Cultures and Stimulation Protocols

High-Density Hippocampal and Cortical Cultures

Neuronal high-density hippocampal cultures were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (0.06% (w/v); 15 min incubation at 37°C; GIBCO - Life Technologies) and deoxyribonuclease I (5.36 mg/ml) in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS: 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4 \cdot 2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES, and 0.001% phenol red). The hippocampi were then washed with Hanks' balanced salt solution containing 10% fetal bovine serum (GIBCO - Life Technologies) to stop trypsin activity, and transferred to Neurobasal medium (GIBCO - Life Technologies) supplemented with SM1 supplement (1:50 dilution, STEMCELL Technologies), 25 µM glutamate, 0.5 mM glutamine, and 0.12 mg/ml gentamycin (GIBCO - Life Technologies). The cells were dissociated in this solution and then plated in 6-well plates (8.9×10^4 cells/cm^2) coated with poly-D-lysine (0.1 mg/ml). The cultures were maintained in a humidified incubator of 5% CO_2/95% air at 37°C for 2 weeks. At DIV 7 the cells were fed with the same Neurobasal medium described above, but

without glutamate addition. At DIV 14-15 the cultures were stimulated with 50 ng/ml BDNF (Peprotech) for 30 min before preparation of cell extracts as described in section high-density culture extracts.

Neuronal high-density cultures of rat cortical neurons were prepared from the cortices of E18-E19 Wistar rat embryos. Briefly, cortices where washed with ice-cold HBSS three and five times, prior and after trypsin (0.06% (w/v), 10 min at 37ºC) treatment, respectively. Cells were mechanically dissociated, no more than 10-15 times with HBSS. After counting, the cells were plated with neuronal plating medium [Minimal Essential Medium (MEM-Sigma-Aldrich) 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-Fluoro-2′-deoxyuridine (5-FDU, Sigma-Aldrich) to the medium. The cultures were maintained in a humidified incubator with 5% CO_2/95% air, at 37°C.

Neuronal Low-Density Hippocampal Cultures

Neuronal low-density hippocampal cultures were prepared as previously described (Kaech and Banker 2006). Briefly, hippocampi were dissected from E18 rat embryos and the cells were dissociated using trypsin (0.06% (w/v); 15 min incubation at 37°C; GIBCO - Life Technologies). 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 [Minimum Essential Medium (MEM, Sigma-Aldrich) 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, neuronal cultures were treated with 10 µM 5-Fluoro-2′-deoxyuridine (Sigma-Aldrich) after 3 DIV. Cultures were maintained in a humidified incubator with 5% CO_2/95% air at 37°C for up to 3 weeks, feeding the cells once per week with the same Neurobasal medium described above, but without glutamate added. At DIV 14-15 the neurons were stimulated for 30 min or 60 min with 50 ng/ml BDNF (Peprotech). Where indicated, cells were pre-treated for 45 min with a protein synthesis inhibitor, cycloheximide (50 µg/mL, Merck-Millipore), or with the vehicle dimethyl sulfoxide (DMSO, 1:1000 dilution, Sigma-Aldrich), as control. At DIV 21-22 the cells were subjected to a chemical long-term potentiation (cLTP) protocol, as described in section chemical long-term potentiation protocol. After stimulation (BDNF or cLTP), cells were fixed and the immunocytochemistry protocol was performed as described in section immunocytochemistry.

Chemical Long-term Potentiation (cLTP) protocol

cLTP was induced as previously described (Ahmad *et al.* 2012, Ribeiro *et al.* 2014). 21-22 DIV low-density hippocampal cultures were thoroughly washed twice with extracellular solution (ECS) containing (in mM): 150 NaCl, 2 CaCl2, 5 KCl, 10 HEPES, 30 Glucose, 0.001 TTX, 0.01 strychnine,

0.03 picrotoxin, pH 7.4. After washing, neurons were stimulated with or without glycine (300 µM) at room temperature for 3 min in ECS, in the presence or absence of a scavenger of extracellular ligands of TrkB receptors TrkB-Fc (1 µg/ml, R&D Systems). The cells were then incubated for 20-25 min in ECS (no added glycine), in a 5% CO_2/95% air incubator, at 37°C; where indicated this incubation was performed in the presence of TrkB-Fc (1 µg/ml) (R&D Systems). The cells were then fixed and the immunocytochemistry protocol was performed as described in section immunocytochemistry.

Isolation of Synaptoneurosomes and Stimulation Protocol

Synaptoneurosomes were prepared as previously described with slight modifications (Yin *et al.* 2002). Briefly, 4-6 hippocampi were dissected from adult Sprague-Dawley rats and tissue was minced with scissors and homogenized with Kontes Dounce Tissue Grinder in a buffer containing 0.32 M sucrose, 10 mM HEPES-Tris pH 7.4 and 0.1mM EGTA, using first a pestle with large clearance, 0.889–0.165 mm (8-10 strokes), followed by a small clearance pestle, 0.025–0.076 mm (8-10 strokes). After centrifugation for 3 min at 1000 x *g*, the supernatant was collected and passed initially through nylon membranes (150 and 50 µm, VWR) and finally through an 8 µm pore size filter (Millipore, MA). The flow-through was centrifuged for 15 min at 10 000 x *g*, and the resulting pellet was resuspended in incubation buffer (in mM: 8 KCl, 3 $CaCl_2$, 5 Na_2HPO_4, 2 $MgCl_2$, 33 Tris, 72 NaCl, and 100 sucrose). All the procedures were performed at 4°C.

Before stimulation, synaptoneurosomes were pre-warmed at 30ºC during 5 min in the same buffer. Incubation with BDNF (50 ng/mL, Peprotech) was performed at the same temperature, and a control experiment in the absence of the neurotrophin was also performed for each time point considered. Where indicated, synaptoneurosomes were pre-incubated with cycloheximide (50 µg/mL, Merck-Millipore) or with the vehicle, DMSO (1:1000 dilution, Sigma-Aldrich), for 15 min at 30ºC. Synaptoneurosomes were then briefly centrifuged in a Minispin microcentrifuge (Eppendorf) for 30 s, and the pellet was resuspended in RIPA buffer supplemented as indicated for the extract preparation (see section high-density culture extracts), followed by sonication and protein quantification using the BCA method. For polysome extraction the pellet was resuspended in Mammalian Lysis Buffer (MLB) supplemented as indicated for isolation of polysomes.

Isolation of Polysomes

Synaptoneurosomes prepared from adult rats (12-13 weeks) were lysed in 800 µl of MLB [15 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 0.3 M NaCl, 0.5 mM DTT, 0.1 mg/ml cycloheximide (Merck-Millipore) and 1% Triton X-100] supplemented with a cocktail of protease inhibitors (0.1 mM PMSF; CLAP: 1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml antipain, 1 µg/ml pepstatin; Sigma-Aldrich) and 50U/ml of RNase inhibitor (SUPERaseIn™, Ambion Applied Biosystems). Membranous structures were removed by spinning at 12 000 x *g* for 10 min. The resulting supernatant was loaded on a 10-50% linear sucrose gradient [prepared in 20 mM Tris-HCl, pH 8, 140 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mg/ml cycloheximide and 5U/ml of RNase inhibitor (SUPERaseIn™, Ambion Applied Biosystems)] and spun at 35 000 rpm for 190 min (4°C) using a SW41 rotor (Beckman Coulter). Each gradient was separated into 11 fractions, with approximately 1.2 ml each, using a BioLogic LP System (Bio-Rad). For western blot analysis, equal volumes (30 µl) of each fraction isolated from the 10-50% linear

sucrose gradient were denatured using the same denaturing buffer described in section high-density culture extracts. For polysome-associated RNA analysis, RNA was extracted as described in section extraction of polysome-associated RNA.

High-Density Culture Extracts

Cultures of hippocampal or cortical neurons with 14-15 DIV (high-density), stimulated or not with 50 ng/mL of BDNF (Peprotech) during 30 min, were washed twice with ice-cold phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl,1.8 mM KH_2PO_4, 10 mM $Na_2HPO_4.2H_2O$, pH 7.4). The cells were then lysed with RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EGTA, 1 % Triton, 0.5% DOC and 0.1 % SDS, pH 7.5) supplemented with a cocktail of protease inhibitors (0.1 mM PMSF; CLAP: 1 µg/mL chymostatin, 1 µg/mL leupeptin, 1 µg/mL antipain, 1 µg/mL pepstatin; Sigma-Aldrich) and phosphatase inhibitors (50 mM NaF and 1.5 mM Na_3VO_4). The extracts were then sonicated and centrifuged at 16 100 x *g* for 10 min at 4ºC.

The protein content in the supernatants was quantified using the bicinchoninic acid (BCA) assay kit (Pierce, Thermo Fisher Scientific). Proteins were then denatured with 5x concentrated denaturing buffer [62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (v/v) SDS, 0.01% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol (added fresh)] and boiled for 5 min. The proteins of interest were then analyzed by Western Blot.

Gel Electrophoresis and Western Blot

Proteins were resolved by SDS-PAGE in 10% polyacrylamide gels. For Western blot analysis, proteins were transferred onto a PVDF membrane (Millipore) by electroblotting (40V, overnight at 4ºC). The membranes were blocked for 1 h at room temperature in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% (v/v) Tween-20 (TBS-T), and 5% (w/v) low-fat milk or BSA. Membranes were probed during 1 h, at room temperature, or overnight, at 4ºC, with the primary antibodies diluted in TBS-T containing 0.5% (w/v) low fat milk or 3% (w/v) BSA. Following several washes, membranes were incubated for 1 h with alkaline phosphatase conjugated secondary antibodies (anti-mouse, anti-rabbit or anti-goat, depending on the primary antibody host species) at room temperature, washed again and incubated with chemifluorescent substrate, ECF (GE Heathcare) for 5 min (maximum) at room temperature. Membranes were scanned with the Storm 860 scanner (GE Heathcare) and quantified using the ImageQuant software under linear exposure conditions. When necessary, the membranes were stripped (0.2 M NaOH for 20 min) and reprobed.

Extraction of Polysome-associated RNA

Polysomes isolated from synaptoneurosomes and their associated RNA content were precipitated at -20ºC overnight by adding an equal volume of isopropanol to each fraction. After centrifugation of each fraction at 16 400 x *g*, during 20 min at 4ºC, the polysome-associated RNAs present in the pellet fractions were immediately isolated, using TRizol reagent (Invitrogen) and following the manufacturer's specifications. After addition of chloroform and phase separation, the RNA was precipitated by addition of isopropanol. The precipitated RNA was washed twice with 75%

ethanol in RNase free-water, centrifuged, air-dried and resuspended in 10-20 µl of RNase-free water (GIBCO - Invitrogen). The RNA concentration was determined using NanoDrop (Thermo Scientific) and samples were stored at -80°C until further use.

Assessment of RNA Quality

Quality of polysome-associated RNAs was assessed using Experion™ Automated Electrophoresis System (Bio-Rad) following the manufacturer's instructions and the RNAs with low quality (RNA quality indicator, RQI<8) were discarded. The same system was used to evaluated the relative levels of ribosomal RNAs, 18S and 28S, in all fractions (pre-monosomes, monosomes and polysomes) collected from linear sucrose gradients.

Reverse Transcription

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

Primer Design

Primers for qRT-PCR were designed by Beacon Designer 7 software (Premier Biosoft International, Palo Alto, CA). The following considerations were taken: 1- GC content about 50%; 2- annealing temperature (T_a) between 55 ± 5°C; 3- secondary structures and primer dimers were avoided; 4- primer length 18-24 bp; 5- final product length 100-200 bp. See table 2.3 for the list of primers and corresponding sequences:

Table 2.3. Primer sequences used in RT-qPCR.

Genes	Primer Forward Sequence (5'- 3')	Primer Reverse Sequence (5'- 3')
Gria1 (GluA1)	ACT ACA TCC TCG CCA ATC TG	AGT CAC TTG TCC TCC ATT GC
Arc	GAG TTC AAG AAG GAG TTT	CAC ATA CTG AAT GAT CTC
PTK2B (Pyk2)	GTA GAT AGC ATT GTG TTA G	ACT ATT GAT TAA GCA TAC TG
RNA 18S	GCT CCT TAC CTG GTT GAT CC	AAT TAC CAC AGT TAT CCA AGT AGG
Actin	ACT ACA TCC TCG CCA ATC TG	AGT CAC TTG TCC TCC ATT GC

Gria1, GluA1, *glutamate receptor ionotropic AMPA* 1; *Arc, activity-regulated cytoskeleton-associated protein; PTK2B,* Pyk2 *proline-rich tyrosine kinase 2; RNA 18S, Ribonucleic Acid 18S.*

Quantitative Real-Time PCR (RT-qPCR)

The relative amount of polysome-associated mRNAs was quantified using RT-qPCR. Quantitative PCR was performed using the SsoFast™ EvaGreen Supermix (172-5201; Bio-Rad). 2 µl of 1:5 diluted cDNA was used and the final concentration of each primer was 250 nM in a final volume of 20 µl. The thermocycling reaction was initiated with activation of Taq DNA polymerase by heating at 95°C during 30 s, followed by 45 cycles of a 10 s denaturation step at 95°C, a 30 s annealing step at the optimal primer temperature of annealing, and a 30 s elongation step at 72°C. The fluorescence was measured after the extension step by the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). After the thermocycling reaction, the melting step was performed with slow heating, starting at 55°C and with a rate of 0.5°C per 10 s, up to 95°C, with continuous measurement of fluorescence to allow the detection of nonspecific products.

Analysis of RT-qPCR Data

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

Cloning of Small hairpin RNAs

Two small hairpin RNA (shRNA) constructs were designed against Pyk2, identified as shRNA ShA2-Pyk2:

GATCCCCGCCGTCATCTTCACGGACAGATTCAAGAGATCTGTCCGTGAAGATGACGGCTTTTTGGAAA

and ShA4-Pyk2:

GATCCCCCGTATCCTCAAGGTCTGCTTCTTCAAGAGAGAAGCAGCCTTGAGGATACGTTTTTGGAAA

were inserted into a pSUPER vector system (Sigma-Aldrich, Portugal). As a control shRNA, a construct with a scrambled sequence, GATCCCCTTCAAGAGAGATGAACGCTCTGGATGCGCGCATCCAGAGCGTTCATCTTTTTA, that lacked homology to any known mammalian mRNAs, was used. To confirm Pyk2 down-regulation, the rat C6 glioma cell line was transfected with the following shRNAs: sh1-Scramble, shA2-Pyk2 and sh4-Pyk2 for 24 and 72h, using the calcium phosphate transfection protocol. The C6 cell line was purchased from America Tissue Type Collection (Manassas, VA; Catalog # CCL-107). Cells were cultured in DMEM/F12 (Sigma-Aldrich, Portugal) supplemented with sodium bicarbonate (14.2mM), 10% fetal bovine serum, 100U/ml of penincilin and

100 µg/ml of streptomycin in 150 cm^2 tissue culture flasks at 37 ºC in a humidified atmosphere of 5% CO_2. For the calcium phosphate transfection protocol, 4µg of each shRNA per well (6 multi-well) was diluted in Tris-EDTA transfection buffer (10 mM Tris-HCl and 2.5 mM EDTA, pH 7.3). Briefly, 2 M $CaCl_2$ solution was then added, dropwise, to the plasmid DNA-containing solution and this mixture was added to an equivalent volume of 2x HEPES-buffered transfection solution (280 mM NaCl, 1.5mM Na_2HPO_4, 50 mM HEPES, pH 7.05). The mixture was vortexed gently, and the precipitates were allowed to develop at room temperature for 30 min, protected from light, and vortexed every 5 min. The precipitates were then added onto the culture medium and after 4h incubation, the medium was removed and replaced. Cells were then returned to a 5% CO_2/95% air incubator at 37°C, to allow expression of the transfected construct for the desired period of time. After transfection, protein extracts were prepared in lysis buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100 supplemented with 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL chymostatin, 1 µg/mL leupeptin, 1 µg/mL antipain, 1 µg/mL pepstatin (CLAP) and a cocktail of phosphatase inhibitors (1x, Roche, Portugal)]. After centrifugation at 16 100 x *g* for 10 min at 4ºC, protein in the supernatant was quantified using the BCA assay kit (Pierce, Termo Fisher Scientific), and the samples were denatured with 5x concentrated denaturating buffer [62.5 mM Tris-HCl (pH 6.8), 10% (v/v) Glicerol, 2% (v/v) SDS, 0.01% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol], and boiled at 95ºC for 5 min. Pyk2 levels were assessed by immunoblot (as previously described in section gel electrophoresis and western blot).

The knockdown of Pyk2 was also confirmed by transfection of neuronal low-density hippocampal cultures with the designated constructs, as described in the next section. The results were evaluated using immunocytochemistry as described in section immunocytochemistry.

Table 2.4. shRNA-Pyk2 sequences.

Gene	Sequence
PTK2B (Pyk2) shRNA A2	GCC GTC ATC TTC ACG GAC AGA
PTK2B (Pyk2) shRNA A4	CGT ATC CTC AAG GTC TGC TTC

Neuron transfection

Constructs were recombinantly expressed in primary cultures of hippocampal neurons at 11-12 DIV using the calcium phosphate transfection protocol (Jiang *et al.* 2004) with minor alterations. Plasmid (2 µg per coverslip) was diluted in Tris-EDTA transfection buffer (10 mM Tris-HCl and 2.5 mM EDTA, pH 7.3). Briefly, a $CaCl_2$ solution (2.5 M in 10 mM HEPES) was then added, dropwise, to the plasmid DNA-containing solution to give a final concentration of 250 mM $CaCl_2$. This was then added to an equivalent volume of HEPES-buffered transfection solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 11 mM dextrose, 42 mM HEPES, pH 7.2). The mixture was vortexed gently for 2–3 s. The precipitated DNA was added dropwise to the coverslips, and the cultures were incubated with the precipitate for 1.5 h in the presence of kynurenic acid (2 mM). Each coverslip was then transferred to a fresh well of the 12-well plate containing 1 ml of conditioned culture medium with kynurenic acid (2

mM), slightly acidified with HCl (~5 mM final concentration), and the plate was returned to a 5% CO2/95% air incubator at 37°C, for 15 min. Coverslips were then transferred to the original dish containing the conditioned medium. The cells were then returned to a 5% CO2/95% air incubator at 37°C, to allow expression of the transfected construct. Protein expression was typically for 72h, and the experiments were performed at DIV 14-15, depending the day of transfection (DIV 11-12).

Viral Production and Neuron Infection

pTRIP vectors were used to generate lentiviral shRNA vectors **(table 2.5)** for hnRNPK knockdown following the methods previously described (Janas *et al.* 2006). Briefly, lentiviruses were generated by triple calcium-phosphate transfection of pTRIPshRNA (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 Dr. Ramiro Almeida (CNC, Coimbra).

HEK293T cells were 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 dropwise added to 2x HBS 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 h and were further incubated for about 60 h 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 (Beckman Coulter, SW 41Ti rotor) for 120 min at 22ºC (Beckman Coulter, OptimaTM 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 multiplicity of infection (MOI) were determined using high-density cultures of cortical neurons, and using the following formulas:

$$\text{Titer (TU/mL)} = \frac{\text{Target Cell Number } \times \% \text{ of GFP} - \text{Positive Cells}}{\text{Volume of Viral Supernatant (mL)}/100}$$

$$\text{MOI} = \frac{\text{Volume of Viral Supernant (mL)} \times \text{Titer (TU/mL)}}{\text{Target Cell Number}}$$

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 fresh culture medium without glutamate after that period. Coverslips with low-density hippocampal neuronal cultures growing over a layer of astroglia cells were transferred to sterile 12 multi-well 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 and processed for immunocytochemistry. When indicated, cells were processed for Western Blot.

Table 2.5. shRNAs sequences targeting the rat mRNA of hnRNPK (sh5 and sh6) or scrambled sequence (sh1).

shRNA	Target Sequence	Sense oligos	Anti-Sense oligos
sh1	None	GATCCCC **GATGAACGCTCTGGATGCG** TTCAAGAGA **CGCATCCAGAGCGTTCATC** TTTTTGGAAA	AGCTTTTCCAAAAA **GATGAACGCTCTGGATGCG** TCTCTTGAA **CGCATCCAGAGCGTTCATC** GGG
sh5	980 - 998 GAG AUC UCA UGG CUU ACG A	GATCCCC **GAGATCTCATGGCTTACGA** TTCAAGAGA **TCGTAAGCCATGAGATCTC** TTTTTGGAAA	AGCTTTTCCAAAAA **GAGATCTCATGGCTTACGA** TCTCTTGAA **TCGTAAGCCATGAGATCTC** GGG
sh6	1201 - 1219 GUA ACU AUU CCC AAA GAU U	GATCCCC **GTAACTATTCCCAAAGATT** TTCAAGAGA **AATCTTTGGGAATAGTTAC** TTTTTGGAAA	AGCTTTTCCAAAAA **GTAACTATTCCCAAAGATT** TCTCTTGAA **AATCTTTGGGAATAGTTAC** GGG

Immunocytochemistry

To label surface GluN2B-containing NMDA receptors, live neurons (low-density hippocampal cultures) were incubated for 15 min at 37ºC with an antibody against an extracellular epitope of the GluN2B N-terminus diluted in conditioned neuronal culture medium. Neurons were then fixed for 15 min in 4% sucrose and 4% paraformaldehyde in PBS at room temperature, and permeabilized with PBS + 0.3% (v/v) Triton X-100 for 5 min, at 4ºC. Neurons were then incubated in 10% (w/v) BSA in PBS for 30 min at 37ºC to block nonspecific staining, and incubated with the appropriate primary antibody diluted in 3% (w/v) BSA in PBS (overnight, 4ºC) . After washing 6-7 times in PBS, cells were incubated with the secondary antibody diluted in 3% (w/v) BSA in PBS (45 min, 37ºC). The coverslips were mounted using fluorescent mounting medium from DAKO (Glostrup). In experiments aiming at labeling only intracellular proteins, neurons were fixed immediately after stimulation (BDNF or cLTP) as described above and the same procedure was followed.

Quantitative Imaging Analysis

Fluorescence imaging was performed on a Zeiss Axio Observer Z.1 microscope using a 63x 1.4 NA oil objective and a Zeiss Axio Imager Z.2 microscope using 63x 1.4 NA oil objective. Images were quantified using the Fiji image analysis software. For quantitation, sets of cells were cultured and stained simultaneously, and imaged using identical settings. The region of interest was randomly selected, and the dendritic length was measured based on MAP2 staining. The protein signals were analyzed after setting the thresholds, and the recognizable puncta under those conditions were included in the analysis. For each experiment, similar threshold levels were used to quantify the number, area and the integrated intensity of puncta in dendrites. Measurements were performed in 3–6 independent preparations, and at least 10 cells per condition were analyzed for each preparation. In the case of transfected and infected cells overexpressing sh1-Scramble, sh5-hnRNPK, sh6-hnRNPK, GFP, GFP-hnRNPK, shA2-Pyk2, shA4-Pyk2, Flag-Empty, Pyk2-Y402F and Pyk2-WT, only neurons positive for infection/transfection markers were selected.

Pyk2, PSD-95, vGlut1 and hnRNPK_GFP

To quantify Pyk2, PSD-95, vGlut1 and hnRNPK signals, digital images were subjected to a user defined intensity threshold to select puncta, and the immunoreactivity was quantified for puncta intensity, number, and area for the selected region. The synaptic Pyk2 puncta were selected by their overlap with thresholded PSD-95 signal. The results were represented per dendritic length.

hnRNPK

To quantify hnRNPK signals, digital images were subjected to a user defined intensity threshold to select puncta and measured for puncta intensity. The results were represented per dendritic length.

Surface GluN2B in non-transfected cells

To analyze GluN2B surface expression in non-transfected cells, the PSD-95 and vGlut1 signals were thresholded and their colocalization was determined. The surface GluN2B signal was measured after thresholds were set so that recognizable puncta were included in the analysis. Surface GluN2B signal present in glutamatergic synapses was obtained by measuring the surface GluN2B puncta positive for both PSD-95 and vGlut1. The results were represented per density of excitatory synapses (number of positive PSD-95-vGlut1 puncta that colocalized per dendritic length).

Surface GluN2B in transfected cells

To quantify the surface GluN2B immunoreactivity, digital images were subjected to a user defined intensity threshold, to select puncta and measured for puncta intensity, number, and area for the selected region. The synaptic GluN2B puncta were selected by their overlap with thresholded vGlut1 signal. The results were represented per dendritic length.

Statistical Analysis

The results are presented as average values ± s.e.m. Statistical differences were calculated using non-parametric tests for most experiments; when parametric tests were used, it was not possible to calculate the distribution of de samples due an insufficient number of *n*. Mann-Whitney test or Student´s *t* test were used to compare statistical differences between two groups of samples. Comparisons between multiple groups were performed with the Kruskal-Wallis analysis of variance followed by Dunn's Multiple Comparison test or one-way ANOVA analysis of variance followed by Bonferroni´s multiple comparison test, as indicated in the figure captions.

Chapter 3

BDNF induces local protein synthesis in dendrites and synaptic accumulation of Pyk2 by a mechanism involving hnRNPK

Pedro M. Afonso, Eduardo Morais, Carlos B. Duarte

In preparation

Summary

Long-term synaptic potentiation is considered the cellular correlate of learning and memory. In highly polarized cells, such as neurons, dendritic localization of mRNAs and their subsequent translation at stimulated synapses contributes to experience-dependent remodeling of synapses and thereby to the formation of long-term memories. BDNF plays a key role in translation-dependent late-phase of LTP in the hippocampus. However, only a small number of mRNAs are known to be locally translated in response to BDNF. In this work we aim to identify new transcripts to be synaptically translated in hippocampal neurons upon BDNF treatment. We found that Pyk2, proline-rich tyrosine kinase 2, is locally translated at the synapse upon stimulation of hippocampal neurons with BDNF. Pyk2 is a kinase that plays a unique role in Ca^{2+} signaling with important functions in both LTP and LTD. In the present study we report that Pyk2 accumulates in dendrites and synapses upon BDNF treatment by a mechanism dependent on protein synthesis. Moreover, we observed that genetic manipulation the levels of hnRNPK, a RNA-binding protein that binds the Pyk2 transcript, affected Pyk2 expression in dendrites. Knockdown of hnRNPK abolished BDNF-induced increase in Pyk2 in dendrites, whereas overexpression of the RNA-binding protein resulted in increased levels of Pyk2, mimicking the effect of BDNF. We also found that endogenous BDNF released upon NMDAR-mediated chemical LTP resulted in a similar increase in synaptic Pyk2, by a TrkB-dependent mechanism. Taken together, our findings strongly suggest that activity-induced release of BDNF may promote local translation of *Pyk2* mRNA in dendrites, transported to the synapse by hnRNPK. The resulting synaptic accumulation of Pyk2 may contribute to neurotrophin-mediated synaptic plasticity events.

