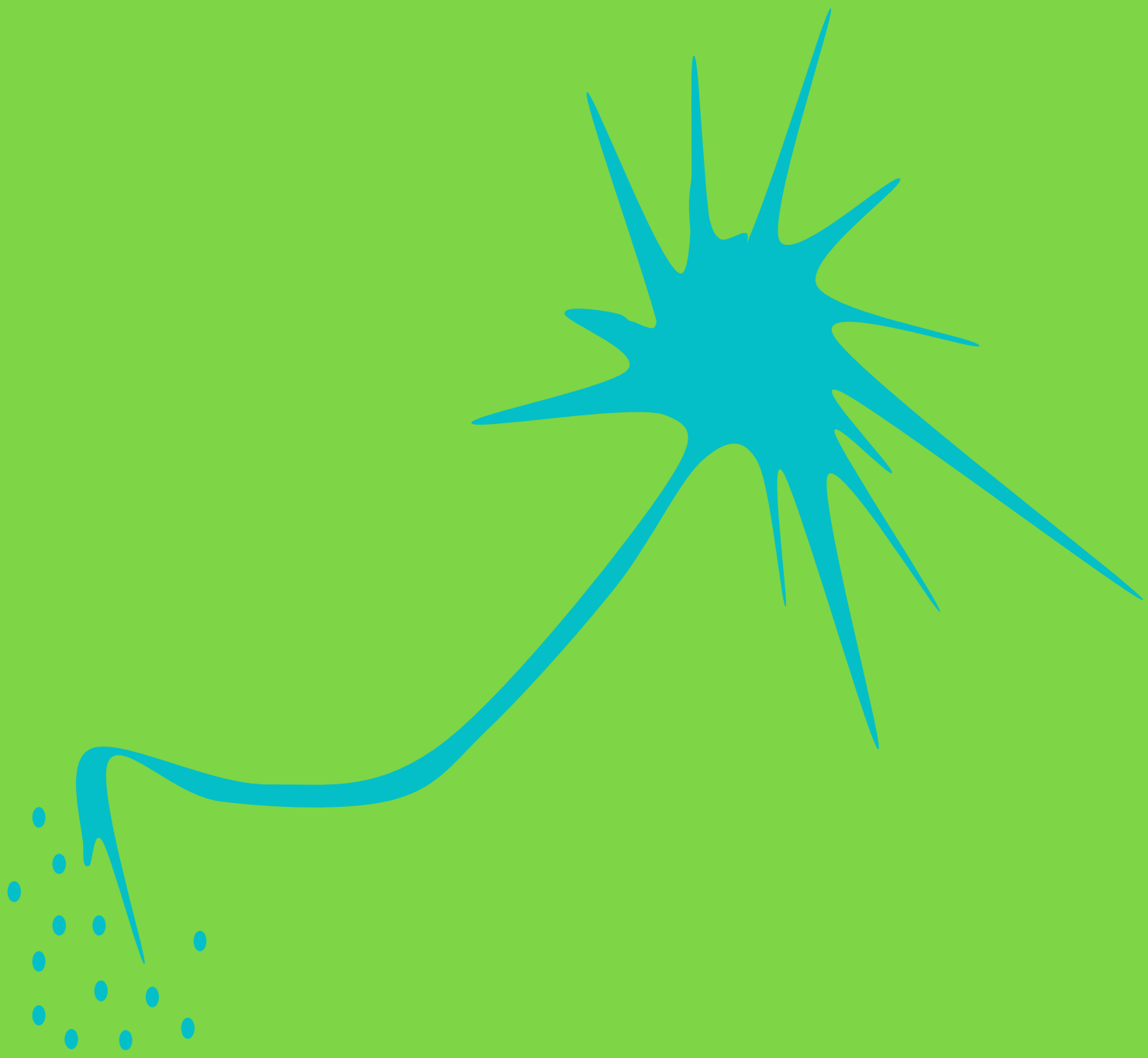
Regulação da expressão dos receptores ionotrópicos do glutamato pelo BDNF em neurónios do hipocampo



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Universidade de Coimbra, 2007

Regulação da expressão dos receptores ionotrópicos do glutamato pelo BDNF em neurónios do hipocampo

Regulation of the expression of ionotropic glutamate receptors by BDNF in hippocampal neurons

Dissertação apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra para prestação de provas de Doutoramento em Biologia, na especialidade de Biologia Celular

Margarida Alexandra Vaz Caldeira **Coimbra 2007**

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Abbreviations

ADAR	adenosine deaminases acting on RNA
ABP	AMPA receptor binding protein
AKAP	A-kinase anchoring protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	analysis of variance
4-AP	4-aminopyridine
APS	adaptor protein with a pleckstrin homology and a SH2 domain
ARMS	ankyrin-rich membrane-spanning
ATPA	(RS)-2-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl)propionic acid
BDNF	brain-derived neurotrophic factor
BCA	bicinchoninic acid
CaMKII	calcium/calmodulin-dependent protein kinase II
$[Ca^{2+}]_i$	intracellular free Ca^{2+} concentration
cAMP	cyclic adenosine monophosphate
CLAP	chymostatin, leupeptin, antipain and pepstatin
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CREB	cAMP response element-binding protein
DAG	diacylglycerol
DL-APV	(D,L)-2-amino-5-phosphonovaleric acid
DIV	days in vitro
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
DOC	deoxycholic acid
DTT	dithiothreitol
ECF	enhanced chemifluorescence
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ERK	extracellular signal-regulated kinase
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GRIP	glutamate receptor interacting protein

GST	glutathione S-transferase
HBSS	Hank's balanced salt solution
HEK	human embryonic kidney
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIAP	human inhibitor of apoptosis protein
IAP	inhibitor of apoptosis protein
$Ins(1,4,5)P_3$	inositol 1,4,5-trisphosphate
IPSC	inhibitory postsynaptic current
JNK	c-Jun N-terminal kinase
KA	kainate
kDa	kilodalton
LTP	long-term potentiation
LTD	long-term depression
MAGE	melanoma antigen
MAGUK	membrane-associated guanylate kinase
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NADE	p75NTR-associated cell death executor
NAIP	neuronal apoptosis-inhibitory protein
Narp	neuronal activity regulated pentraxin
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NRAGE	neurotrophin receptor interacting MAGE homologue
NRIF	neurotrophin receptor interacting factor
NSF	N-ethylmaleimide-sensitive fusion protein
NRSE	neuron-restrictive silencer element
NT3	neurotrophin-3
NT4	neurotrophin-4
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK	3-phosphoinositide-dependent kinase
PDZ	postsynaptic density 95-discs large-zona occludens 1

PICK1	protein interacting with C kinase
PI3-K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PSD	postsynaptic density protein
PTB	phosphotyrosine-binding
PTK	protein tyrosine kinase
PVDF	polyvinylidene difluoride
RACK1	receptor for activated protein kinase C-1
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SAP	synapse-associated protein
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-poliacrylamide gels
SEM	standard error of the mean
SGK3	serum and glucocorticoid-inducible kinase 3
SH2	Src homology 2
SH-PTP	Src homology PTP
SNARE	SNAP receptor
SNT	suc-1-associated neurotrophic factor target
TM	transmembrane region
TNF	tumor necrosis factor
Trk	tropomyosin-related kinase
TRP	transient receptor potential
XIAP	X-linked inhibitor of apoptosis protein

x

Resumo

O glutamato é responsável pela maioria da transmissão sináptica excitatória no cérebro através da sua ligação a receptores do glutamato dos tipos ionotrópico e metabotrópico, os quais desempenham um papel muito importante em alterações fisiológicas da eficácia sináptica. Os receptores ionotrópicos do glutamato formam canais iónicos e são agrupados em três subtipos com base na sua estrutura molecular, farmacologia e propriedades electrofisiológicas: receptores AMPA, NMDA e cainato. Os receptores do tipo AMPA e cainato medeiam a resposta sináptica rápida ao glutamato, enquanto que os receptores do tipo NMDA são responsáveis por uma resposta mais lenta. O número, composição e localização dos receptores de glutamato nos neurónios, bem como a sua fosforilação, são factores determinantes na resposta dos neurónios ao neurotransmissor glutamato. Deste modo, a expressão funcional dos receptores pode ser regulada não apenas ao nível da transcrição e da tradução, mas também ao nível da sinapse.

O BDNF é uma proteína que pertence à família das neurotrofinas, um grupo de proteínas envolvido em mecanismos de plasticidade sináptica importantes para a aprendizagem e memória. As neurotrofinas são também importantes para a sobrevivência, diferenciação, crescimento e morte neuronal. Os efeitos do BDNF são parcialmente mediados pela ligação ao seu receptor, TrkB, seguida da interacção de proteínas adaptadoras com o receptor e activação de várias cascatas de sinalização intracelular.

Neste estudo foi investigado o efeito do BDNF nos receptores do tipo AMPA e NMDA em culturas primárias de neurónios de hipocampo. As células foram mantidas em cultura durante 7 dias e, posteriormente, incubadas com BDNF. Ensaios de *Western Blot* mostraram que a neurotrofina induz aumentos distintos na quantidade de proteína das subunidades GluR1, GluR2 e GluR3 dos receptors AMPA. Pequenos aumentos foram observados após 15 min de estimulação com BDNF, enquanto que os efeitos máximos ocorreram à 1 h, 2 h e 30 min de incubação para o GluR1, GluR2 e GluR3, respectivamente. Para períodos mais longos (24 h) de incubação com a neurotrofina, a abundância destas subunidades diminuiu para valores semelhantes ao controlo. O BDNF induziu também aumentos na abundância das subunidades NR1, NR2A e NR2B dos receptors NMDA. Os

níveis da subunidade NR1 aumentaram de um modo contínuo e duradouro face à estimulação com BDNF (30 min até às 12 h de estimulação). A quantidade de NR2A aumentou apenas quando a estimulação com BDNF foi de longa duração (de 3 h a 12 h), enquanto que os níveis de NR2B aumentaram para tempos curtos de estimulação com BDNF (1 h a 3 h). Estes incrementos estão relacionados com o aumento da transcrição induzido pelo BDNF, como comprovam os estudos com inibidores específicos da transcrição (α-amanitina e actinomicina D) e os aumentos do mRNA observados para as diferentes subunidades através do ensaio de PCR em tempo real. A inibição da tradução com emetina ou anisomicina também impediu os aumentos na quantidade das subunidades dos receptores AMPA e NMDA induzidos pela incubação com BDNF. A estimulação com BDNF de culturas de neurónios de hipocampo com 14 dias aumentou também os níveis das subunidades dos receptores NMDA, mas não teve qualquer efeito nas subunidades dos receptores AMPA. Contudo, quando se quelatou o BDNF endógeno presente no meio extracelular com a proteína de fusão Trk-IgG, os níveis de proteína da subunidade GluR1 diminuíram em relação ao controlo. No entanto, não foram observados quaisquer efeitos para as subunidades GluR2, NR1, NR2A e NR2B em neurónios de hipocampo com 14 dias. A incubação de neurónios de hipocampo em cultura durante 7 dias com a neurotrofina NT3 fez também aumentar os níveis de proteína das subunidades NR2A e NR2B.

Ensaios de biotinilação revelaram que a aplicação de BDNF aumenta a expressão à superfície dos neurónios das subunidades GluR1, NR1, NR2A e NR2B. O aumento verificado na membrana plasmática para a subunidade GluR1 foi abolido pela emetina, sugerindo que o endereçamento do GluR1 para a membrana está dependente da síntese de novas proteínas. O aumento da expressão das subunidades AMPA na membrana relaciona-se com os resultados obtidos nos estudos de electrofisiologia, que mostraram que o BDNF também induz o endereçamento da subunidade GluR1-GFP para a sinapse em fatias de hipocampo que sobrexpressam esta proteína. Neste sistema, o BDNF induziu a fosforilação do receptor Trk, bem como da Ser 831 do GluR1, mas não da Ser 845 do GluR1 ou da Ser 880 do GluR2. A fosforilação da Ser 831 da subunidade GluR1 foi impedida pela queleritrina e pelo KN93, que inibem a PKC e a CaMKII, respectivamente. A incubação com BDNF também ampliou o aumento da concentração intracelular de cálcio em resposta à estimulação com NMDA, em culturas primárias de hipocampo.

Os aumentos na abundância das várias subunidades dos receptores AMPA em neurónios em desenvolvimento e o seu endereçamento para a membrana, em particular para a sinapse, podem explicar, pelo menos em parte, as alterações sinápticas induzidas pelo BDNF. Os aumentos dos níveis das subunidades dos receptores NMDA, quer totais quer na membrana plasmática, e o aumento da resposta ao NMDA na concentração intracelular de cálcio podem explicar também efeito do BDNF na transmissão sináptica e na plasticidade sináptica.

Abstract

Glutamate mediates most excitatory neurotransmission in the brain through the activation of ionotropic and metabotropic glutamate receptors, which play an important role in physiological modifications of synaptic efficacy. Ionotropic glutamate receptors form ion channels, and are grouped into three main subtypes on the basis of their molecular, pharmacological and electrophysiological characteristics: AMPA, NMDA and kainate receptors. AMPA and kainate receptors primarily mediate rapid responses to glutamate, whereas NMDA receptors mediate a slower phase of neurotransmission. The number, composition, and location of glutamate receptors in neurons, together with receptor phosphorylation, are critically important factors in determining the neuron's response to the neurotransmitter glutamate. The functional expression of the receptors may thus be regulated at the transcription and translation level and locally, at the level of the individual synapses.

BDNF is a protein that belongs to the neurotrophin family of trophic factors. This class of proteins is involved in the mechanisms of synaptic plasticity, which are important for learning and memory formation. Neurotrophins are also important for neuronal survival, differentiation, growth and apoptosis. The effects of BDNF are mediated, in part, by binding to its receptor, TrkB, and this leads to the recruitment of several intracellular adaptor proteins and activation of various signalling pathways.

In this study, we investigated the effect of BDNF on AMPA and NMDA receptors in hippocampal neurons. As demonstrated by Western Blot, stimulation of 7 DIV cultured hippocampal neurons with BDNF induced a rapid and transient increase of the protein levels of the AMPA receptor subunits GluR1, GluR2 and GluR3, with small effects already observed after 15 min of BDNF stimulation. Maximal effects were observed upon incubation with BDNF for 1 h, 2 h and 30 min for GluR1, GluR2 and GluR3, respectively. For longer periods (24 h) of incubation with the neurotrophin, the abundance of these subunits decreased to values similar to the control. BDNF also differentially upregulated the NMDA receptor subunits NR1, NR2A and NR2B. The levels of the NR1 subunits were increased by BDNF in a long-lasting manner (30 min to 12 h of incubation). NR2A protein levels were upregulated for longer periods of BDNF incubation (3 h to 12 h), while NR2B protein levels

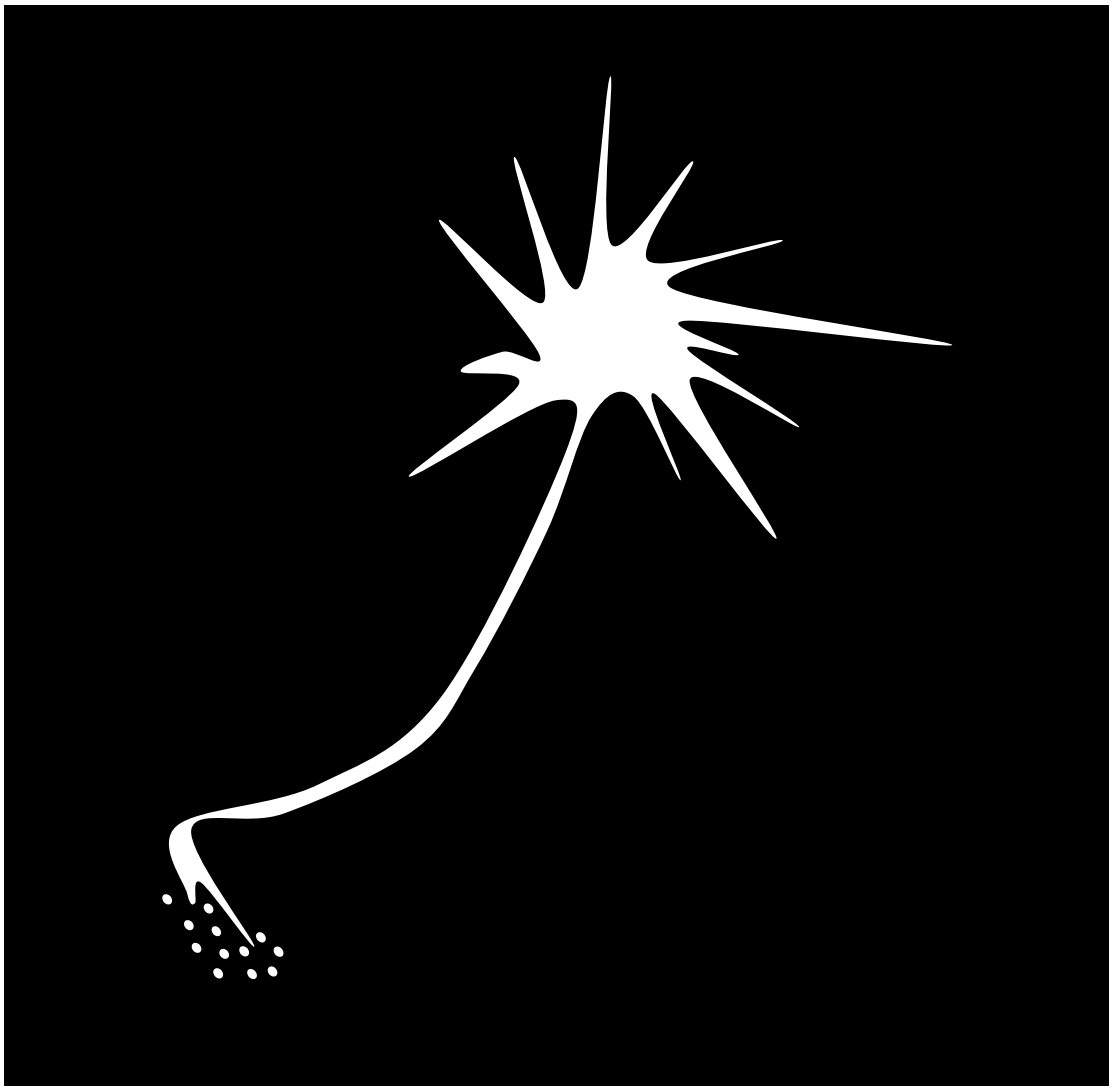
were increased by short periods of stimulation with BDNF (1 h to 3 h). These effects required transcription activity, as demonstrated by the use of specific transcription inhibitors (α-amanitine and actinomycin D), and by the enhancement of the mRNA levels of these subunits, observed by real time PCR. Translation inhibitors (emetine and anisomycin) were also tested and were found to inhibit the upregulation of the subunit protein levels induced by BDNF. Fourteen DIV hippocampal neurons were also treated with BDNF, but no effects were observed for AMPA receptor subunits. In contrast, NMDA receptor subunits were found to be upregulated by BDNF in 14 DIV hippocampal neurons, with a kinetics similar to that observed for 7 DIV cultures. Moreover, chelation of endogenous extracellular BDNF with TrkB-IgG fusion protein decreased GluR1 protein levels, but was without effect on GluR2, NR1, NR2A and NR2B in 14 DIV cultures of hippocampal neurons. Incubation of 7 DIV cultured hippocampal neurons with NT3 also increased the protein levels of the NMDA receptor subunits NR2A and NR2B.

Biotinylation assays demonstrated that incubation of hippocampal cell cultures with BDNF increases the number of plasma membrane-associated GluR1, NR1, NR2A and NR2B. The BDNF-induced increase of GluR1 subunits associated with the plasma membrane was blocked by emetine, indicating that delivery of GluR1 to the plasma membrane was dependent on the synthesis of new proteins. The increase in the surface expression of AMPA receptor subunits correlates with the results obtained in electrophysiology studies, which showed synaptic delivery of GluR1 receptors induced by BDNF, in hippocampal slices overexpressing the GluR1-GFP subunit. In this system, BDNF was shown to promote phosphorylation of the Trk receptor as well as Ser 831 in the GluR1 subunit, but not Ser 845 in GluR1 or Ser 880 in GluR2 subunit. BDNF-induced phosphorylation of GluR1 on Ser 831 was abrogated by chelerythrine and KN93, which inhibit PKC and CaMKII, respectively. BDNF also upregulated the NMDA-induced increase in the intracellular concentration of calcium, in hippocampal neuronal cultures.

The observed increase in the abundance of AMPA receptors in developing neurons and their delivery to the membrane, as well as the phosphorylation, cell surface and synaptic delivery of AMPA receptors, may explain some of the synaptic effects of BDNF. Furthermore, the upregulation of NMDA receptor subunits induced by BDNF, and the increase in the intracellular calcium response to NMDA following exposure to the

neurotrophin may also explain, at least in part, the short- and long-term effects of BDNF on synaptic plasticity.

Chapter 1. Introduction



1.1 The synapse and glutamatergic transmission

Synapses are specialized cell-cell junctions that allow neurons to communicate with each other and with other neuronal targets. Upon neuronal stimulation, neurotransmitters are released from the presynaptic terminal into the synaptic cleft. Neurotransmitters diffuse across this intercellular space and bind to neurotransmitter receptors on the postsynaptic membrane to induce a postsynaptic response. Synapses can be classified as excitatory or inhibitory depending on the effect of presynaptic stimulation on the postsynaptic potential. Stimulation of excitatory synapses induces an excitatory postsynaptic potential (EPSP) that depolarizes the post-synaptic membranes towards the threshold required for activation of an action potential. Conversely, activation of inhibitory synapses hyperpolarizes the post-synaptic membrane away from the threshold potential [(inhibitory postsynaptic potential; IPSP); (reviewed in Sheng and Lin, 2001)].

Glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system (CNS) and plays an important role in a large number of CNS functions. This neurotransmitter is believed to underlie learning processes and memory formation, and is also involved in several neurological disorders, such as epilepsy and ischemia (Dingledine et al., 1999).

Glutamate is a nonessential amino acid that does not cross the blood-brain barrier. It was early considered an excitatory neurotransmitter since 1) it is stored in synaptic vesicles in the presynaptic terminal, from where it can be released into the synapse in a calcium-dependent manner; 2) glutamate containing vesicles are specifically associated with excitatory synapses; 3) high-affinity glutamate transporters are present in the pre-synaptic membrane, as well as in the neighboring glial cells, providing a mechanisms for rapid glutamate uptake and signal termination; 4) exogenously applied glutamate induces an excitatory postsynaptic response identical to that elicited by presynaptic stimulation and, finally, 5) a high concentration of glutamate receptors is specifically found at the postsynaptic site in excitatory synapses (reviewed in Sheng and Lin, 2001).

1.1.1 Glutamate Receptors

Glutamate receptors are transmembrane proteins that bind specifically to glutamate on the extracellular side of the membrane. Upon binding of glutamate, glutamate receptors are activated and transduce this signal into intracellular responses. Depending on their functional and pharmacological properties glutamate receptors can be divided in several classes. Glutamate receptors can be subdivided into ionotropic (ligand-gated cation channels) and metabotropic (coupled to G-protein second messenger systems) receptors.

Table 1.1- Ionotropic receptor subunits and pharmacology

Receptor Family	Subunit	Agonists	Antagonists
AMPA	GluR1 GluR2 GluR3 GluR4	Glutamate AMPA Kainate (S)-5-fluorowillardiine	CNQX NBQX GYKI53655
Kainate	GluR5 GluR6 GluR7 KA1 KA2	Glutamate Kainate ATPA	CNQX LY294486 LY382884 NS 102
NMDA	NR1 NR2A NR2B NR2C NR2D NR3A NR3B	Glutamate Aspartate NMDA Glycine	D-APV (or D-AP5) 2R-CPPene MK-801 Ketamine Phencyclidine

There are three groups of metabotropic glutamate receptors, distinguished based on their sequence homology, signal transduction mechanisms and agonist selectivity. Group I includes mGluR1 and mGluR5, which are positively coupled to phospholipase C (PLC).

Activation of these receptors increases the plasma membrane levels of diacylglycerol (DAG) and the intracellular abundance of inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$], thereby activating protein kinase C (PKC) and releasing intracellular Ca^{2+}, respectively. Group II metabotropic glutamate receptors includes mGluR2 and mGluR3, and group III includes mGluR4, mGluR6-8, which are negatively coupled to adenylyl cyclase, inhibiting cyclic adenosine monophosphate (cAMP) formation (reviewed in Pin and Acher, 2002).

The ionotropic glutamate receptors have been classified into three distinct subgroups based upon their pharmacology, as well as molecular and electrophysiological properties (see table 1.1). These are the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), *N*-methyl-D-aspartate (NMDA), and kainate (KA) receptors (Dingledine et al., 1999; Mayer, 2004). However, it is important to note that AMPA receptors are also responsive to kainate. Ionotropic glutamate receptors form channels permeable to cations, and binding of glutamate to the receptors leads to membrane depolarization.

1.1.1.1 AMPA receptors

AMPA receptors mediate fast and short-lasting excitatory synaptic neurotransmission in the vertebrate CNS. They are ligand-gated ion channels, permeable to Na^+ and K^+ and, depending on their subunit composition, they may also be permeable to Ca^{2+} (Swanson et al., 1997). The cation flux through AMPA receptor channels permits a net depolarization of the membrane (reviewed in Sheng and Lin, 2001). AMPA receptors are composed of four possible subunits, named GluR1 to GluR4, which associate in different stoichiometries to form receptors with distinct properties. Each subunit has a molecular weight of approximately 100 kDa and possesses three transmembrane segments (TM1, TM3 and TM4) in addition to a specific membrane-inserted hydrophobic region (TM2 region) that forms the cation permeable channel pore. The N-terminal domain of each receptor subunit is extracellular while the C-terminal segment is intracellular. Each monomer carries its own glutamate-binding domain composed by two extracellular segments (Fig. 1.1). AMPA receptor subunits are homologous in their extracellular and transmembrane regions, but are distinct in their intracellular cytoplasmatic C-termini, which contain the phosphorylation sites

and are responsible for the interaction with different intracellular proteins (Gomes et al., 2003; Palmer et al., 2005). GluR1, GluR4 and a splice variant of GluR2 (GluR2L) have long cytoplasmatic C-termini, whereas GluR2, GluR3 and an alternative splice form of GluR4 (GluR4c) have shorter cytoplasmatic domains. There is a high homology among long forms, as well as among short forms of AMPA receptor subunits. In the adult hippocampus, receptors made of GluR1/GluR2 and GluR3/GluR2 predominate (Wenthold et al., 1996), whereas GluR4 is present in the immature hippocampus (Zhu et al., 2000). Receptors composed of subunits with short cytoplasmatic C-termini (GluR2/GluR3) cycle continuously in and out of the synapse, while receptors containing subunits with long cytoplasmatic C-termini (GluR1, GluR2L and GluR4) are delivered to synapses upon synaptic activity (Hayashi et al., 2000; Zhu et al., 2000; Shi et al., 2001; Kolleker et al., 2003).

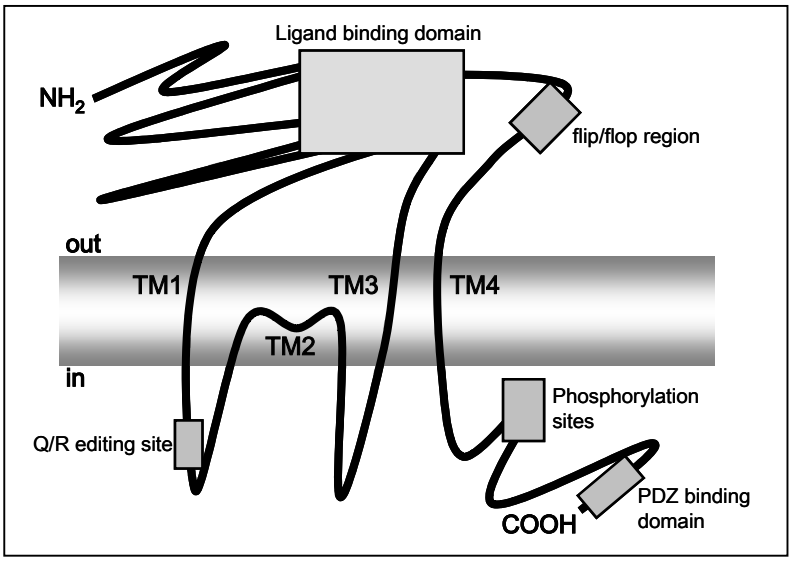


Figure 1.1- Schematic representation of the topology of an AMPA receptor subunit. Each subunit consists of an extracellular N-terminal domain, four transmembrane regions (TM1-4), and an intracellular C-terminal domain. The ligand-binding site is a conserved amino acid pocket formed from a conformational association between the N-terminus and the loop linking TM3 and TM4. A flip/flop alternative splice region is present within the TM3/TM4 loop. TM2 forms an intracellular re-entrant hairpin loop, which contributes to the cation pore channel and is also the site for Q/R RNA editing in the GluR2 subunit. The intracellular C-terminal contains the phosphorylation sites and conserved sequences that have been shown to interact with a number of intracellular proteins, including PDZ domain-containing proteins.

Splice variants

Besides the alternative splicing at the region encoding the C-terminus of GluR2 and GluR4, to give rise to long or short isoforms, all four AMPA receptor subunits can also undergo alternative splicing in an extracellular region, between TM3 and TM4 domains, to generate “flip” or “flop” splice variants (Sommer et al., 1990) (see figure 1.1). Flip forms of AMPA receptor subunits are mainly expressed in embryonic stages of the CNS development, while flop forms are widely expressed in the adult brain. Alternative splicing modifies the kinetics of the receptors, with flip splice variants desensitizing four times slower than flop forms (Koike et al., 2000), and the pharmacological properties of the channel, conferring different sensitivity to allosteric modulators (Sekiguchi et al., 1998; Shen and Yang, 1999; Kessler et al., 2000).

RNA Editing

Ribonucleic acid (RNA) editing of glutamate receptors is a post-transcriptional mechanism that involves the enzymatic deamination of specific adenosines in the pre-mRNA of the glutamate receptor subunits, performed by specific RNA adenosine deaminases (ADARs). This event gives rise to the replacement of a gene-encoded amino acid by a different one, which modifies the physiological properties of the ion channel (Barbon et al., 2003). In the GluR2 subunit, the RNA editing mechanism evokes the change of a crucial glutamine in the pore-forming region (Q/R site; see figure 1.1) of GluR2 subunit by an arginine. This Q/R substitution confers low Ca^{2+} permeability and low single-channel conductance to AMPA receptors containing the edited GluR2 subunit, because of the positive charge and size of the arginine residue (Burnashev et al., 1992; Burnashev et al., 1996; Swanson et al., 1997). Conversely, GluR1, GluR3 and GluR4 subunits exist mainly in the non-edited form and when expressed as homo- or heteromers among themselves, the resultant channels are permeable to Ca^{2+}. The glutamine residue located on the channel pore of GluR1, GluR3 and GluR4 confers inward-rectifying properties to these AMPA receptors, allowing the influx of cations. However, at positive potentials, intracellular polyamines enter the channel pore and block the efflux of cations through the channels. Because GluR2-containing AMPA receptors exist mainly in the edited form, the positive charge of the arginine present at the Q/R site prevents intracellular polyamines from entering

the pore, enabling the efflux of cations through the channel at depolarized potential and thus conferring the non-rectifying properties characteristic of these receptors.

Interacting Proteins

AMPA receptor surface expression at the postsynaptic membrane is dynamically regulated through multiple protein interactions. The AMPA receptor interacting proteins include PDZ (for Postsynaptic Density 95-Discs Large-Zona Occudens 1) domain and non-PDZ domain-containing proteins. PDZ domains are modular protein interaction motifs that bind in a sequence-specific fashion, predominantly to short C-terminal peptides. Each PDZ domain binds only one ligand. Specific selectivity of PDZ motifs for different binding peptides is thought to be dependent upon small changes in the size and geometry of the hydrophobic pocket (Palmer et al., 2005). PDZ-domain containing proteins that bind AMPA receptors include the GRIP/ABP (Glutamate Receptor Interacting Protein/AMPA Receptor Binding Protein), PICK1 (Protein Interacting with C Kinases), syntenin, SAP97 (Synapse-Associated Protein) and Shank3. The group of AMPA receptor-binding proteins lacking PDZ domains includes stargazin, Lyn tyrosine kinase, Narp (Neuronal Activity-Regulated Pentraxin), NSF (N-Ethylmaleimide-Sensitive Fusion Protein), PKCγ, AP2 and the 4.1 protein. Proteins binding to specific AMPA receptor subunits and their role are summarized in table 1.2.

Phosphorylation

Phosphorylation of ligand-gated ion channels can regulate the properties of the channel, its intermolecular interactions and trafficking of the protein. The regulation of AMPA receptor by phosphorylation adds a further complex level of receptor modulation beyond subunit composition, splice variants, and other post-translational modifications (Carvalho et al., 2000; Gomes et al., 2003). Phosphorylation of AMPA receptors can occur under resting conditions or in response to specific changes in synaptic activity, and each AMPA receptor subunit has its own kinase profile.

