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Regulation of synaptic transmission by BDNF

Effects on NMDA receptor-mediated mEPSCs

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Carlos B. Duarte (Departamento de Ciências da Vida, Universidade de Coimbra) e da Doutora Miranda Mele (Centro de Neurociências e Biologia Celular, Universidade de Coimbra)

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Abbreviations

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

BDNF, brain-derived neurotrophic factor

$[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration

CaMKII, Ca^{2+} - and calmodulin-dependent protein kinase II

CaMKIV, Ca^{2+} - and calmodulin-dependent protein kinase IV

CHX, cycloheximide

CNS, central nervous system

Con G, conantokin G

CREB, cAMP response element-binding protein

DIV, days in vitro

EAAT, excitatory amino acid transporter

EPSC, excitatory postsynaptic currents

FAK, focal adhesion kinase

FERM, F for 4.1 protein, E for ezrin, R for radixin and M for moesin

G-proteins, guanine nucleotide-binding proteins

HD, Huntington's disease

hnRNPs, heterogeneous nuclear ribonucleoproteins

iGluR, ionotropic glutamate receptor

KAR, kainate receptor

KI region, K-protein-interactive region

LTP, long-term synaptic potentiation

MAP2, microtubule-associated protein type 2

mEPSC, miniature excitatory postsynaptic currents

mGluR, metabotropic glutamate receptor

MSN, medium spine neurons

NGF, nerve growth factor

NLS, nuclear localization signal

NT-3, neurotrophin-3

NT-4/5, neurotrophin-4/5

NMDA, N-methyl-D-aspartate

NMDAR, N-methyl-D-aspartate receptor

p75^{NTR}, p75 pan-neurotrophin receptor

PH, pleckstrin homology

PI3-K, phosphatidylinositide 3-kinases
PLC γ , phospholipase C, gamma isoform
PSD, postsynaptic density
PSD95, postsynaptic density protein-95
Pyk2, proline-rich tyrosine kinase 2
STEP, STEP (striatal-enriched protein tyrosine phosphatase
Trk, tropomyosin-receptor kinase
TRPC channels, transient receptor potential canonical channels
vGluT1, vesicular glutamate transporter, type 1

Key words

Synaptic plasticity, NMDA receptors, mEPSC, neurotrophins, BDNF

Abstract

Brain derived neurotrophic factor (BDNF) plays an important role in long-term synaptic potentiation (LTP) in the hippocampus, partially due to the upregulation of translation activity. However, the molecular mechanisms are not fully understood. In this work we investigated the effect of BDNF on the surface expression of N-methyl-D-aspartate receptors, which play a key role in synaptic plasticity. Stimulation of cultured hippocampal neurons with BDNF induced the synaptic accumulation of GluN2B-containing NMDAR, as determined by immunocytochemistry with an antibody against the N-terminal region of the receptor subunit and by colocalization with pre- and post-synaptic markers, the vesicular glutamate transporter type 1 (vGlut1) and postsynaptic density protein 95 (PSD-95), respectively. The effect of BDNF on the amplitude and frequency of NMDAR-mediated miniature excitatory postsynaptic currents (mEPSCs) was also evaluated under the same conditions by voltage-clamp recordings. Incubation of cultured hippocampal neurons with BDNF upregulated the amplitude of NMDA receptor-mediated mEPSC, but did not change the rise and decay time. The enhancement in the amplitude of the currents was abrogated by the GluN2B competitive antagonist Conantokin G, in agreement with the results obtained in the immunocytochemistry experiments. Antibody-feeding experiments showed no significant alteration in the rate of internalization of GluN2B-containing NMDA receptors following incubation of the neurotrophin, suggesting that a different mechanism is involved. The BDNF-evoked upregulation of GluN2B surface expression, as well as the increase in the amplitude of mEPSC, were abrogated by the protein synthesis inhibitor cycloheximide, suggesting a role for local translation activity. Based on unpublished observations from the laboratory showing a key role for the hnRNP K (heterogeneous nuclear ribonucleoprotein K) RNA binding protein in the regulation of protein synthesis by BDNF, we characterized the role of this protein in the neurotrophin-induced upregulation of NMDA receptor mediated mEPSC. Transfection of hippocampal neurons with an shRNA against hnRNP K suppressed the effects of BDNF on the amplitude of mEPSC. Considering the available evidence showing that BDNF upregulates Pyk2 protein levels in hippocampal neurons by a mechanism dependent on hnRNP K, we hypothesized that de novo synthesis of the kinase could play a role in the regulation of the synaptic expression of GluN2B-containing NMDA receptors by BDNF. Transfection of hippocampal neurons with an shRNA specific for Pyk2 abrogated the BDNF-evoked increase in the amplitude of the NMDA receptor-mediated mEPSC, showing a role for the kinase as a mediator of the TrkB-BDNF signaling to regulate the activity of NMDA receptors.

BDNF also increased the frequency of NMDA receptor mediated mEPSC in cultured hippocampal neurons, by a mechanism sensitive to cycloheximide and possibly dependent on the presence of hnRNP K. This shows a presynaptic response to BDNF in glutamatergic synapses, which requires protein synthesis.

Together, the results show a novel effect of BDNF in the regulation of the synaptic expression of NMDA receptors that is mediated by the RNA binding protein hnRNP K, as well as by the Pyk2 tyrosine kinase. These alterations in the surface expression of NMDA receptors may play an important role in LTP in the hippocampus.

Resumo

O factor neurotrófico derivado do cérebro (BDNF) desempenha um papel importante na potenciação sináptica de longa duração (LTP) no hipocampo, em parte através da regulação da síntese proteica. No entanto, os mecanismos moleculares envolvidos não estão inteiramente esclarecidos. Neste trabalho investigámos o efeito do BDNF na expressão superficial dos receptores do glutamato do tipo N-metil-D-aspartato (NMDAR), os quais desempenham um importante papel nos mecanismos de plasticidade sináptica. A estimulação de neurónios do hipocampo em cultura com BDNF induziu a acumulação sináptica de receptores NMDA contendo a subunidade GluN2B, detectada com recurso a experiências de imunocitoquímica usando um anticorpo com afinidade para o terminal amínico desta subunidade (domínio extracelular), e através da colocalização com os marcadores pré- (transportador vesicular do glutamato [vGlut1]) e pós- sinápticos (PSD-95). O efeito do BDNF na amplitude e na frequência das correntes miniatura excitatórias pós-sinápticas espontâneas (mEPSCs) foi estudado através de *voltage-clamp*. A estimulação com BDNF aumentou a amplitude das mEPSCs mediada pelos receptores NMDA, não tendo sido observado qualquer efeito no tempo de elevação e de decaimento das correntes. O aumento da amplitude das mEPSCs em resposta ao BDNF foi inibido na presença do antagonista competitivo para a subunidade GluN2B, Conantoquina G, de acordo com os resultados obtidos nas experiências de imunocitoquímica. Ensaio utilizando a técnica de *antibody-feeding* mostraram que não existe qualquer alteração estatisticamente significativa na taxa de internalização dos receptores NMDA contendo a subunidade GluN2B em neurónios incubados com BDNF, o que sugere a participação de um mecanismo alternativo nas alterações da expressão superficial dos receptores. O aumento da expressão superficial das subunidades GluN2B, bem como das mEPSCs, em neurónios estimulados com BDNF, foram completamente inibidos na presença do inibidor da síntese proteica cicloheximida, sugerindo um papel importante da síntese proteica na resposta à estimulação com a neurotrofina. Tendo em conta resultados não publicados do nosso laboratório que mostram um papel importante da hnRNP K (ribonucleoproteína nuclear heterogénea do tipo K) na regulação da síntese proteica promovida pelo BDNF, caracterizámos a função desta proteína no aumento das mEPSCs mediadas pelos receptores NMDA em resposta à estimulação com BDNF. O efeito da neurotrofina no aumento das mEPSCs foi suprimido em neurónios infectados com um shRNA específico para a hnRNPK. Tendo em conta estudos anteriores que mostraram um aumento dos níveis da proteína Pyk2 em neurónios de hipocampo estimulados com BDNF, por um mecanismo dependente de hnRNPK, colocámos a hipótese da síntese de novo da cinase representar um passo

essencial na regulação da expressão sináptica dos receptores NMDA contendo a subunidade GluN2B em resposta à estimulação com BDNF. A transfecção de neurónios do hipocampo com um shRNA específico para a Pyk2 anulou o efeito do BDNF nas mEPSC mediadas pelos receptores NMDA, demonstrando um papel central desta cinase na sinalização pelo complexo TrkB-BDNF e na regulação da actividade dos recetores NMDA.

Foi também observado um aumento na frequência das mEPSC mediada pelos receptores NMDA em resposta ao BDNF, através de mecanismo sensível à ciclohexamida. Estes resultados mostram um efeito pré-sináptico do BDNF nas sinapses glutamatérgicas, bem como a necessidade da síntese proteica para a ocorrência destas alterações. Além disso, os resultados obtidos mostraram que o efeito do BDNF requer a presença da hnRNPK.

Em conclusão, os resultados obtidos mostram um efeito nunca reportado do BDNF na expressão sináptica dos recetores NMDA, e em particular o papel da hnRNPK e da Pyk2 como intermediários nestes efeitos da neurotrofina. Estas alterações na expressão superficial dos recetores NMDA deverão desempenhar um papel importante na regulação da LTP no hipocampo.

Palavras-chave

Plasticidade sináptica, receptores NMDA, mEPSC, neurotrofinas, BDNF

Introduction

The brain is the center of the nervous system and the most complex biological structure known. The human brain contains more than 10^{11} (100 billion) neurons which are connected with each other at the synapses, forming a wide and complex network in the CNS (Central Nervous System). Information is propagated within neurons in the form of electrical signals, while the propagation of information between them is made through specialized junctions named synapses. The extremely fast communication between neurons at the synapses is a unique feature of neurons.

The cells of the nervous system can be divided in two categories: nervous cells or neurons, involved in synaptic transmission, and glial cells, which provide support to neurons and play a role in their development (Purves, 2004). The structure of a neuron is composed by the following compartments: (i) a cell body (or soma), which contains the nucleus and organelles, (ii) the dendrites, which form an arborized net that receives information from neighbor cells at the synapses and (iii) the axon, which is an extension of few microns from the cell body specialized in sending signals (coming from the dendrites) to other neurons. Once these electrical signals that travel along the axon arrive at the nerve terminal, they lead to the release of neurotransmitters, which are the chemical signals involved in neuron-to-neuron communication. The physical and chemical processes that occur at the synaptic level, decoded from actions potential, are called synaptic transmission. There are two different types of synaptic transmission: chemical transmission, which is mediated by neurotransmitters involved in the communication between the pre- and post-synaptic neuron, and the less common but faster electrical transmission (Purves, 2004). In the electrical synapses the communication between neighbor neurons is mediated by the flow of cations through adjacent channels present in the membrane of each of the neurons (Decrock et al., 2015). The release of neurotransmitters to the synaptic cleft in chemical synapses is mediated by Ca^{2+} influx through voltage-gated channels, which gives rise to a transient increase in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) within the pre-synaptic terminal, leading to the fusion of synaptic vesicles with the pre-synaptic membrane. The pre-synaptic neuron releases a “quantum” of neurotransmitter into the synaptic cleft through exocytosis, a process that is mediated by several proteins (Rizo and Xu, 2015). After the release to the synaptic cleft, the neurotransmitters can bind specific receptors on the post-synaptic membrane before being degraded, recycled or they may diffuse to non-synaptic regions.

Introduction

1.1. Glutamatergic synapses

Glutamate is the major excitatory neurotransmitter in the mammalian CNS (Watkins and Jane, 2006). It is synthesized from α -ketoglutarate or glutamine (McKenna, 2007) and is then accumulated into small synaptic vesicles through the activity of specific vesicular glutamate transporters (vGluts) (Takamori, 2006). These synaptic vesicles are accumulated at the active zones within the nerve terminal, and are readily exocytosed following presynaptic activation. At the synaptic cleft neurotransmitters bind to specific receptors localized on the postsynaptic membrane thereby regulating the activity of the target neuron. Furthermore, glutamate may also interact with presynaptic receptors which modulate neurotransmitter release.

There are two major classes of postsynaptic receptors for glutamate: the ionotropic glutamate receptors (iGluRs), which are linked to ion channels, and the metabotropic glutamate receptors (mGluRs), so called because their effects are mediated through regulation of guanine nucleotide-binding proteins (G-proteins) (Hollmann and Heinemann, 1994). Ionotropic glutamate receptors are classified in three categories, named NMDA (N-methyl-D-aspartate) receptors (NMDAR), AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (AMPA), and kainate receptors (KAR), which possess different molecular, electrophysiological and pharmacological properties (see below). Metabotropic glutamate receptors belong to the class of seven transmembrane domain receptors. They possess an extracellular N-terminal domain, which contains the neurotransmitter binding site, and an intracellular C domain where the G-protein binds. Neurotransmitter binding to metabotropic receptors activates G-proteins, which dissociate from the receptors before interacting with the effector proteins (Rondard and Pin, 2015).

Under normal physiological conditions, the activation of glutamate receptors at the synapse is a transient process, since the neurotransmitter is rapidly removed through uptake by excitatory amino acid transporters (EAATs). These transporters are particularly active in astrocytes, allowing the accumulation of glutamate into these cells. Glutamate is metabolized to glutamine which is then transported from the astrocytes to the nerve terminals, where it can be used to synthesize glutamate (McKenna, 2007).

The structure and function of the postsynaptic region in excitatory synapses are highly specialized, and they are usually located on the edge of a dendritic spine (Sheng and Kim, 2011). The synaptic region at the tip of the spines is typically electron-dense when observed in electromicrographs, and therefore it was named postsynaptic density (PSD) (Sheng and Kim, 2002). The PSD contains a

variety of scaffolding and signaling proteins, which allow the attachment of receptors and other molecules in the postsynaptic membrane (through interaction with actin filaments) and the efficient coupling to the regulation of signaling pathways. A large number of proteins have been identified to reside in the post-synaptic density (close to 400) (Peng et al., 2004). These proteins are dynamic, undergoing a continuous turnover by mechanisms that are regulated in response to changes of neuronal activity (Sheng and Kim, 2011).

1.1.1. NMDA receptors

NMDAR have been shown to play important roles in numerous physiological processes in the mammalian brain, including in synaptic transmission, synaptic plasticity and in neuronal development. Furthermore, alterations in the activity of NMDAR have been related with several acute and chronic brain disorders, including stroke, epilepsy, depression, schizophrenia, Alzheimer's disease, Parkinson's disease and in Huntington's disease (Lau and Zukin, 2007; Sanz-Clemente et al., 2013a; Zhou and Sheng, 2013). These receptors are widely expressed in the CNS and are mainly found on the postsynaptic region. However, NMDAR are also present at the extrasynaptic region as well as at the presynaptic terminal.

The NMDAR channels are activated by glutamate and by NMDA, and require the presence of glycine (or D-serine) which binds to an allosteric site on the receptor as co-agonist. The receptor channel is highly permeable to Ca^{2+} , but it also allows the flux of Na^{+} and K^{+} ions. NMDAR are characterized by their sensitivity to the membrane potential, and their activity requires the depolarization of the postsynaptic membrane to remove the Mg^{2+} blockade of the channel (Mayer et al., 1984). Therefore, activation of NMDAR is also dependent of the stimulation of AMPAR, which are colocalized on the postsynaptic membrane. A high-frequency presynaptic stimulation is required to release the amount of glutamate necessary to induce the AMPAR-mediated depolarization of the membrane and to remove the Mg^{2+} ions from the NMDAR channel. Given the inhibition by Mg^{2+} , NMDA receptors do not contribute to the EPSC (excitatory postsynaptic currents) until Mg^{2+} is removed from the channel to allow the flow of ions (Umemiya et al., 1999; Purves, 2004). Furthermore, these receptors are characterized by a relatively slow gating kinetics and contribute to the late component of EPSC.

Introduction

NMDAR are assembled in the endoplasmic reticulum and are transported to the plasma membrane once the quality control mechanisms have checked the correct folding of the subunits. This family of ionotropic glutamate receptors is comprised by heteromeric channels formed by the assembly of different subunits: GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A and GluN3B (Fig. 1.1). These subunits are differentially expressed in the CNS and throughout development. The GluN1 and GluN3 subunits bind glycine (and D-serine), whereas glutamate (and NMDA) interact with GluN2 (Furukawa and Gouaux, 2003; Furukawa et al., 2005; Yao et al., 2008). Most receptors are tetrameric structures containing two GluN1 subunits and two GluN2 subunits (Traynelis et al., 2010). Although it has been suggested that NMDAR form diheteromeric structures, recent studies suggest that a significant fraction of the native receptors are triheteromers formed by the assembly of two GluN1 subunits with two different GluN2 subunits. Accordingly, it was calculated that more than 50% of the total NMDAR present in the hippocampus as well as in the cortex of the adult rodent brain are GluN1/GluN2A/GluN2B triheteromers (Sheng et al., 1994; Luo et al., 1997; Al-Hallaq et al., 2007; Rauner and Kohr, 2011; Tovar et al., 2013). Early observations suggested 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 as additional studies showed the presence of GluN2A in the extrasynaptic compartment in cultured neurons (e.g. (Thomas et al., 2006)), and GluN2B was also found at the postsynaptic density (Shinohara et al., 2008). Furthermore, live imaging studies showed that GluN2-containing NMDAR can diffuse in and out of the synapse, thereby regulating the relative distribution of the various NMDAR subunits (Groc et al., 2004; Groc et al., 2006; Groc and Choquet, 2008; Triller and Choquet, 2008; Choquet and Triller, 2013; Ladepeche et al., 2014). GluN2B-containing NMDAR are more mobile (about 250 fold) than GluN2A, and this mobility is regulated by different mechanisms as indicated below.

