Signalling mechanisms affecting regulated neurotrophin secretion in hippocampal neurons

Dissertation
Zur Erlangung des Grades
“Doktor der Naturwissenschaften“

Am Fachbereich Biologie
Der Johannes Gutenberg-Universität Mainz

Richard Kolarow

Mainz, 2015

Dekan:

1. Berichterstatter:

2. Berichterstatter:

Tag der mündlichen Prüfung: 8. Juli 2015

1. INTRODUCTION

With almost 1000 billion neurons and an estimated 10 trillion synaptic connections, the human brain is the most complex existing structure of a living organism and is often referred to as the last frontier of biology. The questions of the ontogeny of the nervous system and of the remodelling of synapses in the mature brain are related to the discovery and characterization of neurotrophins. From the mid 1930s to the mid 1950s, target-derived signals were hypothesized to control neuronal development by maintaining differentiating neurons alive and by travelling retrogradely in axons to their respective centres. The experiments of Levi-Montalcini and Hamburger paved the way for the identification of a diffusible "nerve growth promoting factor". Released *in vitro* from mouse tumours, this factor was able to induce massive outgrowth of nerve fibres from co-cultured nervous tissue explants. When the factor called NGF was scavenged by an antiserum, the same explants degenerated, confirming its trophic effect on neurons, as well as its role during the development of the vertebrate nervous system (reviewed in Levi-Montalcini, 1987). Thirty years later, in an attempt to identify new neurotrophic factors, Barde and colleagues (1982) isolated the second neurotrophin from pig brains by a million-fold purification factor. BDNF showed similar properties as NGF in an *in vitro* survival assay with sensory neurons. Around the year 1990, the next two members of the neurotrophin family, NT-3 and NT-4 were discovered by molecular cloning, based on gene sequence homology (Maisonpierre et al., 1990; Hallböök et al., 1991). The first evidence for an effect of neurotrophins on synaptic plasticity came from the laboratory of Poo (Lohof et al., 1993), showing rapid potentiation of cholinergic currents at the *Xenopus* neuromuscular junction after applying BDNF or NT-3 (the milestones of neurotrophin research are reviewed in Squire, 2008).

Since then, numerous studies have confirmed that neurotrophins have important functions in synaptic plasticity, including long-term potentiation (LTP), an experience-dependent, long-lasting increase in synaptic strength that is crucial for learning and memory. Especially BDNF is known to be a crucial mediator of synaptic plasticity in the central nervous system. In the last decade, it emerged that understanding the molecular mechanisms of neuronal BDNF secretion and monitoring the specific actions of secreted BDNF on individual synapses is of fundamental importance to uncover the role of this neurotrophin in synaptic plasticity in more detail (Lessmann, 2003).

1.1. Overview of tissue-specific actions of neurotrophins

Neurotrophins are synthesized and secreted from neurons and from innervated non-neuronal tissues (e.g. muscles, visceral organs). In the nervous system, neurotrophins are also encountered in glial cells (Schwann cells: Verderio et al., 2006; astrocytes: Bergami et al., 2008; microglia: Ulmann et al., 2008; oligodendrocytes: Bagayogo et al., 2009). However, neurotrophins are expressed in virtually all viscera (Katoh-Semba et al., 1996; Lommatzsch et al., 2005). They are notably encountered in endothelial cells (Snapyan et al., 2009), immune T- and B-cells (Kerschensteiner et al., 1999; Fauchais et al., 2008), Langerhans Islets (Rosenbaum et al., 1998; Lucini et al.; 2003), thymus (García-Suárez et al., 2003), testis (Müller et al., 2006; Perrard et al., 2007) and ovarian follicles (Paredes et al., 2004). This presence outside the nervous system reflects the plethora of localized biological roles that neurotrophins can have. In inflammatory or injured tissues, they are secreted by immune cells and endothelial cells to support cell survival as well as angiogenesis (Ulmann et al., 2008). In the pancreas, an autocrine function on hormonal secretion by beta and alpha cells is suggested for NGF and BDNF, respectively. The presence of all neurotrophins in several cell types in the mature testis suggests a supportive role in gametogenesis. Nevertheless, neurotrophins have been best characterized in neuronal tissue. Indeed, the different functions of neurotrophins are essential to the development and the maintenance of both the central and peripheral nervous systems.

1.1.1. Neurotrophins and the development of the vertebrate nervous system

Neurotrophin-deficient mice strains all show neuronal losses. Sources of neurotrophins can direct the growth of sensory and mixed afferents in cell cultures and in organotypic cultures of model embryos (e.g. Tucker et al., 2001). All four neurotrophins are able to elicit chemo-attraction on growing axons, but they are differentially required for individual neuronal populations at different developmental stages. For example, neurons of the dorsal root, trigeminal and sympathetic ganglions depend in majority on NGF supply (Crowley et al., 1994; Smeyne et al., 1994). NGF-deficient mice display severe sensory deficits, together with a complete loss of nociception. BDNF and NT-4 both account for the development of nodose-petrosal ganglions, which control sensory information from cardiovascular, respiratory and gastrointestinal systems (Conover et al., 1995). BDNF is mostly responsible for the development of vestibular neurons (Ernfors et al., 1994a; Jones et al., 1994). NT-3 strongly controls the growth of cochlear

and of proprioceptive Ia afferents. Peripheral motor neurons are less dependent on neurotrophins and particularly individual populations of motor neurons like those targeted by Ia afferents require NT-3 expression (Ernfors et al., 1994b; Fariñas et al., 1994; Klein et al., 1994).

It has been shown that neurotrophin-induced signalling is linked to activity-dependent survival of cortical neurons, by making use of different model systems. BDNF was shown to be implicated in survival of primary cortical neurons, an effect that was enhanced by depolarization (Ghosh et al., 1994). Similarly, inhibition of electrical activity and neurotrophin receptor signalling were both correlated with apoptosis in neonatal cortical slices (Heck et al., 2008). During early development of the CNS, neurotrophins obviously promote proliferation, differentiation and migration of neuronal precursor cells. In prenatal rodent organotypic cultures, tangential migration of cells from the ganglionic eminence to the neocortex is markedly reduced by inhibition of BDNF/NT-4-induced signalling (Polleux et al., 2002). Precursor cells of the neuroepithelium express both, BDNF and NT-3. Using *in utero* transfection methods on this tissue, Bartkowska and co-workers (2007) could disrupt BDNF and NT-3 signalling mechanisms in single proliferating precursor cells. The consequence at embryonic stage E16 was a significant decrease in the number of migrating neural precursors that relied on reduced proliferation and was not due to increased apoptosis. According to these observations, the intracellular signalling mechanisms of BDNF and NT-3 were also found to regulate neurogenesis in the neuroepithelium *in vivo*. Actions of neurotrophins on neuronal precursors continue in the adult brain, where migrating neuroblasts follow blood vessels via a BDNF-dependent signalling mechanism (Snapyan et al., 2009).

In addition to their classical role as trophic factors in neuronal survival, proliferation and differentiation during embryonic and early postnatal development, neurotrophins also regulate dendritic morphology (e.g. McAllister et al., 1996 and 1997; Horch et al., 1999 and 2002), assembly of the cytoskeleton, membrane trafficking, membrane fusion, the formation of synapses, as well as synaptic function in the developing and mature nervous system (reviewed in Huang and Reichardt, 2001; and in Gottmann et al., 2009).

1.1.2. BDNF regulation of synapse formation and developmental synapse maturation

BDNF is one of the key modulators of glutamatergic and GABAergic synapses. The actions of BDNF on synaptogenesis and on neurotransmission have been well investigated. Before axo-dendritic contacts can form, synaptogenesis requires axon

arborization. In dissociated and in organotypic hippocampal cultures, the number of axon branches and of synapses formed by neurons depends strongly on BDNF signalling (Martínez et al., 1998; Vicario-Abejón et al., 1998). The maturation of GABAergic synapses seen in these model systems seems to depend exclusively on BDNF, whereas other neurotrophins could compensate the lack of BDNF to allow the formation of glutamatergic synapses (Vicario-Abejón et al., 1998; Kohara et al., 2007). This is seen especially in BDNF knock-out mice, which have deficits in the development of inhibition, but do not show significant changes in the number of functional glutamatergic terminals (Korte et al, 1995; Patterson et al. 1996; Itami et al., 2003; Abidin et al., 2008; Hong et al., 2008).

1.1.2.1. Presynaptic effects of BDNF in synaptogenesis and synapse maturation

Following the formation of axo-dendritic contacts, it is postulated that target-derived BDNF acts on the presynaptic cells, thereby contributing to the cellular recognition process between the axon terminals and their target cells. In this manner the stabilization of the newborn synapses is thus facilitated (Vicario-Abejón et al., 2002; Gottmann et al., 2009). A strong presynaptic stabilizing effect of BDNF on the differentiation of synaptic vesicle pools, together with an increase in the number of docked vesicles have been reported. These effects were found in cultured neurons chronically exposed to BDNF and are associated with enhanced quantal neurotransmitter release and increased frequencies of AMPA- and GABA-mediated miniature currents (Vicario-Abejón et al., 1998; Tyler and Pozzo-Miller, 2001; Collin et al., 2001; Paul et al., 2001).

Similarly, the maturation of silent synapses can be induced by BDNF. Silent synapses are morphologically inconspicuous glutamatergic synapses, characteristic of the immature neocortex that lack electrophysiologically detectable functional activity. They can either be pre- or postsynaptically silent. In the former case, it is the result of very immature vesicle release properties, thus limiting neurotransmitter release. In the latter case, fully developed presynaptic vesicle pools are present, but if stimulated when the postsynaptic neuron is close to the resting potential, there are no postsynaptic responses. This is due to missing postsynaptic AMPA receptors and because of the Mg^{2+} block on NMDA receptors at hyperpolarized membrane potential. Functional vesicle cycling is absent in presynaptically silent synapses, but can be induced by enhanced activity. Using the BDNF scavenger TrkB-Fc (compare Shelton et al., 1995) or BDNF knock-out

in early postnatal neocortical neurons revealed an important role of BDNF in regulating maturation of presynaptic vesicle cycling (Walz et al., 2006).

1.1.2.2. Postsynaptic effects of BDNF in synaptogenesis and synapse maturation

In addition to having presynaptic effects, BDNF application can affect glutamatergic postsynaptic sites by regulating the number and distribution of AMPA and NMDA receptors (Caldeira et al., 2007a/b). The conversion of postsynaptically silent synapses into functional ones through membrane insertion of AMPA receptors has been proposed to involve postsynaptic autocrine BDNF signalling in the developing mouse barrel cortex (Itami et al., 2003). The dynamic changes in the trafficking of AMPA and NMDA receptors seen at postsynaptically silent synapses can either strengthen or weaken synaptic transmission and are also important for hippocampal LTP and LTD (Liao et al., 1995; Isaac et al., 1995; Shi et al., 1999). An involvement of BDNF has been established in early events during synaptic maturation and in synaptic plasticity. Although much progress has been achieved for the understanding of these phenomena, the exact pre- and postsynaptic mechanisms of BDNF action remain to be elucidated in many cases (reviewed in Gottmann et al., 2009).

1.2. Neurotrophin expression and receptor signalling

1.2.1. Evolution and regulation of neurotrophin genes

1.2.1.1. Evolution of neurotrophin genes

The role of neurotrophins in the establishment of complex and well-adapted vertebrate nervous systems may be reflected by the evolution of their genes. The family of vertebrate neurotrophin genes most probably derived from duplications of a common ancestral gene. Thus, NGF, BDNF, NT-3 and NT-4/5 are considered as paralogues. The four neurotrophin paralogues are found in most classes of vertebrates, but some differences exist between classes in the “set” of neurotrophins available. Notably, NT-4/5 seems to be absent in birds. NT-6/7, found in bony fishes and not in other vertebrates, is considered an orthologue of NGF, because it evolved from a common ancestral neurotrophin gene by means of speciation. Two atypical neurotrophin orthologues, Lf-NT and Mg-NT, were characterized in primitive, jawless fishes (Hallböök et al., 2006). In invertebrates, nucleotide sequence orthologues of neurotrophins have been identified in the sea urchin and the acorn worm, whereas sea

squirt species appear to lack such orthologues. These findings suggest that ancestral neurotrophins were already present at the beginning of the evolution of deuterostomes, but were lost in tunicates (Fig. 1.1). The absence of homologues of neurotrophins and of their receptors in sea squirts evolved possibly from their sessile life mode, which limits requirements to their nervous systems (Bothwell et al., 2006). The recent discovery of genes distantly related to mammalian neurotrophins in *Drosophila* provide new arguments for the hypothesis of an existing neurotrophin signalling system in the last shared ancestor of protostomes and deuterostomes, *Urbilateria*. The proteins encoded by these *Spätzle* genes (Spz) are structurally related to vertebrate neurotrophins. Some of these genes were recently renamed Drosophila neurotrophins because their protein products present neurotrophic properties (Zhu et al., 2008; Sutcliffe et al., 2013).

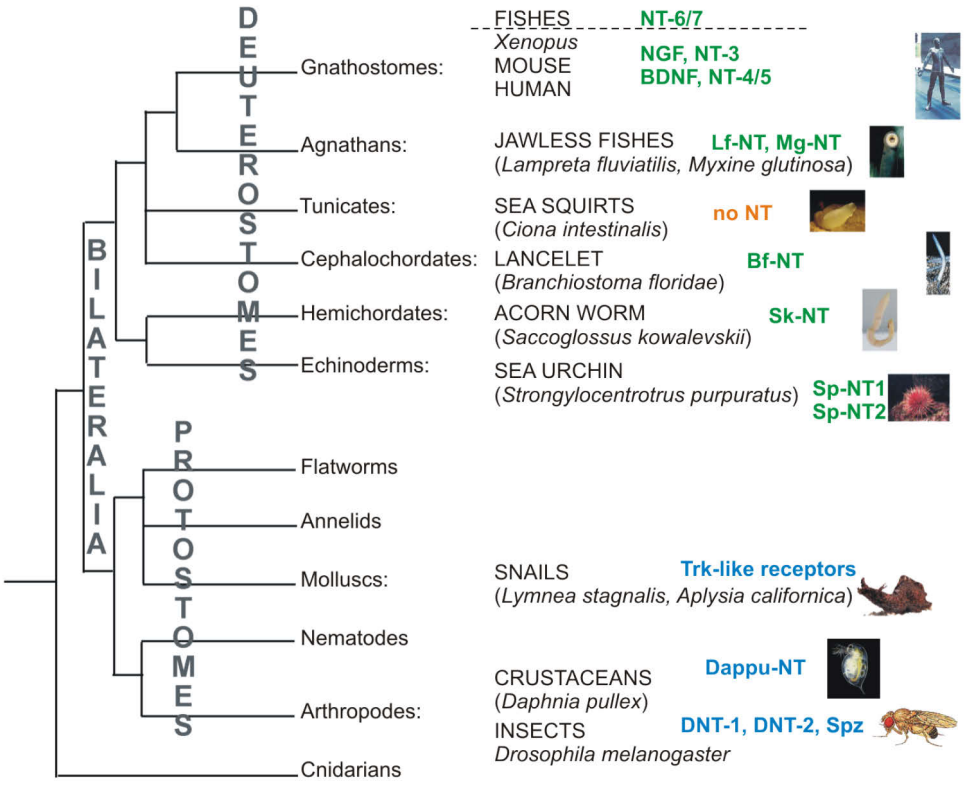

Figure 1.1: Schematic phylogenic tree of animal evolution presenting the known neurotrophins. The names of neurotrophin homologues characterized in deuterostomes are highlighted in green. Neurotrophins are missing and thought to have been lost in tunicates, represented by *Ciona*. Neurotrophin- and neurotrophin receptor-related sequences found in protostomes are highlighted in blue. Receptors distantly related to vertebrate Trks are present in molluscs, represented by *Aplysia*. Neurotrophin-related sequences have been found in the insect *Drosophila* and more recently in *Daphnia* and other species of crustaceans (adapted from: Halböök et al., 2006; Zhu et al., 2008; Wilson, 2009).

1.2.1.2. Transcriptional and posttranscriptional regulation of the BDNF gene

The overall genomic organization of neurotrophins is similar in all vertebrates. Neurotrophin genes have one large major exon encoding the entire neurotrophin precursor with N-terminal signal peptide, prepro-NT. The 3´ major exon is preceded by one or several smaller 5´ non-coding exons. The number of non-coding exons is at least seven in rodents and eleven in humans (Fig. 1.2). Because many exons have unique promoters, the rodent and the human BDNF loci can possibly give rise to at least 11 and 17 alternatively spliced transcripts, respectively (Timmusk et al., 1993 and 1995; Aid et al., 2007; Pruunsild et al. 2007). They all encode for the same mature BDNF protein, but additional translation start codons could lead to extended N-terminal sequences with alternative signal peptide upstream of the canonical prepro-BDNF sequence. Also, the use of an alternative polyadenylation site in the 3´ exon results in *Bdnf* mRNAs with long and short 3´ untranslated regions (3´UTRs) and consequences on translation that will be mentioned later on.

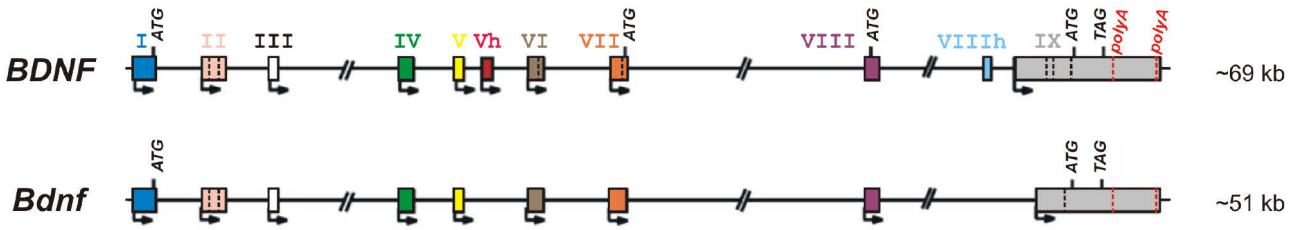

Figure 1.2: Rodent *Bdnf* and human *BDNF* gene structures. Rodent *Bdnf* exons homologous to human *BDNF* exons are shown as colored boxes in the same colour for both genes. Introns are shown as black lines. Arrows indicate transcription start sites. ATG and TAG mark the position of the translational start and stop codons, respectively. Black dashed lines in exons indicate additional splicing sites. Red dashed lines in exon IX indicate alternative polyadenylation sites (adapted from Pruunsild et al., 2007).

The BDNF gene transcripts are differentially expressed during development and across tissues. Exon I-, II-, IV- and VI-containing BDNF gene transcripts are the most highly expressed in the adult brain (Aid et al., 2007; Pruunsild et al. 2007).

In mammalian neurons, a variety of mechanisms tightly control expression of BDNF. At the transcriptional level chromatin remodelling (Timmusk et al., 1999; Zuccato et al., 2003; Lubin et al., 2008), tissue-specific promoters with diverse response elements and transcription factors binding to these elements allow for sophisticated regulation (Timmusk et al., 1993 and 1995; CREB: Shieh et al., 1998; USF1/2: Tabuchi et al., 2002; CaRF: Tao et al., 2002; Pruunsild et al., 2011). It is well established that neuronal activity plays an important role in these mechanisms and the expression of BDNF gene

transcripts can increase markedly after a few hours of depolarization or enhanced neurotransmission (Zafra et al., 1990 and 1992). For example, mouse and human BDNF gene transcripts bearing exon I and IV are expressed under the activity-dependent binding of the transcription factors CREB, USF1/2, ARNT2 and NPAS4 to their respective response elements in promoter regions I and IV, as shown in rat cortical cultures (Pruunsild et al., 2011). Notably, upregulation of BDNF gene promoters after depolarization of cortical neurons through calcium-mediated pathways has been reported. Upstream of exon IV, promoter IV is upregulated via binding of the transcription factors CREB and CaRF to the calcium response elements, CRE and CaRE1, respectively (Shieh et al., 1998; Tao et al., 2002). Mutation of CRE can impair activity-inducible transcription from promoter IV in transgenic mice, suggesting that CREB has a key role in the assembly of a multifactorial transcription complex at *Bdnf* promoter IV (Fig. 1.3; Hong et al., 2008).

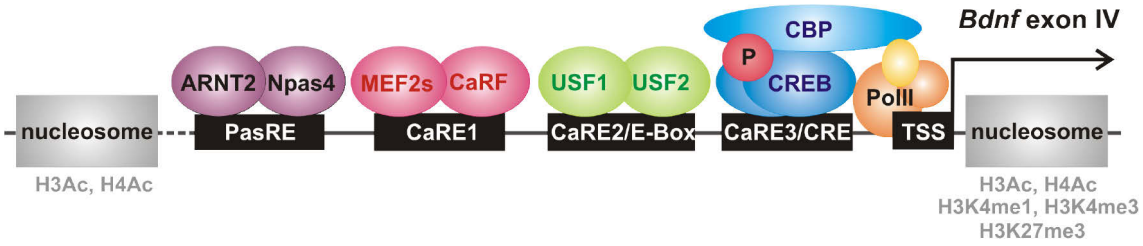

Figure 1.3: Transcription factors associated with the response elements inducing *Bdnf* promoter IV (West, 2011). The line shows an expansion of the region upstream of *Bdnf* exon IV. The black boxes indicate the transcription start site (**TSS**), the calcium response elements (**CaRE**) and the bHLH–PAS transcription factor response element (**PasRE**). The response elements are found within a nucleosome-free region of the proximal promoter, flanked by two well-positioned nucleosomes. The H3 and H4 histones in these nucleosomes are modified by acetylation (Ac) and methylation (me1 or me3) at specific lysine residues (Musumeci and Minichiello, 2011). Aryl hydrocarbon Receptor Nuclear Translocator protein 2 (**ARNT2**) and Neuronal Per-Arnt-Sim Domain Protein 4 (**NPAS4**) bind to PasRE (Pruunsild et al., 2011). Calcium-Response Factor (**CaRF**) and Myocyte Enhancer Factor 2 family members (**MEF2s**) bind to CaRE1 (Lyons, Schwarz and West, 2012). Upstream Stimulatory Factors (**USF-1/2**) and Cyclic AMP –responsive element-binding proteins (**CREB**) bind respectively to CaRE2 and CaRE3 (Scheme adapted from West, 2011; Lubin et al., 2008; Lyons and West, 2011).

Like many genes involved in neuronal development, the transcription of the BDNF gene is also subject to epigenetic regulation via activity-dependent chromatin remodelling and DNA methylation. Modifications of histones by methylation of lysine residues and deacetylation induce changes in the secondary structure of chromatin nucleosomes that regulate the availability of DNA elements for transcription factor binding. Methylation of cytosine residues in CpG dinucleotides has the same effect on *Bdnf* transcription, by

recruiting the transcriptional repressor MeCP2 to so-called CpG islands (Lubin et al., 2008). In primary neurons and in intact brain at rest, MeCP2 inhibits *Bdnf* transcription, but neuronal activity leads, via calcium signalling, to a phosphorylated form of MeCP2 that is released from promoter IV, thereby permitting *Bdnf* promoter IV–dependent expression (Chen et al., 2003; Zhou et al., 2006).

At the posttranscriptional levels of expression, the relations of the different BDNF gene transcripts to the different functions of the BDNF protein remain poorly understood, but other mechanisms of regulation do exist. In human, but not in rodent, the *BDNF* locus contains an *antiBDNF* or *BDNF-AS* gene that produces several antisense transcripts. *antiBDNF* transcripts are thought to regulate BDNF synthesis by forming dsRNA duplexes with the *BDNF* transcripts (Liu et al., 2005; Pruunsild et al., 2007). Also, as mentioned above, the length of 3´UTRs directs the translation and subcellular localization of *Bdnf* mRNAs. The short 3´UTR is carried by the majority of *Bdnf* transcripts, which are restricted to the soma and are the primary source for BDNF synthesis. The long 3´UTR is responsible for targeting *Bdnf* mRNA into dendrites for local translation. Notably, translation from the long 3´UTR mRNA is activity-dependent and fast. At basal levels of neuronal activity, the long 3´UTR causes mRNA to form translationally repressed granules with localizing ribonucleoproteins. This mechanism is proposed to control further actions of BDNF to adapt neuronal function in response to elevated activity (An et al., 2008; Lau et al., 2010). Another posttranscriptional regulation of expression could be that prepro-BDNF isoforms with different N-terminal signal peptides are produced, according to *in silico* predictions for rodent exon I and IX and human exons I, V, VII and IX. Still is it unknown if such isoforms undergo cell-specific trafficking that could lead to differential maturation of the BDNF protein (Aid et al., 2007; Pruunsild et al. 2007).

1.2.2. Structure, dimerization and processing of neurotrophins

The mature mammalian neurotrophins NGF, BDNF, NT-3 and NT-4 are small homodimeric proteins with a molecular weight of approximately 14 kDa per monomer. On the basis of the structural motif they share, they have been classified as a subfamily in the superfamily of cystine knot cytokines. Each monomer chain contains four anti-parallel ß-strands, separated by three ß-hairpin loops (Figure 1.4). Three intrachain disulfide bridges form the cystine knot, providing considerable structural stability to the neurotrophins. The additional NH_2-terminal pro-domain in immature pro-neurotrophins

is devoid of a well-defined tertiary structure. The pro-domain takes a compact globular conformation and could protect the protein from proteolytic digestion, so participating in protein half-life regulation (Paoletti et al., 2011).