The role of GluR1 phosphorylation in synaptic plasticity has been studied in great detail (see section 1.1.2 *Glutamate receptors and synaptic plasticity*). GluR1 is phosphorylated in multiple sites at the C-terminus. PKC and Ca^{2+}-calmodulin dependent protein kinase II (CaMKII) phosphorylate Ser 831 (Roche et al., 1996; Barria et al., 1997; Mammen et al., 1997), while protein kinase A (PKA) phosphorylates Ser 845 (Mammen et

al., 1997). Recently, it was also found that PKC may phosphorylate GluR1 at Ser 818 (Boehm et al., 2006). Differential GluR1 phosphorylation on Ser 831 and Ser 845 occurs according to activity, leading to changes in synaptic efficacy. Phosphorylation of AMPA receptors at the postsynaptic density by CaMKII has been implicated in synaptic unsilencing and enhancement, in long-term potentiation (LTP) (Palmer et al., 2005), and in neuronal damage after brain ischemia (Takagi et al., 2003) and inflammation (Guan et al., 2004), due to an increase in the AMPA receptor-mediated currents and/or modulation of the interaction between GluR1 and a PDZ-containing protein (Palmer et al., 2005). Additionally, CaMKII activity is necessary for targeting of GluR1-containing receptors to synapses independently of Ser 831 phosphorylation (Hayashi et al., 2000; Esteban et al., 2003). PKA phosphorylation leads to potentiation of homomeric GluR1 peak currents, by increasing the peak open probability of the receptor channels, and leads to LTP in naïve synapses. GluR1 phosphorylation at Ser 845 by PKA was also shown to be necessary, but not sufficient, for synaptic targeting of GluR1 homomeric receptors, and was suggested to stabilize GluR1 at the plasma membrane (Lee et al., 2003). Phosphorylation at both CaMKII and PKA sites controls synaptic incorporation of these receptors (Esteban et al., 2003). Furthermore, phosphorylation of GluR1 by PKC at Ser 818 is increased during LTP and is also critical for LTP expression (Boehm et al., 2006). Fyn, a Src family of tyrosine kinases, can also phosphorylate GluR1 *in vitro*, protecting the receptors from calpain truncation (Rong et al., 2001). These phosphorylations may therefore regulate receptor integrity and modulate location and channel properties (Rong et al., 2001).

The GluR2 subunit is phosphorylated by PKC at Ser 880, in the PDZ binding site. This phosphorylation differentially regulates binding of ABP/GRIP1 and PICK1, with a decrease in ABP/GRIP1 binding, but not PICK1, in response to phosphorylation. Phosphorylated GluR2 recruits PICK1 to synapses, causing the release of the GluR2-PICK1 complex from synapses, facilitating its internalization (Chung et al., 2000) and long-term depression (LTD) (Xia et al., 2000; Chung et al., 2003). Furthermore, binding of ABP to GluR2 prevents PKC phosphorylation. GluR2 is also phosphorylated on tyrosine residues (Ahmadian et al., 2004). The Src family of tyrosine kinases phosphorylates GluR2 on Tyr 876, resulting in a decreased interaction between GluR2 and GRIP1, possibly leading to endocytosis of the receptors (Hayashi and Huganir, 2004).

Table 1.2- AMPA receptor interacting proteins and their functions

AMPA Receptor Interacting Protein	AMPA Receptor Interacting Subunit	Function
GRIP/ABP	GluR2, GluR3, GluR4c, GluR4	-Receptor traffic to dendrites -Membrane surface anchoring -Intracellular receptor stabilization
PICK1	GluR2, GluR3, GluR4c, GluR4	-Receptor clustering -Receptor internalization -NMDA-induced AMPA receptor down-regulation
Syntenin	GluR1, GluR2, GluR4, GluR2c, GluR3c	-Unknown
SAP97	GluR1	-Stabilization and synaptic incorporation -Recruitment of protein kinases and phosphatases through AKAP -Association with many proteins -Trafficking along the early secretory pathway -May be necessary for CaMKII to drive synaptic delivery of GluR1
Shank3	GluR1	-Synaptic trafficking during development
Stargazin	GluR1, GluR2, GluR4	-Targeting receptors to the membrane surface
Lyn	GluR1, GluR2, GluR3, GluR4c	-Activation of MAPK pathway upon AMPA receptor stimulation
Narp	GluR1, GluR2, GluR3	-Receptor clustering and excitatory synapse formation
NSF	GluR2, GluR3, GluR4c	-Regulates the delivery and removal of GluR2-containing AMPA receptors
PKCγ	GluR4	-Facilitates receptor phosphorylation
AP2	GluR2	-NMDA-induced AMPA receptor internalization
4.1N	GluR1, GluR4	-Surface receptor stabilization

(reviewed in Gomes et al., 2003; Palmer et al., 2005; Uchino et al., 2006)

The GluR4 subunit can be phosphorylated by PKC at Ser 842 (Carvalho et al., 1999), increasing the synaptic delivery of recombinant receptors (Correia et al., 2003;

Esteban et al., 2003), and at Thr 830 (Carvalho et al., 1999). Ser 842 can also be phosphorylated by PKA, modulating the surface expression of the receptor (Gomes et al., 2004). PKC phosphorylation also increases influx of Ca^{2+} through activated AMPA receptor channels in cultured retinal neurons, where GluR4 is the main AMPA receptor subunit (Carvalho et al., 1998; Carvalho et al., 2002).

Trafficking of AMPA receptors

AMPA receptor localization is a highly dynamic process, with AMPA receptors recycling in and out of synapses under a variety of situations related to synaptic plasticity and development. AMPA receptor trafficking in and out of the synapses is one of the core mechanisms for rapid changes in the number of functional receptors during synaptic plasticity (see section 1.1.2 *Glutamate receptors and synaptic plasticity*). AMPA receptors are transported along the dendrite through several kinesin motors and through protein linkers between AMPA receptors and microtubular motor proteins. Afterwards, AMPA receptors are delivered into specialized dendritic membrane by exocytosis (reviewed in Groc and Choquet, 2006). Exocytosis of receptors, either coming from a pool of newly synthesized receptors or from a pool of recycling receptors, not only is required for the insertion in basal conditions, but also appears to be required for LTP (Park et al., 2004). Receptors could be either directly exocytosed at the synapse or first exocytosed in the extrasynaptic membrane, followed by lateral diffusion at the neuronal surface and trapping at synaptic sites (Tardin et al., 2003). These events are interdependent and regulated by neuronal activity and interaction with scaffolding proteins. However, recent findings (Ashby et al., 2006; Yang et al., 2006) support the second hypothesis.

1.1.1.2 Kainate receptors

Together with AMPA receptors kainate receptors are responsible for the fast neurotransmission in the CNS, since they also activate and desensitize very rapidly in response to glutamate. Moreover, AMPA and kainate receptors share several agonists and antagonists that can interact with both receptors. Therefore, AMPA and kainate receptors

are usually called non-NMDA receptors. However, the discovery that 2,3-benzodiazepines, particularly GYKI53655, antagonize AMPA, but not kainate receptors (Paternain et al., 1995), has become a tool to elucidate the roles of both receptor types in synaptic transmission.

Two kainate receptor subunits were first cloned, KA1 and KA2, which showed high affinity to kainate. However, they do not form functional ion channels when homomerically expressed in host cells. Three further kainate receptor subunits were subsequently cloned, GluR5, GluR6 and GluR7. These receptor subunits can form functional homomeric ion channels when recombinantly expressed. In addition, high affinity kainate receptor subunits (KA1 and KA2) are able to form functional heteromeric assemblies with GluR5 and GluR6 kainate receptors. Kainate receptor subunits are structurally related with AMPA receptor subunits (Fig. 1.1), also containing an extracellular N-terminal followed by three transmembrane domains (TM1, TM3 and TM4), in addition to a membrane loop (TM2) and an intracellular C-terminal (Madden, 2002). Similarly to the AMPA receptor subunits, the kainate receptor subunits undergo alternative splicing and/or RNA editing (Dingledine et al., 1999). RNA editing also occurs at the Q/R site in the pore region of GluR5 and GluR6, but not on GluR7. All splice variants differ on the sequence of their cytoplasmatic C-terminal domain (reviewed in Bleakman and Lodge, 1998; Jaskolski et al., 2005).

In addition to the activity as ligand-gated ion channels, kainate receptors may signal through activation of G proteins, regulating voltage-dependent Ca^{2+} channels (Rodriguez-Moreno and Lerma, 1998; Lauri et al., 2005). Although it is still unknown how the kainate receptors ion channel are coupled to G proteins, an intermediate protein is likely involved. This noncanonical signalling accounts for the inhibitory activity of kainate receptors (reviewed in Lerma, 2006).

Kainate receptors maybe present at the postsynaptic and at the presynaptic membrane. Postsynaptic kainate receptors mediate part of the synaptic currents at certain synapses (Rodriguez-Moreno et al., 1997), while presynaptic receptors modulate the release of glutamate and GABA in a bidirectional fashion (Rodriguez-Moreno et al., 1997), therefore exerting a tight control of the excitability of brain networks.

1.1.1.3 NMDA receptors

NMDA receptors mediate a relatively slow and long-lasting excitatory postsynaptic current component (EPSC). They are ligand-gated ion channels selectively permeable to divalent cations, particularly to Ca^{2+}. The NMDA receptor family is made up of NR1, NR2 and NR3 subunits (Cull-Candy et al., 2001). NR1 is essential for the NMDA receptor function, but the glutamate–binding site is present in the NR2 subunit. Therefore, functional NMDA receptors are heteromeric complexes containing both NR1 and NR2 subunits (Prybylowski and Wenthold, 2004). Although it is still controversial, it has been suggested that each NMDA receptor represents a tetramer of two NR1 and two NR2 subunits (Prybylowski and Wenthold, 2004). NR3 subunits can assemble with NR1-NR2 complexes to depress NMDA receptor responses and can also assemble with NR1 alone to form excitatory glycine receptors, insensitive to glutamate or NMDA, calcium impermeable and resistant to magnesium blockade (Chatterton et al., 2002).

NMDA receptor diversity arises from alternative splicing at three sites in the NR1 messenger RNA (mRNA), generating eight distinct NR1 splice variants. The existence of four NR2 subunits (NR2A-D) and two NR3 subunits also contributes to this diversity. Each NR2 subunit is encoded by its own gene, and is unable to form functional channels on its own, but greatly enhances NMDA receptor function when coexpressed with NR1. NR2A and NR2B subunits form high conductance channels and are highly expressed in the mammalian forebrain (Cull-Candy et al., 2001). NR2B subunit expression predominates early in development, whereas NR2A expression increases with development (Stephenson, 2001). The NR2C subunit expression is confined mainly to the mature cerebellum whereas the NR2D subunit is particularly abundant in the young thalamus (Cull-Candy et al., 2001). Regional and developmental regulation of NR2 subunit expression underlies much of the diversity of NMDA receptor responses in the CNS (Wenthold et al., 2003).

Each one of the NMDA receptor subunits shares a similar topology with the AMPA receptor subunits. They also contain an extracellular N-terminus, followed by three membrane-spanning domains (TM1, TM3 and TM4) and one membrane loop (TM2), resulting in an intracellular C-terminus (Mori and Mishina, 1996).

NMDA receptors possess several different modulatory sites (see figure 1.2). A glycine-binding domain is conferred by the NR1 subunits, and must also be occupied for the channel to open. Because neither glycine nor glutamate acting alone can open the receptor channel, they are referred to as coagonists of the NMDA receptors. These receptors also have a voltage-dependent magnesium-binding site within the channel, which blocks the NMDA receptor pore at the membrane resting potential. This voltage-dependent magnesium block allows receptor activation only at depolarized membrane potentials (see figure 1.2) (Cull-Candy et al., 2001). Another important endogenous allosteric inhibitor of NMDA receptor activation is pH. The frequency of NMDA receptor channel openings is reduced by protons over the physiological pH range. Furthermore, NMDA receptors are endowed with one or more modulatory polyamine binding sites. Occupancy of one of the polyamine sites relieves tonic proton block and, thus, potentiates NMDA receptor activation in a pH-dependent manner. However, at higher concentrations polyamines act on the extracellular site to produce a voltage-dependent block of the ion channel, inhibiting receptor activation (Scatton, 1993).

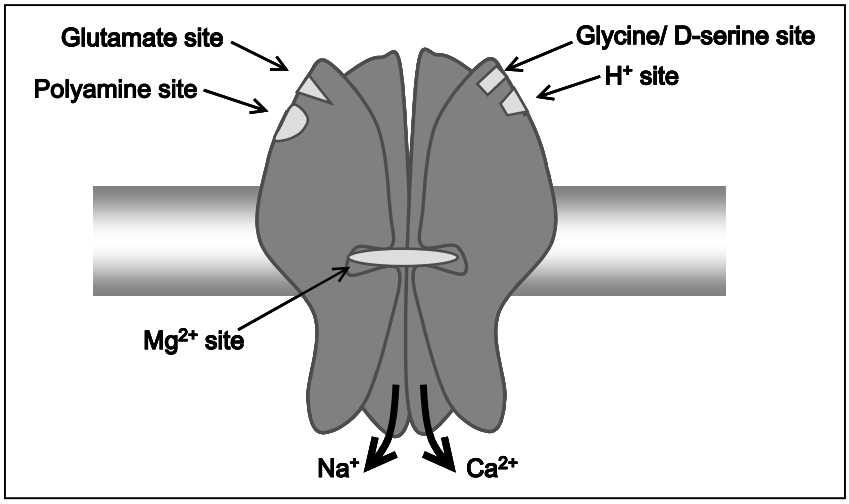


Figure 1.2- Schematic structure of NMDA receptors, indicating the modulatory sites that control their activity.

In addition to the regulatory mechanisms discussed above, an interesting form of Ca^{2+}-dependent inactivation of NMDA receptors is brought by calmodulin. The complex

Ca^{2+}-calmodulin formed upon Ca^{2+} entry through the receptor channel interacts with the C-terminus of the NR1 subunit, inactivating the receptor by reducing the channel opening frequency and opening time (Ehlers et al., 1996).

Interacting Proteins

Similarly to AMPA receptors, the NMDA receptors interact with numerous proteins, which affect their functional properties, trafficking, and synaptic distribution. One of the most highly studied interactions is with the membrane-associated guanylate kinases (MAGUKs). MAGUKs are a family of proteins highly expressed in neurons that includes SAP102, SAP97, postsynaptic density protein-93 (PSD-93) and PSD-95. The NMDA-MAGUK interaction is mediated by the PDZ binding domain of the NR2 subunits and the first or second PDZ domains of MAGUKs (reviewed in Hung and Sheng, 2002; McGee and Bredt, 2003). Although the NR2A and NR2B subunits contain identical PDZ-binding domains, there are differences in the preferences for MAGUK interactions between NR2A and NR2B (Townsend et al., 2003). NR2 subunits lacking the PDZ-binding domain show decreased expression at the synapse, suggesting that MAGUKs stabilize NMDA receptors at the synapse (Barria and Malinow, 2002; Prybylowski et al., 2002). Besides this function, MAGUKs are also involved in the trafficking of NMDA receptors to the synapse through binding to kinesin motor proteins (reviewed in Prybylowski and Wenthold, 2004).

As described above, calmodulin may also interact with the NR1 subunit of NMDA receptors, promoting their inactivation. Furthermore, the complex Ca^{2+}-calmodulin activates CaMKII (Hudmon and Schulman, 2002), which also interacts with NMDA receptors. This kinase is activated by NMDA receptor-mediated Ca^{2+} influx (Lisman et al., 2002), and interaction with the NR2B subunit appears to stabilize CaMKII in an active conformation (Bayer et al., 2001).

The protein receptor for activated protein kinase C-1 (RACK1) is another critical interactor with the NR2B subunit that controls ethanol and Fyn kinase modulation of the NMDA receptor (Yaka et al., 2003).

Phosphorylation

The activity of NMDA receptors is also regulated through phosphorylation by various protein kinases. PKC phosphorylates the NR1 and NR2A subunits of NMDA receptors at

consensus sites located within their intracellular C-terminal tails. The NR1 phosphorylation sites are Ser 889, Ser 890, Ser 896 and Ser 897, which are phosphorylated following exposure to phorbol esters (Tingley et al., 1997). However, the functional consequences of these biochemical events are not well understood. In human embryonic kidney-293 (HEK293) cells expressing NR1/NR2A, the activation of endogenous PKC by phorbol 12-myristate 13-acetate (PMA) increases NMDA receptor desensitization (Jackson et al., 2006). In *Xenopus* oocytes injected with rat brain RNA, stimulation with phorbol esters dramatically enhance NMDA receptor responses (Urushihara et al., 1992). PKC phosphorylation of NMDA receptors was also found to be responsible for moving the receptors from synaptic to extrasynaptic sites in neurons (Groc et al., 2004).

NMDA receptors are also phosphorylated at the C-terminus of NR2B, on Tyr 1472, which interferes with binding to PSD-95 and promotes clathrin-dependent endocytosis, presumably by facilitating the binding of AP2 to NR2B (Vissel et al., 2001). Dephosphorylation of Tyr 837 on NR1, and Tyr 842 on NR2A might affect AP2 binding, promoting clathrin-mediated endocytosis in a similar way (Vissel et al., 2001). Tyr dephosphorylation may also cause extrasynaptic NMDA receptor rundown, although the specific Tyr residues responsible for this effect are still unknown (Li et al., 2002).

Other kinases and phosphatases have been shown to regulate the NMDA receptor function, including PKA (Raman et al., 1996), CaMKII (Soderling and Derkach, 2000) and Src family of protein kinases (Wang and Salter, 1994).

Synaptic Localization

Although NMDA receptors are concentrated at the postsynaptic membrane, a second population of receptors, extrasynaptic NMDA receptors, has been identified and may have important functional roles, distinct from those of synaptic receptors (Groc and Choquet, 2006). Synaptic receptors can be replaced rapidly through lateral diffusion in the plasma membrane, presumably by extrasynaptic receptors (Tovar and Westbrook, 2002). On the other hand, synaptic and extrasynaptic NMDA receptors differ in their subunit composition, with extrasynaptic receptors containing mainly NR2B subunits, whereas synaptic receptors are endowed with NR2A subunits (Tovar and Westbrook, 1999). NMDA receptors also appear to be present at immature synapses before AMPA receptors (Petralia et al., 1999), producing “silent synapses”, which do not respond to synaptically released glutamate in the

absence of sufficient depolarization to remove the Mg^{2+} block of NMDA receptor. Synaptic and extrasynaptic receptors are also differently regulated by phosphorylation (Li et al., 2002).

Neurons have a large pool of unassembled (intracellular) NR1 subunits, whereas NR2 subunits are assembled with NR1 and are mainly found on the cell surface (Wenthold et al., 2003). Overexpression of NR2 subunits by transfection increases the number of surface NMDA receptor channels in cerebellar granule cells (Prybylowski et al., 2002), suggesting that the production of functional NMDA receptors is limited, at least in part, by the availability of NR2 subunits.

During development, changes in the subunit composition of NMDA receptors are also found. Hippocampal or cortical neurons express NR2B early in development, when NMDA receptors are predominantly nonsynaptic, whereas NR2A appears later in the development and increases over time, being incorporated at synapses (Tovar and Westbrook, 1999; Guillaud et al., 2003).

NMDA receptor trafficking

On the surface of living neurons there are mobile and immobile pools of NMDA receptors, at both synaptic and extrasynaptic sites (Groc et al., 2004). NMDA receptors can move from extrasynaptic to synaptic sites on the cell surface (Tovar and Westbrook, 2002), suggesting that the number of NMDA receptors at synapses might be rapidly modulated by lateral diffusion in the plasma membrane (Sharma et al., 2006). For example, Groc and collaborators reported that PKC activation increases diffusion of synaptic and extrasynaptic NMDA receptors (Groc et al., 2004). NMDA receptors may also move between intracellular compartments and the plasma membrane of neurons. Trafficking of NMDA receptor is regulated by an endoplasmatic reticulum-retention motif in the C-terminus of NR1, and their surface expression is facilitated by an interaction with a protein containing a PDZ domain (Standley et al., 2000). At synapses, NMDA receptors are stabilized by interaction with PSD-95, but they can be rapidly internalized in response to the binding of L-glutamate (Roche et al., 2001) or glycine (Nong et al., 2003). Similarly to the AMPA receptors, the NR1/NR2B receptor complexes may be constitutively inserted into synapses early in the development and replaced by NR1/NR2A receptors in an activity-dependent manner (Barria and Malinow, 2002). As described above, the association between NMDA receptors and PSD-95 and their consequent endocytosis is regulated by tyrosine phosphorylation. The surface expression of

NMDA receptor is also regulated by PKC (Lan et al., 2001b). Accordingly, activation of metabotropic glutamate receptor increases the surface expression of NMDA receptor in *Xenopus* oocytes via regulated exocytosis, probably through PKC activation (Lan et al., 2001a).

1.1.2 Glutamate receptors and synaptic plasticity

LTP and LTD are forms of synaptic plasticity thought to underlie learning and memory processes (Braunewell and Manahan-Vaughan, 2001; Lynch, 2004). LTP refers to a persistent increase in efficacy of synaptic transmission, following a short period of pre-synaptic high-frequency stimulation (tetanic stimulation) or pairing pre-synaptic low-frequency stimulation with the depolarization of the postsynaptic membrane. Conversely, LTP is characterized by a reduction in the synaptic strength, following low-frequency synaptic stimulation (Fig. 1.3).

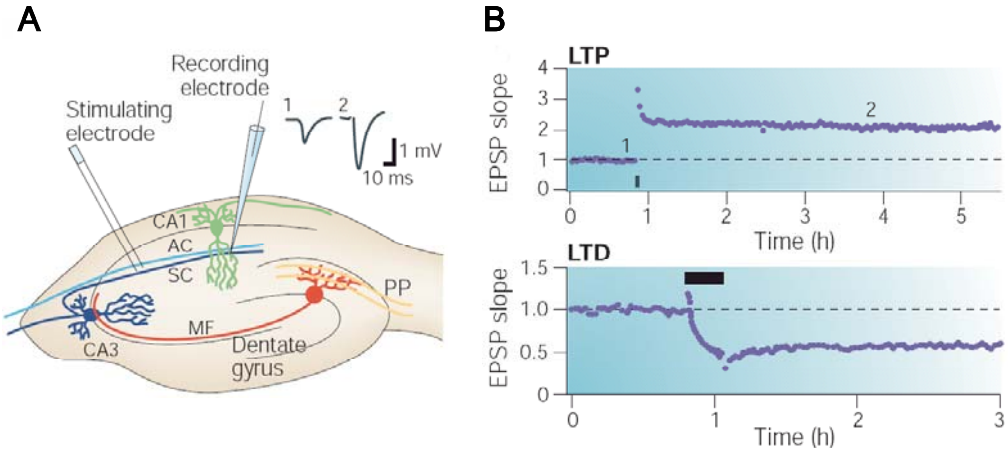


Figure 1.3- Synaptic plasticity in the hippocampus. A) Schematic diagram of the rodent hippocampal slice preparation, showing the main excitatory pathways (AC, associational/commissural; MF, mossy fibre; PP, perforant path; SC, Schaffer collateral). Typical electrode placements for studying synaptic plasticity at Schaffer collateral-commissural synapses are indicated. The traces are field EPSPs recorded before (1) and during (2) LTP. **B)** Time-course plots showing alterations in field EPSP (rising slope normalized to baseline) against time, during LTP (100 Hz stimulation, 1 s, baseline intensity) or after the induction of *de novo* LTD (1 Hz stimulation, 15 min, baseline intensity). The black bar represents the time of the stimulus and the numbers (1 and 2) indicate the time points illustrated in A) (Collingridge et al., 2004).

NMDA receptors are the most important known trigger for the long-term modification of synaptic strength. In the hippocampus, both plasticity mechanisms, LTP and LTD, require activation of NMDA receptors and Ca^{2+} influx through these receptors. The raise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to different levels is a signal that activates various signalling pathways: LTP induction is thought to be mediated by CaMKII activation (Silva et al., 1992), while LTD requires calcineurin (also known as phosphatase 2B) activation (Mulkey et al., 1994). These effects may cause either insertion or removal of AMPA receptors from the postsynaptic membrane, respectively (Beattie et al., 2000; Lin et al., 2000; Lu et al., 2001; Collingridge et al., 2004).

LTP

One defining feature of LTP is its dependence on high levels of postsynaptic Ca^{2+}. The primary source of Ca^{2+} influx during the induction of LTP are the NMDA receptors, which become active following binding of synaptically released glutamate and plasma membrane depolarization. The massive and transient increase in the $[Ca^{2+}]_i$ lead to activation of kinases, like CaMKII. Alterations in NMDA receptor trafficking may further contribute to LTP and LTD. An increase in NMDA receptor surface expression has been reported after the induction of LTP in adult CA1 synapses (Grosshans et al., 2002). Conversely, a form of LTD that is induced by metabotropic glutamate receptor activation characterized by internalization of NMDA receptors (Snyder et al., 2001).

Analysis of the subunit composition of the NMDA receptors has revealed that hippocampal LTP relies on the expression of NR2A, since gene disruption of this subunit resulted in impairment of LTP in hippocampus (Sakimura et al., 1995). Moreover, overexpression of the NR2B subunit was found to be associated with enhanced LTP (Tang et al., 1999). Association between active CaMKII and synaptic NMDA receptors is also required for synaptic plasticity. Synaptic NR2B-containing NMDA receptors bind CaMKII with high affinity, while those containing NR2A subunits bind with low affinity. Replacement of NR2B with NR2A was found to decrease LTP, an effect that was reversed by mutations on the NR2A subunit that increased the affinity for the active CaMKII. Therefore, association of active CaMKII and NR2B or NR2A may control different forms of synaptic plasticity (Barria and Malinow, 2005).

NMDA receptor activation may also contribute to synaptic plasticity by affecting AMPA receptor trafficking, but this effect depends on the subunit composition of NMDA receptors (Kim et al., 2005). In mature cultured neurons, NR2A-containing NMDA receptors promote, whereas NR2B containing NMDA receptors inhibit, the surface expression of GluR1, primarily by regulating its surface insertion. Moreover, in mature neurons, NR2B is coupled to inhibition of the Ras-ERK pathway, which drives surface delivery of GluR1 (Kim et al., 2005).

Synaptic AMPA receptor levels can be rapidly regulated. Electrophysiology studies in the hippocampal CA1 region showed, for the first time, the existence of excitatory synapses containing NMDA receptors but not AMPA receptors (Isaac et al., 1995; Liao et al., 1995). When these called “silent synapses” were depolarized, to enable NMDA receptor activation, AMPA receptor-mediated responses were then detected, suggesting that AMPA receptors could be rapidly inserted into synapses, making them active. This delivery of AMPA receptors and consequent synaptic activation may play an important role in the potentiation of synaptic transmission during LTP. Early in development, spontaneous neuronal activity drives GluR4-containing AMPA receptors to functional or silent synapses (Zhu et al., 2000), through a mechanism dependent on PKA phosphorylation of GluR4 Ser 842 (Esteban et al., 2003). Ser 842 phosphorylation is necessary and sufficient for synaptic delivery of GluR4 homomeric AMPA receptors, and is thought to relieve a retention signal that traps the receptor intracellularly (Esteban et al., 2003).

Furthermore, studies using sensory neurons showed that recruitment of AMPA receptors to silent synapses was inhibited by blocking the interaction of GluR2-GluR3 subunits with GRIP (Li et al., 1999b), indicating that glutamate receptor interacting proteins also play an important role in receptor translocation to the membrane.

As described above, in the hippocampus, most AMPA receptors are tetramers composed of GluR1-GluR2 or GluR2-GluR3 subunits. Induction of LTP promotes the rapid translocation of GFP-tagged GluR1 subunits to dendritic spines in hippocampal organotypical cultures, and this process is dependent on the activation of NMDA receptors (Shi et al., 1999). The same experimental approach also revealed that GluR2-containing AMPA receptors are constitutively delivered to the synapses and cycle continuously

(Malinow and Malenka, 2002), whereas GluR1-containing AMPA receptors are inserted into the synapse in response to high frequency stimulation with consequent NMDA receptor activation and stimulation of CaMKII (Shi et al., 1999; Hayashi et al., 2000). The GluR4 subunit plays the role of GluR1 at immature synapses (Zhu et al., 2000). This activity-dependent synaptic delivery of AMPA receptors is relevant in the mechanism of LTP, with GluR1 being dominant over GluR2, since heteromeric receptors containing GluR1 and GluR2 subunits behave like the GluR1 subunit (Hayashi et al., 2000; Passafaro et al., 2001; Shi et al., 2001).

Phosphorylation of AMPA receptor subunits may also mediate synaptic plasticity by affecting the electrophysiological properties of the receptors, in addition to the effect on synaptic trafficking and surface expression. Phosphorylation of GluR1 on Ser 831 and Ser 845 increases the apparent single channel conductance (Derkach et al., 1999), and the apparent opening-channel probability (Banke et al., 2000), respectively, and may therefore mediate, at least in part, the potentiation of synaptic transmission during the early phase of LTP (Fig 1.4).

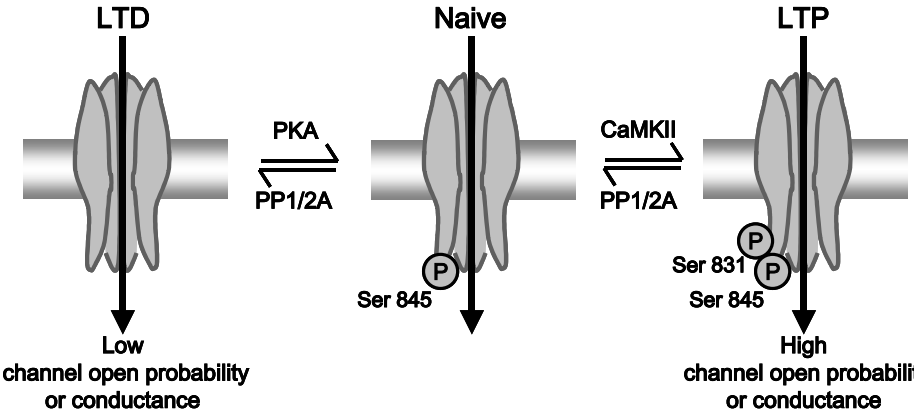


Figure 1.4- Schematic illustration of the effect of GluR1 phosphorylation as a bi-directional switch in synaptic plasticity.

GluR1 is phosphorylated by CaMKII during LTP in the hippocampus (Barria et al., 1997; Lee et al., 2000), and postsynaptic expression of a constitutively active form of CaMKII

in hippocampal slices enhanced synaptic transmission and prevented further LTP induction (Pettit et al., 1994; Lledo et al., 1995), suggesting that CaMKII and LTP enhance synaptic transmission through the same mechanism. However, mutation of the CaMKII phosphorylation site Ser 831 in GluR1 for an alanine revealed that Ser 831 phosphorylation is not necessary for GluR1 delivery to synapses (Hayashi et al., 2000), suggesting that CaMKII-induced synaptic delivery of homomeric GluR1 receptors is dependent on GluR1 C-terminal interaction with PDZ-domain containing proteins (Hayashi et al., 2000). Recently, the association of GluR1-SAP-97-4.1N complex was suggested to be necessary for actin-mediated synaptic targeting of GluR1-containing AMPA receptors (Rumbaugh et al., 2003). Moreover, inhibition of CaMKII, or inhibition of calpains, suppressed the potentiation of synaptic transmission by NMDA in hippocampal slices, and the associated increase in the number of AMPA receptors present in synaptic membranes (Broutman and Baudry, 2001). Recently, the small GTPase Ras, a downstream effector of CaMKII, was shown to mediate the NMDA receptor and CaMKII signalling that drives synaptic delivery of AMPA receptors with long cytoplasmic tails during LTP (Zhu et al., 2002). Ras activity is thought to act through the p42/44 mitogen-activated protein kinase (MAPK) on the delivery of AMPA receptor subunits with a long C-termini (Zhu et al., 2002). Ras is known to activate phosphatidylinositol 3-kinase (PI3-K) and, therefore, activation of Ras by CaMKII may lead to activation of AMPA receptors bound to PI3-K, a lipid kinase necessary for glycine-induced delivery of AMPA receptors to the plasma membrane.

In addition to Ser 831, GluR1 is also phosphorylated on Ser 845 when LTP is induced in hippocampal CA1 region (Lee et al., 2000). Phosphorylation of this site by PKA is required for synaptic incorporation of GluR1. However, PKA activation alone is not sufficient to induce synaptic delivery of GluR1 in hippocampal slices, indicating that PKA and CaMKII act in parallel to induce the translocation of the receptor to the membrane (Hayashi et al., 2000; Esteban et al., 2003; Lee et al., 2003). Recent studies showed that PKC can also phosphorylate GluR1 on Ser 818, and phosphorylation of this site is increased during LTP and is important for LTP expression (Boehm et al., 2006).