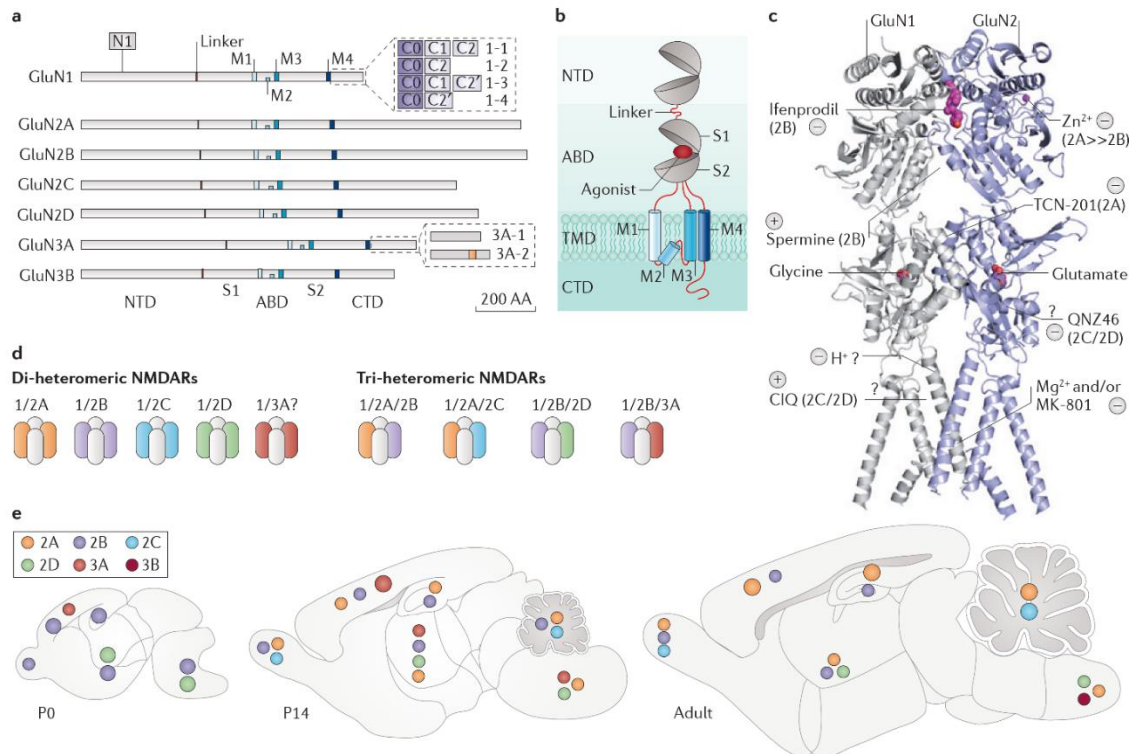


Figure 1.1. NMDAR subunit diversity, structure and expression. (a) Seven NMDAR subunits have been identified: GluN1, GluN2A – GluN2D and GluN3A and GluN3B. The heterogeneity in NMDAR subunits is further enhanced by alternative splicing of GluN1 and GluN3A subunits. M1 – M4 indicate membrane segments. (b) All GluN subunits share a modular architecture that is made of four distinct domains: the N-terminal domain (NTD), the agonist-binding domain (ABD) which binds to glycine or D-serine in GluN1 and GluN3, and glutamate in GluN2, the transmembrane domain (TMD) containing the ion channel, and an intracellular C-terminal domain (CTD). (c) NMDAR are endowed with multiple binding sites for extracellular small-molecule ligands acting as subunit-selective allosteric modulators. A model of a GluN1/GluN2 heterodimer based on the X-ray crystal structures of GluN1/GluN2B NTDs³⁹, GluN1/GluN2A ABDs⁴⁰ and the AMPA receptor GluA2 pore region 43 is shown. The + and – signs indicate positive and negative allosteric modulators, respectively. Question marks (?) indicate uncertainty concerning the exact location of the binding site. (d) A sample of the various populations of di-heteromeric and tri-heteromeric NMDARs that are thought to exist in the CNS is shown. (e) The developmental profile of GluN subunit expression in the mouse brain at day of birth (postnatal day 0 (P0)), 2 weeks following birth (P14) and at the adult stage. Adapted from (Paoletti et al., 2013).

Introduction

The subcellular distribution of GluN2A and GluN2B subunits is a relevant issue since they confer different properties to the NMDAR. Receptors containing the former subunit are characterized by a faster kinetics when compared with the receptors formed by the assembly of GluN1+GluN2B (Vicini et al., 1998; Gray et al., 2011). Furthermore, studies performed in an heterologous system showed that GluN2A-containing NMDAR have a higher open probability and a faster deactivation time (Vicini et al., 1998; Erreger et al., 2005). These results were supported by more recent studies performed in CA1 pyramidal neurons (Gray et al., 2011).

Different posttranslational modifications have been shown to regulate GluN2A and GluN2B-containing NMDAR, including palmitoylation, nitrosylation and phosphorylation (Sanz-Clemente et al., 2013a; Lussier et al., 2015). Despite the homology between GluN2A and GluN2B in their C-terminal region, they are differentially regulated by phosphorylation (Fig. 1.2). The activity of GluN2B-containing NMDAR is regulated by phosphorylation of several different residues, mediated by distinct protein kinases which regulate the surface expression of the receptors. The Fyn/Src-mediated phosphorylation of Tyr1472 upon synaptic activity prevents the internalization of NMDAR by impairing the interaction with the clathrin adaptor AP-2 which mediates the internalization of the receptors. Therefore, synaptic activity increases GluN2B surface expression by preventing their endocytosis (Lavezzari et al., 2003; Prybylowski et al., 2005; Sanz-Clemente et al., 2010). A recent study showed that Fyn mediated GluN2B phosphorylation on Tyr1070 up-regulated the phosphorylation of the receptor subunit at Tyr-1472 (Lu et al., 2015). The phosphorylation of GluN2B by Fyn/Src is facilitated by the interaction of the kinases with PSD scaffold proteins (e.g. PSD and SAP102), which maintains a pool of kinase molecules at the synapse. GluN2B phosphorylation by casein kinase 2 (on Ser1480) antagonizes the effect of Fyn/Src by decreasing the interaction of the receptors with the scaffold proteins (Chung et al., 2004; Chen et al., 2012). Therefore, GluN2B phosphorylation on Ser1480 enhances the diffusion of NMDAR to extrasynaptic sites. At the same time, this redistribution of the receptors downregulates GluN2B phosphorylation on Tyr1472. Synaptic activity also enhances the interaction of GluN2B with the Ca²⁺- and calmodulin-dependent protein kinase II (CaMKII), with a consequent phosphorylation of the receptor subunit on Ser1303. However, this is a transient process since GluN2B phosphorylation on this residue decreases the affinity of the receptors for CaMKII (O'Leary et al., 2011; Sanz-Clemente et al., 2013b). Since casein kinase 2 interacts with the active form of CaMKII, the interaction of the latter kinase with GluN2B

induced by synaptic activity also favors the phosphorylation on Ser1480, which dissociate the receptors from the scaffold proteins. The GluN2B Ser1303 and Ser1166 residues are also phosphorylated by protein kinase C and PKA, respectively. The phosphorylation of GluN2B by PKA enhances NMDAR-dependent Ca^{2+} permeability (Aman et al., 2014). GluN2A containing NMDAR are also regulated by phosphorylation, but the residues involved are upstream of the C-terminal end of the protein. Dyrk 1a mediated phosphorylation of GluN2A on Ser1048 also increases the surface expression of the receptors by decreasing the rate of internalization (Grau et al., 2014), but the mechanisms involved still remain to be elucidated. Furthermore, it remains to be investigated how the different regulatory mechanisms interact in the regulation of the surface distribution of NMDAR contain GluN2B and GluN2A subunits.

1.1.2. AMPA receptors

AMPA are the ionotropic glutamate receptors that mediate the fast component of excitatory neurotransmission. They are characterized by their high affinity for AMPA and are permeable to Na^+ and K^+ ; depending on their subunit composition AMPAR may also display some permeability to Ca^{2+} (Fig. 1.3). Similarly to other ionotropic receptors, AMPA receptors are also composed by different but highly homologous subunits, named GluA1, GluA2, GluA3, GluA4, which are encoded by four different genes (Hollmann and Heinemann, 1994). These receptors are tetrameric assemblies, and the presence of GluA2 subunits makes the receptors impermeable to Ca^{2+} (Burnashev et al., 1992).

AMPA receptors are very dynamic entities, and their traffic in and out of the synapse plays a key role in activity-dependent plasticity of glutamatergic synapses. These alterations in the synaptic distribution of AMPAR are mediated by posttranslational modifications of their subunits, namely by phosphorylation in the C-terminal region, which regulates the interaction with intracellular proteins (Shepherd and Huganir, 2007; Kessels and Malinow, 2009; Bassani et al., 2013; Chater and Goda, 2014).

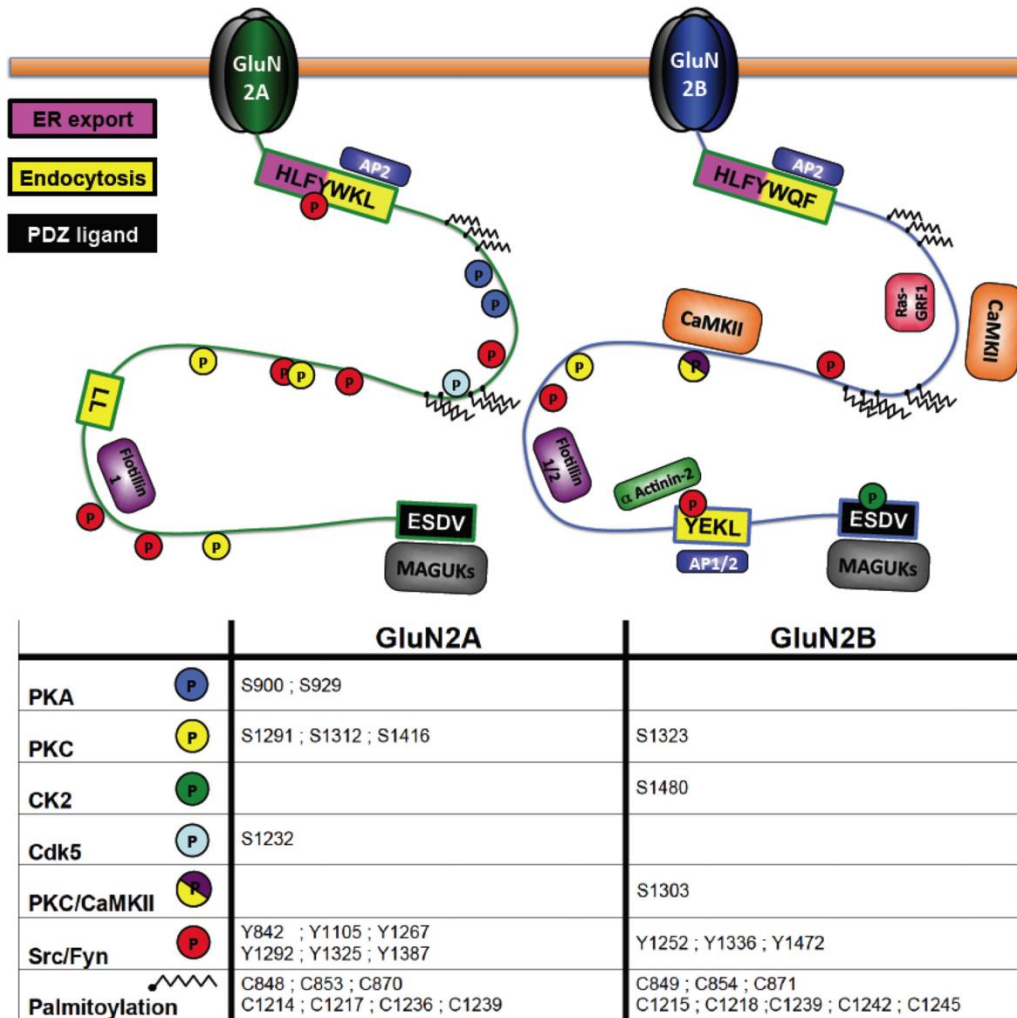


Figure 1.2. GluN2A and GluN2B regulatory motifs present in the C-terminal region of the NMDAR subunits. Phosphorylation and palmitoylation sites are represented, as well as protein-protein interaction domains. Not all phosphorylation sites are indicated in the figure.

1.2. Plasticity of glutamatergic synapses

The brain is continuously subjected to modifications due to the experience. Glutamatergic and GABAergic synapses can adjust their strength depending on the pattern of activation. Pioneering studies performed by Bliss, Gardner-Medwin and Lomo showed that high-frequency stimulation of the perforant path fibers enhances synaptic transmission between the stimulated axons and the dentate areas of the hippocampus (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). This

phenomenon is named long-term synaptic potentiation (LTP) and is thought to underlie learning and memory formation.

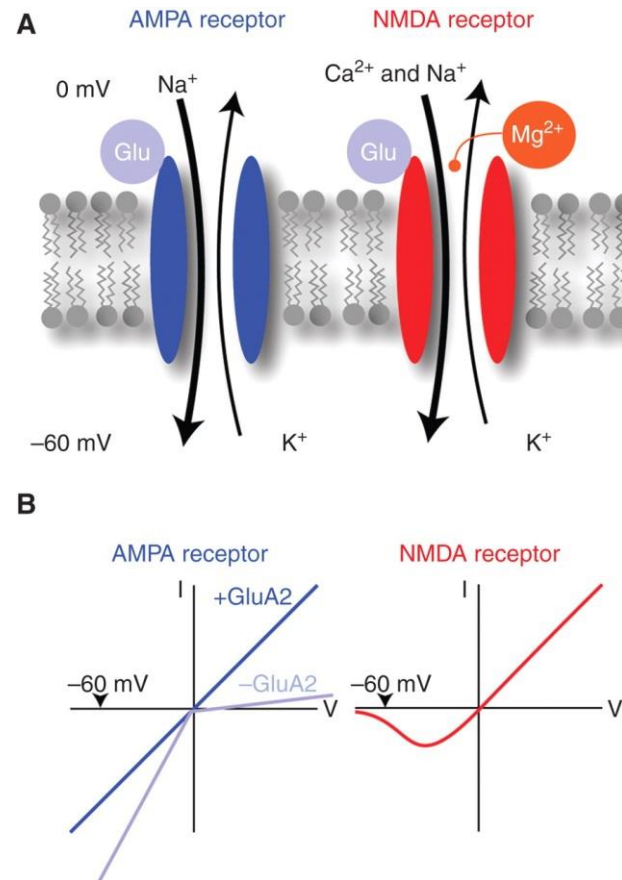


Figure 1.3. Schematic representation of AMPA and NMDA receptor channels and their biophysical properties. **A.** Glutamate binding to AMPAR induces a strong influx of sodium ions whereas a lower amount of potassium ions leave the neuron, causing a depolarization of the membrane. NMDA receptors are also permeable to calcium but only if the magnesium is removed from the pore of the channel by a net 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 without GluA2. NMDA receptors have a complex I–V curve because Mg²⁺ blocks the pore at negative potentials. Adapted from (Luscher and Malenka, 2012).

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There are different forms of synaptic plasticity: short-term plasticity, which refers to a form of rapid, activity-dependent modulation of synaptic efficacy (Hennig, 2013), and long-term plasticity, a slow, activity-dependent modulation of synaptic strength involved in long-term memory formation and in learning (Morris, 2003). The two forms of long-term plasticity, named respectively long-term potentiation and depression (LTP and LTD), are related to an increase or decrease of the synaptic response, respectively, and can be induced by distinct neuronal activity paradigms. For example, stimulation of an axonal fiber of the perforant pathway with a frequency higher than 100 Hz, for a few seconds, causes the influx of a high amount of Ca^{2+} through NMDAR, resulting in LTP. In contrast, a stimulus with 5-10 Hz induces the entry of lower amounts of Ca^{2+} , leading to LTD (Yashiro and Philpot, 2008). Under the former conditions, the influx of Ca^{2+} through NMDAR stimulates the post-synaptic activation of CaMKII, a protein kinase that is self-phosphorylated after dissipation of the $[\text{Ca}^{2+}]_i$ within the spine. Activation of this and other signaling pathways induce locally the insertion of more AMPA receptors into the membrane with a consequent strengthening of the synapse. Structural changes are also induced, including the formation of new synaptic boutons on post-synaptic neuron (Fig. 1.4). The activation of local protein synthesis at the synapse and alterations in gene expression play a key role in making these changes permanent (Shonesy et al., 2014).

In contrast to the response to stimulation protocols that induce LTP, low-frequency presynaptic stimulation may induce a small increase in the postsynaptic $[\text{Ca}^{2+}]_i$ which activates preferentially protein phosphatases (Purves, 2004). The resulting dephosphorylation of target proteins may contribute to decrease synaptic strength. It was hypothesized that the synaptic content in GluN2A and GluN2B subunits may determine the threshold for induction of LTP vs LTD (Yashiro and Philpot, 2008). According to this hypothesis, synapses with a higher GluN2B content may show a more robust increase in the $[\text{Ca}^{2+}]_i$ for the same conditions of stimulation and/or the entry of Ca^{2+} may be more efficiently coupled to the activation of CaMKII, thereby inducing LTP. In these synapses, a very weak stimulation would be required to preferentially activate protein phosphatases, thereby inducing LTD. In contrast, synapses characterized by a higher GluN2A/GluN2B ratio may show a limited entry of Ca^{2+} , and/or a less efficient activation of CaMKII, requiring a stronger stimulation in order to undergo LTP.

1.2.1. BDNF signaling and synaptic plasticity

Nerve growth factor (NGF) was discovered in the early 1950s due to its trophic (survival- and growth-promoting) effects on sensory and sympathetic neurons (Levi-Montalcini and Hamburger, 1951). However, later studies also showed the presence and function of NGF in the CNS (Levi-Montalcini et al., 1996). The discovery of NGF suggested the existence of analog proteins, which could play similar roles in other regions of the nervous system. This family of proteins is named neurotrophins, because they have a fundamental role in the survival of their target cells, and in addition to NGF, includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Chao, 2003).

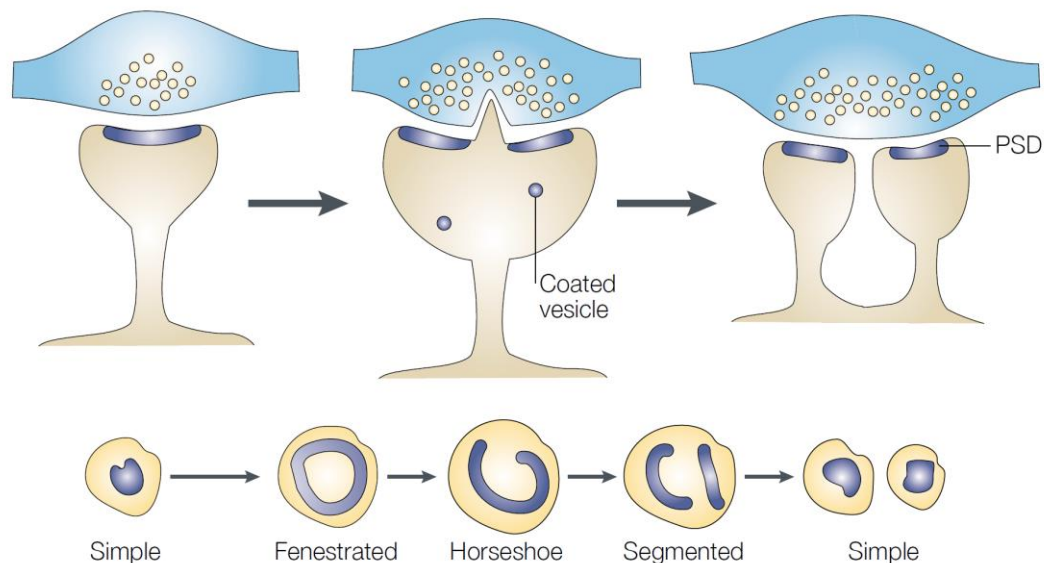


Figure 1.4. A model of changes in spine morphology after LTP. Schematic depiction of changes in dendritic spine morphology associated with long-term potentiation (LTP). Electron-microscopic analysis of putatively activated spines indicates that LTP induction results in a transient increase in 'perforated' postsynaptic densities (PSDs), followed by an increase in the number of glutamate receptors on the presynaptic terminal and multiple spine boutons (MSBs). Adapted from (Hering and Sheng, 2001).