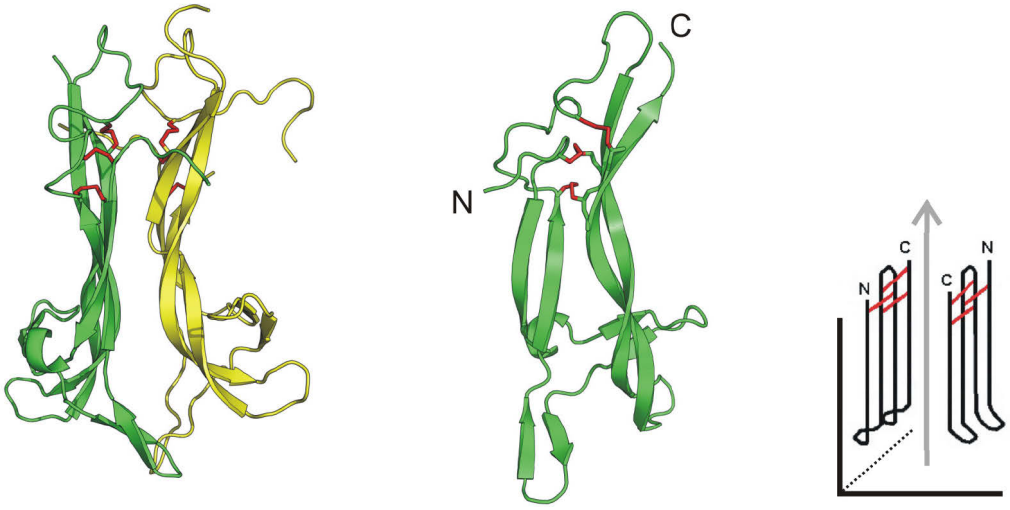

Figure 1.4: Structure of the neurotrophin dimer. **Left**: Front view of the BDNF/ NT-4 heterodimer. **Middle**: view of the hydrophobic side of the BDNF monomer. **Right**: Scheme of monomers' orientation in a neurotrophin dimer (fig. 4 in: Sun and Davies, 1995); the molecular symmetry axis of the dimer is represented by an arrow. N and C label respectively the NH_2- and carboxy-end. The knotted disulfide bonds are represented by red sticks in all 3 sketches. To date, the tertiary structure of BDNF and pro-BDNF can only be extrapolated from the structural analysis of heterodimers or from the other neurotrophins. The BDNF/NT-4 heterodimer corresponds to the structure published by Robinson et al. (1999) and available under the PDB entry 1B8M at: http://www.ebi.ac.uk/pdbe/. For modeling, we used the PyMOL Molecular Graphics System (Version 1.3 Schrödinger, LLC) available at: http://pymol.org.

Neurotrophins dimerize in a head-to-head orientation of the monomers (Figure 1.4). The dimer interface consists mainly of hydrogen bonds and of hydrophobic interactions. The hydrophobic residues are structurally conserved, allowing the formation of heterodimers. Although bioactive heterodimers of BDNF/pro-NT-3, BDNF/NT-3 and BDNF/NT-4 are easily formed *in vitro*, their biological role *in vivo* still remains uncertain since dimerization would require simultaneous expression of the different neurotrophins (Sun and Davies, 1995; Robinson et al., 1999; Farhadi et al., 2000; Hibbert et al., 2003). The dimerization can take place spontaneously in the endoplasmic reticulum, after cleavage of the short signal peptide and N-glycosylation of the pro-domain. N-glycosylation of nascent peptides through oligosaccharyltransferase participates to quality control of protein folding in the ER (reviewed in Moremen, Tiemeyer and Nairn, 2012).

Subsequently, pro-neurotrophins can undergo further posttranslational modifications on their way through the Golgi apparatus and the trans-Golgi network, before ending up in secretory vesicles. The maturation process of neurotrophins is not entirely characterized yet, but some features can be emphasized according to the classical concept of protein processing: 1) the pro-domain can be cleaved off by various endoproteases. Especially the pro-protein convertases PC1 and PC2, and the membrane-bound furin can execute this step in neurons, either in the Golgi lumen, in the trans-Golgi network or in secretory vesicles (reviewed in Seidah and Prat, 2012); 2) Exopeptidases are expected to process NH_2- and carboxy-ends of neurotrophins. Carboxypeptidase E is a classic exopeptidase, which membrane-bound form serves as a sorting receptor that facilitates targeting to the regulated pathway of secretion (Lou et al., 2005; Park et al., 2008);. 3) NH_2-terminal acetylation and carboxy-terminal α-amidation of neurotrophins in secretory granules remain elusive, although the vast majority of proteins undergo these processes. N-terminal acetylation can occur co-translationally or post-translationally and participates in protein half-life regulation (Bradshaw et al., 1998; Varshavsky, 2011). C-terminal α-amidation is thought to enhance bioactive potency by rendering secreted peptides more hydrophobic (reviewed in Eipper et al., 1993).

This classical view of protein processing has been challenged by the detection of uncleaved pro-neurotrophins released in the extracellular space (Lee et al., 2001; Mowla et al., 2001). Several clues indicate that the pro-domain has not just a function of protecting the protein from proteolysis, but that it also changes subcellular protein targeting and bears information for activation of the pro-apoptotic signalling (e.g. Lee et al., 2001; Brigadski et al., 2005). Therefore, knowing the site of pro-neurotrophin cleavage under physiological conditions is essential to understand neurotrophin action. After exocytosis, pro-neurotrophins can undergo cleavage by extracellular endoproteases, including the plasmin and diverse matrix-metalloproteinases (Lee et al., 2001; Pang et al., 2004; Bruno and Cuello, 2006). In secondary cell lines overexpressing BDNF, the uncleaved pro-BDNF is released together with mature BDNF, possibly because the levels of the protein saturate the capacity of processing of these cells (e.g. Heymach et al., 1996; Haubensak et al., 1998; Mowla et al., 2001; Chen et al., 2004). In primary neurons, endogenous pro-BDNF can be detected in intracellular compartments, showing a rapid conversion to the mature protein, without quantifiable levels of released pro-BDNF. Specifically, using hippocampal cells from knock-in mice expressing myc-tagged BDNF instead of native BDNF, Matsumoto and colleagues

(2008) could not detect pro-BDNF in the supernatant of those cells. However, in another study using a similar model of knock-in mice, prominent pro-BDNF immunoreactivity was detected in the supernatant of the primary cells (Yang et al., 2009).

It remains controversial which form of neurotrophins is preferentially released by neurons *in vivo*. Cell culture conditions might have been decisive in the two studies mentioned above. For instance, glial cells could also provide for extracellular exopeptidases or even release pro-BDNF themselves. Nevertheless, the contribution of both forms of BDNF to long-term plasticity are thought to be opposed, with endogenous pro-BDNF inducing long-term depression of synaptic potentials (Woo et al., 2005), whereas mature BDNF induces long-term potentiation after extracellular cleavage of pro-BDNF through plasmin (Pang et al., 2004). As the type of neurotrophin receptor activated appears to be instrumental for the form of synaptic plasticity evoked (Minichiello et al., 2002; Rösch et al., 2005), the physiology of BDNF processing and the resulting BDNF protein involved in plasticity remain elusive (reviewed in Lessmann and Brigadski, 2009).

1.2.3. Signal transduction through Trk receptors and p75^{NTR}

The pleiotropic nature of neurotrophins depends on the multiplicity of receptors and signalling pathways they can activate. A dual receptor system composed of p75^{NTR} and Trk receptors controls responsiveness to NGF, BDNF, NT-3 and NT-4. Related to the family of TNF receptors, p75^{NTR} binds with equal affinity to all neurotrophins. The tyrosine kinase receptors of the Trk family exhibit ligand selectivity. NGF binds preferentially to TrkA, BDNF and NT-4 bind preferentially to TrkB and NT-3 binds to TrkC. NT-3 can bind with lower affinity to TrkA and TrkB. This simplified view is counterbalanced by the possibility of p75^{NTR} acting as a co-receptor for Trk receptors. In this case, the ligand selectivity of Trk receptors is enhanced. Uncleaved pro-neurotrophins bind with higher affinity to p75^{NTR} than mature neurotrophins and pro-neurotrophins can activate p75^{NTR}, leading to apoptosis in neurons. These effects have been shown at least in sympathetic, cortical and hippocampal neurons (Bamji et al. 1998; Friedman, 2000; Lee et al., 2001; Brann et al., 2002; Palmada et al., 2002; Song et al., 2010). The association of the co-receptor, sortilin, with p75^{NTR} has been shown as essential for apoptosis induction following pro-neurotrophin binding (Nykjaer et al., 2004; Teng et al., 2005).

Canonical full-length Trk receptors are primarily activated by neurotrophin binding, leading first to the dimerization of the receptors. Subsequent autophosphorylation of cytoplasmic tyrosine residues creates recruitment sites for adaptor proteins that mediate initiation of three main signalling cascades (for detailed reviews on neurotrophin receptor signalling see: Huang and Reichardt, 2003; Segal, 2003). Phosphotyrosine-515 on TrkB (in mouse and rat, Y516 in humans) and analogous sites on TrkA (Y496) and TrkC (Y516) interact with Shc, Frs2 and other adaptor proteins, which provide mechanisms for activation of Ras/Raf/Erk and PI3-kinase/Akt signalling pathways. The activity of the PI3-kinase/Akt signalling cascade inhibits the biological function of various pro-apoptotic proteins. The neurotrophin-induced activation of the Ras/Raf/MEK/Erk signalling pathway promotes mainly neuronal differentiation and prolonged survival of neurons (Riccio et al., 1999). Phosphotyrosine-816 on TrkB (mouse and rat, Y817 in humans) and similarly positioned sites on TrkA (Y791) and TrkC (Y834) recruits and activates directly PLC-γ1. TrkB-mediated PLCγ-1/PKC signalling exerts an important influence on neurite outgrowth (Stephens et al, 1994) as well as on synaptic plasticity (Minichiello et al., 2002).

The extracellular ratio between neurotrophins and pro-neurotrophins could be decisive for the fate of a cell. An excess of unprocessed neurotrophins would lead to the activation of the $p75^{NTR}$/sortilin complex with effects opposing the activation of TrkB (associated to $p75^{NTR}$). This suggests that regulation of neurotrophin processing could be the bottleneck of neurotrophin action (compare Le and Friedman, 2012; reviewed in Schweigreiter, 2006).

1.3. The regulation of synaptic plasticity by BDNF in mammals

1.3.1. BDNF regulation of short-term plasticity

Synaptic plasticity can be defined as a use- or activity-dependent change in the amplitude of synaptic potentials. Short-term forms of synaptic plasticity, like augmentation, paired-pulse facilitation (PPF) and post-tetanic potentiation (PTP), are sensitive to the presence of neurotrophins.[1] This was first shown by Poo and co-workers

[1] "At most synapses, repetitive high frequency stimulation […] initially is dominated by a [fast] growth in successive [postsynaptic potential] amplitudes [within about 1 s], called facilitation. […] when two stimuli are given with very close spacing, the second [response] can be as much as twice the amplitude of the first. This is called paired-pulse facilitation. At most synapses, a slower phase of increase in efficacy […] called augmentation, succeeds facilitation [within several seconds]. Finally, some synapses display a third phase of growth in [postsynaptic potential] amplitude that last minutes [called potentiation]. Potentiation often is visible in isolation only long after a tetanus and is thus called post-tetanic potentiation." (Squire, 2008, p176).

at the cultured *Xenopus* neuromuscular junction (Lohof et al., 1993). Exposure to BDNF and NT-3, but not NGF, could enhance evoked cholinergic transmission within 10 minutes. In light of many synaptic effects found on mammalian neurons thereafter, it was soon proposed that repetitive synchronous neuronal activity can enhance the expression, secretion and actions of neurotrophins at synapses to modify synaptic strength and thus can provide a causal connection between neuronal activity and synaptic plasticity (Bonhoeffer, 1996).

In brain slices and in cultures of dissociated neurons, acute application of BDNF, and to different degrees NT-3 and NT-4, was shown to rapidly enhance the strength of glutamatergic synapses (Lessmann et al., 1994; Kang and Schuman, 1995; Scharfman, 1997; Carmignoto et al., 1997; Lessmann and Heumann 1998; Li et al., 1998b). The application of BDNF or NT-3 was accompanied by reduced PPF, a mechanism of presynaptic origin, but there is evidence that both, presynaptic and postsynaptic effects of neurotrophins on glutamatergic synapses co-exist (Levine et al., 1995; Akaneya et al., 1997). In addition, catalytic TrkB receptors are localized at both pre- and postsynaptic sites (Drake et al., 1999; Aoki et al., 2000), supporting a role of BDNF signalling at both synaptic sites. BDNF was shown to increase the amplitude of evoked EPSCs (Lessmann et al., 1994; Lessmann and Heumann, 1998; Berninger et al., 1999; Schinder et al., 2000; Tyler et al., 2006), as well as the frequency, but not the amplitude, of miniature EPSCs (Lessmann et al., 1994; Lessmann and Heumann, 1998; Li et al., 1998b; Collin et al., 2001; Tyler and Pozzo-Miller, 2001), suggesting a presynaptic site of action. In addition, variance analysis of evoked EPSCs strongly supported this assumption (Lessmann and Heumann, 1998). Meanwhile, presynaptic effects by endogenous BDNF have been confirmed (Walz et al., 2006; Tyler et al., 2006; Shen et al., 2006; Magby et al., 2006). In line with these findings, deletion of the BDNF gene leads to pronounced synaptic fatigue, fewer docked synaptic vesicles, and reduced expression levels of presynaptic markers (Figurov et al., 1996; Gottschalk et al., 1998; Pozzo-Miller et al., 1999). The mechanism responsible for NT-induced presynaptic changes in transmitter release has been attributed to post-translational modifications of the presynaptic secretion machinery. Thus, presynaptic, but not postsynaptic overexpression of dominant-negative TrkB receptors abolishes the transient potentiation induced by BDNF (Li et al., 1998a). Hippocampal neurons with a mutated form of the vesicular trafficking protein, Rab3a, exhibit a complete impairment of the acute response to BDNF (Thakker-Varia et al., 2001). Consistent with presynaptic

modulation, it was shown that BDNF-induced enhancement of PTP implies an increase of the releasable pool of neurotransmitter vesicles through ErK-dependent phosphorylation of synapsin I (Jovanovic et al., 2000; Valente et al., 2012). Also, the actin motor protein, Myo6, is activated by TrkB via association with the adaptor protein GIPC1, during BDNF-induced neurotransmitter release (Yano et al., 2006). Overall, these data validate that proteins regulating the transport and the recycling of synaptic vesicles are targeted by presynaptic BDNF/TrkB signalling, thus strengthening glutamate release.

Acute BDNF application can also affect postsynaptic responses. BDNF regulates both the number and distribution of AMPA and NMDA receptors inserted in the membrane (Caldeira et al., 2007a/b). In hippocampal neurons, BDNF triggered a selective enhancement of the NMDA component of glutamate currents through phosphorylation of the NMDA receptor subunit, NR2B (Lin et al., 1998; Crozier et al., 1999). The regulation of the NR2B subunit by BDNF/TrkB has been shown to involve the PTP1D phosphatase, the Src protein family kinase Fyn, CaMKII and to lead to an increased opening probability of the NMDA receptor (Lin et al., 1999; Xu et al., 2006). The postsynaptic modulation of the glutamate current depends on the activity state of the postsynaptic cell and on endogenous BDNF as well, characterizing it as a permissive effect (Crozier et al., 2008).[2] However, acute application of BDNF was able to also directly activate postsynaptic Ca^{2+} currents, concomitantly inducing LTP in the dentate gyrus of hippocampal slices (Kovalchuk et al., 2002). The BDNF-induced postsynaptic Ca^{2+} influx seen here originated most probably from NMDA receptors, but a role for TRPC channels in the mediation of BDNF/TrkB signalling was shown in different neuronal preparations as well (Li et al., 1999; Li et al., 2005; Jia et al., 2007). In hippocampal cells, the activation of TRPC3 channels by exogenous BDNF was able to elicit a slow rising Ca^{2+} current that was dependent on TrkB, PLC and intact intracellular Ca^{2+} stores (Amaral and Pozzo-Miller, 2007). Locally applied and endogenous BDNF induces synaptic Ca^{2+} signals that are also dependent on active voltage-gated Na^{+} channels but the subtype of channels involved in this signalling remains undetermined to date (compare Lang et al., 2007). In the hippocampal CA1 area, cumulative evidence shows fast activity-dependent postsynaptic effects of BDNF

[2] Neurotrophins can have either an instructive or permissive role in activity-dependent synaptic modification in developing and adult brains. The instructive role means that modification is a consequence of NTs acting at the synapse to directly modify presynaptic transmitter release, postsynaptic sensitivity or synaptic morphology. The permissive role means that modification is induced by other factors in association with neuronal activity, whereas NTs carry out housekeeping functions that are necessary for the modification of the synapse (Bonhoeffer, 1996 and 1998; but see Lessmann, 1998).

on the cytoskeleton and the morphology of dendritic spines (Rex et al., 2007; Tanaka et al., 2008). By monitoring F-actin labelling during theta burst stimulation, Rex and colleagues (2007) could demonstrate BDNF-dependent changes of spine cytoskeleton at the onset of LTP. Tanaka et al. (2008) found that release of endogenous BDNF associated to NMDAR activation contributes to spine head volume increase. The modified pairing stimulation model used herein is assumed to favour the induction of hippocampal LTP in similar experimental conditions.[3] As changes in synaptic strength, like LTP, are often associated with changes in the shape of dendritic spines, the results of above functional studies suggest a mechanistic role of BDNF in LTP through restructuring of dendritic spines.

1.3.2. BDNF and LTP

The long-term adaptive synaptic processes LTP and LTD are persistent changes in synaptic strength elicited by specifically patterned stimuli. They can last for hours, even days, and are believed to take a central part not only in memory formation, but also in drug addiction, mood stability and chronic pain. Like LTP at the hippocampal CA3-CA1 synapse, most forms of LTP are associative and NMDA receptor-dependent.[4] The course of LTP is usually described by induction, early-LTP (E-LTP, lasting one to two hours), followed by late-phase LTP (L-LTP). E-LTP is protein synthesis-independent whereas L-LTP develops only through *de novo* synthesis of mRNA and proteins and with structural synaptic changes.

Depletion of BDNF in studies using gene knock-outs or inhibitors of BDNF/TrkB signalling has shown that BDNF is significantly involved in the expression of LTP in the hippocampal CA1 region (Korte et al., 1995, 1996; Figurov et al., 1996; Patterson et al., 1996; Pozzo-miller et al., 1999), in layers II-III of the visual cortex (Abidin et al., 2006), in the somatosensory cortex (Itami et al., 2003), in the striatum (Jia et al., 2010), and recently also in the lateral amygdala and the hippocampal CA3 area (Meis et al., 2012; Schildt et al., 2013). Otherwise, application of exogenous BDNF has reliably shown to induce long-term enhancement of glutamatergic transmission in the hippocampal CA1 (Kang and Schuman, 1995 and 1996), in the dentate gyrus ("BDNF-LTP": Messaoudi et al., 1998) and the developing visual cortex (Jiang et al.,

[3] The pairing protocols consist of simultaneous stimulation of presynaptic fibres with lasting or intermittent depolarization of the postsynaptic cell. They differ from historical high frequency protocols which consist of solely presynaptic activation, like e.g. tetanic stimulation (1 s at 100 Hz) or theta burst stimulation (4 bursts at 100 Hz repeated 10 times or more).

[4] Associativity of LTP refers to the observation that when a tetanic stimulation of a single input is insufficient for the induction of LTP, simultaneous stimulation of a stronger input will induce LTP at both inputs. Associativity is possibly a manifestation of a Hebbian mechanism taking place at the CA3-CA1 synapse (Squire, 2008, p1142-1143).

2001). These different approaches have revealed very diverse contributions of BDNF/TrkB signalling to LTP. Depending on the stimuli used, on the neuronal circuit and on the time window observed, LTP expression can either be completely, or partially reliant on the BDNF/TrkB pair (reviewed in Bramham and Messaoudi, 2005; Gottmann et al., 2009; Edelmann et al., 2013). Therefore, in order to elucidate the exact function of BDNF in LTP, it is necessary to discriminate between permissive/ modulatory[5] and instructive/ mediatory actions of endogenously released BDNF, and between effects of endogenous BDNF on different phases and forms of LTP. Normal BDNF/TrkB signalling at the CA3-CA1 synapse prevents synaptic fatigue, a reduction in EPSP amplitude observed in response to consecutive stimuli during high frequency stimulation (HFS, see Figurov et al., 1996). A presynaptic origin of this effect was assumed by Xu and colleagues (2000), who observed a reduction of HFS-induced CA1 E-LTP equivalent in CA1 conditional TrkB knock-outs and in transgenic mice with globally decreased TrkB expression. A further decrease in CA1 E-LTP in this latter strain was obtained by application of a scavenger of endogenous BDNF, TrkB-IgG. By using a stimulus weaker than HFS, a so-called pairing protocol, the authors could show that BDNF/TrkB signalling can be dispensable in the induction of LTP (Xu et al., 2000). This finding illustrates well a permissive presynaptic effect on E-LTP that relied on changes in basal glutamatergic transmission. This permissive effect of BDNF was similarly seen by direct comparison of the LTP outcome after different stimulus frequencies in BDNF knock-outs and wild type mice and also in induction of PTP (200 Hz vs. 50 Hz: Zakharenko et al., 2003; but compare Valente et al., 2012).

The impairment of CA1 LTP found in homozygous $trkB^{cre-/cre-}$ and $trkB^{PLC-/PLC-}$ mice strains suggested an instructive role of BDNF (Minichiello et al., 2002). The localization of endogenous BDNF signalling herein was not questioned at first, but specifically inhibition of BDNF/TrkB/PLCγ signalling at both pre- and postsynaptic sites disrupted LTP almost entirely, with a significant reduction of E-LTP and L-LTP. Local expression of dominant negative variants of PLCγ at either pre- or postsynaptic neurons alone did not reduce LTP, indicating mixed pre- and postsynaptic BDNF/TrkB signalling at CA3-CA1 synapses (Gärtner et al., 2006). By using a pairing protocol, exogenous BDNF application, as well as BDNF/TrkB inhibitors, Mohajerani et al. (2007) showed that a presynaptic component of E-LTP was dependent on presynaptic

[5] "Mediators differ from modulators in that their function is directly influenced by the electrical activity that induces LTP, whereas modulators could regulate the processes and mediators of LTP but their level or functions are not necessarily regulated by the LTP-inducing activity." (Park and Poo, 2013)

BDNF/TrkB signalling and that activation of postsynaptic Erk was taking place simultaneously downstream of BDNF/TrkB. The view of co-existing pre- and postsynaptic instructive effects of BDNF in LTP has been reinforced as well by studies in other circuits (see Edelmann et al., 2013).

Also, strong lines of evidence point out to a limited time window for the presence of endogenous BDNF in the synaptic cleft to induce LTP at hippocampal CA1 (e.g., Kang et al., 1997, Kossel et al., 2001). The use of locally photoactivable caged antibodies against BDNF allowed defining this period at ± 2 min around the HFS preceding LTP. Although this effect, seen by Kossel and colleagues (2001) was modest, BDNF was shown to have a fast-acting instructive role in the expression of E-LTP. By the use of different BDNF scavengers, Kang and colleagues (1997) could show that stable L-LTP requires intact BDNF/TrkB signalling in the period between 30 and 60 min after the HFS inducting LTP, but not later.

Nevertheless, the importance of timing seen for the temporal availability of BDNF, as well as for the stimulation pattern on LTP expression and maintenance underline the need for more physiology-related stimuli in the study of LTP. If BDNF is to function as a trigger of LTP, we have to take into account that the necessary amount of secreted BDNF must be tightly regulated by activity at the associated synaptic sites. As BDNF can be released from single hippocampal cells upon certain electrical patterns (e.g. Hartmann et al., 2001; Kuczewski et al., 2008), more observations are to come from studies using so-called spike-timing dependent protocols, which have been able to induce LTP efficiently in many brain regions (reviewed in Edelmann et al., 2013, Edelmann et al., 2015, in press).

1.4. What do we know about BDNF exocytosis?

BDNF can reasonably be considered a key mediator and modulator of synaptic plasticity in the central nervous system. To date, our knowledge of the exact location and time point of action of BDNF is surprisingly modest. We have to understand the molecular mechanisms of BDNF secretion and monitor the specific actions of secreted BDNF on individual synapses in order to uncover the role of BDNF in synaptic plasticity in more detail (see Lessmann, 2003).

1.4.1. Cellular targeting and transport of BDNF

Newly synthesized BDNF can be sorted into at least two distinct types of secretory vesicles. Vesicles of the constitutive pathway of secretion are formed continuously and release their cargo by default when reaching the plasma membrane. Granules of the regulated pathway of secretion, the so-called dense core vesicles (DCVs), release their cargo following a stimulus, which can consist of electrical activity, glutamate application, or depolarization (reviewed in Burgoyne and Morgan, 2003). In neurons, NGF and NT-4 are preferentially targeted to the constitutive secretory pathway, whereas BDNF and NT-3 are targeted most efficiently to the regulated pathway (Wu et al., 2004; Brigadski et al., 2005). The information contained in the pro-sequences necessarily affects the cellular targeting of neurotrophins. The replacement of the pro-sequence of NT-4 by the pro-sequence of BDNF redirects NT-4 to the regulated secretory pathway in primary neurons. Similarly, a naturally occurring valine to methionine mutation in BDNF, at the position 66 of the pro-domain, impairs targeting and regulated release of BDNF in hippocampal neurons (Egan et al., 2003; Chen et al., 2004; Jiang et al., 2009). Recently, a role for the Val66 mutation could be postulated in binding to a protein complex assembling Huntingtin associated protein 1 (HAP1) and the sorting receptor, sortilin. Indeed, there is evidence for existing interactions between HAP1 and the residues of proBDNF at position 65-90 (Wu et al, 2010; Yang et al., 2011). Normal Huntingtin (htt) promotes the transport of BDNF-containing vesicles along microtubules. Interestingly, mutated htt, which causes Huntington disease, impairs regulated secretion of Val66BDNF, but not of Met66BDNF (Gauthier et al., 2004; del Toro et al., 2006). HAP1 seems to be the possible “missing link” between BDNF and Huntingtin. How HAP1, which belongs to the extravesicular transport machinery, could reach intravesicular BDNF, is still unknown. Equally, the pro-domain of BDNF binds to sortilin in the trans-Golgi network, sorting BDNF to the regulated pathway of secretion (Chen et al., 2005), but the absence of HAP1 from a HAP1/Sortilin/proBDNF complex directs BDNF to the endosome for proteolysis (Evans et al., 2011; Yang et al., 2011). However, the residue E148 in the mature portion of BDNF ensures interaction with the transmembrane sorting receptor, CPE. BDNF is dramatically redirected to the constitutive pathway when this recognition site is mutated, because the cytoplasmic tail of CPE associates with the microtubule transport machinery (Lou et al., 2005; Park et al., 2008). Certainly, sorting of BDNF and its precursor to DCVs depends on the above mentioned protein interactions with prosequence and mature sequence. Studies on

interaction-dependent conformation changes of the microtubule-associated machinery will help clarify these mechanisms, but once sorted, what is the destination of BDNF vesicles for secretion?