Exocytosis has been implicated in the continual exchange of a substantial pool of AMPA receptors at the spine surface, although recent findings suggest that lateral diffusion is also involved (Ashby et al., 2006). Cleavage and inactivation of soluble NSF attachment

protein receptors (SNAREs), a multiproteic complex of the membrane fusion machinery, by postsynaptic application of botulinum toxin, reduced LTP in the hippocampal CA1 region, indicating that membrane fusion events in the postsynaptic membrane are required for LTP (Lledo et al., 1998). Moreover, induction of LTP in cultured hippocampal neurons by stimulation of postsynaptic NMDA receptors with glycine evoked a rapid insertion of AMPA receptors at the surface of dendritic membranes, and an increased clustering in synaptic regions. Both processes are blocked by tetanus toxin (Lu et al., 2001), indicating that AMPA receptors are inserted into synapses via SNARE-dependent exocytosis during LTP. Synaptic potentiation induced by brief application of NMDA to rat hippocampal slices was also associated with increased levels of GluR1 and GluR2-GluR3 subunits in synaptic membrane preparations (Broutman and Baudry, 2001). This effect was inhibited by brefeldin A (Broutman and Baudry, 2001), a drug that inhibits exocytosis of newly synthesized protein from the Golgi apparatus to the cell membranes, suggesting that the upregulation in the number of plasma membrane-associated AMPA receptors requires a functional secretory pathway. Recent studies also reported that recycling endosomes supply AMPA receptors for LTP and provide a mechanistic link between synaptic potentiation and membrane remodeling during synapse modification (Park et al., 2004).

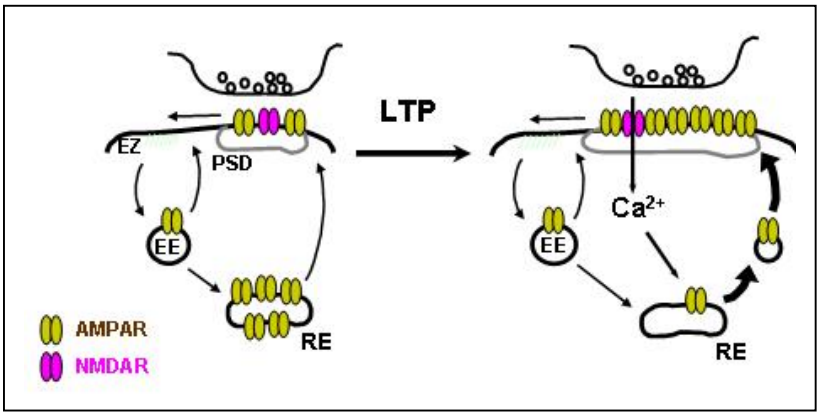


Figure 1.5- Schematic model for the mobilization of AMPA receptors during LTP. In resting conditions, AMPA receptor cycling is supported by trafficking of endosomal vesicles (EE). After LTP induction, NMDA receptors present in the post-synaptic density (PSD) are activated, raising the $[Ca^{2+}]_i$, which activate several kinases. One of these kinases is CaMKII that phosphorylates AMPA receptors present in recycling endosomes (RE), inducing the traffic of newly synthesized AMPA receptor to the postsynaptic membrane (from www.ehlerslab.org).

LTD

The release of small amounts of glutamate, lower the those required to induced LTP, activate only a small percentage of NMDA receptors, thereby increasing the postsynaptic [Ca^{2+}] to levels bellow the threshold for eliciting LTP. Similarly, when the density of NMDA receptors is low, small increases in the postsynaptic [Ca^{2+}] are induced and may be sufficient to activate phosphatase 2B and generate LTD (Bear and Abraham, 1996).

Cerebellar LTD involves down-regulation of functional synaptic AMPA receptors, which could be due to a loss of receptors from postsynaptic sites or to changes of single channel properties, such as kinetics, unitary conductance, glutamate activity and/or channel opening probability (Wang and Linden, 2000). The generation of LTD *in vivo* was also found to decrease the number of AMPA receptors in synaptoneurosomes, providing further evidence for the role of AMPA receptor endocytosis (Heynen et al., 2000). LTD induced by low frequency stimulation of cultured hippocampal neurons is also associated with a decrease in synaptic GluR1 subunits, due to the activation of NMDA receptors. These findings further suggest that AMPA receptors are removed from the post-synaptic membrane during LTD (Carroll et al., 1999). Application of NMDA to hippocampal slices also induces LTD (Lee et al., 1998), and occludes synaptically evoked LTD, suggesting that these two LTD-inducing stimuli occur through the same mechanism. In cultured hippocampal neurons, NMDA-induced LTD is primarily mediated by activation of extra-synaptic NMDA receptors (Lu et al., 2001).

The mechanisms involved in receptor internalization following the induction of LTD in hippocampal CA1 neurons was investigated using peptides that block the interaction of the C-terminal region of GluR2/3 with PDZ proteins (Daw et al., 2000; Kim et al., 2001). Post-synaptic intracellular perfusion with a peptide that disrupts the interaction of GluR2 with GRIP1, GRIP2/ABP and PICK1 inhibited LTD, but the role played by PICK1 is still controversial (Daw et al., 2000; Kim et al., 2001). Thus, induction of LTD in hippocampal slices increases GluR2 phosphorylation on Ser 880, which is within the GluR2 C-terminal PDZ binding domain (Kim et al., 2001). Phosphorylation of Ser 880 by PKC was shown to disrupt the interaction of GluR2 with GRIP, but still enables PICK1 interaction (Chung et al., 2000; Matsuda et al., 2000). Therefore, it was proposed that phosphorylation of the GluR2 subunit during LTD may prevent its interaction with GRIP1 and GRIP2/ABP at the synaptic

plasma membrane. This may allow the receptors to be internalized, and then stabilized by interaction with PICK1 (Kim et al., 2001). The internalization of AMPA receptors during homosynaptic hippocampal CA1 LTD is mediated by clathrin (Man et al., 2000) and requires the participation of AP2, a clathrin adapter complex that associates with GluR2 (Lee et al., 2002). In contrast, chemically-induced LTD in the CA1 region of the hippocampus causes a persistent decrease in the phosphorylation of GluR1 on Ser 845, but not of Ser 831 (Lee et al., 1998; Lee et al., 2000). Furthermore, reversal of LTP with low frequency stimulation is associated with a dephosphorylation of GluR1 on Ser 831, but not of Ser 845, indicating that the phosphorylation site modulated by low frequency stimulation depends on the previous experience of the synapse (Lee et al., 2000; Huang et al., 2001). Accordingly, induction of LTD in a hippocampal cell culture model is also correlated with a selective reduction in the number of GluR1 subunits clustered at synapses (Carroll et al., 1999).

In hippocampal slices, Rap1, a member of the Ras superfamily of small GTPases, mediates the NMDA receptor-dependent removal of synaptic AMPA receptors (GluR2/GluR3 subunits) during LTD, through p38 MAPK activation (Zhu et al., 2002). Furthermore, NR2A-containing NMDA receptor activation stimulates Rap2, which was shown to control synaptic removal of AMPA receptors with long cytoplasmatic tails (GluR1/GluR2L) during depotentiation. This effect of Rap2 is mediated by activation of c-Jun N-terminal kinase (JNK) rather than by extracellular signal-regulated kinase 1 and 2 (ERK1/2) or p38 MAPK (Zhu et al., 2005).

Cerebellar LTD is also associated with changes in the phosphorylation and trafficking of AMPA receptor subunits. Stimuli inducing LTD in cultured cerebellar granule neurons cause GluR2 phosphorylation at Ser 880, thereby reducing the affinity of the receptor subunit to GRIP (Matsuda et al., 2000; Xia et al., 2000), as reported in hippocampal neurons (see above). This may explain the disruption of GluR2 postsynaptic clusters followed by internalization of the protein observed under the same experimental conditions (Matsuda et al., 2000). Studies using peptides designed to disrupt the interaction PICK1-GluR2/3, or using antibodies directed against the PDZ domain of PICK1, as well as expression of mutant PICK1-Glutathione S-transferase (GST) fusion proteins, showed that PICK1 binding to GluR2/3 plays an important role in cerebellar LTD (Xia et al., 2000). Expression of cerebellar LTD is also thought to require clathrin-mediated internalization of AMPA receptors (Wang and Linden, 2000), which depends upon the carboxy-terminal region

of GluR2/3 (Xia et al., 2000). Furthermore, LTD in the cerebellum requires PKC, which is crucial for the internalization of AMPA receptors in these cells (Xia et al., 2000), in contrast with the hippocampus where the kinase is not required for internalization of GluR2/3 (Kim et al., 2001).

1.2 Neurotrophins

Neurotrophins are a family of proteins that are essential for the development of the vertebrate nervous system. They mediate the survival, differentiation, growth, and apoptosis of neurons, as well as changes in synaptic activity (Lewin and Barde, 1996).

Neurotrophins are synthesized as precursor proteins (pro-neurotrophins), with about 32 kDa, which are cleaved within the trans-Golgi network and/or in immature secretory vesicles to produce the mature proteins (Mowla et al., 2001). Pro-neurotrophins are cleaved by furin at a highly conserved dibasic amino acid cleavage site to release the carboxy-terminal mature proteins. However, some pro-neurotrophins may be released to the extracellular compartment, being biologically active (reviewed in Lu et al., 2005), and then cleaved extracellularly by plasmin (Lee et al., 2001). The mature proteins are about 14 kDa in size, form stable non-covalent dimers and are normally expressed at very low levels during development (Mowla et al., 2001). The neurotrophin family of trophic factors includes the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4).

1.2.1 Expression, transport and secretion of neurotrophins

Expression

Neurotrophin expression is regulated during development, but persists in many parts of the adult brain. It is well accepted that the expression of neurotrophins, in particular NGF and BDNF, is regulated by activity. Thus, increases of the BDNF mRNA levels are observed upon LTP-inducing stimuli (Patterson et al., 1992; Castren et al., 1993). Moreover, neuronal

depolarization of cultured neurons with glutamate or KCl also increases the level of BDNF and NGF mRNA (Lu et al., 1991; Zafra et al., 1991; Lindholm et al., 1994). In contrast, inhibition of neuronal activity by γ-aminobutyric acid (GABA) decreases the level of neurotrophin mRNA (Lindholm et al., 1994; Berninger et al., 1995). Seizure activity also induces a rapid increase in NGF and BDNF mRNA in the hippocampus and cerebral cortex (Gall and Isackson, 1989; Zafra et al., 1990; Ernfors et al., 1991). Interestingly, neuronal expression of NT3 and NT4 is not regulated by activity (Ernfors et al., 1991).

Although, transcription of neurotrophin genes occurs in the cell body, the BDNF mRNA may also be found at dendrites where translation machinery is also available (Steward and Levy, 1982; Kang and Schuman, 1996; Casadio et al., 1999).

Transport and secretion

Neurotrophins that are synthesized and packed into vesicles in the soma are then transported to presynaptic axon terminals or to postsynaptic dendrites for local secretion. Postsynaptic dendrites that synthesize neurotrophins release them locally. Thus, the synaptic levels of neurotrophins may be regulated by the transport and targeting of neurotrophin-containing vesicles to the synapse, or by local translation of neurotrophin mRNA (Lu, 2003). Therefore, neurotrophins may be transported anterogradely, from the soma to the axon terminals, and accumulated presynaptically, from where they may be released in response to neuronal firing, to act on the postsynaptic neuron. Alternatively, neurotrophins may be secreted postsynaptically at dendrites (Hartmann et al., 2001), taken up by the presynaptic terminal and then retrogradely transported to the soma. Both models may co-exist *in vivo*, since there is evidence for both presynaptic and postsynaptic neurotrophin release (Poo, 2001).

Synaptic activity also regulates the secretion of neurotrophins, in addition to the effect on their expression. In hippocampal slices or dissociated cell cultures, depolarization induced by veratridine, glutamate, KCl or patterned electrical stimulation evoked secretion of NGF and BDNF (Blochl and Thoenen, 1995; Goodman et al., 1996; Balkowiec and Katz, 2000). Finally, neurotrophins themselves can function as the regulatory signal for neurotrophin secretion (Canossa et al., 1997; Kruttgen et al., 1998). Evidences for anterograde transport and secretion of neurotrophins are provided by the observed BDNF enrichment in a vesicular fraction isolated from brain synaptosomes (Fawcett et al., 1997),

and by results showing that exogenous application of NT3 into the eye of chick embryos leads to an uptake of the neurotrophin by retinal ganglion cells, followed by anterograde transport to axon terminals, where it is released and then taken up by the tectum cell (von Bartheld et al., 1996). The finding that BDNF is widely distributed in nerve terminals, even in brain areas lacking BDNF mRNA, such as striatum, and that inhibition of axonal transport by colchicine or de-afferentation depleted BDNF in these areas, further suggests that BDNF is transported anterogradely (Conner et al., 1997; Fawcett et al., 1997). Experiments using BDNF-Green fluorescence protein (GFP) also showed that the fusion protein was present in dendrites and in axons of transfected cortical neurons, and live imaging experiments revealed both anterograde and retrograde BDNF-GFP movements (Kohara et al., 2001). Moreover, BDNF-GFP was found on untransfected postsynaptic neurons innervated by BDNF-GFP-positive axons, demonstrating transfer of BDNF from pre- to postsynaptic neurons (Kohara et al., 2001). High-frequency activation of glutamatergic synapses also triggers the release of BDNF-GFP from synaptically localized secretory granules, and this effect depends on activation of postsynaptic ionotropic glutamate receptors and on postsynaptic Ca^{2+} influx. BDNF-GFP is also released from extrasynaptic dendritic vesicle clusters, suggesting that a possible spatial restriction of BDNF release to specific synaptic sites can only occur if the postsynaptic depolarization remains local (Hartmann et al., 2001). The somatodendritic localization of BDNF as well as BDNF and TrkB (the BDNF receptor; see next section) mRNAs in hippocampal neurons also suggests a postsynaptic release of this neurotrophin (Goodman et al., 1996; Tongiorgi et al., 1997). Moreover, KCl depolarization induces a shift of BDNF and TrkB mRNAs to more distal dendritic locations, with a concomitant increase in BDNF and TrkB protein levels in the distal portion of dendrites (Tongiorgi et al., 1997). This again confirms the postsynaptic release of BDNF.

The neurotrophin NGF was also found to be retrogradely transported in central and peripheral nervous system (Hendry et al., 1974; Korsching and Thoenen, 1983). Moreover, strong evidences for postsynaptic secretion of NT4 were provided in studies using *Xenopus* nerve-muscle cultures (Wang and Poo, 1997), where activity-dependent synaptic potentiation occurs when NT4 is overexpressed at the postsynaptic myocyte but not at the presynaptic neuron (Wang and Poo, 1997).

1.2.2 Neurotrophins and their receptors

Neurotrophins activate two distinct types of transmembrane receptors, the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (RTKs) and the p75 receptor, a member of tumor necrosis factor (TNF) receptor family. Each neurotrophin shows binding specificity for particular Trk receptors. NGF binds preferentially to TrkA, BDNF and NT4 to TrkB, and NT3 to TrkC. These interactions are of high affinity. NT3 can also bind to TrkB and TrkA receptors, although with lower affinity (Fig. 1.6) (Dechant, 2001; Patapoutian and Reichardt, 2001). Unlike Trk receptors, the p75 receptor binds all neurotrophins, although with similar and lower affinity. However, this receptor has a significantly higher affinity for the unprocessed forms of NGF and BDNF, and binding of these pro-neurotrophins is thought to be involved in apoptotic cell death (Chao and Bothwell, 2002; Teng et al., 2005). p75 receptors may also interact with Trk receptors (Gargano et al., 1997; Yano and Chao, 2000) and this interaction is thought to increase Trk specificity towards its preferred neurotrophin (Esposito et al., 2001).

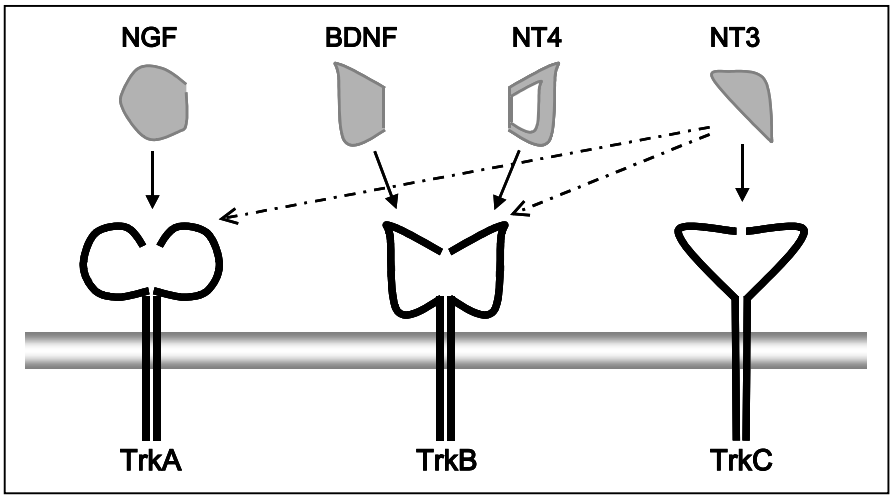


Figure 1.6- Interaction of the different neurotrophins with the respective Trk receptors. NGF is specific for the TrkA receptor, BDNF and NT4 interact specifically with TrkB, and NT3 binds preferentially to TrkC, although it can also bind to TrkA and TrkB with lower affinity.

A new neurotrophin receptor was recently identified, Sortilin, which belongs to the family of Vps10p-domain transmembrane receptors and was previously characterized as a

receptor for the neuropeptide neurotensin. Sortilin has been shown to bind both unprocessed NGF (Nykjaer et al., 2004) and BDNF (Teng et al., 2005). As for Trk receptors, the p75 receptor associates with sortilin when co-expressed in 293 cells, and the receptor complex displays an increased affinity to proNGF (Nykjaer et al., 2004). However, it is still unclear whether sortilin acts merely as a co-receptor together with the p75 receptor, or whether it can trigger signalling cascades of its own.

1.2.3 Signalling mechanisms induced by neurotrophins

Neurotrophins bind as dimers to Trk receptors, leading to the dimerization and transphosphorylation of the receptors on tyrosine residues located in the intracellular domain, and subsequent activation of cytoplasmatic signalling pathways. Trk receptors transmit positive signals such as enhanced survival and growth, in contrast with p75 receptors, which transmit both positive and negative signals. The effects generated by both neurotrophin receptor types can either augment or oppose each other (Chao, 2003).

Trk activation results in the phosphorylation of several conserved tyrosine residues present in the cytoplasmatic domains of each receptor. Transphosphorylation of human TrkA on Tyr 670, Tyr 674 and Tyr 675, localized in the activation loop, further potentiates tyrosine kinase activity. Phosphorylation of addional tyrosine residues creates docking sites for proteins containing phosphotyrosine-binding domains (PTB) or Src homology 2 (SH2) domains. The intracellular signalling events activated by these adaptor proteins include Ras-Raf-ERK, PI3-K-Akt (also known by protein kinase B), PLCγ-Ca^{2+}, NF-κB, and atypical protein kinase C pathways (Foehr et al., 2000; Kaplan and Miller, 2000; Huang and Reichardt, 2003) (Fig. 1.7).

Tyr 490 and Tyr 785 are the major phosphorylated tyrosine residues on the TrkA receptor (and analogous residues on other Trks) away from the kinase activation domain (Stephens et al., 1994). Phosphotyrosine 490 interacts with Shc, fibroblast growth factor receptor substrate 2 (Frs2), and other adaptors. Binding of the adaptor Shc and its phosphorylation provides mechanisms for activation of the Ras-ERK pathway, through recruitment of Grb2 and Sos. However, the activation of this signalling cascade is transient

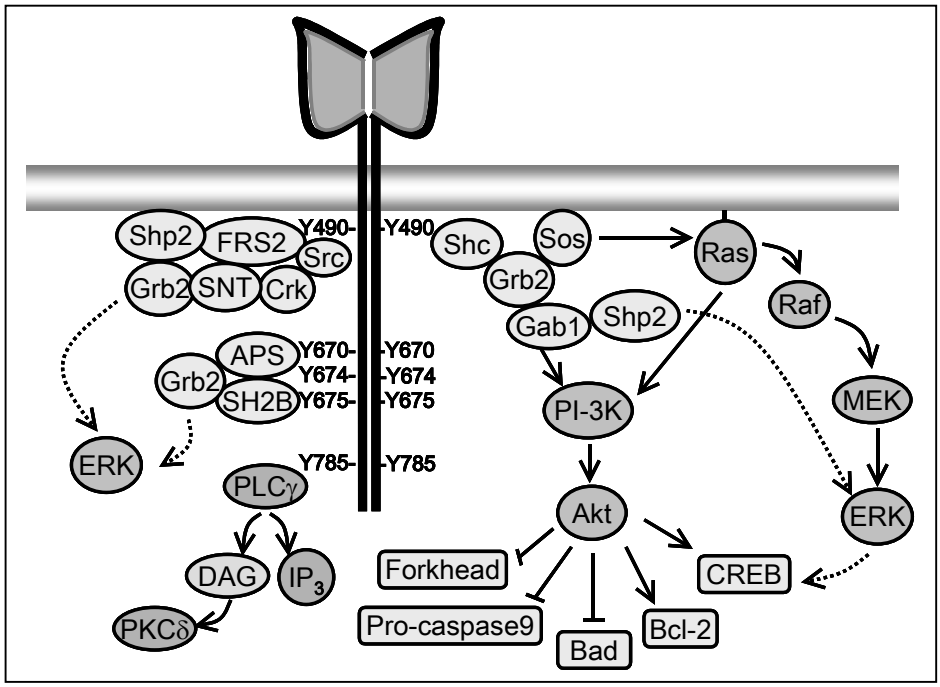


Figure 1.7- Schematic diagram of Trk receptor-mediated signal transduction pathways. Neurotrophin binding to a Trk receptor stimulates its dimerization and transphosphorylation, resulting in the recruitment of proteins that interact with specific phosphotyrosine residues in the cytoplasmatic domains of Trk receptors. These interactions trigger the activation of signalling pathways, such as the Ras, PI3-K, and PLCγ pathways, and ultimately result in activation of gene expression, neuronal survival, and neurite outgrowth (adapted from Huang and Reichardt, 2003).

and its termination appears to be caused by ERK phosphorylation of Sos, resulting in the dissociation of the Sos-Grb2 complex (Kao et al., 2001). The binding of Shc to TrkA Tyr 490 also activates the PI3-K pathway, either by direct interaction of Ras with PI3-K (Rodriguez-Viciana et al., 1994), or through the recruitment of the adaptor protein Gab1 (Holgado-Madruga et al., 1997). Gab1 can additionally recruit the SH2 domain-containing protein tyrosine phosphatase (Shp2) that is responsible for a sustained enhancement of the activity the Ras-ERK cascade (Yamada et al., 1999; Shi et al., 2000) (Fig. 1.7). Interaction of TrkA phosphotyrosine 490 with Frs2 leads to the phosphorylation of this adaptor protein, creating new binding sites for the adaptor proteins Grb2, Crk, Sph2 and Src, in addition to the cyclin-dependent kinase substrate suc-1-associated neurotrophic factor target (SNT) (Meakin et al., 1999) (Fig. 1.7). TrkA phosphorylation on Tyr 785 induces phosphorylation on tyrosine

residues and consequent activation of PLCγ, leading to the generation of DAG and $Ins(1,4,5)P_3$ (Fig. 1.7). $Ins(1,4,5)P_3$ promotes the release of Ca^{2+} from intracellular stores, activating Ca^{2+} dependent enzymes, such as the protein kinase CaMK. DAG stimulates DAG-regulated PKC isoforms, such as PKCδ that can be activated by NGF, and is required for NGF-induced ERK activation and neurite outgrowth (Corbit et al., 1999).

Two additional proteins, the adaptor protein with a pleckstrin homology and a SH2 domains (APS) and SH2B, bind to phosphotyrosine residues within the activation loop of all Trk receptors (Qian et al., 1998). These proteins are phosphorylated by Trk receptors at tyrosine residues, recruiting Grb2, which provides a link between ERK and PI3-K activation (Qian et al., 1998).

Recently, neurotrophins have been shown to induce the phosphorylation of the ankyrin-rich membrane-spanning protein (ARMS), a 220 kDa membrane spanning protein, at Tyr 1096, through activation of the Trk receptors, in primary cultures of cerebrocortical neurons and PC12 cells. This phosphorylation was important for binding of ARMS to CrkL, a known binding partner of ARMS, through CrkL SH2 domain (Arevalo et al., 2006a). Phosphorylation of Tyr 1096 also leads to a long lasting MAPK response and to PC12 cells differentiation upon NGF treatment. However, mutation of Tyr 1096 impairs prolonged MAPK activation and PC12 cells differentiation (Arevalo et al., 2006a).

Activation of PI3-K by Ras stimulates the activity of several other signalling proteins, including the serine/threonine kinase Akt, which is activated through phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK1) and PDK2. In neurons, direct targets of Akt are the proteins Bad, an inhibitor of the Bcl-2 anti-apoptotic protein, pro-caspase-9, which is cleaved into the pro-apoptotic caspapse-9, and Forkhead, a transcription factor that induces apoptosis by increasing the levels of Fas ligand (FasL; see figure 1.7). Phosphorylation of these Akt substrates suppresses apoptosis. Bad phosphorylation induces its association with 14-3-3, and prevents it from associating with and inactivating the anti-apoptotic Bcl-2 and Bcl-XL proteins (Datta et al., 1997; del Peso et al., 1997). Akt can also reduce the proteolytic cleavage of pro-caspase-9, thereby inhibiting caspase-9 activity (Fujita et al., 1999). Phosphorylation of the Forkhead transcription factor by Akt also suppresses its activity. Another potential target of PI3-K is the IAP (inhibitor of apoptosis) family of caspase inhibitors, which includes X-linked inhibitor of apoptosis (XIAP), neuronal apoptosis-inhibitory

protein (NAIP), and human inhibitor of apoptosis protein (HIAP). Therefore, activation of Ras and consequent activation of the signalling pathway consisting of PI3-K and Akt is the major regulator of neuronal survival by inhibiting the activity of apoptotic proteins (Kaplan and Miller, 2000).

In contrast with the effects of the PI3-K pathway, the Ras-ERK pathway induces neuronal survival by stimulating the activity or expression of anti-apoptotic proteins, including Bcl-2 and Bcl-XL, and the transcription factor CREB (cAMP response element-binding protein; see figure 1.3). CREB has been shown to regulate genes whose products are essential for normal differentiation, survival and plasticity of neurons *in vitro* and *in vivo*. This pathway in neurons is also important for synaptic plasticity and long-term potentiation (reviewed by Lonze and Ginty, 2002).

Trk splicing

The diversity of Trk receptors is further increased by differential splicing of their mRNAs, generating different isoforms of each one of the Trk receptors. These splice variants have truncations or insertions in the tyrosine kinase domains, affecting the specificity of Trk receptor responsiveness. An isoform of the TrkA receptor lacking a short insert on the juxtamembrane region is activated efficiently only by NGF, in contrast with the receptors containing this insert, which may also be activated by NT3 (Clary and Reichardt, 1994). Truncated TrkB isoforms can reduce the activation of full-length TrkB receptors by acting either as dominant negatives or BDNF scavengers (reviewed in Dechant, 2001). However, truncated TrkB receptors appear to be capable of initiating their own signalling mechanisms since expression of truncated TrkB.T1 and TrkB.T2 receptors triggers BDNF-mediated signalling in the absence of the full-length form (Baxter et al., 1997). In glial cells, activation of TrkB.T1 by BDNF was shown to activate a G protein that stimulates PLC, leading to the production of $Ins(1,4,5)P_3$, Ca^{2+} release from intracellular stores and Ca^{2+} entry from the extracellular space (Rose et al., 2003).

From the physiological point of view, the expression of full-length or truncated TrkB on visual cortex slices differentially regulates dendritic arborization (Yacoubian and Lo, 2000). The truncated TrkB was also shown to regulate the surface expression of full-length TrkB (Haapasalo et al., 2002). TrkC receptor isoforms containing peptide insertions in their

PTK domain are phosphorylated in response to their ligands, but have different signalling potential (Garner and Large, 1994).

p75 receptor

Neurotrophin binding to p75 receptors promotes survival of some cells and apoptosis of others, and affects the axon outgrowth both *in vivo* and *in vitro*. p75 exerts these diverse actions through a set of signalling pathways largely distinct from those activated by Trk receptors. Prosurvival pathways activated by p75 receptor include NFκB and Akt (Roux et al., 2001; Wooten et al., 2001), whereas the proapoptotic mediators include the JNK-p53 signalling cascade, activation of sphingomyelinase, and association with adaptor proteins (neurotrophin receptor interacting factor- NRIF, neurotrophin receptor-interacting MAGE [melanoma antigen] homologue- NRAGE, p75 receptor-associated cell death executor- NADE) that directly promote cell cycle arrest and apoptosis (Mukai et al., 2000; Salehi et al., 2000; Whitfield et al., 2001; Harrington et al., 2002). p75 receptors also activate the small G proteins Rac and Rho that directly affect growth cone motility (Harrington et al., 2002).

The intracellular signalling activated by p75 neurotrophic receptors may be affected by simultaneous stimulation of Trk receptors. For example, Trk stimulation and subsequent Ras activation suppresses the p75-induced activation of the JNK cascade and the p53-dependent apoptotic pathway (Mazzoni et al., 1999). Also, the sphingomyelinase activity induced by p75 receptors is completely suppressed by Trk signalling through PI3-K (Bilderback et al., 2001). On the other hand, p75 activation may facilitate Trk activation, either by presenting the neurotrophin to Trk receptors or by inducing a favorable conformational change in the receptor (Chao and Bothwell, 2002).

Neurotrophin receptor internalization

Ligand engagement of neurotrophins to Trk receptors stimulates their internalization into vesicles through clathrin-dependent and independent endocytosis (Grimes et al., 1997; Howe et al., 2001). This internalization appears to require Trk phosphorylation and PI3-K signalling (Howe et al., 2001), and the vesicles are then transported to the cell body. Some of the biological effects of neurotrophins require that signals be conveyed over long distances, from the nerve terminal to the cell body (Ginty and Segal, 2002). Both Trk and

p75 receptors undergo retrograde and anterograde transport, and several proteins are associated with both receptors during transport. Since the transported receptors are thought to keep the interaction with the neurotrophin, signalling activity persists after internalization (Grimes et al., 1997). For example, activation of Rap1 is completely dependent on Trk internalization, since this protein is localized intracellularly in the endocytic vesicle-like structures (York et al., 2000). Internalization of the receptors may also control their desensitization, and a rapid loss of neuronal response to BDNF is observed following stimulation with the neurotrophins, due to a rapid degradation of internalized TrkB receptors. Because the pathways activated by NGF and BDNF are differentially regulated, this type of desensitization is not observed for TrkA and NGF (Carter et al., 1995; Sommerfeld et al., 2000). Recently, the identification of a specific E3 ubiquitin ligase molecule (Nedd4-2) that promotes TrkA ubiquitination and downregulation of the receptor in an NGF-dependent manner represents a potential mechanism to control the levels of Trk receptors in neurotrophin-responsive neurons. Although the structure of the various Trk receptors is highly similar, this event is specific to TrkA receptors, since the site of E3 ubiquitin ligase association is not conserved in TrkB and TrkC receptors (Arevalo et al., 2006b)

Thus, the specificity of neuronal responses to neurotrophins can arise from differences in the type of receptor, differential splicing, and the availability of p75 receptors.

1.2.4 Modulation of synaptic transmission by neurotrophins

Activation of Trk receptors regulates the expression and activities of ion channels, neurotransmitter receptors and other tyrosine kinases, and modulates exocytosis and endocytosis of synaptic vesicles. Some of these effects are observed in seconds to minutes and clearly do not require protein synthesis, while other actions do involve regulation of gene expression through control of transcription factors. Protein phosphorylation, localization of proteins and organelles, local regulation of protein translation and the control of gene expression affects function of the synapse, and these mechanisms are attractive candidates

to mediate the important roles that neurotrophins play in regulating synaptic plasticity in the hippocampus.

Neurotrophin effects on neurotransmitter release

Neurotrophins have been reported to enhance excitatory synaptic transmission at central synapses (Kim et al., 1994; Lessmann et al., 1994; Kang and Schuman, 1995; Levine et al., 1995; Carmignoto et al., 1997; Li et al., 1998; Messaoudi et al., 1998; Sherwood and Lo, 1999), and to suppress inhibitory transmission in both slice and dissociated cell cultures (Kim et al., 1994; Tanaka et al., 1997; Frerking et al., 1998). These effects appear to rely on the modulation of pre-synaptic activity, possibly by an effect on the neurotransmitters release.

Application of NT4 induced a transient augmentation of evoked synaptic currents and a transient increase in the frequency of miniature excitatory postsynaptic currents in cultured hippocampal neurons (Lessmann et al., 1994). However, the amplitudes of the AMPA receptor mediated miniature EPSC were unaffected, suggesting a presynaptic action of BDNF and NT-4. Accordingly, acute addition of BDNF to rat hippocampal nerve terminals, was shown to enhance the K^+-evoked glutamate release, by a mechanism involving Ca^{2+} entry through the voltage-sensitive calcium channels (Canas et al., 2004). Similarly, application of exogenous BDNF to isolated adult rat hippocampal nerve terminals was found to moderately potentiate the K^+-evoked release of glutamate, possibly by activating PLCγ (Pereira et al., 2006). In contrast, BDNF inhibited K^+-evoked GABA release from hippocampal synaptosomes, by a mechanism independent of Ca^{2+} influx through voltage-sensitive calcium channels. Moreover, blockade of the GABA transporters prevented the inhibitory action of BDNF upon GABA release, indicating that the effect of the neurotrophins is mediated by inhibition of the reversal of the GABA transporters present in the nerve terminals (Canas et al., 2004).