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BDNF and its receptor (see below) are widely expressed in the CNS, especially in the hippocampus. This neurotrophin has been shown to play an important role in the regulation of synaptic transmission and in LTP in the hippocampus and in other brain regions. Furthermore, evidence is also available showing a role for BDNF in specific forms of memory (Leal et al., 2014). The *bdnf* gene (in humans mapped to chromosome 11p) has 11 exons and 9 promoter sequences which are differentially activated depending on the brain region (Pruunsild et al., 2007), giving rise to several dozen transcripts. BDNF, NGF, NT-3 and NT-4/5 have in common about 50% amino acid identity. All the neurotrophins are synthesized from a precursor protein (pro-neurotrophin) which is cleaved by specific proteases to generate the mature form of the molecule. The precursor form of BDNF, pre-pro-BDNF, is converted into pro-BDNF by removal of a signal peptide, and the protein is then transported from the trans-Golgi network into secretory granules, where it can be cleaved by a specific enzyme, prohormone convertase 1 (PC1), giving rise to the mature form of BDNF (Chao and Bothwell, 2002). The release of BDNF is mainly due to a Ca^{2+} -dependent mechanism, which contributes to the exocytosis of BDNF-containing vesicles. It has been hypothesized that some of the neurotrophin molecules may also be released in the precursor form. In this case, extracellular proteases (e.g. plasmin or metalloproteinases) may be responsible for the cleavage of proBDNF, giving rise to the mature form of the neurotrophin. To what extent BDNF/proBDNF are released from the nerve terminal and/or from dendritic spines upon synaptic stimulation also remains to be established (Leal et al., 2014; Leal et al., 2015).

Activation of two distinct classes of membrane-bound receptors mediates the physiological responses to the neurotrophins, namely the p75 pan-neurotrophin receptor ($p75^{NTR}$) and the tropomyosin-receptor kinase (Trk) receptors (TrkA-TrkC). Trk receptors belong to the family of tyrosine kinase receptors and specifically mediate the response to different neurotrophins (Patapoutian and Reichardt, 2001): NGF binds to TrkA receptors, BDNF and NT4/5 bind to TrkB receptors, while NT3 binds to TrkC (Reichardt, 2006). These receptors undergo dimerization upon neurotrophin binding, which is followed by transphosphorylation on several tyrosine residues. This allows the initiation of several parallel signaling pathways, after receptor interaction with proteins containing phosphotyrosine binding domains. Trk receptors are typically coupled to the activation of the Ras/ERK pathway, the PI3-K (phosphatidylinositol 3-kinase)/Akt pathway and phospholipase C γ (PLC γ). The latter enzyme is activated by Trk receptor mediated tyrosine phosphorylation, leading to the formation of diacylglycerol and inositol 1,4,5-trisphosphate, and downstream activation of PKC and mobilization of Ca^{2+} from intracellular stores, respectively (Chao, 2003).

TrkB receptors for BDNF exist in a full-length (TrkB.FL) form as well as in truncated (TrkB.T1, TrkB.T2) forms lacking the kinase domain. Although most of the functions of BDNF have been associated to the long form of the protein, a role for the truncated forms has been proposed in mediating the effects of BDNF in the activation of glial $[Ca^{2+}]_i$ signalling (Rose et al., 2003). Furthermore, it was shown that TrkB.T1 receptors regulate the morphology and the cytoskeleton of astrocytes (Ohira et al., 2005; Ohira et al., 2007). A recent study also showed a role for TrkB.T1 in the modulation of glycine transporters in astrocytes (Aroeira et al., 2015).

The early studies concerning the effect of BDNF on synaptic transmission showed an increased in the frequency of miniature excitatory postsynaptic currents (mEPSCs) in *Xenopus* neuromuscular cultures (Lohof et al., 1993). Furthermore, the expression of neurotrophins and their receptors was found to increase following high-frequency stimulation under conditions that induce LTP in the dentate gyrus of awake rats (Bramham et al., 1996). This suggested a fundamental role for BDNF in synaptic plasticity. Since then, the effects of BDNF in synaptic regulation, including a role in LTP, have been reported in many studies.

There are different forms of LTP according to the area investigated in the hippocampus and the induction protocol used. The first evidence linking BDNF to LTP was in knock-out mice deficient in BDNF, which showed an impairment in LTP (at Schaffer collateral—CA1 synapses), as well as in learning and memory (Patterson et al., 1996). These studies also showed that addition of recombinant BDNF completely reversed the deficits in LTP and improved basal synaptic transmission (Patterson et al., 1996). Similarly, other reports showed the impairment of LTP in hippocampal CA1 synapses under the following conditions: (i) using an anti-BDNF antibody or with TrkB-IgG, which sequesters the free neurotrophin in the medium (Figurov et al., 1996; Kang et al., 1997; Chen et al., 1999) and (ii) with an antibody against TrkB receptors (Kang et al., 1997; Korte et al., 1998). Furthermore, using knockin mice carrying a point mutation either in the Shc- or at the PLC γ -docking sites on TrkB, it was possible to characterize the pathways that mediate the BDNF-TrkB signaling in LTP (Minichiello et al., 2002). These studies showed that the effect of TrkB on LTP is mediated via recruitment of PLC γ and subsequent induction of CREB (cAMP response element-binding protein) and CaMKIV (Ca $^{2+}$ - and calmodulin-dependent protein kinase IV) phosphorylation. Furthermore, in studies where PLC γ signaling was inhibited by overexpressing the PLC γ pleckstrin homology (PH) domain with a Sindbis virus vector it was found that simultaneous activation of pre- and post-synaptic TrkB receptors and their downstream signaling mechanisms is required to induce LTP of

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CA1 synapses (Gartner et al., 2006). A major question in the field is the compartment (pre- or post-synaptic) involved in the release of BDNF upon high-frequency stimulation. Endogenous BDNF was detected in the presynaptic terminal, suggesting that the major source of BDNF regulating LTP in the hippocampus may be presynaptic (Dieni et al., 2012; Andreska et al., 2014). However, numerous studies have shown a preferential postsynaptic release of BDNF in hippocampal neurons (Matsuda et al., 2009; Edelman et al., 2014).

Once BDNF is released, it binds presynaptic TrkB receptors, recruiting synaptic vesicles to the synapse and enhancing the exocytosis of vesicles packed with the neurotransmitter glutamate (Pozzo-Miller et al., 1999; Jovanovic et al., 2000; Pereira et al., 2006; Madara and Levine, 2008; Melo et al., 2013) (Fig. 1.5). Furthermore, BDNF has been shown to exert short- and long-term effects on the post-synaptic cell. Studies performed in hippocampal organotypic cultures showed that BDNF enhances the delivery of GluA1-containing AMPA receptors to the synapse within 30 min (Caldeira et al., 2007b), and an upregulation in the surface distribution of NMDA receptors was also observed in cultured hippocampal neurons stimulated with the neurotrophin (Caldeira et al., 2007a) (Fig. 1.5). These early effects of BDNF, mediated by post-translational modifications of synaptic proteins, are followed by delayed but longer lasting responses which involve an upregulation of translation activity (Leal et al., 2014; Leal et al., 2015). The machinery required for local protein synthesis is available at the synapse (Steward and Levy, 1982), and the mRNAs that code for a large number of synaptic proteins are transported along dendrites in RNA granules. BDNF stimulation disassembles these granules, and this may contribute to make the transcripts available for translation. At later time-points the effects of BDNF on the synaptic proteome are also dependent on transcription activity (Leal et al., 2014; Leal et al., 2015) (Fig. 1.5).

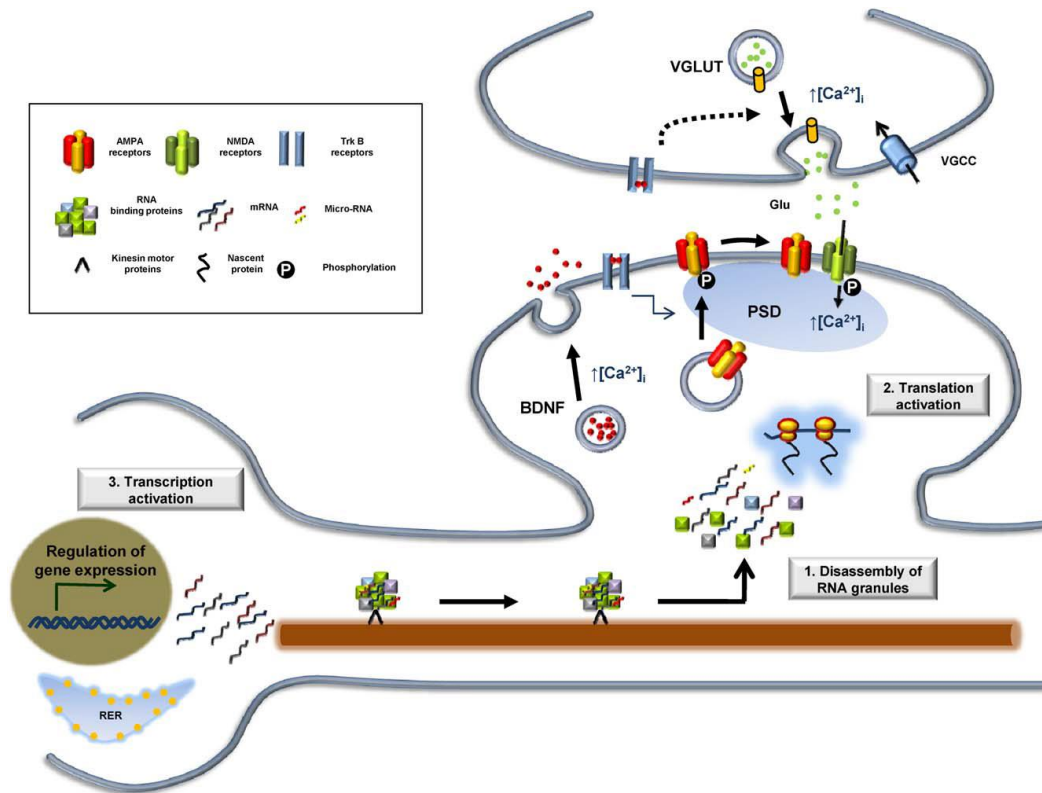


Fig. 1.5. BDNF regulates glutamatergic synaptic transmission by acting at the pre- and post-synaptic level. BDNF sequestered in secretory vesicles present in the post-synaptic region is released by a Ca^{2+} -dependent mechanism, following activation of glutamate receptors. BDNF acts on pre-synaptic TrkB receptors, potentiating glutamate release, and exerts short- and long-term effects in the post-synaptic cell. BDNF induces the translocation of AMPA receptors to the synapse and increases the activity of NMDA receptors by phosphorylation-dependent mechanisms. Furthermore, BDNF induces local protein synthesis at the synapse from mRNAs transported along dendrites in RNA granules, by promoting the disassembly of the granules (**1**) and activating the translation machinery (**2**). Additional effects of BDNF include the regulation of RNA transport along dendrites, which is mediated by kinesin motor proteins, and activation of gene expression (**3**). Adapted from (Santos et al., 2010).

1.3. Proline-rich tyrosine kinase 2

Proline-rich tyrosine kinase 2 (Pyk2) is a nonreceptor tyrosine kinase which belongs to the FAK (focal adhesion kinase) family of protein kinases. The human Pyk2 gene is located on chromosome 8 (8p21.1; gene reference: *ptk2b*) (Herzog et al., 1996; Inazawa et al., 1996), and is mainly expressed in cells derived from hematopoietic lineages and in the CNS (Avraham et al., 1995; Lev et al., 1995;

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Avraham et al., 2000). Recent studies showed that the kinase shares a similar domain structure with FAK (homologous non-receptor tyrosine kinases), exhibiting approximately 45% sequence identity, particularly in the central kinase domain (approximately 60%). Although the structure of Pyk2 is not fully defined, the major domains have been identified and characterized. These domains include: an N-terminal FERM (F for 4.1 protein, E for ezrin, R for radixin and M for moesin) domain, a central catalytic domain, a number of proline rich sequences, and a C-terminal focal adhesion targeting domain (Fig. 1.6).

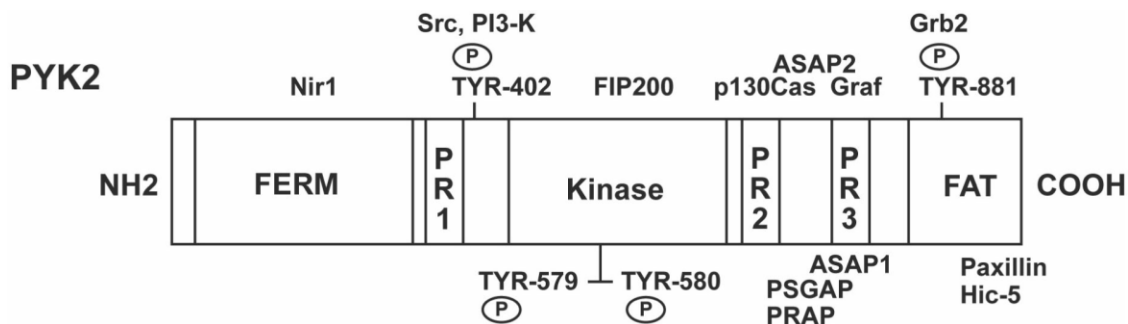


Figure 1.6. Schematic representation of Pyk2 functional domains and interacting proteins. Pyk2 contains an N-terminal FERM domain, a central kinase domain, three proline rich motifs (PR1, Pr2, PR3), and a C-terminal focal adhesion targeting domain (FAT). Phosphorylation of Tyr-402 is as a binding site 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 allow the 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).

There are several features that distinguish FAK and Pyk2: Pyk2 is typically activated in response to stimuli that increase the $[Ca^{2+}]_i$, whereas FAK is mainly activated ensuing integrin mediated adhesion to the extracellular matrix (ECM) (Lev et al., 1995). Pyk2 also contributes to the signaling of G-protein-coupled receptors.

1.3.1. Pyk2 and hippocampal synaptic plasticity

Numerous signaling players contribute to synaptic plasticity. Among those molecules, Pyk2 plays an important role in LTP in the hippocampus (Lu et al., 1998; Xiong et al., 1999; Huang et al., 2001; Salter and Kalia, 2004). The kinase interacts directly with the NMDAR complex, together with Src (Lu et al., 2000; Huang et al., 2001) (Fig. 1.7). Furthermore, studies performed in hippocampal slices showed that Pyk2 is activated by PKC following stimulation of NMDAR (Siciliano et al., 1996). The activation of Pyk2 resulted from the increase in the $[Ca^{2+}]_i$, and potentiated NMDAR currents (Lu et al., 2000; Huang et al., 2001). The NMDAR-mediated entry of Ca^{2+} causes a postsynaptic clustering of Pyk2 and autophosphorylation of the kinase by a mechanism that was dependent on the Ca^{2+} - and calmodulin-stimulated binding to PSD-95 (Bartos et al., 2010). The interaction between Pyk2 and PSD-95 is necessary, because injection of the peptide GST-tagged SH3 domain 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 required for LTP induction, and may depend upon downstream activation of Src to upregulate NMDAR activity. Accordingly, tyrosine phosphorylation of Pyk2, and its interaction with Src, are enhanced under conditions of synaptic stimulation that induce LTP (Huang et al., 2001). A model was recently proposed according to which following NMDAR stimulation, or under conditions that induce chemical LTP, Pyk2 activates Src and this kinase phosphorylates PSD-95 on Tyrosine523, thereby enhancing the interaction between Pyk2 and PSD-95. This facilitates the Pyk2-mediated phosphorylation (and activation) of Src, which activate GluN2A-containing NMDAR (Zhao et al., 2015).

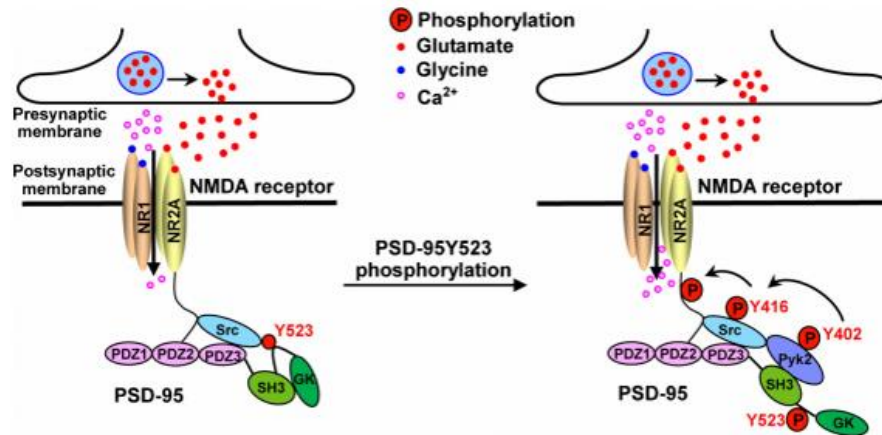


Figure 1.7. Model for the role of PSD-95Y523 phosphorylation in NMDA receptor upregulation. Stimulation of NMDAR leads to rapid Src-mediated phosphorylation of PSD-95 at Y523, opens up the intramolecular interaction between SH3 and GK domains of PSD-95, and facilitates the PSD-95-Pyk2 interaction, activates Pyk2 and Src, and subsequently results in the tyrosine phosphorylation of GluN2A (NR2A), which ultimately upregulates the function of NMDAR and contributing for LTP. Adapted from (Zhao et al., 2015).

The activity of Pyk2 is negatively regulated by the protein phosphatase STEP (striatal-enriched protein tyrosine phosphatase), which dephosphorylates the kinase on Tyrosine402 (Xu et al., 2012). Studies performed in STEP KO mice showed a significant upregulation in the phosphorylation of the GluN2B NMDAR subunit (Tyrosine1472), together with a significant improvement in the performance in learning and memory tasks that are dependent on the hippocampus (Venkitaramani et al., 2011). Furthermore, an upregulation in GluN1/GluN2B NMDAR and in GluA1/GluA2-containing AMPA receptors was observed in hippocampal synaptosomal fractions, which may account for the improved cognitive functions (Venkitaramani et al., 2011).

1.4. Heterogeneous Nuclear RiboNucleoProteins (hnRNPs)

In eukaryotic cells, a new transcript produced by RNA polymerase II undergoes additional modification before a functional and mature mRNA is produced. These pre-mRNAs are named heterogeneous nuclear ribonucleic acids (hnRNAs) and are assembled into pre-mRNPs (pre-mRNA-protein complexes). Before leaving the nucleus, the transcripts undergo several modifications, including 5' capping, splicing, 3' cleavage and polyadenylation. For most mRNAs, these tasks are carried out co-transcriptionally by proteins that interact with the C-terminal domain of RNA

polymerase II (Steward and Schuman, 2001). The resulting mRNP complexes are then exported to the cytoplasm, after crossing the interchromatin space (Bjork and Wieslander, 2011). Research in the last 25 years showed the biological importance of gene regulatory processes at the post-transcriptional level (mRNA processing, mRNA stability and turnover, and translation). Some of the most important questions that are still being discussed are: (i) how RNA-binding proteins recognize specific RNA sequences, (ii) which effects do they have on RNA structure and processing dynamics, and (iii) how do they regulate physiological events. These RNA-binding proteins typically have different functions: they may participate both in the processing of heterogeneous nuclear RNAs (hnRNAs) into mature mRNAs and regulate gene expression as trans-factors (Dreyfuss et al., 1993; Chaudhury et al., 2010).