Introduction

Long-term potentiation is the most studied form of synaptic plasticity and it is considered the cellular correlate of learning and memory. There are many players involved in this form of plasticity, including membrane receptors, scaffold proteins, cytoskeleton proteins and RNA-binding proteins (Wheal *et al.* 1998, Sheng and Kim 2011). Proteins of the postsynaptic density have not only been described to play an important role in synaptic plasticity phenomena (Iasevoli *et al.* 2013), but also in disease contexts, such as in psychiatric disorders (Gong and Lippa 2010, Iasevoli *et al.* 2013, Nithianantharajah and Hannan 2013) The postsynaptic density is comprised by several proteins that interact directly or indirectly to maintain the normal synaptic transmission and to mediate synaptic plasticity events (Sheng and Kim 2011, Iasevoli *et al.* 2013). One of the best characterized postsynaptic density proteins with a preponderant effect in synaptic plasticity is PSD-95. It was described that PSD-95 and Pyk2 can directly interact (Seabold *et al.* 2003), and a recent study proposed that interfering with the direct binding of PSD-95 to Pyk2 was sufficient to prevent induction of LTP in the CA1 region of the hippocampus (Bartos *et al.* 2010). Pyk2 is a focal adhesion kinase (FAK) family member, highly expressed in the central nervous system, which is activated in response to neuronal activity (Siciliano *et al.* 1996), activation of protein kinase C and increases of intracellular calcium (Alier and Morris 2005).

Neurotrophins have also been identified as key players in synaptic plasticity. In particular, BDNF was shown to be sufficient for LTP induction in the hippocampus (Kang and Schuman 1996, Messaoudi *et al.* 2002, Ying *et al.* 2002). The mature form of BDNF acts preferentially at TrkB receptors and its role in induction of LTP is known to require local protein synthesis (Kang and Schuman 1996, Leal *et al.* 2015). Dendritic protein synthesis plays an important function in neurons because of their high compartmentalization, with a consequent requirement of a local control of the proteome (Sutton and Schuman 2006). The control of local translation in dendrites contributes to synaptic plasticity phenomena, as well as to the maintenance of a normal brain function (Sutton and Schuman 2006, Swanger and Bassell 2013). Several mRNAs coding for synaptic localized proteins were already reported to be translated upon BDNF treatment, such as *GluA1*, *Arc*, *CaMKIIα* and *Homer2* (Schratt *et al.* 2004, Takei *et al.* 2004). BDNF has also been implicated in the regulation of the translation machinery thereby enhancing dendritic translation (Leal *et al.* 2014). However, the identity of mRNAs that are translated in dendrites in response to BDNF treatment is still poorly understood.

RNA-binding proteins (RBPs), especially those belonging to the hnRNPs family, have been reported to accumulate at postsynaptic densities in response to neuronal activity (Zhang *et al.* 2012, Leal *et al.* 2014). hnRNPK regulates dendritic spine density and LTP induction by a mechanism involving activation of the ERK cascade and GluA1 (S845) phosphorylation, with a consequent upregulation in the surface expression of GluA1-containing AMPA receptors (Folci *et al.* 2014). RBPs regulate many aspects of RNA metabolism through binding and incorporating transcripts into specialized ribonucleoprotein particles (RNPs) (Sossin and DesGroseillers 2006). It is widely accepted that mRNAs can be transported in a silent form along dendrites, as a part of RNPs, and they are translated in the vicinity of the synapses upon appropriate stimulation. Translation of these mRNAs enriches the postsynaptic compartment in specific proteins that may contribute to LTP or LTD (Leal *et*

al. 2014). In the present study, we investigated the role of BDNF on synaptic protein synthesis. We found that *Pyk2* mRNA is locally translated at the synapse upon BDNF treatment, by a mechanism that depends on hnRNPK, leading to an accumulation of the protein in postsynaptic compartments.

Results

BDNF treatment increases local protein translation in synaptoneurosomes

Previous studies showed that BDNF stimulation increases translation activity in cultured cortical neurons, with a maximum effect after 20 min (Schratt *et al.* 2004). Here, we investigated the effect of BDNF on local protein synthesis at the synapse, using adult rat (10-12 weeks) hippocampal synaptoneurosomes, a subcellular fraction containing the pre- and postsynaptic regions (Yin *et al.* 2002). Synaptoneurosomes were stimulated or not with BDNF (50 ng/mL) for 20 min, and polysomes together with their associated mRNAs were isolated using a linear sucrose (10-50%) gradient **(Fig. 3.1A)**. Polysome profiles were acquired by continuous measurement of the absorvance at 254 nm, and were composed of peaks corresponding to monosome (40S, 60S and 80S ribosome) and polysome (mRNAs associated with increasing number of actively translating ribosomes) fractions **(Fig. S3.1)**. Previous reports have shown that increased mRNA translation results in a shift of mRNA and ribosomal subunits into the polysomal fractions (Schratt *et al.* 2004). To evaluate the effect of BDNF on local translation in hippocampal synaptoneurosomes, we compared the peaks corresponding to polysome fractions after separation in a linear sucrose gradient. We found that 20 min of stimulation with BDNF increased the magnitude of the polysome peak **(Fig. 3.1B)**, suggesting that BDNF induces dendritic protein synthesis in hippocampal synapses.

Schratt and colleagues identified 48 mRNAs that are translated in cultured cortical neurons (14 DIV) upon BDNF treatment by a mechanism sensitive to rapamycin (Schratt *et al.* 2004). One of the transcripts identified codes for Pyk2, a protein kinase that regulates LTP and LTD (Huang *et al.* 2001, Hsin *et al.* 2010). Therefore, we next asked whether Pyk2 was locally translated at the synapse in response to BDNF. To address this question, we isolated polysome-associated mRNAs from synaptoneurosomes stimulated or not with BDNF for 20 min. The levels of *Pyk2* mRNA and other well-known targets of BDNF, the *Arc* and *GluA1* transcripts, were assessed using RT-qPCR. We found an enrichment in polysome-associated *Pyk2* mRNA, as well as in *GluA1* and *Arc* mRNAs, further proving an enrichment in polysome fractions after BDNF treatment **(Fig. 3.1C)**. As expected, an upregulation in the 18S ribosomal RNA was also observed. As negative control we measured the relative levels of *actin* mRNA, which did not significantly change **(Fig. 3.1C)**.

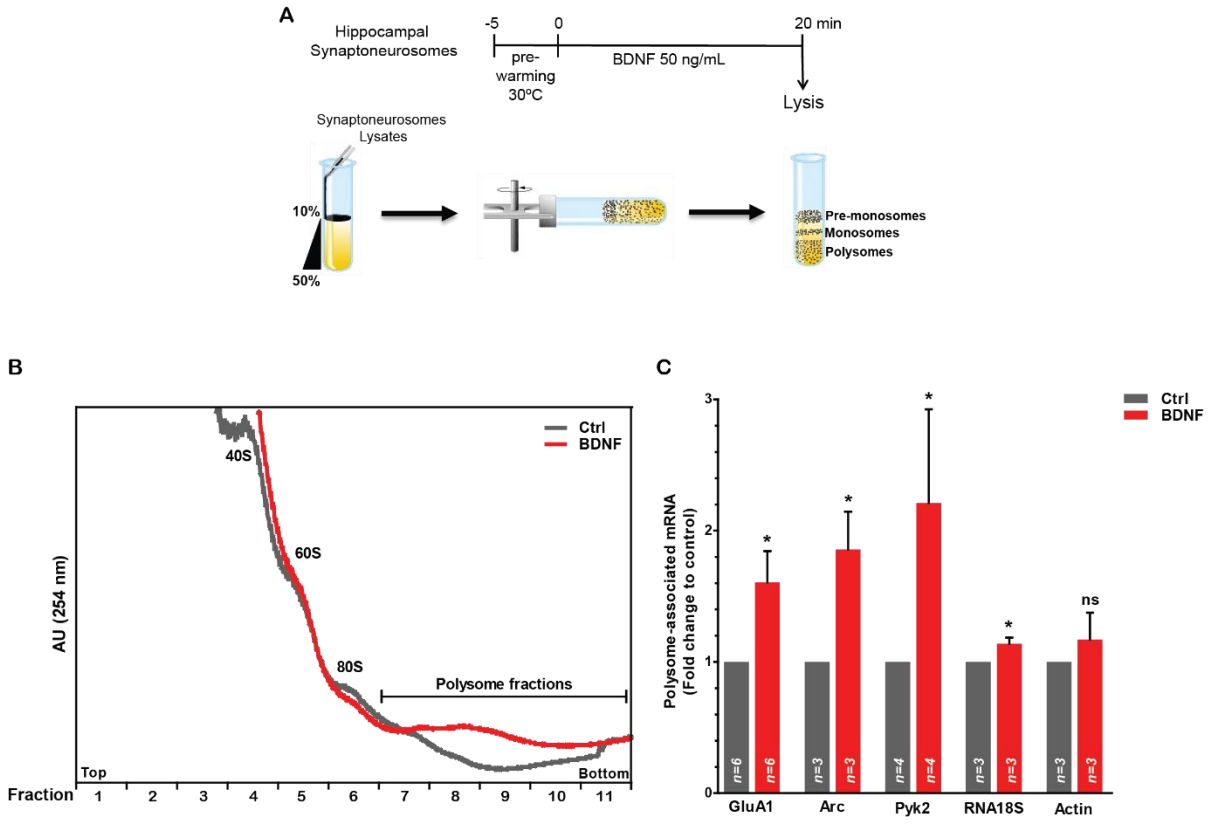


Figure 3.1. BDNF treatment increases local protein translation in synaptoneurosomes. A. Schematic representation of hippocampal synaptoneurosome stimulation paradigm and the sucrose gradient used in the isolation of polysomes. Hippocampal synaptoneurosomes were stimulated or not with BDNF (50 ng/mL) and polysomes and their associated mRNAs were fractionated into high density fractions, using a continuous linear 10-50% sucrose gradient. **B**. Representative polysome profile isolated from synaptoneurosomes treated or not with BDNF (50 ng/mL) for 20 min. **C.** BDNF upregulates specific polysome-associated transcripts, including *Pyk2* mRNA. After polysome fractionation, the associated mRNAs were isolated as described in the methods section. 500 ng of polysome-associated mRNAs were reverse transcribed and the relative amount of specific mRNAs (*GluA1, Arc, Pyk2, RNA18S and β-Actin*) was evaluated by RT-qPCR using specific primers. The results were normalized for *Gapdh* mRNA levels. Error bars represent s.e.m. The statistical significance was calculated using the unpaired *t* test ($^{ns}P > 0.05$, $^{*}P < 0.05$). *n* represents the number of independent synaptoneurosomal preparations.

BDNF stimulation increases Pyk2 protein levels in synaptoneurosomes in a protein-synthesis dependent manner

To further investigate the BDNF-induced increase in local translation of *Pyk2* mRNA at the synapse, we measured the effect of the neurotrophin on total Pyk2 protein levels in hippocampal synaptoneurosomes isolated from adult (10-12 weeks) rats. Synaptoneurosomes were stimulated with BDNF during 20, 30 and 40 min (time course), and the synaptic Pyk2 protein levels were assessed by immunoblot and compared with respective time control **(Fig. 3.2A)**. BDNF increased synaptic Pyk2 protein levels, after 20 and 30 min of stimulation **(Fig. 3.2B,C)**. To determine whether the BDNF-induced upregulation in Pyk2 protein levels was dependent on protein synthesis, synaptoneurosomes were stimulated with the neurotrophin (50 ng/mL) for 20 min in the presence of cycloheximide (50 µg/mL). In agreement with the results obtained in the analysis of polysome fractions **(Fig. 3.1C)**, we observed that cycloheximide (CHX) completely abolished the effects of BDNF on synaptic Pyk2 protein levels **(Fig. 3.2E,F)**, indicating that the synaptic increase of Pyk2 induced by BDNF was dependent on protein synthesis.

The results described above show an effect of BDNF in the upregulation of Pyk2 in the synaptic compartment in hippocampal neurons, complementing the previous evidence pointing to a role of the neurotrophin in the regulation of total Pyk2 levels in cortical neurons (Schratt *et al.* 2004). To determine whether BDNF also induces an overall increase in Pyk2 protein synthesis in cultured hippocampal neurons, the cells were stimulated with BDNF (50 ng/mL) for 20 min and the Pyk2 levels were evaluated by Western Blot. In contrast with the results obtained in hippocampal synaptoneurosomes, BDNF did not affect Pyk2 total protein levels in hippocampal neurons **(Fig. 3.2G,H)**, suggesting a specific compartmentalized effect of BDNF. Taken together, these results show that BDNF induces a specific increase in synaptic Pyk2 mediated by new protein synthesis, likely occurring within the dendritic compartment.

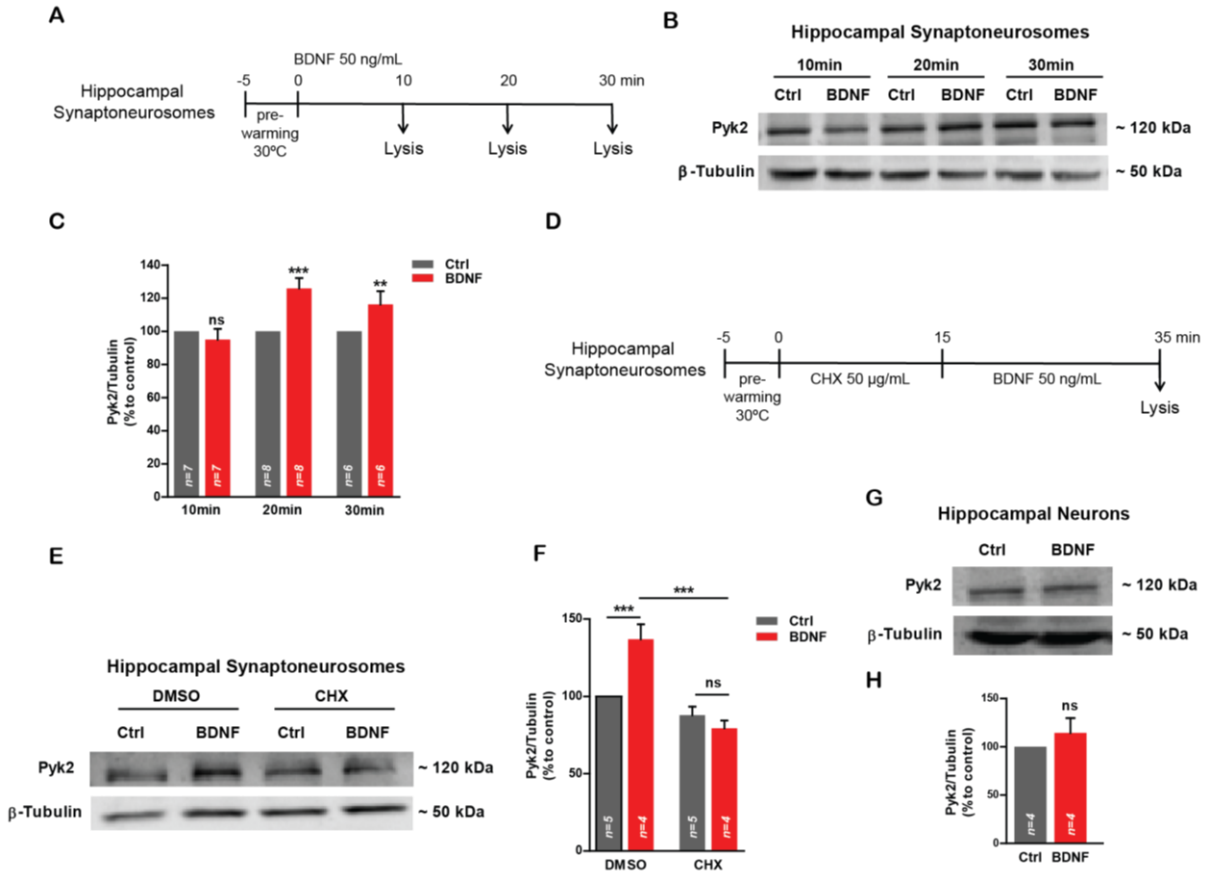


Figure 3.2. BDNF stimulation increases Pyk2 protein levels in synaptoneurosomes in a protein synthesis-dependent manner. **A,D.** Schematic representation of hippocampal synaptoneurosome stimulation paradigms. **B,C.** BDNF induces a significant increase in synaptic Pyk2 protein levels after 20 and 30 min of stimulation as assessed by Western Blot. Hippocampal synaptoneurosomes were stimulated or not with BDNF (50 ng/mL) during 10, 20 and 30 min, after a pre-warming (30ºC) step of 5 min. A representative image is shown in panel **(B)** and **(C)** shows the average ± S.E.M. results. **E,F.** BDNF-induced increase in synaptic Pyk2 requires local protein-synthesis, as assessed by Western Blot. Pre-incubation of synaptoneurosomes with CHX (50 µg/mL) abolished the BDNF-induced increase in synaptic Pyk2 protein levels. After pre-warming, synaptoneurosomes were pre-incubated (15 min) with CHX (50 µg/mL) or vehicle, DMSO (dilution 1:1000), before stimulation (or not, Control) with BDNF (50 ng/mL) for 20 min at 30ºC, as indicated. A representative image is shown in panel (E), and (F) shows the average ± s.e.m. results. Levels of Pyk2 (total or synaptic) were normalized to β-Tubulin levels. The statistical significance was calculated using the one-way ANOVA ($P < 0.0001$) followed by the Bonferroni´s multiple comparison test (**E-F)** or unpaired *t* test (**B-C, G-H**) ($^{ns}P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). *n* represents the number of independent experiments, performed in different preparations. *CHX- Cycloheximide, DMSO-Dimethyl Sulfoxide (vehicle).*

BDNF increases Pyk2 protein levels in dendrites of hippocampal neurons, in a protein synthesis-dependent manner

Ca^{2+} influx through NMDA receptors induces a dendritic accumulation of the Pyk2 protein in cultured hippocampal neurons (Bartos *et al.* 2010). Based on the results shown in the previous sections, we asked whether BDNF could increase dendritic Pyk2 levels by a mechanism dependent on protein synthesis. To address this question, hippocampal neurons (14-15 DIV) were stimulated with BDNF (50ng/mL) for 30 or 60 min, in the presence or in the absence of cycloheximide (50 µg/mL), and Pyk2 protein levels were evaluated by immunocytochemistry. In control experiments, hippocampal neurons were incubated in culture conditioned medium, in the presence or in the absence of the translation inhibitor. After fixation, cells were immunostained for Pyk2 and MAP2 **(Fig. 3.3A)**, and the images obtained were analyzed for the number of Pyk2 puncta **(Fig. 3.3B)**, area of puncta **(Fig. 3.3C)** and intensity of puncta **(Fig. 3.3D)** per dendritic length (MAP2). The results obtained were similar for all parameters analyzed. BDNF induced a dendritic accumulation of Pyk2 at 30 or 60 min or incubation, and this effect was abolished by pre-treatment with cycloheximide. Therefore, we can conclude that BDNF-induced upregulation of dendritic Pyk2 in hippocampal neurons requires protein synthesis.

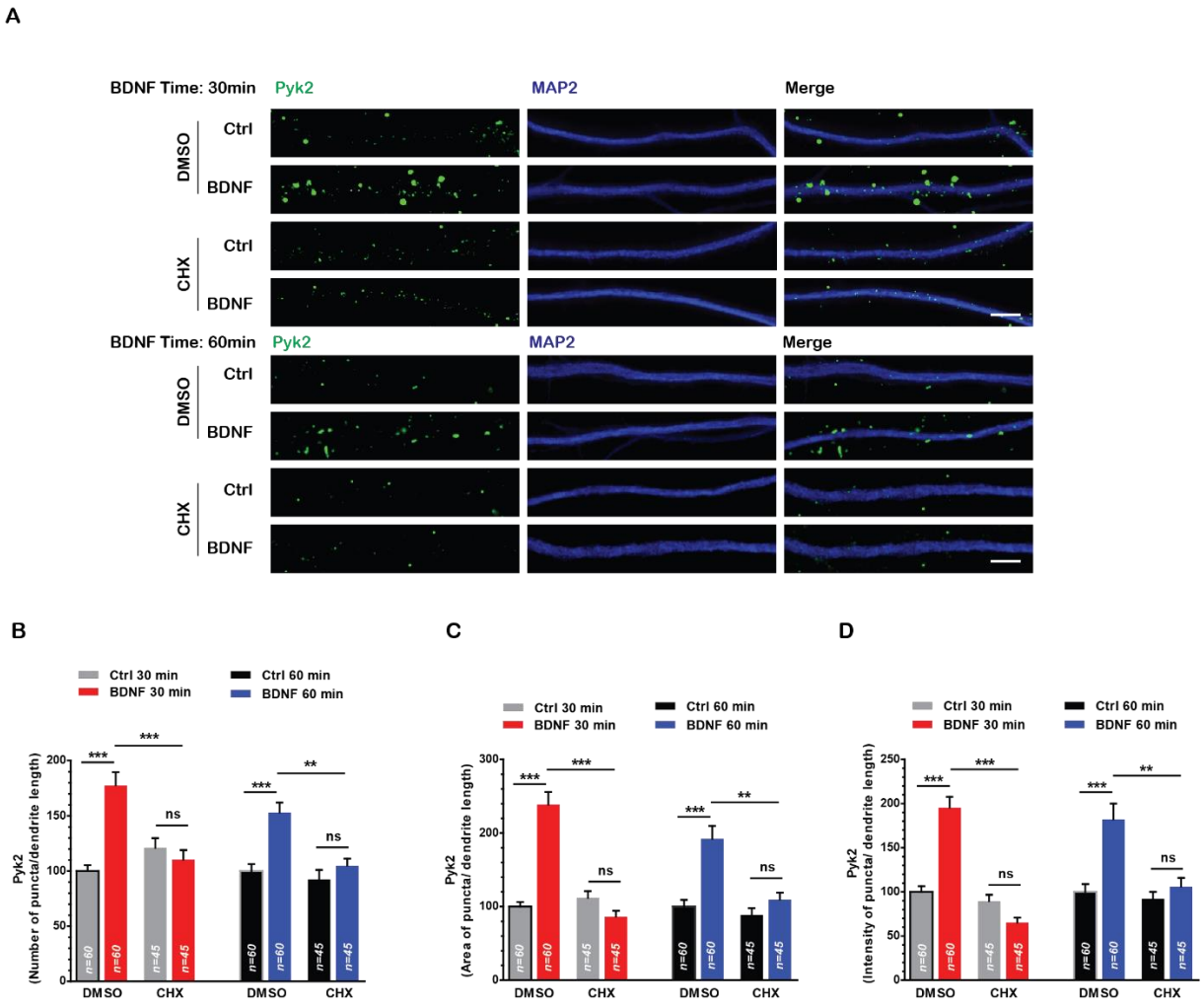


Figure 3.3. BDNF stimulation increases total dendritic Pyk2 levels in a protein synthesis-dependent manner. A. Hippocampal neurons (DIV 14-15) were pre-incubated with CHX (50 µg/mL) or vehicle DMSO (dilution 1:1000) for 45 min, before stimulation with BDNF (50 ng/mL) during 30 or 60 min, as indicated. After BDNF treatment, neurons were immediately fixed and immunostained for Pyk2 and MAP2. Neurons were analyzed for dendritic Pyk2 number of puncta (**B**), area of puncta (**C**) and intensity of puncta (**D**) per dendritic length. Results are expressed as percentage of the respective DMSO control (at 30 and 60 min). Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{ns}P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). *n* represents the number of neurons analyzed from at least three independent experiments, performed in different preparations. *CHX- Cycloheximide, DMSO- Dimethyl Sulfoxide (vehicle).* Scale bar = 5 µm.

Multiple lines of evidence suggest that PSD-95 and Pyk2 work together to mediate synaptic plasticity through a mechanism involving NMDA receptors (Bongiorno-Borbone *et al.* 2005, Bartos *et al.* 2010, Zhao *et al.* 2015). Moreover, Ca^{2+} influx through NMDA receptors was shown to induce Pyk2 clustering at synapses, namely at postsynaptic terminals (Bartos *et al.* 2010). To determine whether stimulation with BDNF also induces the synaptic accumulation of Pyk2, hippocampal neurons (DIV 14-15) were stimulated with BDNF for 30 or 60 min, and the cells were immunostained for Pyk2, PSD-95 and MAP2 **(Fig. 3.4A)**. Images were analyzed for synaptic **(**PSD-95 colocalized; arrowheads in **Fig 3.4A)** Pyk2 number of puncta **(Fig. 3.4B)**, area of puncta **(Fig. 3.4C)** and intensity of puncta **(Fig. 3.4D)** per dendritic length (MAP2). The results were similar for the three parameters analyzed, showing a specific increase in synaptic clustering of Pyk2 after stimulation with BDNF **(Fig. 3.4 A-D)**.

Given the role of translation activity in BDNF-induced dendritic accumulation of Pyk2, we also determined whether protein synthesis was important in the observed synaptic accumulation of the kinase in hippocampal neurons incubated with the neurotrophin. Hippocampal neurons were stimulated with the neurotrophin in the presence or in the absence of cycloheximide (50 µg/mL), and the synaptic expression of the kinase was evaluated as described above. The results of Fig. 4A-D show that the BDNF-induced increase in synaptic clustering of Pyk2 is also dependent on protein-synthesis. However, when the cells were stimulated with the neurotrophin for 60 min in the presence of the translation inhibitor there was still a small but significant upregulation in the number of synaptic puncta containing Pyk2, possibly due to a redistribution of preexisting protein to synapse. Using the same paradigm of stimulation, we also evaluated the percentage of synaptic Pyk2 (% of colocalization Pyk2-PSD-95) **(Fig. 3.4E)** and the percentage of synapses containing Pyk2 (% of colocalization PSD-95-Pyk2) **(Fig. 3.4D)** in hippocampal neurons stimulated with BDNF, in the presence or in the absence of cycloheximide. We found an increase in the percentage of synaptic Pyk2 and in the percentage of synapses containing Pyk2 at 30 and 60 min following BDNF application, and these alterations were completely abrogated by pre-treatment of neurons with cycloheximide **(Fig. 3.4E,F)**.