The neurotrophin NGF was shown to rapidly and robustly enhance K^+-evoked acetylcholine release from basal forebrain cultures. This effect persisted for several hours after NGF removal and was dependent on TrkA signalling, Ca^{2+}, and voltage-gated calcium channels, but not dependent on *de novo* protein synthesis (Auld et al., 2001).

BDNF was also shown to induce a rapid and transient release of glutamate from cultured cortical neurons by a mechanism independent of extracellular Ca^{2+} entry, but

dependent on intracellular Ca^{2+} (Takei et al., 1998). Surprisingly, botulinum toxin B was found not to inhibit the BDNF-induced release of glutamate, indicating that this secretion of glutamate was through a non-exocytotic pathway, presumably by reverse transport of glutamate transporters (Takei et al., 1998). These results contrast with the effect of BDNF and NT3 on neurotransmitter release from hippocampal neurons, where the neurotrophins potentiate the depolarization-induced increases in the cytosolic Ca^{2+} (Berninger et al., 1993; Stoop and Poo, 1996), that eventually results in changes in the efficacy of synaptic vesicle exocytosis (Pozzo-Miller et al., 1999). Rapid and transient glutamate and aspartate release induced by BDNF were also observed in cultured cortical, hippocampal, striatal and cerebellar neurons (Numakawa et al., 1999). NGF and NT-3 also elicited neurotrophin-induced release of glutamate as well as BDNF from the cerebellar granule neurons. This release was dependent on intracellular Ca^{2+} mobilization. However, because these cells do not possess TrkA mRNA, the results suggest that NGF-elicited release was not mediated by Trks (Numakawa et al., 1999). Other evidences have implicated synaptic vesicle-associated proteins like synapsin (Jovanovic et al., 2000), synaptophysin and synaptobrevin (Pozzo-Miller et al., 1999) as downstream targets of the BDNF signalling pathway.

In *Xenopus* nerve-muscle cultures overexpression of NT4 induced a high level of spontaneous synaptic activity and enhanced evoked synaptic transmission. The NT4 effects involved a potentiation of presynaptic neurotransmitter release as well as a lengthening of the mean burst duration of postsynaptic low conductance acetylcholine channels (Wang and Poo, 1997).

Neurotrophins may also affect synaptic transmission in developing synapses. Indeed, application of BDNF or NT3 (but not NGF) to *Xenopus* nerve-muscle cultures enhanced the spontaneous and evoked transmitter secretion, and this effect persisted for as long as the factor was present (Lohof et al., 1993). In cultures of sympathethic neurons or PC12 cells, withdrawal of NGF from the culture medium resulted in a gradual collapse of filapodia in the neuritic growth cone, and reintroduction of NFG caused the reappearence of active filapodia (Phelan et al., 1991). In the developing visual cortex, BDNF was shown to accelerate the maturation of inhibitory pathways and overexpression of BDNF in transgenic mice increases the number of synapses in sympathetic ganglia (Huang et al., 1999).

Postsynaptic effects of neurotrophins

In addition to the presynaptic effects, neurotrophins may also modulate the postsynaptic activity. Accordingly, BDNF rapidly enhanced synaptic efficacy through an increased postsynaptic responsiveness via a phosphorylation-dependent mechanism (Levine et al., 1995). BDNF application to cultured hippocampal neurons dramatically increased the spontaneous firing rate, as well as the frequency and amplitude of excitatory postsynaptic currents. Interestingly, NT3 potentiates GABA-induced postsynaptic currents in cultured immature hypothalamic neurons in a developmental stage during which GABA induces depolarizing currents (Gao and van den Pol, 1999). However, in mature hypothalamic neurons and cortical neurons, where GABA evokes inhibitory hyperpolarizing currents, NT3 has an inhibitory action on GABA-induced currents (Gao and van den Pol, 1999).

Role of neurotrophins in synaptic plasticity

The effects of neurotrophins on synaptic transmission are relevant to understand the mechanisms underlying LTP or LTD. Thus, genetic deletion of BDNF in mice disrupted normal induction of LTP in the CA1 region of the hippocampus (Korte et al., 1995; Xie et al., 2000), and this defect was rescued by reintroducing BDNF, through transfection of hippocampal slices with BDNF-expressing adenovirus or by supplying exogenous BDNF (Korte et al., 1996; Patterson et al., 1996). Moreover, chelation of endogenously secreted BDNF with TrkB–IgG or using antibodies against BDNF reduces LTP (Figurov et al., 1996; Kang et al., 1997), and the same LTP phenotype is observed in TrkB mutant mouse lines (Minichiello et al., 1999). Therefore, BDNF seems to be a permissive factor required for the induction, expression or maintenance of LTP, rather than a signal to potentiate basal synaptic transmission in the CA1 region of the hippocampus. This is supported by the finding that BDNF reduces tetanus-induced depression of transmitter release at CA3–CA1 synapses of young rats, allowing sufficient postsynaptic activation for the induction of LTP (Figurov et al., 1996). At dentate synapses, pairing of a brief puff of BDNF with a weak presynaptic stimulation also elicits a robust LTP (Kovalchuk et al., 2002). Furthermore, exogenous BDNF was found to block LTD induced by low frequency stimulation and to enhance tetanus-induced LTP in slices of visual cortex (Akaneya et al., 1996; Huber et al., 1998; Kinoshita et al., 1999), without affecting basal synaptic transmission. BDNF is also required for the

maintenance of late phase potentiation in the CA1 region of the hippocampus after the induction of LTP (Kang et al., 1997; Korte et al., 1998).

The signalling pathways contributing to the effect of TrkB receptors on LTP in the hippocampus were characterized by preventing PLCγ signalling concomitantly at the presynaptic and postsynaptic level (Gartner et al., 2006). This blocking was shown to reduce LTP to levels comparable with those in TrkB and BDNF knock-out mice. However, blockade of presynaptic or postsynaptic PLCγ signalling alone did not result in a significant reduction of LTP (Gartner et al., 2006). Moreover, mutant mice expressing TrkB receptors lacking the PLCγ docking site were found to have deficits on LTP. BDNF stimulation of primary neurons derived from PLCγ mutant mice fully retained their ability to activate MAP kinases, but the induction of CREB and CaMKIV phosphorylation was strongly impaired (Minichiello et al., 2002).

Mutant mice expressing TrkB receptors lacking the Shc binding site were also a valuable tool in the understanding of the signalling mechanisms involved in BDNF modulation of certain forms of synaptic plasticity (Korte et al., 2000; Minichiello et al., 2002). This mutation did not affect the induction-rate or magnitude of early LTP and late LTP induced by theta-burst or tetanus stimulation, suggesting that the Shc-binding site in the TrkB receptor and its downstream signalling cascade are not required for LTP at Schaffer-collateral/CA1 synapse in mouse hippocampus (Korte et al., 2000; Minichiello et al., 2002). In contrast, a conditional mutation of the Trk gene results in deficits in memory acquisition and consolidation in several hippocampus-dependent learning tasks.

Modulation of synaptic currents

Neurotrophins have been shown to influence both the frequency and amplitude of synaptic currents in various experimental systems. Thus, BDNF and NT3 produce rapid increases in synaptic strength in nerve-muscle synapses, as well as increases in excitatory postsynaptic currents in hippocampal neurons (Lohof et al., 1993; Kang and Schuman, 1995; Levine et al., 1995). In contrast, BDNF was found to block postsynaptic AMPA receptor-mediated currents in a subset of nucleus tractus solitarius cells (Balkowiec et al., 2000). The catalytic activity of TrkB receptors is required for the decrease in AMPA receptor activity, implying that there might be a close association between TrkB and AMPA receptors. The

exo- and endocytosis of AMPA receptors, which determine activity dependent changes of synaptic efficacy, could also be influenced by BDNF signalling.

Increasing number of interactions between Trk receptors and ion channels are being discovered. Electrophysiological measurements in cultured rat olfactory bulb neurons showed that acute BDNF exposure induces the tyrosine phosphorylation of $K_v1.3$ channels and suppresses their currents, although no effect is observed in the channel kinetics of inactivation or deactivation (Tucker and Fadool, 2002). In contrast, chronic exposure (days) of the same cells to BDNF increased the magnitude of $K_v1.3$ current and accelerated the inactivation and deactivation of the channel (Tucker and Fadool, 2002). Therefore, depending on the stimulation used, BDNF may have depolarizing (acute stimulation) or hyperpolarizing (chronic stimulation) effects in cultured rat olfactory bulb neurons.

A depolarizing response to BDNF may also be mediated through activation of the $Na_v1.9$ sodium channels (Blum et al., 2002), since an inward sodium current was detected within milliseconds of BDNF treatment in hippocampal neurons. The time course of $Na_v1.9$ activation by phosphorylation is considerably longer than the observed response to BDNF, suggesting that TrkB and $Na_v1.9$ exist in a complex. According to this model, a change in the conformation of TrkB receptors may lead to the activation of the $Na_v1.9$ channels. Low concentrations of BDNF and NT4 also activate a Na^+ conductance as rapidly as glutamate, in the CA1 area of the hippocampus and in the brain cortex and cerebellum (Kafitz et al., 1999). This rapid response to BDNF or NT4 again suggests that the Na^+ channels are very likely to physically associate with TrkB receptors. Activation of Na^+ channels and the resulting inward currents lead to membrane depolarization and gate voltage–dependent Ca^{2+} channels, thereby inducing Ca^{2+} influx. Accordingly, BDNF and NT4 have been shown to activate postsynaptic Ca^{2+} currents in dentate granule cells (Bramham and Messaoudi, 2005). Dendritic spines appear to be the exclusive site of rapid activation of these voltage–dependent Ca^{2+} channels by BDNF and, together with the resulting influx of Ca^{2+} through NMDA receptors, induce robust LTP (Bramham and Messaoudi, 2005).

Trk receptor activation has also been shown to activate several members of the transient receptor potential family of cation channels (TRPC). Many members of this family have previously been shown to be activated through PLC. TRPC3 is a non-voltage-gated and store-operated cation channel that is highly expressed in brain regions where TrkB receptors are found (Li et al., 1999a). Treatment of pontine neurons with BDNF resulted in

an increase in cation inward currents, through a PLC-dependent mechanisms (Li et al., 1999a)

Activity dependent effects

Synaptic modulation by neurotrophins also depends on a cytoplasmic signalling activity and on the neuronal electrical activity. This is supported by the finding that synaptic potentiation by BDNF is greatly facilitated by presynaptic activity at developing neuromuscular junctions (Boulanger and Poo, 1999). Brief depolarization (or spiking) of the presynaptic neuron in the presence of a low BDNF concentration resulted in a marked potentiation of spontaneous and evoked transmitter secretion, whereas exposure to either a low BDNF concentration or depolarization alone had no effect. This depolarization of the presynaptic terminal was mediated by an elevation of cAMP levels (Boulanger and Poo, 1999). Thus, electrically active nerve terminals may be more susceptible to synaptic potentiation by secreted neurotrophins than inactive terminals. This may constitute a mechanism for activity dependent synapse refinement. High-frequency neuronal activity and synaptic transmission were also shown to elevate the number of TrkB receptors on the surface of cultured hippocampal neurons (Du et al., 2000), and may therefore facilitate the synaptic action of BDNF. Neuronal or synaptic activity is also known to promote the effects of neurotrophins on dendritic arborization in cortical slices (McAllister et al., 1996) and the survival of cultured retinal ganglion cells (Meyer-Franke et al., 1995). In the latter case, the activity elevates cAMP levels to enhance the responsiveness of the neuron to neurotrophins, apparently by recruiting extra TrkB receptors to the plasma membrane (Meyer-Franke et al., 1998). Taken together, these findings indicate that the response of a given synapse to BDNF may depend on its recent history.

BDNF modulation of AMPA receptors

The effect of BDNF on AMPA receptors has been investigated mainly in studies where neurons were subjected to chronic stimulation with the neurotrophin. Daily application of BDNF increases the protein expression of AMPA receptor subunit GluR1 and GluR2/GluR3 in cultured rat neocortical neurons (Narisawa-Saito et al., 1999a), through a mechanism dependent on the activity of Fyn, a non-receptor-type protein tyrosine kinase belonging to the Src-family (Narisawa-Saito et al., 1999b). BDNF was also shown to trigger a

surface translocation of AMPA receptors by an exocytotic process, within a period of 3 h, as determined using an [^{3}H]AMPA binding assay. Studies performed in a transfected cell line suggested that the BDNF-evoked translocation of GluR2-containing AMPA receptors to the membrane depends on the interaction of GluR2 with NSF (Narisawa-Saito et al., 2002). Similarly, chronic treatment of neocortical neurons with BDNF elevated total and surface GluR1 and GluR2/3 subunits of the AMPA receptors (Nagano et al., 2003). However, no changes in the messenger RNA (mRNA) for the AMPA receptor subunits were detected in cerebral neocortical neurons subjected to chronic stimulation with BNDF (Narisawa-Saito et al., 1999a). In contrast, BDNF treatment for two days increased the promoter activity of a fragment of the GluR2 gene in a transfected human neuroblastoma cell line (differentiated cell line SH-SY5Y) (Brene et al., 2000). This effect was accompanied by increased expression of endogenous GluR2 protein in those cells, and appeared to be mediated via neuron-restrictive silencer element (NSRE) (Brene et al., 2000).

Chronic stimulation of rat neocortical neurons with BDNF was also shown to upregulate the expression of some postsynaptic density proteins, which interact with AMPA receptors (SAP97, GRIP1, Pick1), and to increase the mRNA levels of GRIP1 and PICK1, but with no changes on SAP97 mRNA (Jourdi et al., 2003). Conversely, BDNF gene disruption reduced the levels of SAP97, GRIP1, and Pick1 protein in neocortex. Under the same conditions there was an increase in the interaction between GluR1 and SAP97, as well as between GluR2 and GRIP1, which may play a role in the upregulation of AMPA receptors by BDNF (Jourdi et al., 2003).

BDNF was also shown to play a role in the regulation of AMPA receptor activity. Multipolar cortical GABAergic neurons chronically treated with BDNF showed a marked increase in the inward membrane currents evoked by AMPA (Nagano et al., 2003). However, in this case, the sustained stimulation of neurotrophin receptors from early in development may have caused changes in cell phenotype. Also, under these conditions no effect on mRNA for the receptor subunits was observed. On the other hand, long-term treatment of hippocampal cultures with BDNF potentiated excitatory transmission by augmenting the amplitude of AMPA receptor-mediated miniature EPSCs (Bolton et al., 2000). In contrast, BDNF was shown to strongly inhibit postsynaptic AMPA receptor-mediated currents in a large subset of newborn nucleus tractus solitarius neurons (Balkowiec et al., 2000). These BDNF induced-changes in synaptic activity may be due to the insertion or removal of AMPA

receptors from potentiated and depressed synapses (Carroll et al., 1999; Lissin et al., 1999), respectively, or to changes in the phosphorylation state of AMPA type glutamate receptors (Wu et al., 2004).

Translation of mRNA may also be modulated by BDNF, through stimulation of TrkB receptors coupled to activation of the mammalian target of rapamycin (mTOR). This regulation of the translation initiation complex (Bramham and Messaoudi, 2005) can be activated by the PI3-K-Akt and the Ras-Raf-ERK pathways, which act downstream of TrkB receptor activation (see section 1.2.3 Signalling mechanisms induced by neurotrophins). Inhibition of mTOR with rapamycin prevents forms of protein synthesis-dependent synaptic activity and blocks both late LTP induced by high-frequency stimulation and BDNF-induced LTP at CA3-CA1 synapses (Tang et al., 2002; Cammalleri et al., 2003). The effects of mTOR may be, at least in part, mediated by an upregulation of GluR1 protein level, since BDNF was shown to upregulate this receptor subunit in synatoneurosomes through the mTOR–PI3-K pathway (Schratt et al., 2004). Therefore, this pathway may contribute to increase locally the amount GluR1 after high-frequency stimulation (Schratt et al., 2004).

BDNF was also found to induce gene expression through phosphorylation and activation of the transcription factor CREB (Finkbeiner et al., 1997) and CREB-binding sites are present in the promoter region of GluR1. However, there are no evidences for a role of CREB in the regulation of GluR1 promoter activity (Borges and Dingledine, 2001).

Studies in a heterologous system showed that the expression of GluR2 can be regulated by BDNF, through a mechanism involving a NRSE present within the GluR2 promoter (Brene et al., 2000). Another transcription factor that controls GluR2 expression is nuclear factor of activated T-cells isoform 4 (NFATc4) (Weik et al., 2005). In hippocampal neurons, BDNF activates NFAT-dependent transcription via TrkB receptors, PLCγ signalling and calcineurin (Groth and Mermelstein, 2003). However, this transcription factor was shown to bind to the gene encoding the GluR2 subunit and to repress GluR2 gene expression, decreasing the abundance of GluR2 mRNA and protein (Weik et al., 2005). These results contrast with the effects observed in cerebrocortical neurons chronically exposed to BDNF, where no change in the mRNA for GluR2 was observed (Narisawa-Saito et al., 1999a).

BDNF modulation of NMDA receptors

Less is known about the effect of BDNF on NMDA receptors. BDNF increases NMDA single channel open probability in cultured hippocampal neurons (Levine et al., 1998; Levine and Kolb, 2000), probably by enhancing the phosphorylation of NR1 and NR2B subunits in hippocampal and cortical neurons (Suen et al., 1997; Lin et al., 1998; Alder et al., 2005). The effect of the neurotrophin on the activity of NMDA receptors in cultured hippocampal neurons is sensitive to inhibitors of NR2B (Crozier et al., 1999; Levine and Kolb, 2000) and, accordingly, BDNF was shown to acutely increase tyrosine phosphorylation of NR2B (but not NR2A) subunits in cortical and hippocampal postsynaptic densities (Lin et al., 1998), and in cultured hippocampal neurons (Alder et al., 2005). Phosphorylation of NR2B may be mediated by Fyn, a member of the Src family, since this kinase is activated by TrkB receptors (Narisawa-Saito et al., 1999b), and increases currents mediated by recombinant NMDA receptors (Kohr and Seeburg, 1996). Fyn was also suggested to contribute to the increase of glutamatergic synaptic transmission by BDNF (Alder et al., 2005). The BDNF-induced phosphorylation of NR2B may contribute, at least in part, to the observed tyrosine phosphorylation of this receptor subunit in LTP in the hippocampal CA1 region (Nakazawa et al., 2001).

The activity of NMDA receptors in CNS neurons was shown to increase following intracellular application of recombinant Src (Wang and Salter, 1994; Kalia et al., 2004; Salter and Kalia, 2004), and Fyn-mediated interaction between BDNF signalling and NMDA receptors may play an important role in spatial learning and memory (Mizuno et al., 2003). The signalling activity induced by BDNF may also affect NMDA receptors through PKC, which modulates NMDA receptor trafficking and gating in cultured hippocampal neurons (Lan et al., 2001b). In fact, activation of Trk receptors promotes PLCγ activity, giving rise to DAG, which activates PKC.

In addition to these rapid effects of BDNF on NMDA receptors, recent studies showed that BDNF increases the translation of the NR1 subunit mRNA in cultured cerebrocortical neurons (Schratt et al., 2004), suggesting that the neurotrophin may also regulate the abundance of NMDA receptors in the hippocampus. NR1 expression is regulated by different transcription factors, including the NF-κB (Liu et al., 2004) and CREB (Lau et al., 2004), and the latter is a major mediator of neuronal neurotrophin responses (Finkbeiner et al., 1997). NR2B expression is also regulated by CREB (Rani et al., 2005), in

addition to AP-1 (Qiang and Ticku, 2005), which may also be activated by BDNF-induced signalling (Li et al., 2004). NT4 was shown to upregulate NR2A through the immediate early transcription factor Egr-1 in cultured cerebrocortical neurons (Choi et al., 2004).

1.3 Objectives of the present study

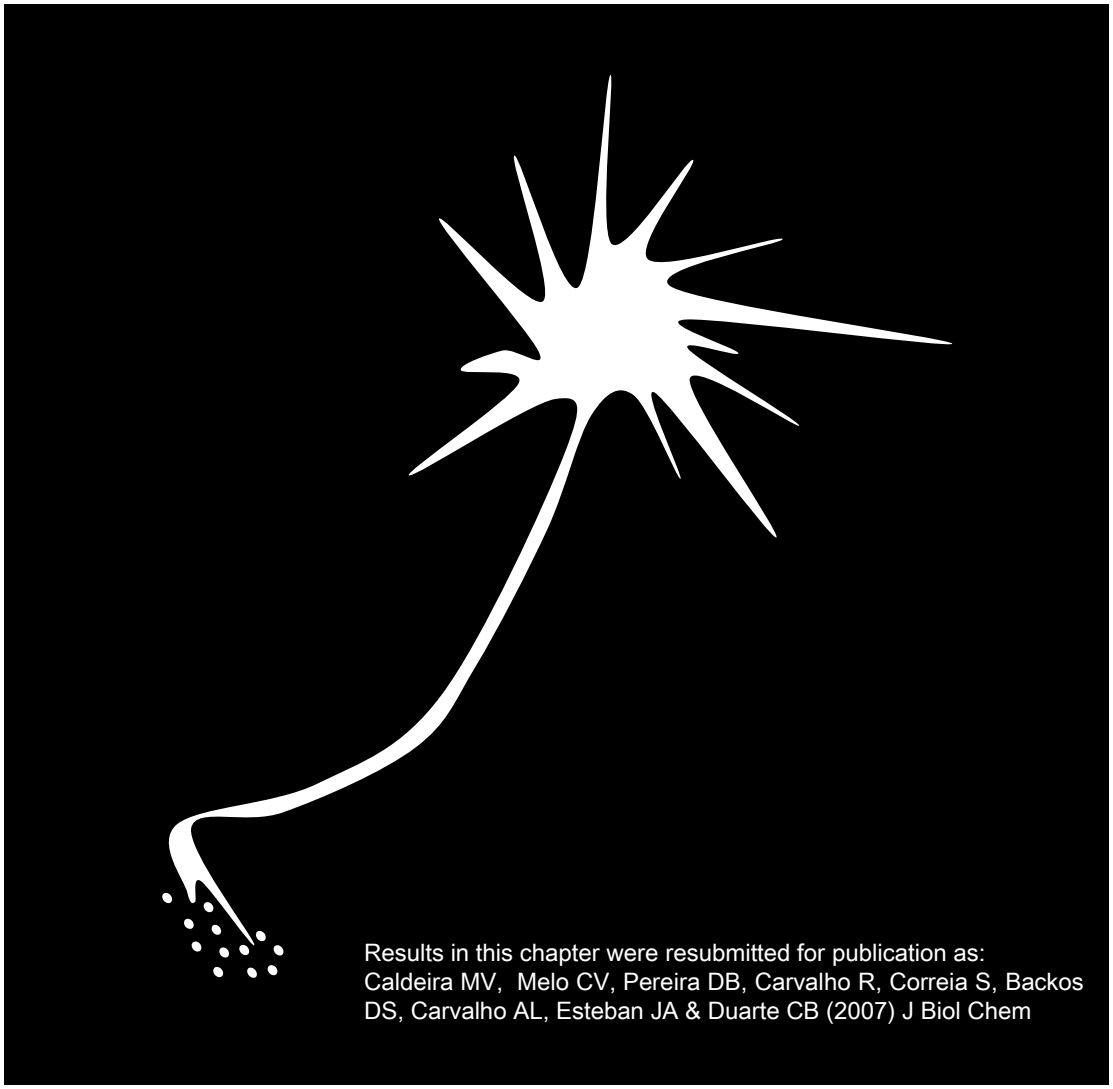
The main goal of this study was to investigate the effect of BDNF on AMPA and NMDA receptors, which may account, at least in part, for the role of the neurotrophins in synaptic plasticity. Hippocampal cultures were chosen as an experimental model since AMPA and NMDA receptors are highly expressed in this region and because synaptic plasticity in the hippocampus is though to underlie memory formation and learning. BDNF, besides controlling neuronal growth and development, is also responsible for modifications on synaptic transmission and localization of the BDNF-Trk complex in glutamatergic synapses makes this system an attractive regulator of excitatory transmission and plasticity. Therefore, it was of interest to further investigate the relationship between BDNF activity and AMPA and NMDA receptors.

The study of the effect of BDNF on glutamate receptors was divided in two different, but interconnected parts, which are included in chapters 2 and 3. The first focuses on the effect of BDNF on AMPA receptors, and the second concerns the effect of the neurotrophin on NMDA receptors. In both cases, we first studied the effect of BDNF on the abundance of AMPA and NMDA receptor subunits by Western blot. Because BDNF was shown to upregulate several AMPA and NMDA receptor subunits, and since BDNF is known to modulate translation and transcription (Finkbeiner et al., 1997; Schratt et al., 2004; Bramham and Messaoudi, 2005), two different translation and transcription inhibitors were used and the levels of AMPA and NMDA receptor subunits were evaluated. This question was further addressed by performing real-time PCR experiments to measure the mRNA levels of the different subunits.

Since a large pool of receptors is intracellular and therefore unable to respond to the neurotransmitter glutamate, we have also examined the effect of BDNF on plasma

membrane expression of AMPA and NMDA receptor subunits by performing biotinylation assays. Since BDNF induced the translocation of AMPA and NMDA receptors to the plasma membrane, functional studies were performed in order to determine whether the neurotrophin upregulates the activity of the receptors. Thus, the effect of BDNF on NMDA receptor activity was studied by measuring the $[Ca^{2+}]_i$ response to addition of the NMDA receptor agonists in single-cell cultured hippocampal neurons, using the Fura-2 fluorescent probe. Synaptic delivery of AMPA receptors upon BDNF stimulation was also studied by electrophysiology techniques in cultured hippocampal slices overexpressing GluR1-GFP, by infection with Sindbis virus. Since BDNF was shown to promote the synaptic delivery of the GluR1 subunit, the phosphorylation of specific residues on this subunit was also examined.

Chapter 2. BDNF regulates the expression and synaptic delivery of AMPA receptor subunits in hippocampal neurons



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BDNF REGULATES THE EXPRESSION AND THE SYNAPTIC DELIVERY OF AMPA RECEPTOR SUBUNITS IN HIPPOCAMPAL NEURONS

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Running title: Modulation of AMPA receptors by BDNF

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BDNF plays an important role in synaptic plasticity in the hippocampus, but the mechanisms involved are not fully understood. The neurotrophin couples synaptic activation to changes in gene expression underlying long-term potentiation and short-term plasticity. Here we show that BDNF acutely upregulates GluR1, GluR2 and GluR3 AMPA receptor subunits in 7 DIV cultured hippocampal neurons. The increase in GluR1 and GluR2 protein levels in developing cultures was impaired by K252a, a Trk inhibitor, and by translation (emetine and anisomycin) and transcription inhibitors (α-amanitine and actinomycin D). Accordingly, BDNF increased the mRNA levels for GluR1 and GluR2 subunits. Biotinylation studies showed that stimulation with BDNF for 30 min selectively increased the amount of GluR1 associated with the plasma membrane, and this effect was abrogated by emetine. Under the same conditions, BDNF induced GluR1 phosphorylation on Ser831 through activation of PKC and CaMKII. Chelation of endogenous extracellular BDNF with TrkB-IgG selectively decreased GluR1 protein levels in 14 DIV cultures of hippocampal neurons. Moreover, BDNF promoted synaptic delivery of homomeric GluR1 AMPA receptors in cultured organotypic slices, by a mechanism independent of NMDA receptor activation. Taken together, the results indicate that BDNF upregulates the protein levels of AMPA receptor subunits in hippocampal neurons and induces the delivery of AMPA receptors to the synapse.

INTRODUCTION

Neurotrophins are essential for the development of the vertebrate nervous system, modulate synaptic function and play an important role in synaptic plasticity (Poo, 2001; Vicario-Abejon et al., 2002). Brain-derived neurotrophic factor (BDNF) has been implicated in activity-dependent synaptic plasticity, particularly in long-term potentiation (LTP) induced by high-frequency stimulation. Accordingly, LTP is impaired in the hippocampal CA1 region of animals deficient in BDNF, but it can be rescued by supplying the neurotrophin (Korte et al., 1995; Korte et al., 1996; Patterson et al., 1996). Chelation of endogenous BDNF also prevents the induction of LTP by theta burst stimulation and reduces late-phase LTP induced by high-frequency stimulation (Figurov et al., 1996; Kang et al., 1997). Furthermore, the late-phase LTP induced by tetanic stimulation was not observed in slices from BDNF-knockout mice and was also abrogated when TrkB receptors were

blocked (Korte et al., 1998). Taken together, the available evidences point to a direct role of BDNF in the early and late phases of LTP.

Binding of BDNF to TrkB receptors is followed by activation of intracellular signalling pathways, including the Ras/extracellular signal-regulated protein kinase-, phospholipase C-γ (PLCγ)-, phosphatidylinositol-3-kinase/Akt- and Src-pathways (Narisawa-Saito et al., 1999b; Kaplan and Miller, 2000; Huang and Reichardt, 2003). TrkB receptors are located on axon terminals and in the post-synaptic density of glutamatergic synapses (Drake et al., 1999; Aoki et al., 2000; Pereira et al., 2006), but whether the effects of BDNF on synaptic plasticity are mediated by pre- and/or post-synaptic receptors is not fully elucidated. BDNF was originally shown to induce a long-lasting potentiation of excitatory synaptic transmission in the hippocampal CA1 region, acting at a presynaptic site [(Kang and Schuman, 1995); for conflicting results see (Figurov et al., 1996; Patterson et al., 1996; Tanaka et al., 1997; Frerking et al., 1998; Gottschalk et al., 1998)], and subsequent studies showed that BDNF was selectively required for those forms of LTP that recruit a presynaptic component (Zakharenko et al., 2003). However, activation of postsynaptic TrkB receptors generates intracellular Ca^{2+} concentration transients in dentate granule cells, which induce LTP when paired with weak synaptic stimulation (Kovalchuk et al., 2002). The enhancement of synaptic transmission by BDNF observed in the dentate gyrus *in vivo* (Messaoudi et al., 1998) may also be due, at least in part, to the activation of post-synaptic receptors (Messaoudi et al., 2002; Ying et al., 2002). Nevertheless, a postsynaptic role of BDNF in synaptic plasticity, and the potential mechanisms involved, remain controversial.

The number, composition and location of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors in neurons, together with receptor phosphorylation, are critically important factors in determining the neuronal response to glutamate, and play an important role in the mechanisms of synaptic plasticity (Gomes et al., 2003). AMPA receptors are formed by the association of GluR1-GluR4 subunits, and their delivery to the synapse is tightly controlled by the intracellular signalling activity. In this work, we characterized the effect of BDNF on the abundance of AMPA receptors in cultured hippocampal neurons and on their cellular distribution. Furthermore, we investigated the effect of BDNF on the synaptic delivery of GluR1-containing AMPA receptors in CA1 hippocampal neurons, which could account for the postsynaptic effects of the neurotrophin in the early phase of LTP.

EXPERIMENTAL PROCEDURES

Hippocampal cultures- Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (0.06%, 15 min, 37 ºC; GIBCO Invitrogen, Paisley, UK) and deoxyribonuclease I (5.36 mg/ml), 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 $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and 0.001% phenol red). The hippocampi were then washed with HBSS containing 10% fetal bovine serum (BioWhittaker Europe, Belgium), to stop trypsin activity, and transferred to Neurobasal medium (GIBCO Invitrogen) supplemented with B27 supplement (1:50 dilution; GIBCO Invitrogen), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin. The cells were dissociated in this solution and were then plated in 6 well plates ($91.6x10^3$

cells/cm^2), coated with poly-D-lysine (0.1 mg/mL). The cultures were maintained in a humidified incubator of 5% CO_2/95% air, at 37 °C, for seven or fourteen days. Cultures were stimulated with 100 ng/ml BDNF (kind gift from Regeneron, Tarrytown, NY) for the indicated periods of time. When appropriate, 200 nM K252a, 2.0 μM emetine, 2.0 μM anisomycin, 1.5 μM α-amanitine, 1.5 μM actinomycin D (Calbiochem, La Jolla, CA), 5 μM chelerythrine (Sigma, Sintra, Portugal) or 10 μM KN-93 (Sigma) were added 30 min before stimulation, as indicated. Scavenging of endogenous extracellular BDNF was performed with TrkB-IgG (1 μg/ml; Sigma) for 24 h.

Hippocampal slices were prepared from young rats (postnatal days 5–6) and cultured on semiporous membranes as previously described (Gahwiler et al., 1997).

Preparation of extracts- Hippocampal neurons were washed twice with ice-cold PBS buffer and once more with PBS buffer supplemented with 1 mM DTT and a cocktail of protease inhibitors (0.1 mM PMSF, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin; Sigma-Aldrich Química, Sintra, Portugal). The cells were then lysed with RIPA (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5) supplemented with with 50 mM NaF, 1.5 mM sodium orthovanadate and the cocktail of protease inhibitors. After centrifugation at 16,100 g for 10 min, protein in the supernatants was quantified using the BCA method, and the samples were diluted with a 2x concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue).