Analysis of the molecular composition of mRNP complexes identified the following components: hnRNPs (heterogeneous nuclear ribonucleoproteins), nuclear cap binding proteins, splicing machinery, SR proteins (involved in RNA splicing), 3'-end processing machinery, transcription-export complexes, mRNA export adaptors and receptors, in addition to gene-specific components, among other elements (Bjork and Wieslander, 2011). A proteomics characterization of the components present in RNA granules isolated from the developing brain identified ribosomal proteins, RNA-binding proteins and microtubule-associated proteins (Elvira et al., 2006). In a different study, a total of 42 proteins were identified in a preparation of RNA granules isolated from the rat brain; some of these proteins are known to play roles in RNA transport, protein synthesis and translational silencing (Kanai et al., 2004). Among other functions, these RNA granules play a key role in the delivery of mRNAs along dendrites, where they are translated. In particular the synthesis of proteins in the vicinity of the synapses contributes to the late phase of LTP (Schuman et al., 2006).

hnRNPs are proteins that interact with hnRNAs from the moment they are produced by RNA polymerase until they mature into mRNAs, forming large complexes (Gall, 1956; Dreyfuss et al., 1993). Furthermore, many of these proteins remain associated with the transcripts while they are transported through the nuclear pores and, therefore, they undergo a continuous cycle of transport in and out of the nucleus. Proteins belonging to the hnRNP class of RNA-binding proteins play important roles in the maturation of mRNA, being involved in the process of splicing and in the export, localization, translation and stability on the transcripts (Dreyfuss et al., 2002) (Table I).

Table I. hnRNP proteins. RBD, RNA-binding domain or RNA recognition motif (RRM); RGG, arginine/glycine-rich box. *Molecular mass (kDa) was estimated from SDS-polyacrylamide gel electrophoresis. (Dreyfuss et al., 2002).

<i>Proteins</i>	<i>Domain structure</i>	<i>kDa*</i>	<i>Possible functions</i>
A1	2XRBD, RGG	34	mRNA splicing, mRNA export, Telomere biogenesis
A2/B1	2XRBD, RGG	36/38	mRNA splicing, mRNA localization
C1/C2	1XRBD	41/43	mRNA splicing, mRNA stability
F	3XRBD	53	mRNA splicing
K	3XKH, RGG	62	Transcription, Translational regulation
L	4XRBD	68	mRNA export, mRNA stability
U	RGG	120	Nuclear retention

The hnRNP family of proteins is composed of more than 20 proteins. Some of the most abundant hnRNP proteins are described in Table I. Analysis of cDNA sequence of hnRNPs shows a modular structure, comprised by one or more RNA-binding motifs and at least one auxiliary domain that regulates protein–protein interactions and the subcellular localization of the protein (Han et al., 2010; Geuens et al., 2016) (Fig. 1.8).

1.4.1. hnRNP K

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) has a wide range of functions, both in the nucleus and in the cytoplasm, including mRNA silencing, transcription, splicing, and regulation of mRNA stability and translation (Han et al., 2010). hnRNP K is a 65 kDa protein composed by 463 amino acids, which contains different regions with defined functions (Fig. 1.8): three KH domains, involved in the RNA and dsDNA binding, a NLS (nuclear localization signal), a KNS (nuclear shuttling domain) and a KI region (K-protein-interactive region), responsible for numerous protein-protein interactions (Bomsztyk et al., 2004). Mass spectrometry analysis of the hnRNP K interactome in a rat hepatoma cell line showed 114 binding partners, which included kinases and proteins controlling

mRNA splicing, transcription and translation processes (Mikula et al., 2006). The KI region of hnRNP K includes, for instance, proline-rich docking sites that interact with SH3 domains, a characteristic of Src-family kinases (Van Seuning et al., 1995). Several serine, threonine and tyrosine residues are found along the hnRNP K amino acid sequence, which constitute putative phosphorylation sites (Schullery et al., 1999; Ostrowski et al., 2000; Ostareck-Lederer et al., 2002). The regulation of the phosphorylation state of some of these residues is important to relieve translational silencing (Ostareck-Lederer et al., 2002).

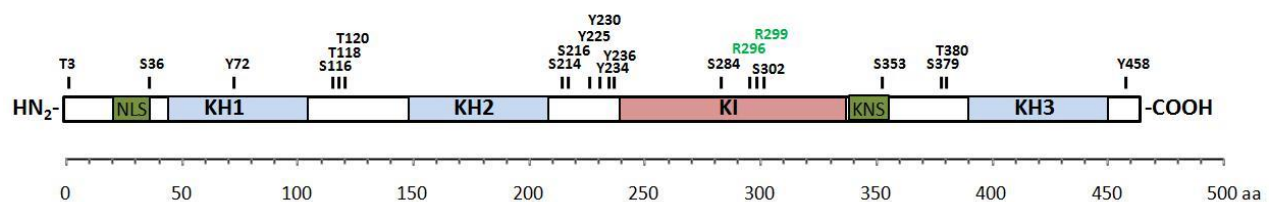


Figure 1.8. hnRNP K structure and covalent modification sites. The residues subjected to phosphorylation (black) and methylation (green) are represented above the structure. NLS, nuclear localization signal; KH, K homology domains; KI, K interactive region; KNS, nuclear shuttling domain. From (Ostareck-Lederer et al., 2002; Mikula et al., 2006).

hnRNP K has been shown (i) to participate in development processes, (ii) to be recruited in response to DNA damage and oxidative stress, and (iii) to play a role in disease conditions (Geuens et al., 2016). In the nervous system, hnRNP K is involved in the regulation of multiple genes that code for proteins of the axon cytoskeleton. Therefore, it is not surprising that the protein was found to be essential for axonogenesis (Liu et al., 2008; Liu and Szaro, 2011). More recently, hnRNP K was reported to play a role in the regulation of dendritic outgrowth, spine density and long-term potentiation, in cultured hippocampal neurons. Knockdown of hnRNP K caused deficits in chemical LTP, an effect that was attributed to the inhibition of ERK signaling, with consequent reduction of GluA1 S845-phosphorylation and impairment on GluA1 surface expression (Folci et al., 2014).

Objectives

Local protein synthesis at the synapse plays an important role in the effects of BDNF on the late phase of LTP in hippocampus. However, the identity of the proteins that are translated and how the transcripts arrive at the synapse is not fully understood. Previous results from our laboratory showed that BDNF enhances the dendritic distribution of the hnRNP K protein (Leal, 2014). Furthermore, BDNF-TrkB signaling was found to induce the dissociation of a large number of transcripts from the hnRNP K protein in cultured hippocampal neurons and in synaptoneuroosomes, suggesting that the protein plays an important role in the regulation of protein synthesis at the synapse (Comprido, 2011). One of the transcripts that may interact with hnRNP K by a mechanism that is sensitive to BDNF-TrkB signaling is the Pyk2 mRNA (Comprido, 2011). Considering the role of Pyk2 in the regulation of the phosphorylation state of GluN2B (Xu et al., 2012; Zhao et al., 2015; Xu et al., 2016), in this work we aimed to address the following questions:

(i) To determine the effect of BDNF on the synaptic expression of GluN2B NMDAR subunits and on the activity of synaptic GluN2B-containing NMDAR. Previous studies have shown that BDNF increases the surface distribution of NMDAR subunits in cultured hippocampal neurons (Caldeira et al., 2007a), but whether those alterations are extended to the synaptic compartment had not been established. The effect of BDNF on the synaptic distribution of NMDAR in cultured hippocampal neurons was investigated by immunocytochemistry with an antibody against GluN2B subunits. The alterations in the activity of synaptic NMDAR were evaluated by analyzing the NMDAR-mediated mEPSC and using appropriate pharmacological tools.

(ii) To investigate the putative role of translation activity and of hnRNP K in the BDNF-induced alterations in the activity of synaptic NMDAR.

(iii) To validate previous results from our laboratory suggesting that activation of BDNF-TrkB signaling dissociates the Pyk2 mRNA from hnRNP K, and to determine the role of Pyk2 in the BDNF-evoked alterations in the activity of synaptic NMDAR. The participation of hnRNP K and Pyk2 in the BDNF-evoked alterations in NMDAR-mediated mEPSCs was investigated by expression of specific shRNAs in cultured hippocampal neurons.

The ultimate goal of this work is to better understand the pre- and post-synaptic effects of BDNF in hippocampal neuron glutamatergic synapses. This should also contribute to elucidate the

Objectives

mechanisms whereby BDNF contributes to the protein synthesis-dependent mechanisms involved in LTP.

Materials and Methods

2.1 Cultures of hippocampal neurons

High density cultures of hippocampal neurons were prepared from Wistar rat embryos (E18-E19). Briefly, after careful dissection from diencephalic structures, the hippocampi were digested in trypsin (0.06 % [GIBCO - Life Technologies] in Ca^{2+} - and Mg^{2+} -free Hank's 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 $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES, and 0.001 % phenol red]) for 15 min at 37°C with gentle shaking. The hippocampi were then washed with HBSS containing 10 % fetal bovine serum (GIBCO Invitrogen), to stop trypsin activity, and transferred to Neurobasal medium supplemented with SM1 (1:50 dilution; Stem Cell Technologies), 25 μM glutamate, 0.5 mM glutamine, and 50 $\mu\text{g}/\text{ml}$ gentamycin. The cells were dissociated in this solution, and the suspension was filtered (filter of 70 μm) to select the dissociated cells. Dissociated cells were plated at a density of 2.71×10^5 cells/well in a 25-mm dish with poly-D-lysine coated coverslips in Neuronal Basal Medium (NBM) supplemented with SM1, and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 3 days in vitro the cell division inhibitor 5-fluoro-2'-deoxyuridine (FDU, 10 μM ; Sigma-Aldrich) was added to the culture, and the cells were maintained for a total period of 15 - 17 days (Mele et al., 2016).

Low-density hippocampal cultures were prepared as previously described (Kaech and Banker, 2006). Briefly, hippocampi were dissected from E18 Wistar rat embryos and the cells were dissociated using trypsin (0.06 % [GIBCO - Life Technologies] in Ca^{2+} - and Mg^{2+} -free Hank's 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 $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES, and 0.001 % phenol red]) for 15 min at 37°C. Neurons were plated at a final density of 7.5×10^4 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 (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 of glial cells, 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% $\text{CO}_2/95\%$ air at 37 °C for up to 2 weeks, feeding the cells after the first week in culture with Neurobasal medium supplemented with SM1, but without glutamate added.

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2.2 Neuron transfection with calcium phosphate

Transfection of cultured hippocampal neurons with sh4-Pyk2, sh6-hnRNPK or sh1-scramble sequence constructs was performed by the calcium phosphate coprecipitation method. Briefly, hippocampal neurons were incubated with cultured-conditioned medium with 2 mM kynurenic acid (antagonist of ionotropic glutamate receptors; Sigma) for 15 min. Two μg of plasmid DNA was diluted in Tris-EDTA (TE) pH 7.3 and mixed with 2.5 M CaCl_2 . This DNA/TE/calcium mix was added to 10 mM HEPES-buffered saline solution (270 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4 , 11mM dextrose, 42 mM HEPES, pH 7.2). The precipitates were added drop-wise to each well and incubated for 1h 30min at 37 °C, in a humidified incubator with 95% air/5% CO_2 . The cells were then washed with acidic culture medium containing 2 mM kynurenic acid and returned to the 95%air/5% CO_2 incubator for 20 min at 37 °C. Finally, the medium was replaced with the initial culture-conditioned medium, and the cells were further incubated in a 95% air/5% CO_2 incubator for 72 h at 37 °C to allow GFP expression and the downregulation of the protein of interest (Mele et al., 2016). Hippocampal neurons were stimulated or not with BDNF (50 ng/ml) during at least 30 min before recording the NMDA-mEPSCs.

2.3 Electrophysiology

Cultured hippocampal neurons (2.71×10^5 cells/well) with pyramidal morphology (15-17 days in vitro - DIV), were whole-cell voltage-clamped to -60 mV, at room temperature, in a MgCl_2 -free Tyrode's solution containing (in mM): 150 NaCl, 4 KCl, 10 glucose, 10 HEPES, and 2 CaCl_2 , pH 7.35 (310 mOsm). To record and isolate NMDA-receptor-mediated miniature EPSCs, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM ; Tocris [AMPA/kainate receptor antagonist]), bicuculline (10 μM ; Tocris [GABA_A receptor antagonist]), tetrodotoxin (TTX; 500 nM; Tocris [blocker of voltage-gated Na^+ channels]) and glycine (15 μM ; Sigma-Aldrich [co-agonist of NMDA receptors]) were added to the bath solution (Atasoy et al., 2008). The electrode solution had the following composition (in mM): 115 Cs-MeSO₃, 20 CsCl, 2.5 MgCl_2 , 10 HEPES, 0.6 EGTA, 4 $\text{Na}_2\text{-ATP}$, 0.4 Na-GTP, pH 7.3 (300 mOsm; Sigma) (Kessels et al., 2013). Where indicated, hippocampal neurons were pre-incubated with cycloheximide (CHX; 50 $\mu\text{g}/\text{ml}$) or with vehicle (DMSO) (1:1000 dilution) for 45 min before recording the NMDA-receptor mediated miniature EPSC (mEPSC). Pre-incubation with Conantokin G (Con G; 3 μM ; Tocris) (Donevan and McCabe, 2000) was performed for 30 min. When the effect of BDNF was tested, hippocampal neurons were pre-incubated with the neurotrophin (50 ng/ml) for at least 30 min before recording the NMDA-receptor-mediated mEPSCs.

Recording electrodes were made of borosilicate glass capillaries and pulled on a horizontal stage Sutter Instrument P-97 puller (resistances: 3-4 M Ω). Seals (1-10 G Ω) were formed by applying gentle suction to patch pipettes. Cells were held for a period of 5 min and the baseline for the analysis of NMDA receptor-mediated mEPSCs was manually determined as the average current level of silent episodes during a recording. Whole-cell recordings from hippocampal neurons were performed using an Axon CNS, Multiclamp 700B amplifier, an Axon Digidata 1550 A acquisition board, and pClamp software (version 10.5; Molecular Devices). Rise and decay time were calculated as the average of at least four cells for each experimental condition, as indicated in the figure captions.

2.4 Immunocytochemistry analysis of GluN2B surface expression

To label surface GluN2B-containing NMDA receptors, low-density hippocampal cultures were incubated with an antibody against an extracellular epitope of the receptor subunit diluted in conditioned neuronal medium (Alomone Labs; 1:100 [#AGC-003]). Incubation with the primary antibody was performed for 15 min at 37°C, before fixation of the cells. Neurons were then fixed in 4% sucrose/paraformaldehyde (in PBS) for 15 min at RT, and permeabilized with 0.3 % (v/v) Triton X-100 (in PBS) for 5 min at 4°C. Non-specific staining was blocked by incubation with 10 % (w/v) BSA in PBS for 30 min at 37°C, and the preparation was then incubated with the following primary antibodies: anti-PSD95 (1:200, mouse; Thermo Scientific #7E3-1B8), anti-vGlut1 (1:5000, guinea-pig; Millipore #AB5905) and anti-MAP2 (1:10000, chicken; Abcam #ab5392). Incubation with the primary antibodies under the latter conditions was performed in 3 % (w/v) BSA in PBS (overnight, at 4°C). After washing 6 - 7 times in PBS, the cells were incubated with the secondary antibodies (AMCA-conjugated anti-chicken [1:200; Jackson Immunoresearch #103-155-155]; Alexa 647-conjugated anti-Guinea-pig [1:500; ThermoFisher Scientific #A-21450]; Alexa488-conjugated anti-rabbit [1:1000; ThermoFisher Scientific #A-11034]; Alexa568-conjugated anti-mouse [1:1000; ThermoFisher Scientific #A-11004] diluted in 3 % (w/v) BSA prepared in PBS, during 45 min (T = 37°C). The coverslips were then mounted on slides with a fluorescence mounting medium (DAKO).

Fluorescence imaging was performed on a Zeiss Axio Imager Z.2 microscope using a 63X and 1.4 NA oil objective. Quantification of the fluorescence signal was performed using the Fiji image analysis software. The cells used in each set of experiments was 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 specific fluorescence signals were analyzed after setting the thresholds, and the puncta detected under those conditions were included in the analysis. For each

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experiment, similar threshold levels were used to quantify the number, area and the integrated intensity of puncta in dendrites. At least 10 cells were analyzed per condition for each preparation.

2.5 Fluorescence assay of receptor internalization

Cultured hippocampal neurons (15 DIV) were treated with cycloheximide (CHX; 50 µg/ml) or vehicle (DMSO) (1:1000 dilution) for 45 min at RT in culture conditioned medium. During the last 10 min of incubation, the medium was supplemented with a high concentration (1:100) of an antibody directed against the N-terminus (an extracellular epitope) of the GluN2B NMDAR subunit (Alomone Labs #AGC-003). During this period the cells were then stimulated or not with BDNF (50 ng/ml). After this incubation period, hippocampal neurons were washed with PBS at 37°C, to remove the unbound antibody, and were further incubated in an antibody-free conditioned medium for 15 min at 37 °C to allow the internalization of antibody-bound receptors. Cycloheximide, BDNF or DMSO were maintained in the medium during the internalization period. The preparations were then fixed in 4% sucrose/paraformaldehyde (in PBS) for 15 min at RT. Next, neurons were exposed to a saturating concentration (1:300) of the first of two secondary antibodies (Alexa Fluor 488 goat anti-rabbit; ThermoFisher #A-11034) for 1h at RT. After permeabilization (0.25 % Triton X-100 for 5 min) the cells were blocked with 10 % BSA in PBS (1h at RT) before incubation with the second secondary antibody (Alexa Fluor 568 goat anti-rabbit, 1:500 ThermoFisher #A-11036) for 1h at RT. This strategy allows distinguishing the surface receptors from those receptors that have been internalized before fixation (Goodkin et al., 2005). The coverslips were then mounted on slides with a fluorescence mounting medium (DAKO) (Mele et al., 2016). Images were acquired on an Axio Observer 2.1 fluorescence microscope (Zeiss) coupled to an Axiocam HRm digital camera, using a 63x oil objective and were quantified using the ImageJ image analysis software. The ratio of internalization was calculated using the internalized antibody signal/total antibody signal ratio. The same settings were used to acquire the images from each batch of experiments. Incubation of the preparations in the absence of primary antibodies gave no specific signal.