BDNF was shown to induce the recruitment of PSD-95 to dendrites (Yoshii and Constantine-Paton 2007) and to spines by a mechanism dependent on microtubule invasion (Hu *et al.* 2011). In order to rule out a contribution of the redistribution of PSD-95 in the BDNF-induced synaptic accumulation of Pyk2 described above, we analyzed the distribution of the postsynaptic marker in hippocampal neurons stimulated with the neurotrophin for different periods of time. The changes in the number **(Fig. S3.2B)**, area **(Fig. S3.2C)** and density **(Fig. S3.2D)** of PSD-95 puncta per dendritic length were evaluated following stimulation with BDNF for 30 or 60 min. Under the latter conditions there was indeed an increase in PSD-95 number, area and intensity of puncta, but no significant

differences in PSD-95 number of puncta were detected after 30 min of stimulation with BDNF. Therefore, we can conclude that alterations in the dendritic distribution of PSD-95 do not account for the observed alterations in Pyk2 expression at the synapse in hippocampal neurons stimulated with BDNF for 30 min. The observed alteration in the number of PSD-95 puncta after 60 min of stimulation with BDNF makes more difficult the interpretation of the results obtained regarding the synaptic distribution of Pyk2 under these conditions. Therefore, in all experiments shown below, hippocampal neurons were stimulated with BDNF for 30 min.

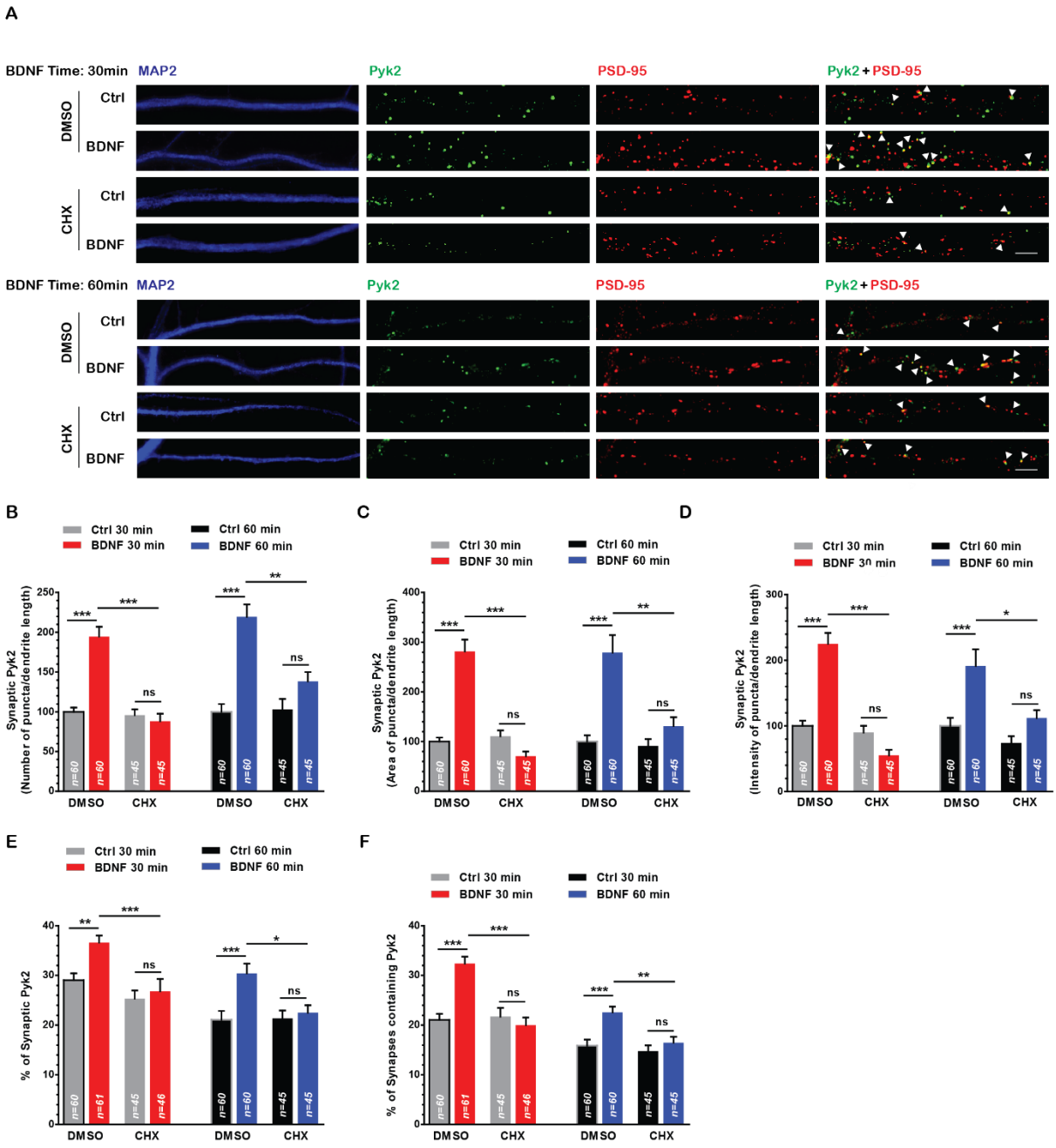


Figure 3.4. BDNF treatment increases synaptic Pyk2 levels and the percentage of synapses containing Pyk2, in a protein synthesis-dependent manner. A. Hippocampal neurons (DIV 14-15) were pre-incubated with CHX (50 µg/mL) or vehicle DMSO (dilution 1:1000) for 45 min, before stimulation with BDNF (50 ng/mL) during 30 or 60 min, as indicated. After BDNF treatment, neurons were immediately fixed and immunostained for Pyk2, PSD-95 and MAP2. Neurons were analyzed for synaptic (PSD-95-colocalized) Pyk2 number of puncta (**B**), area of puncta (**C**) and intensity of puncta (**D**) per dendritic length. Results are expressed as percentage of the respective DMSO control (at 30 and 60 min). The percentage of synaptic Pyk2 (number of Pyk2 puncta-colocalized with PSD-95/ total Pyk2 number of puncta) and the percentage of synapses containing Pyk2 (number of PSD-95 puncta-colocalized with Pyk2/ total PSD-95 number of puncta) are shown in panels (**E**) and (**F**), respectively. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). *n* represents the number of neurons analyzed from at least three independent experiments, performed in different preparations. *CHX- Cycloheximide, DMSO- Dimethyl Sulfoxide (vehicle).* Arrowheads: Pyk2-PSD-95 colocalized puncta. Scale bar = 5 µm.

hnRNPK mediates BDNF-induced increase in dendritic Pyk2 protein levels

Previous studies performed in *Xenopus laevis axons* showed that knockdown of hnRNPK reduces the efficiency of neurofilament-M (*NF-M*) and *GAP-43* mRNAs export from the nucleus, and inhibited their loading onto polysomes for translation. These alterations were associated to the suppression of NF-M and GAP-43 protein expression, with implications in axon outgrowth and regeneration (Liu *et al.* 2008, Liu *et al.* 2012). Previous results from our laboratory, based on the microarray analysis of the transcripts that dissociate from hnRNPK following BDNF treatment of hippocampal neurons, suggested that (i) *Pyk2* mRNA interacts with hnRNPK, and (ii) this interaction is weakened following stimulation with the neurotrophin. In preliminary qPCR experiments we confirmed that the *Pyk2* mRNA is present in hnRNPK immunoprecipitates from cultured hippocampal neurons (data not shown). Therefore, we hypothesized that hnRNPK could be involved in BDNF-induced upregulation in dendritic localized Pyk2 protein. To test this hypothesis, two different shRNA (sh5-hnRNPK and sh6-hnRNPK) were designed, generated and incorporated in a lentivirus vector, to knockdown hnRNPK. As control, a scramble sequence (sh1-Scramble) lacking homology to any known mammalian mRNAs, was used. The efficiency of hnRNPK knockdown was confirmed by immunoblot **(Fig. S3.3A)** and immunocytochemistry **(Fig. S3.3B,C)** using cortical and hippocampal neurons, respectively. The results of the validation experiments indicated that the construct sh6-hnRNPK was more efficient than sh5-hnRNPK5 in knocking down Pyk2 when analyzed after 4 days of expression **(Fig. S3.3A-C)**. Therefore, in the experiments described below hippocampal neurons were infected at DIV10 with sh1-Scramble or sh6-hnRNPK, and stimulation with BDNF (50 ng/mL; 30 min) was performed 4 days later **(Fig. 3.5A)**. After fixation, neurons were immunostained for Pyk2, GFP (infection marker) and MAP2, and the results were analyzed for Pyk2 number of puncta **(Fig. 5B)**, area of puncta **(Fig. 3.5C)** and intensity of puncta **(Fig. 3.5D)** per dendritic length (MAP2). hnRNPK knockdown (sh6-RNPK) suppressed the effect of BDNF on the dendritic distribution of Pyk2 **(Fig. 3.6A-D)**, while no effect was observed in hippocampal neurons transduced with sh1-Scramble.

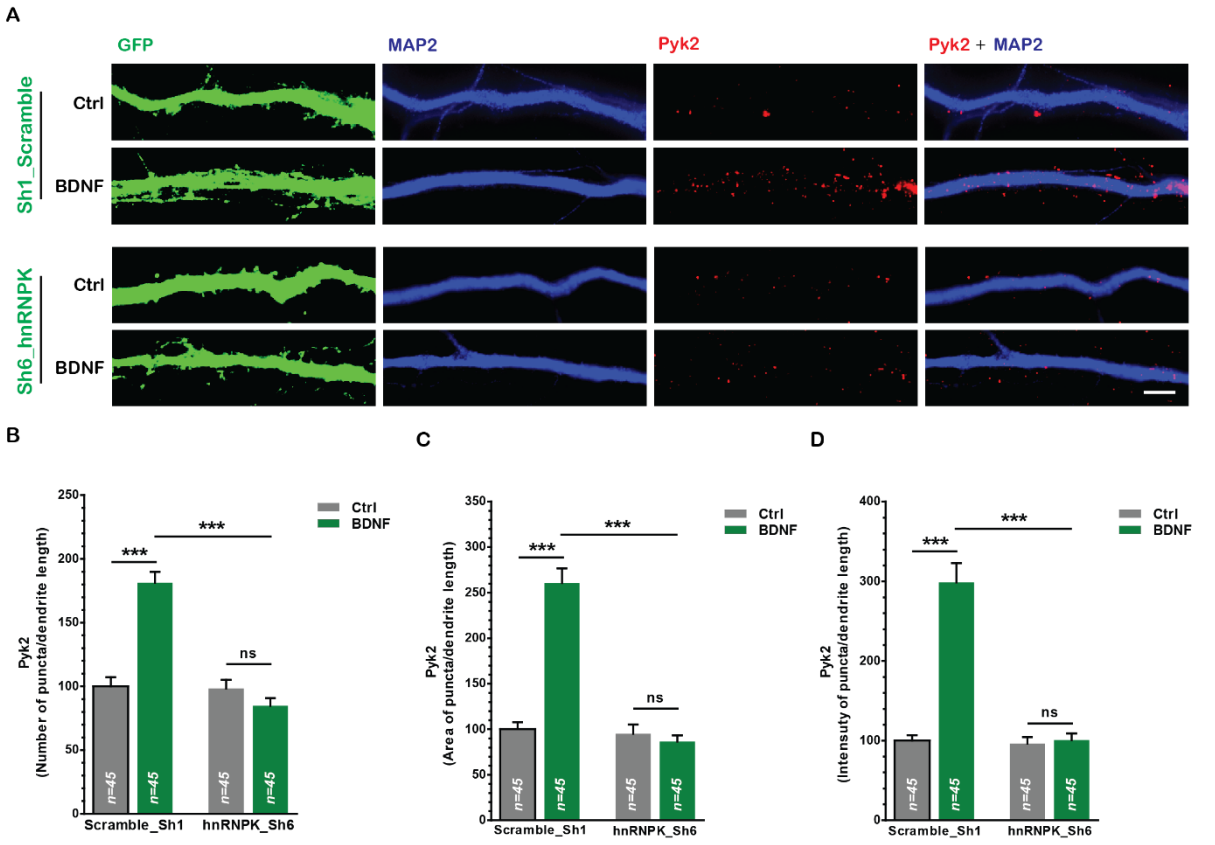


Figure 3.5. Knockdown of hnRNPK completely abolishes the BDNF-induced upregulation of dendritic Pyk2 protein levels. A. Hippocampal neurons were infected (DIV 10) with sh1-Scramble or sh6-hnRNPK. At DIV 14, neurons were stimulated or not with BDNF (50 ng/mL) during 30 min, as indicated. After BDNF treatment, neurons were immediately fixed and immunostained for Pyk2, GFP and MAP2. Neurons were analyzed for dendritic Pyk2 number of puncta (**B**), area of puncta (**C**) and intensity of puncta (**D**) per dendritic length. Results are expressed as percentage to sh1-Scramble control. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{ns}P > 0.05$, $^{***}P < 0.001$). *n* represents the number of neurons analyzed from three independent experiments, performed in different preparations. Scale bar = 5 µm.

To further investigate the role of hnRNPK in BDNF-induced upregulation in dendritic Pyk2, we overexpressed a GFP-tagged hnRNPK construct in hippocampal neurons. The cells were transfected at DIV 11-12 with GFP (GFP-Empty) or GFP-hnRNPK, and at DIV 14-15 neurons were treated or not with BDNF (50 ng/mL) for 30 min. After fixation, neurons were immunostained for Pyk2, GFP (transfection marker) and MAP2 **(Fig. 3.6A)**. Three parameters were analyzed with similar results, Pyk2 number of puncta **(Fig. 3.6B)**, area of puncta **(Fig. 3.6C),** and intensity of puncta **(Fig. 3.6D)** per dendritic length (MAP2). Overexpression of hnRNPK increased Pyk2 protein levels in dendrites, similar to the effect resulting from BDNF treatment **(Fig. 3.6A-D)**. However, under these conditions BDNF did not change Pyk2 expression levels. As expected, BDNF upregulated dendritic Pyk2 protein levels in hippocampal neurons transfected with GFP.

Preliminary data from our laboratory suggest that BDNF can induce the translocation of hnRNPK from the soma to dendrites (data not shown), which would be expected to provide additional transcripts for local synthesis of Pyk2. We hypothesized that 72 h of GFP-hnRNPK expression could be sufficient to saturate dendrites with this RBP and, therefore, to prevent a further increase in the local abundance of the protein following stimulation with BDNF. This would halt the effect of BDNF on

the transport/regulation/metabolism of *Pyk2* mRNA, ultimately preventing the alterations in Pyk2 dendritic expression. To address this hypothesis, we measured the changes in dendritic GFP-hnRNPK levels following treatment of hippocampal neurons with BDNF. There was no significant effect of BDNF on the dendritic expression of the fusion protein **(Fig. S3.4A-D)**, supporting our hypothesis.

Taken together these findings show a role for hnRNPK in BDNF-induced dendritic increase of Pyk2 by a mechanism likely involving the control of *Pyk2* mRNA nuclear export/transport/loading onto polysomes, which ultimately interferes with BDNF-induced local translation of Pyk2 at dendrites.

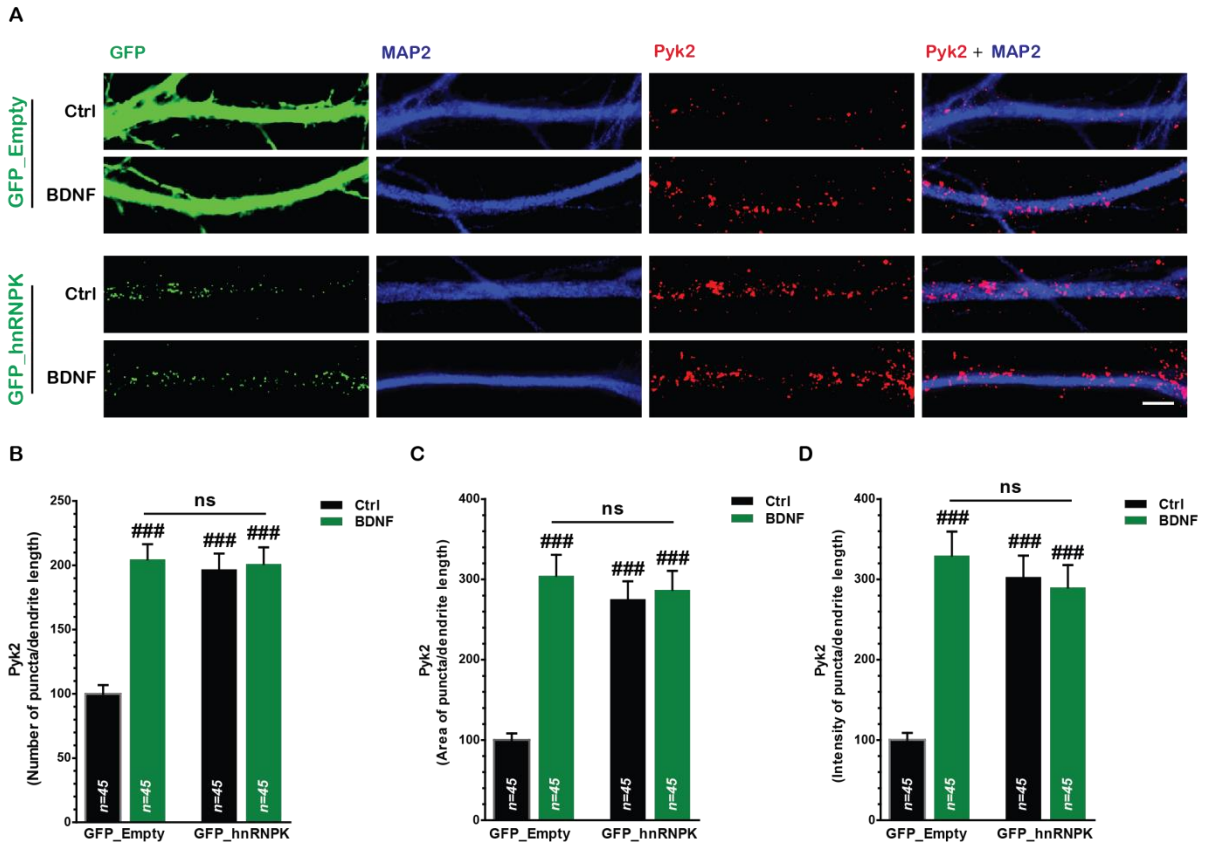


Figure 3.6. Overexpression of hnRNPK induces a similar increase of dendritic Pyk2 levels to that obtained by BDNF treatment. A. Hippocampal neurons were transfected at DIV 11-12 with GFP_Empty or GFP_hnRNPK, and 3 days later were stimulated or not with BDNF (50 ng/mL) during 30 min, as indicated. After BDNF treatment, neurons were immediately fixed and immunostained for Pyk2, GFP and MAP2. Neurons were analyzed for dendritic Pyk2 number of puncta (**B**), area of puncta (**C**) and intensity of puncta (**D**) per dendritic length. Results are expressed as percentage to GFP-Empty control. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{ns}P > 0.05$ between indicated conditions, or $^{###}P < 0.001$ comparing to GFP-Empty control). *n* represents the number of neurons analyzed from three independent experiments, performed in different preparations. Scale bar = 5 µm.

cLTP increases dendritic Pyk2 levels in hippocampal neurons by a BDNF-dependent mechanism

It has been described that LTP induction by high-frequency stimulation induces BDNF release from glutamatergic synapses in a Ca^{2+}-dependent manner (Hartmann *et al.* 2001, Gartner and Staiger 2002). NMDAR-dependent cLTP (chemical LTP) has also been described to promote the release of BDNF in hippocampal cultures (Cho *et al.* 2013). This protocol uses a brief incubation with the

NMDAR co-agonist glycine in an Mg^{2+}-free medium, which is capable of increasing the exocytosis of GluA1-containg AMPA receptors into the synaptic plasma membrane, leading to LTP of AMPAR-mediated excitatory transmission (Ribeiro *et al.* 2014). Given the effect of exogenous addition of BDNF on the dendritic levels of Pyk2, we investigated whether endogenously released BDNF is capable of modulating the dendritic distribution of Pyk2 using a cLTP protocol. Thus, we subjected mature hippocampal neurons (DIV 21) to a cLTP protocol in presence or absence of a scavenger of TrkB receptor ligands (TrkB-Fc). Neurons were immediately fixed and labeled to Pyk2 and MAP2 **(Fig. 3.7A-D)**, and we analyzed the dendritic number of Pyk2 puncta **(Fig. 3.7B)**, area of puncta **(Fig. 3.7C)** and intensity of puncta **(Fig. 3.7D)** per dendritic length (MAP2). Although cLTP did not change significantly the dendritic Pyk2 number of puncta **(Fig 3.7B)**, there was a significant increase in area **(Fig. 3.7C)** and intensity of those puncta **(Fig. 3.7D)**, showing that the protocol used favors the strengthening of pre-existing clusters of Pyk2. TrkB-Fc completely abolished the cLTP-induced increase in Pyk2 dendritic levels (area and intensity), in agreement with the results obtained following addition of exogenous BDNF **(Fig. 3.7C,D)**. Although BDNF did not affect the total number of Pyk2 puncta along dendrites under conditions of cLTP, the number of clusters was significantly decreased in neurons incubated with TrkB-Fc, suggesting a tonic regulation by BDNF **(Fig. 3.7A)**. Altogether these findings suggest that endogenous BDNF released in a context of LTP upregulates Pyk2 protein levels in dendrites of hippocampal neurons.

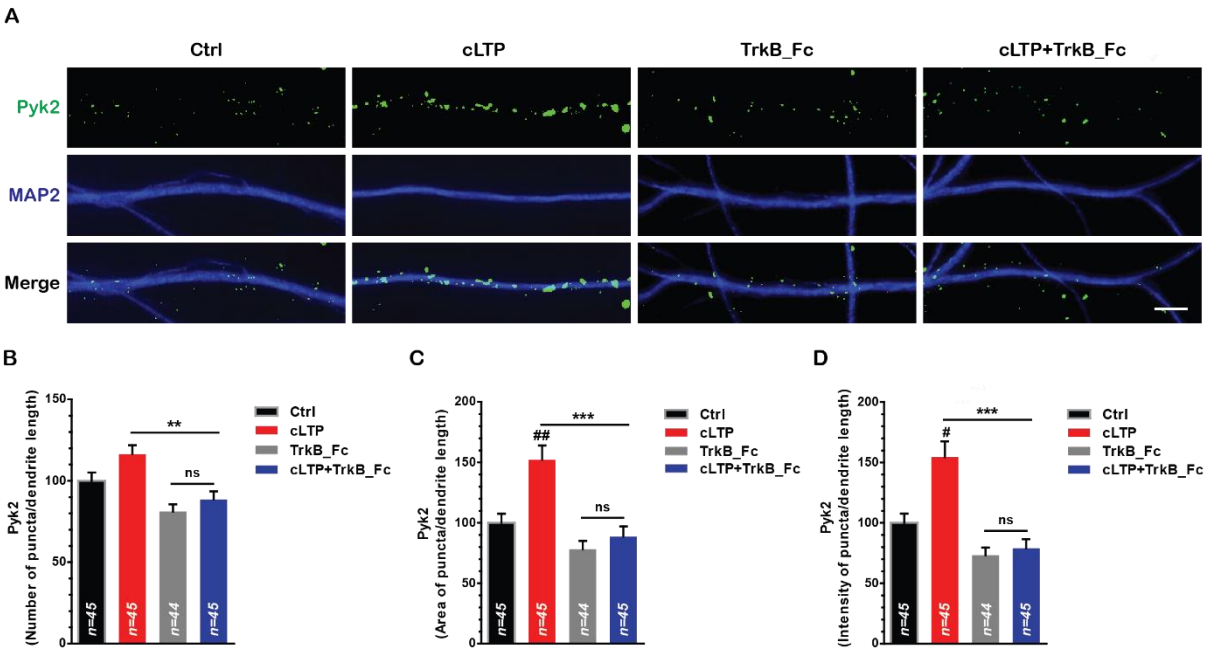


Figure 3.7. cLTP increases dendritic Pyk2 levels in hippocampal neurons by mechanism dependent of BDNF. A. Hippocampal neurons (DIV 21) were subjected or not to a cLTP protocol (300 µM glycine for 3 min, without Mg^{2+}) in presence or absence of TrkB-Fc (1µg/mL), where indicated. Neurons were then incubated at 37º C for 20 min (without glycine) in presence or absence of TrkB-Fc (1ug/mL), and immediately fixed and immunostained for Pyk2 and MAP2. Neurons were analyzed for dendritic Pyk2 number of puncta (**B**), area of puncta (**C**) and intensity of puncta (**D**) per dendritic length. Results are expressed as percentage to control. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{ns}P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between indicated conditions, or $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ comparing to control). *n* represents the number of neurons analyzed from three independent experiments, performed in different preparations. Scale bar = 5 µm.

In additional experiments we investigated whether cLTP specifically modulates Pyk2 protein levels at the synapse. To address this question we used the cLTP paradigm described above, and the hippocampal neurons (DIV 21) were fixed and immunostained for Pyk2, PSD-95 and MAP2. Dendrites were analyzed for the number of synaptic **(**PSD-95 colocalized; arrowheads **Fig 3.8A)** Pyk2 puncta **(Fig. 3.8B)**, area of puncta **(Fig. 3.8C)** and intensity of puncta **(Fig. 3.8D)** per dendritic length (MAP2). In contrast with the results obtained when total dendritic Pyk2 levels were analyzed, cLTP upregulated the number of synaptic Pyk2 puncta **(Fig 3.8A-D)**, and similar effects were observed on the area and intensity of the synaptic Pyk2 puncta. The cLTP-evoked upregulation of Pyk2 was abrogated in the presence of TrkB-Fc (number, area and intensity of puncta), suggesting a role for endogenous BDNF **(Fig. 3.8A-D)**. Moreover, TrkB-Fc decreased synaptic Pyk2 puncta number under resting conditions **(Fig. 3.8D)**, indicating that BDNF may also play a tonic role in the maintenance of synaptic Pyk2 levels.

To determine whether cLTP induces a preferential upregulation of Pyk2 in the synaptic or extrasynaptic compartment, we evaluated the changes in the percentage of synaptic Pyk2 (% of colocalization Pyk2-PSD-95) **(Fig. 3.8E)** as well as in the percentage of synapses containing the kinase (% of colocalization PSD-95-Pyk2) **(Fig. 3.8F)**. The cLTP protocol used upregulated the percentage of synapses containing Pyk2 by a mechanism sensitive to TrkB-Fc **(Fig. 3.8F)**. However, when we analyzed the percentage of synaptic Pyk2, no significant differences between control and cLTP were detected, indicating that the increase in total dendritic expression of the protein follows the same pattern of distribution **(Fig. 3.8E)**. TrkB-Fc treatment significantly reduced the percentage of synaptic Pyk2, indicating that TrkB receptor activation is coupled to the synaptic recruitment of the kinase.