Extracts from treated hippocampal slices were prepared in homogenisation buffer containing protease and phosphatase inhibitors (10 mM HEPES, 500 mM NaCl, 10 mM EDTA, 10 mM NaF, 1 μM Microcystin, 0.1 mM PMSF, 2 μg/ml chymostatin, 2 μg/ml leupeptin, 2 μg/ml antipain, 2 μg/ml pepstatin, 10 μM sodium orthovanadate, and 1% Triton X-100). Samples were centrifuged at 16,100 g for 4 min and the protein present in the supernatant was quantified using the BCA method, and denaturated with denaturating buffer at 95 ºC for 5 min. The proteins of interest were then analysed by Western blot.

Total RNA isolation and reverse transcription for Real-Time PCR- Total RNA from cultured hippocampal neurons was extracted with TRIzol reagent (Invitrogen, Barcelona, Spain), according to the instructions of the manufacturer. The full content of a 6 well cell cluster plate, with 870,000 cells/well (DIV 7), was collected for each experimental condition. For first strand cDNA synthesis, 3 μg of total RNA were reverse-transcribed with AMV Reverse Transcriptase (Roche, Carnaxide, Portugal) using Random Primer $p(dN)_6$ (3.2 μg), dNTPs (1 mM each), $MgCl_2$ (25 mM), RNase inhibitor (50 units) and Gelatine (0.01 μg/μl) in Reaction Buffer (10 mM Tris, 50 mM KCl, pH 8.3) in a total volume of 40 μl. The reaction was performed at 25 ºC for 10 min, followed by 60 min at 42 ºC, for primer annealing to the RNA template and cDNA synthesis, respectively. The Reverse Transcriptase was then denatured during 5 min at 99 ºC, and the sample was cooled to 4 ºC for 5 min and finally stored at -80 ºC until further use.

Real-Time PCR- Real-Time PCR analysis of gene expression was performed using the LightCycler System II (Roche, Portugal). The PCR reactions were performed using LightCycler FastStart DNA Master SYBR Green I (Roche et al.) in 20 μl capillaries. The primers used for amplification of genes encoding AMPA receptor subunits were,

respectively, RGR1F2271- 5'GAA CCA TCC GTG TTT GTT CG3´ and RGR1R2937- 5'TTC CTG TCT GCT CCA GTT AC3´ for GluR1; and RGR2F2522 - 5'GAA GCC TTG TGA CAC CAT GA3´ and RGR2R3008 - 5'AGC CTT GCC TTG CTC CTC AT3´ for GluR2. RGR3F2431 - 5'CAA AGG CTA TGG TGT GGC AA3´ and RGR3R2927 - 5'ACA CCA GGG AGA GTG AAA TC3´ for GluR3; S2288 - 5´TGG AGG GCG TGG CTC GTG TC3´and R2800 - 5'TTG GGG CAG TCA GGG GTA AG3´ for GluR4. The primers used for the amplification of endogenous control gene 18S rRNA were those included in the Applied Biosystems TaqMan Ribosomal RNA Control Reagents Kit (Porto, Portugal). Each primer of a pair was added to the reaction mixture (10 μl) at a final concentration of 0.8 μM with 3 mM $MgCl_2$, in addition to the "Hot Start" LightCycler Fast Start DNA Master SYBR Green I mix (1x) and 1.2 μl of cDNA sample. Thermal cycling was initiated with activation of the FastStart Taq DNA Polymerase by denaturation during 10 min at 95 ºC followed by 45 cycles of a 30 s melting step at 95 ºC, a 5 s annealing step at 58 ºC, and a 25 s elongation step at 72 ºC. All temperature transition rates at 20 ºC/s. After amplification for 45 cycles, at least 10 cycles beyond the beginning of the linear phase of amplification, samples were subjected to a melting curve analysis according to the instructions of the manufacturer in order to confirm the absence of unspecific amplification products and primer-dimers. In all experiments, samples containing no template were included as negative controls.

mRNA quantitative analysis- The mRNA levels of the constitutively expressed housekeeping gene encoding 18S ribosomal RNA were used as a control, in all experiments. The relative changes in the mRNA levels of glutamate receptor subunits in cultured hippocampal neurons were determined using the ΔΔCp method. Accordingly, for each experimental condition (unstimulated neurons and neurons treated with 100 ng/ml BDNF for 30 min or 3 h) the "Crossing point" (Cp) values given by the LightCycler system II software, for each target gene, were subtracted by the respective Cp value determined for the 18S gene from the same sample and condition (ΔCp). This allows normalizing changes in target gene expression. Afterwards, the ΔCp values were subtracted by the respective values of the control for the target gene giving ΔΔCp. The derivation to the formula $2^{-(\Delta\Delta Cp)}$ sets each control at the unity (or 100%), since ΔΔCp (control)=0, and the stimuli conditions at a percentage relative to the control.

Surface biotinylation and precipitation- Hippocampal cell cultures were treated or not with 100 ng/ml BDNF and then incubated with 1 mg/ml EZ-Link™ Sulfo-NHS-SS-biotin (Pierce, Madison, WI) in ice-cold PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, for 30 min (Gomes et al., 2004). The non-bound biotin was removed by washing the cells with PBS containing 100 mM glycine. Cell lysates were obtained as described above, and were incubated with UltraLink Plus™ immobilized streptavidin or UltraLink® immobilized NeutrAvidin™ plus beads (Pierce), for 2 h at 4 °C, under constant agitation. Non-biotinylated proteins were removed by centrifugation at 2,500 g for 3 min, and the beads were washed three times with RIPA buffer. Biotinylated proteins were then eluted with denaturating buffer at 95 °C for 5 min (Fig 6A, C and D), or at 65 °C for 15 min (Fig. 6B). Samples were then processed for Western blotting analysis.

Western blotting- Protein samples were separated by SDS-PAGE, in 6% polyacrylamide gels, transferred to polyvinylidene (PVDF) membranes (BioRad,

Amadora, Portugal), and immunoblotted. Blots were incubated with primary antibodies (overnight at 4 ºC), washed and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20000 dilution; 1 h at room temperature). Alkaline phosphatase activity was visualized by ECF on the Storm 860 Gel and Blot Imaging System (Amersham Biosciences, Buckinghamshire, UK), or by ECL (Fig. 8A). The following primary antibodies were used: anti-GluR1 (1:1500; Upstate, Waltham, MA), anti-GluR2 (1:600; Chemicon International, Temecula, CA), anti-GluR3 (1:200; Zymed, San Francisco, CA), anti-GluR4 (1:200; Chemicon International), anti-pGluR1 Ser831 (1:1000; Chemicon International or 1:1500; Tocris, UK), anti-pGluR1 Ser845 (1:1000; Chemicon International), anti-pGluR2 Ser880 (1:1000; Chemicon International), anti-pTrk (1:1000; Cell Signalling, Beverly, MA) and anti-TrkB (clone 47, 1:1000; BD Bioscience, San Jose, CA). Anti-α-Tubulin (1:1000; Zymed), anti-β-Tubulin I (1:150000; Sigma), anti-Actin (1:20000; Chemicon), and anti-transferrin receptor (1:3000; Zymed) antibodies were used in loading controls.

Electrophysiology- After 3–5 days in culture, the organotypic cultures of rat hippocampal slices, prepared as previously described (Gahwiler et al., 1997), were infected (36 h) with the Sindbis virus expressing recombinant GluR1 or GluR1 plus the constitutive active αCaMKII (as indicated), tagged to GFP. Voltage-clamp whole-cell recordings were obtained from infected and uninfected CA1 pyramidal neurons, under visual guidance using fluorescence and transmitted light illumination. The recording chamber was perfused with 119 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM glucose, 0.1 mM picrotoxin, 0.1 mM (D,L)-2-amino-5-phosphono valeric acid (APV), and 2 μM 2-chloroadenosine (pH 7.4), gassed with 5% CO_2/95% O_2. Patch recording pipettes (4–7 MΩ) were filled with the 115 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 10 mM sodium phosphocreatine, 0.6 mM EGTA, and 0.1 mM spermine (pH 7.25). Whole-cell recordings were carried out with a MultiClamp 700A amplifier (Axon Instruments, Union City, CA). Synaptic responses were evoked with two bipolar electrodes with single voltage pulses (duration of 0.3 ms, up to 20 V). The stimulating electrodes were placed over Schaffer collateral fibers between 200 and 300 μm from the recorded cells. Synaptic AMPA receptor-mediated responses were collected at -60 mV and +40 mV with glass electrodes placed in CA1 stratum radiatum and averaged over 50–60 trials; their ratio was used as an index of rectification. All electrophysiological data were collected with pCLAMP software (Axon Instruments). When the effect of K252a (200 nM) was tested, the drug was added to the perfusion medium 30 min prior to incubation with 100 ng/ml BDNF. Incubation with BDNF was performed from 30 min to 2.5 h. When the effect of NMDA receptor activity on AMPA delivery to the synapse was studied, NMDA receptor mediated responses were blocked pharmacologically using 0.1 mM DL-APV

Statistical Analysis- Statistical analysis was performed using one-way ANOVA analysis of variance followed by the Dunnett's test or Bonferroni test, or using the Student´s t test, as indicated in the figure captions.

RESULTS

Effect of BDNF on the total protein levels of AMPA receptors subunits- To determine whether acute stimulation with BDNF affects the abundance of glutamate receptor

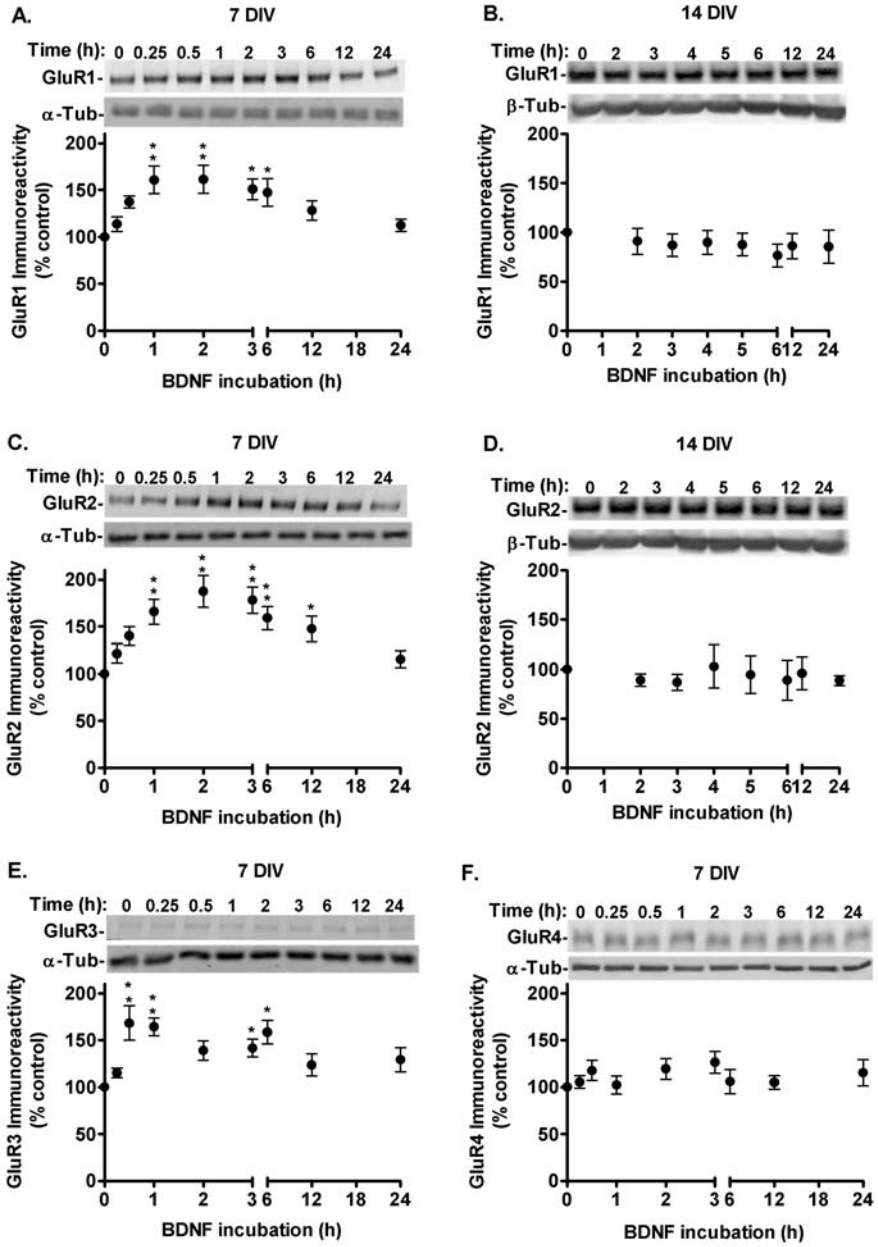

Figure 1- BDNF upregulates the protein levels of the GluR1, GluR2 and GluR3 AMPA receptor subunits in developing cultured hippocampal neurons. 7 DIV (A, C, E, F) or 14 DIV (B, D) cultured neurons were incubated with or without 100 ng/ml BDNF (15 min, 30 min, 1h, 2 h, 3 h, 5 h, 6 h, 12 h and 24 h) as indicated. Total GluR1 (A), GluR2 (C), GluR3 (E) and GluR4 (F) protein levels were determined by Western blot for 7 DIV, and GluR1 (B) and GluR2 (D) protein levels were also determined for 14 DIV. Control (0 h) protein levels of AMPA receptor subunits were set to 100%. α-tubulin or β-tubulin were used as loading controls. The results are the average ± SEM of 5-9 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Dunnett's test. *P<0.05, **P<0.01.

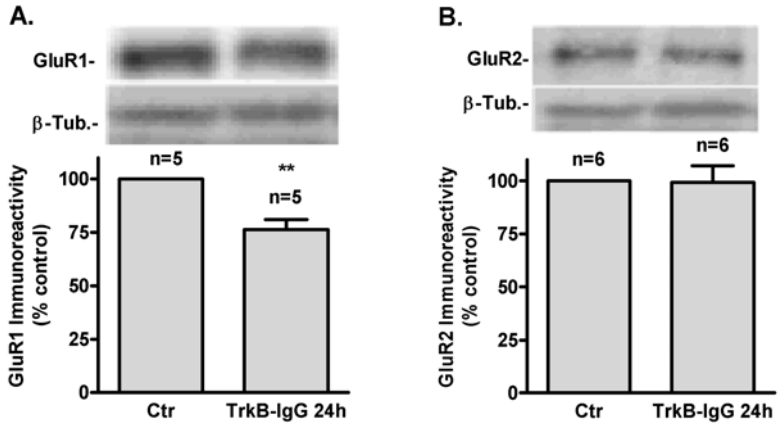

Figure 2- Chelation of endogenous released BDNF with the fusion protein TrkB-IgG decreases the protein levels of GluR1, but was without effect on GluR2 protein levels. Fourteen DIV neurons were incubated with or without 1 μg/ml TrkB-IgG for 24 h. Total GluR1 and GluR2 protein levels were measured by Western blot. Control protein levels of AMPA receptor subunits were set to 100%. β-tubulin was used as loading control. The results are the average ± SEM of 5-6 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Dunnett's test. ** $P<0.01$.

subunits, 7 DIV cultured hippocampal neurons were incubated with or without 100 ng/ml BDNF, for various periods of time (15 min - 24 h). The AMPA receptor subunit (GluR1, GluR2, GluR3 and GluR4) protein levels were determined by Western blotting (Fig.1). BDNF rapidly upregulated GluR1-GluR3 protein levels, in a transient manner, with small effects observed already after 15 min of stimulation with the neurotrophin (Fig. 1A, C and E). In contrast, BDNF did not change GluR4 protein levels (Fig. 1F). The increases in GluR1, GluR2 and GluR3 had distinct kinetics, and the maximal effects were observed after incubation with BDNF for 1 h, 2 h and 30 min, respectively. For longer periods of BDNF stimulation (12 h - 24 h), the abundance of GluR1-GluR3 subunits decreased to values similar to the control. Since there was little GluR3 immunoreactivity with the antibody used, the effect of BDNF on this subunit was not further examined.

In contrast with the results obtained using 7 DIV hippocampal neurons, BDNF did not affect GluR1 and GluR2 protein levels in neurons cultured for 14 DIV, for incubation periods with the neurotrophin of 2 – 24 h (Fig. 1B and D). However, when the endogenous extracellular BDNF was chelated with the fusion protein TrkB-IgG for 24 h, the levels of GluR1 significantly decreased (Fig. 2A), while no changes were observed for the GluR2 subunit (Fig. 2B). These results indicate that endogenous BDNF regulates the GluR1 protein levels in 14 DIV cultures of hippocampal neurons.

Activation of Trk neurotrophin receptors was required for upregulation of GluR1 and GluR2 AMPA receptor subunits by BDNF in 7 DIV cultures, since no effect of the neurotrophin was observed when the experiments were conducted in the presence of K252a (200 nM), an inhibitor of this family of receptors (Fig. 3).

In order to test whether the effect of BDNF was due to an increase in protein synthesis, we used two translation inhibitors, emetine and anisomycin. Hippocampal neurons were stimulated with BDNF for 3 h, in the presence or in the absence of the translation inhibitors. Emetine (2 μM) and

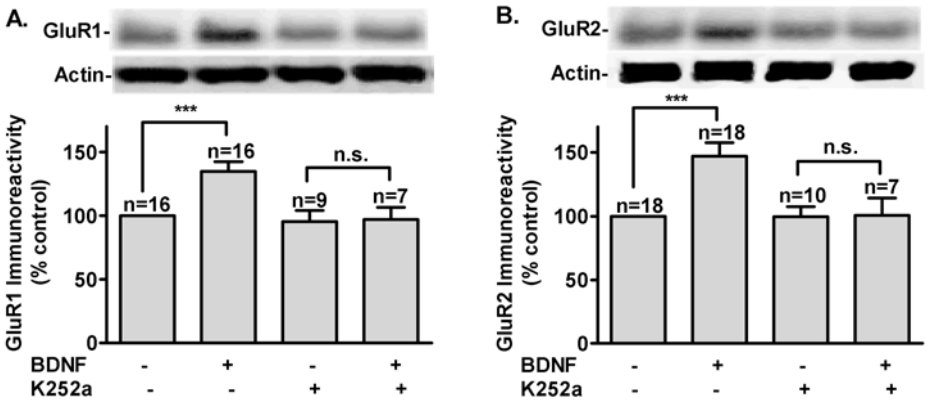

Figure 3- Inhibition of Trk activity blocks the BDNF-induced upregulation of GluR1 and GluR2 protein levels. Neurons (7 DIV) were incubated with or without 100 ng/ml BDNF for 2 h, in the presence or in the absence of K252a (200 nM). When the inhibitor was present it was pre-incubated 30 min before stimulation with BDNF. Total GluR1 and GluR2 protein levels were measured by Western blot. Control (unstimulated) protein levels of AMPA receptor subunits were set to 100%. Actin was used as loading control. The results are the average ± SEM of 7-18 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Bonferroni test. ***P<0.001.

anisomycin (2 μM) abrogated the effect of BDNF on GluR1 and GluR2 subunits (Fig. 4). None of the protein synthesis inhibitors reduced the GluR1 or GluR2 protein levels under control conditions, in agreement with the long half-life of AMPA receptor subunits (Archibald et al., 1998; O'Brien et al., 1998; Huh and Wenthold, 1999). Taken together, our findings indicate that the effect of BDNF on AMPA receptor subunits is mediated by activation of TrkB receptor, and is due to an upregulation of protein synthesis instead of a reduction in protein degradation.

BDNF upregulates AMPA receptor subunits by promoting transcription activity- BDNF may stimulate either transcription of genes (Messaoudi et al., 2002), or stimulate protein synthesis by activating translation cascades (Takei et al., 2001). Therefore, in order to test for the role of transcription in the upregulation of glutamate receptor subunits by BDNF we used two different transcription inhibitors, α-amanitine (1.5 μM) and actinomycin D (1.5 μM). Both transcription inhibitors blocked the effect of BDNF on GluR1 and GluR2 protein levels, but were without effect on the abundance of the receptor subunits in the absence of the neurotrophin (Fig. 5). In agreement with these observations, real-time PCR using the SYBR green assay showed that BDNF stimulation for 30 min increased significantly the mRNA levels for GluR1, and when the cells were incubated with the neurotrophin for 3 h a significant increase in the mRNA for GluR1 and GluR2 was also observed (Fig. 6). Taken together, the results indicate that BDNF regulates GluR1 and GluR2 AMPA receptor subunits at the transcription level. In contrast with the results obtained for GluR1 and GluR2, no effect of BDNF was observed on the mRNA levels for GluR3 and GluR4.

Effect of BDNF on GluR1 and GluR2 protein levels at the plasma membrane- Because a significant percentage of glutamate receptors expressed in neurons is intracellular (Hall and Soderling, 1997), and therefore do not participate in the response to the

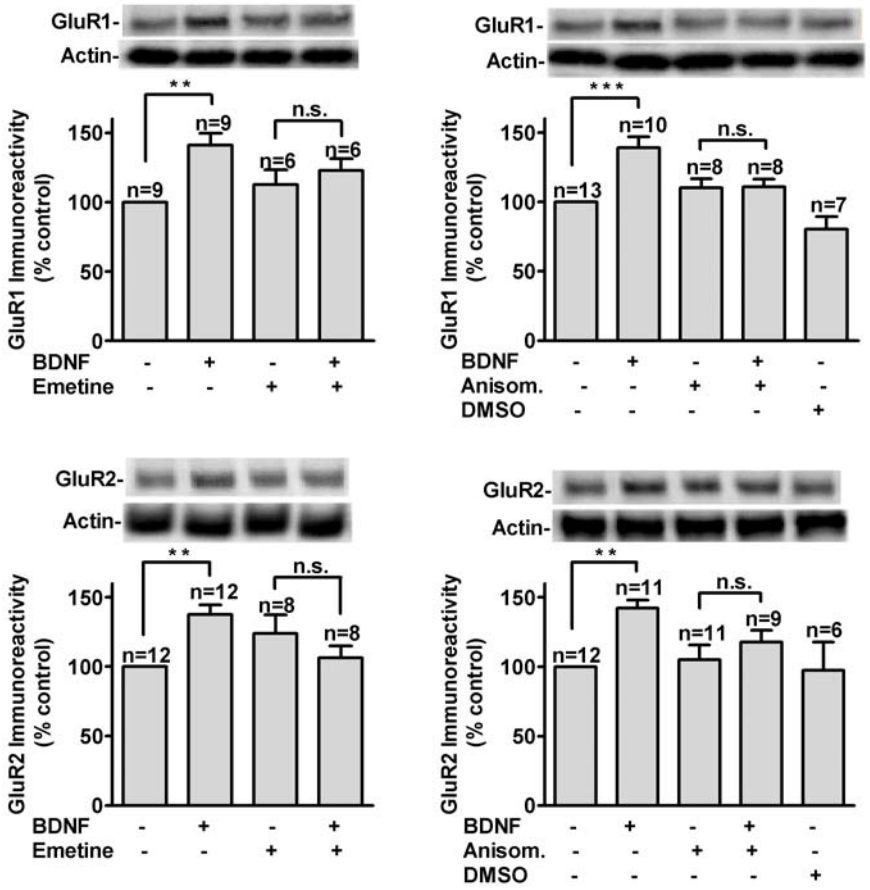

Figure 4- Translation inhibitors impair the BDNF-induced upregulation of GluR1 and GluR2 protein levels. Neurons (7 DIV) were incubated with or without 100 ng/ml BDNF for 3 h, in the presence or in the absence of emetine (2.0 μM) or anisomycin (2.0 μM). When the inhibitors were used the cells were pre-incubated with the compounds for 30 min before stimulation with BDNF. Total GluR1 and GluR2 protein levels were measured by Western blot. Control (unstimulated) protein levels of AMPA receptor subunits were set to 100%. Actin was used as loading control. The results are the average ± SEM of 6-12 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Bonferroni test. $^{**}P<0.01$, $^{***}P<0.001$.

neurotransmitter glutamate, we tested whether BDNF can affect the translocation of AMPA receptor subunits to the plasma membrane. To study the effect of BDNF on the cell surface distribution of the AMPA receptor subunits GluR1 and GluR2, 7 DIV cultured hippocampal neurons were treated with or without 100 ng/ml BDNF (30 min, 3 h and 24 h). Following treatment, proteins on the cell surface were biotinylated, collected with streptavidin or neutravidin-coupled beads, and subjected to Western blotting (Fig. 7). GluR1 protein associated with the plasma membrane was markedly increased by BDNF treatment during 30 min when compared with non-treated cells. However, incubation with BDNF for 3 h was without effect (Fig. 7A). The increase observed by BDNF treatment during 30 min was blocked by emetine (Fig. 7B), indicating that the

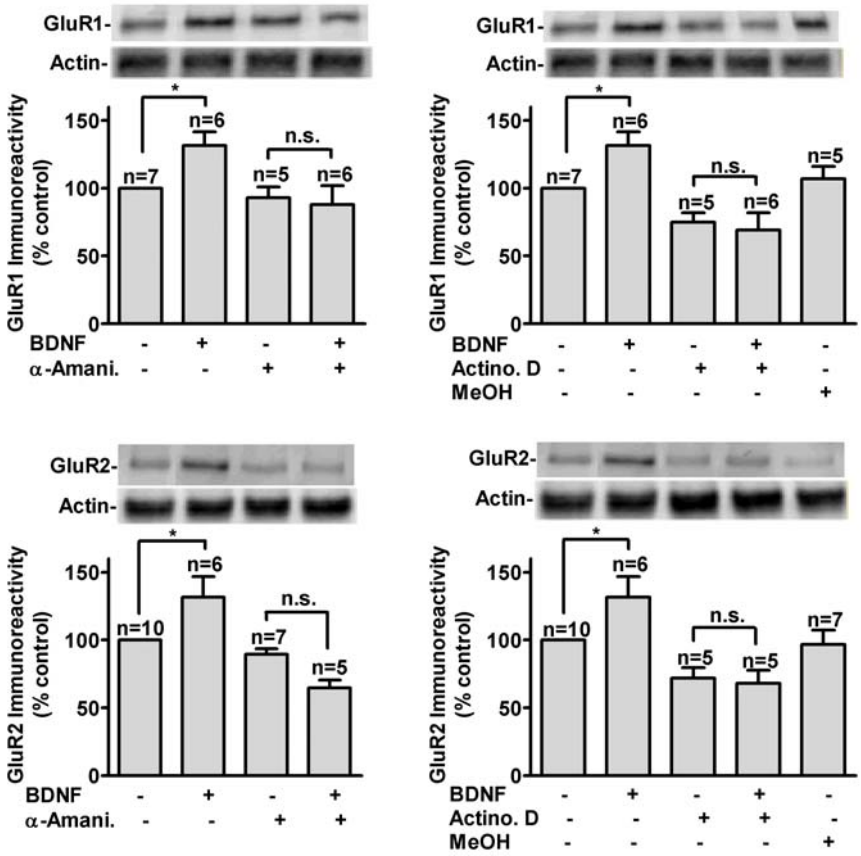

Figure 5- Transcription inhibitors prevent the BDNF-induced upregulation of the GluR1 and GluR2 protein levels. Neurons (7 DIV) were incubated with or without 100 ng/ml BDNF for 3 h, in the presence or in the absence of α-amanitine (1.5 μM) or actinomycin D (1.5 μM). When the inhibitors were used the cells were pre-incubated with the compounds for 30 min before stimulation with BDNF. Total GluR1 and GluR2 protein levels were measured by Western blot. Control (unstimulated) protein levels of AMPA receptor subunits were set to 100%. Actin was used as loading control. The results are the average ± SEM of 5-10 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Bonferroni test. *P<0.05

BDNF-induced upregulation in the amount of plasma membrane-associated GluR1 requires *de novo* protein synthesis. In contrast, BDNF did not affect significantly the GluR2 protein levels at the plasma membrane (Fig. 7D). These results indicate that BDNF has a differential effect on the traffic of AMPA receptor subunits in developing cultured hippocampal neurons. Also, the distinct time-courses for the increase in surface and total GluR1 expression suggest that the effects of BDNF on total GluR1 content and receptor trafficking may be mediated by separate signalling pathways. In contrast with the results obtained in developing neurons, BDNF did not affect the surface expression of GluR1 in 14 DIV cultured hippocampal neurons (Fig. 7C).

The delivery of GluR1 subunits to

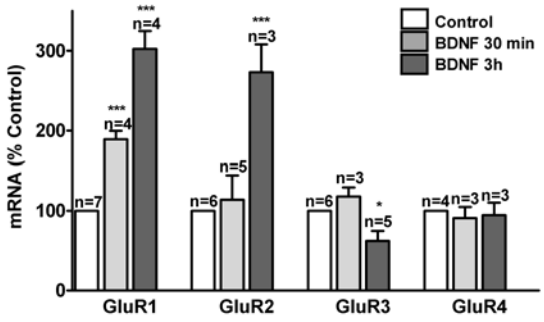

Figure 6- BDNF increases the mRNA levels of the GluR1 and GluR2 AMPA receptor subunits. The variation of GluR1 and GluR2 mRNA levels was assayed by SYBR Green Real-Time PCR of total RNA samples, converted to cDNA in reactions normalized to contain equal amounts of mRNA. The cells (7 DIV) were incubated in the presence or in the absence of 100 ng/ml BDNF, during 30 min (gray columns) or 3 h (black columns). The results are presented as mean percentage ± SEM compared to the control (unstimulated), and normalized to the reference gene 18S, and are the average ± SEM of 3-7 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Dunnett's test. *P<0.05, ***P<0.001.

the synapse is regulated by phosphorylation in the C-terminal region. GluR1 is phosphorylated on Ser831 during LTP, although this phosphorylation is not sufficient to induce synaptic delivery of AMPA receptors (Roche et al., 1996; Hayashi et al., 2000; Lee et al., 2000; Gomes et al., 2003). Stimulation of cultured hippocampal neurons (7 DIV) with 100 ng/ml BDNF increased the phosphorylation of GluR1 on Ser831, as determined by Western blot using a phosphospecific antibody (Fig. 7E). However, since the total amount of GluR1 present in the cells was also increased upon stimulation with the neurotrophin, the percentage of total protein that was phosphorylated was not significantly changed (Fig. 7F; see also Fig. 1A). To better understand which signalling pathways may be involved in GluR1 phosphorylation following Trk receptor activation, and since Ser831 is a phosphorylation site for protein kinase C (PKC) and Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) (Roche et al., 1996; Gomes et al., 2003), the effect of the kinase inhibitors chelerythrine (5 μM) and KN-93 (10 μM) was tested. Inhibition of PKC and CaMKII with chelerythrine and KN-93, respectively, inhibited GluR1 phosphorylation on Ser831, suggesting that both pathways contribute to the effect of BDNF (Fig. 7E).