2.6. hnRNP K Immunoprecipitation

High-density cultures of hippocampal neurons were prepared as described above, and at DIV 15 the cells were incubated for 20 min in the presence or in the absence of 50 ng/ml BDNF in a humidified incubator with 5 % CO₂/95 % air at 37°C. The cells were then washed twice with ice-cold phosphate

buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4 , 10 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, pH 7.4) before lysis 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 $\mu\text{g}/\text{ml}$ chymostatin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{mL}$ antipain, 1 $\mu\text{g}/\text{ml}$ pepstatin; Sigma-Aldrich), phosphatase inhibitors (50 mM NaF and 1.5 mM Na_3VO_4) and 50 U/ml of RNase inhibitor (SUPERaseIn™, Ambion Applied Biosystems). The extracts were then frozen at -80°C . After thawing, the extracts were centrifuged at $16\ 100 \times g$ for 10 min at 4°C , to remove a membrane fraction. Protein content in the supernatants was quantified using the bicinchoninic acid (BCA) assay kit (Pierce, Thermo Fisher Scientific). Antibody-immobilized beads were prepared by incubating 6 μg of anti-hnRNP K or Mouse IgG antibodies with 100 μL of Protein G PLUS-Agarose beads (Santa Cruz Biotechnology), overnight at 4°C . The immobilized antibodies were incubated with 1 mg protein during 1 h at 4°C , and the beads were washed four times (2 min centrifugations, $2\ 000 \times g$) at 4°C with washing buffer, supplemented as described for the lysis buffer. The final pellet, containing the immunoprecipitated hnRNP bound to the antibody-immobilized beads, was used for Western Blot analysis and RNA isolation as described below.

2.7. RNA extraction from immunoprecipitates

RNA contained in pellets resulting from immunoprecipitations were immediately isolated using TRizol reagent (Invitrogen) following the manufacturer's specifications. After addition of chloroform and phase separation, the RNA was precipitated by the 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.

2.6.2 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 25°C for 5 min, followed by 30 min at 42°C , for primer annealing to the template and cDNA synthesis, respectively. The reverse

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transcriptase was then denatured for 5 min at 85°C, and the sample cooled to 4°C, before storage at -20°C until further use. Equal amounts of RNA of each conditions were used to reverse transcription.

2.6.3 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 $55 \pm 5^\circ\text{C}$; 3- secondary structures and primer dimers were avoided; 4- primer length 18-24 bp; 5) final product length 100-200 bp. The following primers were used: (i) Pyk2 - forward, 5'GTAGATAATTGTGTTAG3', reverse, 5'ACTATTGATTAAGCATACTG3'; (ii) GluA1 - forward, 5'ACTACATCC TCGCCAATCTG3', reverse, 5'AGTCACTTGTCTCCATTG3'; (iii) Npas4 - forward, 5'AATGGAGATATTCAGGCT3', reverse, 5'TAGTTATTGGCAGTAATAGG3'.

2.7 Quantitative Real-Time PCR (RT-qPCR)

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

2.7.1 Analysis of RT-qPCR Data

The comparative Ct method ($2^{-(\Delta\Delta\text{Ct})}$) 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.

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. Statistical analysis of the results obtained in the immunocytochemistry experiments was performed by using the Kruskal-Wallis test followed by the Dunn's Multiple Comparison test. Statistical analysis of the results obtained in the recordings of NMDAR mediated mEPSC amplitude and frequency was performed by one-way ANOVA, followed by Dunnett or Bonferroni post-test. Results from fluorescence assay of receptor internalization were evaluated by one-way ANOVA (significance level 0.05, confidence interval 0.95).

Results

3.1. BDNF-induced synaptic expression of GluN2B-containing NMDA receptors

Stimulation of cultured hippocampal and cerebrocortical neurons with BDNF was shown to induce the phosphorylation of GluN2B subunits of NMDAR on Y1472 (Xu et al., 2006; Nakai et al., 2014; Xu et al., 2016). Fyn-mediated phosphorylation of this GluN2B amino acid residue was found to upregulate NMDA receptor surface expression, to enhance the receptor channel activity and to increase synaptic transmission in hippocampal neurons (Xu et al., 2006). Moreover, studies with biotinylation of surface proteins showed that BDNF increases the surface expression of GluN2B subunits in cultured hippocampal neurons at DIV7 (Caldeira et al., 2007a). To investigate whether BDNF upregulates the synaptic expression of GluN2B-containing NMDAR, cultured hippocampal neurons were stimulated with the neurotrophin for 30 min, and the surface receptors were labeled with an antibody that binds specifically to an extracellular sequence located next to the N-terminal region. The synaptic expression of the receptors was evaluated by colocalization with the vesicular glutamate transporter type 1 (vGluT1), a presynaptic marker (Takamori, 2006), and with the postsynaptic density protein-95 (PSD95), a postsynaptic marker of glutamatergic synapses (Huganir and Nicoll, 2013). The dendritic compartment was labeled with an antibody against the protein marker MAP2 (microtubule-associated protein type 2) (Mele et al., 2014).

Surface GluN2B-containing NMDAR show a punctate pattern of distribution, showing a non-homogeneous distribution on the plasma membrane (Fig. 3.1). The effect of BDNF (50 ng/ml; 30 min) on GluN2B synaptic surface levels (arrowheads, Fig. 3.1 A) was assessed by quantifying the number of GluN2B puncta (Fig. 3.1 B), area of puncta (Fig. 3.1 C) and intensity of puncta (Fig. 3.1 D) that colocalized with PSD-95 and vGluT1. BDNF increased the number, area and intensity of surface synaptic GluN2B puncta, i.e. immunoreactivity that colocalized with vGluT1 and PSD95 (Fig. 3.1 B-D).

The BDNF-TrkB signaling is coupled to the regulation of transcription and translation activity (Leal et al., 2014; Leal et al., 2015), and activation of the protein synthesis machinery partly accounts for the effects of BDNF on the synaptic proteome. To determine whether the BDNF-induced synaptic accumulation of GluN2B-containing NMDAR requires de novo synthesis of proteins, hippocampal neurons were stimulated with BDNF in the presence of the translation inhibitor cycloheximide (50 µg/ml) (Pestova and Hellen, 2003), and the synaptic distribution of GluN2B-containing NMDAR was analyzed as described above. The effects of BDNF on the area and intensity of surface synaptic GluN2B were abrogated in the presence of cycloheximide (Fig. 3.1C and D). In contrast, the effects of BDNF on the number of puncta were not affected by inhibition of translation activity (Fig. 3.1B)

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and, therefore, may be mediated by recruitment of receptors from extrasynaptic regions. Together, our findings indicate that BDNF treatment requires protein synthesis to induce a synaptic enrichment of surface GluN2B-containing NMDAR.

3.2. BDNF increases the amplitude and the frequency of GluN2B-mediated mEPSCs

The increased synaptic surface GluN2B expression upon stimulation of hippocampal neurons with BDNF suggests that incubation with the neurotrophin may increase NMDA receptor mediated synaptic activity. To address this hypothesis we tested the effect of BDNF on the amplitude of NMDAR-mediated mEPSCs. These currents were recorded in the absence of presynaptic stimulation and in the presence of TTX, resulting from the spontaneous release of glutamate from nerve terminals. Furthermore, NMDAR-dependent synaptic responses were pharmacologically isolated by blocking AMPA and GABA receptors, and by supplementing the salt solution with the NMDAR coagonist glycine. The postsynaptic NMDAR-mediated component was expressed by using a salt solution lacking Mg^{2+} , which allowed recording the mEPSC activity at a physiological holding potential of -60 mV. Alterations in the number of NMDAR at the synapse are expected to correlate with changes in the amplitude of mEPSC. On the other hand, changes in the frequency of mEPSC are thought to arise from presynaptic alterations in neurotransmitter release.

Incubation of cultured hippocampal neurons with BDNF (50 ng/ml; $t > 30$ min) increased the amplitude of mEPSC, and this effect was abrogated by the GluN2B inhibitor conantokin G (Donevan and McCabe, 2000) (**Fig. 3.2B**). Furthermore, conantokin G significantly decreased the amplitude of NMDA receptor mediated mEPSC in hippocampal neurons under control conditions and after incubation with BDNF. These results show a role for GluN2B-containing receptors in NMDA receptor mediated mEPSC. The conantokin G-insensitive mEPSC, which was insensitive to stimulation with BDNF, may be mediated by NMDAR expressing GluN2A subunits.

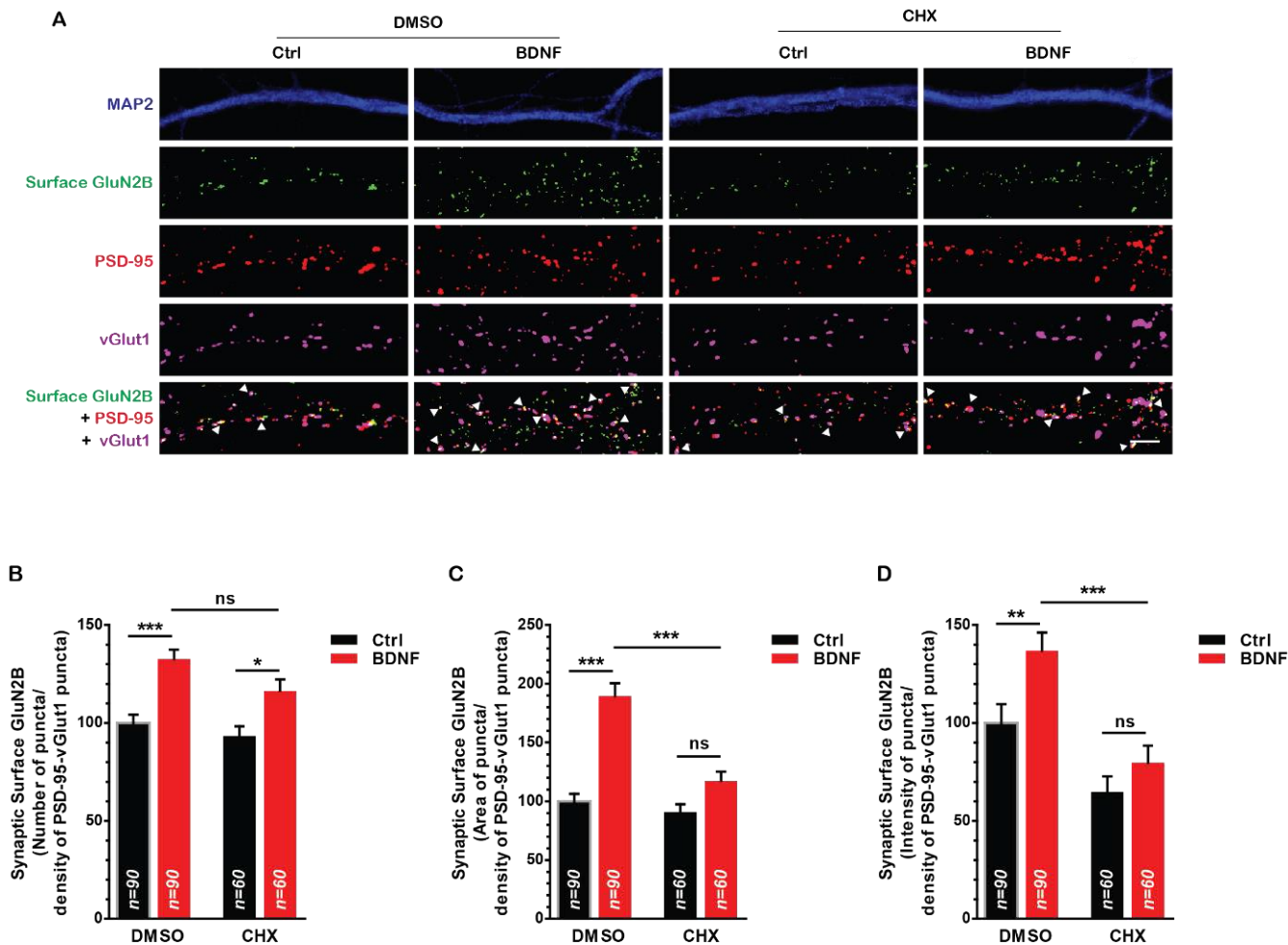


Figure 3.1. BDNF treatment increases the expression of synaptic surface GluN2B-containing NMDA receptors in a protein synthesis-dependent manner. (A) Hippocampal neurons (DIV 14-15) were pre-incubated with CHX (50 μ g/ml) or vehicle DMSO (1:1000 dilution) for 45 min, and were then stimulated or not with BDNF (50 ng/ml) during 30 min, where indicated. After incubation with BDNF, neurons were live immunostained for GluN2B, using an antibody against an extracellular epitope in the GluN2B N-terminus. After fixation neurons were incubated with specific antibodies against 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 results are expressed as percentage to control (DMSO). Error bars represent SEM. 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 μ m.

Results

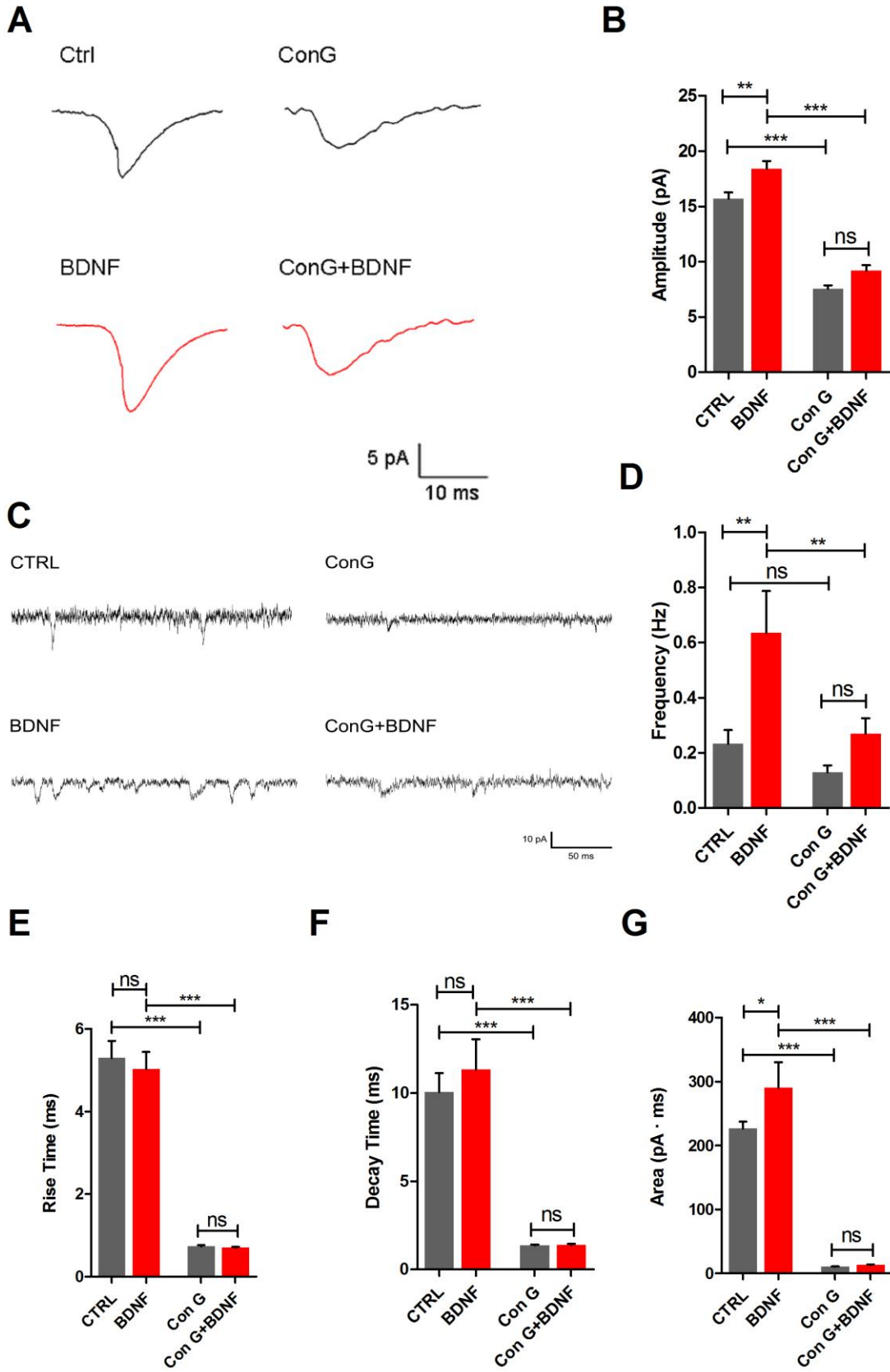


Figure 3.2. BDNF treatment increases the functional expression of synaptic GluN2B-containing NMDA receptors. **(B)** Mean NMDAR-mEPSC amplitude recorded under control conditions and after >30 min of BDNF (50 ng/ml) stimulation, in the presence or in the absence of conantokin G (Con G). The average mEPSC traces recorded are shown in panel **(A)** and representative traces are shown in **(C)**. Cells treated with Con G show no significant increase in the amplitude of NMDA-mEPSC upon BDNF treatment. **(D)** Mean NMDAR-mEPSC frequency recorded under control conditions (n = 13 cells) and after 30 min of BDNF stimulation (n = 13 cells), in the presence (n = 17 cells) or in the absence of Con G (n = 14 cells). Cells treated with Con G show no significant increase in the frequency of NMDA-mEPSC upon BDNF treatment. **(E)** Rise and **(F)** decay time of NMDA-mEPSCs in cultured hippocampal neurons maintained under control conditions or incubated with Con G. Both rise and decay times are decreased (10–90% rise time: before Con G, t = 5.29 ms; after Con G treatment, t = 0.74 ms; 90-10% decay time: before Con G, t = 10.06 ms; after Con G treatment, t = 1.37 ms). **(G)** Mean NMDAR-mEPSC area under the peak recorded under control conditions and after > 30 min of BDNF stimulation, in the presence or in the absence of Con G. Cells treated with Con G show no significant change in the peak area upon BDNF treatment. The results are the average \pm SEM. n represents the number of neurons analyzed in at least three independent preparations. Statistical analysis was performed using the one-way ANOVA followed by the Bonferroni test (^{ns} p > 0.05, * p < 0.05, ***p < 0.001). Con G- Conantokin G.

Stimulation of cultured hippocampal neurons with BDNF also increased the frequency of NMDA receptor mediated mEPSC, showing a presynaptic effect of the neurotrophin (**Fig. 3.2C**), and this effect was also sensitive to the GluN2B antagonist Conantokin G (3 μ M). Under control conditions Conantokin G did not change the frequency of NMDA receptor-mediated mEPSC, but decreased significantly their rise and decay time (**Fig. 3.2D and E, F**), as well as the peak area (**Fig. 3.2G**). The average area under the peaks recorded in the presence of Conantokin G was about 5 % when compared with the control, suggesting that most functional synaptic NMDAR contain GluN2B subunits. Conantokin G also abrogated the effect of BDNF on the area of NMDA receptor mediated mEPSC.