Numerous studies showed that PSD-95 overexpression is sufficient to potentiate synaptic transmission (Schnell *et al.* 2002, Ehrlich and Malinow 2004, Elias *et al.* 2006), suggesting the recruitment of PSD-95 during LTP, which may affect the assessment of the Pyk2 synaptic expression. To determine whether the cLTP protocol used also affects the PSD-95 clustering along dendrites, we analyzed the distribution of the postsynaptic marker in hippocampal neurons subjected to the same protocol used above. The changes in the number **(Fig. S3.5B)**, area **(Fig. S3.5C)** and intensity **(Fig. S3.5D)** of PSD-95 puncta per dendritic length (MAP2) were evaluated. There was a significant increase in number and area of PSD-95 puncta upon cLTP, and these alterations were abrogated in hippocampal neurons incubated with TrkB-Fc. Together, these results indicate that the cLTP protocol used to induce synaptic activity affects PSD-95 and Pyk2 to a similar extent.

Taken together, the results suggest that endogenous BDNF released following synaptic activity increases both the dendritic and synaptic Pyk2 protein levels, in accordance with the effects observed upon exogenous BDNF application. However this paradigm also increased the dendritic PSD-95 levels, perhaps by triggering distinct mechanisms also dependent on BDNF. These findings corroborate the strong association between Pyk2 and PSD-95 already described in the literature (Bongiorno-Borbone *et al.* 2005, Bartos *et al.* 2010, Zhao *et al.* 2015), and show a key role for BDNF in the modulation of Pyk2 and PSD-95 downstream of the NMDAR signaling.

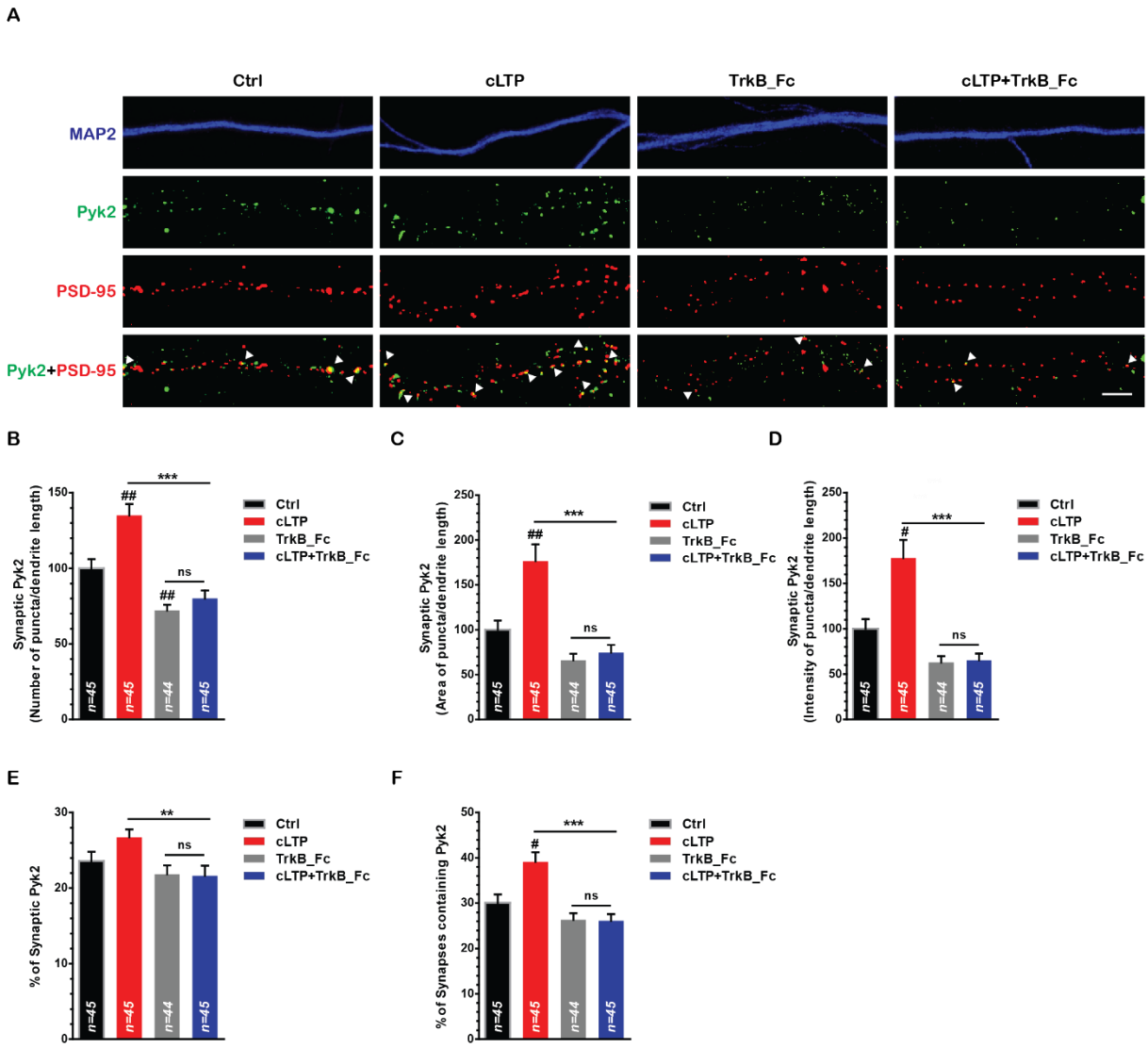


Figure 3.8. cLTP protocol increases synaptic Pyk2 levels and the percentage of synapses containing Pyk2, in a BDNF-dependent manner. A. Hippocampal neurons (DIV 21) were submitted or not to cLTP protocol (300 µM glycine for 3 min without Mg^{2+}) in presence or absence of TrkB-Fc (1µg/mL), where indicated. Then, neurons were kept at 37ºC for 20 min (without glycine) in presence or absence of TrkB-Fc (1ug/mL), and immediately fixated and immunostained for Pyk2, PSD-95 and MAP2. Neurons were analyzed for synaptic (PSD-95-colocalized) Pyk2 number of puncta (**B**), area of puncta (**C**) and intensity of puncta (**D**) per dendritic length. Results are expressed as percentage to control. It was also analyzed the percentage of synaptic Pyk2 (number of Pyk2 puncta-colocalized with PSD-95/ total Pyk2 number of puncta) (**E**) and percentage of synapses containing Pyk2 (number of PSD-95 puncta-colocalized with Pyk2/ total PSD-95 number of puncta) (**F**). Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{ns}P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between indicated conditions or $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ comparing to control). *n* represents the number of neurons analyzed from three independent experiments, performed in different preparations. Arrowheads: Pyk2-PSD-95 colocalized puncta. Scale bar = 5 µm.

Discussion

Herein, we reported the local translation of the tyrosine kinase Pyk2 in hippocampal synapses upon BDNF stimulation. We propose that the BDNF-induced accumulation of Pyk2 in postsynaptic densities involves the RNA-binding protein, hnRNPK, and may contribute to synaptic plasticity events.

In the present study we observed that stimulation of synaptoneurosomes with BDNF upregulated the polysome-associated mRNAs, providing the strongest evidence that BDNF induces local translation. Our results also showed an enrichment in polysome-associated *Pyk2* mRNA, as well as *GluA*1 and *Arc* transcripts; the latter mRNAs are known to be locally translated at the synapse in response to BDNF (Schratt *et al.* 2004, Takei *et al.* 2004). The observed BDNF-induced enrichment in ribosomal RNA 18S associated with polysome fractions validates the model used. In accordance with the results obtained in the analysis of mRNA associated with polysome fractions, incubation of synaptoneurosomes with BDNF upregulated Pyk2 protein levels, showing that the kinase is a novel target for BDNF-induced local translation/regulation. Previous studies showed that BDNF induces dendritic protein synthesis by a mechanism involving TrkB signaling and calpain-2 mediated PTEN degradation, which promotes tuburin/hamartin complex dissociation, increasing the activity of mTOR (Takei *et al.* 2004, Briz *et al.* 2013). Moreover, it was proposed that BDNF-induced local translation is triggered by positive AMPA receptor modulators, which induce BDNF release (Jourdi *et al.* 2009). However, these studies were focused on the BDNF-induced activation/phosphorylation of the translation machinery and mTOR, and did not provide a clear evidence that local translation is increased. The translation of specific proteins at the synapse has been reported in several other studies as follows: (i) BDNF induced the translation of a protein synthesis reporter consisting in GFP flanked by 3´UTR and 5´UTR of CaMKIIα, a known target for local translation (Havik *et al.* 2003, Neant-Fery *et al.* 2012), in intact dendrites of cultured hippocampal neurons (Aakalu *et al.* 2001, Jourdi *et al.* 2009); (ii) live cell imaging studies using a fluorescent TimeSTAMP tag showed that copies of PSD-95 were synthesized in response to local application of BDNF, and preferentially localized to stimulated synapses in rat hippocampal neurons (Butko *et al.* 2012); (iii) studies using synaptoneurosomes showed local translation of Arc, Homer2, GluA1 and CaMKII, in a TrkB-, NMDAR- and/or rapamycin-dependent manner (Yin *et al.* 2002, Schratt *et al.* 2004, Takei *et al.* 2004).

BDNF induces dendritic/synaptic accumulation of proteins with relevant roles in synaptic plasticity such as the GluA1 AMPA receptor subunit (Fortin *et al.* 2012), PSD-95 (Hu *et al.* 2011) and vGlut1 (Melo *et al.* 2013). It was also shown that BDNF-induced GluA1 and vGlut1 accumulation at the postsynaptic membrane and in dendrites, respectively, is dependent on protein synthesis (Fortin *et al.* 2012, Melo *et al.* 2013). As suggested for others proteins, our results indicated that dendritic and in particular synaptic Pyk2 is upregulated after BDNF treatment for 30 - 60 min. This effect was abrogated by pre-treatment of neurons with a protein-synthesis inhibitor, cycloheximide, and similar results were obtained in synaptoneurosomes, showing that BDNF-induced dendritic/synaptic accumulation of Pyk2 is dependent on local protein synthesis in dendrites. However, the effect of BDNF in increasing the number of synaptic Pyk2 puncta was partly insensitive to the cycloheximide, suggesting de novo formation of Pyk2 synaptic clusters through recruitment of an extrasynaptic pool of the kinase.

In agreement with previous reports showing that BDNF induces the transport of PSD-95 to dendrites through PI3K-Akt signaling upon NMDA receptor activation in visual cortical neurons (Yoshii and Constantine-Paton 2007), we also found that BDNF treatment for 60 min (but not for 30 min) increased dendritic levels of PSD-95. In addition, BDNF-mediated increase in dendritic PSD-95 also required protein synthesis. Since PSD-95 is a known target of BDNF-induced local translation (Butko *et al.* 2012), we can hypothesize that the observed increase in dendritic PSD-95 levels after 60 min of BDNF stimulation was mediated by local synthesis of the protein.

A direct interaction between Pyk2 and PSD-95 is crucial to synaptic plasticity, namely for the induction of LTP (Bartos *et al.* 2010). It was proposed that activation of NMDA receptors leads to a rapid Src-mediated phosphorylation of PSD-95 at Y523, opening the intramolecular interaction between the SH3 and GK domains of PSD-95 that facilitates the PSD-95-Pyk2 interaction. Thus, Pyk2 is activated and subsequently promotes the activity of Src, which ultimately upregulates the function of NMDA receptors contributing to synaptic plasticity (Zhao *et al.* 2015). In the present study we observed that synaptic Pyk2 (Pyk2-PSD-95 colocalized puncta) is upregulated by BDNF treatment (30 and 60 min) in hippocampal neurons, strongly suggesting that BDNF may have an important role in the regulation of synaptic plasticity through modulation of NMDAR-PSD-95-Pyk2 signaling.

A total of 2550 transcripts were localized in dendrites/axons from hippocampal slices (Cajigas *et al.* 2012) and those mRNAs can be transported in a silent form along dendrites to allow local protein synthesis (Holt and Schuman 2013). During transport, mRNAs are packaged into RNA granules containing RNA-binding proteins that control not only the transport, but also nuclear export, metabolism and loading of mRNAs onto polysomes (Krichevsky and Kosik 2001, Darnell 2013). RBPs, particularly the hnRNPs family, have been described to accumulate at postsynaptic densities with synaptic activity (Zhang *et al.* 2012). A different study showed that hnRNP A2/B1, an hnRNP family member, was increased in the cell soma and in the dendritic compartment after neuronal activity or BDNF treatment (Leal *et al.* 2014). Another hnRNP family member, hnRNPK, was shown to be involved in the regulation of dendritic spine morphology (Proepper *et al.* 2011, Folci *et al.* 2014) and LTP induction in hippocampal neurons by a mechanism involving the ERK cascade and GluA1 (S845) phosphorylation (Folci *et al.* 2014). Here, we showed that knockdown of hnRNPK abrogates the BDNF-induced increase in dendritic Pyk2. Furthermore, overexpression of GFP-hnRNPK mimicked the effects of BDNF treatment, and application BDNF to neurons overexpressing hnRNPK did not further increase dendritic Pyk2 protein levels. These results may be explained by a saturation of dendrites with GFP-hnRNPK after 72h of construct expression, which may prevent an additional increase following stimulation with the neurotrophin. Taken together, our findings show that the effects of BDNF on the accumulation of Pyk2 in dendrites are mediated by local translation, through a mechanism involving hnRNPK.

BDNF has been implicated in LTP induction as well as in maintenance of late-LTP (Panja and Bramham 2014). The neurotrophin is released upon high-frequency stimulation and is required for both HFS-LTP (Gartner and Staiger 2002) and cLTP (Cho *et al.* 2013). It was proposed that the Ca^{2+}/calmodulin complex formed upon Ca^{2+} influx through NMDA receptors induces Pyk2 binding to PSD-95 and Pyk2 clustering at synapses (Bartos *et al.* 2010). In the present work we compared the effect of endogenous BDNF (released under conditions that induce NMDAR-mediated cLTP) on the

dendritic distribution of Pyk2 with the effect of exogenous addition of the neurotrophin. Notably, we observed that both experimental conditions induced a similar increase in dendritic and synaptic Pyk2 levels. Perhaps the only difference between the exogenous addition of BDNF and cLTP in what concerns the dendritic Pyk2 distribution, was the effect on the percentage of synaptic protein which was significantly upregulated only under the former experimental condition. This differential may be due to the activation of extrasynaptic TrkB receptors under the former experimental conditions. Therefore, we conclude that, in a context of high synaptic activity, there is an accumulation of Pyk2 in postsynaptic densities mediated by BDNF-TrkB signaling. Collectively, our results point to a mechanism in which endogenous BDNF, released by synaptic activity, triggers local translation of Pyk2, which ultimately increases the accumulation of this protein in postsynaptic densities, contributing/regulating synaptic plasticity. The cLTP protocol also affected PSD-95 protein levels at the synapse by a mechanism sensitive to the TrkB-Fc ligand. This alteration at the synapse may have contributed to the observed upregulation of synaptic Pyk2 levels under the same conditions.

Herein we identified a novel protein, Pyk2, which is dendritically synthesized and accumulates at the synapse in response to exogenous and endogenous BDNF by a mechanism involving hnRNPK. The results suggest that BDNF released during LTP induces the dissociation of *Pyk2* mRNA from hnRNPK, allowing its local translation and accumulation of the newly synthesized protein in the postsynaptic compartment. The resulting increase in the association of Pyk2 with PSD-95 may regulate NMDA receptor function and synaptic plasticity events. Additional studies are required to understand the physiological relevance of Pyk2 accumulation at the postsynaptic level during LTP.

Supplementary Figures

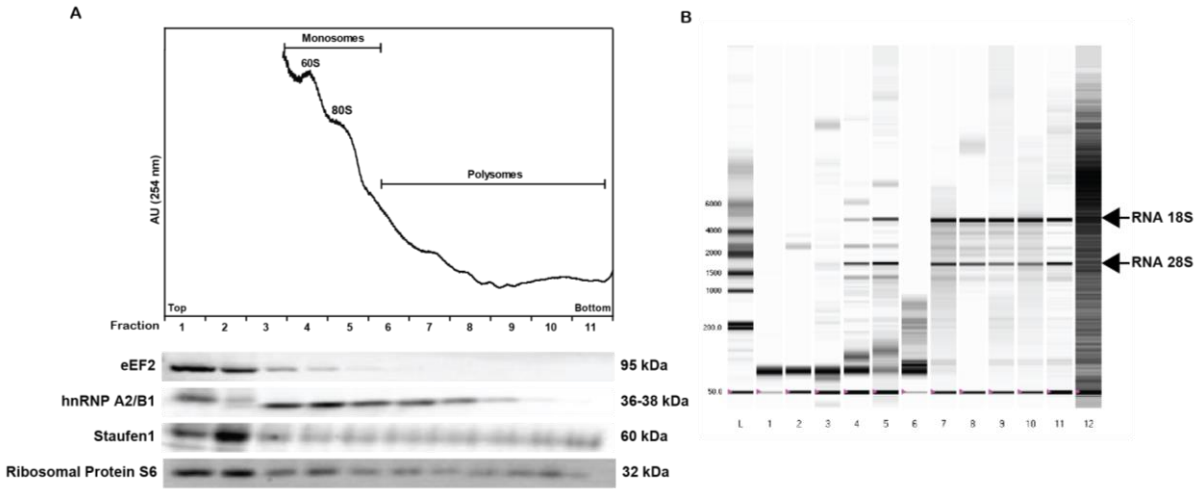


Figure S3.1. Distribution of different proteins involved in protein synthesis, mRNA metabolism, and ribosomal mRNAs, among monosomal, polysomal and non-polysomal fractions at the synapse. **A**. Co-sedimentation of synaptoneurosomal proteins using a 10–50% linear sucrose gradient. The polyribosomes, monosomes and mRNPs (non-polysomal fractions) were detected by UV absorbance at 254 nm and the gradient was collected into 11 fractions. Equal volumes from each fraction were analyzed by SDS-PAGE and Western blot using antibodies that recognize hnRNPA2/B1, Staufen1, eEF2 and the ribosomal protein S6. **B**. Ribosomal RNA 18 and 28S is enriched in polysomal fractions. After separation of synaptoneurosome lysates into 11 fractions of increased density, the associated mRNAs were isolated and the enrichment in ribosomal RNAs 18 and 28S was assessed using Experion™ Automated Electrophoresis System. In both cases (**A** and **B**) the results are representative of two different experiments performed in independent synaptoneurosomal preparations.

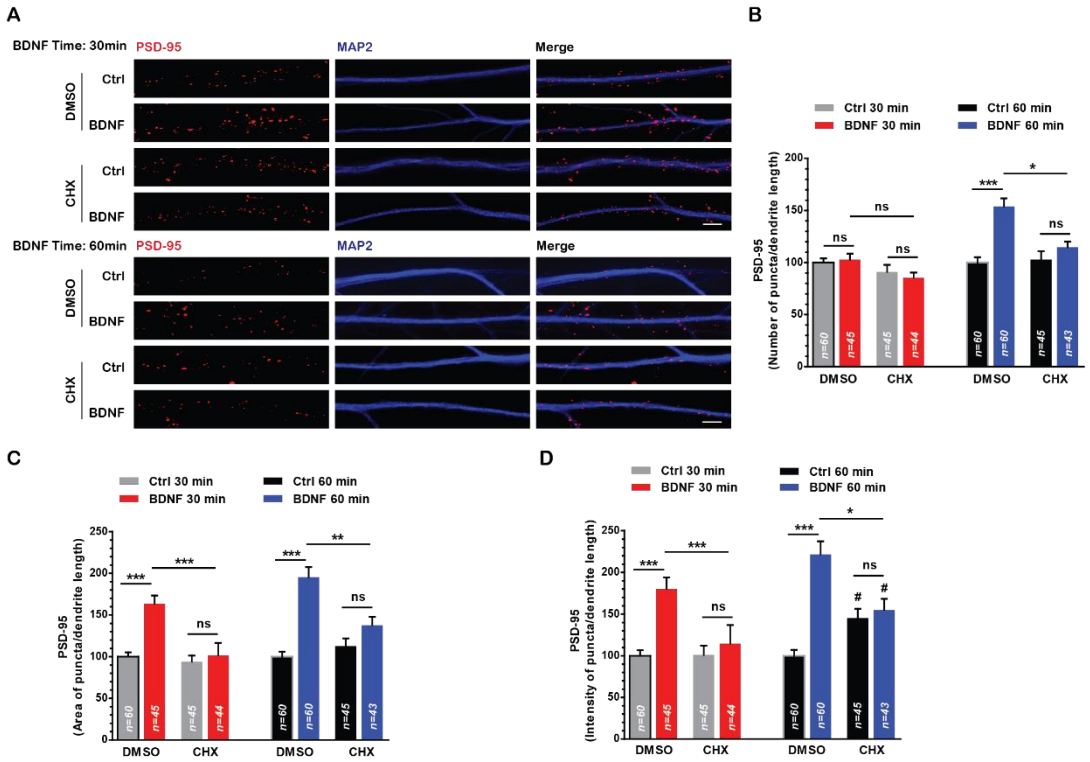


Figure S3.2. Stimulation of hippocampal neurons with BDNF for 60 min increases PSD-95 levels in dendrites, in a protein synthesis-dependent manner. A. Hippocampal neurons (DIV 14-15) were pre-incubated with CHX (50 µg/mL) or vehicle DMSO (1:1000 dilution) for 45 min, and then stimulated or not with BDNF (50 ng/mL) during 30 or 60 min, where indicated. After BDNF treatment, neurons were immediately fixed and immunostained for PSD-95 and MAP2. Neurons were analyzed for dendritic PSD-95 number of puncta (**B**), area of puncta (**C**) and intensity of puncta (**D**) per dendritic length. Results are expressed as percentage to respective DMSO control (30 and 60 min). Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between indicated conditions, or $^{\#}P < 0.05$ comparing to respective control). *n* represents the number of neurons analyzed from at least 3 independent experiments. *CHX- Cycloheximide, DMSO- Dimethyl Sulfoxide (vehicle).* Scale bar = 5 µm.

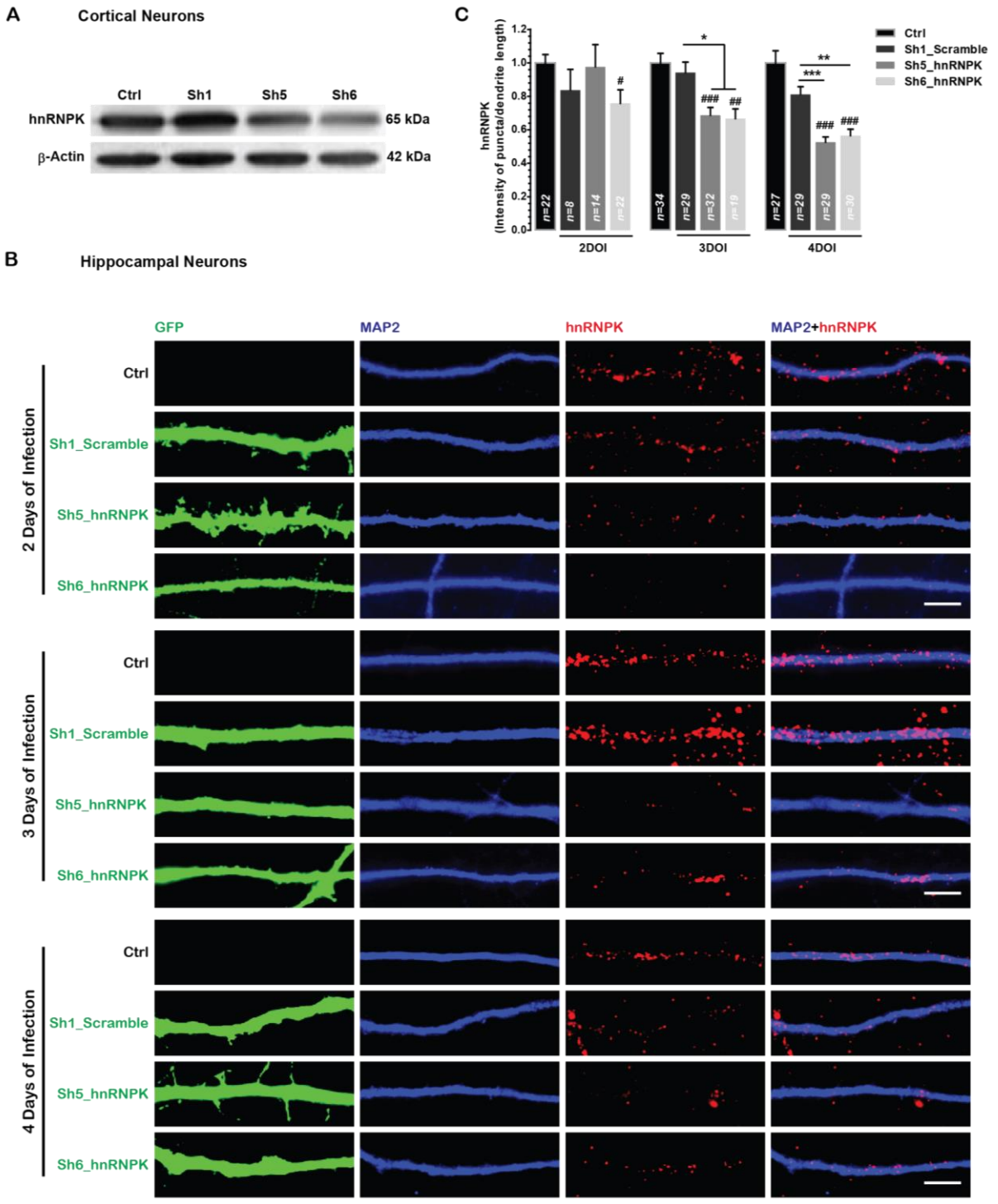


Figure S3.3. Evaluation of hnRNPK knockdown efficiency by two sh-RNA (sh5-hnRNPK and sh6-hnRNPK) in neurons. A. High-density cultures of cortical neurons were infected at DIV 10 (MOI=5) with Sh1_Srcamble, sh5-hnRNPK or sh6-hnRNPK. Protein extracts were prepared at DIV 14, and the levels of hnRNPK protein were assessed by Western Blot. **B-C.** Low-density cultured hippocampal neurons were infected from DIV 10 to DIV 12 (MOI=5) with sh1-Scramble, sh5-hnRNPK and sh6-hnRNPK. Cells were fixed at DIV 14 before immunostaining for hnRNPK, GFP and MAP2. The hnRNPK signal intensity was measured in dendrites and represented per dendrite length. Results are expressed as fold-change to control. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, between indicated conditions, or $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ comparing to control). *n* represents the number of neurons analyzed. Each condition represents two experiments performed in independent preparations, with the exception of the experiments performed in neurons expressing the sh-RNA constructs for 2 days, which represent only one experiment. *DOI- Days of infection*. Scale bar = 5 µm.