1.4.2. The site of BDNF secretion revealed by combined experimental methods

A role for BDNF in the modulation of synaptic strength implies synaptic release for input-specific action of BDNF. However the site of neuronal BDNF release during expression of synaptic plasticity remains elusive. Whether BDNF is released mainly from active postsynaptic sites to induce changes at presynapses (retrograde messenger hypothesis) or released from active axon terminals (anterotrophin hypothesis) is still not clarified.[6] Several recent electrophysiological studies support the view of BDNF as a retrograde messenger. Notably, presynaptic glutamate release is enhanced after postsynaptic tonic depolarization or focal glutamate stimulation of single hippocampal cells. This result is prevented by inhibition of BDNF/ TrkB signalling (Magby et al., 2006; Crozier et al., 2008). A similar retrograde effect has been shown on GABAergic transmission between CA3 neurons and interneurons in hippocampal slices (Gubellini et al., 2005). Functional induction of glutamatergic presynaptic terminals by high frequency stimulation is impaired in $BDNF^{-/-}$ mice. The appearance of new presynapses is critically dependent on BDNF release from the postsynaptic sites (Walz et al., 2006). The co-existence of retrograde and anterograde transported BDNF, plus the simultaneous presence of internalized and of readily releasable BDNF, sometimes in the same neurite, add contradictory information to the riddle of BDNF's site of action. Axonal targeting, as well as dendritic localization of endogenous BDNF has been reported in the brain (for a comprehensive review, see Edelmann et al., 2013).

The very low expression levels in most neurons make detection of endogenous BDNF problematic. In the hippocampal formation, axonal targeting of BDNF is clearly evident in mossy fibers projecting to CA3 pyramidal cells, because of a high expression level in this subregion (Altar and DiStefano, 1998; Danzer and McNamara, 2004; Dieni et al., 2012). Endogenous BDNF is expressed at much lower levels in CA1 pyramidal cells. A recent study, using a genetic knock-in of epitope-tagged BDNF and

[6] The retrograde messenger hypothesis for synaptic plasticity is born out of the classical concept of target-derived neurotrophic factor established in developmental biology (Levi-Montalcini, 1987). According to this theory, neurotrophins are released from the soma and dendrites of the innervated cells upon which the innervating axons terminate. Neurotrophins bind to their receptors at the presynapses, are endocytosed and transported retrogradely to the cell soma where they can initiate signalling leading to survival or, concerning the retrograde messenger hypothesis, to long-lasting changes of synaptic strength (Bonhoeffer, 1996). The anterotrophin hypothesis for synaptic plasticity postulates the preponderance of neurotrophins transported anterogradely towards axon terminals for local paracrine or autocrine signalling (reviewed in Lessmann et al., 2003).

immunohistochemistry with antibodies against the epitope, did indicate exclusive presynaptic staining of BDNF in adult hippocampal slices, with faint staining in the CA1 area (Dieni et al., 2012). However, several other studies did show dendritic localization of BDNF in CA1 pyramidal cells (Wetmore et al., 1991; Dugich-Djordjevic et al., 1995; Conner et al., 1997; reviewed in Lessmann et al., 2003). The activity-dependent targeting of long 3'UTR BDNF mRNA splice variants to dendrites in the hippocampus pleads in favour of timely BDNF translation for BDNF release at nearby sites (Tongiorgi et al., 1997 and 2008; see section 1.2.1.1.). In dissociated cultures of hippocampal neurons, endogenous BDNF has been detected in both axons and dendrites, at synaptic and extrasynaptic sites, with a pronounced somatodendritic pattern of expression (Goodman et al., 1996; Swanwick et al., 2004; Brigadski et al., 2005).

Ectopic expression of GFP-tagged BDNF has been used in recent years by several groups to overcome the hurdle of the detection threshold. In cultured primary neurons, GFP-tagged BDNF and NT-3 (but also NGF and NT-4 to a lesser extent) are both targeted to vesicles of the regulated pathway of secretion, a fact correlating with a distal expression pattern (Wu et al., 2004; Adachi et al.; 2005; Brigadski et al., 2005). GFP-tagged neurotrophins are secreted from postsynaptic as well as extrasynaptic dendritic structures (Hartmann et al., 2001; Kojima et al., 2001; Brigadski et al., 2005; Matsuda et al., 2009). Additional axonal release of GFP-tagged BDNF has been shown for cultured cortical neurons (Kohara et al., 2001) and until recently in hippocampal neurons (Dean et al., 2009; Matsuda et al., 2009). The differences in reported BDNF subcellular localizations seem to depend not only on the local neuronal network, but rather on the level of expression, the developmental stage and the recent history of electrical activity, so that both dendritic and axonal release of BDNF exist (e.g. Yan et al., 1997; reviewed in Lessmann and Brigadski, 2009). The results of an elegant study using ectopic expression of photoactivable GFP (PAGFP) tags are in line with this thesis. Dean and co-workers could selectively "mark" single BDNF-PAGFP vesicles in primary hippocampal neurons and determine their destination. They could show that BDNF vesicles are targeted to both dendritic and axonal synaptic sites, following an increase of neuronal activity under depolarization (Dean et al., 2012).

1.4.3. Time course and molecular mechanisms of regulated BDNF secretion

1.4.3.1. Time course of regulated BDNF secretion

In the regulated secretory pathway and before exocytotic membrane fusion can occur, secretory granules must pass through a number of stages leading to their tight attachment to the plasma membrane. These stages consist of ATP-dependent priming, tethering and docking. Priming steps involve reorganization of the actin cytoskeleton for initial granule recruitment and the ATP-dependent modification of the fusion machinery. Tethering is the initial attachment of granules to the plasma membrane that precedes a true docked interaction, in which granules are ready for exocytosis. (reviewed in Burgoyne and Morgan, 2003). Several studies, using cell capacitance or amperometric measurements on secondary cell lines, identified that different pools of secretory granules undergo exocytosis according to the stage they were in. The sequence of granules fusion in these cells shows an immediate exocytotic burst, followed by a short rapid phase and a prolonged sustained phase.

In spite of the important findings they allowed by offering a high temporal resolution, these methods can not help resolve the site of BDNF exocytosis in neurons. In the contrary, detection of BDNF by techniques of immunostaining allows localizing the release sites, but it only provides a snapshot of the pools of DCVs in a dynamic process. Therefore ectopic expression of GFP-tagged BDNF has been used in recent years to visualize the dynamics of BDNF secretion at high spatiotemporal resolution. The dendritic and somatic secretion of BDNF measured using GFP tags is one order of magnitude slower than release of glutamate in the same cells. In the same study with hippocampal neurons, NGF and NT-3 were released at a similar speed and NT-4 slightly faster (Brigadski et al., 2005). Other neuropeptides like tPA are released at a similar time scale as neurotrophins (Silverman et al., 2005; Lochner et al. 2006). The reason for this slow secretion rate is that the whole cargo of neuropeptides can not exit the granule immediately after opening of the fusion pore. Acidic pH and high intragranular Ca^{2+} concentration allow the formation of a scaffolding matrix of chromogranins/ secretogranins with quasi-crystalline state. The granular granin matrix enables storage of the neuropeptides in a highly compact fashion (see Chanat and Huttner, 1991; Angleson et al., 1999). Dissolution and release of the peptide core can be accelerated by prior neutralization of intragranular pH with drugs, like monensin (Han et al. 1999; Brigadski et al., 2005). Moreover, the molecular weight of the neuropeptide can limit both the speed of unbinding of a neuropeptide from the core and the speed of

diffusion through the fusion pore. For example, the neuropeptide Y (11 kDa) is released about 40 times faster than tPA (63kDa; Barg et al., 2002; Taraska et al., 2003; Perrais et al., 2004). In addition to the granin matrix and the molecular weight of BDNF, the secretion rate of BDNF is limited by the delay of fusion pore opening, which is related to the number of stages DCVs have to pass beforehand.

1.4.3.2. Mechanisms participating in regulated BDNF exocytosis

Various molecular determinants are involved in the processes leading to the fusion of DCVs with the plasma membrane. They depend initially on the mode of action of the stimulus. Regulated neuronal BDNF secretion can be induced by depolarization through electrical activity, glutamate application or elevated extracellular K^+ (e.g. Goodman et al., 1996; Griesbeck et al., 1999; Hartmann et al., 2001). Following a depolarizing stimulus, a well established trigger of the secretion process is the rise of the intracellular Ca^{2+} concentration through Ca^{2+} entry and/or through Ca^{2+}-induced Ca^{2+} release from internal stores (e.g. Griesbeck et al., 1999; Balkowiec and Katz, 2002; reviewed in Lessmann et al., 2003; Lessmann and Brigadski, 2009). Increase of Ca^{2+} levels above a critical threshold activate Ca^{2+} sensors like synaptotagmin-I (Syt-I) to regulate the fusion machinery composed of SNARE proteins. Applied to DCVs, the details of this mechanism and the function of other synaptotagmin isoforms remain controversial. As we know from SVs, Ca^{2+}-bound Syt-I associates at the same time to the vesicular and the plasma membrane while bringing the SNARE complex to overpass the energy barrier for fusion-convenient conformation change. Intriguingly, the Ca^{2+}-insensitive synaptotagmin isoform Syt-IV has been shown to negatively regulate fusion of co-localizing BDNF vesicles so that Syt-IV can be considered as a site specific “brake” for BDNF release (Dean et al., 2009 and 2012). Further types of Ca^{2+}-binding proteins, like CaMKII or Calpain can control Ca^{2+} influx through regulation of Ca^{2+} channels and could consequently regulate exocytosis. Other kinds of positive stimuli activate G protein coupled receptors, leading to activation of protein kinase C and of the cAMP-dependent protein kinase A (Canossa et al., 2001; Balkowiec and Katz, 2002; Santi et al. 2006). These kinases could also regulate exocytosis through the control of Ca^{2+} influx (reviewed in Dai, Hall and Hell, 2009) or through phosphorylation of proteins participating in the regulation of exocytosis (reviewed in Burgoyne and Morgan, 2003). In hippocampal neurons, also BDNF signalling was found to induce BDNF release through Trk receptors in an autocrine manner (Canossa et al., 1997 and 2001), whereas nitric oxide signalling played an inhibitory role (Canossa et al., 2002).

1.5. Aim of this thesis

As we consider the role of BDNF and other neurotrophins in the establishment and the function of the vertebrate CNS, we are led to ask ourselves how such molecular mechanisms can be translated into information and thus contribute to learning and memory. The aim of this thesis is to shed a modest light on the mechanistic details of regulated BDNF secretion in hippocampal neurons, by using pharmacology, fluorescent tags and live cell imaging. We chose to target signalling cascades known to be involved in synaptic plasticity (published in Kolarow et al., 2007; 2014). This step is necessary for a better understanding of the contribution of BDNF to synaptic plasticity.

2. MATERIALS

2.1. Substances

8-Bromo-cAMP	Sigma
Acetic acid	Sigma
Agar (select)	Sigma
Agarose	Invitrogen
ARAC	Sigma
B27	Invitrogen
Bafilomycine A1	Sigma
BES	Sigma
BME	Invitrogen
Boric acid	Sigma
BSA	Merck
CaCl 97 %	Riedel-de Haen
CaCl ($2H_2O$)	Merck
Carbogen	Merck
Cyclopiazonic acid	Alexis Biochemicals
D(+)-Glucose	Merck
$_{D,L}$-APV	Tocris Bioscience / Sigma
DMEM	Invitrogen
DMSO	Sigma
DNQX	Sigma
dNTPs	Boehringer Mannheim
Ethanol	Baker
EDTA	Sigma
EGTA	Sigma
FCS	Biochrom / Invitrogen
FM4-64	Molecular Probes
Gluconic acid	Janssen Chimica
Glucose	Merck
Glutamax	Invitrogen
Glycerin	Riedel de Haen
Glycine	Roth
HEPES	Roth
Human recombinant BDNF	Alomone Labs
Human recombinant NT-3	Alomone Labs
Hydrochloric acid	Sigma
Insulin	Invitrogen
K252a	Alomone Labs
KCl	Merck
KH_2PO_4	Roth
KN-62	Tocris Bioscience
KN-92	Sigma
KN-93	Sigma
KOH	Sigma
$_{L}$-Glutamate	Sigma
Mg-ATP	Sigma
MgCl ($6H_2O$)	Merck
Na_2HPO_4	Merck
NaH_2PO_4	Merck
Na_3-GTP	Sigma
NaCl	Merck
NaOH	Sigma

Neurobasal medium	Invitrogen
Nifedipine	Sigma
Penicillin-Streptomycin-Glutamine	Invitrogen
p-Formaldehyd	Sigma
Phenol red (sodium salt)	Sigma
Polyornitine hydrobromide	Sigma
Potassium gluconate	Janssen
Pyruvate	Sigma
Rp-cAMPS	Tocris Bioscience
Ryanodine	Alexis Biochemicals
Sodium acetate	Sigma
Sodium pyruvate	Invitrogen
Sucrose	Sigma
Tetrodotoxin	Alomone Labs
Thapsigargine	Tocris Bioscience
Tris base	Sigma
Trypan blue	Sigma
Trypsin 2.5 %	Invitrogen
Trypsin-EDTA	Invitrogen
SybrGold	Molecular probes
Yeast extract	Gibco BRL
Loading Dye	MBI Fermentas
DNA Ladder	MBI Fermentas

2.2. Enzymes and Proteins

DNase I	Roche Diagnostics
Bovine serum albumin (BSA)	Serva
RNase A	Boehringer Mannheim
Taq-Hot-Start-Polymerase	MBI Fermentas
Taq-Polymerase	MBI Fermentas

2.3. Devices

AOTF controller	Visitech International
Bath controller	Luigs & Neumann
Borosilicate glass	Harvard
CCD IR Camera	Till Photonics
CCD Camera PCO	CCD Imaging
CCD Camera Sensys 1401E	Photometrics
Centrifuge Megafuge 1.0R Rotor 2252	Heraeus
Centrifuge 5415C, Sorvall Rotor SS34	Eppendorf
CO_2 incubators HeraCell 240	Heraeus
Drying oven	Heraeus
Electrode puller PP-830	Narishige
Epifluorescence microscope BX51WI	Olympus
Epifluorescence microscope IX70	Olympus
Fluorescence lamp - U-RFL-T Burner	Olympus
Handwheel SM5	Luigs & Neumann
Laser	Laser Physics
Light microscope CK40	Olympus
Light microscope	Zeiss
Light source KL1500 electronic	Schott
Magnetic stirrer - MR 3001	Heidolph
Micromanipulator	Luigs & Neumann
Neubauer cell counting chamber	Brand
Oscilloscope TDS 210 Two channel Digital	Textronik

Osmometer	Knauer
Patch Clamp amplifier EPC-8	HEKA
PCR-Cycler Mastercycler Personal	Eppendorf
pH-Meter	Inolab
Photometer	Eppendorf
Piezo system	Jena
Pipettes	Eppendorf
Isolated Pulse Stimulator 2100	A-M Systems
Shutter	UniBlitz Electronics
Laminar flow cabinet	Heraeus
Thermomixer 5436	Eppendorf
Video Monitor WV-BM 1410	Panasonic
Water bath	Memmert

2.4. Molecular biology kits

EndoFree Plasmid Maxi Kit	Qiagen
DNeasy Kit	Qiagen

2.5. Softwares

Imaging Metaview	Universal Imaging Corporation
Statistics MS Excel	Microsoft Corporation
Sigmaplot	SPSS Science
Clampfit PClamp	Molecular Device
Figure layout CorelDRAW!	Corel Corporation
Word processor MS Word	Microsoft Corporation
Patch Clamp Clampex	Molecular Device

2.6. Goods

Coverslips	Laborservice Brenzinger
Cryotubes	Nunc
flexiPERM con A / Lumox dishes	Greiner Bio-One
Plasticware	Eppendorf, Greiner, Nunc, Sarstedt
Sterile filters Filtropur S, 0.2 µm	Fluka
Cell culture flasks	Sarstedt, Nunc

2.7. Buffers, solutions and media

ACSF	90	mM	NaCl
	25	mM	$NaHCO_3$
	10	mM	Glucose
	2.5	mM	KCl
	2.5	mM	$CaCl_2$
	2	mM	$MgCl_2$
	1.25	mM	Na_2HPO_4
Agarose-Gel	1.5	%	Agarose in TBE
2× BBS	50	mM	BES pH 7.6
	280	mM	NaCl
	1.5	mM	$Na_2HPO_4 \cdot 7\ H_2O$

BME /10% FCS	10	%	FCS
	5	mM	Glucose
	1	mM	Glutamax
	10	mM	HEPES
	300	µl	Insulin
	250	µl	Penicillin-Streptomycin
			in BME
Borate buffer	0.15 M		Boric acid
			pH 8.35
D,L-APV stock solution	50	mM	D,L-APV
			in 1 M NaOH
DMEM /10 % FCS	10	%	FCS
	5	mM	Glucose
	1	mM	Glutamax
	10	mM	HEPES
	300	µl	Insulin
	250	µl	Penicillin-Streptomycin
			in DMEM
DNase-Stock solution	6	mg/ml	DNase I
			in PBS (-/-)
DNQX Stock solution	50	mM	DNQX
			in DMSO
Expyr	2	mM	$CaCl_2$
	10	mM	Glucose
	10	µM	Glycine
	15	mM	HEPES
	5	mM	KCl
	1	mM	$MgCl_2$
	110	mM	NaCl
			pH 7.3
FM4-64® Stock solution	10	mM	FM4-64®
			in DMSO
FM4-64® in HBS-/-	1	mM	FM4-64®
FM4-64® Dye solution	10	µM	FM4-64®
			in HBSK50 (2/1)
HBS (-/-)	20	mM	HEPES
	4	mM	KCl
	100	mM	NaCl
	1	mM	Na_2HPO_4
			pH 7.3
HBS (2/1)	2	mM	$CaCl_2$
	10	mM	Glucose
	10	µM	Glycine
	1	mM	$MgCl_2$
			in HBS (-/-)

HBS (0/3)	10	mM	Glucose
	10	µM	Glycine
	3	mM	$MgCl_2$
			in HBS (-/-)
HBSK50 (2/1)	2	mM	$CaCl_2$
	10	mM	Glucose
	10	µM	Glycine
	20	mM	HEPES
	50	mM	KCl
	1	mM	$MgCl_2$
	89	mM	NaCl
	1	mM	Na_2HPO_4
			pH 7.3
Insulin Stock solution	12.5	g/l	Insulin
			in 0.01 M HCl
Inpyr	0.2	mM	$CaCl_2$
	0.6	mM	EGTA
	15	mM	HEPES
	1	mM	$MgCl_2$
	5	mM	NaCl
	90	mM	Potassium Gluconate
	4	µM	ATP
	1	µM	Na_3GTP
			pH 7.2
MPBS (-/-)	1	mg/ml	BSA
	6	µg/ml	DNase I
	10	mM	Glucose
	1	mM	Glutamax
	4	mM	NaOH
	1.25	ml	Penicillin-Streptomycin
	5	mg/ml	Phenol red
	1	mM	Pyruvate
	in	500 ml	PBS
MPBS (+/+)	0.25	mM	$CaCl_2$
	5.8	mM	$MgCl_2$
			in MPBS (-/-)
NB/B27	10	ml	B27
	1	mM	Glutamax
	1.25	ml	Penicillin-Streptomycin
	in	500 ml	Neurobasal medium
PBS (-/-)	2	mM	KH_2PO_4
	150	mM	NaCl
	8	mM	Na_2HPO_4
PBS (+/+)	0.9	mM	$CaCl_2$
	0.5	mM	$MgCl_2$
			in PBS (-/-)

SybrGold	0.08	%	SybrGold in TBE buffer
TBE buffer	89 89 2	mM mM mM	Tris base pH 8.0 Boric acid EDTA
Trypan blue solution	5	g/l	Trypan blue in PBS (-/-)

3. METHODS

3.1. Cell culture

3.1.1. Primary cell culture

3.1.1.1. Preparation of hippocampal neurons and cortical astrocytes from rats (Brewer and Cotman, 1989; Brewer et al., 1993)

Postnatal P0-P3 rats were used for the preparation of hippocampal neurons and cortical astrocytes. All work was done under sterile conditions. At first, the anaesthetized rats were decapitated. The scalps were then opened with a scalpel and unfolded to the sides. The uncovered skullcaps were cut at the chondral junction points, above the eyes and along the center line. Using two forceps, the halves of the skullcaps were opened to the sides. The brains were then extracted with a spoon and transferred into a cooled drop of MPBS (+/+) on a SYLGARD-coated preparation dish. The hippocampi (three per eppendorf) and cortices (two per eppendorf) were isolated and transferred to an eppendorf containing 500 µl ice cold MPBS (+/+).

After completion of the tissue preparation, the buffer was discarded and the tissues were incubated with 0.25 % trypsin in MPBS (-/-) for several minutes in a thermomixer at 37 °C / 800 rpm. The incubation periods are given in Table 3.1.

Table 3.1: Incubation times for the trypsinization of rat neuronal tissues.

Tissue	Stage	Incubation time with trypsin
Hippocampus	P0-P1 P2 P3	8 min 10 min 12 min
Cortex	P0-P1 P2 P3	15 min 16 min 18 min

The duration of successful trypsinization increases with rising age of the rats. Hippocampal tissue dissociates more easily than cortical tissue.

The following steps took place in ice cold medium. After sedimentation of the trypsinized tissues, the supernatants were transferred in 3 ml DMEM/10 % FCS. One ml of MPBS (-/-) was added to the remaining tissue, which was dissociated with a pipette. After sedimentation, the whole procedure was repeated two times.

The afterwards used “pre-plating” allows the separation of the neuronal and glial cells by making use of the different adhesive properties of the two cell types. Non-neuronal cells attach to the bottom of the dish. The supernatant contains the neurons. The glial cells of the cortical tissue are needed for the culture of the astrocyte islands, on which the hippocampal neurons are finally sown. Microcultures of neurons that form *in vivo*-like interactions can be obtained with this method (Lessmann and Heumann, 1997), making investigations on small neuronal networks possible.
The cell number was determined with trypan blue. Two to three million cells were sown on 6 cm culture dishes in a final volume of 3 ml and were incubated at 37 °C. The incubation period depends on the age of the rats and on the kind of tissue (see table 3.2).

Table 3.2: Incubation periods for the pre-plating of the triturated neuronal tissue.

Tissue	Stage	Pre-plating time
Hippocampus	P0 P3	65 min 55 min
Cortex	P0 P3	60 min 50 min

The adhesion of the astrocytes takes place faster with cortical tissue than with hippocampal tissue. The incubation period for the pre-plating decreases with increasing age of the rats.

Following the appropriate incubation time, the supernatant was discarded and 3 ml BME/10 % FCS was added to the dish containing the cortical cells. The cells were kept for 2 to 4 weeks in the incubator until the passaging of the astrocytes (see paragraph 3.1.1.2). The suspension of hippocampal neurons was transferred into a small tube and the dish with glial cells was discarded. The suspension was centrifuged for 10 min at RT and 1000 rpm. The supernatant was discarded and the sediment resuspended in 3 ml DMEM/10 % FCS (prewarmed, 37 °C). The favoured cell density was sown on glial microislands (see 3.1.1.2). A medium change with NB/B27 was performed 4-24 hours later.

3.1.1.2. Passaging of rat cortical astrocytes (Lessmann and Heumann, 1997 and 1998)

Passaging of cortical astrocytes was performed 2 weeks after preparation of the cortices (see 3.1.1.1). At this time point the astrocytes had formed confluent cultures in the dishes. The confluent astrocyte cultures were washed once with 1.5 ml DMEM/FCS 10 % and a mix of 1.5 ml EDTA solution (1 mM) with 30 µl trypsin

solution (2.5 %) was added. After an incubation time of 15 – 30 min (37 °C, 5 % CO_2 und 100 % humidity), the cells detached from the dish bottom. The cells were resuspended in 3 ml DMEM/FCS 10 % and the dish bottom was washed with an additional 1 ml of DMEM/FCS 10 %. The suspension was centrifuged at 900 rpm for 10 min at RT, the supernatant discarded and the pellet resuspended in 3 ml DMEM/FCS 10 %. After cell counting in a Neugebauer cell chamber, 80.000 cells were sown on glass coverslips (12 mm Ø). The glass coverslips had been soaked for many hours in 100 % ethanol, then flamed and transferred to a 3.5 cm culture dish. Two to three days after seeding the astrocytes, 3-4 µM ARAC was added. One to two weeks after passaging, the adherent astrocytes formed single microisland groups with an average diameter of 100 μm^2. The hippocampal neurons were sown on these cultures.

3.1.2. Transfection of primary neurones with the calcium phosphate precipitation method (Chen and Okayama, 1988; Kohrmann et al., 1999)

Primary neurons were transfected by Ca^{2+} phosphate precipitation after 8 DIV. The transfected cells were used for experiments 2 – 4 days after the Ca^{2+} phosphate precipitation. For transfection, all solutions were cooled to 4 °C to enable an efficient formation of the precipitate. At first, 3 µg of plasmid DNA were prepared in an eppendorf tube and 32.5 µl of sterile cell culture water was added. 4.5 µl of a 2.0 M $CaCl_2$ solution and 40 µl of 2×BBS buffer were added consecutively to the plasmid DNA solution. 800 µl of preconditioned medium from DIV8 neuronal cells was pipetted into a second reaction tube. The neurons were returned to the incubator and the preconditioned culture medium was incubated for 5 min at RT. The plasmid DNA solution was then added dropwise to the preconditioned culture medium under continuous stirring. The neurons were removed from the incubator again and the residual preconditioned medium was carefully replaced with the transfection solution, but was not discarded. Both the residual preconditioned medium (1.0 – 1.2 ml) and the primary cells were taken back to the incubator for 2.5 h. DNQX (10 µM) and $_{D,L}$-APV (50 µM) were added to reduce excitotoxicity.

After this incubation the cells were washed twice with 37 °C warm HBS (0/3) buffer. Then 1 ml preincubated NB/B27 medium and 1 ml of the residual preconditioned medium were pipetted carefully into the cell culture dish. The transfected neurons were maintained in the incubator until use.

3.1.3. Co-transfection with synaptic marker proteins

The construction and use of a PSD-95-DsRed expression plasmid was described by Brigadski et al. (2005) and intact synaptic targeting of the resulting protein was confirmed by co-localization of PSD-95-DsRed with the green fluorescent activity-dependent label of synapses, FM 1-43. To identify postsynaptic structures in BDNF-GFP and NT-3-GFP expressing hippocampal neurons, cells were co-transfected with the respective NT-GFP construct, together with PSD-95-DsRed (DNA ratio, GFP:DsRed = 1:1; not more than 3 µg per dish in total).

3.1.4. Polyornithine coating of coverslips

Coverslips were sterilized for 5 h at 130 °C. Once cooled down to RT, the coverslips were transferred to a polyornithine solution (1 mg/ml polyornithine in borate buffer). After 4 h incubation in this solution, the coverslips were washed three times in water, singly dried and placed in sterile cell culture dishes.