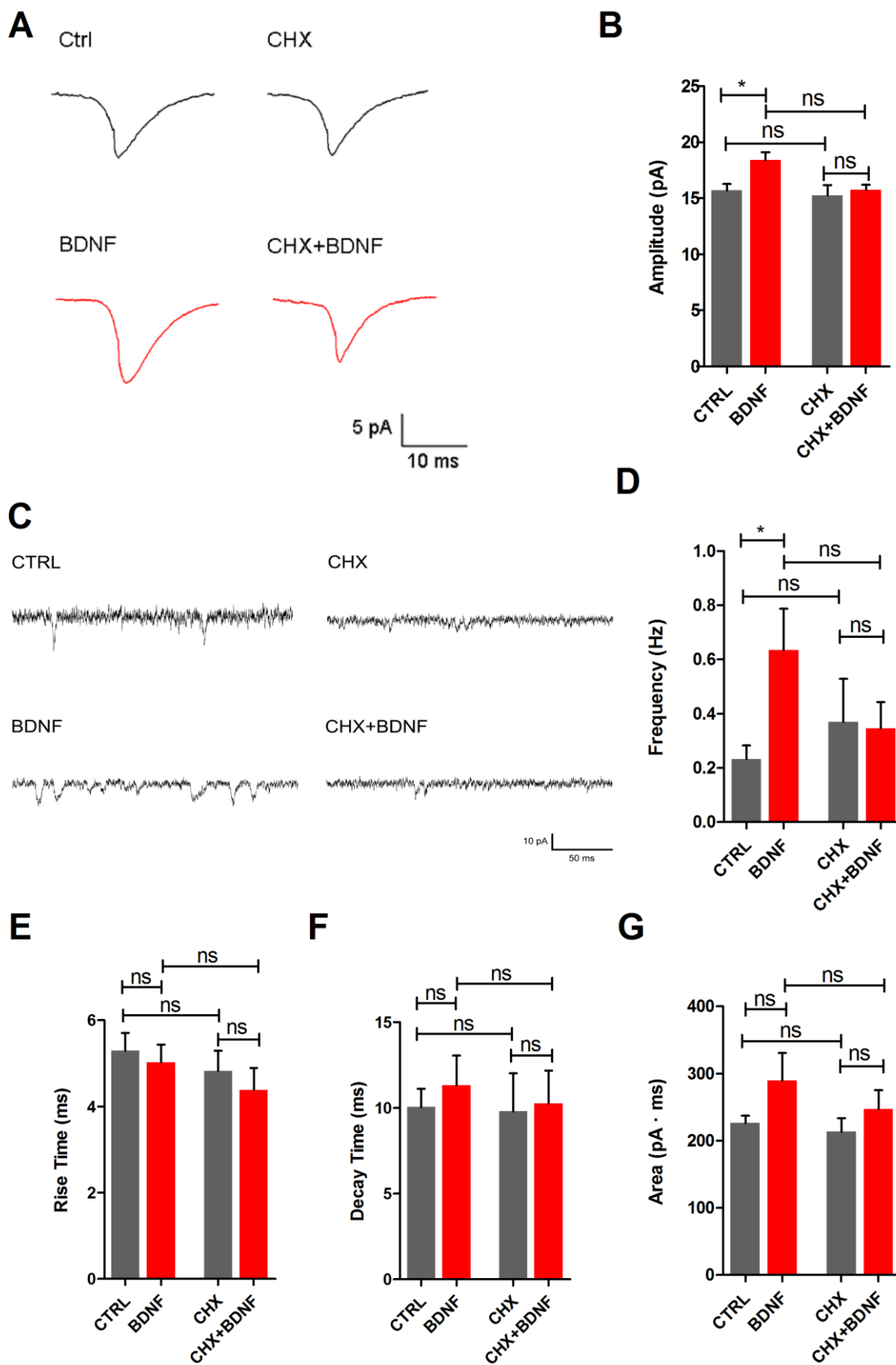
Together, the immunocytochemistry and electrophysiology experiments presented in this section show that BDNF upregulates the synaptic expression of GluN2B-containing NMDAR.

3.3 BDNF stimulation increases the amplitude and the frequency of GluN2B-mediated mEPSC by a protein synthesis-dependent mechanism

The results of **Fig. 3.1** showing a BDNF-induced increase in synaptic expression of GluN2B by a mechanism dependent of protein synthesis suggest that translation activity may be required for the effects of the neurotrophin on the amplitude of NMDA receptor mediated mEPSC in cultured hippocampal neurons. To address this hypothesis we investigated the effect of translation inhibition with cycloheximide on BDNF (>30 min)-evoked increase in the amplitude of NMDAR-mediated mEPSC recorded at -60 mV. The results of **Fig. 3.3B** show that cycloheximide abrogated the BDNF-induced upregulation of mEPSC amplitude, showing a role for protein synthesis in the neurotrophin-induced changes in the activity of NMDAR.

The effects of BDNF on the frequency of NMDAR-mediated mEPSC were abrogated in the presence of cycloheximide (**Fig. 3.3C, D**), but inhibition of protein synthesis was without effect on the mEPSC frequency under control conditions. Furthermore, inhibition of translation did not change the rise and decay time of the NMDA-mEPSCs under resting conditions (**Fig. 3.3E, F**), as well as the area under the peak (**Fig. 3.3G**). Although additional experiments are required, the results obtained also suggest that inhibition of protein synthesis abrogates the effect of BDNF on the peak area.

Together, our findings indicate that BDNF increases the amplitude and frequency of NMDAR-mediated mEPSCs by a protein synthesis-dependent mechanism. The effect of BDNF on the functional expression of synaptic NMDAR is in accordance with the results obtained in the immunocytochemistry experiments, by labeling surface synaptic GluR2B NMDA receptor subunits.



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Figure 3.3. BDNF stimulation may increase the amplitude and frequency of NMDA receptor-mediated mEPSCs by a protein synthesis-dependent mechanism. (A, B) Mean NMDAR-mEPSC amplitude recorded under control conditions (n = 13 cells) and after 30 min of BDNF stimulation (n = 13 cells), in the presence (n = 6 cells) or in the absence (n = 6 cells) of cycloheximide (CHX). Cells treated with CHX show no significant increase in the amplitude of NMDA-mEPSC upon BDNF treatment. The average mEPSC traces recorded are shown in panel **(A)** and representative traces are shown in **(C)**. **(D)** Mean NMDAR-mEPSC frequency recorded under control conditions and after 30 min of BDNF stimulation, in the presence or in the absence of CHX. Cells treated with CHX showed no significant increase in the frequency of NMDA-mEPSC upon incubation with BDNF. **(E)** Comparison of rise **(E)** and **(F)** decay times of NMDA-mEPSCs, before and after CHX treatment. The rise and decay times were not affected by protein synthesis inhibition (10–90% rise time: before CHX, t = 5.29 ms; after CHX treatment, t = 4.83 ms; 90–10% decay time: before CHX, t = 10.06 ms; after CHX treatment, t = 9.81 ms). **(G)** The area of the peaks recorded for NMDA-mEPSC was not affected by BDNF, and by protein synthesis inhibition. The results are the average \pm SEM of the indicated number of experiments. n represents the number of neurons analyzed in at least three independent preparations. CHX- cycloheximide. Statistical analysis was performed using the one-way ANOVA followed by the Bonferroni test (ns p > 0.05, ***p < 0.001).

3.4. BDNF-induced alteration in the GluN2B surface expression is not due to a decrease in the rate of the internalization

The results of the previous sections show a synaptic accumulation of GluN2B-containing NMDAR in cultured hippocampal neurons stimulated with BDNF. These results may be attributed to an increased synaptic delivery of the receptors and/or to a reduction in the rate of internalization. To address the latter hypothesis we used an antibody feeding assay, which allows distinguishing the cell-surface and the internalized pools of native GluN2B-containing NMDAR. Cell-surface GluN2B subunits on living cultured hippocampal neurons were labeled with an antibody that binds to the N-terminal region of the protein, which is extracellular.

Under resting conditions surface GluN2B subunits showed a constant rate of internalization in the neurite compartment, for about 15 min, when about 2.8 % of the receptors were internalized. In the soma compartment the internalization of the receptors reached a plateau after 5 min, at about 2 % of the total. In both compartments there was a pool of NMDAR containing GluN2B subunits, corresponding to more than 50 % of the labeled proteins (not shown), which was stable and did not undergo internalization (**Fig. 3.4A, B**). Therefore, in all other experiments the internalization of

GluN2B-containing NMDAR was followed for 15 min, after a 10 min incubation period in the presence of the anti-GluN2B antibody; these were the most appropriate conditions to study the trafficking of the receptors in the neurite compartment.

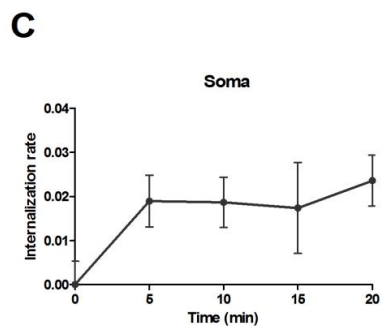
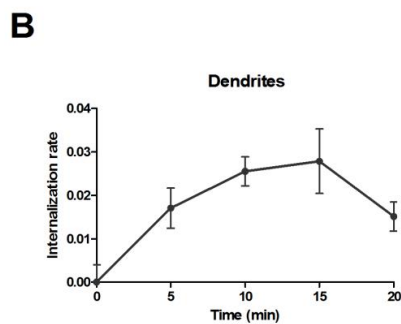
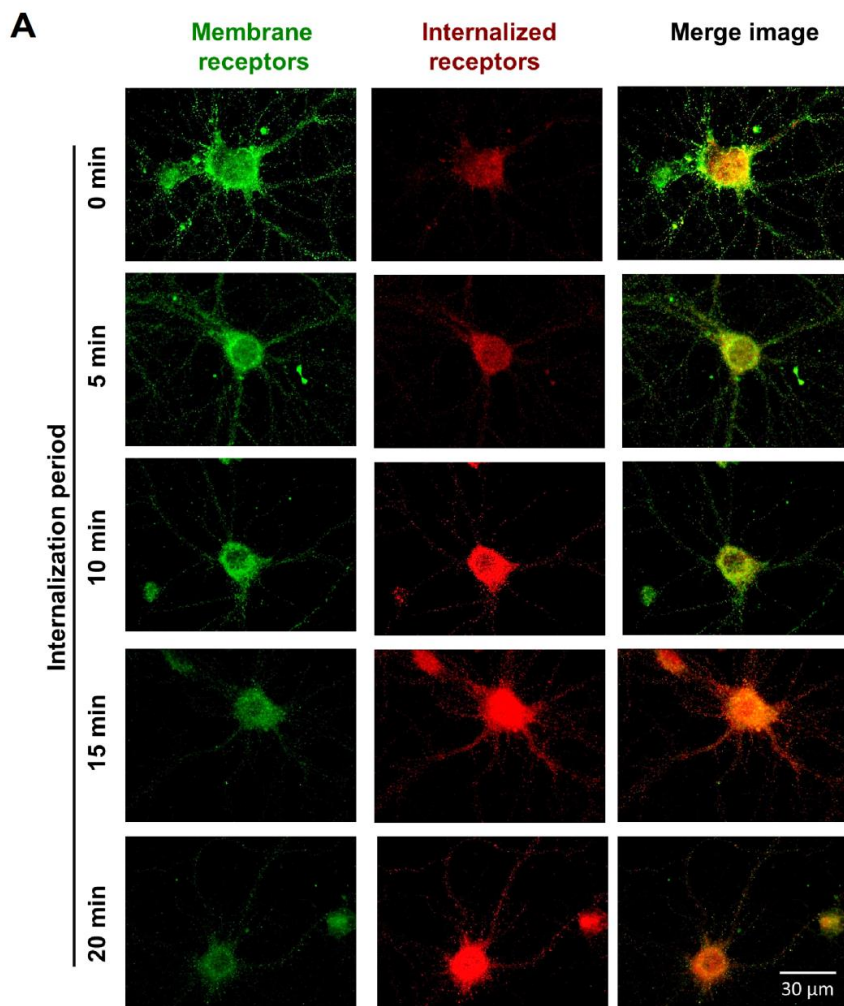


Figure 3.4. Internalization of GluN2B-containing NMDA receptors under resting conditions. Receptor internalization was assessed through an antibody-feeding assay and analyzed by fluorescence microscopy in hippocampal neurons (DIV 15-17) labelled with an antibody against the N-terminal region of GluN2B. The cells were incubated with the antibody for 10 min at 37 °C and the internalization of the antibody was allowed for the indicated periods of time. After quantification of the images at the dendrites (B) and soma (C) compartments, the results were expressed as a ratio of internalized receptors/total receptor immunoreactivity. Representative images are shown in panel (A). The results are the average \pm SEM of 7 different experiments, performed in independent preparations. No significant difference was observed between the experimental conditions tested, as determined by one-way ANOVA, followed by Dunnett's test.

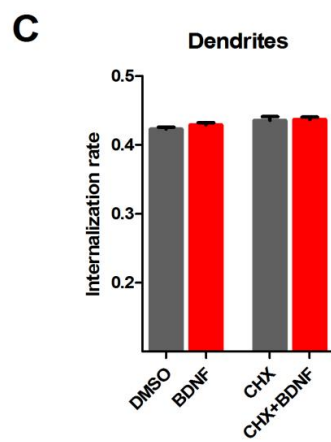
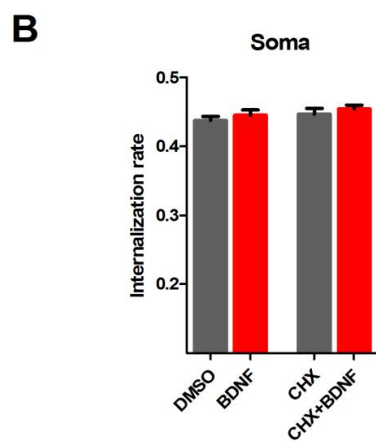
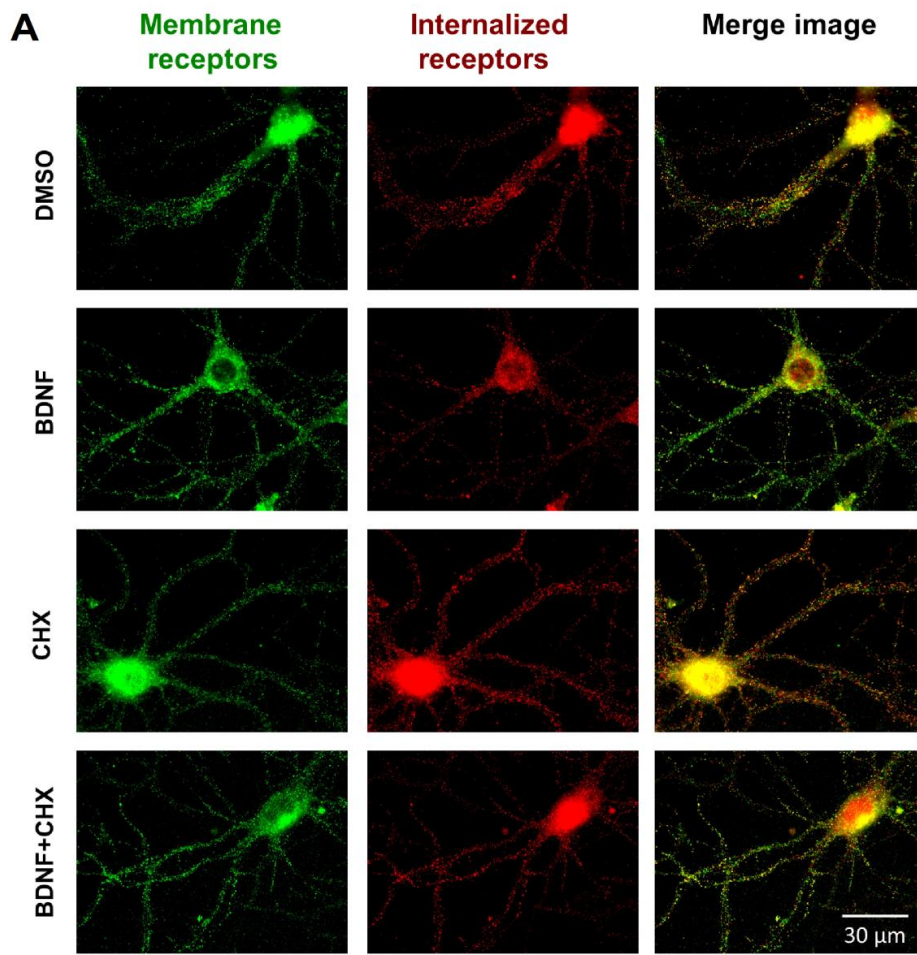
When the effect of BDNF on GluN2B internalization was tested, the cells were incubated with the neurotrophin for 25 min, during the incubation with the antibody and the receptor internalization period. The population of surface receptors and the internalized GluN2B-containing NMDAR were differentially labeled as indicated in the methods section. Immunocytochemistry analysis of the two populations of receptors showed no significant differences between control conditions and hippocampal neurons stimulated with BDNF. Furthermore, inhibition of protein synthesis with cycloheximide (35 min preincubation before addition of the primary antibody to the medium) was without effect on the rate of receptor internalization (**Fig. 3.5**). Together, these results suggest that the BDNF-induced alteration in the GluN2B surface expression is not due to a decrease in the rate of the internalization of NMDAR.

3.5 Signaling mechanisms underlying BDNF-induced synaptic expression of NMDAR

3.5.1 hnRNP K mediates the BDNF-induced increase in NMDAR-mediated mEPSCs

A microarray analysis of the transcripts that interact with hnRNP K showed a large number of mRNAs that dissociate from this RNA binding protein following BDNF treatment of cultured hippocampal neurons (Comprido, 2011). These results suggest that hnRNP K plays an important role in the regulation of translation activity by BDNF. Therefore, we hypothesized that hnRNP K could be involved in BDNF-induced increase in NMDA receptor-mediated mEPSCs. To test this hypothesis, hippocampal neurons were transfected with a specific shRNA (sh6-hnRNP K; target sequence [1201-

1219]: GUAACUAUUCCTCAAAGAUU) to knockdown hnRNP K. As control we used a scramble sequence (sh1-Scramble) lacking homology to any known mammalian mRNAs.