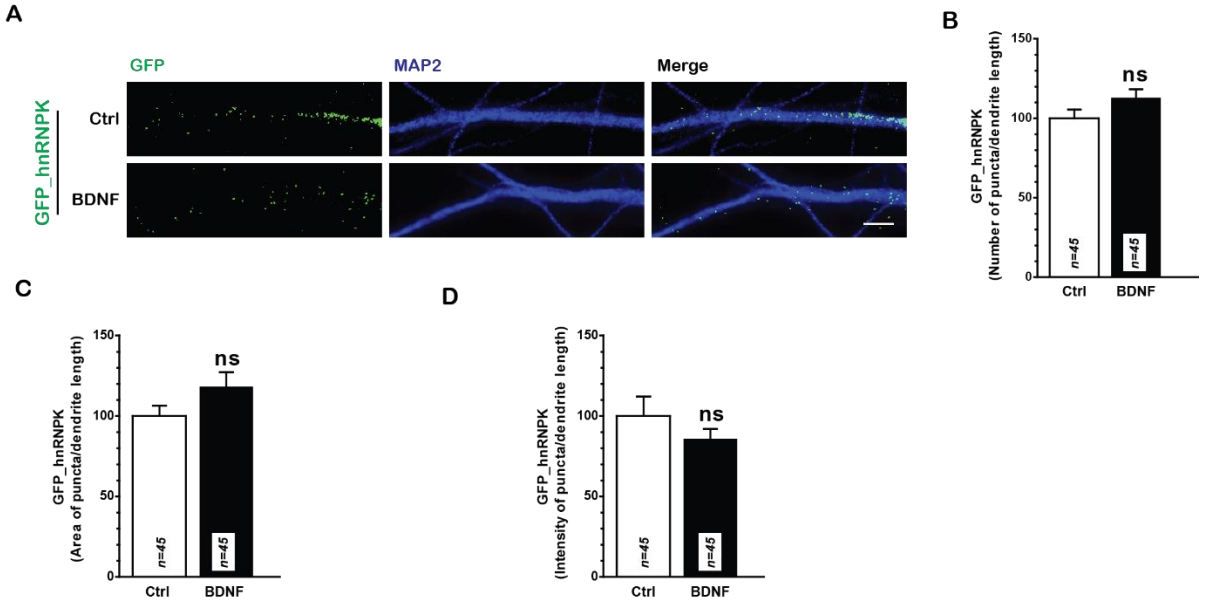


Figure S3.4. BDNF treatment does not increase GFP_hnRNPK dendritic expression in cultured hippocampal neurons. A. Hippocampal neurons were transfected at DIV 11-12 with GFP-hnRNPK. At DIV 14-15 neurons were stimulated or not with BDNF (50 ng/mL) during 30 min, where indicated. After BDNF treatment, neurons were immediately fixed and immunostained to GFP and MAP2. Neurons were analyzed for dendritic GFP-hnRNPK number of puncta (**B**), area of puncta (**C**) and intensity of puncta (**D**) per dendritic length. Results are expressed as percentage to control. Error bars represent s.e.m. The statistical significance was calculated using the Mann-Whitney test ($^{ns}P > 0.05$). *n* represents the number of neurons analyzed from three independent experiments. Scale bar = 5 µm.

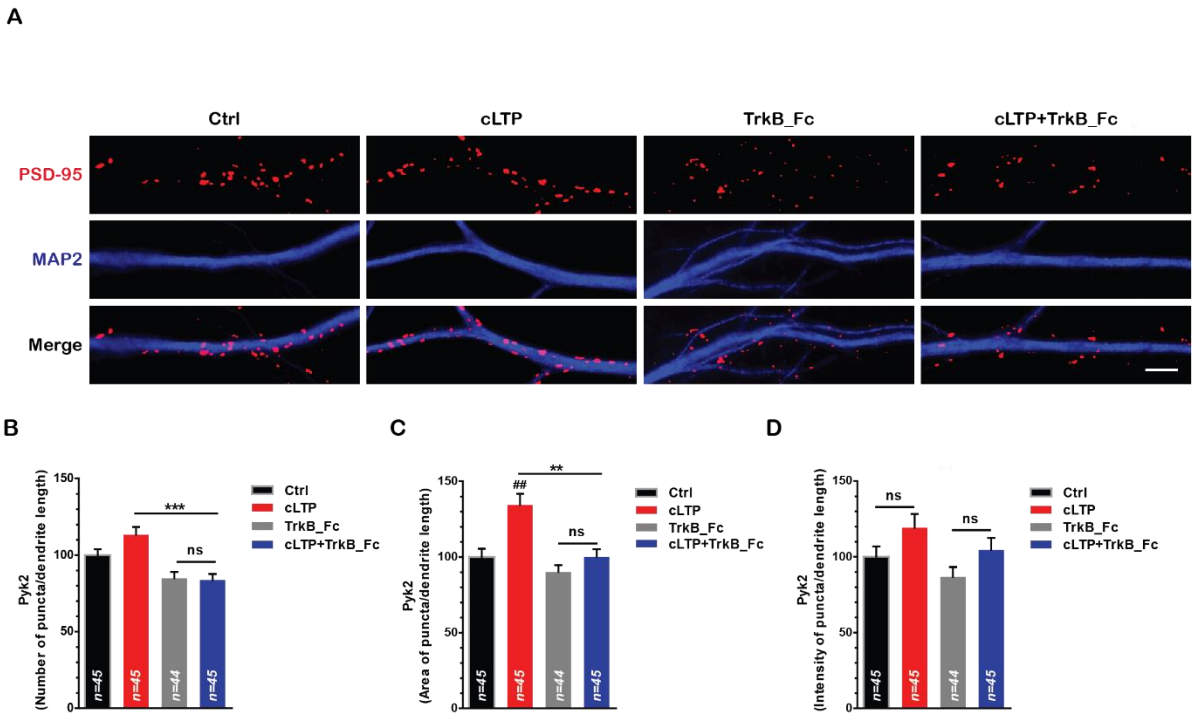


Figure S3.5. cLTP increases dendritic PSD-95 levels in a BDNF-dependent manner. A. Hippocampal neurons (DIV 14-15) were submitted to a cLTP protocol (300 µM glycine for 3 min, without Mg^{2+}) in presence or absence of TrkB-Fc (1µg/mL), where indicated. Then, neurons were kept at 37ºC for 20 min (without glycine) in presence or absence of TrkB-Fc (1µg/mL), and immediately fixed and immunostained to PSD-95 and MAP2. Neurons were analyzed for dendritic PSD-95 number of puncta (**B**), area of puncta (**C**) and intensity of puncta (**D**) per dendritic length. Results are expressed as percentage to control. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{ns}P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between indicated conditions, or $^{\#\#}P < 0.01$ comparing to control). *n* represents the number of neurons analyzed from three independent experiments. Scale bar = 5 µm.

Chapter 4

BDNF increases the surface expression of GluN2B-containing NMDA receptors by a mechanism involving Pyk2

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In preparation

Summary

NMDAR activity plays a key role in learning and memory, and overexpression of the NMDAR subunit GluN2B has been shown to improve synaptic plasticity and memory formation. However, the mechanisms contributing to the regulation of GluN2B surface expression are not fully understood. It has been shown that the neurotrophin BDNF induces the phosphorylation of GluN2B at Y1472, which may regulate its membrane trafficking. Here, we report that BDNF increases the surface expression of GluN2B-containing NMDA receptors by a mechanism involving protein synthesis. Moreover, we found that knockdown of Pyk2, a kinase located in postsynaptic densities which regulates NMDAR activity, completely abolishes the BDNF-induced increase in surface GluN2B expression. The maintenance of these NMDA receptors on the surface of hippocampal neurons under resting conditions also requires Pyk2. Additionally, we observed that BDNF induces a rapid and transient increase in synaptic activation of Pyk2, which is required for the increase in surface GluN2B expression induced by the neurotrophin, as well as for the maintenance of the surface GluN2B under resting conditions. Overexpression of wild-type Pyk2 was sufficient to mimic the effects of BDNF on the surface expression of GluN2B-NMDAR. Taken together, our results suggest a mechanism by which BDNF induces synaptic activation/accumulation of Pyk2 which regulates the surface levels of GluN2B-containing NMDA receptors. The BDNF-induced surface expression of NMDA receptors may partly account for the effect of the neurotrophin in LTP. This work may contribute to the development of a novel therapeutic target to restore the learning and memory deficits characteristic of several brain disorders.

Introduction

LTP and LTD are two forms of synaptic plasticity which depend on the activation of NMDAR (Malenka and Bear 2004). Different levels and/or kinetics of NMDAR-mediated calcium influx, which ultimately trigger distinct signaling pathways, are thought to selectively induce each of the two forms of synaptic plasticity (Malenka and Bear 2004). LTP is induced by an increase of the postsynaptic $[Ca^{2+}]_i$ which activates the calcium/calmodulin-dependent kinase II and induces AMPAR insertion into the postsynaptic membrane (Malenka and Bear 2004, Lisman *et al.* 2012, Lisman and Raghavachari 2015). Induction of LTD requires a lower $[Ca^{2+}]_i$ increase, which is coupled to the internalization of AMPAR by a mechanism involving the activation of phosphatases, such as calcineurin (Malenka and Bear 2004, Xia and Storm 2005, Collingridge *et al.* 2010).

Functional NMDAR are heterotetrameric complexes formed by two glycine-binding GluN1 subunits assembled with two subunits belonging to the glutamate-binding GluN2 (A, B, C or D subtypes) or glycine-binding GluN3 (A or B subtypes) class (Dingledine *et al.* 1999, Cull-Candy and Leszkiewicz 2004). The biological properties of NMDAR are deeply linked to their composition and an upregulation of GluN2B expression is associated with an improvement of synaptic plasticity and memory formation in mice (Crair and Malenka 1995, Tang *et al.* 1999). Several different animal models were developed with upregulated GluN2B expression levels, using different experimental strategies, such as altered GluN2B synthesis, transport, or degradation, and have been reported to exhibit improved synaptic plasticity and memory (Lee and Silva 2009). The control of the molecular machinery that regulates the trafficking, degradation and subcellular distribution of NMDAR is not fully understood, but it has been proposed that phosphorylation of NMDAR subunits, including GluN2B, is crucial for the regulation of such processes (Ma and Jan 2002, Wenthold *et al.* 2003, Chen and Roche 2007).

Neurotrophins are considered key intermediates in synaptic plasticity events. In particular, BDNF/TrkB signaling has been proposed to be essential and sufficient for LTP induction and maintenance, both *in vitro* (Kang and Schuman 1996) and *in vivo* (Messaoudi *et al.* 1998, Panja and Bramham 2014, Panja *et al.* 2014), by a mechanism involving protein synthesis (Leal *et al.* 2015). TrkB receptors for BDNF are coupled to the activation of the Ras/ERK, PLC-γ and PI3-k/Akt pathways (Reichardt 2006, Carvalho *et al.* 2008, Minichiello 2009, Yoshii and Constantine-Paton 2010). BDNF has also been reported to be important in NMDAR-mediated synaptic plasticity. In fact, it was observed that NMDAR-mediated Ca^{2+} influx is important for BDNF release (Cho *et al.* 2013, Park *et al.* 2014) and BDNF/TrkB signaling regulates the phosphorylation/function of NMDA receptor subunits (Levine and Kolb 2000, Xu *et al.* 2015). Thus, BDNF induces the phosphorylation of GluN2B subunit of NMDA receptors at Y1472 by a mechanism involving BDNF/TrkB/Akt signaling and girdin, with implications in synaptic plasticity and memory (Xu *et al.* 2006, Nakai *et al.* 2014). Moreover, whole-cell recordings performed in hippocampal slices showed enhanced NMDAR-mediated currents after fast perfusion with BDNF (Xu *et al.* 2006, Ji *et al.* 2010) and acute BDNF treatment increased surface expression of GluN2B-NMDAR in cultured hippocampal neurons (Caldeira *et al.* 2007). However, the molecular mechanisms involved in the regulation of NMDAR by BDNF have not yet been elucidated.

In the work described in chapter 3 we proposed that BDNF induces dendritic synthesis of Pyk2 by a mechanism involving hnRNPK, thereby promoting Pyk2 accumulation at the synapse. Pyk2 is a kinase widely expressed in the central nervous system, which belongs to the FAK family (Lipinski and Loftus 2010). This kinase has been involved in both LTD and LTP (Huang *et al.* 2001, Hsin *et al.* 2010) by a mechanism requiring the regulation of NMDAR function through activation/binding to PSD-95 (Bartos *et al.* 2010, Zhao *et al.* 2015). Studies performed in an animal model (*STEP* KO mice) with increased levels of Pyk2 and phosphorylated GluN2B showed an enhanced performance in hippocampal-dependent learning and in memory tasks (Venkitaramani *et al.* 2011). Recently, two studies proposed a mechanism by which BDNF/TrkB signaling induces a proteasome-mediated degradation of the phosphatase $STEP_{61,}$ with concomitant increase in Pyk2, ERK1/2 and GluN2B phosphorylation, which contribute to synaptic plasticity (Saavedra *et al.* 2015, Xu *et al.* 2015). However, the molecular mechanism by which BDNF regulates Pyk2 and NMDAR function remains to be elucidated. The objective of this work was to uncover the molecular mechanism underlying the potentiation of NMDAR by BDNF. We propose that BDNF increases surface expression of GluN2B-containing NMDA receptors by a mechanism that requires Pyk2.

Results

BDNF stimulation increases the synaptic surface expression of GluN2B-containing NMDA receptors by a mechanism dependent on protein synthesis

Recent evidence suggests that BDNF/TrkB/Akt signaling is coupled to the phosphorylation of the GluN2B subunit of NMDA receptors at Y1472 in hippocampal and cerebrocortical neurons (Xu *et al.* 2006, Nakai *et al.* 2014, Xu *et al.* 2015). It was also proposed that this phosphorylation can mediate the upregulation of GluN2B receptors in synaptic fractions (Venkitaramani *et al.* 2011). Moreover, biotinylation of plasma membrane proteins showed that BDNF increases the surface expression of GluN2B receptors in cultured hippocampal neurons (DIV7) (Caldeira *et al.* 2007). Herein, we investigated whether the effect of BDNF on the surface expression of GluN2B subunits along dendrites could be mediated by protein synthesis. Indeed, the mRNA for GluN2B is present in dendrites (Cajigas *et al.* 2012), suggesting that the protein may be locally translated, and a similar mechanism was shown to mediate the effect of BDNF on the surface expression of the GluA1-AMPA receptor subunit (Fortin *et al.* 2012).

The surface expression of GluN2B NMDAR subunits in hippocampal neurons (DIV14-15) was investigated by live staining with an antibody against an extracellular epitope in the N-terminal region of the protein **(Fig. 4.1A)**. After fixation and permeabilization of the plasma membrane, neurons were labeled for MAP2, and the preparations were analyzed for the surface GluN2B number of puncta **(Fig. 4.1B)**, area of puncta **(Fig. 4.1C)** and intensity of puncta **(Fig. 4.1D)**, per dendritic length (MAP2). Stimulation with BDNF (50 ng/mL) during 30 min increased the surface expression of GluN2B-containing NMDA receptors, and this effect was abrogated when the cells were incubated with cycloheximide (50 µg/mL). These results indicate that the BDNF-induced increase in GluN2B surface expression in the dendritic compartment requires protein synthesis **(Fig. 4.1A-D)**. However, it is not clear whether the effects of BDNF depend on the synthesis of the receptor subunit itself and/or of an intermediary protein required for the translocation of GluN2B. The former hypothesis is suggested by evidence showing that BDNF increases total protein levels of GluN2B subunits in cultured hippocampal neurons by a mechanism sensitive to transcription and translation inhibitors (Caldeira *et al.* 2007).

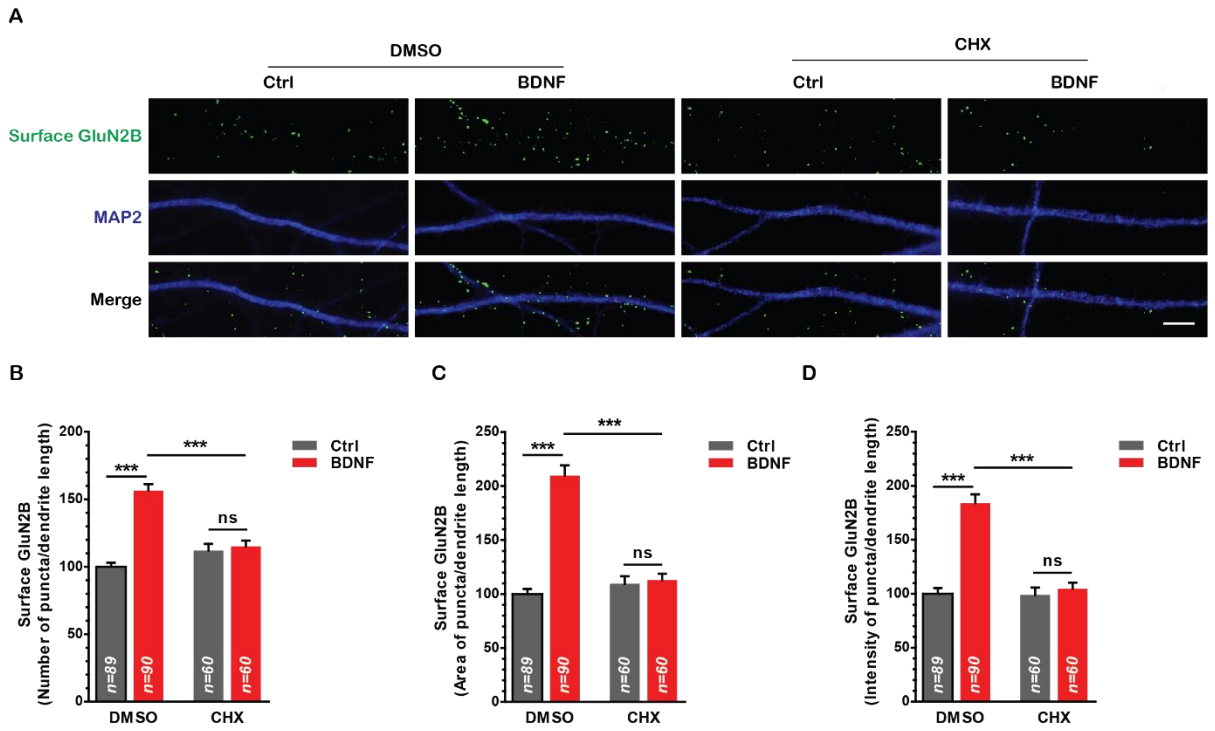

Figure 4.1. BDNF stimulation increases total surface expression of GluN2B-containing NMDA receptors in a protein synthesis-dependent manner. A. Hippocampal neurons (DIV 14-15) were pre-incubated with CHX (50 µg/mL) or vehicle DMSO (1:1000 dilution) for 45 min, and then stimulated or not with BDNF (50 ng/mL) during 30 min, where indicated. Following BDNF treatment, neurons were live immunostained for GluN2B, using an antibody against an extracellular epitope in the GluN2B N-terminus. After fixation, neurons were stained for MAP2. Neurons were analyzed for surface GluN2B number of puncta (**B**), area of puncta (**C**) and intensity of puncta (**D**) per dendritic length. Results are expressed as percentage to DMSO control. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{ns}P > 0.05$ $^{***}P < 0.001$). *n* represents the number of neurons analyzed from at least four independent experiments, performed in independent preparations. *CHX- Cycloheximide, DMSO-Dimethyl Sulfoxide (vehicle).* Scale bar = 5 µm.

The results of Fig. 4.1 show that acute BDNF treatment increases surface expression of GluN2B-containing NMDA receptors, in agreement with the results obtained using surface biotinylation (Caldeira *et al.* 2007). We then investigated whether BDNF treatment also increases surface expression of GluN2B subunit at synapses in mature neurons, and whether this effect would require protein synthesis. To address these questions we used the same stimulation paradigm used in thee experiments of Fig. 4.1. After stimulation, neurons were live immunostained for GluN2B, using an antibody against extracellular epitope in the GluN2B N-terminus and then fixed and labeled for PSD-95, vGlut1 and MAP2 **(Fig. 4.2A)**. We first evaluated the density of excitatory synapses after 30 min of stimulation with BDNF, in presence or absence of a protein synthesis inhibitor, since previous evidence suggested that BDNF, *per se*, is capable of increasing both axonal vGlut1 puncta (Melo *et al.* 2013) and dendritic PSD-95 puncta (Yoshii and Constantine-Paton 2007). The density of excitatory synapses was assessed by quantifying the number of clusters showing colocalization of PSD-95-vGlut1 **(Fig. 4.2E)** per dendritic length. Acute stimulation with BDNF increased the density of excitatory synapses in a protein synthesis-dependent manner **(Fig. 4.2E)**. Thus, our results confirm previous reports, indicating that BDNF is a key player in the regulation of excitatory glutamatergic synapses by a mechanism dependent on protein synthesis.

The effect of BDNF on GluN2B synaptic surface levels (arrowheads, **Fig. 4.2A**) was then assessed by quantifying the surface GluN2B number of puncta **(Fig. 4.2B)**, area of puncta **(Fig. 4.2C)** and intensity of puncta **(Fig. 4.2D)** that colocalized with PSD-95 and vGlut1. Since we previously observed an increase in the density of excitatory synapses following BDNF treatment, the results were normalized to the density of synapses (PSD-95-vGlut1-colocalized). BDNF increased the area and intensity of surface synaptic GluN2B puncta, and this effect was blocked by the protein synthesis inhibitor cycloheximide **(Fig. 4.2C,D)**. Furthermore, BDNF upregulated the number of synaptic surface GluN2B puncta by a mechanism that is partly independent on protein synthesis, possibly involving the activation of specific kinases and the recruitment of receptors from extrasynaptic regions **(Fig. 4.2B)**.

Together, our findings indicate that BDNF treatment requires protein synthesis to induce a synaptic enrichment of surface GluN2B-containing NMDA receptors.

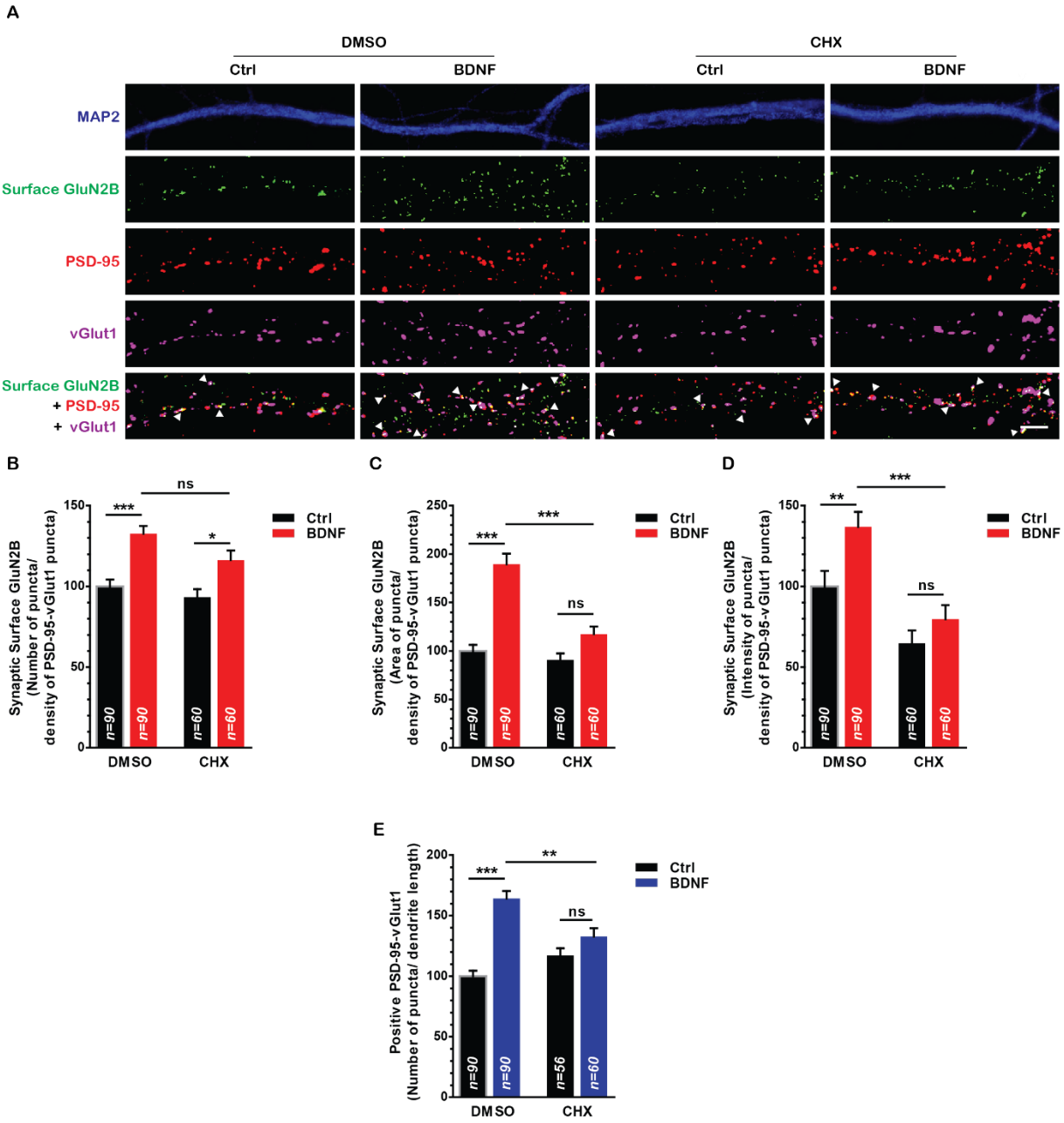

Figure 4.2. BDNF treatment increases the expression of synaptic surface GluN2B-containing NMDA receptors in a protein synthesis-dependent manner. A. Hippocampal neurons (DIV 14-15) were pre-incubated with CHX (50 µg/mL) or vehicle DMSO (1:1000 dilution) for 45 min, and were then stimulated or not with BDNF (50 ng/mL) during 30 min, where indicated. Following BDNF treatment, neurons were live immunostained for GluN2B, using an antibody against an extracellular epitope in the GluN2B N-terminus. After fixation neurons were immunostained for PSD-95, vGlut1 and MAP2. Neurons were analyzed for synaptic (PSD-95-vGlut1-colocalized) surface GluN2B number of puncta (**B**), area of puncta (**C**) and intensity of puncta (**D**) per density of excitatory synapses (number of puncta PSD-95-vGlut1-colocalized/per dendrite length). The number of excitatory synapses (number of puncta PSD-95-vGlut1 colocalized) per dendritic length was also analyzed (**E**). Results are expressed as percentage to DMSO control. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). *n* represents the number of neurons analyzed from at least four independent experiments, performed in independent preparations. *CHX- Cycloheximide, DMSO- Dimethyl Sulfoxide (vehicle).* Arrowheads: surface GluN2B-PSD-95-vGlut1 colocalized puncta. Scale bar = 5 µm.