Effect of BDNF on synaptic delivery of AMPA receptors- We next examined the role of BDNF in the trafficking of AMPA receptors into the synapse, in CA1 neurons of rat hippocampal organotypic slices expressing GluR1-GFP. Overexpression of GluR1-GFP, with a Sindbis virus expression system, leads to the formation of homomeric AMPA receptors containing the GluR1 subunit (Hayashi et al., 2000). These GluR2-lacking receptors are inwardly rectifying (Boulter et al., 1990; Hollmann et al., 1991; Verdoorn et al., 1991), and therefore their recruitment to the synapse increases the inward currents at –60 mV, with no effect on the outward currents. Hence, synaptic delivery of GluR1-containing AMPA receptors to the synapse can be monitored as an increase in the ratio between the currents at –60 mV versus the currents at +40 mV (rectification index) (Hayashi et al., 2000). As expected, overexpression of GluR1 in CA1 hippocampal neurons did not increase the rectification index of synaptic responses (Fig. 8A and B; compare first and second column in Fig. 8B), confirming that GluR1 is not spontaneously delivered into synapses (Hayashi et al., 2000). In marked contrast, the rectification index of homomeric GluR1-expressing neurons was significantly increased upon incubation with BDNF (100 ng/ml) for 30 min to 3 h (Fig. 8A and 7B;

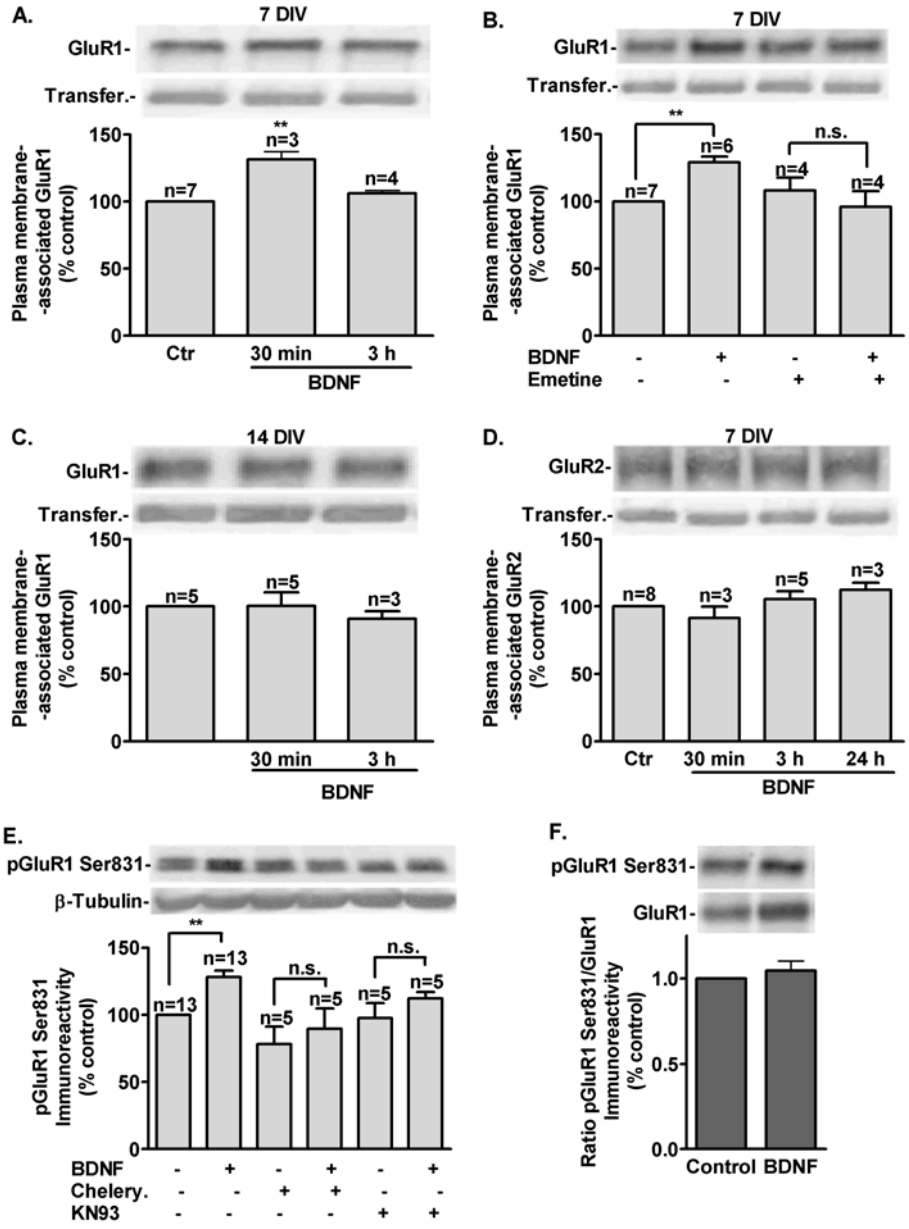

Figure 7- BDNF increases the GluR1 subunit in the plasma membrane, but not the GluR2 subunit, in developing cultured hippocampal neurons. 7 DIV (A, B, D) or 14 DIV (C) cultured neurons were treated with or without 100 ng/ml BDNF (30 min, 3 h and 24 h) as indicated. In panel B, 7 DIV neurons were pre-incubated or not with emetine (2.0 μM), for 30 min, before stimulation with BDNF, for 30 min, in the presence or in the absence of the inhibitor. Following treatment, cell surface proteins were labelled by biotinylation, followed by precipitation with streptavidin beads. The abundance of each subunit in the plasma membrane was then determined by Western blot. Control (unstimulated) levels of AMPA subunits protein was set to 100%. The transferrin receptor was used as loading control. The effect of BDNF on GluR1 phosphorylation on Ser831 (pGuR1 Ser831) is shown in panel E. Seven DIV

compare second and forth column in Fig. 8B). BDNF had no significant effect on the rectification index of non-infected cells (Fig. 8B; compare first and third column). The effect of BDNF was similar to that observed in cells where GluR1 was coexpressed with a constitutively active form of αCaMKII (tCaMKII; Fig. 8A and 7B; compare forth and seventh columns in Fig. 8B), which is known to induce synaptic delivery of GluR1-containing AMPA receptors (Hayashi et al., 2000; Poncer et al., 2002). Inhibition of the Trk receptors with K252a (200 nM) completely prevented synaptic delivery of AMPA receptors induced by BDNF (Fig. 8B; compare forth and fifth columns). Taken together, the results indicate that BDNF induces a very efficient delivery of GluR1 homomeric receptors into synapse and that this effect is mediated by binding of BDNF to Trk receptors.

Tetanic stimulation was shown to induce a rapid delivery of GFP-GluR1-containing AMPA receptors into dendritic spines and cause receptor clustering in dendrites by a mechanism requiring activation of NMDA receptors (Shi et al., 1999). In order to determine whether synaptic delivery of GFP-GluR1-containing AMPA receptors induced by BDNF requires activation of NMDA receptors, experiments were performed in the presence of the NMDA receptor antagonist APV. Pre-incubation of the slices with APV (100 μM) for 30 min did not affect the increase in the rectification index induced by BDNF (Fig. 8B; compare forth and sixth column).

Stimulation of hippocampal slices with BDNF (100 ng/ml) for 30 min increased GluR1 phosphorylation on Ser831 (Fig. 9A e B), in agreement with the results obtained in monolayer cultures of hippocampal neurons (Fig. 7E). In contrast, no change in GluR1 phosphorylation was detected on Ser845 (Fig. 9A). The activation of Trk receptors by BDNF was confirmed by Western blot, using an antibody that recognizes phosphotyrosine 490 in TrkA, a residue conserved in the other Trk receptors. Stimulation of hippocampal slices with BDNF for 30 min significantly increased Trk (presumably TrkB) phosphorylation. Control experiments showed that the total GluR1 and Trk protein levels did not change significantly in the slices under the experimental conditions used (Fig. 9A). The effect of BDNF on GluR1 phosphorylation was specific, since no phosphorylation of GluR2 on the PKC phosphorylation site [Ser 880; (Gomes et al., 2003)] was observed (Fig. 9A).

DISCUSSION

It is well known that BDNF plays an important role in synaptic plasticity (Poo, 2001; Lu, 2003; Alder et al., 2005), particularly in LTP induced by high-frequency stimulation (Chen et al., 1999; Schuman, 1999; Ernfors and Bramham, 2003), which late phase requires transcription activation and protein synthesis (Nguyen and Kandel, 1996). Accordingly, LTP is impaired in the hippocampal CA3-CA1 region of *bdnf* null mutant and forebrain-specific *trkB* knockout mice

Figure 7 (cont)- cultured hippocampal neurons were incubated for 30 min with or without BDNF, in the presence or absence of chelerythrine (5 μM) or KN-93 (10 μM). The phosphorylation of GluR1 on Ser831 was measured by Western blot, using a phosphospecific antibody. Control (unstimulated) phosphorylation of GluR1 was set to 100%. β-tubulin was used as loading control. The total amount of GluR1 was determined after striping the membranes, and the ratio between pGluR1 Ser831 and the total amount of GluR1 is plotted in panel F. The results are the average ± SEM of 3-13 independent experiments, as indicated, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Dunnett's (A, C and D) or Bonferroni test (B, E). **P<0.01.

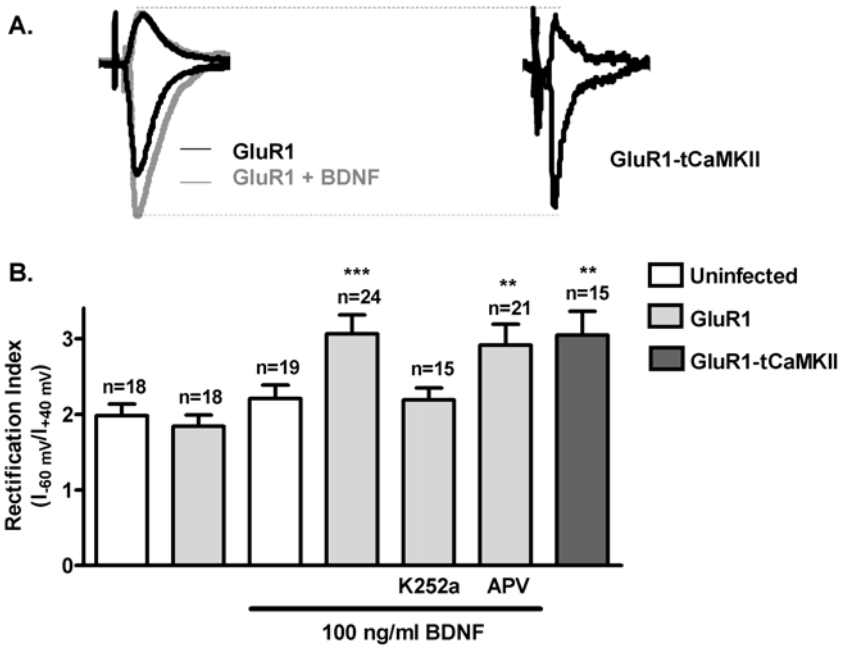

Figure 8- BDNF increases the delivery of the AMPA receptors to the synapse. AMPA receptor-mediated responses were recorded at -60 mV and +40 mV from CA1 neurons and the rectification index was calculated as the ratio between the responses at these holding potentials ($I_{-60\ mV}/I_{+40\ mV}$). A) AMPA receptor-mediated responses at -60 mV and +40 mV in neurons infected with Sindbis virus expressing recombinant GluR1 or GluR1 plus the constitutive active αCaMKII tagged to GFP. GluR1 infected cells were incubated (grey line) or not (black line) with 100 ng/ml BDNF. B) Rectification values were obtained from uninfected neurons, and from neurons infected with recombinant GluR1 or GluR1 plus the constitutive active αCaMKII (as indicated), tagged to GFP. Where indicated the cells were stimulated with 100 ng/ml BDNF for 30 min - 3 h. Since no difference was found between the results obtained for the various preincubation periods tested, the results were pooled together. The effect of 200 nM K252a and 0.1 mM DL-APV on the BDNF-induced increase on the rectification index was also tested by perfusing the hippocampal slices with these compounds for 30 min prior to BDNF incubation and during the recordings. The results are the average ± SEM of the indicated number of experiments. Statistical analysis was performed using the Students *t* test. **P<0.01, ***P<0.001.

(Korte et al., 1995; Patterson et al., 1996; Minichiello et al., 1999; Xu et al., 2000). In the later case, a compromised learning ability was also shown (Minichiello et al., 1999). However, the mechanisms whereby BDNF contributes to LTP are not fully understood. In this study, we show that Trk receptor activation (presumably TrkB) by BDNF rapidly upregulates GluR1 and GluR2 protein levels in cultured hippocampal neurons, by increasing transcription activity. Although BDNF also upregulated GluR3 protein levels, no effect on the mRNA for this receptor subunit was observed. The neurotrophin also induced phosphorylation of GluR1 on Ser831, most likely by activating PKC and CaMKII, and promoted synaptic delivery of GluR1-containing AMPA receptors in the CA1 region of the hippocampus.

Short incubation periods with BDNF (30 min) upregulated GluR1 and GluR2

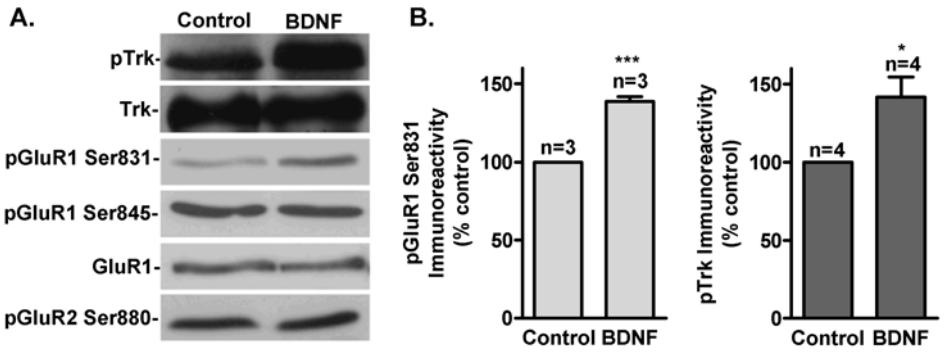

Figure 9- BDNF induces the phosphorylation of GluR1 on Ser 831. A) Hippocampal slices were stimulated with 100 ng/ml, for 30 min, and Trk, GluR1 and GluR2 phosphorylation was analysed by western blot, using phosphospecific antibodies. BDNF induces phosphorylation of Trk receptor, and GluR1 phosphorylation at Ser831 but not at Ser845. GluR2 phosphorylation at Ser 880 is not affected by BDNF (panels A and B). The phosphorylation of GluR1 on Ser831 and the phosphorylation of Trk were normalized to GluR1 and Trk receptor protein levels, respectively. The results are the average ± SEM of 3-4 independent experiments. Statistical analysis was performed using the Students *t* test. *P<0.05, ***P<0.001.

protein levels to about the same extent in cultured hippocampal neurons (Fig. 1A and C), but selectively increased the amount of GluR1 subunits associated with the plasma membrane (Fig. 7). These findings indicate that the delivery of GluR1 and GluR2 subunits to the membrane is differentially regulated, as shown for synaptic delivery of the AMPA receptor subunits in the adult hippocampus (Passafaro et al., 2001; Shi et al., 2001). Furthermore, we found that the BDNF acutely increases GluR1 subunit associated with the plasma membrane by enhancing the translation activity (Fig. 7B). This may be due to the delivery of newly synthesized receptors to the plasma membrane and/or to an increased stability of the plasma membrane-associated receptors by interaction with protein(s) synthesized following Trk receptor stimulation. The acute effects of BDNF on the plasma membrane GluR1 protein levels may require continuous signalling activity by Trk receptors, since after 3 h stimulation with BDNF, when Trk receptors are desensitized to some extent (Almeida et al., 2005), the receptor subunits found in the membrane were similar to the control. In contrast with the results obtained in 7 DIV cultures, when synaptogenesis is particularly active (Fletcher et al., 1991), addition of BDNF had no effect on GluR1 and GluR2 protein levels in hippocampal neurons cultured for 14 DIV (Fig. 1B, D). In these cultures, chelation of endogenous extracellular BDNF with the fusion protein chimera TrkB-IgG selectively decreased the GluR1 levels, indicating that BDNF has a tonic effect on GluR1 protein levels (Fig. 2). These results suggest that the lack of effect of BDNF on total GluR1 protein levels in 14 DIV hippocampal cultures (Fig. 1B), and on the total amount of surface receptors (Fig. 7C), may be due the activity of endogenous BDNF, which controls to some extent the abundance of GluR1, thereby precluding an effect of exogenous addition of the neurotrophin. This may also explain the results showing no effect of BDNF on total GluR1 protein levels in the organotypic slices (Fig. 9A).

Electrophysiology studies have also shown that BDNF induces synaptic delivery of GluR1-containing AMPA receptors in CA1 neurons of hippocampal organotypic slices

expressing GluR1-GFP, by a mechanism independent of NMDA receptor activity (Fig. 8B). This contrasts with the role of NMDA receptors in synaptic delivery of GluR1-containing AMPA receptors following tetanic stimulation (Shi et al., 1999). The synaptic delivery of GluR1 induced by BDNF was sensitive to the Trk inhibitor K252a, and was associated with the phosphorylation of the protein in Ser831, the CaMKII and PKC phosphorylation site (Gomes et al., 2003). Interestingly, GluR1 phosphorylation in Ser831 was also observed upon induction of LTP in naïve synapses (Lee et al., 2000). In contrast, BDNF did not affect GluR1 phosphorylation in Ser845, which is preferentially phosphorylated upon high-frequency stimulation of previously depressed synapses (Lee et al., 2000). Since GluR1 phosphorylation in Ser831 is not sufficient to induce synaptic delivery of AMPA receptors (Hayashi et al., 2000), the effect of BDNF may also involve GluR1 phosphorylation on Ser818. Phosphorylation of this site is significantly increased during hippocampal LTP, playing an important role in synaptic incorporation of GluR1 (Boehm et al., 2006). Alternatively, BDNF may induce the phosphorylation of a regulatory protein, leading to the release of a retention interaction and allowing the incorporation of GluR1 subunits into the synapse. Phosphorylation of GluR1 on Ser831 may also allow the interaction with a protein that anchors the receptor at the plasma membrane.

The effect of BDNF on synaptic delivery of GluR1 was similar to that observed in cells where a constitutively active form of αCaMKII was coexpressed, suggesting that analogous signalling mechanisms may be involved. In fact, treatment of cultured hippocampal neurons also increased the phosphorylation of GluR1 on Ser831 and this effect was abolished by inhibition of PKC and CaMKII, suggesting that both pathways are involved in the BDNF-induced GluR1 phosphorylation and possibly on the delivery of AMPA receptors to the synapse. In agreement with the role of PKC and CaMKII in BDNF-induced GluR1 phosphorylation, activation TrkB receptors stimulates the PLCγ pathway, giving rise to diacylglycerol, which activates PKC, and inositol-1,4,5-trisphosphate, which mobilizes Ca^{2+} from intracellular stores (Kaplan and Miller, 2000; Huang and Reichardt, 2003). Interestingly, this signalling pathway is involved in the synaptic changes resulting from local application of BDNF to the *Xenopus laevis* optic tectum, which cause rapid modifications of synaptic inputs at the dendrites of retinal ganglion cells by upregulating AMPA receptors at the retinal ganglion cells (Du and Poo, 2004). Furthermore, recruitment of PLCγ by active TrkB receptors was shown to play a role in hippocampal LTP (Minichiello et al., 2002).

Although activation of NMDA receptors induces a rapid delivery of GluR1 containing AMPA receptors to the synapse, and their clustering in dendrites (Shi et al., 1999), this does not account for the effect of BDNF since we found no effect of the NMDA receptor antagonist APV on BDNF-induced delivery of GluR1 subunits to the synapse. Interestingly, inhibition of NMDA receptors was also shown not to affect BDNF-induced LTP at medial perforant path→granule cell synapses in the rat dentate gyrus, in contrast with the high frequency stimulation-induced LTP, which depended on the activation of NMDA receptors (Messaoudi et al., 2002).

In addition to the rapid effects on delivery of GluR1 subunits to the synapse in hippocampal CA1 neurons, and to the plasma membrane in developing cultured hippocampal neurons, BDNF also rapidly upregulated GluR1, GluR2 and GluR3 protein levels in the latter preparation. This effect was transient, most likely due to the desensitisation of the Trk receptors, followed

by a decrease in intracellular signalling activity (Sommerfeld et al., 2000; Almeida et al., 2005). The upregulation in AMPA receptor subunits induced by BDNF was due to an increase in transcription activity, as demonstrated by the effect of transcription inhibitors, followed by synthesis of the receptor subunits. The role of transcription activity on the effect of BDNF on the protein levels of the GluR1 and GluR2 subunits is not surprising, since BDNF is known to regulate transcription of several genes (Ernfors and Bramham, 2003; Groth and Mermelstein, 2003) and to modulate transcription during the late phase LTP (Messaoudi et al., 2002). In agreement with the present findings, chronic treatment of cultured cerebrocortical neurons with BDNF also increased GluR1 and GluR2/3 protein levels, but no effect on the mRNA for the receptor subunits was found under these conditions (Narisawa-Saito et al., 1999a; Nagano et al., 2003). However, in this case, the sustained stimulation of neurotrophin receptors from early in development may have caused changes in cell phenotype.

The signalling mechanisms involved in the rapid increase in the transcription of GluR1 and GluR2 in hippocampal neurons exposed to BDNF remain to be identified. Previous studies have shown GluR1 protein levels may be regulated by the serum- and glucocorticoid-inducible kinase 3 (SGK3), one of the downstream targets of phosphatidylinositol-3-kinase (Strutz-Seebohm et al., 2005), and by the tyrosine kinase Fyn, which also regulates GluR2/3 (Narisawa-Saito et al., 1999b). Studies in a heterologous system showed that the expression of GluR2 can be regulated by BDNF, through a mechanism involving a neuron-restrictive silencer element (NRSE) present within the GluR2 promoter (Brene et al., 2000). These results are in agreement with our results, and contrast with the effects observed in cerebrocortical neurons chronically exposed to BDNF, where no change in the mRNA for GluR2 was observed (Narisawa-Saito et al., 1999a).

In addition to the effect of BDNF on the mRNA levels for GluR1 and GluR2, which accounted for the upregulation in the AMPA receptor subunits in cultured hippocampal neurons, a recent study showed that BDNF also increased GluR1 protein levels in synaptoneurosomes (Schratt et al., 2004). This effect, mediated by a mammalian target of rapamycin (mTOR) – phosphatidylinositol 3-kinase-dependent pathway, may contribute to increase locally the amount GluR1 after high-frequency stimulation. Delivery of these receptors to the synapse may contribute to LTP. Chronic stimulation of neocortical neuronal cultures with BDNF also increased the abundance of the AMPA receptor interacting proteins SAP97, GRIP1, and PICK1 (Jourdi et al., 2003). Under the same conditions there was an increase in the interaction between GluR1 and SAP97, as well as between GluR2 and GRIP1, which was suggested to play a role in the upregulation of AMPA receptors by BDNF (Jourdi et al., 2003). It remains to be determined whether BDNF induces a local synthesis and increase in the protein levels of these AMPA receptor interacting proteins, which may contribute to stabilize the receptors at the synapse.

In conclusion, our results strongly suggest that BDNF plays a direct role in the early phase of synaptic plasticity by triggering the delivery of GluR1 subunits to the synapse. Furthermore, BDNF rapidly upregulated AMPA receptor subunits in developing hippocampal neurons, and induced an overall increase in the number of receptors associated with the membrane, at a period of active synaptogenesis.

FOOTNOTES

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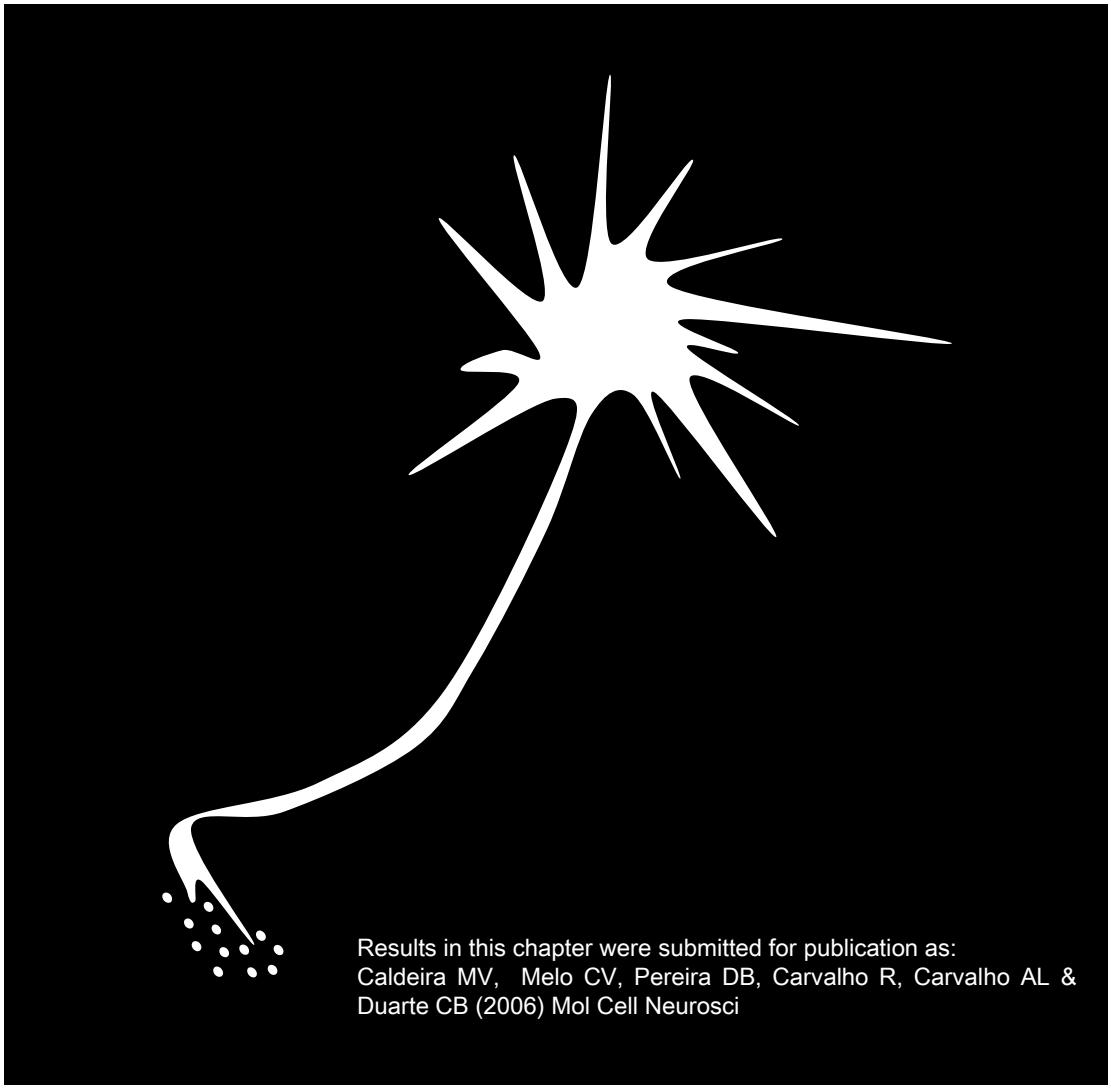
Chapter 3. BDNF regulates the expression and traffic of NMDA receptors in cultured hippocampal neurons



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BDNF REGULATES THE EXPRESSION AND TRAFFIC OF NMDA RECEPTORS IN CULTURED HIPPOCAMPAL NEURONS

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The neurotrophin BDNF regulates the maturation of the postsynaptic density of hippocampal synapses and is a key modulator of activity-dependent modifications of synaptic strength in the CNS. Physiological and biochemical evidence implicates the NMDA glutamate receptor as one of the targets for BDNF modulation. In the present study, we investigated the effect of BDNF on the expression and plasma membrane abundance of NMDA receptor subunits. Acute stimulation of hippocampal neurons with BDNF differentially upregulated the protein levels of the NR1, NR2A and NR2B NMDA receptor subunits, by a mechanism sensitive to transcription and translation inhibitors. Accordingly, BDNF also increased the mRNA levels for NR1, NR2A and NR2B subunits. The neurotrophin NT3 also upregulated the protein levels of NR2A and NR2B subunits, but was without effect on NR1 subunit. The amount of NR1, NR2A and NR2B proteins associated with the plasma membrane of hippocampal neurons was differently increased by BDNF stimulation for 30 min or 24 h. The rapid upregulation of plasma membrane-associated NMDA receptor subunits was correlated with an increase in NMDA receptor activity. The results indicate that BDNF increases the abundance of NMDA receptors and their delivery to the plasma membrane, thereby upregulating receptor activity in cultured hippocampal neurons.

INTRODUCTION

The neurotrophin brain-derived neurotrophic factor (BDNF) promotes neuronal survival and differentiation, and regulates synaptic transmission and plasticity [reviewed in (Bramham and Messaoudi, 2005; Kalb, 2005; Lu et al., 2005)]. BDNF rapidly potentiates excitatory synaptic transmission in cultured cerebrocortical and hippocampal neurons, in part by enhancing presynaptic neurotransmitter release (Lessmann and Heumann, 1998; Takei et al., 1998). Post-synaptically, BDNF enhances glutamatergic synaptic transmission by regulating the phosphorylation of NMDA (N-methyl-D-aspartate) receptors (Suen et al., 1997; Lin et al., 1998; Alder et al., 2005), thereby enhancing NMDA receptor activity (Levine et al., 1998; Song et al., 1998), increasing synaptic clustering of NMDA receptors in cultured hippocampal neurons (Elmariah et al., 2004), and upregulating AMPA receptor protein levels (Narisawa-Saito et al., 1999a). Accordingly, BDNF has been implicated in activity-dependent synaptic plasticity,

including the early- and late phases of long-term potentiation (LTP) induced by high-frequency stimulation [reviewed in (Bramham and Messaoudi, 2005)]. Activity-dependent changes in synaptic strength are thought to underlie learning and memory formation (Lynch, 2004).

NMDA receptors are glutamate, glycine and voltage-dependent ion channels characterized by their high calcium permeability. In the hippocampus, NMDA receptors are heteromeric complexes composed of at least one NR1 subunit and one or more of the two subunits, NR2A and NR2B (Sheng et al., 1994). Both NR1 and NR2 subunits are required to form a functional ionotropic receptor (Meguro et al., 1992; Monyer et al., 1994; Dingledine et al., 1999), but different NR2 subunits confer distinct kinetic properties to the NMDA receptors (Monyer et al., 1994). Some NMDA receptors may also include an NR3 subunit (either NR3A or NR3B) as part of the tetrameric structure (Chatterton et al., 2002). Before synapse formation in developing hippocampal neurons, NMDA receptors consist predominantly of NR1 and NR2B subunits (Tovar and Westbrook, 1999). NR2B seems to remain in NMDA receptors that are primarily extrasynaptic after synapse formation. In contrast, NR2A subunit progressively increases its expression (Monyer et al., 1994; Sheng et al., 1994; Margottil and Domenici, 2003) and is incorporated at synaptic sites (Stocca and Vicini, 1998; Tovar and Westbrook, 1999). The mechanisms controlling the switch in the composition of synaptic NMDA receptors from NR2B- to NR2A-containing receptors are not well understood, but studies in cortical neurons showed that the expression of NMDA receptor subunits is regulated by neuronal activity (Muzet and Dupont, 1996).
BDNF enhances the phosphorylation of NR1 and NR2B subunits in hippocampal and cortical neurons (Lin et al., 1998), and increases the open probability of NMDA receptor channels (Levine et al., 1998; Levine and Kolb, 2000). Phosphorylation of NR2B by Fyn was suggested to contribute to the increase of glutamatergic synaptic transmission by BDNF (Alder et al., 2005), and tyrosine phosphorylation of the NR2B subunit was also associated with LTP in the hippocampal CA1 region (Nakazawa et al., 2001). In addition to these rapid effects of BDNF on NMDA receptors, recent studies showed that BDNF increases the translation of the mRNA for NR1 in cultured cerebrocortical neurons (Schratt et al., 2004), suggesting that the neurotrophin may also regulate the abundance of NMDA receptors in the hippocampus. Although NMDA receptors were initially thought to be relatively stable in the membrane, recent studies have indicated that their surface expression is dynamic and regulated (Nong et al., 2004). Thus, the BDNF-induced upregulation of glutamatergic activity may also be due to the delivery of NMDA receptors to the plasma membrane. In the present study we investigated the short- and long-term effects of BDNF on the cellular and plasma membrane abundance of NMDA receptor subunits in cultured hippocampal neurons, and correlated the results with the activity of the receptors.

EXPERIMENTAL METHODS

Hippocampal cultures- Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18-E19 Wistar rat embryos, after treatment with trypsin (0.06%, 15min, 37ºC; GIBCO Invitrogen, Paisley, UK) and deoxyribonuclease I (5.36 mg/ml), 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 $Na_2HPO_4.2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES and

0.001% phenol red). The hippocampi were then washed with HBSS containing 10% fetal bovine serum (BioWittaker Europe, Belgium), to stop trypsin activity, and transferred to Neurobasal medium (GIBCO Invitrogen) supplemented with B27 supplement (1:50 dilution; GIBCO Invitrogen), 25 μM glutamate, 0.5 mM glutamine and 0.12 mg/ml gentamycin. The cells were dissociated in this solution and were then plated in 6 well plates ($91.6x10^3$ cells/cm^2), coated with poly-D-lysine (0.1 mg/mL), or on poly-D-lysine coated glass coverslips, at a density of $37.5x10^3$ cells/cm^2. The cultures were maintained in a humidified incubator of 5% CO_2/95% air, at 37 °C, for seven days or fourteen days. Cultures were stimulated with 100 ng/ml BDNF (kind gift from Regeneron, Tarrytown, NY), 100 ng/ml heat inactivated (5 min, 95ºC) BDNF or with 100 ng/ml NT3 (Peprotech, London U.K.) for the indicated periods of time. When appropriate, 2.0 μM emetine, 2.0 μM anisomycin, 1.5 μM α-amanitine or 1.5 μM actinomycin D (Calbiochem, La Jolla, CA) were added 30 min before stimulation, as indicated. Scavenging of endogenous extracellular BDNF was performed with TrkB-IgG (1 μg/ml; Sigma) for 24 h or 48 h.

Cerebellum cultures- Cerebellar granule neurons were isolated from the cerebella of 7 days-old Sprague Dawley rats as previously described (Schousboe, 1999), with minor modifications. Briefly, following digestion (15 min, 37 ºC) with 0.2% trypsin and 0.045 mg/ml deoxyribonuclease (Sigma) in Mg^{2+}-free Na^+-salt solution (120 mM NaCl, 5 mM KCl, 1.2 KH_2PO_4, 13 mM glucose,15 mM HEPES, 0.3% BSA, pH 7.4), and dissociation in 0.03% STI (soybean trypsin inhibitor; Sigma) and 0.04 mg/ml deoxyribonuclease prepared in Na^+-salt solution (120 mM NaCl, 5 mM KCl, 1.2 KH_2PO_4, 1.2 mM $MgSO_4$, 13 mM glucose,15 mM HEPES, 0.3% BSA, pH 7.4), the dissociated cells were centrifuged at 196 *g*, and washed with basal medium Eagle (BME, Sigma), supplemented with 25 mM KCl, 30 mM glucose, 26 mM $NaHCO_3$, 1% penicillin/streptomycin and 10% fetal calf serum (BioWittaker Europe). Neurons were then plated on 6 well plates ($34.4x10^4$ cells/cm^2), coated with poly-D-lysine (0.1 mg/mL), and cultured in supplemented BME. Approximately 24 h after plating, 10 μM cytosine-1-β-D-arabino-furanoside (Sigma) was added to the culture medium to prevent the glial proliferation. The cultures were maintained in a humidified incubator of 5% CO_2/95% air, at 37 °C, for eight days. Cultures were stimulated with 100 ng/ml BDNF (kind gift from Regeneron) for the indicated periods of time.