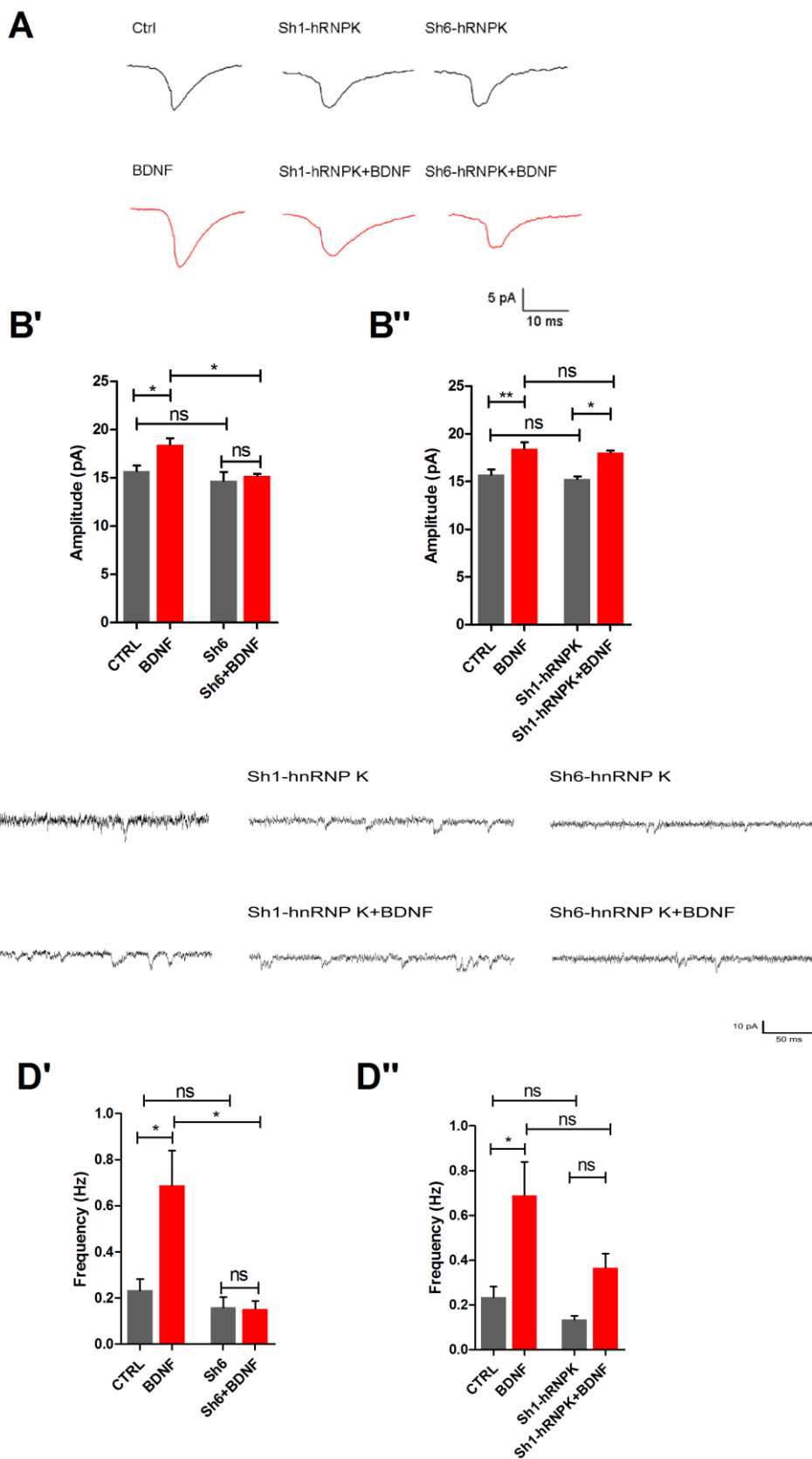


Results

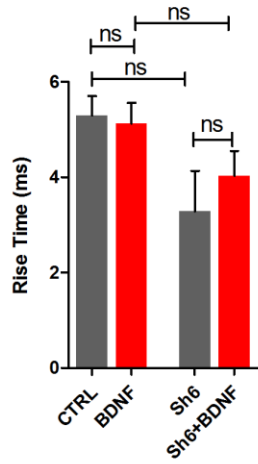
Figure 3.5. BDNF does not affect the rate of internalization of GluN2B-containing NMDA receptors in cultured hippocampal neurons. Hippocampal neurons (DIV 14-15) were pre-incubated with CHX (50 µg/ml) or vehicle DMSO (1:1000 dilution) for 35 min, and were then stimulated or not with BDNF (50 ng/ml) during 25 min, where indicated. When the effect of BDNF was tested, the cells were live immunostained for GluN2B during the first 10 min of incubation with the neurotrophin, using an antibody against an extracellular epitope in the GluN2B N-terminus. After quantification of the images at the soma (B) and neurite (C) compartments, the results were expressed as a ratio of internalized receptors/total receptor immunoreactivity. Representative images are shown in panel (A). The results are the average \pm SEM of 4 different experiments, performed in independent preparations. Statistical analysis was performed using the one-way ANOVA followed by the Bonferroni test.

The use of sh6-hnRNP K to downregulate hnRNP K in cultured hippocampal neurons has been validated in a previous study (Rodrigues, 2013). Transfection was performed 72 h before measurement of the NMDAR-mediated mEPSCs, and the transfected cells were identified by the expression of GFP. Downregulation of hnRNP K with sh6-hnRNP K abrogated the effect of BDNF on the amplitude of NMDAR-mediated mEPSC (**Fig. 3.6B**). In contrast, a small but not significant increase in mEPSC amplitude was observed in cells transfected with the control shRNA (sh1-hnRNP K). Additional experiments are required to confirm these observations.

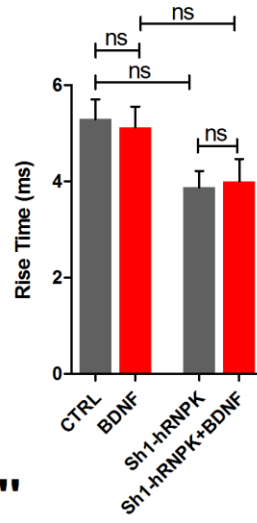
Downregulation of hnRNP K with sh6-hnRNP K also suppressed the BDNF-induced increase in the frequency of NMDA receptor mediated mEPSC, but no effect was observed under resting conditions (**Fig. 3.6D**). However, these results are not very conclusive and additional experiments should be performed, since the control shRNA used (sh1-hnRNP K) also reduced the frequency of NMDAR-mediated mEPSC. As described in Figure 3.3, BDNF was without effect on the decay and rise time of NMDA receptor mediated mEPSC, and similar results were observed in hippocampal neurons expressing reduced levels of hnRNP K (**Fig. 3.6E and F**). Although the results suggest that hnRNP K is important to maintain the effect of BDNF on the area of the NMDA receptor mediated mEPSC, they are not conclusive at this point since the effect of the specific shRNA was similar to the scramble control (**Fig. 3.6G**).



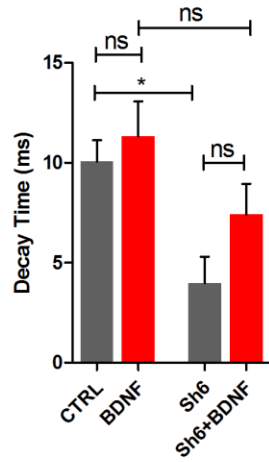
E'



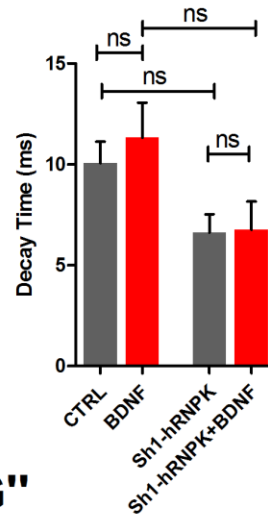
E''



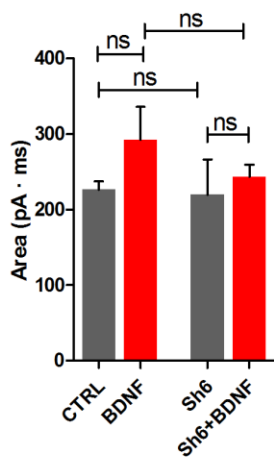
F'



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G'



G''

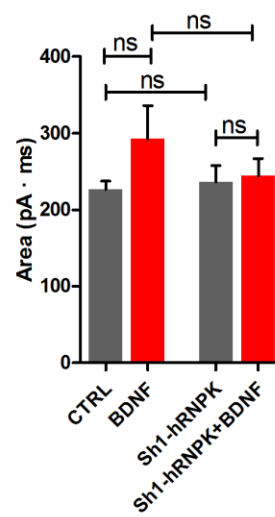


Figure 3.6. hnRNP K mediates the BDNF-induced increase in the amplitude of NMDAR mEPSCs. (A, B) Mean NMDAR-mEPSC amplitudes recorded under control conditions (n = 13) and after 30 min of BDNF stimulation (n = 14), in hippocampal neurons transfected with sh1-hRNPK scramble (n = 6) and sh6-hRNP K (n = 6). Cells transfected with sh6-hRNP K show no significant increase in the amplitude of NMDA-mEPSC upon BDNF treatment. The average mEPSC traces recorded are shown in panel (A) and representative traces are shown in (C). (D) Mean NMDAR-mEPSC frequency recorded under control conditions and after 30 min of BDNF stimulation, in cells transfected with sh1-hRNP K or with sh6-hRNP K. Cells transfected with sh6-hRNP K show no significant increase in the frequency of NMDA-mEPSC upon BDNF treatment. (E, F) Comparison of rise and decay times of NMDA-mEPSCs under control conditions and after >30 min of BDNF stimulation, in hippocampal neurons transfected with sh1-hRNP K (scramble) or with sh6-hRNP K. Both rise and decay times show no significant difference (10–90% rise time: sh1-hRNPK scramble, t = 3.51 ms; sh6-hRNPK, t = 3.28 ms; 90-10% decay time: sh1-hRNP K scramble, t = 5.76 ms; with cells sh6-hRNP K, t = 3.98 ms). (G) Area of NMDAR mediated mEPSCs. Error bars represent the average \pm SEM for the indicated number of experiments performed in at least three independent preparations. Statistical analysis was performed using the one-way ANOVA followed by the Bonferroni test (*p < 0.05).

3.6 BDNF stimulation decreases the interaction of hnRNP K with PYK2 mRNA

The Pyk2 tyrosine kinase is an important regulator of GluN2B-containing NMDAR (Pelkey et al., 2002; Snyder et al., 2005; Xu et al., 2012). Previous microarray studies showed that Pyk2 mRNA was among the transcripts that co-immunoprecipitated with hnRNP K in extracts prepared cultured hippocampal neurons (Comprido, 2011). Furthermore, the same study showed a decrease in the amount of Pyk2 mRNAs that co-immunoprecipitated with hnRNP K in hippocampal neurons stimulated with BDNF (**Fig. 3.7A**) (Comprido, 2011). Together, these results suggest that BDNF-TrkB signaling modulates the interaction of hnRNP K with the Pyk2 mRNA, and may therefore regulate the translation of the kinase. To validate these results we performed qPCR experiments to determine the relative abundance of Pyk2 mRNA in hnRNP K immunoprecipitates prepared from extracts of hippocampal neurons, under control conditions and following stimulation with BDNF (50 ng/ml; 20 min). The results were compared with those obtained for the GluA1 and NPAS4 mRNA. The interaction of GluA1 transcripts with hnRNP K were previously shown to be regulated by the BDNF-TrkB signaling pathway, while the interaction of NPAS4 mRNA is known to be insensitive to stimulation with the neurotrophin (Comprido, 2011). Stimulation of cultured hippocampal neurons with BDNF decreased the coimmunoprecipitation of Pyk2 mRNA with hnRNP K by about 70 % (**Fig.**

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3.7B). As expected based on previous observations, BDNF stimulation also decreased the coimmunoprecipitation of GluA1 mRNA, although the effect was not statistically significant in this set of experiments, while no effect was observed for the NPAS4 mRNA. Control experiments showed a single band when the immunoprecipitates were analyzed by western blot (Comprido, 2011). Furthermore, when IgG was used in the immunoprecipitation no detectable amounts of GluA1, Pyk2 and NPAS4 mRNA were detected (Comprido, 2011).

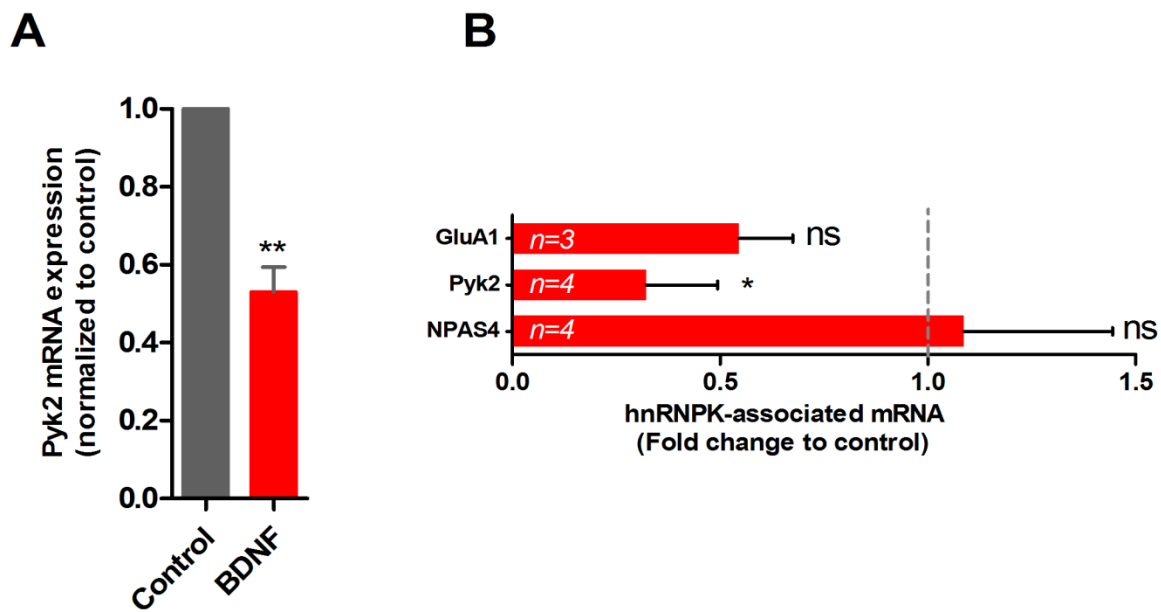


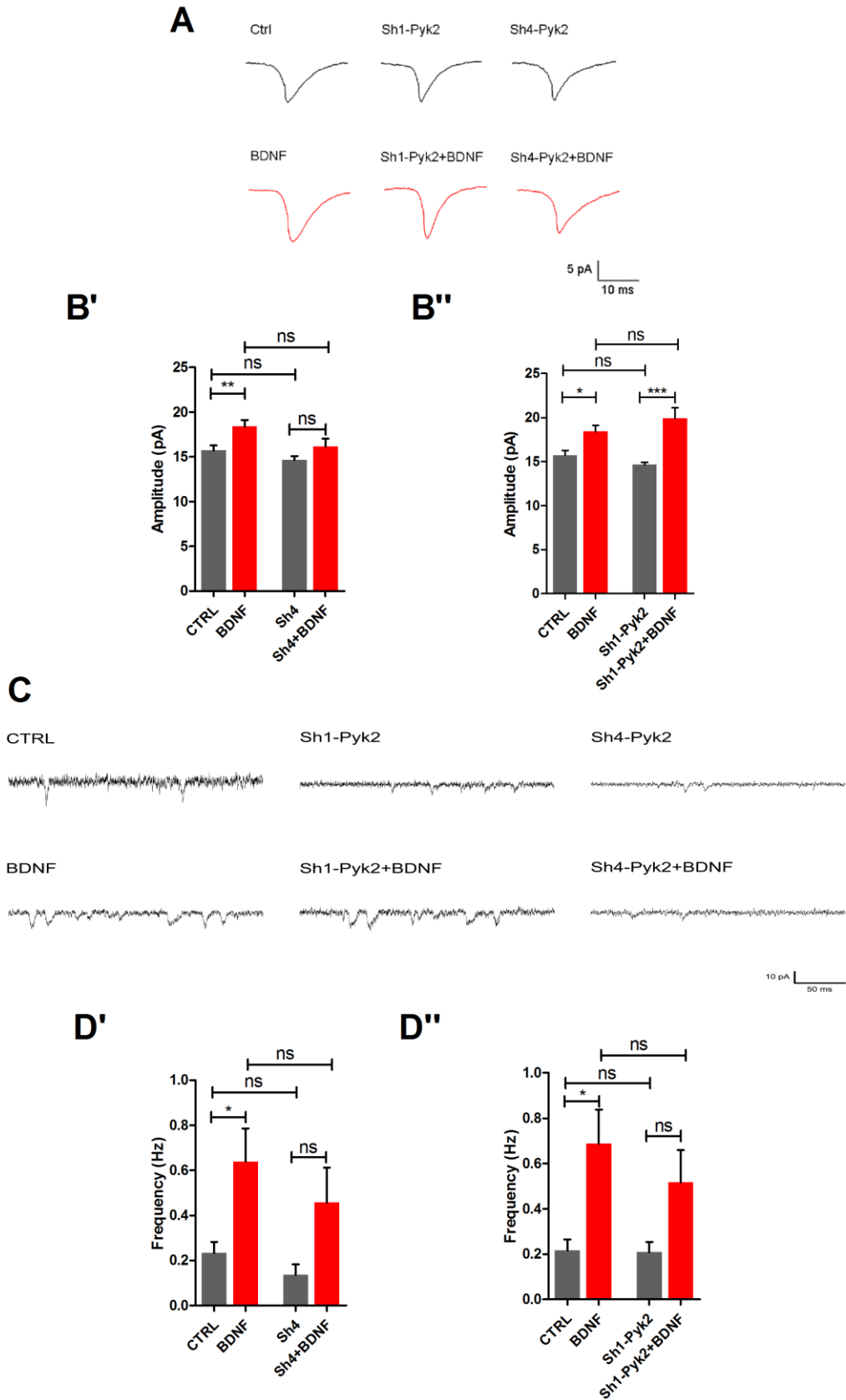
Figure 3.7. BDNF stimulation decreases the interaction of hnRNP K with PYK2 mRNA. (A) A microarray analysis of the transcripts that coimmunoprecipitate with hnRNP K in homogenates from cultured hippocampal neurons show a reduction in the relative amount of Pyk2 mRNA upon stimulation of cells with BDNF (from (Comprido, 2011)). When the effect of BDNF was tested, the cells were stimulated with the neurotrophin (50 ng/ml) for 10 min. (B) The relative amount of Pyk2, GluA1 and NPAS4 transcripts coimmunoprecipitated with hnRNP K from neuronal hippocampal cultures, stimulated or not with BDNF (50 ng/ml) for 20 min, was assessed by RT-qPCR. The results are expressed as fold change to control and are the average \pm SEM of the indicated number of independent experiments performed in distinct preparations. Statistical analysis was performed using the Student's *t* test. ns - not significant as compared with the control; **p* < 0.05, as compared with the control.

3.5 Pyk2 mediates the BDNF-induced upregulation of NMDA receptor-mediated mEPSCs

To further investigate the putative role of Pyk2 in the BDNF-induced upregulation of the functional expression of synaptic NMDAR we transfected cultured hippocampal neurons with sh1-scramble or sh4-Pyk2 (Zhang et al., 2014) at DIV 12, and NMDAR-mediated mEPSC were measured at DIV 15, under control conditions and upon stimulation with BDNF (50 ng/ml; > 30 min). A downregulation of Pyk2 had no effect on the NMDAR-mediated mEPSC under control conditions, but abrogated the upregulation in the current amplitude induced by BDNF (**Fig. 3.8A, B**). The results obtained with the scramble shRNA show a similar effect of BDNF on the amplitude of the NMDA receptor-mediated currents, but additional experiments should be performed since the effect of BDNF did not reach statistical significance.

Analysis of the NMDAR mediated currents in hippocampal neurons transfected with sh4-Pyk2 showed no difference in the frequency of mEPSC under resting conditions, but downregulation of the kinase decreased the effects of BDNF (**Fig. 3.8D**). As expected, transfection of hippocampal neurons with a scramble sequence (sh1-Pyk2) was without effect on the frequency of NMDAR-mediated mEPSC. Furthermore, downregulation of Pyk2 did not change the rise and decay time of the NMDAR mediated mEPSC, and no differences were observed in hippocampal neurons transfected with BDNF (**Fig. 3.8E, F**). Finally, although the results suggest that Pyk2 is important for the effect of BDNF on the area of the NMDAR mediated mEPSC, the results are not conclusive at this point (**Fig. 3.8G**).