Basal and BDNF-induced upregulation of surface GluN2B-containing NMDA receptors requires Pyk2

Previous studies proposed that Pyk2 can regulate the activity of group 2 subunits of NMDA receptors, namely GluN2A and GluN2B, by interaction with PSD-95 and activation of Src and Fyn (Bartos *et al.* 2010, Saavedra *et al.* 2015, Xu *et al.* 2015, Zhao *et al.* 2015). Herein, we investigated the role of Pyk2 in BDNF-induced surface expression of GluN2B-containing NMDA receptors using two shRNAs to knockdown Pyk2 *in vitro* (shA2-Pyk2 and shA4-Pyk2). To evaluate the efficiency of Pyk2 knockdown, we transfected rat C6 glioma cells and hippocampal neurons (DIV12) with shA2-Pyk2, shA4-Pyk2 or with a scramble shRNA (sh1-scramble) which lacks homology to any known mammalian mRNA. Knockdown efficiency was evaluated by immunoblot after 24 and 72 hours of transfection for the C6 cell line **(Fig. S4.1A,B),** and by immunocytochemistry for hippocampal neurons at 72 hours after transfection **(Fig. S4.1C-F)**. Transfection of the shA2 and shA4 constructs resulted in a robust knockdown of Pyk2 protein after 72 hours of expression in the C6 cell line and hippocampal neurons **(Fig. S4.1A-F)**.

To determine the role of Pyk2 on BDNF-induced upregulation of GluN2B surface expression, hippocampal neurons were transfected with sh1-Scramble, shA2-Pyk2 or shA4-Pyk2, and 72 hours later (DIV 15) were stimulated with the neurotrophin (50 ng/mL) for 30 min. Immediately after stimulation, neurons were live immunostained for GluN2B, using the antibody described in the previous section, and after fixation and permeabilization the cells were labeled for GFP (transfection marker) and MAP2. The results were analyzed for surface GluN2B number of puncta **(Fig. 4.3B)**, puncta area **(Fig. 4.3C)** and puncta intensity **(Fig. 4.3D)** per dendritic length (MAP2). Knockdown of Pyk2 completely eliminated the effects of BDNF on the surface expression of GluN2B subunit, including the alteration in the surface GluN2B number of puncta, puncta area and puncta intensity. Furthermore, in the absence of Pyk2 we observed a decrease on basal levels of surface GluN2B when compared with sh1 transfected control cells **(Fig. 4.3A-D)**. These results indicate that similarly to the BDNF-induced increase in surface GluN2B expression, the maintenance of GluN2B-NMDAR on the membrane under resting conditions requires the Pyk2 protein. Altogether our findings show a key role for Pyk2 in the regulation of the surface expression of GluN2B-containing NMDA receptors, both under resting conditions and following stimulation with BDNF.

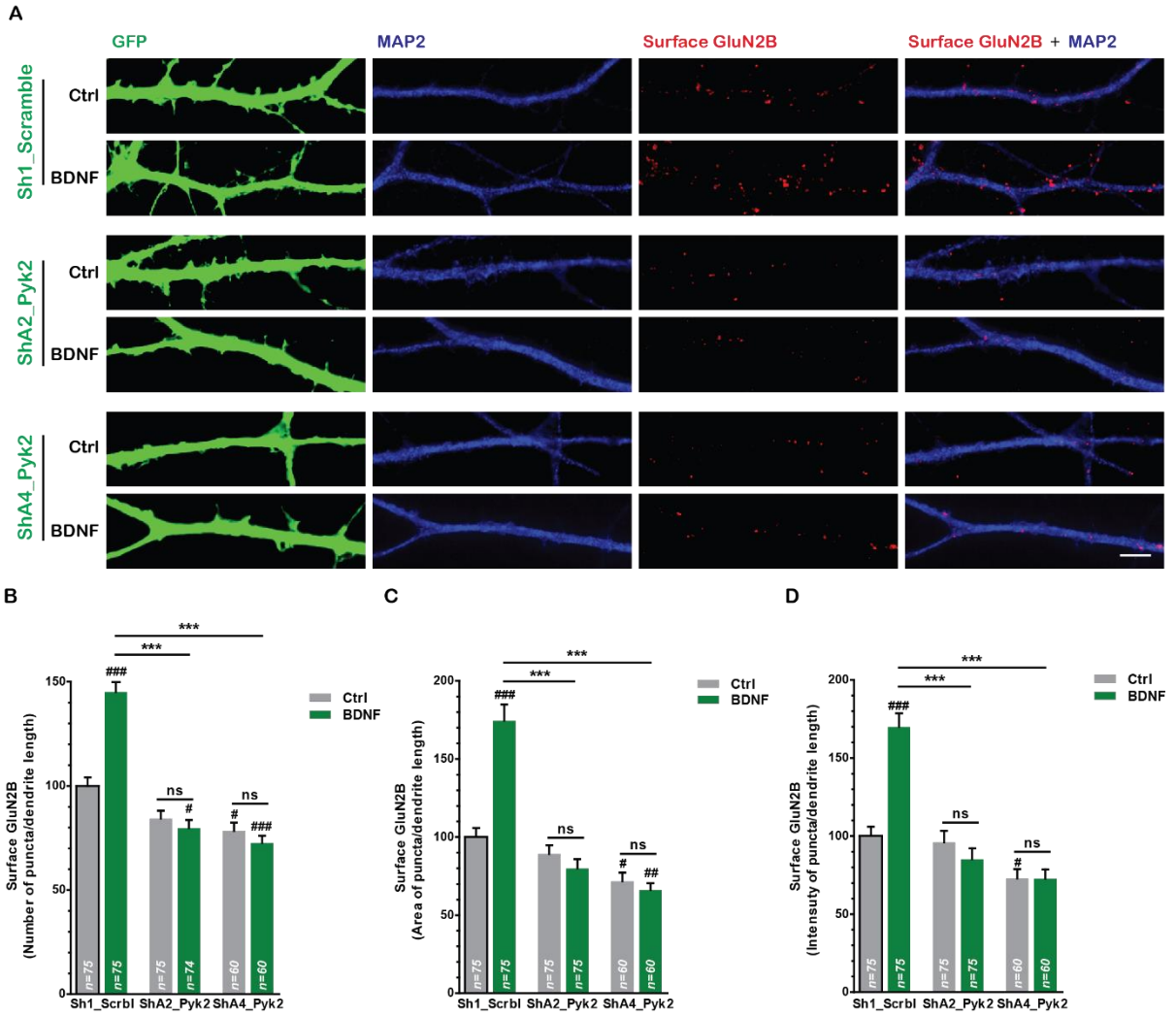

Figure 4.3. Basal and BDNF-induced increase in total surface expression of GluN2B-containing NMDA receptors require Pyk2. A. Hippocampal neurons were transfected with sh1-Scramble, shA2-Pyk2 or shA4-Pyk2 at DIV 12, and were then stimulated or not (DIV 15) with BDNF (50 ng/mL) during 30 min, where indicated. Following BDNF treatment, neurons were live immunostained for GluN2B, using an antibody against an extracellular epitope located in the GluN2B N-terminus. After fixation and permeabilization neurons were immunostained for GFP and MAP2. Neurons were analyzed for total surface GluN2B number (**B**), area (**C**) and intensity (**D**) of puncta per dendritic length, and are expressed as percentage to sh1-Scramble control. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{ns}P > 0.05$, $^{***}P < 0.001$ between indicated conditions or $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ comparing to sh1-Scramble control). *n* represents the number of neurons analyzed from at least four independent experiments, performed in independent preparations. Scale bar = 5 µm.

In additional experiments we investigated whether Pyk2 also plays a role in BDNF-induced upregulation of GluN2B surface expression in synaptic compartments. The shRNAs described above were used to downregulate Pyk2 expression before stimulation with BDNF (50 ng/mL) for 30 min, and surface GluN2B was analyzed by live immunostaining. After fixation and permeabilization, neurons were labeled for GFP (transfection marker), vGlut1 and MAP2. The levels of synaptic surface GluN2B per dendritic length (MAP2) were quantified as the number **(Fig. 4.4B)**, area **(Fig. 4.4C)**, and intensity **(Fig. 4.4E)** of puncta that colocalized with VGlut1 **(**arrowheads in **Fig. 4.4A)**. BDNF induced a significant increase in synaptic surface GluN2B-containig NMDA receptors in neurons transfected with sh1-Scramble construct **(Fig. 4.4A-D)**. Knockdown of Pyk2 completely abolished the effects of the

neurotrophin **(Fig 4.4A-D)**, suggesting that Pyk2 is also responsible for the upregulation of post-synaptic GluN2B-NMDAR after acute treatment with BDNF.

To determine whether BDNF affects preferentially the synaptic pool of surface GluN2 receptor subunits, we analyzed the effect of the neurotrophin on the (i) percentage of synaptic surface GluN2B (number of surface GluN2B puncta-colocalized with vGlut1) **(Fig. 4.4D)** and (ii) the percentage of synapses containing surface GluN2B (number of vGlut1 puncta co-localized with surface GluN2B) **(Fig. 4.4E)**. Knockdown of Pyk2 allowed investigating whether these effects required the activity of this protein kinase. The percentage of synaptic surface GluN2B was not changed significantly in hippocampal neuron stimulated with BDNF, under control conditions and in neurons expressing reduced levels of Pyk2 **(Fig. 4.4D)**. In contrast, the percentage of synapses containing surface GluN2B was significantly upregulated in hippocampal neurons stimulated with BDNF by a mechanism dependent on Pyk2. Accordingly, knockdown of Pyk2 abrogated the BDNF-induced upregulation in the number of synapses containing GluN2B **(Fig. 4.4E)**. Taken together, these results indicate that BDNF-induced increase on surface GluN2B at the synapse accompanies the overall increase in the surface expression of this receptor subunit evoked by BDNF. These findings may be related with the protocol used since bath application of BDNF activates the synaptic and extrasynaptic pools of TrkB receptors.

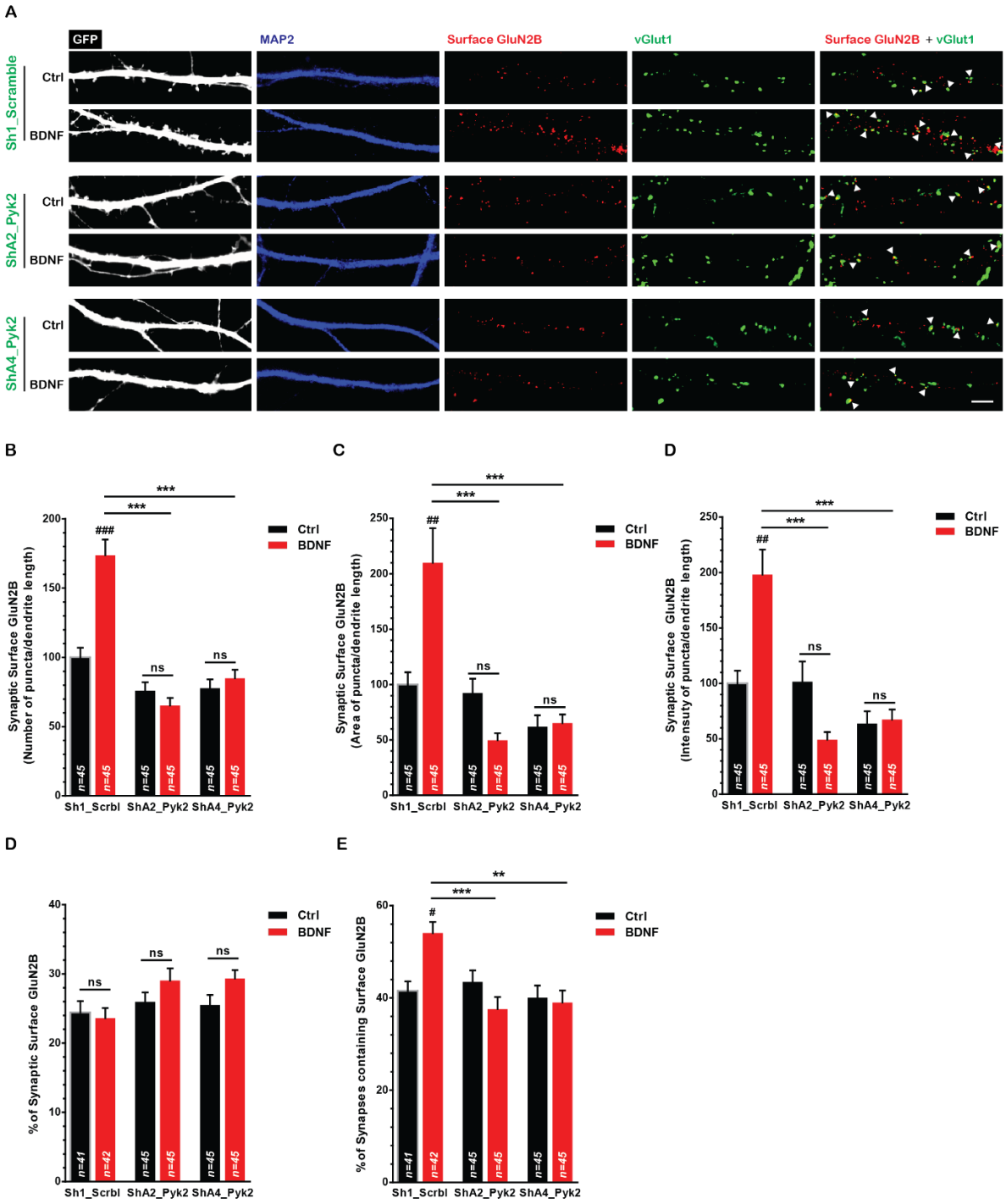

Figure 4.4. BDNF-induced increased levels of synaptic surface GluN2B-containing NMDA receptors requires Pyk2. A. Hippocampal neurons were transfected with sh1-Scramble, shA2-Pyk2 or shA4-Pyk2 at DIV 12, and were then stimulated or not (DIV 15) with BDNF (50 ng/mL) during 30 min, where indicated. Following BDNF treatment, neurons were live immunostained for GluN2B, using an antibody against an extracellular epitope in the GluN2B N-terminus. After fixation and permeabilization neurons were immunostained for vGlut1, GFP and MAP2. The results were analyzed for the number (**B**), area (**C**) and intensity (**D**) of surface GluN2B puncta per dendritic length that colocalize with the synaptic marker vGlut1, and are expressed as percentage to Sh1-Scramble. The percentage of surface synaptic GluN2B (number of surface GluN2B puncta-colocalized with vGlut1/ total surface GluN2B number of puncta) (**E**) and percentage of synapses containing surface GluN2B (number of vGlut1 puncta-colocalized with surface GluN2B/ total vGlut1 number of puncta) (**F**) was also analyzed. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test ($^{ns}P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between indicated conditions or $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ comparing to sh1-Scramble control). *n* represents the number of neurons analyzed from three independent experiments, performed in independent preparations. Arrowheads: surface GluN2B- vGlut1 colocalized puncta. Scale bar = 5 µm.

BDNF stimulation increases synaptic activation/phosphorylation of Pyk2

Pyk2 is a kinase widely expressed in the central nervous system and it is known that auto-phosphorylation of tyrosine 402 regulates its kinase activity (Lipinski and Loftus 2010). Following NMDAR activation Pyk2 can be auto-phosphorylated by a mechanism involving a direct interaction with PSD-95. The Pyk2-PSD95 interaction also contributes to the accumulation of the kinase at post-synaptic densities, contributing to the regulation of NMDAR activity (Bartos *et al.* 2010, Zhao *et al.* 2015). Recent studies have suggested that BDNF upregulates Pyk2 phosphorylation through a downregulation of striatal-enriched protein tyrosine phosphatase 61 ($STEP_{61}$) (Xu *et al.* 2014, Saavedra *et al.* 2015, Xu *et al.* 2015). Based on these previous reports we hypothesized that BDNF could activate/phosphorylate Pyk2 in the synaptic compartments in hippocampal neurons, leading to a downstream regulation of NMDAR. To address this question, synaptoneurosomes from adult (10-12 weeks) rats were stimulated with BDNF (50 ng/mL) for 10, 20 or 30 min, and Pyk2 activity/ phosphorylation (Y402) was analyzed by immunoblot by comparison with the respective time control **(Fig. 4.5A)**. BDNF stimulation for 20 - 30 min increased total Pyk2 phosphorylation levels **(Fig. 4.5B)**. Since the results of Chapter 3 showed an upregulation of total Pyk2 protein levels in synaptoneurosomes stimulated with BDNF, we investigated whether a change in the total abundance of the kinase could account for the observed BDNF-evoked increase in Pyk2 phosphorylation in synaptoneurosomes. Analysis of the pPyk2/Pyk2 ratio showed a transient increase after 10 - 20 min of stimulation with the neurotrophin **(Fig. 4.5C)**. To determine whether the effects of BDNF on Pyk2 phosphorylation were specifically observed at the synapse we performed similar experiments in cultured hippocampal neurons. In this case, 20 min stimulation with BDNF did not change total Pyk2 protein levels, and no alterations in the phosphorylation of the kinase were observed **(Fig. 4.5D)**. Similarly, no significant alterations were observed in the pPy2/total Pyk2 ratio **(Fig. 4.5F)**. Taken together, these results indicate that Pyk2 undergoes a rapid and transient activation at the synapse following stimulation with BDNF, possibly by a mechanism involving dendritic Pyk2 synthesis.

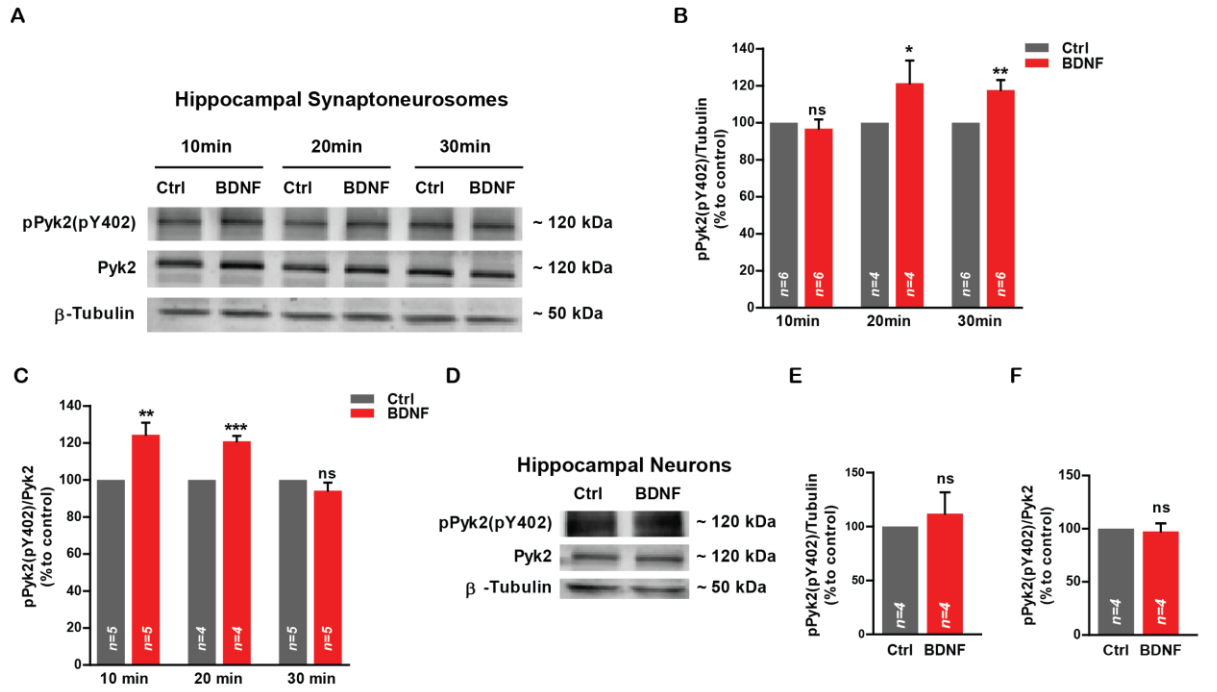

Figure 4.5. BDNF stimulation increases synaptic activation/phosphorylation of Pyk2. **A-C.** Western Blot analysis of synaptic pPyk2 (Y402) levels. Hippocampal synaptoneurosomes were stimulated or not with BDNF (50 ng/mL) during 10, 20 and 30 min, after a pre-warming (30ºC) of 5 min. Proteins were extracted and then resolved and analyzed by immunoblot, using antibodies against pPyk2 (Y402), Pyk2 and β-Tubulin. **B.** BDNF induces a significant increase in synaptic levels of pPyk2 (Y402) after 20 and 30 min of stimulation. In this analysis, synaptic pPyk2 (Y402) levels were normalized to β-Tubulin. **C.** Stimulation with BDNF also induced a specific synaptic phosphorylation/activation of Pyk2. In this analysis synaptic pPyk2 (Y402) levels were normalized to synaptic Pyk2. **D-F.** Western Blot analysis of total pPyk2 (Y402) levels. Hippocampal neurons (high-density cultures; 14-15DIV) were stimulated or not with BDNF (50 ng/mL) during 20 min. After protein extraction the lysates were resolved and analyzed by immunoblot, using antibodies against pPyk2 (Y402), Pyk2 and β-Tubulin. BDNF does not affect the total levels of pPyk2 (**E**) and the specific activation/phosphorylation of the kinase (**F**). Error bars represent s.e.m. The statistical significance was calculated by unpaired *t* test ($^{ns}P >$ 0.05, $^{*}P < 0.05$ $^{**}P < 0.01$, $^{***}P < 0.001$). *n* represents the number of independent experiments, performed in distinct preparations.

Basal and BDNF-induced increased levels of total surface GluN2B-containing NMDA receptors require activation/phosphorylation of Pyk2

To determine whether BDNF-induced increase in GluN2B surface expression is mediated by activation/phosphorylation of Pyk2, the effect of the neurotrophin was investigated in hippocampal neurons transfected with a phospho-mutant form of Pyk2 (Pyk2-Y402F), which has no kinase activity, or with the wild-type form of Pyk2 (Pyk2-WT). As a control, hippocampal neurons were transfected with the Flag vector (Flag-Empty). Stimulation with BDNF (50 ng/mL) was performed during 30 min, and the cells were then live immunostained with a GluN2B antibody against an extracellular epitope located at the N-terminus. After fixation and permeabilization neurons were labeled with antibodies against Flag (transfection marker) and MAP2 **(Fig. 4.6A)**. Images were analyzed for total number **(Fig. 4.6B)**, area **(Fig. 4.6C)** and intensity **(Fig. 4.6D)** of surface GluN2B puncta per dendritic length (MAP2). The results were similar for the three parameters analyzed: overexpression of Pyk2-Y402F completely abolished the BDNF-induced increase in surface GluN2B expression observed in neurons transfected with Flag-Empty **(Fig. 4.6A-D)**. Moreover, in accordance with the results obtained for Pyk2 knockdown **(Fig 4.3A-D)**, overexpression of the phospho-mutant Pyk2 (Y402F) also decreased the

surface expression GluN2B under resting conditions **(Fig. 4.6A-D)**. These results clearly suggest that activation/phosphorylation of Pyk2 is required for the maintenance and for BDNF-induced increase in total surface GluN2B expression. Surprisingly, we also observed that overexpression of the wild-type (Pyk2-WT) form of Pyk2 mimicked the effects of the neurotrophin on surface GluN2B-NMDAR. These findings indicate that the role of BDNF in the regulation of surface expression of these receptors is mediated not only by activation of Pyk2, but also by promoting the dendritic/synaptic accumulation of the kinase (see chapter 3). Furthermore, BDNF treatment of neurons overexpressing Pyk2 did not further increase surface GluN2B expression **(Fig. 4.6A-D)**, further confirming this hypothesis.

Together, these results strongly suggest that BDNF increases GluN2B total surface expression by two mechanisms: 1) BDNF increases the dendritic/synaptic levels of Pyk2, which upregulates the surface expression of GluN2B-containing NMDA receptors; 2) BDNF-activated signaling induces the phosphorylation of Pyk2, which is required to enhance GluN2B surface expression. Pyk2 activity is also required to maintain GluN2B surface expression under resting conditions.