Preparation of extracts- Hippocampal and cerebellar granule neurons were washed twice with ice-cold PBS and once more with PBS buffer supplemented with 1 mM DTT and a cocktail of protease inhibitors (0.1 mM PMSF, CLAP: 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin; Sigma-Aldrich Química, Sintra, Portugal). The cells were then lysed with RIPA (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EGTA, 1% Triton, 0.5% DOC and 0.1% SDS at a final pH 7.5) supplemented with the cocktail of protease inhibitors. After centrifugation at 16,100 *g* for 10 min, protein in the supernatants was quantified using the BCA method, and the samples were denaturated with 2x concentrated denaturating buffer (125 mM Tris, pH 6.8, 100 mM glycine, 4% SDS, 200 mM DTT, 40% glycerol, 3 mM sodium orthovanadate, and 0.01% bromophenol blue), at 95 ºC for 5 min. NMDA receptor subunits were then analysed by Western blot.

Total RNA isolation and reverse transcription for Real-Time PCR- Total RNA from 7 DIV cultured hippocampal neurons was extracted

with TRizol reagent (Invitrogen, Barcelona, Spain), according to the instructions of the manufacturer. The full content of a 6 well cell cluster plate, with 870,000 cells/well (DIV 7), was collected for each experimental condition. For first strand cDNA synthesis, 3 μg of total RNA were reverse-transcribed with AMV Reverse Transcriptase (Roche, Carnaxide, Portugal), using Random Primer $p(dN)_6$ (3.2 μg), dNTPs (1 mM each), $MgCl_2$ (25 mM), RNase inhibitor (50 units) and Gelatine (0.01 μg/μl), in Reaction Buffer (10 mM Tris, 50 mM KCl, pH 8.3) and in a total volume of 40 μl. The reaction was performed at 25 ºC for 10 min, followed by 60 min at 42 ºC, for primer annealing to the RNA template and cDNA synthesis, respectively. The Reverse Transcriptase was then denatured during 5 min at 99 ºC, and the sample was cooled to 4 ºC for 5 min and finally stored at -80 ºC until further use.

Real-Time PCR- Real-Time PCR analysis of gene expression was performed using the LightCycler System II (Roche, Portugal). The PCR reactions were performed using LightCycler FastStart DNA Master SYBR Green I (Roche et al.) in 20 μl capillaries. The primers used for amplification of genes encoding NMDA receptor subunits were, respectively, RNFRIF2079 - 5´TAC ACT GCC AAC TTG GCA GCT TTC3' and RNRIR2591 - 5´CAT GAA GAC CCC TGC CAT GTT3´ for NR1, RNRZAF1961 -5´TGG CTG CCT TCA TGA TCC A3´ and RNRZAR2312 - 5´TGC AGC GCA ATT CCA TAG C3´ for NR2A, and RNRZBF1040 - 5´GGA TCT ACC AGT CTA ACA TG3´and RIRNRZBR1602 - 5´GAT AGT TAG TGA TCC CAC TG3´ for NR2B. The primers used for the amplification of endogenous control gene 18S rRNA were those included in the Applied Biosystems TaqMan Ribosomal RNA Control Reagents Kit (Porto, Portugal). Each primer of a pair was added to the reaction mixture (10 μl) at a final concentration of 0.8 μM with 3 mM $MgCl_2$, in addition to the "Hot Start" LightCycler Fast Start DNA Master SYBR Green I mix (1x) and 1.2 μl of cDNA sample. Thermal cycling was initiated with activation of the FastStart Taq DNA Polymerase by denaturation during 10 min at 95 ºC, followed by 45 cycles of a 30 s melting step at 95 ºC, a 5 s annealing step at 58 ºC, and a 25 s elongation step at 72 ºC (all temperature transition rates at 20 ºC/s). After amplification for 45 cycles, at least 10 cycles beyond the beginning of the linear phase of amplification, samples were subjected to a melting curve analysis according to the instructions of the manufacturer in order to confirm the absence of unspecific amplification products and primer-dimers. In all experiments, samples containing no template were included as negative controls.

mRNA quantitative analysis- The mRNA levels of the constitutively expressed housekeeping gene encoding 18S ribosomal RNA were used as a control, in all experiments. The relative changes in the mRNA levels of glutamate receptor subunits in cultured hippocampal neurons were determined using the ΔΔCp method. Accordingly, for each experimental condition (unstimulated neurons and neurons treated with 100 ng/ml BDNF for 30 min or 3 h) the "Crossing point" (Cp) values given by the LightCycler system II software, for each target gene, were subtracted by the respective Cp value determined for the 18S gene for the same sample and condition (ΔCp). This allows normalizing changes in target gene expression. Afterwards, the ΔCp values were subtracted by the respective values of the control for the target gene giving ΔΔCp. The derivation to the formula $2^{-(\Delta\Delta Cp)}$ sets each control at the unity (or 100%), since ΔΔCp (control) =0, and the stimuli conditions as a percentage relative to the control.

Surface biotinylation and precipitation- Hippocampal cell cultures (7 DIV) were treated or not with 100 ng/ml BDNF and then incubated with 1 mg/ml EZ-Link™ Sulfo-NHS-SS-biotin (Pierce, Madison, WI) in ice-cold PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, for 30 min (Gomes et al., 2004). The non-bound biotin was removed by washing the cells with PBS containing 100 mM glycine. Cell lysates were obtained as described above, and were incubated with UltraLink Plus™ immobilized streptavidin beads (Pierce), for 2 h at 4 °C, under constant agitation. Non-biotinylated proteins were removed by centrifugation at 2500×g for 3 min, and the beads were washed three times with RIPA buffer. Biotinylated proteins were then eluted with denaturating buffer at 95 °C for 5 min. Samples were then processed for Western blotting analysis.

Western blotting- Protein samples were separated by SDS-PAGE, in 6% polyacrylamide gels, transferred to polyvinylidene (PVDF) membranes (BioRad, Amadora, Portugal), and immunoblotted. Blots were incubated with primary antibodies (overnight at 4 ºC), washed and exposed to alkaline phosphatase-conjugated secondary antibodies (1:20000 dilution; 1 h at room temperature). Alkaline phosphatase activity was visualized by ECF on the Storm 860 Gel and Blot Imaging System (Amersham Biosciences, Buckinghamshire, UK). The following primary antibodies were used: anti-NR1 (1:200, Chemicon International, Temecula, CA), anti-NR2A (1:750, Chemicon International, Temecula, CA or 1:300, BD Transduction Laboratories, Erembodegem, Belgium) and anti- NR2B (1:400, BD Transduction Laboratories). Anti-β-Tubulin I (1:700000; Sigma) and anti-transferrin receptor (1:3000; Zymed, South San Francisco, CA) antibodies were used as loading controls.

Single cell $[Ca^{2+}]_i$ measurements- Changes in the $[Ca^{2+}]_i$ were assessed by monitoring the Fura-2 (Invitrogen-Molecular Probes, Leiden, The Netherlands) fluorescence ratio (F340/F380). Seven DIV hippocampal neurons were loaded in a Mg^{2+}-free Na^+-salt solution (132 mM NaCl, 4 mM KCl, 2.5 mM $CaCl_2$, 6 mM Glucose, 10 mM Hepes, 10 mM $NaHCO_3$, pH 7.4) containing 5 μg/ml Fura-2/AM, 0.2% pluronic acid F-127 (Invitrogen-Molecular Probes) and 0.1% fatty acid-free bovine serum albumin, in the presence or absence of 100 ng/ml BDNF, for 40 min at 37 ºC. After incubation, the glass coverslips were mounted on a RC-20 chamber in a PH3 platform (Warner Instruments, Hamden, CT), at room temperature, on the stage of an inverted fluorescence microscope Axiovert 200 (Zeiss). Neurons were continuously perfused with a Mg^{2+}-free Na^+-salt solution, for 5 min, and were then stimulated with 100 μM NMDA and 10 μM glycine, or with 30 mM KCl (Na^+ was isoosmotically replaced by KCl), for 30 s. After stimulation neurons were again perfused with a Mg^{2+}-free Na^+-salt solution, for 10 min. Solutions were added to the cells by a fast-pressurized (95% air, 5% CO_2 atmosphere) system (AutoMate Scientific, Inc, San Francisco, CA). The cells were alternately excited at 340 and 380 nm using a Lambda DG4 apparatus (Sutter Instruments Company, Novato, CA). Changes in the fluorescence ratio of Fura-2 were acquired with a 40x objective and a CollSNAP HQ digital camera (Roper Scientific, Tucson, AZ), and processed using the MetaFluor software (Universal Imaging Corporation, Downingtown, PA). The results are presented as the ratio of fluorescence intensities after excitation at 340 nm and at 380 nm.

Statistical Analysis- Statistical analysis was performed using one-way ANOVA analysis of variance followed by the Dunnett's test, or

Bonferroni test, or using the Student´s t test, as indicated in the figure captions.

RESULTS

Effect of BDNF on the total protein levels of NMDA receptor subunits- To evaluate the acute effects of BDNF on the abundance of NMDA receptor subunits, 7 DIV cultured hippocampal neurons were incubated with or without 100 ng/ml BDNF, for various periods of time (15 min - 24 h). The NMDA receptor subunit NR1, NR2A and NR2B protein levels were determined by Western Blotting (Fig. 1A, C and E). BDNF upregulated NR1 subunits rapidly and in a sustained manner. Significant effects were observed after 30 min to 12 h incubation with BDNF. The neurotrophin also did upregulate NR2A and NR2B subunits, but with a distinct kinetics. NR2A protein levels were significantly increased for longer periods of incubation with BDNF (3 h to 12 h), while NR2B protein levels were enhanced by brief BDNF stimulation periods (1 h to 3 h). After 24 h of stimulation with BDNF the NR1 and NR2A protein levels were still slightly higher than the control, whereas a small reduction of NR2B was observed. NR2C and NR2D subunits were undetectable (data not shown), consistent with the lack of these NMDA receptor subunits in the hippocampus [e.g. (Janssens and Lesage, 2001)]. BDNF also upregulated the amount of NR1, NR2A and NR2B subunits in more mature hippocampal neurons, cultured for 14 DIV [(Fletcher et al., 1991); Fig. 1B, D and F]. In these cells, BDNF induced a sustained increase in NR1 protein levels, particularly for incubation periods of 5 h, 6 h and 24 h. The NR2A subunit was also upregulated by BDNF for long periods of incubation with BDNF (6 h, 12 h and 24 h). In contrast, the NR2B subunit was upregulated only by short incubations with BDNF, as observed for 7 DIV cultures (Fig. 1F). Activation of Trk neurotrophin receptors was required for upregulation of NR1, NR2A and NR2B receptor subunits by BDNF, since no effect was observed when neurons were incubated with heat-inactivated BDNF (Fig. 2A, B and C). In contrast with the results obtained with the hippocampal neurons, incubation of cultured cerebellar granule cells with 100 ng/ml BDNF, for 2 h or 6 h, did not increase the protein levels of NR1, NR2A and NR2B subunits (Fig. 2D, E and F). These results indicate that the BDNF-induced upregulation of the NMDA receptor subunits is specific for hippocampal neurons.

Cultured hippocampal neurons produce BDNF and release the neurotrophin in an activity-dependent manner (Hartmann et al., 2001; Balkowiec and Katz, 2002). In order to determine whether endogenous BDNF modulate the levels of NMDA receptor subunits in cultured hippocampal neurons we used the TrkB-IgG fusion protein that chelated the endogenous extracellular BDNF. Incubation of hippocampal neurons with TrkB-IgG for 24 h or 48 h did not affect significantly the levels of NR1, NR2A and NR2B subunits (Fig. 3).

Incubation of hippocampal neurons with a different neurotrophin, neurotrophin-3 (NT3), which binds preferentially to the TrkC receptors (Dechant, 2001), also increased the protein levels of NR2A and NR2B subunits (Fig. 4B and C), but was without effect on NR1 subunit (Fig. 4A), indicating that this neurotrophin also modulates the levels of the NMDA receptor subunits.

Since BDNF is known to upregulate translation [e.g. (Takei et al., 2001)], we studied whether *de novo* protein synthesis could account for the BDNF-induced increase in NR1, NR2A and NR2B NMDA receptor subunits. We used two translation inhibitors, emetine and anisomycin, and the hippocampal neurons were stimulated with BDNF for 3 h in the presence or in the

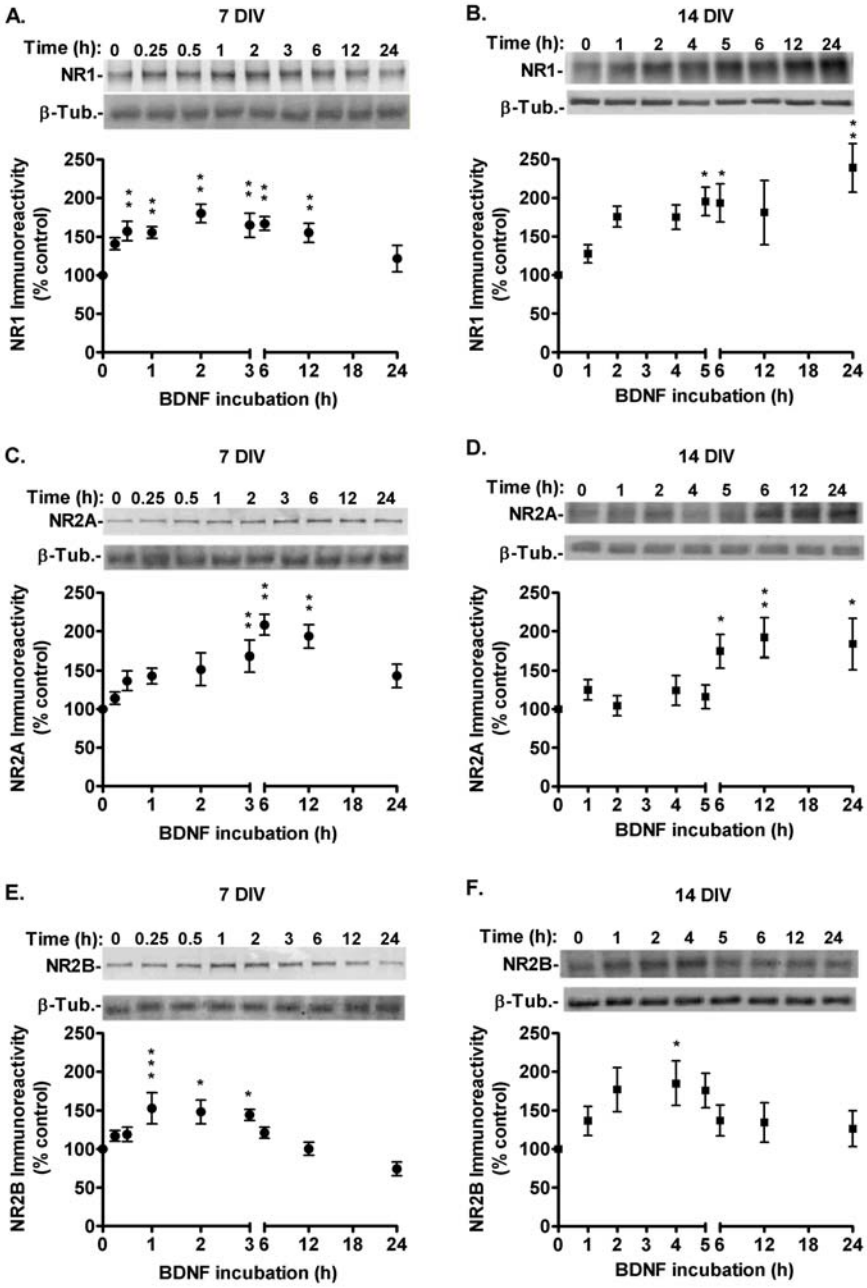

Figure 1- BDNF upregulates the protein levels of the NR1, NR2A and NR2B NMDA receptor subunits cultured hippocampal neurons. Seven DIV or 14 DIV cultured hippocampal neurons were incubated with or without 100 ng/ml BDNF (15 min, 30 min, 1h, 2 h, 3 h, 4 h, 5 h, 6 h, 12 h and 24 h), as indicated. Total NR1, NR2A and NR2B protein levels were determined by Western blot. Control (0 h) protein levels of NMDA receptor subunits were set to 100%. β-tubulin was used as loading control. The results are the average ± SEM of 5-9 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Dunnett's test. *P<0.05, **P<0.01.

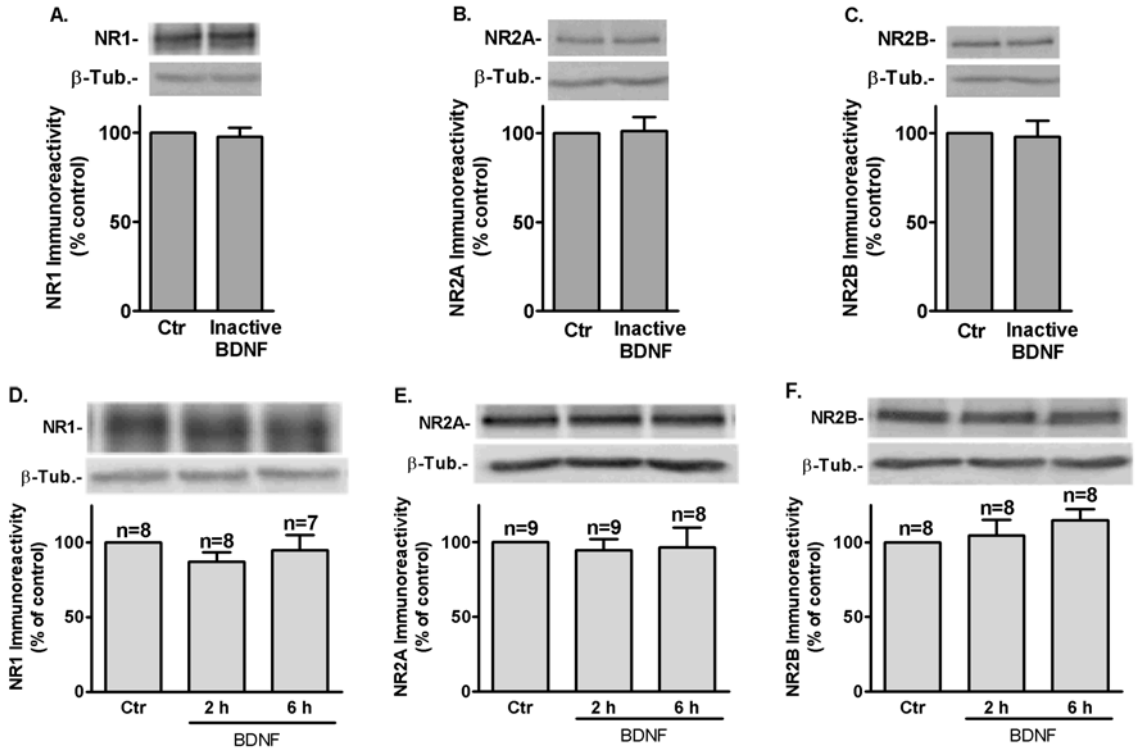

Figure 2- Heat-inactivated BDNF does not upregulate of NR1, NR2A and NR2B protein levels (A, B, C). Hippocampal neurons were incubated with or without 100 ng/ml heat-inactivated BDNF (95 ºC, 5 min), for 3 h. NR1, NR2A and NR2B protein levels were measured by Western blot. BDNF does not affect the protein levels of NR1, NR2A and NR2B subunits in cultured cerebellar granule neurons (D, E, F). The cells were incubated with or without 100 ng/ml BDNF (2 h and 6 h), and the NR1, NR2A and NR2B protein levels were measured by Western blot. Control (unstimulated) protein levels of NMDA receptor subunits were set to 100%. β-tubulin was used as loading control. The results are the average ± SEM of 4-9 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Dunnett´s test.

absence of the protein synthesis inhibitors. Pre-incubation of cells with emetine (2 μM) or anisomycin (2 μM) abolished the effect of BDNF on NR1, NR2A and NR2B subunits (Fig. 5). None of the protein synthesis inhibitors altered the NR1, NR2A and NR2B protein levels under control conditions. These results are in agreement with the long half-life determined for the NR2A/B subunits in cultured cerebellar granule cells, and with the turn-over rate of NR1 in the same preparation (Huh and Wenthold, 1999). Taken together, the results indicate that BDNF affects NMDA receptor subunits by increasing protein synthesis and suggest that a reduction in protein degradation is not involved.

Effect of BDNF in the transcription activity of NMDA receptor subunits- The effects of BDNF on protein synthesis may be due to an increase in transcription activity (Messaoudi et al., 2002) and/or to direct regulation of the protein synthesis machinery (Takei et al., 2001). Therefore, the role of transcription in the BDNF-induced upregulation of NR1,

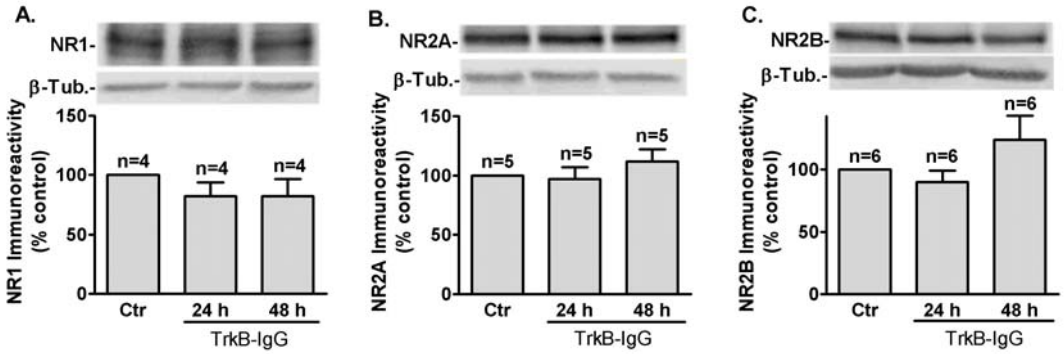

Figure 3- Chelation of endogenous released BDNF with the fusion protein TrkB-IgG does not affect the protein levels of NR1, NR2A and NR2B. Fourteen DIV neurons were incubated with or without 1 μg/ml TrkB-IgG for 24 h and 48 h and the total NR1, NR2A and NR2B protein levels were measured by Western blot. Control protein levels of NMDA receptor subunits were set to 100%. β-tubulin was used as loading control. The results are the average ± SEM of 4-6 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Dunnett's test.

NR2A and NR2B protein levels was investigated using two transcription inhibitors, α-amanitine and actinomycin D. As for the translation inhibitors, pre-incubation of the cultures with α-amanitine (1.5 μM) or actinomycin D (1.5 μM) abolished the BDNF-induced upregulation of the NR1, NR2A and NR2B protein levels, but was without effect on the abundance of the NMDA receptor subunits in the absence of BDNF (Fig. 6). To further confirm that the effects of BDNF occur at the transcription level, real-time PCR using the SYBR green assay was performed (Fig. 7). Stimulation with BDNF for 30 min or 3 h significantly increased the mRNA levels of NR1 and NR2A, although the effect was less significant in the latter incubation period. A delayed increase in the mRNA levels for NR2B was also observed after 3 h of stimulation with BDNF (Fig. 7). These results point to a regulation of NR1, NR2A and NR2B NMDA receptor subunits by BDNF at the transcription level. Moreover, they clearly correlate the several fold increase in NR1 and NR2A mRNA with the sustained raise in the respective protein levels. On the other hand, the transient increase in NR2B protein levels induced by BDNF is also correlated with a relatively weaker effect on the mRNA levels of this specific subunit.

Effect of BDNF on NMDA receptor subunit protein levels at the plasma membrane- In cerebellar granule neurons there is a large intracellular pool of NR1 subunits, whereas most NR2A and NR2B subunit proteins are present on the cell surface (Huh and Wenthold, 1999). Thus, in these cells the NR2 subunit availability determines the number of cell surface receptors (Prybylowski et al., 2002). This may also apply to hippocampal neurons, where the majority of NR2B is associated with the plasma membrane, whereas a significant fraction of NR1 is intracellular (Hall and Soderling, 1997). Therefore, we investigated whether the upregulation of NMDA receptor subunits by BDNF affects the NR1 and NR2 protein levels in the plasma membrane of cultured hippocampal neurons. The cell surface distribution of the NMDA receptor subunits NR1, NR2A and NR2B was determined by biotinylation of cell surface

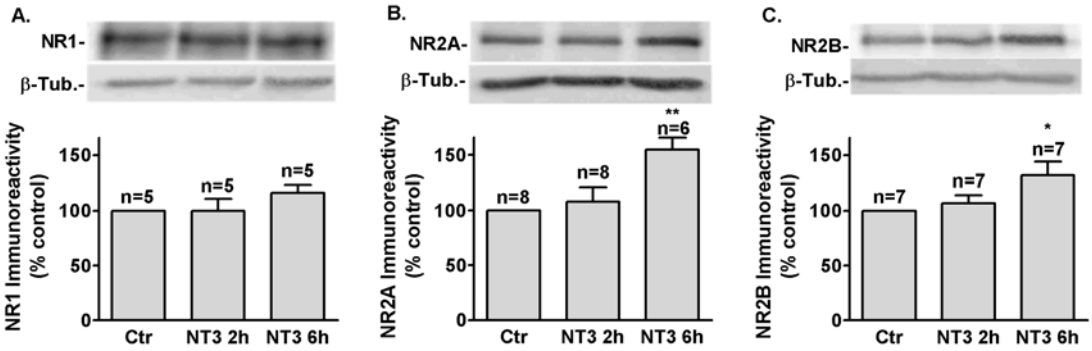

Figure 4- Incubation of hippocampal neurons with NT3 increased the protein levels of NR2A and NR2B, but was without effect on NR1 protein levels. Seven DIV neurons were incubated with or without 100 ng/ml NT3 for 2 h and 6 h and total NR1, NR2A and NR2B protein levels were measured by Western blot. Control (unstimulated) protein levels of NMDA receptor subunits were set to 100%. β-tubulin was used as loading control. The results are the average ± SEM of 5-8 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Dunnett's test. *P<0.05, **P<0.01.

proteins, under control conditions and after treatment with 100 ng/ml BDNF. Surface proteins were then collected with streptavidin-coupled beads, and subjected to Western blotting (Fig. 8). BDNF treatment during 30 min markedly increased NR1 and NR2B proteins associated with the plasma membrane, whereas longer incubations with the neurotrophin (24 h) were required to significantly increase surface NR2A. These data suggest that BDNF differentially regulates the translocation of the NMDA receptors with different subunit compositions to the plasma membrane in cultured hippocampal neurons.

Effect of BDNF on the NMDA-induced $[Ca^{2+}]_i$ changes- NMDA receptors are cation channels, permeable to Na^+ and Ca^{2+}, and activation of these receptors increases the intracellular free calcium concentration ($[Ca^{2+}]_i$) [e.g. (Duarte et al., 1996)]. The effect of BDNF on the activity of NMDA receptors was investigated in single cultured hippocampal neurons, by Fura-2 imaging. Stimulation of cultured hippocampal neurons with NMDA, in a Mg^{2+}-free medium, increased the Fura-2 fluorescence ratio (F340/F380) (Fig. 9). When the cells were pre-incubated with BDNF for 40 min there was an increase in the NMDA-induced $[Ca^{2+}]_i$ rise. The $[Ca^{2+}]_i$ response to activation of NMDA receptors is due to Ca^{2+} entry through the receptor channels and to Ca^{2+} influx through voltage-gated Ca^{2+} channels (Duarte et al., 1996). To determine whether the effect of BDNF on the responses to NMDA is due to an increase in the activity of voltage-gated Ca^{2+} channels, rather than to a direct change in the activity of the receptors, we studied the effect of the neurotrophin on the initial $[Ca^{2+}]_i$ changes caused by KCl depolarization. Perfusion of the cells with a solution where NaCl was isoosmotically replaced by 30 mM KCl rapidly increased the $[Ca^{2+}]_i$, but the magnitude of the response was not affected by pre-incubation of the cells with BDNF. Taken together, these results strongly suggest that the BDNF-induced increase in the $[Ca^{2+}]_i$ responses to NMDA are due, at least in part, to an upregulation of the plasma membrane-associated receptors.

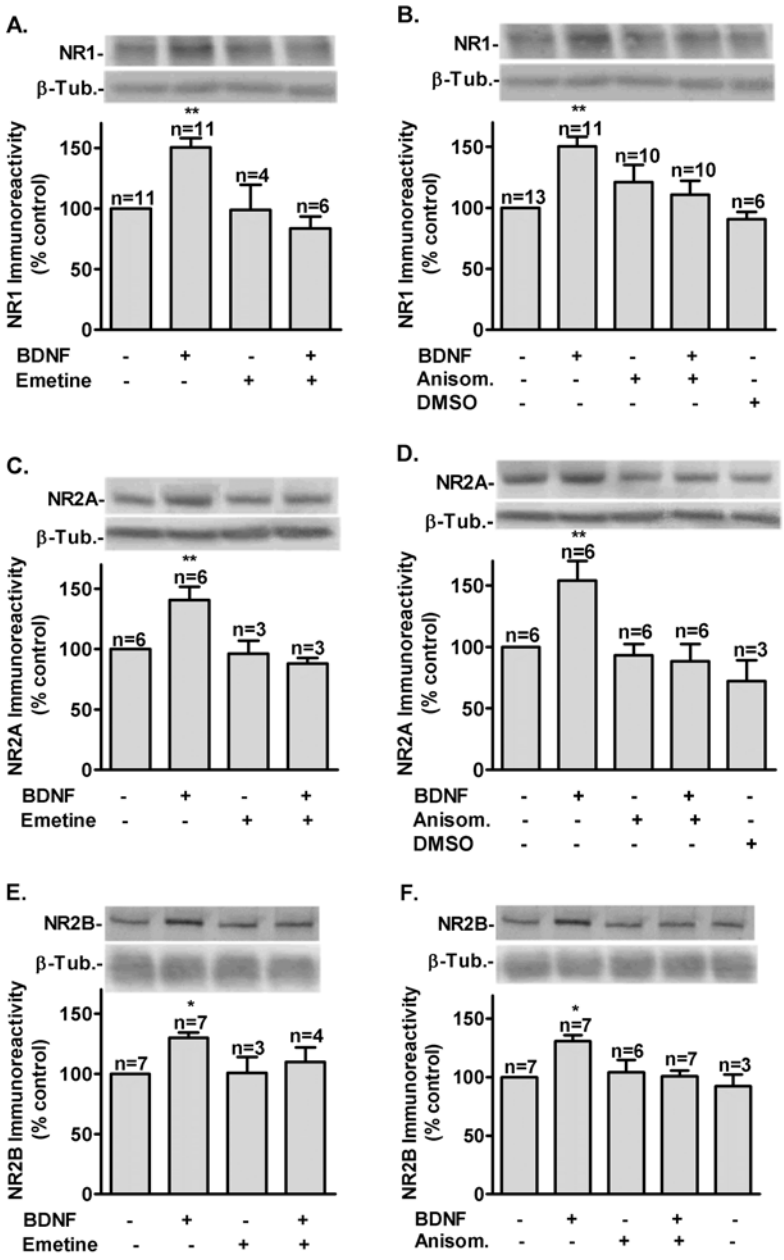

Figure 5- Translation inhibitors impair the BDNF-induced upregulation of NR1, NR2A and NR2B protein levels. Seven DIV hippocampal neurons were incubated with or without 100 ng/ml BDNF for 3 h, in the presence or in the absence of emetine (2.0 μM) or anisomycin (2.0 μM). When the inhibitors were used the cells were pre-incubated with the compounds for 30 min before stimulation with BDNF. Total NR1, NR2A and NR2B protein levels were measured by Western blot. Control protein levels of NMDA receptor subunits were set to 100%. β-tubulin was used as loading control. The results are the average ± SEM of 6-12 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Dunnett's test. *P<0.05, **P<0.001.