3.2. Molecular biology

3.2.1. Competent bacteria

Competent bacteria were prepared according to the calcium chloride method. To this end, 2 ml LB medium were inoculated with either a single colony or an aliquot of bacterial stock culture (E. coli K12 C600) and incubated overnight in a warm air shaker at 37 °C and 170 rpm. After 20 hours, 200 ml LB medium were inoculated with 1 ml of the preculture and incubated 2 h until reaching an OD (A_{600}) of 0.4. The bacteria were immediately centrifuged for 10 min at 4000 rpm, 4 °C (Heraeus Sepatech, 2252 rotor). The supernatant was discarded and the pellet was carefully resuspended in 60 ml ice cold, sterile 50 mM $CaCl_2$ solution (avoiding bubble formation). After centrifugation, the pellet was carefully resuspended in 3.2 ml ice cold 50 mM $CaCl_2$ solution and incubated for 1 h at 4 °C. The suspension was mixed up to 1 volume 40 % (v/v) glycerine in 50 mM $CaCl_2$. The bacteria were then aliquoted (300 µl), nitrogen-cooled and stored at -80 °C.

3.2.2. Transformation

Competent bacteria were slowly thawed on ice. About 1 µg of DNA was carefully added to 100 µl competent bacteria for transformation. After 15 min incubation on ice, the bacteria were heat-shocked for 100 s at 42 °C. 500 µl of antibiotic-free LB Medium was added and the bacteria were incubated for 1 h at 42 °C. The bacteria suspension was plated on a 10 cm LB agar dish including antibiotics according to the resistance of

the plasmid used. The dish was incubated upside down overnight at 37 °C. Single colonies were used to make glycerine stock cultures.

3.2.3. Midipreparation of plasmid DNA

Successful transfection of neurons requires especially pure, endotoxin-free plasmid DNA. Midipreparations were performed with the EndoFree maxi kit according to manufacturer's instructions. The purified plasmid DNA was resuspended in water and the concentration was determined photometrically.

3.2.4. Glycerine stock culture

A preculture in LB medium was prepared with a single bacterial colony. One and a half (1.5) ml of preculture was centrifuged, resuspended in 0.5 ml LB medium and the suspension was mixed with 0.5 ml sterile glycerine. The glycerine stock culture was kept at -80 °C.

3.2.5. Electrophoresis

The separation of DNA fragments was performed using a horizontal electrophoresis setup and 1.5 % agarose gels. Agarose was dissolved in TBE buffer by boiling in the microwave oven. The liquid agarose was poured into the sealed electrophoresis equipment and a comb was added in order to form pockets. 6× loading dye was added to the DNA samples. 100 bp DNA Ladder was used as a molecular size marker. Electrophoresis took place for approximately 40 min with 4.5 – 6 V/cm electrode gap. The gel was subsequently stained with SybrGold solution according to the manufacturer's instructions.

3.3. Microscopy

3.3.1. Epifluorescence microscopy

Coverslips with transfected cells were transferred into Lumox dishes with folio bottom and inspected with an inverted microscope (Olympus IX 70), using 40× (NA, 1.0) and 100× (NA, 1.35) oil immersion objectives. Image capture was performed with a cooled CCD camera (SenSys 1401E; 12 bit dynamic range). The electronic shutter (UniBlitz, Electronics) was controlled by the Metavue software (Visitron System). GFP fluorescence was detected with narrow excitation (450 – 490 nm) and emission (dichroic mirror: 495 nm; 500 – 550 nm) band pass filters. The DsRed and FM4-64 fluorescence, respectively, were detected with a custom-built filter set (excitation: 530 – 550 nm; dichroic mirror: 570 nm; emission: 590 -650 nm). The exposure times for time-

lapse recordings (between 0.3 and 1.5 s when using the 40× objective) were adjusted for every cell so that no area of the CCD chip was driven into saturation. Processing of images was performed using Metavue and Adobe Photoshop software without compromising the evident primary image information.

3.3.2. Confocal microscopy

In some experiments, co-localization of BDNF-GFP and PDS-95-DsRed was evaluated with a Nipkow spinning disk confocal System (Visitech) attached to a conventional fluorescence microscope (Olympus BX51 WI) equipped with a high-aperture water immersion objective (60×; NA, 0.9). Full-frame image capture was performed with a cooled CCD camera (CoolSnap HQ; 12 bit dynamic range, Roper Scientific). The electronic shutter (UniBlitz, Electronics) was controlled over the Metamorph software (Visitron System). Green and red fluorescence were excited with the 488 nm and 568 nm lines of a Kr/Ar laser.

3.3.3. Osmolarity measurements

Osmolarity is a measure of the concentration of osmotically active particles per volume unit. Osmolarity is an important parameter of solution for insuring viability when, for instance, living cells are subjected to frequent changes of solutions during the experiment. There is a linear relation between freezing point depression und osmolarity. Freezing point depression corresponds to the difference between the freezing point of pure water and the freezing point of the aqueous solution to measure. An aqueous solution freezing at -1.858 °C has a concentration of 1°Osmol/kg.

Osmolarity was measured with a freezing point depression osmometer (KNAUER). Pure water and a NaCl solution with an osmolality of 400 mOsm/kg were first used to calibrate the instrument. The solutions were supercooled below the freezing point, between -5 °C and -8 °C, without stirring. The device initiated crystallization by automatic stirring during and, if crystallization completely occurred, displayed the concentration of the solution in mOsm/kg.

3.3.4. Perfusion system for the study of neurotrophin secretion

For the reason that BDNF is an adhesive polycationic protein and for correct time-lapse imaging of activity-dependent secretion, it was necessary to quickly wash off the secreted neurotrophins from the cell membrane. The cells were continuously superfused with a gravity-driven laminar local superfusion system, allowing exchange of successively applied solutions within 10 s.

The superfusion system consisted of an inflow pipette which was positioned close to the cell with micromanipulators. The opening of the inflow pipette was approximately 50 µm in diameter. The inflow pipette was connected through Teflon tubings with several fluid reservoirs filled with the different solutions. The Teflon tubings as well as the inflow pipette had to be filled bubble-free with solution to allow correct functioning of the system. An outflow pipette permitted continuous suction of solution from the system. The flow of a glucose containing solution, adjusted in a water-filled dish, acted as control to adjust the flow rate of the system prior to the experiment. The appropriate flow rate was characterized by pear-shaped stream lines.

3.3.5. Videomicroscopic study of neurotrophin secretion (Hartmann et al., 2001)

Real-time imaging of synaptic secretion of GFP-tagged NTs was performed as described with the superfusion system (described in 3.3.4): coverslips of transfected hippocampal neurons were mounted into a Lumox dish and maintained between folio bottom and a FlexiPerm silicone ring. Cells with synaptically localized NT-GFP were locally superfused with HBS, containing 2 mM Ca^{2+} and 1 mM Mg^{2+} [HBS $^{(2/1)}$] throughout the experiment.

After a 5 min control period (i.e., superfusion with HBS), cells were depolarized for 1-5 min by superfusing 50 mM K^+ containing HBS at RT [HBSK50 $^{(2/1)}$, replacing an equal amount of Na^+] and lastly superfused for 5 min with HBS $^{(2/1)}$. If not stated otherwise, the superfusion solutions contained DNQX (10 µM), $_{D,L}$-APV (50 µM), and gabazine (5 µM) to inhibit postsynaptic glutamate and GABA receptors. Time-lapse images were acquired at 10 s intervals and illumination of the sample was restricted to image capture.

Data analysis was performed with Metaview software. Regions containing postsynaptic clusters of NT-GFP were analyzed over time for changes in intracellular fluorescence intensity. The corresponding average fluorescence intensities were standardized to the measured value at the start of each stimulation ($t = 0$ in graphs). Background fluorescence levels of a void region in the same field of view was recorded in parallel and subtracted afterwards. Due to differences in the level of BDNF-GFP expression between individual cells, the illumination strength and exposure times differed from cell to cell, resulting in different levels of photobleaching during control periods. Photobleaching was corrected for by monoexponential fit extrapolation of the fluorescence decrease during the initial 5 min control period. Measured fluorescence values at a given time point were normalized to the corresponding value of this

extrapolated bleaching curve, thus providing normalized values of the fluorescence decrease caused by the release of NT-GFP. This fluorescence decrease has been shown previously to reflect tetanus toxin- and Ca^{2+}-sensitive release of neurotrophin. Time-lapse data from cells with more than 20 % photobleaching during the 5 min of control superfusion were discarded. All experiments were performed at RT (~22 °C).

3.3.6. Videomicroscopic study of styryl dyes secretion

Optic methods can be used to monitor the synaptic vesicle cycle, i.e. the rapid activity-dependent exocytotic events or intracellular vesicle trafficking, in living organisms. Fluorescent membrane markers with lipophilic properties, like styryl dyes, are therefore frequently used (for an overview see Cochilla et al., 1999). Live presynaptic terminals were labelled with the styryl dye FM 4-64 as follows: at 10–12 DIV, coverslips with hippocampal neurons were incubated for 1-2 min at RT with 10 µM FM 4-64 in HBSK50 $^{(2/1)}$ to allow vesicle staining. Depolarization of the cells through high K^+-triggered synaptic vesicle exocytosis and subsequent dye internalization through endocytosis. Cells were washed four times in dye-free HBS $^{(0/3)}$ and then transferred into the chamber of an inverted microscope for recordings. Real-time imaging and analysis of activity-dependent fluorescence decrease were performed as described above for neurotrophin secretion (section 3.3.5), except that images were captured at a shortened interval of 4 s.

4. RESULTS

4.1. Induction of postsynaptic secretion of neurotrophins

The use of neurotrophin-GFP fusion proteins has enabled us to answer unsolved questions about the localization and the conditions of hippocampal secretion of neurotrophins. Previous protein-biochemical work in the laboratory of Volkmar Lessmann (Haubensak et al., 1998; Brigadski et al., 2005) has confirmed the correct expression, the processing and the biological activity of the constructs used in our experimental model. An indication of the subcellular localization of BDNF was given by a study of Matthias Hartmann, who showed that 96 % of BDNF-GFP containing vesicles are localized in dendrites (Hartmann et al., 2001).

Two major expression patterns exist for NT-GFP constructs that are transfected in primary hippocampal neurons. BDNF-GFP and NT-3-GFP are localized in distal dendritic vesicles in more than 80 % of the transfected expressor cells, whereas proximal expression is predominant for NGF-GFP and NT-4-GFP. The work of Tanja Brigadski (2005) has suggested that the respective preprodomains of neurotrophins play a role in the differential expression of NT-GFP constructs. This detailed comparative study, using co-transfection of NT-GFP with synaptic PSD-95-fusion proteins, as well as immunocytochemistry, has shown that NT-GFP proteins are localized at postsynapses in close vicinity to active glutamatergic terminals (Brigadski et al., 2005). In the following experiments, we therefore chose to focus on BDNF and NT-3 because of the greater occurrence of distal expression, i.e. targeting the regulated pathway of secretion, when using these constructs.

4.1.1. Neurotrophin exocytosis

The BDNF-EGFP^{M1A} construct cloned by Matthias Hartmann (BDNF-GFP) was used to transfect mouse primary hippocampal neurons (see Appendix for plasmid maps). BDNF-EGFP^{M1A} does not contain the start codon of the EGFP protein. Therefore, the occurrence of neurons expressing plasmatic EGFP after transfection with this construct was ruled out (personal observation). In order to confirm the localization of BDNF or NT-3 at glutamatergic synapses, BDNF-GFP or NT-3-GFP were co-expressed with PSD-95-DsRed in primary hippocampal neurons. After transfection at 8-9 DIV, clusters of neurotrophin vesicles were found co-localizing with PSD95 at 10-15 DIV. (Fig. 4.1 A-B). Although a majority of these clusters were shown to be synaptic, extrasynaptic

localization of BDNF vesicles was also observed, including mainly single vesicles in movement (Fig. 4.4).

As indicated by immunocytochemistry, ectopic expression yielded an approximately fourfold overexpression of the GFP-tagged neurotrophin, compared to endogenous levels of neurotrophin (Fig. 4.1 C-D, contribution of Tanja Brigadski in Kolarow et al., 2007).

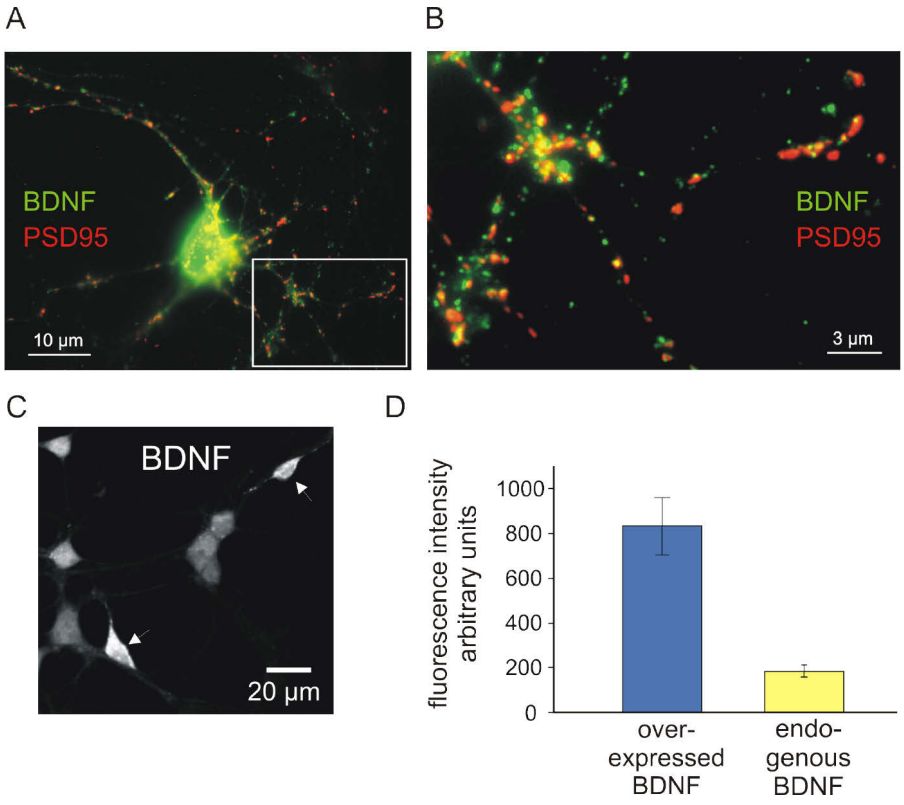

Figure 4.1: Localization of BDNF vesicle clusters and BDNF expression levels. **A**: Hippocampal neurons were co-transfected at 8 DIV with BDNF-GFP and PSD-95-DsRed. Co-localization of both proteins was monitored at 10-11 DIV. **B**: Boxed area in A, shown at higher magnification. Postsynaptic vesicle clusters of BDNF (green) were identified by co-localization with PSD-95 (Red). **C:** confocal image of BDNF fluorescence. Hippocampal neurons were co-transfected at 8 DIV with BDNF and GFP and immunostained at 10 DIV with a polyclonal antibody directed against BDNF. Arrows indicate the cells overexpressing BDNF. **D:** Quantification of BDNF fluorescence intensity in the soma of transfected and untransfected cells. The average fluorescence intensity of the soma of BDNF overexpressing cells (n = 8) is 4.6 times greater than the fluorescence intensity of untransfected cells (n = 18). Error bars represent SEM. **Figure and legend adapted from Kolarow et al. 2007**.

4.1.1.1. Measure of depolarization-induced BDNF and NT-3 release at postsynapses

In order to investigate activity-dependent exocytosis of BDNF, we first chose isolated transfected cells with the distal expression pattern, due to the fact that proximal expressor cells show only constitutive exocytosis (Brigadski et al., 2005). Secondly, we monitored only cells of which neurites stayed in focus under superfusion. After 5 min superfusion with control HBS, cells were depolarized for 1–5 min with HBSK50 $^{(2/1)}$. One important condition of our experimental model was the blockade of synaptic transmission (with 50 µM gabazine; 50 µM $_{D,L}$-APV, 10 µM DNQX) to exclude indirect effects of presynaptic released glutamate and GABA via activation of ionotropic receptors. We did not use inhibitors of metabotropic glutamate receptors, as mGluRs do not mediate neurotrophin secretion during depolarization with high K^+ (Hartmann et al., 2001).

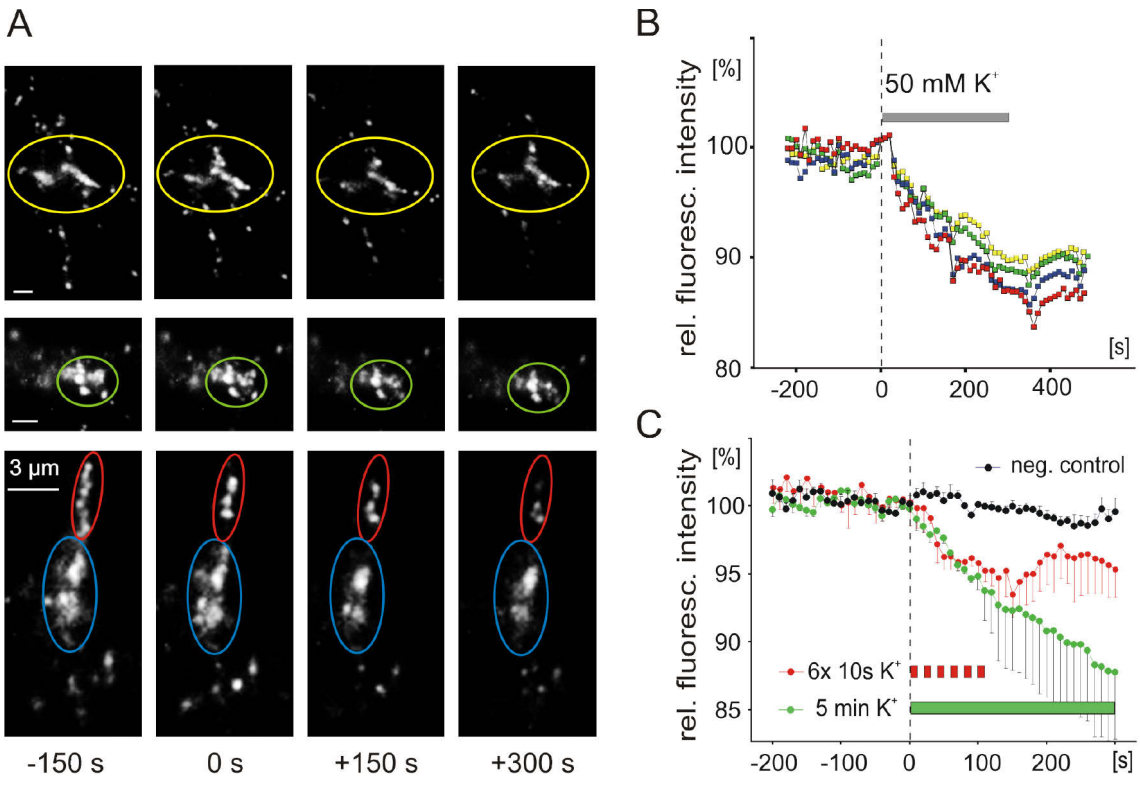

Figure 4.2: Investigation of postsynaptic secretion of BDNF and NT-3. **A**: Time course of BDNF release in response to depolarization with elevated K^+ (50 mM, 300 s). The pictures show postsynaptic BDNF-GFP vesicle clusters at indicated time points (0 s = start of depolarization, compare D). Note the gradual decrease of intracellular BDNF-GFP following the start of depolarization (+ 150 s, + 300 s). **B**: Time course of intracellular fluorescence of regions of interest in C. Note the decrease in intracellular fluorescence during stimulation (bar). **C**: Postsynaptic BDNF secretion in response to different depolarization paradigms (red: 6x 10 s elevated K^+ at 10 s intervals; green: 300 s K^+). Comparable degrees of BDNF secretion at 120 s are evident. **Figure and legend adapted from Kolarow et al. 2007**.

Under these conditions the postsynaptic neuron is isolated from synaptic network effects and the pharmacological effects on neurotrophin release can be directly related to the postsynaptic release process of neurotrophins itself.

On average, roughly 10 % of stored neurotrophin content of the secretory vesicles were released upon high K^+ stimulation (Fig. 4.3 A, B: BDNF-GFP 90.6 ± 1.3 % [n = 19 cells, 138 ROIs]; NT-3-GFP 89.6 ± 1.3 % [n = 16 cells, 123 ROIs]; 50 mM K^+ vs. negative control significantly different with $p < 0.0001$). The average time constants (monoexponential fit) of secretion for BDNF (173 ± 33 s), and NT-3 (238 ± 25 s) were similar to previously published values (Brigadski et al., 2005). To characterize the minimal requirements to elicit release, we asked if a shorter pattern of depolarization could be as effective as a 5 min depolarization to induce significant postsynaptic neurotrophin secretion. We found that six short applications (10 s each) of elevated K^+ with 10 s intervals also induced a significant loss of postsynaptic NT-GFP fluorescence at 120 s (Fig. 4.2 C). Considering a common time point for both the continuous and the sequential stimulations at 120 s, the remaining fluorescence intensity amounted to 95.2 ± 2.2 % (6x 10 s stimulation, 4 cells, 34 ROIs), compared to 93.7 ± 2.3 % (5 min stimulation, 3 cells, 27 ROIs; both significantly different from negative control with $p < 0.05$). The initial time course was similar under both conditions, but after 5 min depolarization the amount of secreted BDNF was larger than after the short sequential depolarization (remaining fluorescence intensity, respectively 87.8 ± 4.3 % [3 cells, 27 ROIs] and 95.3 ± 1.7 % [4 cells, 34 ROIs]). Therefore the continuous 5 min stimulation was used as positive control in all of the following experiments, for allowing a higher sensitivity in detecting postsynaptic secretion of neurotrophins. It could be argued that the strength of the stimulus is unphysiological and could kill the cells, leading to unphysiological release of BE. However, using propidium iodide, Matthias Hartmann (2001) showed that this stimulus was not prejudicial to the integrity of hippocampal neurones. Thus, all subsequent experiments were performed using the 5 min depolarization protocol. Since the efficiency of release varied between different preparations (range: 8-15 % release), matched positive controls (i.e. 300 s elevated K^+ + GBZ/ APV/ DNQX) were included in each set of experiments. During the stimulation, we did not detect “clouds” of fluorescence around BDNF-GFP vesicles as described by other groups (Xia et al., 2009), but we did observe heterogeneous and dynamic variability of the background fluorescence that could originate in part from released BDNF-GFP itself.

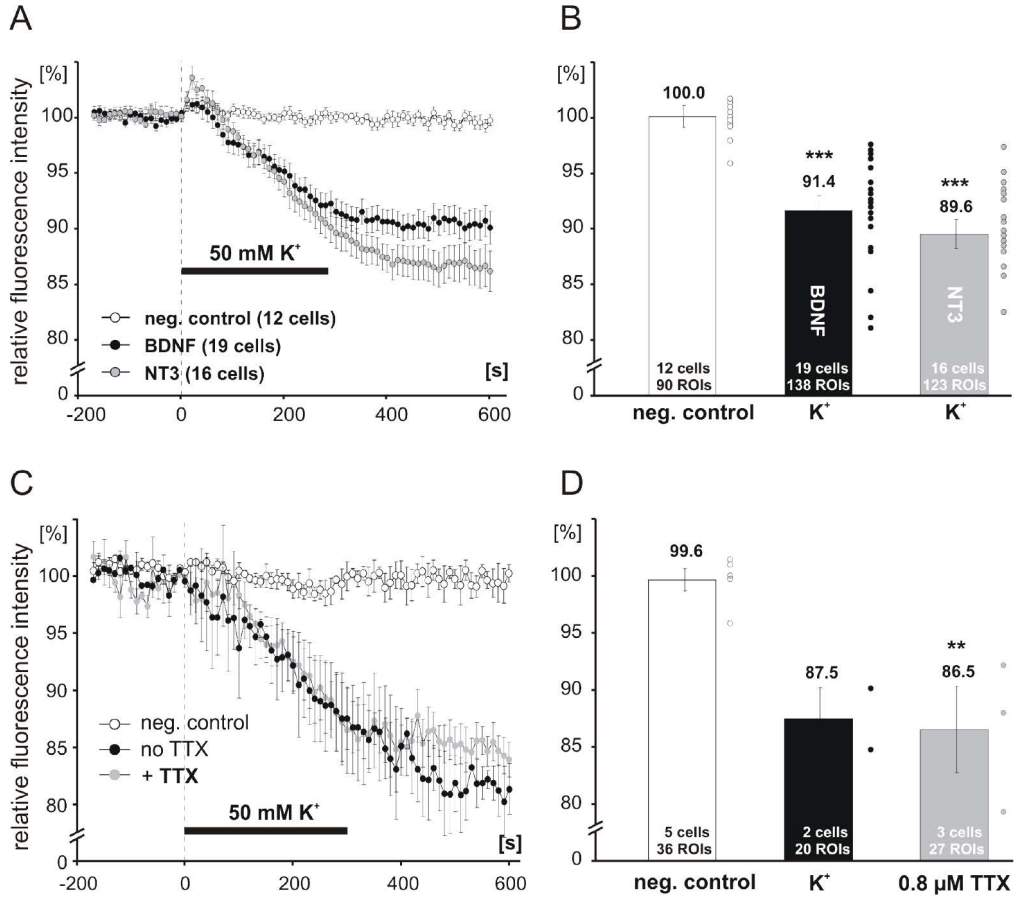

Figure 4.3: **A**: Average time course of postsynaptic BDNF-GFP and NT-3-GFP release in response to depolarization with elevated K^+ (50 mM, 300 s). **B**: Average residual fluorescence of postsynaptic BDNF-GFP and NT-3-GFP after 300 s depolarization. Experiments were performed in the presence of 10 µM DNQX, 200 µM D,L-APV, and 10 µM gabazine, to avoid secondary effects via transmitter secretion. ***p vs. negative control < 0.0001. **C**: Time course of elevated K^+-induced secretion of BDNF in the absence or presence of tetrodotoxin (TTX, 0.8 µM). **D**: Residual intracellular fluorescence of BDNF-GFP after 300 s depolarization for conditions as indicated. **p vs. negative control < 0.01. Note the independence of BDNF secretion from active voltage-gated Na^+ channels. Error bars represent SEM. **Kolarow et al. 2007, modified**.

In hippocampal mass primary cultures, extracellular Na^+ has been shown to be a prerequisite for neurotrophin secretion induced by elevated K^+ (Androutsellis-Theotokis et al., 1996; Blöchl and Thoenen, 1995; Kojima et al., 2001). Therefore we tested whether the postsynaptic secretion process itself requires opening of TTX sensitive voltage-gated Na^+ channels. As is evident from figure 4.3 (C and D), tetrodotoxin did not block neurotrophin secretion induced by elevated K^+. This shows that the postsynaptic secretion of neurotrophins in our experimental model occurs independently of action potential firing and of action potential driven activity of the synaptic network.