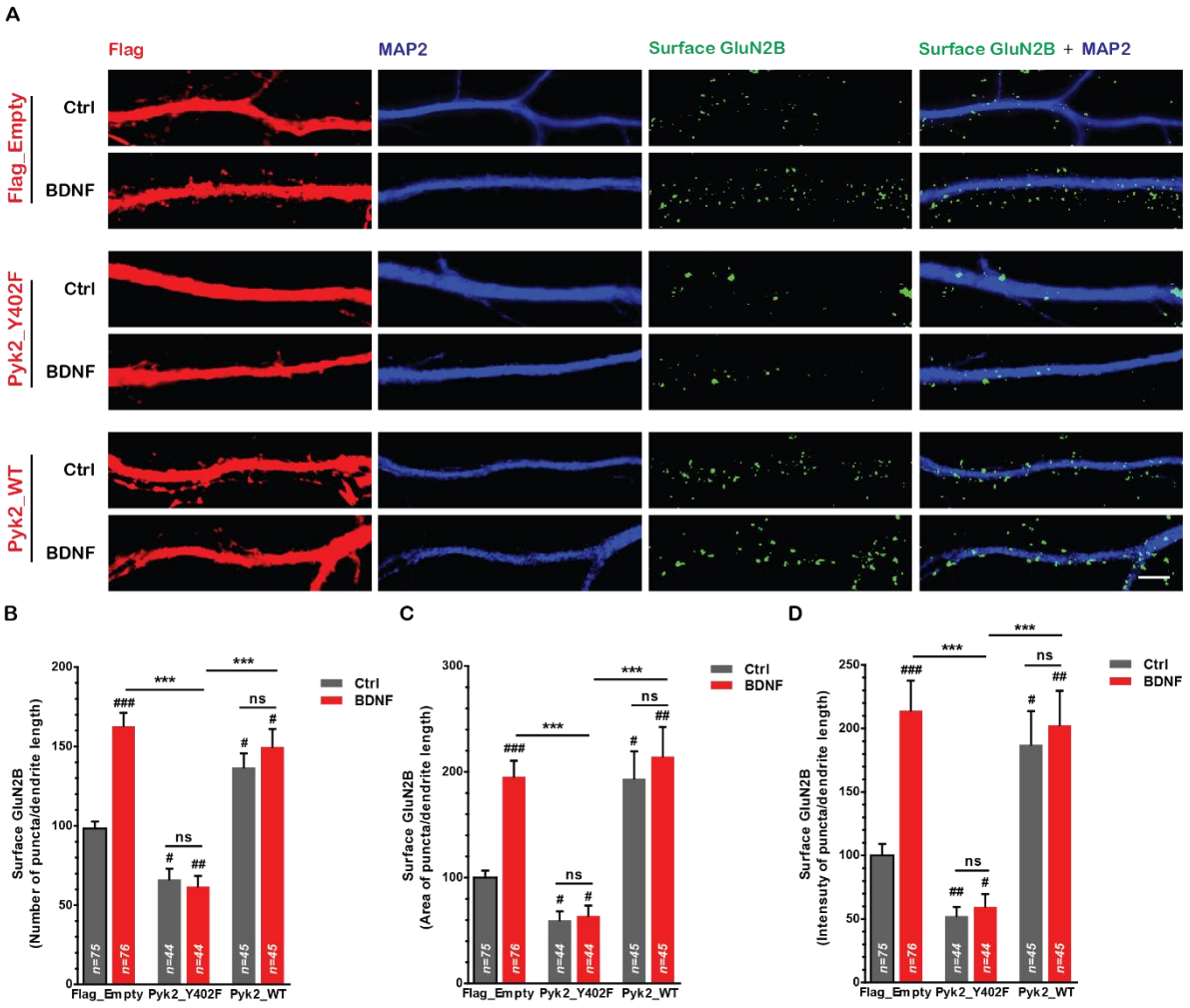

Figure 4.6. Basal and BDNF-induced increased levels of total surface GluN2B-containing NMDA receptors require activation/phosphorylation Pyk2. A. Hippocampal neurons were transfected with sh1-Scramble, shA2-Pyk2 or shA4-Pyk2 at DIV 12, and were then stimulated or not (DIV 15) with BDNF (50 ng/mL) during 30 min, as indicated. Following BDNF treatment, neurons were live immunostained for GluN2B, using an antibody against an

extracellular epitope in the GluN2B N-terminus. After fixation and permeabilization neurons were immunostained for Flag and MAP2. Neurons were analyzed for total surface GluN2B number (**B**), area (**C**) and intensity (**D**) of puncta per dendritic length. Results are expressed as percentage to Flag-Empty control. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test (***$P < 0.001$ between indicated conditions or $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ comparing to Flag-Empty control). *n* represents the number of neurons analyzed from at least three independent experiments, performed in independent preparations. Scale bar = 5 µm.

Discussion

Herein we reported that Pyk2 is a key player in the control of the trafficking of GluN2B-containing NMDAR in hippocampal neurons. Furthermore, we showed that BDNF induces the synaptic accumulation of GluN2B subunits by a mechanism involving Pyk2 accumulation and activation at the synapse. We also found that Pyk2 is important for the maintenance of this population of NMDA receptors on the surface of mature hippocampal neurons under resting conditions.

It has been reported that the trafficking and function of NMDAR is regulated by phosphorylation of their subunits (Wang *et al.* 2014). This mechanism may account, at least in part, for the BDNF-induced alteration in GluN2B surface expression observed in the present work. Accordingly, recent studies have shown that BDNF increases the phosphorylation of GluN2B on Y1472 through a mechanism involving the proteasome-mediated degradation of the phosphatase STEP (Nakai *et al.* 2014, Saavedra *et al.* 2015, Xu *et al.* 2015). GluN2B Y1472 is located in the C-terminal region of the protein and phosphorylation of this residue by Fyn/Src kinases regulates the internalization of the receptors by a mechanism involving the clathrin protein adaptor AP-2. Phosphorylation of Y1472 negatively regulates the endocytosis of GluN2B-NMDAR by blocking the binding of AP-2, and therefore increases the surface expression of the receptors (Lavezzari *et al.* 2003, Prybylowski *et al.* 2005, Sanz-Clemente *et al.* 2010). GluN2B phosphorylation on Y1472 also favors the accumulation of NMDAR at the synapse, while phosphorylation at Y1336 increases their extrasynaptic distribution (Grosshans *et al.* 2002, Prybylowski *et al.* 2005, Goebel-Goody *et al.* 2009). Together, these evidence suggest that GluN2B phosphorylation on Y1472 may account for the Pyk2 mediated effects of BDNF on the surface expression of NMDAR. Phosphorylation of the same GluN2B residue was also observed upon HFS-induced LTP in the hippocampus and amygdala that is essential for learning and memory (Nakazawa *et al.* 2001, Nakazawa *et al.* 2006, Isosaka *et al.* 2008). Casein kinase II (CKII) and Cyclin-dependent kinase 5 (Cdk5) have also been reported to regulate the trafficking of GluN2B-containing NMDARs by targeting different phosphorylation sites (Chung *et al.* 2004, Hawasli *et al.* 2007, Zhang *et al.* 2008, Plattner *et al.* 2014).

We also found that the BDNF-evoked increase in the surface expression of GluN2B-containing NMDAR is dependent on protein synthesis. This is surprising since it was proposed that GluN2A- but not GluN2B-containing NMDA receptors are inserted into the plasma membrane of rat hippocampal neurons after induction of neuronal activity, by a mechanism dependent on dendritic synthesis of the receptors (Swanger *et al.* 2013). This may suggest that translation activity may be important for the synthesis of a regulatory protein that mediates the effect of BDNF on the surface expression of GluN2B-containing NMDAR. However, since the *GluN2B* mRNA is also expressed in synapses (Cajigas *et al.* 2012), additional experiments should be performed to determine whether local synthesis of the receptors mediates the effect of BDNF on the surface expression of GluN2B-containing NMDAR.

In the present work we also showed that BDNF treatment increases the surface expression of GluN2B-NMDAR specifically at the synapse, and this was accompanied by an upregulation in the density of excitatory synapses. Moreover, we reported that both effects were dependent on protein synthesis. However, it remains to be determined whether the BDNF-induced increase in the number of

excitatory synapses increases the surface expression of GluN2B or, alternatively, if it is the BDNF-induced increase in surface GluN2B that mediates the increase in density of excitatory synapses in hippocampal neurons. A recent study reported that reducing the degradation of NMDAR, by loss of the ubiquitin ligase substrate adaptor protein adaptor Fbxo2, increases NMDAR surface expression and upregulates the synaptic markers PSD-95 and vGlut1 (Atkin *et al.* 2015). These synaptic changes do not manifest as neurophysiological differences or alterations in dendritic spine density in Fbxo2 knock-out mice, but result instead in increased axo-dendritic shaft synapses (Atkin *et al.* 2015). These findings suggest that the surface expression of NMDAR may indeed influence the synapse formation and maintenance.

BDNF-TrkB signaling is coupled to the phosphorylation/ activation of Pyk2 and Src. Pyk2 was previously identified as a key regulator of the activity of NMDAR by a mechanism dependent on a direct interaction with PSD-95, which also mediates the activation of Src (Bartos *et al.* 2010, Zhao *et al.* 2015). The latter kinase phosphorylates the GluN2B subunit on Y1472 (Xu *et al.* 2006, Nakai *et al.* 2014, Zhao *et al.* 2015), thereby mediating the surface retention of NMDAR. Based on our previous results (see chapter 3), we hypothesized that Pyk2 could account for the effects of BDNF on GluN2B surface expression. Accordingly, we found that Pyk2 knockdown abrogates the BDNF-induced increase in total surface GluN2B-containing NMDAR, as well as the effects on the expression of the receptors at the synapse. Furthermore, Pyk2 was required for the maintenance of GluN2B subunits of NMDAR on the dendritic plasma membrane under resting conditions, as well as at the synapse. Knockdown of Pyk2 also reduced the percentage of synapses containing GluN2B-NMDAR. Together, the results suggest that the BDNF-induced upregulation in the surface expression of GluN2B-containing NMDAR at the synapse may be a result of an overall increase in surface expression of this type of receptors. This pattern of response may be due to the protocol of stimulation used, which consisted in a bath application of BDNF for 30 min, with a consequent activation of virtually all TrkB receptors (synaptically and non-synaptically). On the other hand, the results of chapter 3 suggest that the increase in the percentage of synapses containing surface GluN2B-NMDAR upon BDNF treatment may result from the synaptic accumulation of Pyk2 following stimulation with the neurotrophin (see chapter 3). If this is the case, distinct mechanisms may be involved in the Pyk2-mediated regulation of GluN2B surface expression in extrasynaptic sites.

Pyk2 kinase activity is regulated by the phosphorylation state of tyrosine 402. This is the auto-phosphorylation site coupled to the kinase domain responsible for the regulation of the activity of the protein (Lipinski and Loftus 2010). This is one of the mechanisms whereby BDNF regulates Pyk2 in cultured hippocampal neurons since we observed a rapid but transient increase in the activation/phosphorylation of synaptic Pyk2 upon stimulation of synaptoneurosomes with the neurotrophin. A recent study showed that BDNF-mediated degradation of the phosphatase $STEP_{61}$ by the proteasome increases Pyk2 Y402 phosphorylation in rat cortical neurons (Saavedra *et al.* 2015). Interestingly, BDNF-induced activation of Pyk2 was synapse specific, in contrast with previous reports in rat cortical neurons (Xu *et al.* 2015). These differences may be explained by: i) the putative signaling machinery coupled to hippocampal TrkB receptors, namely synaptic versus extrasynaptic; ii) the use of cortical versus hippocampal neurons; iii) and by the use of TrkB agonist [7,8-dihydroxyflavone (DHF)] versus recombinant BDNF that may have different affinities to the TrkB

receptors, namely synaptic or extrasynaptic ones. Notably, we found that Pyk2 phosphorylation in Y402 is required for the BDNF-induced increase in the surface expression of GluN2B in hippocampal neurons. In addition to the effects of BDNF in the activation of Pyk2 at the synapse, the results of chapter 3 also showed an upregulation of synaptic Pyk2 protein levels in hippocampal synaptoneurosomes incubated with the neurotrophin.

Pyk2 activity was found to be important not only to the BDNF-induced upregulation of GluN2B subunits, but also to the maintenance of the surface expression of these receptors under resting conditions. Accordingly, overexpression of Pyk2 WT mimicked the effects of BNDF on the surface expression of GluN2B-NMDAR, suggesting that the upregulation of surface GluN2B receptor subunit induced by the neurotrophin is deeply linked to the kinase protein levels and its activation/phosphorylation. Since GluN2B surface expression was similar in both conditions, it may be hypothesized that BDNF-induced local synthesis of Pyk2 accounts to the cycloheximide sensitivity of BDNF-induced upregulation in plasma membrane associated NMDAR.

Taken together, our results suggest that BDNF increases the surface expression of GluN2B-containing NMDA receptors by a mechanism involving the synaptic activation of Pyk2 and its accumulation in synaptic compartments, most likely by mechanisms dependent on the local dendritic protein synthesis (see chapter 3). The upregulation and activation of Pyk2 at the synapse may lead to a downstream activation of Src, which phosphorylates the C-terminus of the GluN2B subunit on Y1472. GluN2B phosphorylation on this residue retains the GluN2B-NMDAR on the cell surface avoiding its internalization mediated by AP-2/clathrin complex. This novel mechanism, proposes Pyk2 as new target for genetic or pharmacological manipulation to enhance learning and memory tasks, which may be relevant in neurological and psychiatric diseases exhibiting an impairment in cognition.

Supplementary Figures

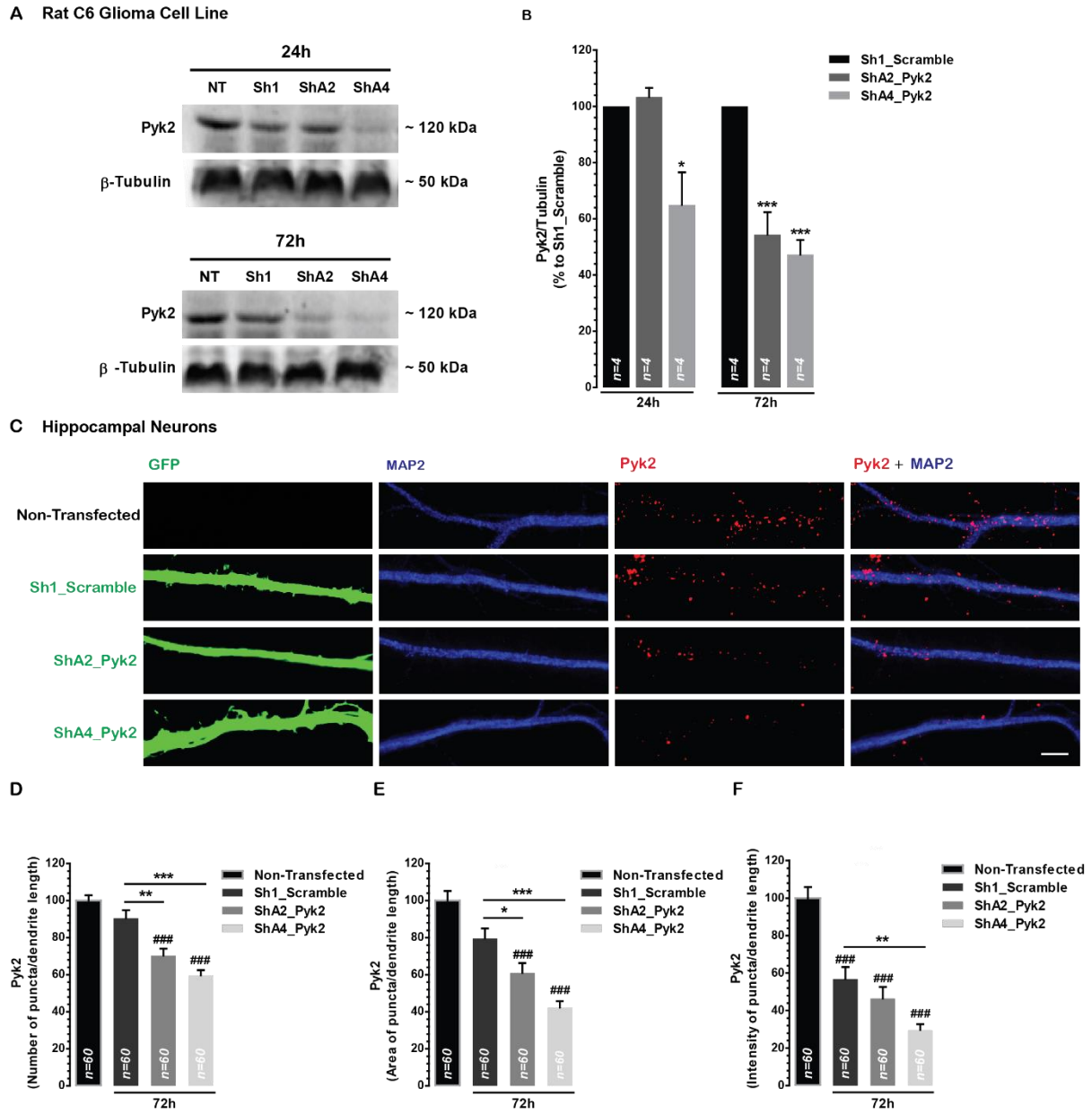

Figure S1. Evaluation of Pyk2 knockdown efficiency by ShA2-Pyk2 and ShA4-Pyk2. **A.** Transfection of the C6 cell line with the following shRNAs: scrambled shRNA, ShA2 or ShA4 (against Pyk2). C6 cells were transfected and protein extracts were prepared 24h or 72 h after transfection. Pyk2 levels were assessed by immunoblot. **B.** Quantification of the results shown in **A**. The graph represents the mean intensity of the bands normalized to β-tubulin, relative to the sh1-Scramble. **C.** Hippocampal neurons were transfected with Sh1-Scramble, shA2-Pyk2 or shA4-Pyk2 at DIV 12. After 72h (DIV 15), neurons were fixed and permeabilized before immunostaining against Pyk2, GFP and MAP2. Neurons were analyzed for dendritic Pyk2 number (**D**), area (**E**) and intensity (**F**) of puncta per dendritic length. Results are expressed as percentage to not-transfected neurons. Error bars represent s.e.m. The statistical significance was calculated using the Kruskal-Wallis test ($P < 0.0001$) followed by the Dunn's Multiple Comparison test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ between indicated conditions or $^{###}P < 0.001$ comparing to non-transfected neurons). *n* represents the number of neurons analyzed four independent experiments. Scale bar = 5 µm. *NT - Non-transfected.*

Chapter 5

GENERAL DISCUSSION AND FUTURE DIRECTIONS

GENERAL DISCUSSION

The current work has focused on molecular mechanisms that may underlie the effects of BDNF on synaptic plasticity on hippocampal neurons. These effects have been widely attributed to the induction of dendritic protein synthesis which is mainly required for the maintenance of L-LTP (Kang and Schuman 1995, Kang and Schuman 1996, Holt and Schuman 2013, Leal *et al.* 2015). This work unravels an important role of BDNF in regulating of the surface expression of GluN2B-NMDA receptors by a mechanism involving the synaptic synthesis/activation of Pyk2. Five major findings are herein reported: i) BDNF induces the synaptic translation and accumulation of Pyk2 by a mechanism involving hnRNPK; ii) NMDAR-mediated synaptic activity induces the dendritic and synaptic accumulation of Pyk2 by a mechanism involving BDNF-TrkB signaling; iii) Acute BDNF application increases GluN2B-NMDAR surface expression and the number of excitatory synapses; iv) BDNF-induced upregulation in surface GluN2B-NMDAR expression requires Pyk2; v) BDNF activation of synaptic Pyk2 is required for the enhancement of GluN2B-containing NMDAR.

BDNF-mediated regulation of synaptic proteome by induction of local protein synthesis in hippocampal neurons

In the first part of the work, and taking advantage of polysome fractionation from synaptoneurosomes isolated from the hippocampus of adult rats, we confirmed previous evidence indicating that BDNF increases translation activity at the synapse. In particular, we found that BDNF upregulates the polysome fraction and decreases the abundance of monosomes, suggesting an enhancement in synaptic translation rates following BDNF application. In addition to the previous reports showing the synaptic translation of *Arc, GluA1, CaMKIIα and Homer2* mRNAs in response to BDNF (Ju *et al.* 2004, Schratt *et al.* 2004, Takei *et al.* 2004), we found that Pyk2 is a new target for BDNF-induced synaptic protein synthesis.

BDNF increases dendritic protein synthesis by activation/phosphorylation of components of the translational machinery, such as S6K, eIF4E and 4E-BP1. Phosphorylation of these three targets of the mTOR signaling, which are present in dendrites, correlates with the initiation of translation activity (Tang *et al.* 2002, Schratt *et al.* 2004, Takei *et al.* 2004). In addition to the effects of BDNF in the upregulation of Pyk2 in synaptoneurosomes, the neurotrophin also upregulated Pyk2 protein levels in dendrites/synapses by a mechanism involving protein synthesis. However, further studies are required to determine whether the transport of *Pyk2* mRNA along dendrites is regulated by BDNF and whether the neurotrophin induces the dendritic translation of Pyk2 in cultured hippocampal neurons. This question may be addressed using a fluorescent in situ hybridization assay to assess the localization of *Pyk2* mRNA in neurons. Furthermore, transfection of hippocampal neurons with a GFP reporter flanked by the 3´UTR and 5´UTR of Pyk2, together with live cell fluorescence imaging experiments, will allow determining whether Pyk2 is locally translated in dendrites in response to BDNF. Additional live-imaging experiments should be performed to determine whether synaptic activity induces an enrichment of Pyk2 into the postsynaptic density, and whether this effect requires

BDNF. This question may be addressed using a glutamate uncaging protocol in hippocampal neurons expressing a fluorescent Pyk2 construct, in the presence of the scavenger of the TrkB ligands.

It has been proposed that mRNAs can be transported along dendrites in a silent form as a part of RNA granules. These are very dynamic structures that regulate mRNA metabolism, from the transcription step until translation takes place (Zeitelhofer *et al.* 2008, Zeitelhofer *et al.* 2008, Arribere *et al.* 2011, Jung *et al.* 2014). RNA granules are mainly comprised by RNA-binging proteins and display fast bidirectional mobility in hippocampal neurons (Tubing *et al.* 2010). These structures were found to accumulate at the synapse upon synaptic activity (Zhang *et al.* 2012), loading dendritically localized mRNAs (e.g. *Septin 7 and CaMKIIα*) (Tubing *et al.* 2010). A previous microarray study performed in our laboratory (unpublished data) to analyze the effect of BDNF on the interaction of hnRNPK with RNAs in cultured hippocampal neurons, identified the *Pyk2* mRNA as a candidate transcript to be associated with this RNA binding protein; stimulation of BDNF-TrkB signaling was found to decrease the interaction between the *Pyk2* mRNA and hnRNPK. In this work we observed that the knockdown of hnRNPK abolishes the BDNF-induced synaptic accumulation of Pyk2 in cultured hippocampal neurons, while overexpression of the RNA binding protein results in the accumulation of Pyk2 in dendrites, mimicking the effect of stimulation with BDNF. These results, together with the key role played by protein synthesis in Pyk2 dendritic/synaptic accumulation, lead us to conclude that hnRNPK is likely involved in the control of the dendritic transport and translation of *Pyk2* mRNA.

Additional experiments are required to confirm that the binding of *Pyk2* mRNA to hnRNPK is synaptically regulated by BDNF. This may be performed by evaluating the effect of stimulation with BDNF on the levels of *Pyk2* mRNA co-immunoprecipitated with hnRNPK from rat hippocampal synaptoneurosomes. In non-neuronal cells, it was proposed that hnRNPK phosphorylation can regulate protein-protein and protein-RNA interaction (Ostrowski *et al.* 2000). Moreover, it was shown that Src phosphorylation of hnRNPK inhibits in a reversible manner the binding of hnRNPK to the differentiation control element (DICE) of the 15-lipoxigenase (LOX) mRNA 3' UTR, *in vitro*, and specifically derepresses the translation of DICE-bearing mRNAs *in vivo* (Ostareck-Lederer *et al.* 2002). Furthermore, it was shown that LTP in the hippocampus requires ERK1/2-mediated hnRNPK phosphorylation on S284, and the cytoplasmic accumulation of the protein (Folci *et al.* 2014). Recent studies also showed an ERK1 phosphorylation site on *Xenopus* hnRNPK (S257; homologous with S284 of human hnRNPK), which regulates NF-M protein expression and axon outgrowth (Hutchins *et al.* 2015). Therefore, it will be of interest to understand whether the binding of *Pyk2* mRNA to hnRNPK is regulated by BDNF through hnRNPK phosphorylation,

The role of Pyk2 in BDNF-induced increase in GluN2B-NMDAR

In the second part of the work we uncovered a role for Pyk2 in BDNF-induced increase in the surface expression of GluN2B-NMDAR in hippocampal neurons. As previously discussed these results are in agreement with the BDNF-induced potentiation of NMDAR currents (Xu *et al.* 2006, Ji *et al.* 2010), by a possible mechanism involving the GluN2B phosphorylation on Y1471 by Src (Xu *et al.* 2006, Nakai *et al.* 2014) and degradation of $STEP_{61}$ through the UPS (Saavedra *et al.* 2015, Xu *et al.*

2015). Therefore, it is likely that the effects on surface expression of GluN2B-NMDAR are mediated by GluN2B Y1472 phosphorylation through the BDNF/TrkB/Pyk2/Src axis.

The results obtained also showed that protein synthesis is required for BDNF-induced upregulation in surface GluN2B. A previous study reported that GluN2A, but not the GluN2B subunit, is dendritically translated and inserted in the post-synaptic membrane upon synaptic activity in hippocampal neurons (Swanger *et al.* 2013). These results suggest that translation activity may be required for the synthesis of an intermediary protein, which can stabilize GluN2B-containing NMDAR on the membrane, rather than for the "de novo" synthesis of receptor subunit. Accordingly, in the first part of our work we identified Pyk2 as a novel mRNA likely translated in dendrites in response to the stimulation with BDNF and knockdown of the kinase abolished the effects of BDNF on the surface expression of GluN2B-containing NMDAR. Moreover, Pyk2 was found to be required for the maintenance of this population of NMDAR in the membrane under resting conditions, in agreement with the results showing that overexpression of Pyk2 mimicked the BDNF-induced increase in plasma membrane-associated GluN2B-NMDAR. Altogether these findings show that Pyk2 is a new player in neurotrophin-induced regulation of NMDAR trafficking and surface expression.

In this study, using hippocampal synaptoneurosomes and cultured hippocampal neurons, we observed that BDNF-induced Pyk2 activation/phosphorylation occurs specifically at the synapse. These results are in agreement with previous reports showing that Pyk2 is recruited to synapse and can be activated by direct interaction with PSD-95 (Bartos *et al.* 2010, Zhao *et al.* 2015). Using two different strategies to knockdown Pyk2 activity, a "kinase dead" construct of Pyk2 (Y402F) and transfection with specific shRNAs, we characterized a role for the kinase in BDNF-induced increase in the surface expression of GluN2B. Furthermore, both strategies showed that the activation/ phosphorylation of Pyk2 is required for the maintenance of the receptor surface expression under resting conditions. These results clearly indicate that the synaptic activity of Pyk2 is required for the BDNF-mediated increases on the surface levels of GluN2B-containing NMDA receptors. Furthermore, multiple evidence have suggested that BDNF may induce the phosphorylation and regulation of NMDA receptors through Src and Fyn kinases (Xu *et al.* 2006, Nakai *et al.* 2014, Zhao *et al.* 2015). Additional studies are required to determine whether Src or Fyn are involved in the BDNF-mediated trafficking of GluN2B-NMDAR and whether Pyk2 is upstream these kinases as previously proposed (Zhao *et al.* 2015).