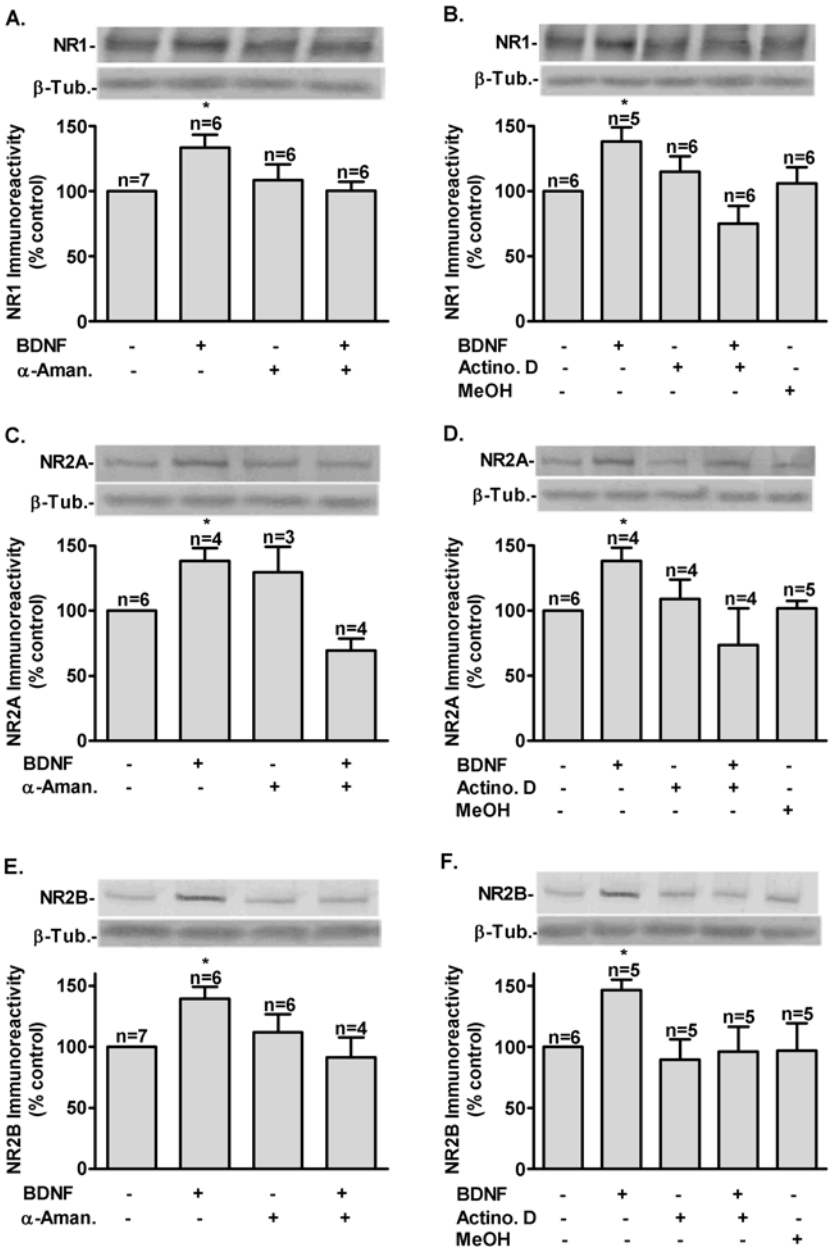

Figure 6- Transcription inhibitors prevent the BDNF-induced upregulation of the NR1, NR2A and NR2B protein levels. Seven DIV cultured hippocampal neurons were incubated with or without 100 ng/ml BDNF for 3 h, in the presence or in the absence of α-amanitine (1.5 μM) or actinomycin D (1.5 μM). When the inhibitors were used the cells were pre-incubated with the compounds for 30 min before stimulation with BDNF. Total NR1, NR2A and NR2B protein levels were measured by Western blot. Control protein levels of NMDA receptor subunits were set to 100%. β-tubulin was used as loading control. The results are the average ± SEM of 5-10 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Dunnett's test. *P<0.05.

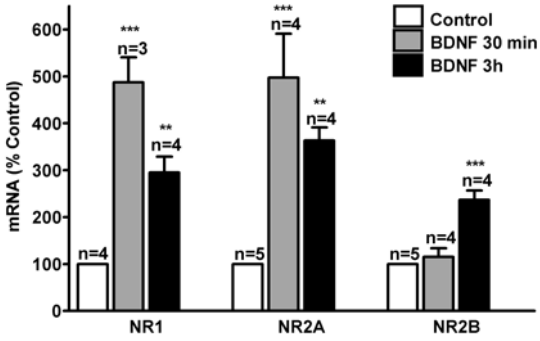

Figure 7- BDNF increases the mRNA levels of the NR1, NR2A and NR2B NMDA receptor subunits. The variation of NR1, NR2A and NR2B mRNA levels was assayed by SYBR Green Real-Time PCR of total RNA samples, converted to cDNA in reactions normalized to contain equal amounts of mRNA. The cells were incubated in the presence or in the absence of 100 ng/ml BDNF, during 30 min (gray columns) or 3 h (black columns). The results are presented as mean percentage ± SEM compared to the control (unstimulated), and normalized to the reference gene 18S, and are the average ± SEM of 3-7 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Dunnett's test. **P<0.01, ***P<0.001.

DISCUSSION

BDNF has been shown to play important roles in the regulation of the glutamatergic synaptic transmission and in the early- and late-phases of LTP [reviewed in (Bramham and Messaoudi, 2005)], but the underlying mechanisms are still not fully understood. The rapid effects of BDNF on the post-synaptic responses to glutamate have been largely attributed to the phosphorylation of NMDA receptor subunits, which increases receptor activity (Levine et al., 1998; Levine and Kolb, 2000). In the present study, we showed that BDNF also induces a rapid delivery of NR2B-containing NMDA receptors to the plasma membrane, which correlated with an increased $[Ca^{2+}]_i$ response to the activation of the receptors. Furthermore, we showed that BDNF differentially upregulates the NR1, NR2A and NR2B NMDA receptor subunits in cultured hippocampal neurons through an increase in transcription activity. The NT3, which binds to a different Trk receptor (TrkC), increased the protein levels of NR2A and NR2B subunits, but was without effect on NR1. The results showing the BDNF-induced rapid delivery of NR1 and NR2 subunits to the plasma membrane contribute to the growing evidence that the surface expression of NMDA receptors is dynamic and regulated (Lan et al., 2001; Roche et al., 2001), as previously documented for AMPA receptors (Gomes et al., 2003). If BDNF has a similar effect on the delivery of NMDA receptors to the synapse, this may account, at least in part, for the role of the neurotrophin in synaptic plasticity. The delivery of NMDA receptors to the synapse plays an important role in long-term potentiation, although the NR2 subunit involved may depend on the development stage (Barria and Malinow, 2005; Kim et al., 2005).

Stimulation of hippocampal neurons with BDNF induced a rapid delivery of NR1- and NR2B-containing NMDA receptors to the plasma membrane, but no increase in NR2A was observed for short incubations with the neurotrophin (Fig. 8). This upregulation in plasma membrane-associated NMDA receptor subunits correlated with an increase in the $[Ca^{2+}]_i$ responses mediated by the receptor (Fig. 7). The simultaneous effect on the delivery of NR1 and NR2 subunits was as expected, since both subunits are required to form a functional NMDA receptor (Meguro et al., 1992; Monyer et al., 1994; Dingledine et al., 1999), and occurred at a time point when the amount of NR2B was still not significantly changed. The rapid effect of BDNF on the traffic of NR1/NR2B subunits changes the ratio of NR2A- and NR2B-containing NMDA receptors associated with the membrane, and is likely

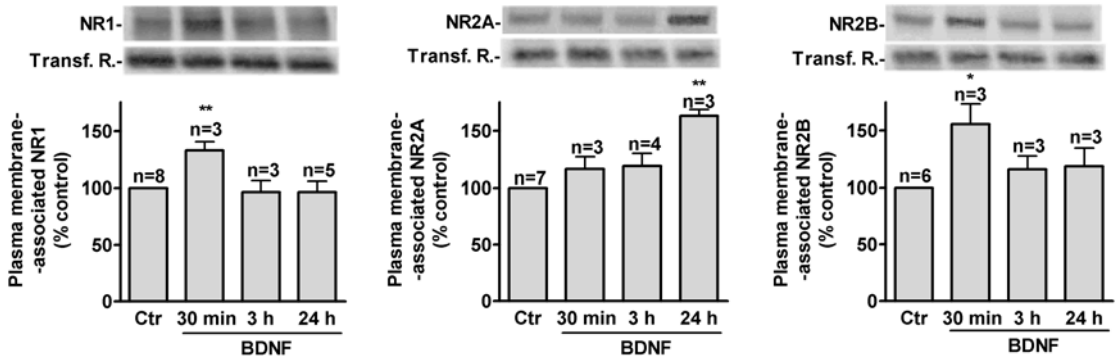

Figure 8- BDNF increases the NR1, NR2A and NR2B subunits in the plasma membrane. Neurons were treated with or without 100 ng/ml BDNF (30 min, 3 h and 24 h). Following treatment, cell surface proteins were labelled by biotinylation, followed by precipitation with streptavidin beads. The abundance of each subunit in the plasma membrane was then determined by Western blot. Control (0 h) expression of NMDA subunits protein was set to 100%. The transferrin receptor was used as loading control. The results are the average ± SEM of 3-8 independent experiments, performed in independent preparations. Statistical analysis was performed by One Way ANOVA, followed by the Dunnett's test. *P<0.05, **P<0.01.

to result in slower excitatory postsynaptic currents (EPSCs) (Monyer et al., 1994). BDNF was previously shown to acutely increase tyrosine phosphorylation of NR2B (but not NR2A) subunits in cortical and hippocampal postsynaptic densities (Lin et al., 1998) and in cultured hippocampal neurons (Alder et al., 2005), and the effect of the neurotrophin on the activity of NMDA receptors in cultured hippocampal neurons is sensitive to inhibitors of NR2B (Crozier et al., 1999; Levine and Kolb, 2000). Our findings indicate that the upregulation of NR2B-containing receptors associated with the membrane accounts, at least in part, for the effect of the neurotrophin on the receptor activity, but the molecular mechanisms involved remain to be determined. The mechanisms mediating the effect of neurotrophins may be similar to those involved in insulin-induced rapid delivery of NMDA receptors to the cell surface, since both receptors have tyrosine kinase activity. The effect of insulin occurs via a SNAP-25 mediated form of SNARE-dependent exocytosis, and does not require direct phosphorylation of the C-terminal tails of the receptor protein, but rather of associated targeting, anchoring, or signalling protein(s) (Skeberdis et al., 2001).

In addition to the effect resulting from the upregulation of plasma membrane-associated receptors, phosphorylation of NMDA receptors may also change their electrophysiological properties, and therefore may contribute to the change in activity induced by BDNF (Fig. 9). BDNF acutely induces phosphorylation of NR1 and NR2B subunits in rat hippocampal postsynaptic densities (Suen et al., 1997; Lin et al., 1998; Alder et al., 2005), and increases NMDA single channel open probability in cultured hippocampal neurons (Levine et al., 1998; Levine and Kolb, 2000). Phosphorylation of NR2B may be mediated by Fyn, a member of the Src family, since this kinase is activated by TrkB receptors (Narisawa-Saito et al., 1999b), and increases currents mediated by recombinant NMDA receptor (Kohr and Seeburg, 1996). The activity of NMDA receptors in CNS neurons was also shown to increase following intracellular

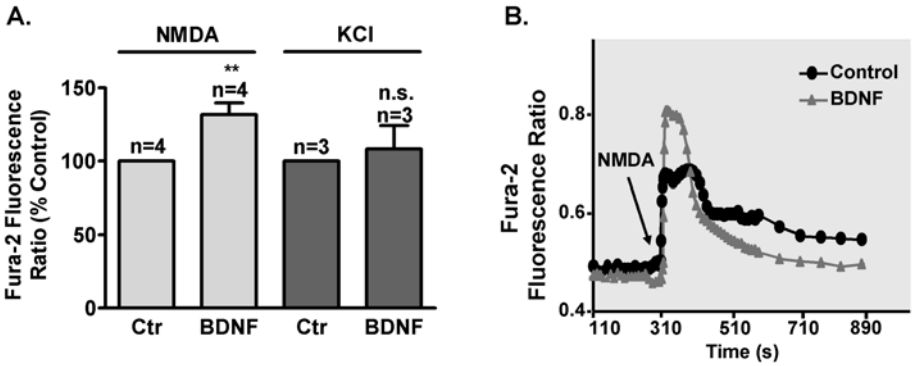

Figure 9- BDNF increases in the $[Ca^{2+}]_i$ responses to NMDA. Cultured hippocampal neurons were loaded with Fura-2/AM fluorescence probe in the presence or in the absence of 100 ng/ml BDNF for 40 min. Following incubation, cells were perfused with Mg^{2+}-free Na^+-salt solution for 5 min and were stimulated with 100 μM NMDA and 10 μM glycine or 30 mM KCl for 30 sec. After stimulation neurons were perfused with Mg^{2+}-free Na^+-salt solution for 10 min. The $[Ca^{2+}]_i$ response to K^+ depolarization or to NMDA-receptor stimulation was monitored in single cells as the ratio between the fluorescence at 340 nm and 380 nm. For each experimental condition, the control $[Ca^{2+}]_i$ responses were set to 100%. The results are the average ± SEM of 3-4 independent experiments, performed in independent preparations. Statistical analysis was performed using Student *t* test. **P<0.01.

application of recombinant Src (Wang and Salter, 1994; Kalia et al., 2004; Salter and Kalia, 2004), and Fyn-mediated interaction between BDNF signalling and NMDA receptors may play an important role in spatial learning and memory (Mizuno et al., 2003). The signalling activity induced by BDNF may also affect NMDA receptors through protein kinase C, which modulates NMDA receptor trafficking and gating in cultured hippocampal neurons (Lan et al., 2001). In fact, activation of Trk receptors promotes PLCγ activity (Chao, 2003), giving rise to diacylglicerol which activates PKC.

In contrast with the short-term effects of BDNF on the surface expression of NR1 and NR2B subunits, longer incubations with BDNF (24 h) increased the amount of NR2A subunits associated with the plasma membrane, but not of NR2B, further indicating that the traffic of the two subunits to the membrane is differentially affected by the neurotrophin. The delayed increase in NR2A in the membrane induced by BDNF may be secondary to the upregulation of the subunit induced by BDNF (Fig. 1) and/or due to changes in abundance of regulatory, motor or anchoring proteins that regulate traffic of the receptor, since after 24 h of stimulation of the Trk receptors with the neurotrophin the receptors are no longer active (Almeida et al., 2005). However, it was surprising not to observe an increase of NR1 subunit associated with the membrane, together with NR2A, after long incubations with BDNF. Although this may suggest that the delivered receptors contain more NR2A than NR1 subunits, this hypothesis is against evidences suggesting that the NMDA receptor channels are formed as dimers of dimers (an NR1 dimmer and a NR2 dimmer) (Schorge and Colquhoun, 2003). This BDNF-induced upregulation of NR2A associated with the plasma membrane after long incubation periods with the neurotrophin resembles the shift from NR2B to NR2A observed in developing neurons (Watanabe et al., 1992; Sheng et al., 1994; Barria and Malinow, 2002; Erisir and Harris, 2003; Kobayashi et al., 2006), resulting in faster excitatory postsynaptic currents (EPSCs) and lower sensitivity to NR2B-selective

antagonists (Carmignoto and Vicini, 1992; Flint et al., 1997). This change is also thought to contribute to the developmental changes in NMDA-receptor mediated plasticity at glutamatergic synapses (Philpot et al., 2001).

In addition to the translocation of NMDA receptor subunits to the plasma membrane, we also found that BDNF upregulated NR1, NR2A and NR2B subunits, by a mechanism involving transcription activation. This is supported by the results showing an increase in the mRNA levels for the three subunits in hippocampal neurons stimulated with BDNF (Fig. 7) and the inhibition of neurotrophin-induced upregulation of NR1, NR2A and NR2B in the presence of transcription inhibitors (Fig. 6). Therefore, although Trk receptor activation by neurotrophins may stimulate protein synthesis directly (Takei et al., 2004), without transcription induction, this mechanism is not involved in the upregulation of NMDA receptor subunits by BDNF in hippocampal neurons. The effects of BDNF on NMDA receptor subunits were transient in 7 DIV cultures, most likely due to the desensitization of the Trk receptors, followed by a decrease in the intracellular signalling activity (Sommerfeld et al., 2000; Almeida et al., 2005). However, in 14 DIV cultures BDNF induced a sustained increase in the NR1 and NR2A protein levels, but not of NR2B (Fig. 1), which may be due to a change in the turn-over rate of the subunits in the more mature cultures. Interestingly, the expression of NR2A but not NR2B subunit is markedly reduced in the developing cortex of BDNF knockout mice (Margottil and Domenici, 2003). NR1 expression is regulated by different transcription factors, including the NF-kappaB (Liu et al., 2004) or CREB (Lau et al., 2004), and the latter is a major mediator of neuronal neurotrophin responses (Finkbeiner et al., 1997). NR2B expression is also regulated by CREB (Rani et al., 2005), in addition to AP-1 (Qiang and Ticku, 2005), which may also be activated by BDNF-induced signalling (Li et al., 2004). Neurotrophin-4/5 was shown to upregulate NR2A through the immediate early transcription factor Egr-1 in cultured cerebrocortical neurons (Choi et al., 2004).

Stimulation of hippocampal neurons with NT3 also upregulated NR2A and NR2B protein levels, but was without effect on NR1. In contrast with the effect of BDNF, NT3 did not change the NMDA receptor subunit protein levels when 2 h of incubation with the neurotrophin was used. Although the effects of both neurotrophins are likely mediated by Trk receptors (TrkB and TrkC, respectively), the differential responses may be due to a distinct cellular localization of the receptors and/or to differences in the magnitude of the signalling responses induced.

The BDNF-induced upregulation of NMDA receptor subunits and activity that we observed in cultured hippocampal neurons contrast with the lack of effect of BDNF in cerebellar granule neurons incubated with the neurotrophin for 2 h or 6 h (Fig. 2D, E and F), indicating that BDNF has a specific effect on hippocampal neurons. These results contrast with the previously reported downregulation of the NR2A receptor subunits evoked by BDNF in cerebellar granule neurons (Brandoli et al., 1998). This discrepancy may be due to differences in the composition of the culture medium, as well as the use of a distinct concentration of BDNF.

In conclusion, our work shows that BDNF differentially upregulates the plasma membrane associated NMDA receptors, and the protein levels of the receptor subunits in cultured hippocampal neurons. NMDA receptors play an important role in synaptic plasticity, and there is a selective activation of NMDA receptors in specific circuits during

memory formation (Tang et al., 1999; Lynch, 2004). The activation of NMDA receptors, together with non-NMDA receptors, induces the synthesis and release of BDNF (Zafra et al., 1990; Hughes et al., 1993) which may further promote NMDA receptor activity by increasing the number of receptors associated with the membrane. The resulting increase in calcium entry through the NMDA receptors will also regulate numerous other downstream signalling pathways, leading to both short-term and long-term neuronal changes (Hardingham and Bading, 2003). If BDNF induces a rapid synaptic delivery of NR2B-containing receptors to the synapse, similarly to the effect observed on the overall surface expression of NMDA receptor subunits, this may be important to allow binding of active CaMKII to synaptic NMDA receptors, thereby contributing to synaptic potentiation (Barria and Malinow, 2005). This model, proposed for developing synapses, contrasts with the role played by NR2A-NMDA receptors in promoting surface expression of GluR1-containing AMPA receptors, a key event in LTP in more mature synapses (Malinow and Malenka, 2002). Interestingly, BDNF also caused a delayed increase in NR2A associated with the plasma membrane.

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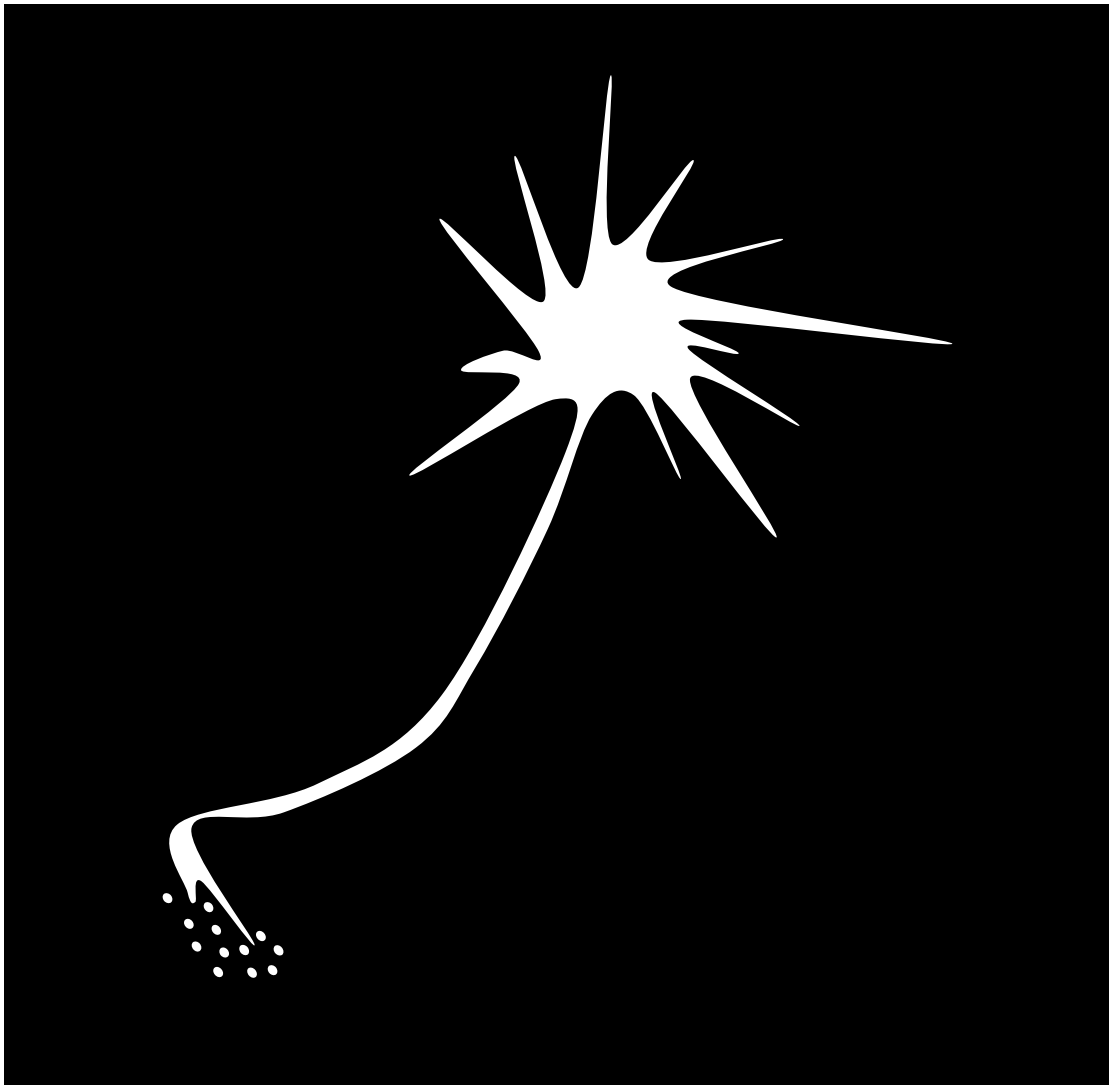
Chapter 4. General Conclusions



General conclusions

BDNF plays important roles in the regulation of the glutamatergic synaptic transmission, particularly in synaptic plasticity (Lu, 2003; Alder et al., 2005). However, the underlying mechanisms are not fully understood. In the present work, we studied the effect of BDNF on AMPA and NMDA receptors in cultured hippocampal neurons, which may contribute, at least in part, to synaptic plasticity.

In this study, we show that BDNF differentially increases the total amount of GluR1, GluR2 and GluR3 subunits of AMPA receptors, and NR1, NR2A and NR2B subunits of NMDA receptors. In 14 DIV cultures of hippocampal neurons, the endogenous extracellular BDNF was also shown to be important in the upregulation of the GluR1 protein levels. Incubation of 7 DIV hippocampal neurons with BDNF also upregulated the mRNA levels of GluR1, GluR2, NR1, NR2A and NR2B, and pre-incubation of neurons with two different transcription inhibitors (α-amanitine and actinomycin D) or two translation inhibitors (emetine and anisomycin) abolished the upregulation of the protein levels of AMPA receptor and NMDA receptor subunits. Thus, we conclude that BDNF increases the abundance of AMPA and NMDA receptor subunits through a mechanism dependent on the activation of transcription. The effect of BDNF on GluR1 and GluR2 protein levels may be mediated by CREB, a transcription factor activated by TrkB (Finkbeiner et al., 1997), since GluR1 and GluR2 gene promoters possess a CREB binding site (Borges and Dingledine, 2001). However, the role of CREB in the regulation of the genes encoding for these subunits remains to be investigated. In contrast, CREB was shown to regulate the expression of NR1 and NR2B (Lau et al., 2004; Rani et al., 2005). The transcription factors NF-κB and AP1, which are known to be activated by BDNF signalling (Liu et al., 2004; Qiang and Ticku, 2005), are also involved in the expression of these subunits (Liu et al., 2004; Qiang and Ticku, 2005). Therefore, BDNF acting through TrkB receptors may induce the activation of several transcription factors, regulating the expression of AMPA and NMDA receptors. It remains to be determined the signalling pathways used by BDNF to regulate the expression of AMPA and NMDA receptor subunits, and which specific transcription factors are activated by BDNF in the modulation of AMPA and NMDA receptor subunits expression.

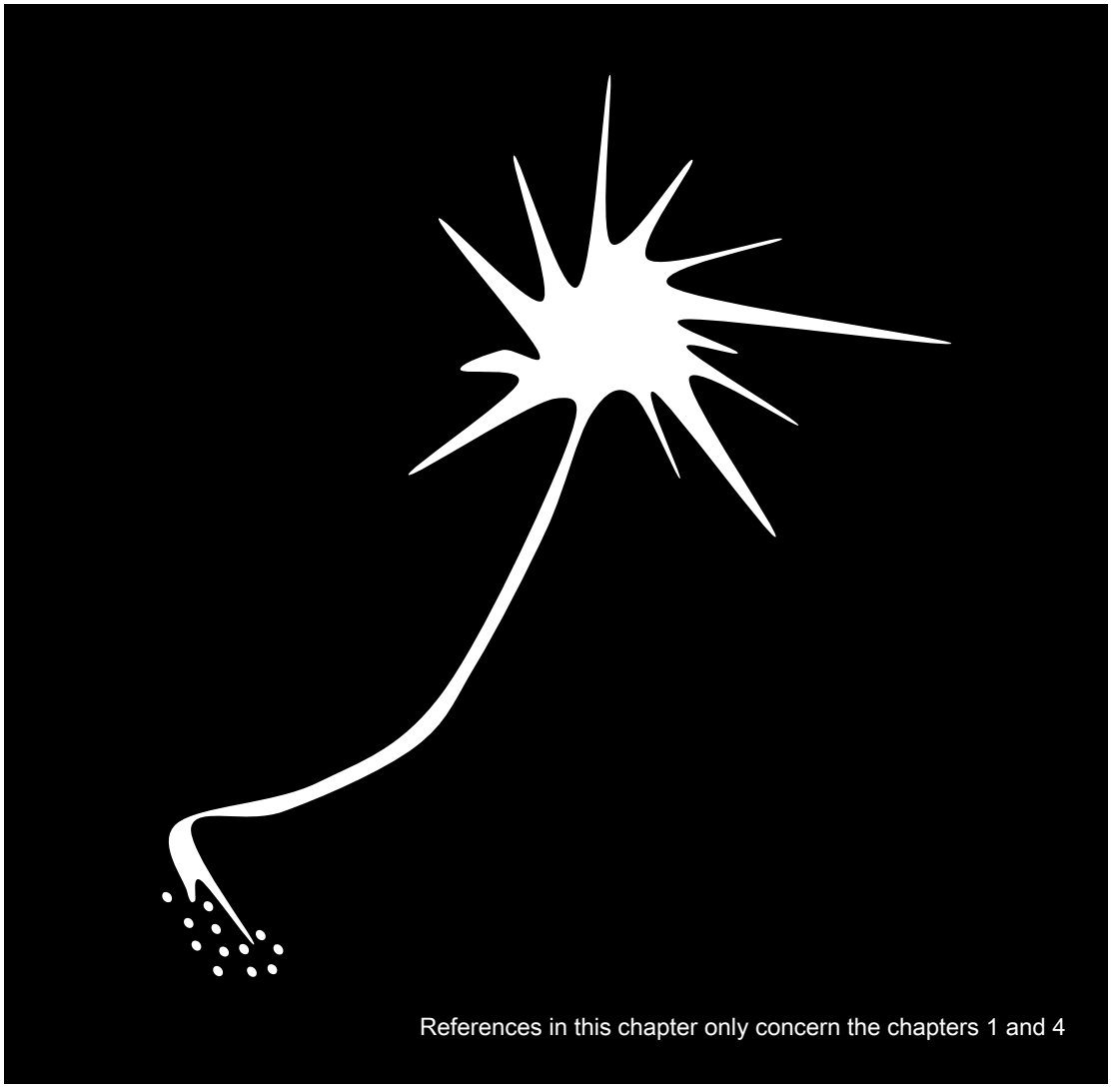
We also showed that BDNF increases the abundance of GluR1, NR1, NR2A and NR2B in the plasma membrane of cultured hippocampal neurons. Since the observed effects were quite fast (within 30 min of BDNF incubation for GluR1 subunit) and translation dependent (for GluR1 and GluR2 subunits), it will be of interest to distinguish between the contribution of local dendritic synthesis and soma mRNA translation to the observed upregulation of AMPA and NMDA receptor subunits. The surface increase in the GluR1 protein levels was further supported by the electrophysiology studies in hippocampal slices that showed synaptic delivery of GluR1-GFP homomeric receptors by BDNF stimulation. The plasma membrane enrichment in NMDA receptor subunits upon BDNF stimulation was also correlated with the increases in NMDA-induced changes in $[Ca^{2+}]_i$. Besides supporting the idea that BDNF induces the delivery of NMDA receptor subunits to the plasma membrane, the increased $[Ca^{2+}]_i$ response to NMDA stimulation in the presence of BDNF may also be due to an effect of BDNF on the activity of NMDA receptors by phosphorylation of the receptor subunits. Therefore, it will be of interest to determine weather the effects of BDNF are mediated by delivery of receptors to the plasma membrane, presumably by enhancing the exocytosis of NMDA-containing vesicles, and/or to phosphorylation of the receptor subunits. For this purpose, the NMDA-induced $[Ca^{2+}]_i$ responses should be studied in the presence of exocytosis inhibitors, such as botulinum toxin or N-ethylmaleimide. These studies would further clarify the mechanism by which BDNF modulates synaptic plasticity. In fact, the BDNF-induced increase in the plasma membrane associated-NMDA receptors and the concomitant increase in the $[Ca^{2+}]_i$ responses to receptor stimulation may activate CaMKII, which phosphorylates the GluR1 subunit of AMPA receptors on Ser 831 (Mammen et al., 1997), and constitutes a signal for synaptic delivery of AMPA receptors (Shi et al., 1999). Accordingly, BDNF was shown to induce synaptic delivery of AMPA receptors in GluR1-GFP infected hippocampal slices. However, this AMPA receptor delivery to the synapse was shown to be independent on the NMDA receptor activity, suggesting that BDNF itself, through binding to its receptor, induces the delivery of AMPA receptors through activation of CaMKII and/or other protein kinases. In fact, in this study we observed an increase in the phosphorylation levels of GluR1 on Ser 831 in both cultured hippocampal neurons and slices. This residue is also known to be phosphorylated by PKC (Roche et al., 1996). The activation of this kinase by TrkB receptors may also contribute to the synaptic delivery of GluR1-containing AMPA receptors. Similarly to the effects observed in

hippocampal slices, BDNF also induced phosphorylation of GluR1 on Ser 831 in cultured hippocampal neurons, and this phosphorylation was shown to be mediated by PKC or CaMKII. Interestingly, exposure of hippocampal slices to BDNF did not induce phosphorylation of GluR1 on Ser 845. Since phosphorylation of Ser 831 is known to be necessary, but not sufficient to induce synaptic delivery (Hayashi et al., 2000), the identification of new GluR1 phosphorylation sites should be helpful to understand BDNF-induced synaptic delivery of AMPA receptors. Recently, GluR1 Ser 818 phosphorylation by PKC was shown to be increased during LTP (Boehm et al., 2006), but it remains to be determined whether phosphorylation of this residue plays a role in BDNF-induced synaptic delivery of GluR1-containing AMPA receptors.

Not only subunit phosphorylation accounts for changes in the activity and synaptic distribution of AMPA and NMDA receptors. Changes in the abundance of AMPA and NMDA receptor-binding proteins may also affect their cellular distribution. Therefore, it will be of interest to determine whether BDNF also affects synthesis or induces postranslational modifications of AMPA and NMDA receptor-interacting proteins, thereby promoting the insertion of receptors at the synapse or preventing receptor endocytosis.

The BDNF induced increase in the transcription activity and in the AMPA and NMDA receptor protein levels, together with the delivery of AMPA receptors to the synapse and the increase in the $[Ca^{2+}]_i$ responses to NMDA stimulation provide a strong evidence for the importance of BDNF on the modulation of synaptic activity. In fact, these alterations may, at least partially, account for the short- or long-term effects of BDNF on synaptic transmission important for LTP.

Chapter 5. References



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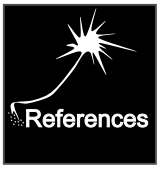

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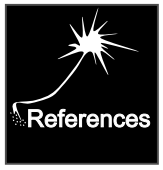

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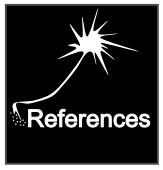

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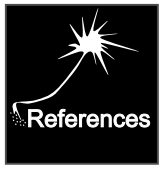

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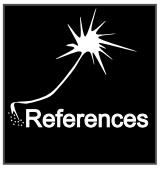

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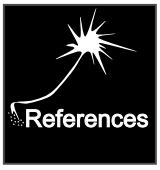

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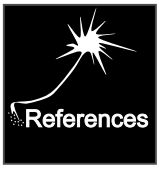

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