4.1.1.2. Size of BDNF-GFP vesicles

The size of the BDNF-GFP vesicles was assessed with the purpose to determine if the puncta of overexpressed BDNF-GFP we monitored were single vesicles. It is highly unlikely that the movement of one BDNF-GFP punctum represents the parallel movement of more than one vesicle. Thus, we chose moving vesicles in order to exclude doublets (Fig. 4.4). The size of isolated BDNF-GFP vesicles was found to be about 440 nm in average, which is larger than the size of neuronal DCVs previously measured by electron microscopy (~160 nm, see Morris et al., 1978).

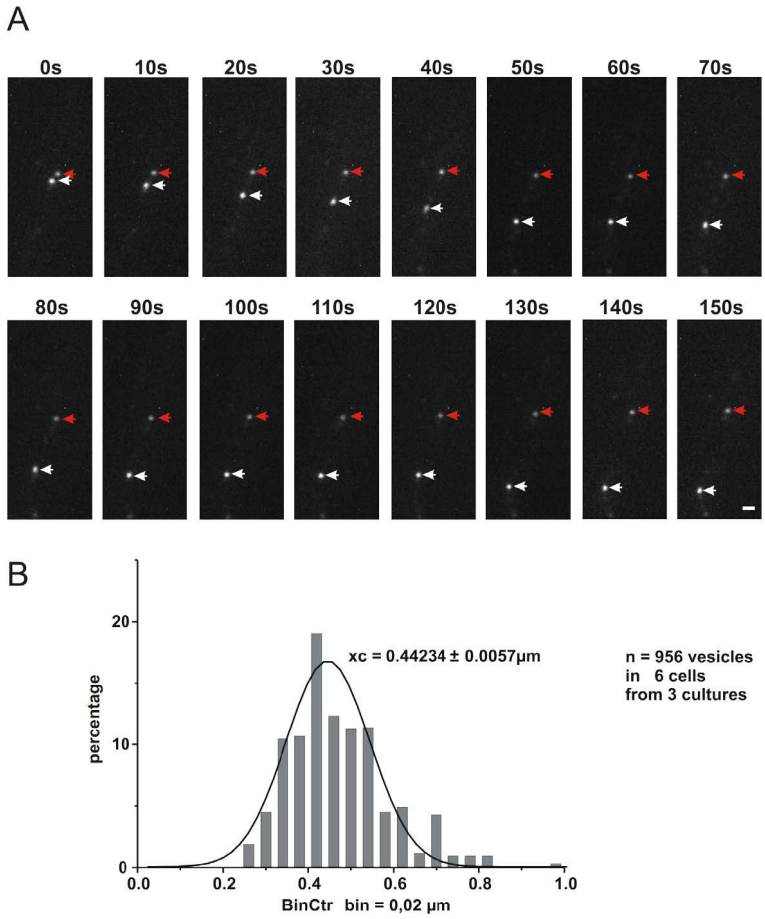

Figure 4.4: Single BDNF-GFP vesicle movement. Hippocampal neurons were transfected with BDNF-GFP at 8 DIV and intracellular GFP fluorescence was monitored using time lapse videomicroscopy with a high aperture (N.A.: 1.35) 100x objective. **A**: 16 consecutive images (taken at a frequency of 0.1 Hz) of single BDNF-GFP vesicles in a dendritic stretch of a hippocampal neuron. The white arrow marks a vesicle that moves along the dendrite. Note that the vesicle moves repetitively in an all-or-none fashion, which would be a highly unlikely event, if the fluorescent spot would consist of two or more vesicles. The red arrow indicates an immobile vesicle in the same dendrite. Scale bar: 1 µm. **B**: The diameter of single moving BDNF-GFP vesicles as shown in A, was measured and plotted in a histogram. 956 vesicles from 6 cells were analysed. A Gaussian curve was fitted to the distribution and yielded a mean vesicle diameter of 0.44 µm. **Figure and legend adapted from Kolarow et al. 2007**.

4.1.1.3. Effect of intravesicular pH on the fluorescence of BDNF-GFP

Recurrently, BDNF-GFP vesicles showed a transient increase preceding the decrease of fluorescence at the onset of stimulation. The intravesicular pH of secretory granules has formerly been shown to be in the range of 5.5-6.0 and the fluorescence of the EGFP protein is known to be pH sensitive (Han et al., 1999; Barg et al., 2002). The observed increase of fluorescence likely corresponded to deprotonation of EGFP due to intragranular pH neutralization, possibly after fusion pore opening. To confirm this assumption, we used Bafilomycin A1, which is known to neutralize intragranular pH by inhibiting the vesicular proton pump. As shown in figure 4.5, this treatment yielded a gradual increase in fluorescence intensity throughout the assay (result at 700 s: negative control 99.6 ± 0.7 %; Baf A1 117.8 ± 5.3 %). This result provides strong evidence that, before exocytosis, BDNF-GFP fluorescence is quenched by the acidic pH in DCVs (compare Brigadski et al., 2005, Fig.13). Even if unquenching of BDNF-GFP fluorescence in single vesicles was consistently observed upon depolarization, approximately 50 % of the monitored fluorescent vesicles were not quenched (personal observation). This likely reveals the heterogeneity of priming stages of synaptic BDNF-GFP vesicles.

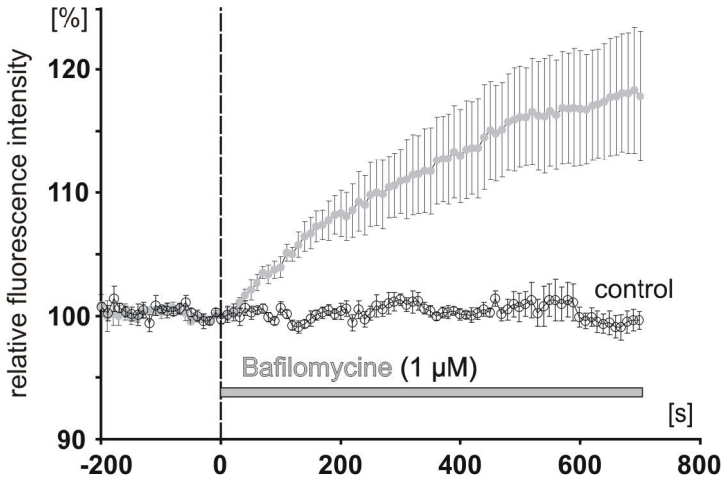

Figure 4.5: Neutralization of intragranular pH increases BDNF-GFP fluorescence. Hippocampal neurons were transfected with BDNF-GFP at 8 DIV and intracellular GFP fluorescence was monitored at 10 DIV using time lapse videomicroscopy. The graph represents the average change in fluorescence intensity in response to acute application of the vesicular proton pump inhibitor Bafilomycine A1 (1 µM; n = 4 cells, 31 ROIs). Note the increase in intravesicular fluorescence intensity (reflecting unquenching of pH-dependent GFP fluorescence) upon neutralization of the intravesicular pH during Bafilomycine A1 application. Error bars represent SEM. **Figure and Legend from Kolarow et al. 2007**.

4.1.1.4. Neurotransmitter release is unaffected by the inhibitors used to test BDNF release

The Ca^{2+} and cAMP signalling inhibitors used in this study for neurotrophin release could potentially interfere with other release processes (see sections 4.4 to 4.8). To exclude the possibility of an indirect modulation of postsynaptic BDNF exocytosis by altered presynaptic transmitter release, the effect of these inhibitors on the secretion of neurotransmitters was investigated under the following identical recording conditions: synaptic vesicles of hippocampal neurons were loaded with the lipophilic dye FM 4-64 and release of the FM dye was taken as an indicator for release of transmitters (Fig. 4.6A and B; see Cochilla et al., 1999).

Pre-incubation of the neurons with 50 µM nifedipine neither significantly changed the time course (Fig. 4.6C: exponential time constant 11.8 ± 3.3 s, n = 6), nor the efficiency of FM 4-64 secretion, as indicated by the average remaining fluorescence after 40 s of depolarization (Fig. 4.6D). Similarly, none of the other drugs tested (thapsigargine, KN-62 and 8-Br-cAMP), had an effect on the time course or the efficiency of release. As the inhibitors of Ca^{2+} and cAMP signalling used in this study did not interfere with neurotransmitter release, these results highlight their specific action on postsynaptic neurotrophin secretion.

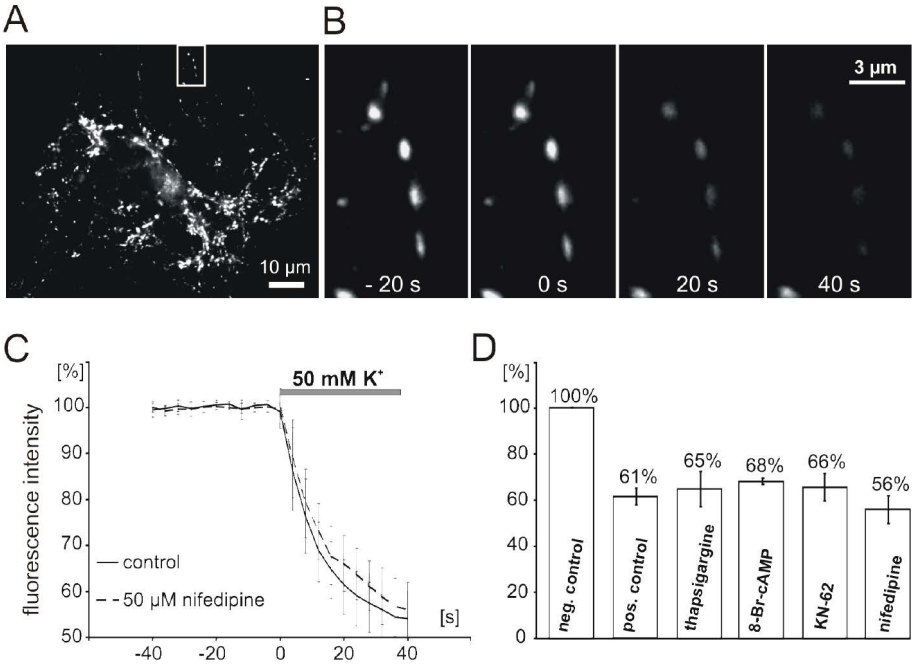

Figure 4.6: Unaltered neurotransmitter release during pharmacological treatments. **A**: Presynaptic terminals of hippocampal neurons were pre-loaded with FM 4-64 at 10 DIV. Presynaptic secretion of FM 4-64 was elicited with elevated K^+ (50 mM, 40 s). **B**: Boxed area in A shown at higher magnification. The pictures show FM 4-64 fluorescence in presynaptic terminals at indicated time points (0 s = start of depolarization, compare C). **C**: Average time course of FM 4-64 release in untreated (control) and in treated neurons (50 µM nifedipine). Note the similar time courses of FM 4-64 secretion, indicating unaltered presynaptic secretion following the drug treatment also used in neurotrophin secretion experiments. **D**: Average residual FM 4-64 fluorescence after 40 s depolarization for different treatments as indicated. All fluorescence values are given relative to the fluorescence levels at the start of the depolarization. Note that the different treatments did not significantly change presynaptic release of transmitters, indicating intact synaptic release of transmitters under the recording conditions. Error bars represent SEM. **Figure and legend from Kolarow et al. 2007**

4.2. Neurotrophin secretion proceeds via a kiss-and-run mechanism

It is known that two different mechanisms can operate when synaptic vesicles fuse with the cell membrane: full-collapse fusion and kiss-and-run (reviewed in An and Zenisek, 2004). A recently developed technique uses the quencher of fluorescence bromophenol blue (BPB; 2 mM), which completely eliminates vesicular GFP fluorescence upon first fusion pore opening (Harata et al., 2006). Therefore, BPB was used to investigate whether postsynaptic secretion of NTs proceeds via full-collapse vesicle fusion or via graded release through repetitively opening vesicle fusion pores. Upon elevated K^+, the presence of BPB markedly speeded the decrease of BDNF-GFP fluorescence: the observed fluorescence loss was steeper at the start of depolarization and was more efficient (remaining fluorescence after 300 s: 77.8 ± 3.1 %, n = 6 cells, 54 ROIs) than in the absence of BPB (90.6 ± 1.3 %, n = 5 cells, 35 ROIs; see Fig. 4.7A).

Focussing on single BDNF-GFP vesicles undergoing exocytosis, the loss of fluorescence in the presence of BPB was complete and was faster than 10 s (i.e., the interval between two image captures Fig. 4.7B). By comparison, in the absence of BPB the slope of the loss of fluorescence in single vesicles lasted over several hundreds of seconds (Fig. 4.7C). This difference suggests that BPB entered the secretory vesicles upon first opening of the fusion pores and immediately quenched the entire GFP fluorescence in these vesicles (compare Harata et al., 2006).

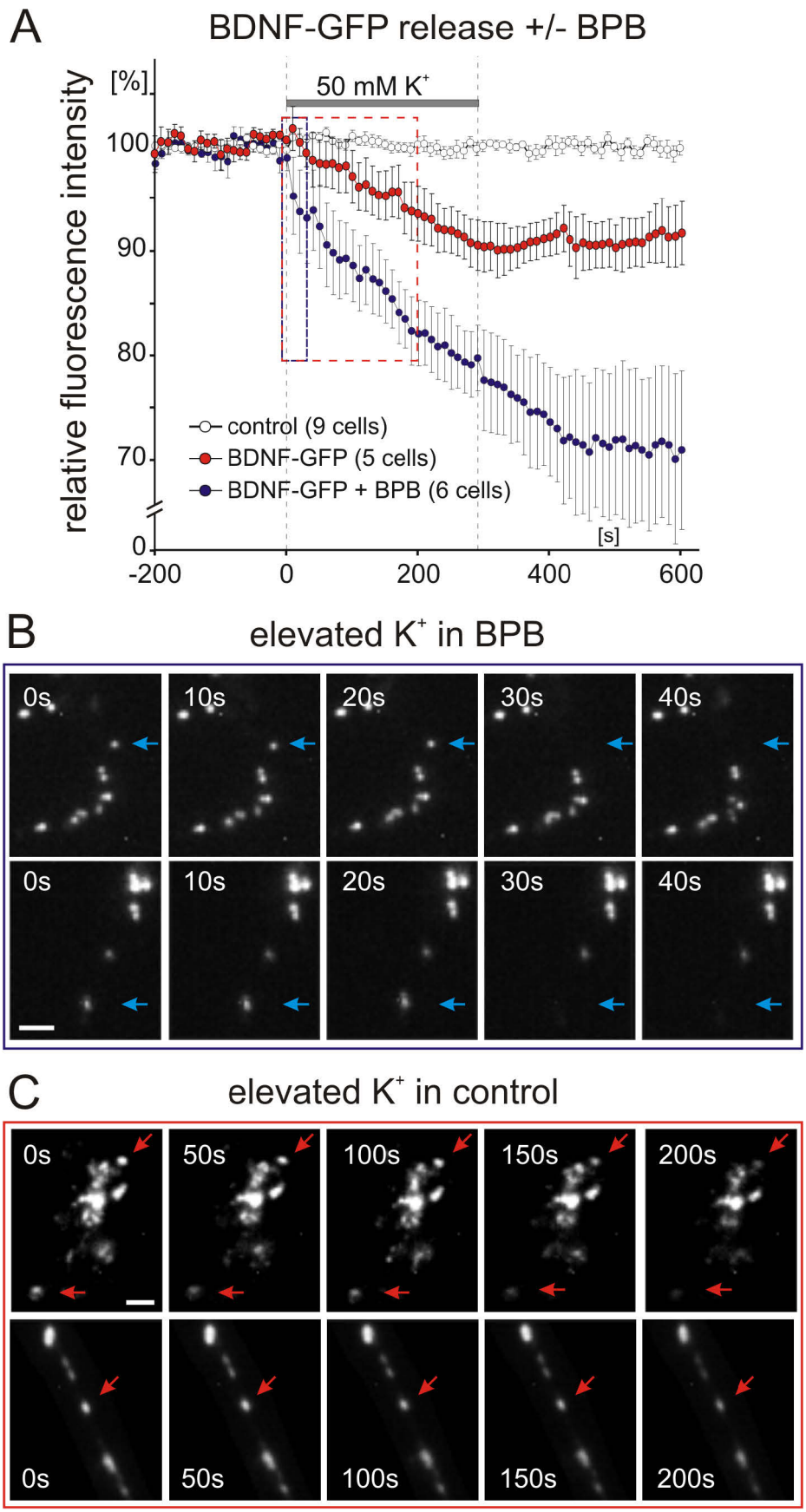

Figure 4.7: Bromophenol blue quenching of GFP fluorescence reveals fast fusion pore opening of BDNF-GFP vesicles. Hippocampal neurons were transfected with GFP-tagged BDNF, and depolarization-induced decrease in intracellular GFP fluorescence was monitored (50 mM K^+, 300 s,

(continuation of figure 4.7 on page 49) postsynaptic receptors blocked with 10 µM DNQX, 50 µM D,L-APV, and 50 µM gabazine). **A**: Average time course of fluorescence decay in the absence/presence of the green fluorescence quencher, bromophenol blue (BPB, 2 mM in all superfusion solutions). In the absence of BPB, the fluorescence decay reflects release of BDNF-GFP. In the presence of BPB the decay represents the kinetics of first fusion pore openings of the BDNF-GFP vesicles (compare text). Note the fast onset of first fusion pore openings compared to the delayed and less pronounced release of BDNF-GFP. **B**: Two representative series of pictures (stippled blue box in A) for a cell measured in the presence of BPB. Note the sudden and complete loss of single vesicles' fluorescence (blue arrows) within a 10 s imaging interval. **C**: Two representative series of pictures (stippled red box in A) for another cell measured in the absence of BPB. Note the graded fluorescence decay of single vesicles (red arrows) during a 200 s imaging interval. The sudden BPB dependent quenching of intravesicular BDNF-GFP fluorescence explains the faster and more complete decrease of the average data for BPB cells in A. Scale bars: 2 µm. **Figure and legend from Kolarow et al. 2007**

The effect of BPB on single vesicle fluorescence could explain another difference observed between both conditions: after the end of the stimulus, the average decay of fluorescence in the presence of BPB lasted about 160 s longer than in its absence (compare the fluorescence loss between t = 300 – 460 s in Fig. 4.7A). This result clearly indicates that fusion pore formation continued (blue curve) and BDNF-GFP remained trapped within secretory vesicles at the same time (red curve). This configuration is possible only when full-collapse fusion of secretory vesicles is not achieved while the fusion pores are being transient.

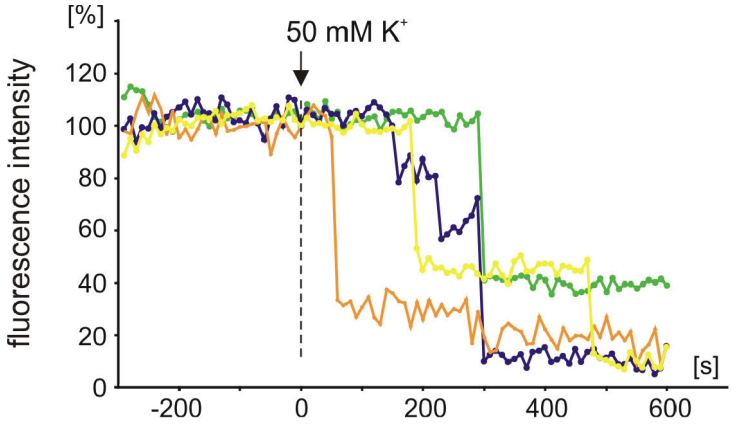

Figure 4.8: Asynchronous fusion pore opening of BDNF-GFP vesicles. All experimental details as in figure 4.7. Recording of fluorescence in single vesicle and vesicle cluster in response to elevated K^+ solution in the presence of BPB. Note the large scatter in delays until first fusion pore opening of individual vesicles in the same dendrite. **Figure and legend adapted from Kolarow et al. 2007**.

Since first fusion pore opening was observed both during and after the stimulation, we asked if fusion events could occur synchronously during stimulation. Figure 4.8 shows a significant variation in the delay between the start of the depolarization and first fusion pore opening for different vesicles of the same cell. This observation was confirmed by a more detailed examination of single vesicles (see Fig. 4.9), indicating asynchronous release of neurotrophins at different release sites. Additionally, the quenching effect of BPB was reversible, and was observed repetitively for the same vesicle, indicating prolonged and/or repeated fusion pore opening of vesicles that remained at the same site of release during ongoing depolarization (Fig. 4.9A-C). The sudden increase in fluorescence of the "green" vesicle is likely to result from intravesicular pH neutralization – possibly via opening of the fusion pore at that moment. Although we cannot definitely prove this assumption, a similar fluorescence increase of GFP-tagged peptides, also for BDNF-GFP, upon fusion pore opening has been described previously (compare Fig. 4.5 and Barg et al., 2002; Brigadski et al., 2005).

Taken together, these results show that the use of BPB in our experiments is an excellent tool for directly measuring fusion rates of secretory vesicles. However, the measurement of secretion efficiency by decline of BDNF-GFP fluorescence intensity after stimulation without BPB is a well established method that provides definite information about the fate of the cargo. Thus, all following experiments were carried out in the absence of BPB. Nevertheless, it can be said that BDNF vesicles fuse with the membrane, slowly releasing their contents via repetitive fusion pore opening or fusion pore dilation.

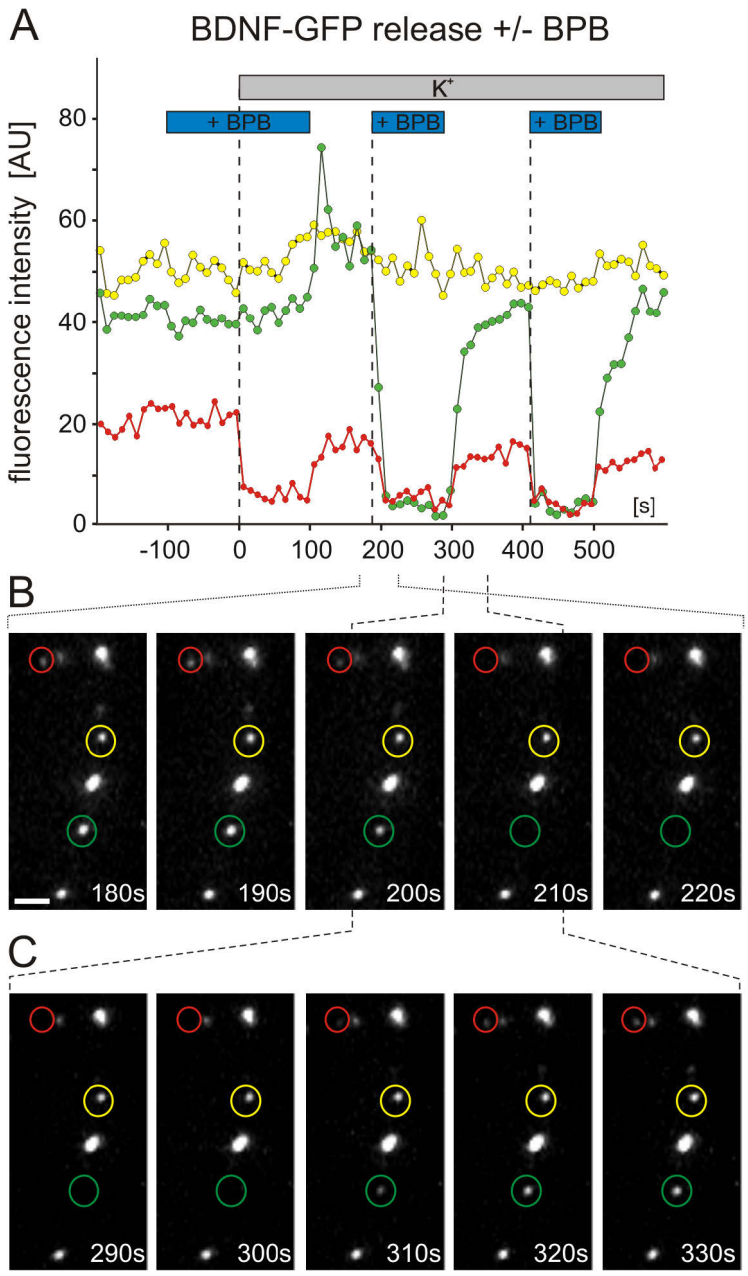

Figure 4.9: Prolonged or repetitive fusion pore opening of BDNF-GFP vesicles. All experimental procedures as in figure 4.7. **A**: Fluorescence intensity of single BDNF vesicles in the colour coded circles in B and C. Bars indicate superfusion conditions. **B, C**: Two image sequences (time windows indicated by stippled brackets) of the vesicles analyzed in A. The repeated disappearance/re-appearance of the vesicles marked in red and green, upon wash-in/washout of BPB, indicates prolonged and/or repetitive fusion pore opening during the depolarization. The transient increase in fluorescence intensity of the "green" vesicle at +100 s is due to reduced quenching of GFP fluorescence after neutralization of intragranular pH through the fusion pore (Han et al., 1999). The unchanged fluorescence of the "yellow" vesicle reflects absence of fusion pore opening for this vesicle. **Figure and legend, Kolarow et al., 2007**.

4.3. NO dependent modulation of postsynaptic release of neurotrophins

Since previous reports suggested a negative effect of NO signalling on global BDNF secretion (Canossa et al., 2002), we wanted to address the question whether a similar regulatory effect could be observed for postsynaptic secretion of BDNF at glutamatergic synapses. We first evaluated whether elevation of intracellular levels of NO could directly initiate the release of neurotrophins with acute application of the NO donor, sodium nitroprusside (SNP, 100 µM, 10 min). As expected, SNP alone did not induce release of either BDNF or NT-3 (remaining intracellular fluorescence for acute SNP: 100.2 ± 0.7 % [n = 5 cells, 22 ROIs]; Fig. 4.10 A, B). However, 5 min pre-incubation with SNP significantly reduced depolarization induced neurotrophin secretion (BDNF + SNP: 92.9 ± 2.1 % [n = 7 cells, 40 ROIs]; BDNF control: 85.2 ± 2.7 % [n = 4 cells, 18 ROIs]; NT-3 + SNP: 94.3 ± 1.0 %, [n = 15 cells, 87 ROIs]; NT-3 control 89.4 ± 1.4 %, [n = 8 cells, 52 ROIs]; $p < 0.05$).