Results



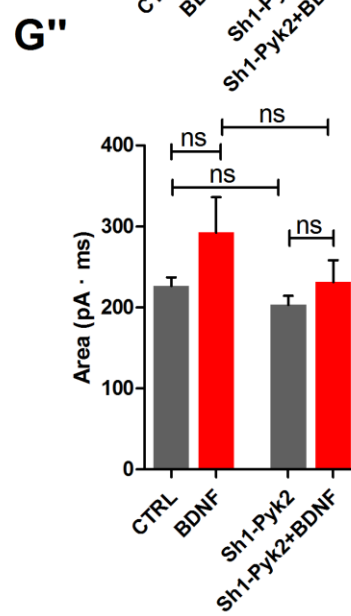
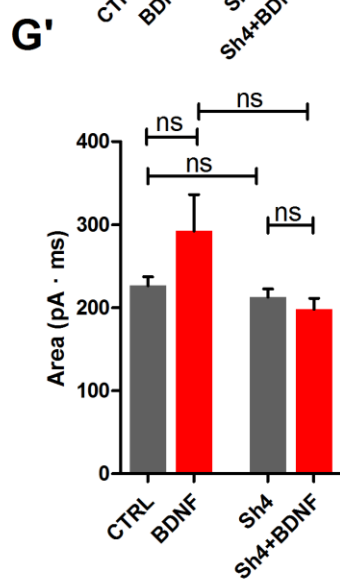
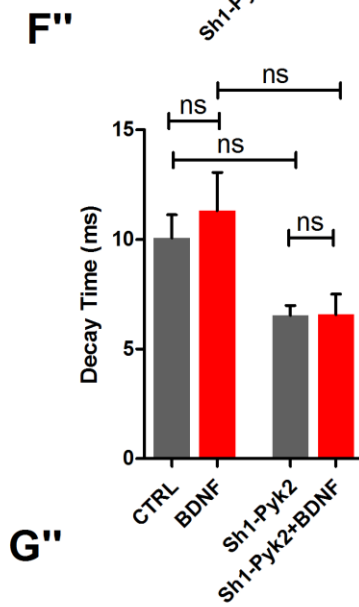
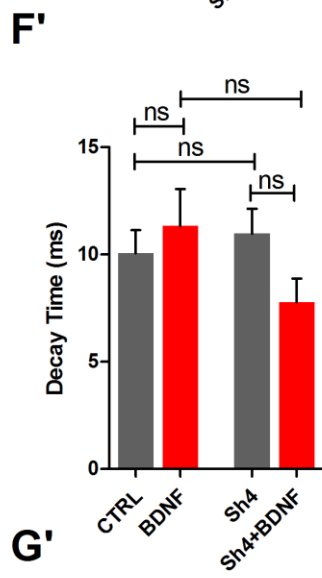
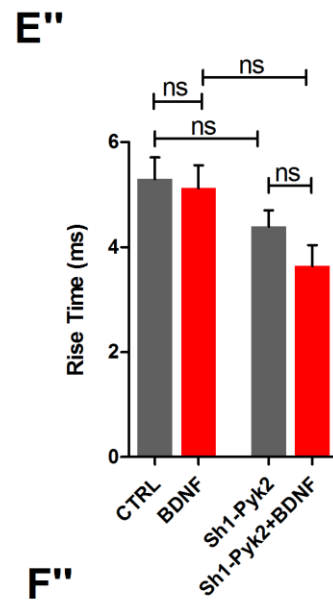
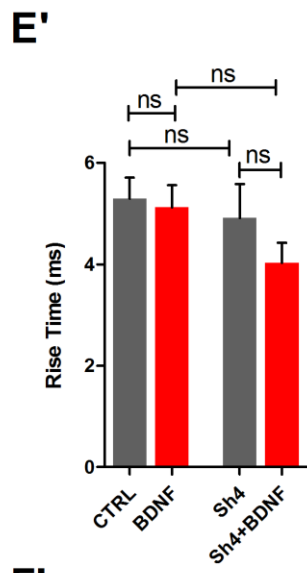


Figure 3.8. BDNF-induced upregulation of NMDAR-mediated mEPSCs is mediated by Pyk2. Hippocampal neurons were transfected with sh1-Scramble (n = 5) (Sh1-Pyk2) or Sh4-Pyk2 (n = 8) at DIV 12, and were then stimulated or not (DIV 15) with BDNF (50 ng/ml) for at least 30 min. Where indicated, NMDAR-mediated mEPSC were measured under control conditions (n = 13) or in cells incubated with BDNF (50 ng/ml; > 30 min) (n = 13). Panels **(A and B)** show the averaged NMDAR mEPSC traces recorded at -60 mV. **(B)** NMDAR-mediated mEPSC amplitude recorded after >30 min BDNF treatment in cells transfected with a shRNA specific for Pyk2 (Sh4-Pyk2) or with a scramble sequence (Sh1-Pyk2). Representative traces are shown in panel **(C)**. No significant effect of BDNF on the amplitude of NMDAR mediated mEPSC was detected in cells transfected with Sh4-Pyk2. **(D)** Mean NMDAR-mediated mEPSC frequency recorded under control conditions and after 30 min of BDNF stimulation, in cells transfected with sh1-Pyk2 or with sh4-Pyk2. Cells transfected with sh4-Pyk2 show no significant increase in the frequency of NMDA-mEPSC upon BDNF treatment. **(E, F)** Comparison of rise and decay times of NMDA-mEPSCs under control conditions and after >30 min of BDNF stimulation, in hippocampal neurons transfected with sh1-Pyk2 (scramble) or with sh4-Pyk2. Both rise and decay times show no significant difference (10–90% rise time: sh1-Pyk2 scramble, t = 4.68 ms; sh4-Pyk2, t = 4.91 ms; 90-10% decay time: sh1-Pyk2 scramble, t = 6.85 ms; with cells sh4-Pyk2, t = 10.97 ms). **(G)** Area of NMDA-mEPSCs. The results are the average \pm SEM of the indicated number of experiments performed in at least three different preparations. Statistical analysis was performed using the one-way ANOVA followed by the Bonferroni test (*p < 0.05).

Discussion and Conclusion

BDNF plays an important role in LTP in the hippocampus, in part by stimulating local protein synthesis at the synapse. However, the molecular mechanisms involved are still not fully understood. In this work we showed an important role for BDNF in enhancing the functional expression of synaptic NMDAR by a mechanism involving protein synthesis and mediated by Pyk2. The major findings herein reported are: i) BDNF treatment increases the surface expression of GluN2B-containing NMDAR in a protein synthesis-dependent manner; ii) BDNF increases the amplitude and the frequency of GluN2B-mediated mEPSCs by a protein synthesis-dependent mechanism; iii) the alteration in the GluN2B surface expression induced by BDNF may be mediated by a mechanism different from an alteration in the rate of internalization; iv) hnRNPk mediates the BDNF-induced enhancement in NMDAR mediated mEPSCs; v) BDNF signaling decreases the interaction of hnRNPk with PYK2 mRNA; vi) BDNF-induced upregulation of the amplitude of NMDAR-mediated mEPSCs is mediated by Pyk2.

Stimulation of cultured hippocampal neurons with BDNF upregulated the amplitude of the NMDAR-mediated mEPSCs, in voltage-clamp recordings from cultured hippocampal neurons, showing a postsynaptic effect of the neurotrophin. Under the same conditions we observed an enhancement of the synaptic expression of GluN2B-containing NMDAR, suggesting that the effects of BDNF on the mEPSC amplitude are mediated by an increase in the number of receptors present at the synapse. This hypothesis is in accordance with the results showing that Conantokin G, a GluN2B-selective competitive antagonist of NMDAR (Donevan and McCabe, 2000), blocked the effects of BDNF on the amplitude of mEPSC. Since the Conantokin G-insensitive component of the mEPSC amplitude was not altered by stimulation with BDNF, the results suggest that the neurotrophin signaling does not affect the synaptic abundance of GluN2A-containing NMDAR. In contrast with the results obtained in the present study, acute exposure to BDNF did not change the amplitude of NMDAR-mediated mEPSC in neocortical and hippocampal brain slices (Madara and Levine, 2008). This suggests that BDNF has a delayed effect on the synaptic accumulation of GluN2B-containing NMDAR, which was expressed upon > 30 min incubation with the neurotrophin, as observed in the immunocytochemistry experiments. Additional studies should be performed to determine the incubation period with BDNF that is required for the synaptic accumulation of GluN2B subunits, as well as for the increase in the amplitude of the mEPSC.

The effects of BDNF on the surface expression of GluN2B, as well as the neurotrophin induced upregulation of NMDAR-mediated mEPSC, were abrogated by the translation inhibitor

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cycloheximide. In contrast, GluN2A- but not GluN2B containing NMDAR are added into the membrane surface of rat hippocampal neurons after induction of neuronal activity, by a mechanism dependent on dendritic synthesis of the receptors (Swanger et al., 2013). The present findings may suggest that translation activity is required for the synthesis of a regulatory protein that mediates the effect of BDNF on the surface expression of GluN2B-containing NMDAR. However, since the presence of GluN2B mRNA has also been described in dendrites (Cajigas et al., 2012), additional studies should be performed to evaluate whether local synthesis of the receptors mediates the effect of BDNF on the surface expression of GluN2B-containing NMDAR. This hypothesis is supported by the results of antibody-feeding experiments which showed no effect of BDNF on the rate of internalization of GluN2B subunits, suggesting that the effect of the neurotrophin on the surface expression of NMDAR is mediated by an increased delivery of receptors to the synapse. However, since the low dynamic range of the signal may have precluded the assessment of possible alterations in the rate of receptor internalization, additional studies should be performed in order to improve the methodology used to analyze the internalization of GluN2B subunits.

Previous studies from our laboratory suggested a key role of hnRNP K in the regulation of dendritic protein synthesis (Morais, 2014), and stimulation of hippocampal neurons with BDNF was shown to induce the dissociation of a large number of transcripts that interact with this RNA binding protein (Comprido, 2011). In this work we found that hnRNP K is a mediator of the effects of BDNF on the amplitude of NMDAR-mediated mEPSC, but it was without effect on the currents recorded under resting conditions. These results indicate that at least part of the machinery involved in the response to the neurotrophin is not involved in the regulation of the surface expression of NMDAR under resting conditions. Although hnRNP K is present at the synapse (Liao et al., 2007; Folci et al., 2014), it remains to be determined whether the effects of the protein on the amplitude of the mEPSC are due the regulation of local protein synthesis. Furthermore, it remains to be determined whether the transcripts that co-immunoprecipitated with hnRNP K (GluA1, Pyk2 and NPAS4) interact directly or indirectly with the protein. In fact, besides the KH domains which are involved in the interaction with mRNAs, hnRNP K also contains a KI region which is responsible for the interaction with other proteins (Bomsztyk et al., 2004). Since some of these binding partners are also RNA binding proteins (Mikula et al., 2006), the observed decrease in the interaction of hnRNP K with the Pyk2 (or GluA1) mRNA following stimulation of hippocampal neurons may be due a decrease in affinity between the two interacting proteins. These results may also be explained based on a putative decrease in the affinity of the hnRNP K binding protein with the associated transcripts. Whether hnRNP K binds

directly to the Pyk2 or GluA1 mRNA can be determined using the electrophoretic mobility shift assay (EMSA).

The effects of BDNF on the amplitude of NMDAR-mediated mEPSC were abrogated in hippocampal neurons transfected with an shRNA specific for Pyk2. The observed dissociation of the Pyk2 mRNA from hnRNP K upon stimulation of hippocampal neurons with BDNF, suggests that *de novo* synthesis of the kinase may be important in the upregulation of the NMDAR-mediated currents. Accordingly, unpublished observations from our laboratory showed that BDNF induces the synaptic accumulation of GluN2B subunits by a mechanism involving Pyk2 accumulation and activation at the synapse (Afonso, 2016). Therefore, inhibition of Pyk2 synthesis by cycloheximide may account for the effect of translation inhibition on the amplitude of NMDAR-mediated mEPSC. There are indeed several pieces of evidence suggesting that the effect of BDNF on the synaptic expression of GluN2B-containing NMDAR may be mediated by Pyk2: (i) BDNF increases the phosphorylation of GluN2B on Tyrosine1472 through a mechanism involving the proteasome-mediated degradation of the phosphatase STEP (Nakai et al., 2014; Saavedra et al., 2016; Xu et al., 2016); (ii) GluN2B phosphorylation on Tyrosine1472 also favors the accumulation of NMDAR at the synapse, while phosphorylation on Tyrosine1336 increases their extrasynaptic distribution (Grosshans et al., 2002; Prybylowski et al., 2005; Goebel-Goody et al., 2009); (iii) Phosphorylation of Tyrosine1472 by Fyn/Src tyrosine kinases negatively regulates the endocytosis of GluN2B-NMDAR by blocking the binding of AP-2, and therefore increases the surface expression of the receptors and synaptic transmission (Lavezzari et al., 2003; Prybylowski et al., 2005; Xu et al., 2006; Sanz-Clemente et al., 2010); (iv) BDNF activates Fyn (Narisawa-Saito et al., 1999); (v) Src family kinases are activated by Pyk2 (Yu et al., 1997; Zheng et al., 1998; Xiong et al., 1999; Huang et al., 2001; Kalia et al., 2004); (vi) BDNF-TrkB signaling was shown to induce Pyk2 phosphorylation (activation) in different biological contexts (Zhang et al., 2010; Xu et al., 2016). Importantly, GluN2B phosphorylation on Tyrosine1472 was also observed upon HFS-induced LTP in the hippocampus and amygdala and is essential for learning and memory (Nakazawa et al., 2001; Nakazawa et al., 2006; Isosaka et al., 2008). Although this working hypothesis to explain the effect of BDNF on the synaptic expression of GluN2B is supported by the available literature, it is not in accordance with the results of the antibody-feeding experiments (see above for discussion).

In contrast with the role of Pyk2 in BDNF-induced enhancement of the amplitude of NMDAR-mediated mEPSC, downregulation of the kinase with a specific shRNA did not change the mEPSC

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amplitude under resting conditions. These results resemble the effect of hnRNP K downregulation, which specifically affected the amplitude of the NMDAR-mediated currents without affecting the mEPSC under resting conditions. Together, this evidence suggest that distinct mechanisms are involved in the regulation of synaptic NMDAR under resting conditions and in response to neuronal activity.

Increased GluA1 surface expression is a hallmark of LTP and brief incubations with BDNF also induce the surface delivery and synaptic incorporation of GluA1-containing AMPA receptors in hippocampal organotypic cultures (Caldeira et al., 2007b). BDNF also induced the surface expression of GluA1-containing receptors in developing hippocampal neurons by a mechanism dependent of protein synthesis, which was mediated by the influx of Ca^{2+} through TRPC (transient receptor potential canonical) 5/6 channels followed by activation of the Ca^{2+} - and calmodulin-dependent protein kinase kinase (CaMKK) (Fortin et al., 2012). Furthermore, CaMKK was shown to upregulate translation activity by stimulation of Akt followed by phosphorylation of mTOR (Fortin et al., 2012). Whether this pathway also contributes to the upregulation of synaptic GluA1-containing receptors, presumably characterized by a higher Ca^{2+} permeability, remains to be investigated. Furthermore, it will be of interest to determine whether a similar pathway contributes to the BDNF-induced upregulation of translation activity that leads to the increase synaptic expression and activity of GluN2B observed in the present study.

The surface delivery of GluA1 subunits of AMPA receptors in cultured hippocampal neurons subjected to a protocol of chemical LTP was also found to require the phosphorylation of hnRNP K. In contrast, under resting conditions this RNA binding protein does not play a role in the regulation of GluA1 surface expression (Folci et al., 2014). These results resemble the role of hnRNP K in the regulation of NMDAR-mediated mEPSC, i.e., it mediates the BDNF-evoked upregulation in the current amplitude, but plays no role under resting conditions. The effect of hnRNP K on the surface distribution of GluA1 after induction of chemical LTP was dependent on the phosphorylation of the RNA binding protein by ERK. Remarkably, hnRNP K was also important for ERK activation under the same conditions, as a positive feedback mechanism (Folci et al., 2014). Since the Ras/ERK pathway is also activated following stimulation of TrkB receptors by BDNF, it will be of interest to determine whether a similar mechanism is involved in the BDNF-induced alteration in NMDAR-mediated mEPSC.

In addition to the postsynaptic effects in enhancing NMDAR-mediated mEPSC, stimulation of hippocampal neurons with BDNF also increased the frequency of events, which reflect presynaptic alterations. This is in agreement with the evidence available pointing to presynaptic effects of the neurotrophin (e.g. (Pozzo-Miller et al., 1999; Jovanovic et al., 2000; Pereira et al., 2006)). Interestingly, the BDNF-evoked upregulation of mEPSC frequency was blocked in the presence of cycloheximide, showing a role for protein synthesis in these alterations. The reported clustering of vesicular glutamate transporters at the synapse induced by BDNF may account, at least in part, for these observations (Melo et al., 2013). Preliminary results also suggested that transfection of hippocampal neurons with an shRNA against hnRNP K abolish the BDNF-induced increase in the frequency of mEPSC. This is an interesting observation considering the low rate of neuronal transfection, which suggest that the presynaptic effects of BDNF may be dependent of alterations in the postsynaptic neurons as a result of hnRNP K downregulation. Such a retrograde mechanism may not operate in hippocampal neurons transfected with the shRNA against Pyk2, since in this case BDNF still increased the frequency of NMDAR-mediated mEPSC.

NMDAR dysfunctions are also involved in various neurological and psychiatric disorders, including stroke, pathological pain, neurodegenerative diseases, Alzheimer's disease, Parkinson's disease, Huntington's disease (HD) and schizophrenia. In particular Huntington's disease is an autosomal dominant genetic disease, carried by the chromosome four, and caused by a CAG triplet repeat expansion in the HTT gene, which encodes huntingtin (Group, 1993). Although the mutated protein is ubiquitously expressed, 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 (Okamoto et al., 2009; Milnerwood et al., 2010). Experiments performed in this HD mouse model showed that the extra-synaptic NMDAR are essentially GluN2B-NMDAR, which correlate with the low levels of BDNF at the corticostriatal synapses due to an impairment in the transport of the neurotrophin (Millecamps and Julien, 2013). A downregulation in BDNF levels was also observed in the dentate gyrus of patients with Alzheimer's disease (AD) (Narisawa-Saito et al., 1996), which may account for an impairment in synaptic plasticity in these patients and contribute to the neuropathological and clinical manifestations of the pathology (Mesulam, 1999). Several studies have pointed to a role for GluN2B-NMDAR in the alteration of synaptic plasticity in AD

Discussion and Conclusion

(Zheng et al., 2009; Sheng et al., 2012). Together, these evidence suggest that the BDNF-induced stimulation of GluN2B-mediated mEPSCs, including the effects on the synthesis, trafficking and activation of Pyk2, might be a good therapeutic target for diseases characterized by an impairment of NMDAR and synaptic loss, such as neurodegenerative diseases.

In conclusion, in this work we have shown that BDNF enhances the functional synaptic expression of GluN2B-containing NMDAR, by a mechanism dependent on protein synthesis and mediated by Pyk2. An important role for GluN2B-containing NMDAR in cognition has been proposed based on studies where the transport of the receptors was increased (Wong et al., 2002) or by decreasing their rate of degradation (Hawasli et al., 2007). Therefore, the effects of BDNF in the enhancement of the GluN2B synaptic expression may play an important role in synaptic plasticity, learning and memory.

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