BDNF treatment is known to upregulate the dendritic/synaptic PSD-95 clusters, as well as the expression of vGlut1 in hippocampal neurons (Yoshii and Constantine-Paton 2007, Hu *et al.* 2011, Melo *et al.* 2013). Interestingly, our results indicate that 30 min of BDNF application is sufficient to increase the density of excitatory synapses in hippocampal neurons. This effect can be attributed to an increase in vGlut1 clusters as well as to an enrichment in the pre-existent PSD-95 clusters. These alterations in excitatory synapses were accompanied by an enhancement of the surface expression of GluN2B-NMDAR in agreement with recent reports suggesting that increased levels of NMDAR surface expression is accompanied by an upregulation in the number of synaptic markers PSD-95 and vGlut1 (Atkin *et al.* 2015). However, it remains to be clarified whether these phenomena are dependent or independent from each other. This question may be addressed by evaluating the levels of vGlut1 in

hippocampal neurons after BDNF treatment in neurons overexpressing two constructs to knockdown Pyk2 (ShA2 and ShA4), which downregulate the surface expression of GluN2B.

In this work, we proposed a model according to which BDNF induces the dendritic synthesis of Pyk2 by a mechanism involving hnRNPK. Dendritically synthesized Pyk2 accumulates in dendrites/synapses, where it is activated by BDNF, ultimately resulting in an increased surface expression of GluN2B-containing NMDA receptors that may contribute to synaptic plasticity, learning and memory **(Figure 5.1)**. The increase in surface expression of GluN2B-NMDAR is likely mediated by the activation of Src by Pyk2, which increases the phosphorylation of the residue Y1472 contained within the YEKL endocytic motif of the GluN2B subunit, thereby blocking the AP-2/clathrin-mediated endocytosis of the receptor. Additional studies are required to understand the functional relevance of the increased levels of GluN2B-containing NMDAR following stimulation of the BDNF-TrkB signaling. Whole-cell patch-clamp recordings will allow determining the role of Pyk2 in the BDNF-induced alterations in NMDAR-mediated currents.

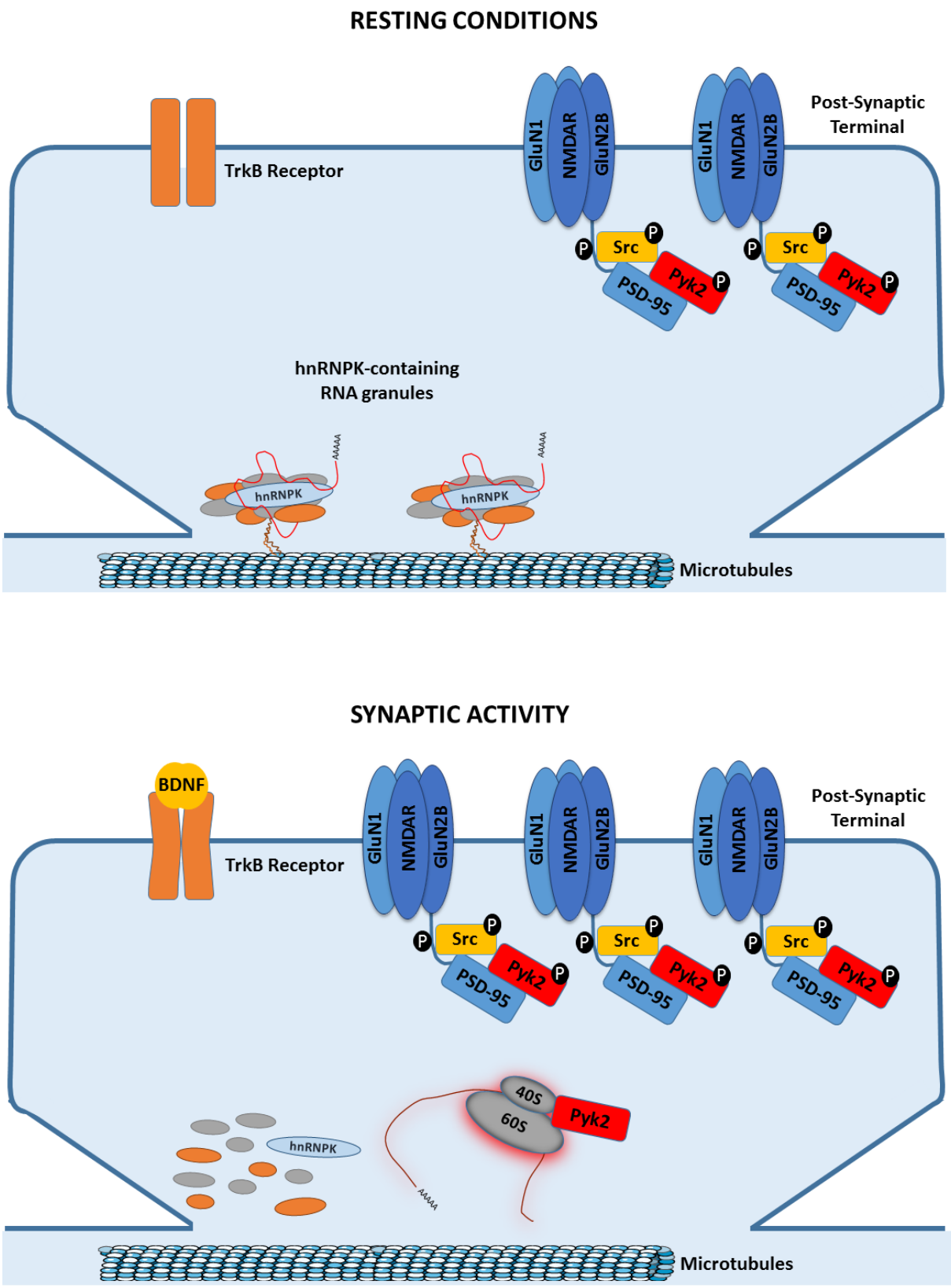

Figure 5.1. Proposed model for BDNF-induced increase in surface expression of GluN2B-containing NMDA receptors. Synaptic activity evokes the release of BDNF, which induces the dendritic synthesis of Pyk2 and synaptic accumulation of activated/phosphorylated Pyk2 (pY402) by a mechanism involving hnRNPK. The synaptic accumulation/activation of Pyk2 increases the surface expression of GluN2B-containing NMDA receptors at post-synaptic membrane, which may account for the BDNF-induced activation of excitatory synapses in hippocampal neurons and BDNF-induced LTP.

The paradoxical role of Pyk2 in synaptic plasticity

The balance between NMDAR-mediated LTP and LTD is believed to be related with distinct levels and/or kinetics of NMDAR-mediated calcium influx, with a differential coupling to signaling transduction mechanisms. It is thought that calcium elevation upon induction of LTP increases the activity of kinases, like CaMKII, and promotes the insertion of AMPA receptors in the post-synaptic membrane, while the $[Ca^{2+}]_i$ rise in LTD leads to AMPAR endocytosis by a mechanism dependent on the activity of serine/threonine phosphatases such as calcineurin (Malenka and Nicoll 1993, Malenka and Bear 2004). It was recently described that LTD also requires CaMKII and its phospho-T286-induced "autonomous" (Ca^{2+}-independent) activity, which is responsible for GluA1 phosphorylation on S567, in contrast with the CaMKII-dependent phosphorylation of GluA1 on S831 induced by LTP (Coultrap *et al.* 2014). Phosphorylation of GluA1 at S567 reduces synaptic GluA1 localization, while phosphorylation of GluA1 S831 has the opposite effect (Coultrap *et al.* 2014). However, NMDAR-mediated plasticity independent of AMPAR have also been proposed (Hunt and Castillo 2012). In particular, induction of LTP at Schaffer collateral - CA1 synapses in rat hippocampal slices increases the surface expression of NMDAR in a PKC and Src-family-dependent manner, without changes in AMPAR levels on the surface (Grosshans *et al.* 2002). In agreement, induction of LTP in the rat DG *in vivo* increases the surface expression of NMDAR (namely GluN1 and GluN2B subunits) in a delayed and protein synthesis-dependent manner (Williams *et al.* 2007). An additional point of divergence between distinct forms of NMDAR-LTP is the requirement of differential kinase activity. PKC (Kwon and Castillo 2008) and Src kinases (Rebola *et al.* 2008) are required for NMDAR-LTP in CA3 neurons, in agreement with results obtained in other synapses (O'Connor *et al.* 1995, Grosshans *et al.* 2002, Li *et al.* 2011), while PKA is required for LTP of NMDAR-mediated excitatory transmission onto dopaminergic neurons of the midbrain (Harnett *et al.* 2009).

Despite all efforts made in the last 30 years, the identity of the Ca^{2+} sensors involved in detecting the post-synaptic Ca^{2+} signals that trigger the different forms of NMDAR-mediated synaptic plasticity remain unclear. NMDAR-LTD requires an increase in intracellular Ca^{2+} for induction and, at least in the DG, the direction of NMDAR plasticity appears to depend on the free Ca^{2+} concentration triggered by the induction protocol (Harney *et al.* 2006). The precise mechanism by which Ca^{2+} elicits NMDAR-LTD is unclear. However, hippocalcin was identified as a Ca^{2+} sensor mediating cholinergic induction of NMDAR-LTD at Schaffer collateral - CA1 synapses, which causes the PSD-95 dissociation from NMDAR, allowing the binding of AP-2 to the receptor subunits thereby initiating dynamin-dependent endocytosis (Jo *et al.* 2010). As described above, it was proposed that CaMKII responds differently in the LTD and LTP contexts, being differentially regulated depending on the magnitude of the $[Ca^{2+}]_i$ changes evoked by each protocol (Coultrap *et al.* 2014). Since CaMKII plays an important role in the regulation of NMDAR surface dynamics (Sanz-Clemente *et al.* 2013, Lussier *et al.* 2015), the balance between NMDAR-LTP and LTD may be regulated by the intracellular Ca^{2+} concentration achieved in response to the stimulation paradigms that evoke different forms of synaptic plasticity.

Pyk2 has been implicated in LTP and LTD. It was shown that Pyk2 upregulates NMDAR function by activating Src in CA1 hippocampal neurons, suggesting that activation of Pyk2 is required for the induction of LTP and may depend on the downstream activation of Src to upregulate NMDAR

(Girault *et al.* 1999, Huang *et al.* 2001). Furthermore, NMDAR stimulation and the resulting entry of Ca^{2+} contribute to the activation of Pyk2 (pY402) through a PKC-dependent mechanism. Binding to PSD-95 also plays a role in the activation of Pyk2, which is required for LTP induction in the hippocampal CA1 region (Bartos *et al.* 2010). Surprisingly, it was found that Pyk2 also plays a role in LTD. In particular, Pyk2 knockdown was found to block LTD but not LTP in hippocampal neurons by a mechanism dependent on its kinase activity (Hsin *et al.* 2010). Furthermore, overexpression of Pyk2 inhibits Rac1 to reduce the number of spines, spine length and the spine width, effects that were not observed in hippocampal neurons overexpressing the kinase "dead" Pyk2 (Suo *et al.* 2012).

In the present study, we proposed a mechanism according to which Pyk2 is involved in the BDNF-induced upregulation in the surface expression of GluN2B-NMDAR. Since BDNF is implicated in LTP induction and maintenance (Leal *et al.* 2015), we hypothesize that Pyk2 may play a key role in this form of synaptic plasticity under the control of BDNF-TrkB signaling. The effects of BDNF induce the synaptic accumulation of Pyk2, which resemble the response to NMDAR activation through a Ca^{2+}- and calmodulin-dependent mechanism, required for LTP induction (Bartos *et al.* 2010). In opposition to these findings, it was shown that membrane depolarization and tetanic stimulation induce nuclear translocation of Pyk2 by a Ca^{2+}- and calcineurin-dependent mechanism, without Pyk2 tyrosine phosphorylation (Faure *et al.* 2007). Since, activation of calcineurin is linked to LTD induction (Morishita *et al.* 2005, Fujiwara *et al.* 2007), these findings suggest that Pyk2 involvement in LTP or LTD may be controlled by the recruitment of Pyk2 to synaptic compartments versus nuclear translocation, respectively.

Additional studies are required to elucidate the mechanisms that can favor LTP and LTD depending on the conditions of neuronal activity, but the previous reports and our evidence suggest that Pyk2 may act as a "sensor" to privilege one of the forms of synaptic plasticity. The present work also showed that overexpression of Pyk2 increases, *per se*, the synaptic surface expression of GluN2B-NMDAR, which is deeply linked to a context of synaptic potentiation, counteracting the previous evidence suggesting that overexpression of Pyk2 does not affect LTP, but instead privileges LTD and spine retraction. The use of different models may explain putative differences in the results previously published. Moreover, the levels of expression of the constructs may influence the balance between LTP versus LTD, since we observed that the amount of Pyk2 is important and sufficient for the regulation of NMDAR surface expression. Whole-cell patch-clamp recordings using Pyk2 constructs under basal conditions and upon BDNF treatment in hippocampal neurons will allow evaluating the activity of NMDA receptors and may contribute to elucidate the role of Pyk2 in the balance between LTP and LTD.

The content of post-synaptic membrane in GluN2A- and GluN2B-containing NMDAR may define the threshold for the induction of LTP or LTD (Yashiro and Philpot 2008). This hypothesis predicts that the same frequency of stimulation may produce different outcomes in synaptic plasticity depending on the ratio of GluN2A/GluN2B at the synapse. According to this model, in synapses containing a higher levels of GluN2B the influx of Ca^{2+} is more likely to activate CaMKII, even with a modest synaptic activity, and therefore to activate LTP pathways. In this type of synapses, only a very weak stimulation would activate calcineurin to induce LTD. In contrast, when GluN2A-containing NMDAR are more abundant at the postsynaptic membrane, Ca^{2+} entry is limited and/or there is less

CaMKII brought to the synapse, thereby increasing the threshold stimulation requirements for LTP induction. Consequently, a low GluN2A/GluN2B ratio favors LTP induction, while synapses with a higher GluN2A/GluN2B ratio are more likely to undergo LTD (Yashiro and Philpot 2008). Taking this into account, we can hypothesize that the effects of BDNF on LTP induction (Leal *et al.* 2015) may be mediated by an increase in synaptic surface GluN2B through synaptic accumulation/activation of Pyk2. GluN2B-containing receptors are also preferentially expressed in smaller dendritic spines (Sobczyk *et al.* 2005, Shinohara *et al.* 2008) which are more likely to undergo LTP than larger spines (Matsuzaki *et al.* 2004). Furthermore, experience-dependent upregulation of the GluN2A/GluN2B ratio increases the threshold needed for LTP induction in the visual cortex (Philpot *et al.* 2007). Altogether, the available evidence suggest a novel mechanism by which BDNF may reduce the threshold required for LTP induction, through the synaptic accumulation/activation of Pyk2 which ultimately increases GluN2B surface expression. However, the contradictory results aforementioned raise the need of additional studies to fully understand the role of Pyk2 in synaptic plasticity, as well as to fully characterize the responses of Pyk2 to different synaptic plasticity paradigms, not only *in vitro* but also *in vivo*. Future studies should also address the regulation of Pyk2 under specific behavioral contexts that privileges learning and memory.

The putative role of Pyk2 in memory and learning

Pharmacological and genetic evidence have shown a role for NMDAR in synaptic plasticity and memory (Morris *et al.* 1986, Sakimura *et al.* 1995, Tsien *et al.* 1996, Kiyama *et al.* 1998, Nakazawa *et al.* 2002, Nakazawa *et al.* 2003, McHugh *et al.* 2007, Lee and Silva 2009, Morris 2013, Cercato *et al.* 2014). In particular, the development of the "*doogie"* mouse, characterized by an overexpression of the GluN2B subunit in the adult forebrain, was the first widely-publicized "smart" mouse (Tang *et al.* 1999). More recently, several approaches were used to enhance cognition in mice by increasing the expression/activity of GluN2B-NMDAR, through an increase in their transport (Wong *et al.* 2002) or by decreasing their degradation (Hawasli *et al.* 2007). The expression of the neurotrophin BDNF has also been genetically manipulated to modulate cognition. The latter studies showed that *BDNF* KO mouse or specific BDNF deletion in the hippocampus results in impaired synaptic plasticity, learning and memory (Korte *et al.* 1996, Patterson *et al.* 1996, Pozzo-Miller *et al.* 1999, Heldt *et al.* 2007). It was also observed that the deletion of TrkB receptor induces phenotypes similar to those exhibited by *BDNF* KO mice (Minichiello *et al.* 1999). In contrast, transgenic neuronal overexpression of tPA, which converts pro-BDNF to mature-BDNF, enhances both LTP and hippocampus-dependent spatial memory (Madani *et al.* 1999, Pang *et al.* 2004).

Herein, we found that overexpression of Pyk2 is sufficient to increase the surface expression of GluN2B-NMDAR. Although no behavioral studies were reported for *Pyk2* knockout mouse (Okigaki *et al.* 2003), *STEP* knockout mice which exhibit an upregulation in the activation/phosphorylation of Pyk2 showed enhanced performance in hippocampal-dependent learning and memory tasks (Venkitaramani *et al.* 2011). In addition, *STEP* knockout mice displayed enhanced phosphorylation of ERK1/2 and GluN2B (Y1472) with concomitant increase in synaptosomal expression of GluN1/GluN2B-NMDAR, providing a potential molecular mechanism for the improved cognitive

performance (Venkitaramani *et al.* 2011). Thus, an extensive synaptic and behavioral characterization of *Pyk2* KO mouse should be performed. It would also be interesting to generate and characterize mice overexpressing Pyk2 in the hippocampus in the case changes are detected in the behavior of the *Pyk2* knockout animals. We also found that an acute application of BDNF increases the total and synaptic surface expression of GluN2B, which may explain the improved performance in spatial orientation learning tasks exhibited by the transgenic mice overexpressing tPA (Madani *et al.* 1999) Furthermore, our findings suggest that the BDNF-induced enrichment of GluN2B-NMDAR at the post-synaptic membrane in hippocampal neurons is mediated by Pyk2. Therefore, it will be of interest to investigate whether Pyk2 genetic or pharmacologic manipulation in the hippocampus of mice with impaired cognitive functions, such as *TrkB* cKO mice, could (totally or partially) revert the phenotype and restores synaptic plasticity properties.

Taken together these findings suggest that Pyk2 may be involved in learning and memory processes, and therefore it may be a good target for intervention in cases where cognition is impaired.

The possible role of Pyk2 in brain disorders

BDNF and NMDAR have been widely implicated in neurodegenerative and psychiatric disorders, including schizophrenia, Huntington and Alzheimer diseases (Zuccato and Cattaneo 2009, Lu *et al.* 2013, Zhou and Sheng 2013), as well as in drug addiction (Autry and Monteggia 2012, Sanz-Clemente *et al.* 2013). In the present study we reported that Pyk2 mediates the regulation of NMDAR by BDNF, suggesting that Pyk2 may be a novel target for these severe disorders.

Huntington´s Disease

NMDAR dysregulation contributes to the pathogenesis of several neurological disorders, including Huntington´s disease (HD) (Arundine and Tymianski 2003, Lau and Zukin 2007, Gladding and Raymond 2011, Zhou and Sheng 2013). HD is a genetic disease caused by a CAG triplet repeat expansion in the *HTT* gene, which encodes huntingtin (Group 1993). The disease is characterized by the degeneration of striatal medium spine neurons (MSN), the major neuronal population in the striatum, which are enriched in NMDAR. Increased levels of extra-synaptic NMDAR are observed in the HD mouse model YAC128, and their activation appears to contribute to the vulnerability of MSN to excitotoxic injury caused by mutant huntingtin protein (mtHTT) (Okamoto *et al.* 2009, Milnerwood *et al.* 2010). Studies performed in this HD mouse model showed that the extra-synaptic NMDAR are mainly GluN2B-NMDAR. Furthermore, this receptor subtype was found to provide a more important contribution to the total NMDA-evoked current in D2 dopamine receptor-containing MSN than in D1-containing MSN in HD mouse model, consistent with the earlier degeneration of D2 MSN in the disease (Jocoy *et al.* 2011). Likewise, crossing GluN2B-overexpressing mice with HD model mice exacerbates the death of MSN (Heng *et al.* 2009). Together, these results suggest that alterations in the balance between synaptic and extra-synaptic GluN2B-containing NMDAR may mediate the excitotoxic damage in HD. Thus, the protein machinery that regulates the localization and trafficking of NMDAR may have an important role in this neurodegenerative condition.

One hypothesis concerning the mechanism whereby mtHTT causes neuronal death proposes that the mutant protein induces mitochondrial defects through selective inhibition of mitochondrial complex II-succinate dehydrogenase (Brouillet *et al.* 1995, Gu *et al.* 1996, Brouillet *et al.* 1998), leading to aberrant Ca^{2+} homeostasis (Panov *et al.* 2002, Panov *et al.* 2005) and consequent calpain activation. Several studies have reported that the Ca^{2+}-dependent protease calpain cleaves the GluN2B C-terminus (Guttmann *et al.* 2001, Guttmann *et al.* 2002) thereby altering the surface NMDAR distribution, increasing its extra-synaptic localization (Gladding *et al.* 2012). Recent studies also showed that synaptic $STEP_{61}$ activity is significantly higher in the YAC128 striatum, which correlates with a decreased GluN2B Y1472 phosphorylation and contributes to the extra-synaptic NMDAR localization in the HD mouse model (Gladding *et al.* 2012). BDNF regulates $STEP_{61}$ activity by promoting its degradation through the UPS (Saavedra *et al.* 2015, Xu *et al.* 2015). Moreover, it was observed that: i) BDNF protein levels are reduced in postmortem of individuals with HD (Ferrer *et al.* 2000); ii) mutation in HTT gene causes decreased BDNF protein levels in striatum (Zuccato *et al.* 2001); iii) *Emx*-$BDNF^{KO}$ mice, which were genetically engineered to be deficient in BDNF production in the cerebral cortex, gradually develop brain damage and behavioral abnormalities in a pattern very similar to mouse models of HD (Baquet *et al.* 2004, Strand *et al.* 2007). These results indicate that BDNF dysregulation in HD may mediate the increased activity in $STEP_{61}$, which regulates the localization of GluN2B-NMDAR. Herein, we found that kinase activity of Pyk2 is a crucial player in the BDNF-mediated regulation of GluN2B-NMDAR trafficking. In addition, this kinase activity is downregulated by $STEP_{61}$ (Venkitaramani *et al.* 2011, Xu *et al.* 2012, Saavedra *et al.* 2015, Xu *et al.* 2015). Therefore, Pyk2 may play an important role in HD and the activity of this kinase in the post-synaptic compartment may be a good candidate for pharmacological or genetic manipulation to ameliorate the symptoms of this disease. However, before reaching this point additional studies are required to specifically understand the putative role of Pyk2 in the pathology.

Alzheimer´s Disease

Early studies have demonstrated that BDNF levels are reduced in the dentate gyrus of patients with Alzheimer´s disease (AD) (Narisawa-Saito *et al.* 1996). More recently, it was proposed that reduced BDNF serum levels are associated with ApoE4, and ApoE4 together with amyloid-β (Aβ) oligomers downregulate BDNF expression through histone deacetylase (HDAC) nuclear translocation (Alvarez *et al.* 2014, Sen *et al.* 2015). Alterations in synaptic plasticity have also been reported in AD, which contribute to the neuropathological and clinical manifestations of the pathology (Mesulam 1999). However, the evidence about the role of BDNF polymorphism in this type of dementia are still inconclusive.

In the present study we proposed that BDNF is an active player in the regulation of NMDAR, in particular the GluN2B-containing receptors. This particular subunit has been widely involved in the AD disease, due to its large extra-synaptic localization. Indeed, multiple lines of evidence have emerged suggesting that the disturbance of synaptic plasticity induced by soluble oligomeric forms of Aβ can be potentiated by the activity of extra-synaptic GluN2B-NMDAR (Sheng *et al.* 2012, Zhou and Sheng 2013). Thus, LTP was found to be impaired, while LTD is facilitated by Aβ (Ondrejcak *et al.* 2010), and

GluN2B antagonists were shown to (i) rescue Aβ-induced impairment of LTP (Li *et al.* 2011, Ronicke *et al.* 2011), (ii) Aβ-induced loss of synapses and synaptic proteins (Liu *et al.* 2010, Ronicke *et al.* 2011) and (iii) Aβ-evoked facilitation of LTD (Li *et al.* 2009) and targeting of Aβ to synapses (Deshpande *et al.* 2009). Studies using non-selective NMDAR antagonists showed similar effects in blocking the Aβ-induced spine loss and impaired LTP (Shankar *et al.* 2007, Rammes *et al.* 2011), suggesting a general role for NMDAR in synaptic dysfunction induced by Aβ oligomers.

More important than the subunit composition of NMDAR is probably their localization. It was proposed that activation of synaptic NMDAR increases the non-amyloidogenic α-secretase-mediated altered amyloid precursor protein (APP) processing and inhibits the release of Aβ (Hoey *et al.* 2009), while activation of extra-synaptic NMDAR increases neuronal production of Aβ (Bordji *et al.* 2010). Therefore, our results showing a BDNF-induced upregulation and activation of Pyk2, with an impact in the trafficking of GluN2B-NMDAR, suggest that BDNF and Pyk2 may play a role in Alzheimer´s disease by regulating the production of Aβ. We also proposed a model according to which Pyk2 is specifically activated at synaptic compartments by BDNF, and this activation was found to be required for the increased GluN2B surface levels. This model privileges the increase in synaptic GluN2B-NMDAR, rather than an effect on the extra-synaptic pool of receptors, in response to BDNF stimulation, which may be therapeutically useful in Alzheimer disease. Therefore, it would be interesting to study the role and the regulation of Pyk2 in AD models, as well as the effect of BDNF-induced increase in density of excitatory synapses observed in our study.

Overall, dysregulation of the machinery involved in the BDNF-mediated control of GluN2B trafficking, including the synthesis and activation of Pyk2, may be a good therapeutic target for diseases characterized by a malfunctioning of NMDAR and synaptic loss, such as neurodegenerative diseases. However, it will also be important to understand how Pyk2 may regulate the balance between LTP and LTD. The answer to these questions will create opportunities to explore the role of BDNF/Pyk2/GluN2B-containing NMDAR axis in several biological contexts.

Chapter 6

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