According to Canossa et al. (2002), impairment of NOS activity transiently increases bulk BDNF secretion. Therefore, we tested if pre-incubation with the NOS inhibitor L-NMMA would interfere with depolarization induced neurotrophin secretion (300 µM L-NMMA, 15 min; a concentration effectively interfering with NO generation by exogenous BDNF). Interestingly, this experiment failed to reveal a significant change of either BDNF or NT-3 secretion (BDNF + L-NMMA: 89.7 ± 2.5 % [n = 10 cells, 64 ROIs]; BDNF control: 89.7 ± 1.4 % [n = 8 cells, 59 ROIs]; NT-3 + L-NMMA: 92.9 ± 0.8 % [n = 7 cells, 51 ROIs]; NT-3 control: 89.5 ± 2.0 % [n = 7 cells, 53 ROIs]; Fig. 4.10 C, D). This result suggests that a robust depolarization, sufficient to trigger release of neurotrophins, can not elevate NO in neurons up to a level that shows a negative feedback on neurotrophin secretion.

Figure 4.10 (on page 54): NO modulation of synaptic release of BDNF and NT-3. Hippocampal neurons were transfected with BDNF-GFP (BDNF) or NT-3-GFP (NT-3) and monitored for neurotrophin secretion. **A, C:** Averaged depolarization-induced (50 mM K^+) release of neurotrophins vs. negative control. **B, D:** Mean residual fluorescence 300 s after stimulation. **A, B:** Pre-incubation with the NO donor SNP (100 µM, 5 min) reduced depolarization induced secretion of neurotrophins.

C, D: Pre-incubation with the NOS inhibitor L-NMMA (300 µM, 5 min) did not change the amount of neurotrophins that was released. Experiments were performed in the presence of 10 µM DNQX, 200 µM D,L-APV, and 10 µM gabazine, to avoid secondary effects via transmitter secretion. A set of cells were superfused with HBS throughout as negative control. *p vs. control < 0.05, **p vs. control < 0.005. Error bars represent SEM. **Figure and legend, Kolarow et al., 2014**.

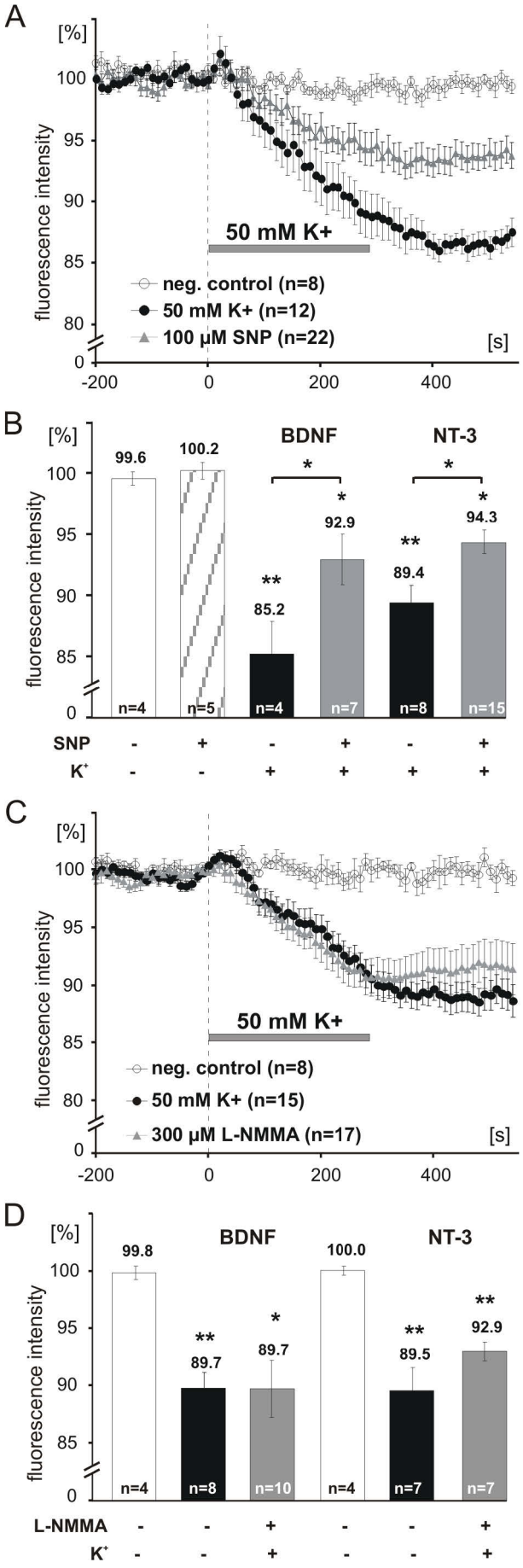

Figure 4.10: NO modulation of synaptic release of BDNF and NT-3 (legend on page 53).

4.4. NT secretion depends on activity of postsynaptic L-type VGCC

As generally admitted for regulated vesicular release of many other substances (Thomas et al., 1990; for a review, see Oheim et al., 2006), postsynaptic secretion of BDNF is thought to be dependent on extracellular Ca^{2+} influx. The successive stimulation in the presence and absence of 2 mM Ca^{2+} in depolarization solution, respectively, provoked BDNF release only when Ca^{2+} was available. This experiment confirms the direct involvement of Ca^{2+} influx in the BDNF release process itself (Fig. 4.11A, compare Hartmann et al., 2001). Moreover, the addition of Ni^{2+} and Cd^{2+} to the depolarization solution, inhibiting high voltage- as well as low voltage-activated VGCCs, blocks BDNF release (Hartmann et al., 2001). The postsynaptic localization, the voltage- and Ca^{2+}-dependence of the BDNF release point to L-type voltage gated Ca^{2+} channels as possible candidates for Ca^{2+} entry (Hell et al., 1993 and 1996, Obermair et al., 2004).

To investigate whether L-type Ca^{2+} channels contribute to the postsynaptic Ca^{2+} influx that induced neurotrophin secretion, we used the selective blocker nifedipine. Pre-incubation of the neurons with 50 µM nifedipine almost completely inhibited secretion of BDNF and NT-3 (BDNF + Nif: 97.6 ± 1.3 % [8 cells, 47 ROIs]; NT-3 + Nif: 97.7 ± 0.8 % [11 cells, 66 ROIs]; BDNF control: 89.6 ± 1.8 % [18 cells, 146 ROIs]; NT-3 control: 88.3 ± 1.0 % [9 cells, 50 ROIS]; Fig. 4.11 B, C). A comparable reduction of BDNF release was also achieved with smaller concentrations of nifedipine (BDNF + 10 µM Nif: 100.5 ± 1.8 % [7 cells, 72 ROIs]; BDNF + 1 µM Nif: 98.4 ± 0.7 % [6 cells, 55 ROIs]). This specific effect of nifedipine provides strong evidence for the role of Ca^{2+} influx through L-type Ca^{2+} channels in mediating postsynaptic NT secretion.

Since BDNF and NT-3 were equally dependent on Ca^{2+} influx, with similar secretion efficiencies and time courses, data for both neurotrophins were pooled in all subsequent experiments.

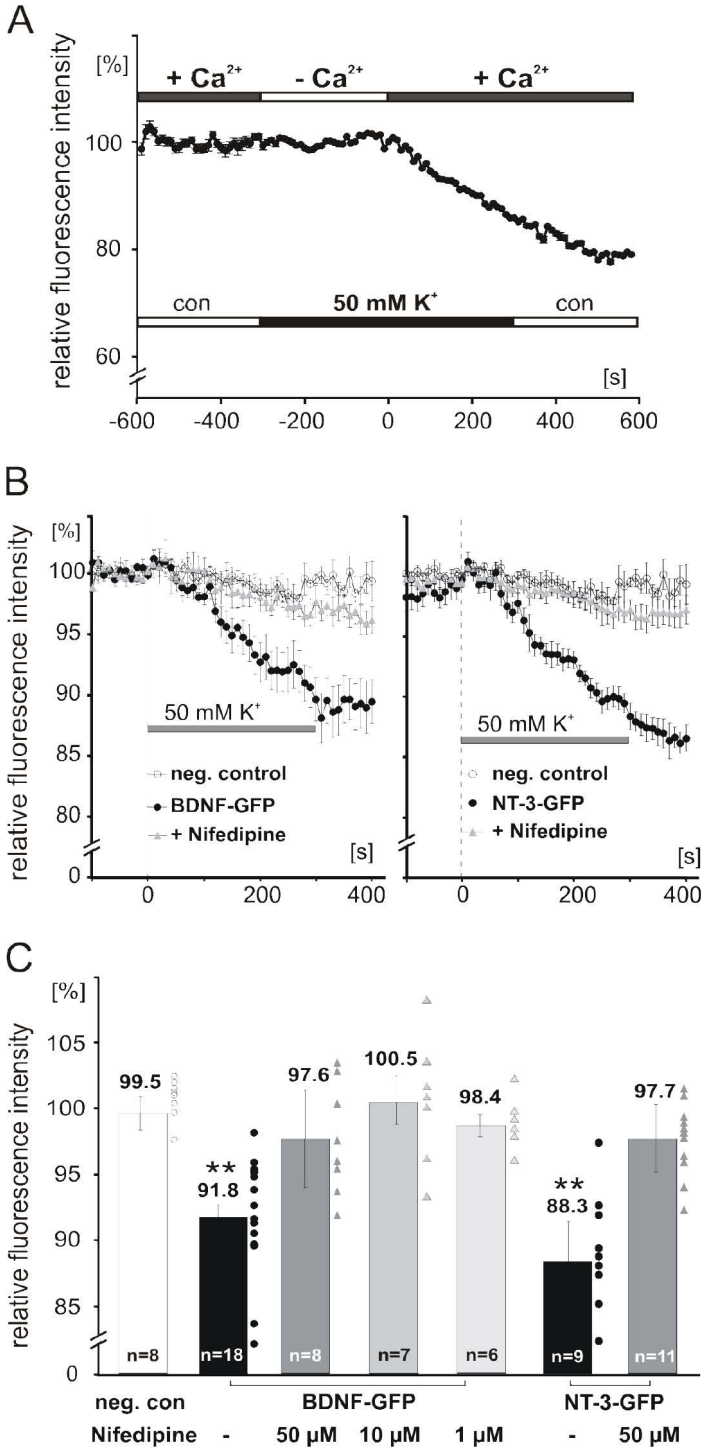

Figure 4.11: Depolarization-induced postsynaptic neurotrophin secretion depends on Ca^{2+} influx. Hippocampal neurons were transfected with GFP-tagged BDNF and NT-3, and depolarization-induced (50 mM K^+, 300 s, postsynaptic receptors blocked with 10 µM DNQX, 50 µM D,L-APV, and 50 µM gabazine) postsynaptic secretion of neurotrophins was measured as the decrease in intracellular fluorescence intensity. **A**: Experiment of Hartmann et al. (2001). Consecutive depolarizations of the same cell in the absence/presence of 2 mM extracellular Ca^{2+} show the dependency of BDNF-GFP secretion on extracellular Ca^{2+} (one cell, 3 ROIs) . **B**: Time course of secretion of BDNF-GFP and NT-3-GFP. Pre-incubation with the L-type Ca^{2+} channel antagonist nifedipine (50 µM, 10 min) significantly inhibited secretion of both neurotrophins ($p < 0.0001$ at $t = 300$ s). **C**: Residual intracellular fluorescence

(continuation of figure 4.11 on page 56): after 300 s stimulation for conditions as indicated (Nif.: nifedipine; **:p <0.0001 compared to negative control). Note the strong dependence of BDNF and NT-3 secretion on the activity of L-type VGCCs. Error bars represent SEM. **Figure and legend from Kolarow et al. 2007**.

4.5. Activation of postsynaptic NMDA receptors can elicit NT secretion

Ca^{2+} can also enter the cell through activated NMDA receptors upon removal of extracellular Mg^{2+}. We questioned whether the influx of Ca^{2+} through this channel can elicit postsynaptic secretion of neurotrophins as efficiently as the influx of Ca^{2+} through L-type VGCCs. Therefore, neurons were stimulated with 300 µM NMDA in the presence of nifedipine, DNQX and gabazine. NMDA evoked a potent release of BDNF-GFP (remaining fluorescence after 300 s: 93.9 ± 1.2 % [11 cells for 110 ROIs] with $p < 0.001$ significantly different from negative control [7 cells for 49 ROIs]). The NMDA-induced release was primarily slower than release upon elevated K^+ stimulation (89.9 ± 1.3 % [8 cells for 82 ROIs]; Fig. 4.12A), but continued steadily after stimulation, and finally reached the same level. From these results we can conclude that Ca^{2+} influx via postsynaptic NMDA receptors is sufficient to induce neurotrophin secretion.

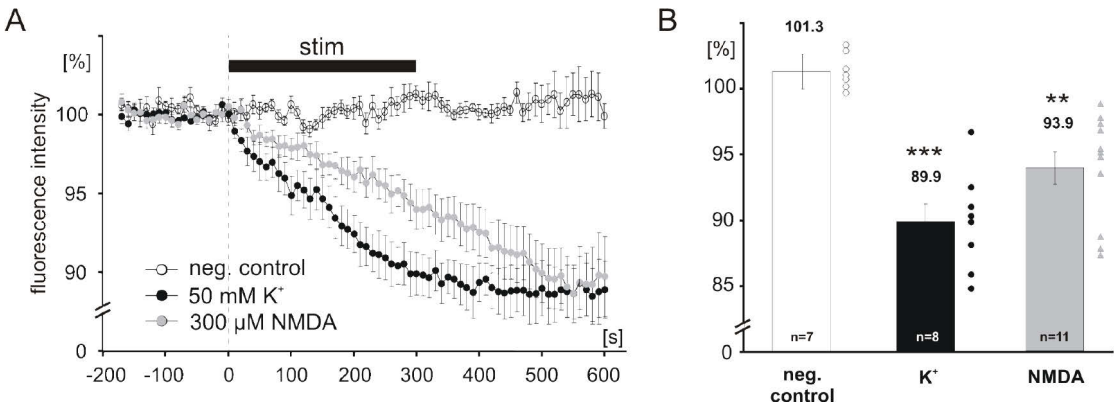

Figure 4.12: Activation of NMDA receptors can trigger postsynaptic secretion of BDNF-GFP. Hippocampal neurons were transfected with GFP-tagged BDNF and postsynaptic secretion of neurotrophins was measured as the decrease in intracellular fluorescence intensity. **A**: Time course of secretion of BDNF-GFP upon application of 300 µM NMDA in saline containing 2 mM Ca^{2+}, 0 mM Mg^{2+}. These experiments were performed in the presence of DNQX (10 µM), gabazine (50 µM) and nifedipine (10µM) to allow for selective observation of NMDAR mediated release. **B**: Average residual BDNF-GFP fluorescence after 300 s depolarization or application of NMDA. **$p < 0.001$ compared to negative control. Error bars represent SEM. **Kolarow et al. 2007, modified**.

4.6. Synaptic neurotrophin secretion is dependent on Ca^{2+} release from internal stores and CaMKII activity

Calcium influx can induce calcium release from internal calcium stores and the resulting increase of $[Ca^{2+}]_i$ could directly control neurotrophin release, as shown in previous studies (Blöchl and Thoenen, 1995; Griesbeck et al., 1999, Balkowiec and Katz, 2002). We asked if depleted calcium stores can prevent BDNF exocytosis. Therefore, we assayed the blocking of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA), which maintains the cytoplasmic-endoplasmic gradient of calcium, using two different drugs. The neurons were pre-incubated with either of the SERCA inhibitors, thapsigargine (10 µM, 10 min) and CPA (30 µM, 5 min), prior to depolarization with elevated K^+. Interestingly, postsynaptic neurotrophin secretion was completely inhibited under these conditions (BDNF + Thapsigargine: 100.3 ± 1.1 % [19 cells, 95 ROIs]; BDNF + CPA: 97.7 ± 2.7 % [6 cells, 48 ROIs]; BDNF control: 91.2 ± 4.8 % [18 cells, 99 ROIs]; Fig. 4.13A, B). This stresses the importance of intracellular Ca^{2+}stores, even though the primary influx of calcium for depolarization-induced secretion was inevitable in our experiments (compare Fig. 4.11). Ryanodine receptors are functionally coupled to L-type VGCCs in neurons and are also localized to dendritic spines (Sharp et al., 1993; Lanner et al., 2010). Since activation of ryanodine receptors is able to induce BDNF release from hippocampal neurons (Balkowiec and Katz, 2002), we tested ryanodine at a concentration inhibitory for ryanodine receptors in our release experiments. At 80 µM, ryanodine also inhibited postsynaptic BDNF release (BDNF + ryanodine: 97.7 ± 2.7 % [6 cells, 50 ROIs], Fig. 4.13A, B), confirming the role of this channel in the release of calcium from internal stores upon depolarization.

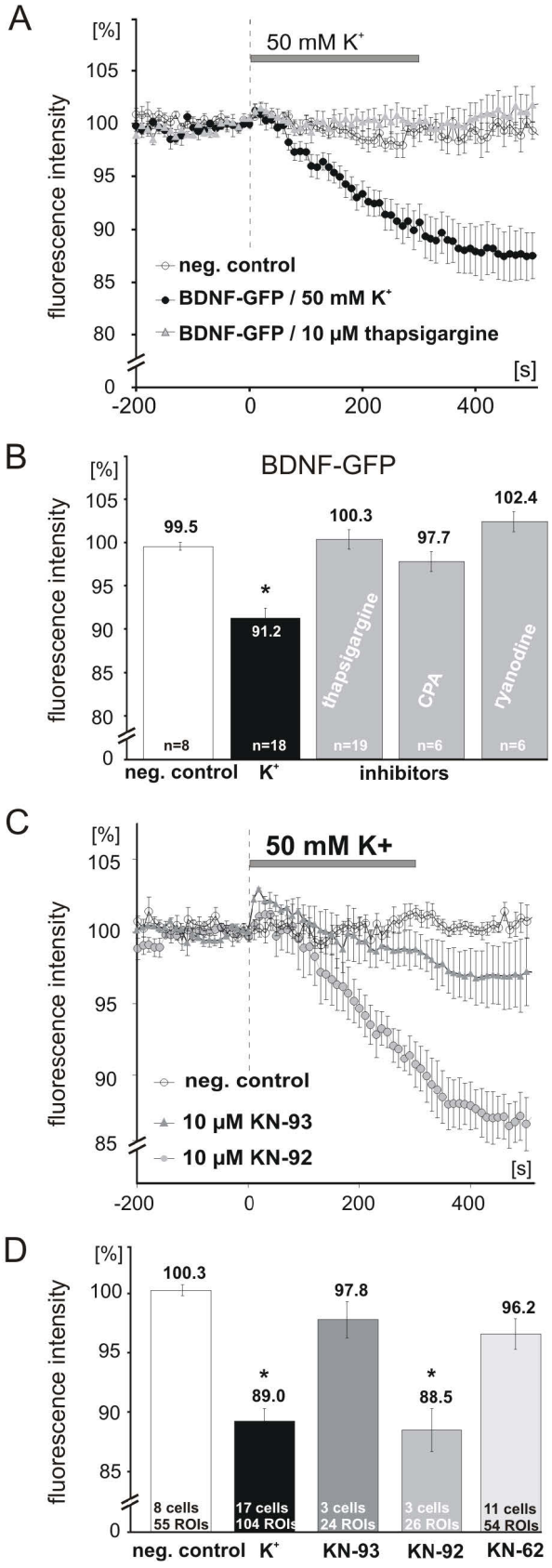

Figure 4.13: Dependence of neurotrophin secretion on intracellular Ca^{2+} stores and CaMKII. Hippocampal neurons were transfected with BDNF-GFP (BDNF) and monitored for postsynaptic neurotrophin secretion, as in Fig. 4.11. **A, C**: Time course of depolarization-induced (50 mM K^+) release of BDNF-GFP. **B, D**: Average residual BDNF-GFP fluorescence after 300 s depolarization.

(continuation of figure 4.13 on page 59) B: Pre-incubation with thapsigargine (10 µM, 10 min), or cyclopiazonic acid (CPA, 30 µM, 30 min) to deplete internal Ca^{2+} stores, or with ryanodine (80 µM, 30 min) to block Ca^{2+}-induced Ca^{2+} release from internal stores significantly inhibited NT secretion (*: $p < 0.0001$). **D**: Pre-incubation with 10 µM KN-62 or 10 µM KN-93 to inhibit αCaMKII both significantly inhibited NT secretion. For KN-62, data from BDNF and NT-3 release were pooled. (*: $p < 0.01$). **Kolarow et al. 2007, modified**.

Postsynaptic activation of αCaMKII is known to regulate synaptic plasticity. Several proteins involved in exocytosis are targets for activated αCaMKII. In order to identify a possible link between αCaMKII and synaptic BDNF, αCaMKII was inhibited by pre-incubating the neurons with 10 µM KN-62 or KN-93 for 30 min. We found that KN-62 strongly inhibited neurotrophin secretion (remaining fluorescence after 300s for BDNF and NT-3 + KN-62: 96.2 ± 1.2 %; BDNF and NT-3 control: 90.1 ± 1.1 %; significantly different with $p < 0.01$; for details see Fig. 4.13C,D), as did KN-93, but not KN-92 (BDNF + KN-93: 97.8 ± 1.6 %; BDNF + inactive isomer KN-92: 88.5 ± 1.8 %; BDNF control 89.0 ± 1.1 %), indicating a pivotal role for αCaMKII activity in postsynaptic secretion of neurotrophins.

4.7. Gating of postsynaptic NT secretion by basal levels of PKA activity

Activation of protein kinase A (PKA) has been suggested previously to regulate BDNF signalling in synaptic plasticity in different neuronal preparations (Ghosh et al, 1994; Meyer-Franke et al., 1995; Boulanger and Poo, 1999), including hippocampal neurons (Patterson et al., 2001). To answer whether postsynaptic PKA signalling can affect neurotrophin secretion, release was investigated first in response to short term superfusion with the membrane permeable cAMP analogue 8-Br-cAMP (100 µM; 5 min). This 5 min period of incubation is long enough to elicit robust cAMP-dependent signalling in our culture system (Lessmann et al., 1997). However, 8-Br-cAMP alone did not evoke any detectable secretion of BDNF or NT-3 under these conditions (Fig. 4.14B). After this pre-incubation with 8-Br-cAMP, depolarization-induced postsynaptic secretion was monitored in the same cells. As shown in figure 4.14A and B, the artificial elevation of intracellular cAMP did not change the time course or the efficiency of neurotrophin secretion (BDNF or NT-3 + 8-Br-cAMP: 93.1 ± 1.6 % residual fluorescence [n = 8 cells, 59 ROIs], BDNF or NT-3 control: 93.3 ± 1.5 % [n = 10 cells, 73 ROIs]).

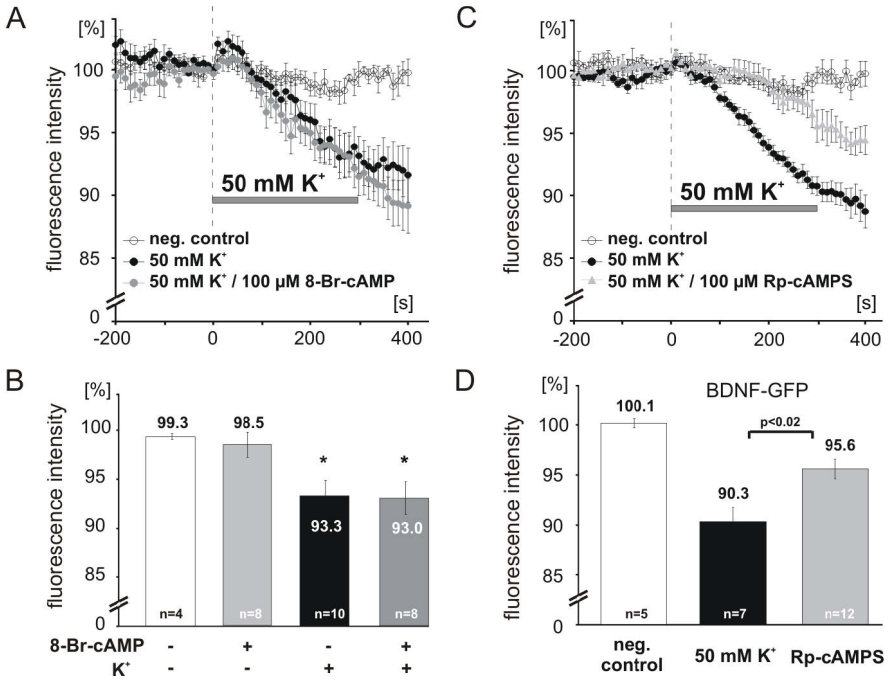

Figure 4.14: Postsynaptic cAMP/PKA activity gates secretion of neurotrophins. Hippocampal neurons were transfected with BDNF-GFP (BDNF) and monitored for neurotrophin secretion as described before. **A, C**: Averaged depolarization-induced (50 mM K^+) release of BDNF vs. negative control. **B, D**: Mean residual fluorescence 300s after stimulation. **A**: Preincubation with the PKA activator 8-Br-cAMP (100 µM, 5 min) did not change depolarization induced BDNF secretion. **B**: Application of 8-Br-cAMP (100 µM) neither induced BDNF secretion (left), nor changed the amount of released BDNF in response to depolarization (right). **C, D**: Preincubation with the PKA inhibitor Rp-cAMP-S (100 µM, 5 min) significantly inhibited and delayed BDNF secretion. Experiments were performed in the presence of 10 µM DNQX, 200 µM D,L-APV, and 50 µM gabazine. Note that inhibition of basal levels of PKA signalling delays secretion of BDNF-GFP. **Fig. and legend, Kolarow et al., 2007**.

On the other hand, interference with basal activity of PKA by pre-incubating cells with the hydrolysation resistant, membrane permeable cAMP antagonist Rp-cAMP-S (100 µM, 5 min) significantly delayed the onset (Fig. 4.14C), and also inhibited the efficiency of depolarization-induced postsynaptic secretion of neurotrophins (residual fluorescence at 300 s for BDNF or NT-3 +Rp-cAMP-S: 95.6 ± 1.0 % [n = 12 cells, 120 ROIs]; BDNF or NT-3 control: 90.3 ± 1.5 % [n = 7 cells, 62 ROIs], significantly different with $p < 0.02$). Taken together, both sets of data suggest that endogenous levels of intracellular cAMP in the neurons are sufficient to gate the postsynaptic release of neurotrophins via activation of PKA (compare Blitzer et al., 1995).

4.8. NT signalling is not involved in the postsynaptic secretion process

In mass cultures of hippocampal neurons, the application of BDNF, NT-4/5 of NT-3 can lead to delayed bulk secretion of neurotrophins (Canossa et al., 1997). To determine whether postsynaptic TrkB or TrkC receptors are involved in the postsynaptic secretion of neurotrophins, saturated concentrations of BDNF (100 ng/ml; 5 min) were applied to postsynaptic structures. Contrary to the experiments of Canossa et al. (1997), these tests were performed in the presence of various transmitter receptor inhibitors (DNQX, APV, GBZ) and of nifedipine (10 µM), to avoid any secondary effects of BDNF via modulation of the synaptic network. Under these conditions, BDNF was unable to elicit neurotrophin secretion (Fig. 4.15A).

Since BDNF-GFP and NT-3-GFP are known to retain full biological activity (Brigadski et al., 2005), we asked if they can sustain their own secretion via autocrine activation of postsynaptic TrkB and TrkC receptors (compare Canossa et al., 1997; Krüttgen et al., 1998). Therefore, neurons were incubated with 200 nM k252a for 30 min to inhibit TrkB and TrkC signalling. BDNF and NT-3 were analyzed separately because of their respective affinities to TrkB and TrkC.

There was no significant reduction of either BDNF-GFP or NT-3-GFP secretion upon blocking of TrkB/TrkC signalling (BDNF-GPF + k252a: 91.5 ± 2.7 %; BDNF-GFP control: 89.8 ± 1.6 %, NT-3-GFP + k252a: 91.8 ± 1.1 %; NT-3-GFP control: 87.9 ± 2.0 %; K252a vs. control not significantly different with $p > 0.05$; Fig. 4.15 B, C). Similarly, the release of BDNF initiated after a short pulsed stimulation did not proceed after 120 s (compare Fig. 4.2), as would have been expected in case of autocrine stimulation of neurotrophin release. The difference between the average time course of fluorescence decay in the presence of BPB and in its absence indicates that autocrine release did not occur in our experimental model (see Fig. 4.3 and 4.7). At the end of a 5 min long stimulation with high K^+, BDNF-GFP release stopped immediately. This makes short term positive autocrine effects of the BDNF-GFP, which was released before, unlikely.

Overall, these data exclude the probability of any direct BDNF-induced BDNF secretion or NT-3-induced NT-3 secretion at postsynaptic sites.

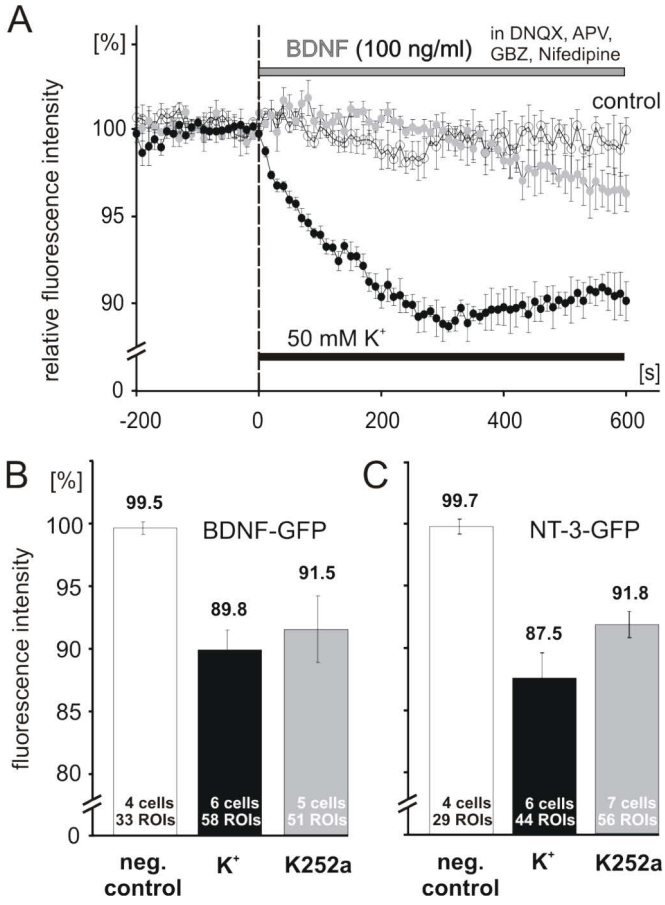

Figure 4.15: Absence of NT-induced NT secretion at postsynaptic sites of hippocampal synapses

Hippocampal neurons were transfected with BDNF-GFP (BDNF) and monitored for neurotrophin secretion, as described above. **A**: Averaged depolarization-induced (50 mM K^+), or BDNF-induced (100 ng/ml) release of BDNF-GFP vs. negative control. All recordings were performed in the presence of 10 µM DNQX, 50 µM D,L-APV, and 50 µM gabazine, to block postsynaptic ionotropic receptors. When BDNF was applied, the extracellular solution contained nifedipine (10 µM), to abolish indirect effects of BDNF on L-type Ca^{2+} channels, and to selectively monitor release that was elicited by BDNF induced release of Ca^{2+} from internal stores. **B**, **C**: Average depolarization-induced (50mM K^+) residual fluorescence, 300 s after start of depolarization, of GFP-tagged BDNF or NT-3 vs. control. Pre-incubation with the Trk kinase inhibitor k252a (200 nM, 30 min) had no significant effect ($p > 0.05$) on depolarization induced NT secretion. **Kolarow et al. 2007, modified**.

5. DISCUSSION

The work presented in this thesis demonstrates important aspects of neurotrophin secretion in hippocampal neurons. First, it sheds light on the mechanism regulating postsynaptic secretion of BDNF and NT-3 when evoked by long-lasting depolarization. Secondly, it provides insight into the temporal dynamics of regulated neurotrophin release. Upon depolarization, release of ectopically expressed BDNF-GFP from postsynaptic sites depends on extracellular Ca^{2+} and not on Na^{+} influx. L-type VGCCs are the entry channels for Ca^{2+} in this case, but activated NMDA receptors can easily initiate the subsequent mechanisms in place of L-type VGCCs. Ca^{2+} influx induces calcium release from internal stores through ryanodine receptors. The rise of intracellular Ca^{2+} must activate CaMKII to elicit release. Efficient BDNF secretion required basal levels of PKA activity, but can not be triggered by enhanced levels of it. BDNF secretion could not be evoked by activation of Trk receptors, ruling out an impact of autocrine BDNF signalling in our experiments. Finally, fusion of individual postsynaptic BDNF granules with the plasma membrane, revealed by GFP quenching, occurred in an asynchronous manner with relatively variable delays of fusion pore opening (published in Kolarow et al., 2007).

5.1. K^{+}-induced postsynaptic neurotrophin release under conditions of blocked synaptic network activity

Neuronal activity induces synaptic BDNF secretion and secreted BDNF, in turn, plays a key role in synaptic plasticity. As synaptic plasticity requires the activation of specific signalling cascades to occur, it was tempting to test if some of these signalling cascades are also relevant for BDNF release, in order to develop a correlation between synaptic plasticity and BDNF release.

As in earlier studies, GFP-tagged BDNF was expressed in a vesicular distribution that is similar to that observed for endogenous BDNF (Hartmann et al., 2001; Adachi et al., 2005). The detected size of isolated BDNF-GFP vesicles was often larger than the earlier reported size of DCVs in neurons (80 to 200 nm from references cited by Crivellato et al., 2005), but was similar to the size of BDNF-pHluorin vesicles, as was measured by Matsuda and colleagues (2009). In the dendrites, BDNF-GFP and NT-3-GFP co-localized with PSD-95-DsRed and were packaged in DCVs of the regulated

pathway of secretion, thus allowing postsynaptic secretion of BDNF-GFP and NT-3-GFP from glutamatergic synapses to be measured with time-lapse fluorescence imaging (compare Brigadski et al., 2005).

A 5 min depolarization with high K^+ has shown to be a robust stimulus, which was defined enough to provide reproducible depolarizations and Ca^{2+} elevations in the observed areas of isolated neurons (see Fig. 4.2A, B and Fig. 4.3A, B). Sustained depolarization with high K^+ is not prejudicial to the integrity of hippocampal cells in culture, as shown in earlier studies (Ghosh et al., 1994; Hartmann et al., 2001). Shortened high K^+ application can also induce BDNF secretion effectively, but the decrease of fluorescence stops directly at the end of the stimulus, at levels that are not compatible with the levels for a convenient statistical power of our assay (see Fig. 4.2C). Therefore, we chose a 5 min high K^+ stimulus with inhibitors of ionotropic glutamatergic and GABAergic neurotransmission. This allowed us to maximize the amount of potential neurotrophin secretion and to detect small effects of the chemical substances we tested.

In the presence of TTX, an inhibitor of voltage-gated sodium channels, depolarization-induced BDNF release could take place. This is at odd with the dependence of neurotrophin secretion on AP firing as reported in other preparations using the same depolarizing stimulus with high K^+ (Blöchl and Thoenen, 1995; Androutsellis-Theotokis et al., 1996; Kojima et al., 2001). Likewise, AP firing has been shown to be instrumental in neurotrophin release evoked by electrical stimulation (Kojima et al., 2001; Gärtner and Staiger, 2002; Balkowiec and Katz, 2002). These previous studies measured bulk secretion of neurotrophins mainly from mass primary cultures involving numerous synaptically interconnected neurons. In these experimental conditions, TTX blocks secretion of BDNF most probably because of an AP driven high basal synaptic network activity due to: 1) the high cell density of these cultures and 2) the unhindered activation of neurotransmitter channels, which reinforces synaptic network activity. The experiments testing TTX in earlier studies were performed in the absence of blockers of neurotransmission, dissimilar to our use of a mix of DNQX/APV/gabazine to isolate the recorded neuron from synaptic network activity (see Fig. 4.3C, D). Consequently, the absence of APs could not impair BDNF release in our experimental setup, because the postsynaptic depolarization itself, caused locally by high extracellular K^+, is the decisive trigger of regulated exocytosis. Our method allies the advantage of a strong

stimulus mimicking an extraordinary tonic state of neuronal activity, without the annoyance of uncontrolled network effects on single cell BDNF release.

5.2. The use of different techniques to unravel the role of vesicle fusion in regulated neurotrophin secretion.

The use of a GFP tag to track the fate of the BDNF cargo requires a correct interpretation of the fluctuations of GFP fluorescence. In this regard, the intraluminal pH of vesicles is of prime importance. The intravesicular pH of native secretory granules has formerly been shown to be in the range of 5.5-6.0 (compare Han et al., 1999). BDNF-GFP fluorescence is quenched by the acidic pH in DCVs. Therefore we asked if sudden brightening of BDNF-GFP might be used to monitor the formation of fusion pores. At the onset of depolarization-evoked exocytosis, BDNF-GFP vesicles recurrently, although not in all experiments, showed a transient increase preceding the decrease of fluorescence. The transient fluorescence increase caused by single vesicle deprotonation at the moment of fusion pore opening was shown to last for less than five seconds, as reported elsewhere (compare Xia et al., 2009), while in our experiments the images were acquired at intervals of 10 seconds. For this reason we took into account that the variability of the occurrence of EGFP brightening in some experiments may be the result of either: 1) a variability of intragranular pH, 2) vesicle size, 3) delayed fusion pore opening, or a combination of two or all of these factors (compare Fig. 4.3 [BDNF/NT-3], Fig. 4.10 [high K^+ vs. SNP], Fig. 4.13 [KN-93] and Fig. 4.14 [high K^+ vs. 8-Br-cAMP]). A lower intragranular pH or bigger vesicles could cause a greater peak of fluorescence that would vanish more slowly. This would cause many delayed fusion events of equally sized vesicles to be better detected in our system. The variability of the signal's sensitivity to pH can be shown by using bafilomycin A1 which neutralizes pH more gradually than monensin, by acting directly on vesicular H^+-ATPase (Fig. 4.5, compare Brigadski et al., 2005). A more precise measurement of the fluctuation of EGFP fluorescence at the onset of stimulation would require higher time resolution and magnification, in order to resolve possible effects of drugs on the initial intragranular pH, or on the delay of fusion pore opening. In addition, the neutralization of intragranular pH was described not to depend exclusively on fusion pore opening (Han et al., 1999). In this regard, quenching EGFP fluorescence with the

non-cytotoxic BPB provides an elegant method to explain the time course of regulated exocytosis by monitoring directly the fusion of vesicles (Harata et al., 2006).

Classically, vesicle exocytosis means that fusion with the plasma membrane leads to full-collapse of the vesicle. Evidence gathered during recent years for a second mode of fusion known as kiss-and-run, "cavicapture" or "flicker" (reviewed in An and Zenisek, 2004). Depending on cell type and recording conditions, this transient mode of fusion has a prevalence varying from 20 % to 80 % for synaptic vesicles (Staal et al., 2004; Harata et al., 2006). The prevalence of kiss-and-run events for DCVs has been less intensively studied in neurons, but they are common in neuroendocrine cells (Klyachko and Jackson, 2002; Taraska et al., 2003; Elhamdani et al., 2006). This mode of exocytosis provides a means for the cell to control the amount of neuropeptide released, with the advantage of keeping the vesicle ready for repetitive release without disassembling of the release machinery in between. Through the observation of immediate quenching of the entire fluorescence of BDNF-GFP vesicles due to the quick entrance of BPB, while at the same time the release of BDNF-GFP ceases, we have shown indirectly that kiss-and-run takes place in hippocampal neurons shortly after stimulation (Fig. 4.7). We can not rule out that the size of the BDNF-GFP dimer (86 kDa vs. 26 kDa for BDNF) is a hindrance for fast release through a transient fusion pore (see Barg et al., 2002). Smaller peptides present in the cargo mixture of hippocampal DCVs might be released more easily by this mode of exocytosis, as shown earlier for DCVs in neuroendocrine cells (i.e. NPY; compare Barg et al., 2002; Taraska et al., 2003 and Perrais et al., 2004). Thus, we must take into account that EGFP fusion proteins might exhibit different release properties than those of the native proteins because of their size and their expression level. Nevertheless, we and others observed substantial release of BDNF-GFP in our experimental model (compare Xia et al., 2009). This indicates that a strong, prolonged stimulation is necessary for a sufficient and durable dilation of the fusion pore.

If DCVs undergo full-collapse fusion and release their BDNF-GFP cargo rather during, than shortly after high K^+ depolarization, this not only shows a switch in the prevailing mode of fusion at the end of depolarization, but also indicates the existence of different pools of DCVs at postsynaptic sites (compare Fig. 4.7 A). If a postsynaptic cluster of BDNF-GFP represents different pools of secretory granules regarding their readiness to undergo regulated exocytosis, asynchronous fusion events can be expected within a

vesicle cluster, because the priming state of DCVs is rate-limiting for the time-course of regulated exocytosis (reviewed in Burgoyne and Morgan, 2003). Vesicles going back and forth in the process that allows them to enter into the readily releasable "primed" state would explain well why we observe asynchronous opening of fusion pores during stimulation at different release sites of a cell (see Fig. 4.8). Recently, Xia and co-workers confirmed the existence of long latencies for stimulus-secretion coupling of DCVs in hippocampal cells monitored by TIRF microscopy[7], a technique that is particularly suited for following the priming steps of single vesicles. They specifically observed that about 50 % of the depolarization-evoked fusion events in neurites occur after the first minute of stimulation (Xia et al., 2009). These latencies before fusion may indicate that many DCVs considered as "docked" to the membrane are not in immediate vicinity to Ca^{2+} channels, lacking the Ca^{2+} trigger that is necessary to undergo fusion at the onset of stimulation. Moreover, we observed numerous motionless DCVs that were reluctant to fusion. These "dead-end docked" vesicles could possibly represent a pool of vesicles with a low probability of priming, as described in a recent model (reviewed in Verhage and Sorensen, 2008).

Depending on their localization on either soma, dendrites or axon, the fusion probability of detectable BDNF-GFP vesicles fluctuates. Although we did not analyse this aspect of BDNF secretion in hippocampal neurons, other research groups have made this observation. Interestingly, Xia et al. (2009) found that DCVs at the soma were more prompt to undergo fusion than DCVs in the neurites while at the same time cargo release was more complete in neurites (~70 % of vesicle fluorescence lost). Also, in primary hippocampal cells, Matsuda and colleagues found striking differences in release properties depending on cellular localization of DCVs: BDNF vesicles located in dendrites underwent rather full collapse fusion in response to a short 10 Hz electrical stimulation than did axonal BDNF vesicles, indicating dendrites as the main site of BDNF secretion (Matsuda et al., 2009). Likewise, preferential activity-driven secretion of progranulin, a growth factor that co-localizes with BDNF in DCVs, has been shown at dendrites, but not at axons (Petoukhov et al., 2013). These findings may be explained

[7] "[A thin layer of light, called the evanescent field,] can form when a beam of light travelling in a medium of high refractive index, such as glass, encounters one of lower refractive index such as the adjoining water or an adherent cell. When the angle of incidence α is small, light is refracted and propagates through the interface. But when α exceeds a certain 'critical angle', light instead undergoes total internal reflection. […] An evanescent field selectively illuminates fluorescent molecules near the interface and leaves more remote structures in the dark." Total internal reflection fluorescence microscopy (TIRFM) makes use of this phenomenon, thus permitting discrimination of structures of interest in a depth of 100 nm from the interface. In these limits, TIRFM suits well to image processes of vesicle exocytosis in very close vicinity of the plasma membrane (Steyer & Almers, 2001).

by subcellular differences in the composition of the pools of DCVs that are responsive to a given stimulus.

As we analyse the time course of BDNF-GFP fluorescence, we must be careful not to confuse the measure of fusion probability in the presence of extracellular BPB and the measure of release efficiency without BPB quenching of GFP. A comparative approach with both conditions in microscopy would permit to clarify which stimulus leads to full-collapse fusion and effective release of the cargo. By comparing the time course of cargo release with the time course of fusion events, we will be able to discriminate the effects of drugs on mechanisms anterior to first fusion from the effects on posterior mechanisms leading to full-collapse in regulated exocytosis.

5.3. Different sources of Ca^{2+} are needed for cytoplasmic Ca^{2+} elevation prior to postsynaptic neurotrophin release

Besides other signalling pathways, intracellular Ca^{2+} rise is the main trigger for regulated peptide exocytosis (references in Burgoyne and Morgan, 2003). Regulated BDNF release requires an elevation of intracellular Ca^{2+} (Hartmann et al., 2001). In excitable cells, membrane depolarizations trigger intracellular Ca^{2+} elevations. Specifically, the depolarization following the raise of extracellular K^+ is expected to cause a sustained increase of the intracellular Ca^{2+} concentration from about 20 nM to up to 1.5 µM in the soma and neurites.[8] The increase of Ca^{2+} in the cell results either through direct opening of VGCCs, or through indirect paths inducing the release of Ca^{2+} from internal calcium stores. Our laboratory has shown an obligatory involvement of extracellular Ca^{2+} entry, whereat the Ca^{2+} necessary for neurotrophin release can enter the cell through VGCCs or ionotropic glutamate receptors (Hartmann et al., 2001). Conversely, other studies have pointed to the instrumental role of calcium release from internal Ca^{2+} stores in that process (Blöchl and Thoenen, 1995 and 1996; Griesbeck et al., 1999; Canossa et al., 2001). Therefore, in order to determine the origin of the intracellular Ca^{2+} increase prior to postsynaptic neurotrophin secretion, we tested different blockers of Ca^{2+} signalling.

Because of their subcellular localization, the possible candidates for Ca^{2+} entry are L-type voltage gated Ca^{2+} channels (VGCCs). L-type VGCCs are stabilized in clusters

[8] Mean value of the bulk cytoplasmic calcium increase measured with the calcium fluorophore Fura-2 AM (Griesbeck et al., 1999; Rusznák et al., 2000; Samuels et al., 2007). The intracellular calcium concentration can theoretically reach 50 to 100 µM in the immediate vicinity of activated calcium channels, depending on the type of channel (reviewed in Rizzuto and Pozzan, 2006).

at postsynaptic sites of somatic and dendritic synapses, especially at dendritic spines, whereas N-type VGCCs are localized predominantly at axon terminals (Westenbroek et al., 1992; Davare et al., 2001; references in Dai et al., 2009). In hippocampal pyramidal neurons, the contribution of L-VGCCs to the generation of Ca^{2+} transients is significant, although variable, in the spines and the dendritic shafts. The amplitude of Ca^{2+} transients in spines is particularly enhanced when L-VGCCs are activated in the gating mode 2, associated with long channel openings and high open probability (Yasuda et al., 2003; Hoogland et al., 2004).[9] Thus, the involvement of L-VGCCs in triggering the release process of neurotrophins is not surprising in our experiment (see Fig. 4.11). Calcium entry through L-VGCCs upon long-lasting depolarization has been confirmed meanwhile for dendritic postsynaptic BDNF release (Kuczewski et al., 2008; Matsuda et al., 2009), while axonal release of BDNF and NT-3 needs activation of N-VGCCs (Balkowiec and Katz, 2002; Wang et al., 2002). It is improbable that N-VGCCs are involved in BDNF/NT-3 release in our model, as Xia and colleagues (2009) demonstrated peptide release under similar stimulation conditions relies strictly on L-type calcium channels.

Another possible source of Ca^{2+} must be considered in the light of results showing that activation of glutamate receptors can induce BDNF secretion (Blöchl and Thoenen, 1996; Marini et al., 1998; Canossa et al., 2001; Hartmann et al., 2001). The reported effects of blockade of AMPA receptors on postsynaptic Ca^{2+} transients obviously indicate that direct activation of AMPA receptors can trigger depolarization-induced Ca^{2+} influx, for example via VGCCs. However, calcium transients detected at dendritic spines during EPSPs are primarily caused by NMDA receptors (Yuste and Denk, 1995; Yuste et al., 1999; Kovalchuk et al., 2000). Therefore we tested another stimulation paradigm by directly activating NMDA receptors in the absence of Mg^{2+}, in order to prevent the Mg^{2+}-dependent block that is specific to these receptors (see Fig. 4.12). This factor can explain why previous studies failed to detect the involvement of NMDA receptors in neurotrophin release. Our data reveal for the first time that activation of postsynaptic NMDA receptors is sufficient to elicit postsynaptic release of BDNF in hippocampal neurons.

In models involving mass primary cultures with numerous synaptically interconnected neurons, Ca^{2+} influx is dispensable when sufficient amounts of glutamate or

[9] L-type VGCCs display three gating modes: mode 0, with channels not opening upon depolarization; mode 1, characterized by multiple short openings; and mode 2, associated with long-lasting openings and brief closings in between. L-type channel blockers typically cause a stabilization of mode 0. Gating mode 2 can be induced by L-type VGCC activators, as by stimulation of Beta-adrenergic receptors or through association of L-type channels with CaMKII (see references in Hoogland et al., 2004).

acetylcholine activate metabotropic receptors (Griesbeck et al., 1999; Balkowiec and Katz, 2002; Blöchl and Thoenen, 1995 and 1996; Canossa et al., 2001; Jourdi et al., 2009). In the absence of extracellular Ca^{2+}, metabotropic glutamate receptors can activate IP_3 receptors, thereafter inducing release of Ca^{2+} from the ER. Finally, this elevation of intracellular Ca^{2+} evokes BDNF / NT-3 secretion. Interestingly, Furutani and colleagues (2006) have demonstrated the existence of a mGlur1/IP_3/BDNF pathway in the cerebellum, which regulates the strength of Purkinje fiber inputs on Purkinje cells (PF-PC). By blocking chronically postsynaptic IP_3 signalling with specific agonists and Sindbis virus constructs, Furutani et al. (2006) could show an attenuation of the synaptic function of these inputs because of the decrease in presynaptic function. Indirectly, their results also suggest a role of retrograde messenger for BDNF at cerebellar PF-PC synapses, BDNF being secreted downstream of mGluR1/ IP_3 signalling. However, works by our laboratory excluded the involvement of metabotropic glutamate receptor signalling during high K^+-induced postsynaptic BDNF release from hippocampal cells (Hartmann et al., 2001). For that reason, we asked if depletion of Ca^{2+} stores would affect BDNF secretion. Interestingly, depletion of Ca^{2+} stores completely blocks BDNF secretion in our experimental model (see Fig. 13A, B). We next asked which mechanism releases Ca^{2+} from internal stores independently from metabotropic glutamate receptors and IP_3 receptors during high K^+-induced depolarization.

According to our results, inhibition of ryanodine receptors (RyRs) is able to prevent regulated Ca^{2+}-dependent postsynaptic BDNF release. In cardiac and skeletal muscle, ryanodine receptors are crucial for the phenomenon of excitation-contraction coupling that ensures an efficient and timely contraction of muscle following excitation. In cardiac muscle, this phenomenon is based on calcium-induced calcium release, or CICR, the Ca^{2+} release through RyR2 after activation by Ca^{2+} influx upon opening of L-type VGCCs. In skeletal muscle, a physical interaction between L-type VGCCs and the ryanodine receptors[10] is required for excitation-contraction coupling and Ca^{2+} release from the sarcoplasmic reticulum, also called voltage-induced calcium release. Excitation-contraction coupling may have its counterpart in other tissues that express L-type VGCCs and ryanodine receptors. There is evidence in neurons for both cardiac-like CICR and skeletal-like protein-protein interactions between L-type VGCCs and

[10] Respectively the skeletal CaV1.1 and RyR1 isoforms. In Cardiac muscle, excitation-contraction coupling is ensured by CaV1.2 and RyR2. All RyR isoforms are found in the mammalian brain: RyR1 appears in the cerebellum; RyR2 is expressed at high levels in Purkinje cells of cerebellum and cerebellar cortex; RyR3 is expressed in hippocampal, thalamic and striatal neurons (reviewed in Lanner et al., 2010). The L-type VGCC brain isoforms CaV1.2 and CaV1.3 are both expressed in somata, dendrites and dendritic spines of hippocampal neurons. CaV1.3 presents a smoothly distribution over the membrane while CaV1.2 is clustered in a pattern typical for synaptic proteins (Hell et al., 1993 and 1996; but see Obermair et al., 2004).

ryanodine receptors (Sharp et al., 1993; references in Lanner et al. 2010). Ca^{2+} release from internal stores might be an important amplification mechanism of neuronal Ca^{2+} transients prior to, or after Ca^{2+} entry. This role could be undertaken through Ca^{2+}-activated or L-VGCCs-coupled ryanodine receptors localized in dendritic shafts and spines.

Smooth ER cisternae are present in nearly 90 % of mature spines and the spine neck builds a barrier for rapid diffusion of free Ca^{2+} through cytoplasm (Spacek and Harris, 1997; Sabatini et al., 2002), so our results suggest that the SER Ca^{2+} store plays a mechanistic role in the spread of Ca^{2+} signals of dendritic origin into the spines to induce postsynaptic release of NTs. As reported recently, application of high K^+ as well as NMDA receptor activation leads to an immediate and reversible "fission" of the dendritic SER in cells of hippocampal slices (Kucharz et al., 2009 and 2011). The herein observed fission-fusion episodes of SER can be interpreted as symptomatic for an adaptive emptying-refilling of this principal Ca^{2+} store upon depolarization and confirm the importance of the SER in the generation of intracellular Ca^{2+} transients. Ryanodine receptors are large conductance channels capable of creating rapid transient increases of cytosolic Ca^{2+}. Their properties make RyRs the ideal candidates for the rapid supply of Ca^{2+} that is necessary for regulated exocytosis of BDNF DCVs. Due to the highly compartmentalized generation of dendritic Ca^{2+} transients, the next challenge will consist in developing paradigms able to discriminate not only the source of Ca^{2+} but also the associated pattern and propagation of Ca^{2+} signals under physiologically defined patterns of activity received by the cell.

5.4. No autocrine loop involved in neurotrophin release

NT/Trk signalling is able to mobilize intracellular Ca^{2+} for subsequent neurotrophin release, as shown by ELISA measurements of bulk secretion of NTs in the hippocampus (Canossa et al., 1997), as well as in neuroendocrine cells (Krüttgen et al., 1998). In these experimental models, activation of TrkA/TrkB/TrkC-mediated PLCγ-1 signalling most probably induces Ca^{2+} release from IP3-sensitive internal stores, independently from extracellular Ca^{2+} influx (Canossa et al., 2001). Thus, the hypothesis that a similar neurotrophin-induced secretion of neurotrophin could account – at least in part – for the regulated postsynaptic exocytosis of NTs in the present experiments, was tested.

However, application of exogenous BDNF under conditions when other sources of Ca^{2+} elevation were eliminated (i.e. in the presence of nifedipine, DNQX and APV) failed to provoke postsynaptic secretion of BDNF-GFP (Fig. 4.15A). Likewise, inhibition of Trk signalling during depolarization altered neither the time course nor the amount of secreted BDNF-GFP and NT-3-GFP, respectively, in these experiments (Fig. 4.15B, C). These data suggest the absence of any direct BDNF-induced BDNF secretion or NT-3-induced NT-3 secretion at postsynaptic sites. Also, the time course of neurotrophin secretion in control conditions indicates that there is no autocrine loop: initiated release never shows to proceed after the end of the depolarization, as would be expected in case of acute autocrine stimulation of neurotrophin release by bioactive GFP-tagged BDNF or NT-3, neither after 120 s nor after 5 min (see Fig. 4.2C and Fig. 4.3A). Overall, these data indicate that the time course and the efficiency of NT secretion at postsynaptic sites in these cultures is not shaped by NT-induced NT secretion, but do not rule out that Trk-mediated intracellular Ca^{2+} elevations can become instrumental for NT secretion at other sites of secretion, like at the growing axon (Cheng et al., 2011).

5.5. CaMKII, cAMP/PKA activity and neurotrophin release

The multifunctional protein kinase CaMKII is a key mediator of synaptic plasticity. CaMKII is activated upon intracellular Ca^{2+} increase and regulates Ca^{2+} -mediated alterations in neuronal function. CaMKII is, however, not a passive mediator of extracellular stimuli, nor of the intracellular Ca^{2+} rise. Due to its particular dodecameric structure and its property of autoregulation, this enzyme can: 1) be activated according to the frequency of intracellular Ca^{2+} transients; 2) become independent of its initial Ca^{2+}-calmoduline activators and autophosphorylate; 3) act as a molecular switch of synaptic plasticity by retaining a trace of past Ca^{2+} transients. It is widely admitted that only the autonomous active form of αCaMKII is able to react to high-frequency patterns of activity[11]. In hippocampal neurons, a brief stimulus promotes a transient and reversible interaction of CaMKII with the NMDA receptor subunit NR2B, whereas a stronger stimulus increases the quantity of CaMKII bound in spines (Bayer et al., 2006). Similarly, translocation of CaMKII to spines occurs upon LTP induction (Otmakhov et

[11] Brain CaMKII, which represents 1-2% of total brain protein, predominantly consists of homomeres or heteromeres of the alpha and the beta isoforms. αCaMKII is exclusively expressed in glutamatergic neurons and is able to autophosphorylate, while key features of βCaMKII are its ability to bind F-actin for the stabilization of the dendritic cytoskeleton and its translocation to the nucleus in order to target immediate early genes (reviewed in: Wayman et al., 2008; Giese and Mizuno, 2013).

al., 2004), and the association of CaMKII with the postsynaptic density correlates with synaptic strength of individual spines (Asrican et al., 2007). This dependency on Ca^{2+} in the context of LTP raised the question whether CaMKII might be directly involved in regulated exocytosis of neurotrophins. We found that inhibiting CaMKII impaired postsynaptic release of BDNF and NT-3 (see Fig. 4.13C, D). Thus, there seems to be a direct causal link between CaMKII and regulated neurotrophin exocytosis. In this regard, CaMKII is known to interact with L-type VGCCs, which have an instrumental role in Ca^{2+} regulated release, as we have shown. However, there currently exists a controversy on the role of CaMKII to be either a facilitator or an inactivator of L-type VGCCs (reviewed in Dai et al., 2009).

We should be cautious about possible side effects and the mode of action of CaMKII inhibitors: i) KN-62 might inhibit the purinergic P2X7 receptor (North and Jarvis, 2013) and KN-93 might inhibit L-type VGCCs (Gao et al., 2006a/b). Still, the common effect of KN-62 and KN-93 and their non-overlapping side effects strongly argue for the inhibition of CaMKII in our cultures; ii) KN-62 or KN-93 work to compete with Ca^{2+}-calmoduline binding to the regulatory domain of CaMKII, meaning that they inhibit Ca^{2+}/CaM-dependent CaMKII, but not autonomous CaMKII activity (Wayman et al., 2008). Consequently, the CaMKII form involved in depolarization-induced neurotrophin secretion should be the Ca^{2+}/CaM-dependent form. We observed no impairment of neurotrophin release when the cells were pre-incubated with the inactive KN-93 analogue KN-92, indicating that CaMKII activation is required at the time of the stimulus. This observation, however, does not rule out the possibility that autonomous activity of CaMKII is maintained for some time during the stimulus. Future experiments addressing the role of CaMKII on postsynaptic neurotrophin release should test new potent inhibitors, e.g. peptides derived from the autoregulatory domain of CaMKII (Wayman et al., 2008).

Besides possibly controlling regulated exocytosis through phosphorylation of L-type VGCCs, CaMKII is also able to phosphorylate multiple targets in the release machinery: syntaxin I, SNAP-25, NSF, alpha-SNAP, synaptotagmin, synaptobrevin, Rabphilin3A (references in Burgoyne and Morgane, 2003). The mechanisms of exocytosis depending on CaMKII activity are still not known in detail, but our data is in line with precedent related results on catecholamine secretion in PC12 cells (Schweitzer et al., 1995), axonal release of NT-3 in the chick optic tectum (Wang et al., 2002) and

Ca^{2+}-evoked dendritic exocytosis in hippocampal neurons (Maletic-Savatic, Koothan and Malinow, 1998).

The contribution of cAMP/ PKA signalling to synaptic plasticity in the hippocampus is well established, particularly for the maintenance of L-LTP at CA3-CA1 synapses. Pharmacological and transgenic inhibitions of PKA both block L-LTP in the CA1 region (Frey et al., 1993; Huang and Kandel, 1994; Abel et al., 1997). Conversely, PKA activation with a cAMP analogue, or with forskolin, an activator of adenylate cyclase, elicits synaptic facilitation (Chavez-Noriega and Stevens, 1992) that can occlude L-LTP (Frey et al., 1993; Huang and Kandel, 1994). At postsynaptic sites of CA1, Blitzer and colleagues (1995) have described a "gating" action of PKA, where exogenous activation of PKA neither induces nor facilitates E-LTP, but inhibition with Rp-cAMPS blocks it. Similarly, while in our experiments activation of PKA does not trigger BDNF secretion per se, the K^+-induced secretion of BDNF is inhibited when the activity of postsynaptic PKA is reduced with Rp-cAMPS (Fig. 4.14). In view of that, our results suggest a "gating" action of PKA, where sustained PKA-dependent phosphorylation of a target protein is needed for efficient postsynaptic release of neurotrophins.

Interestingly, a functional link between L-type VGCCs and actors of the cAMP/PKA pathway has been shown: β-adrenergic activation of L-type VGCCs via cAMP/PKA signalling takes place at dendritic spines of the CA1 (see Hoogland et al., 2004). L-type VGCCs are considered as a target of PKA activity (Dai et al., 2009). Phosphorylation of CaV1.2 channels by PKA proceeds on serine-1928. The cytoplasmic COOH-tail including S1928 can be cleaved-off by the Ca^{2+}-dependent protease calpain. After cleavage, the resulting short form of CaV1.2 is characterized by severalfold potentiated Ca^{2+} currents. The exact mode of regulation of CaV1.2 through PKA and calpain remains to be proved. A current speculative model is that the interaction of the very COOH-terminus with the rest of the channel reduces ion conduction activity and that phosphorylation of S1928 by PKA releases this inhibitory interaction (Dai et al., 2009). This model could explain how PKA "gates" release of neurotrophins at postsynaptic sites through modulation of L-type VGCCs. In others words, PKA could then only enable facilitation of L-type Ca^{2+} currents at basal levels of cAMP.

At present, we can not exclude that the inhibition of PKA limits neurotrophin secretion partially through regulation of L-type VGCCs, rather than through mobilization of readily releasable "primed" DCVs. Like CaMKII, PKA can phosphorylate proteins

which are directly involved in the release machinery: for instance SNAP-25 (Nagy et al., 2004), Rabphilin3A (Lonart and Südhof, 1998), Snapin (Chheda et al., 2001) and tomosyn (Baba et al., 2005; and references in Burgoyne and Morgane, 2003). In order to discriminate the effect of PKA on Ca^{2+} influx and its direct effect on components of vesicle exocytosis, new studies are required. Future experiments addressing the role of PKA in neurotrophin secretion should use combinations of methods, permitting activation of CaV1.2 in gating mode 2 independently from phosphorylation on S1928. According to the model of the COOH-terminus regulated L-type VGCCs, one possible experiment would be the simultaneous use of calpain cleavage or truncation of CaV1.2 and PKA inhibition. The role of PKA on neurotrophin secretion would then primarily shift to a regulation of the release machinery if the gating effect of PKA disappears.

5.6. Interactions between nitric oxide and BDNF signalling pathways (published in Kolarow et al., 2014)

At the molecular level, there is evidence for a direct positive interaction between NO and BDNF signalling pathways, as shown e.g. by the direct activation of TrkB receptors through NO-induced formation of peroxynitrite (Yuen et al., 2000). Interestingly, the small G protein Ras, activated downstream of BDNF/TrkB signalling, is a target for S-nitrosylation, potentially giving rise to NO-induced activation of MAP kinases (Lander et al., 1996). Importantly, several studies showed a postsynaptic localization of both the NO producing nitric oxide synthase (nNOS/eNOS) and the TrkB receptor in the postsynaptic density of glutamatergic synapses of the hippocampus, or of nNOS close to the NMDA receptor complex (Husi et al., 2000). In the CA1 and CA2 layers of rat hippocampus, nNOS is a binding partner for NR2A, a subunit of NMDA receptors, which associate with PSD-95 (Al-Hallaq et al., 2007). The postsynaptic protein PSD-95 is necessary for the association of the nNOS/NMDAR complex, where it binds both proteins of the complex through a PDZ domain (Christopherson et al., 1999; Cui et al., 2007). Also other components of the NO/cGMP/PKG signalling pathway, for instance the NO-sensitive soluble guanylyl cyclase (sGC), are localized in part in postsynaptic densities (Burette et al., 2002). In addition, activated PKG, which is a major effector downstream of NO/cGMP, has been shown to be permissive for regulated exocytosis in non-neuronal cells (Li et al.,2004; Nanamori et al., 2007), thus suggesting the existence of a crosstalk between NO signalling and peptide secretion, also outside the central

nervous system. Taken together, these studies suggest a direct interaction of BDNF and NO signalling pathways in postsynaptic structures, emphasizing that they can act in concert on synaptic plasticity.

In knock-out mice models, the depletion of *Bdnf* or of the gene encoding NOS result both in a partial impairment of CA1-LTP (Korte et al., 1995; Son et al., 1996). Recent measurements on $BDNF^{+/-}$ knock-out slices have shown that additional inhibition of NOS did not further affect the residual component of CA1-LTP (Lessmann et al., 2011). Given that NO and BDNF are regarded as possible retrograde messengers in LTP, it was tempting to speculate that NO could modulate postsynaptic BDNF exocytosis in hippocampal neurons. Our observation that exogenous supply of NO inhibits the secretion of neurotrophins (see Fig. 4.10A, B) is in line with previous observations (Canossa et al., 2002; Santi et al., 2006). It was also reported that exogenous application of activators of the cGMP/PKG signalling pathway, downstream of NOS, similarly decreased basal/spontaneous BDNF secretion (Canossa et al., 2002).

Since depolarization with high K^+ can by itself stimulate NO generation (compare Knowles et al., 1989), the most sensitive test for the contribution of endogenously produced NO on regulated secretion in our experiments was the application of NOS inhibitors during elevated K^+-induced depolarization. As we found that addition of L-NMMA to our cultures had no effect on depolarization-induced secretion of BDNF or NT-3 (Fig. 4.10C, D), these results suggest that the endogenous NO levels reached during a robust depolarization do not reach threshold to inhibit neurotrophin secretion. Our finding that inhibition of endogenous baseline NO production does not affect neurotrophin secretion is at variance with the results of Canossa et al. (2002). This discrepancy can be explained by differences of both our detection and culture systems: e.g. Canossa et al. (2002) measured bulk secretion from mass primary cultures, while we measured depolarization-induced postsynaptic secretion under conditions of blocked synaptic network activity.

The inhibition of neurotrophin secretion seen under elevated NO levels with SNP could possibly result from a modulation of Ca^{2+} permeable ion channels. A study by Petzold et al. (2005) revealed that NO can modulate Ca^{2+} entry in cortical neurons by interacting with NMDARs and VGCCs, that are instrumental in regulated neurotrophin secretion. Importantly, NO-dependent potentiation of L-type Ca^{2+} currents has been found in neurons of the hippocampus (Jian et al., 2007) and of the brain stem (Tozer, Forsythe and Steinert, 2012). Still, the respective contributions of direct S-nitrosylation of

VGCCs through NO-derived free radicals versus phosphorylation of VGCCs as a result of the activated sGC/cGMP/PKG pathway need to be clarified. The target proteins of nitrergic modulation during regulated BDNF secretion could be identified by further pharmacology in comparative studies using the benefits of BDNF-GFP quenching.

5.7. Outlook

New questions arise, as the involvement of key proteins in regulated BDNF secretion is revealed. Still the exact dynamic processes of this phenomenon remain elusive. The relative contributions of the excitatory drive of the synaptic network, diverse sources of Ca^{2+} entry, CaMKII, PKA and NO signalling pathways on BDNF release might not be exclusive, but rather overlap and thus allow compensation as well as potentiation of the initial trigger effect.

Our choice to isolate the recorded cell from synaptic network activity with blockers of neurotransmission allows focusing on intrinsic signalling pathways of the neurotrophin secreting neuron. Nevertheless, future experiments on neurotrophin secretion using GFP imaging with electrical stimulation in dissociated hippocampal cells as well as in hippocampal slices of BDNF-GFP knock-in mice would provide insight into the most physiologically relevant network activity that leads to BDNF secretion in vivo, e.g. LTP-inducing activity and/or backpropagating action potentials.

We performed first BDNF-GFP release experiments in the presence of the fluorescence quencher bromophenol blue that suggest the occurrence of a regulated kiss-and-run mechanism of postsynaptic localized BDNF DCVs. Regardless of the speed of diffusion of neurotrophins through the fusion pore, we are now able to directly compare fusion probability and release efficiency during DCV exocytosis. On the one hand this technique would permit to clarify which stimulus leads to full-collapse fusion and effective release of the cargo. On the other hand, a well-combined pharmacology would allow to identify if CaMKII, PKA, NO and their target proteins act before, during or after priming of BDNF DCVs.

6. ABSTRACT

The mammalian neurotrophins (NTs) BDNF and NT-3 are secreted neuronal growth factors. In addition, NTs are implicated in several forms of activity-dependent synaptic plasticity. Although synaptic secretion of NTs has been described, the intracellular signalling cascades that regulate synaptic secretion of NTs are far from being understood. Analysis of NT secretion at the subcellular level is thus required to resolve the role of presynaptic and postsynaptic NT secretion for synaptic plasticity. In the present work, cultures of dissociated rat hippocampal neurons were transfected with green fluorescent protein-tagged versions of BDNF and NT-3, respectively, and NT vesicles were identified at glutamatergic synapses by co-localization with the co-transfected postsynaptic marker PSD-95-DsRed. Depolarization-induced secretion of BDNF and NT-3 was monitored via live cell imaging. Direct postsynaptic depolarization with elevated K^+ in the presence of blockers of synaptic transmission allowed investigation of the signalling cascades that are involved in the postsynaptic NT vesicle secretion process. It could be shown that depolarization-induced postsynaptic NT secretion is elicited by Ca^{2+} influx, either via L-type voltage-gated calcium channels or via NMDA receptors. Subsequent release of Ca^{2+} from internal stores via ryanodine receptors is required for the secretion process. Postsynaptic NT secretion is inhibited in the presence of KN-62 and KN-93, indicating a critical dependence on the activation of alpha-calcium-calmoduline-dependent protein kinase II (CaMKII). The cAMP/protein kinase A (PKA) signalling inhibitor, Rp-cAMP-S, as well as the NO donor, SNP, impaired NT secretion, whereas elevation of intracellular cAMP levels and the NO synthase inhibitor L-NMMA were without effect. Using the Trk inhibitor k252a, it could be shown that NT-induced NT secretion does not contribute to the NT release process at synapses, and BDNF does not induce its own secretion at postsynaptic sites. Release experiments in the presence of the fluorescence quencher bromophenol blue provide evidence for asynchronous and prolonged fusion pore opening of NT vesicles during secretion. Because fusion pore opening is fast compared with compound release, the speed of NT release seems to be limited by diffusion of NTs out of the vesicle. Together, these results reveal a strong dependence of activity-dependent postsynaptic NT secretion on Ca^{2+} influx, Ca^{2+} release from internal stores, activation of CaMKII, and intact PKA signalling, whereas Trk signalling, activation of Na^+ channels and NO signalling are not required.

7. REFERENCES

Abel T, Nguyen PV, Barad M, Deuel TA, Kandel ER, Bourtchouladze R (1997) Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell 88:615–626.

Abidin I, Eysel UT, Lessmann VJ, Mittmann T (2008) Impaired GABAergic inhibition in the visual cortex of brain-derived neurotrophic factor heterozygous knockout mice. J. Physiol. (Lond.) 586:1885–1901. DOI: 10.1113/jphysiol.2007.148627.

Abidin I, Kohler T, Weiler E, Zoidl G, Eysel UT, Lessmann V, Mittmann T (2006) Reduced presynaptic efficiency of excitatory synaptic transmission impairs LTP in the visual cortex of BDNF-heterozygous mice. Eur. J. Neurosci 24:3519–3531. DOI: 10.1111/j.1460-9568.2006.05242.x.

Adachi N, Kohara K, Tsumoto T (2005) Difference in trafficking of brain-derived neurotrophic factor between axons and dendrites of cortical neurons, revealed by live-cell imaging. BMC Neurosci 6:42. DOI: 10.1186/1471-2202-6-42.

Aid T, Kazantseva A, Piirsoo M, Palm K, Timmusk T (2007) Mouse and rat BDNF gene structure and expression revisited. J. Neurosci. Res 85:525–535. DOI: 10.1002/jnr.21139.

Akaneya Y, Tsumoto T, Kinoshita S, Hatanaka H (1997) Brain-derived neurotrophic factor enhances long-term potentiation in rat visual cortex. J. Neurosci. 17:6707–6716.

Al-Hallaq RA, Conrads TP, Veenstra TD, Wenthold RJ (2007) NMDA di-heteromeric receptor populations and associated proteins in rat hippocampus. J. Neurosci 27:8334–8343. DOI: 10.1523/JNEUROSCI.2155-07.2007.

Altar CA, DiStefano PS (1998) Neurotrophin trafficking by anterograde transport. Trends Neurosci. 21:433–437. DOI: 10.1016/S0166-2236(98)01273-9.

Amaral MD, Pozzo-Miller LD (2007) TRPC3 channels are necessary for brain-derived neurotrophic factor to activate a nonselective cationic current and to induce dendritic spine formation. J. Neurosci 27:5179–5189. DOI: 10.1523/JNEUROSCI.5499-06.2007.

An JJ, Gharami K, Liao G, Woo NH, Lau AG, Vanevski F, Torre ER, Jones KR, Feng Y, Lu B, Xu B (2008) Distinct role of long 3' UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. Cell 134:175–187. DOI: 10.1016/j.cell.2008.05.045.

An S, Zenisek D (2004) Regulation of exocytosis in neurons and neuroendocrine cells. Curr. Opin. Neurobiol. 14:522–530. DOI: 10.1016/j.conb.2004.08.008.

Androutsellis-Theotokis A, McCormack WJ, Bradford HF, Stern GM, Pliego-Rivero FB (1996) The depolarisation-induced release of [125I]BDNF from brain tissue. Brain Res. 743:40–48. DOI: 10.1016/S0006-8993(96)00981-X.

Angleson JK, Cochilla AJ, Kilic G, Nussinovitch I, Betz WJ (1999) Regulation of dense core release from neuroendocrine cells revealed by imaging single exocytic events. Nat. Neurosci. 2:440–446. DOI: 10.1038/8107.

Aoki C, Wu K, Elste A, Len GW, Lin SY, McAuliffe G, Black IB (2000) Localization of brain-derived neurotrophic factor and TrkB receptors to postsynaptic densities of adult rat cerebral cortex. J. Neurosci. Res. 59:454–463. DOI: 10.1002/(SICI)1097-4547(20000201)59:3<454::AID-JNR21>3.0.CO;2-H

Asrican B, Lisman J, Otmakhov N (2007) Synaptic strength of individual spines correlates with bound Ca2+-calmodulin-dependent kinase II. J. Neurosci. 27:14007–14011. DOI: 10.1523/JNEUROSCI.3587-07.2007.

Baba T, Sakisaka T, Mochida S, Takai Y (2005) PKA-catalyzed phosphorylation of tomosyn and its implication in Ca2+-dependent exocytosis of neurotransmitter. J. Cell Biol. 170:1113–1125. DOI: 10.1083/jcb.200504055.

Bagayogo IP, Dreyfus CF (2009) Regulated release of BDNF by cortical oligodendrocytes is mediated through metabotropic glutamate receptors and the PLC pathway. ASN Neuro 1. DOI: 10.1042/AN20090006.

Balkowiec A, Katz DM (2002) Cellular mechanisms regulating activity-dependent release of native brain-derived neurotrophic factor from hippocampal neurons. J Neurosci 22:10399–10407.

Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG, Miller FD (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. J. Cell Biol. 140:911–923. DOI: 10.1083/jcb.140.4.911.

Barde YA, Edgar D, Thoenen H (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J. 1:549–553.

Barg S, Olofsson CS, Schriever-Abeln J, Wendt A, Gebre-Medhin S, Renström E, Rorsman P (2002) Delay between fusion pore opening and peptide release from large dense-core vesicles in neuroendocrine cells. Neuron 33:287–299. DOI: 10.1016/S0896-6273(02) 00563-9.

Bartkowska K, Paquin A, Gauthier AS, Kaplan DR, Miller FD (2007) Trk signaling regulates neural precursor cell proliferation and differentiation during cortical development. Development 134:4369–4380. DOI: 10.1242/dev.008227.

Bayer KU, Lebel E, McDonald GL, O'Leary H, Schulman H, Koninck P de (2006) Transition from reversible to persistent binding of CaMKII to postsynaptic sites and NR2B. J. Neurosci 26:1164–1174. DOI: 10.1523/JNEUROSCI.3116-05.2006.

Bergami M, Santi S, Formaggio E, Cagnoli C, Verderio C, Blum R, Berninger B, Matteoli M, Canossa M (2008) Uptake and recycling of pro-BDNF for transmitter-induced secretion by cortical astrocytes. J. Cell Biol 183:213–221. DOI: 10.1083/jcb.200806137.

Berninger B, Schinder AF, Poo M (1999) Synaptic reliability correlates with reduced susceptibility to synaptic potentiation by brain-derived neurotrophic factor. Learn. Mem 6:232–242.

Blitzer RD, Wong T, Nouranifar R, Iyengar R, Landau EM (1995) Postsynaptic cAMP pathway gates early LTP in hippocampal CA1 region. Neuron 15:1403–1414. DOI: 10.1016/0896-6273(95)90018-7.

Blöchl A, Thoenen H (1995) Characterization of nerve growth factor (NGF) release from hippocampal neurons: evidence for a constitutive and an unconventional sodium-dependent regulated pathway. Eur. J. Neurosci 7:1220–1228. DOI: 10.1111/j.1460-9568.1995. tb01112.x.

Bonhoeffer T (1996) Neurotrophins and activity-dependent development of the neocortex. Curr. Opin. Neurobiol. 6:119–126. DOI: 10.1016/S0959-4388(96)80017-1.

Bonhoeffer T, Shatz CJ (1998) Neurotrophins and Visual System Plasticity. In: Mechanistic relationships between development and learning. Report of the Dahlem Workshop on Mechanistic Relationships between Development and Learning, Berlin, January 19-25, 1997 (Carew TJ, Menzel R, Shatz CJ, eds), pp 93–112. Chichester, New York: Wiley.

Bothwell M (2006) Evolution of the neurotrophin signaling system in invertebrates. Brain Behav. Evol. 68:124–132. DOI: 10.1159/000094082.

Boulanger L, Poo M (1999) Gating of BDNF-induced synaptic potentiation by cAMP. Science 284:1982–1984. DOI: 10.1126/science.284.5422.1982.

Bradshaw RA, Brickey WW, Walker KW (1998) N-terminal processing: the methionine aminopeptidase and N alpha-acetyl transferase families. Trends Biochem. Sci. 23:263–267.

Bramham CR, Messaoudi E (2005) BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. Prog. Neurobiol 76:99–125. DOI: 10.1016/j.pneurobio.2005. 06.003.

Brann AB, Tcherpakov M, Williams IM, Futerman AH, Fainzilber M (2002) Nerve growth factor-induced p75-mediated death of cultured hippocampal neurons is age-dependent and transduced through ceramide generated by neutral sphingomyelinase. J. Biol. Chem. 277:9812–9818. DOI: 10.1074/jbc.M109862200.

Brewer GJ, Cotman CW (1989) Survival and growth of hippocampal neurons in defined medium at low density: advantages of a sandwich culture technique or low oxygen. Brain Res. 494:65–74. DOI: 10.1016/0006-8993(89)90144-3.

Brewer GJ, Torricelli JR, Evege EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J. Neurosci. Res 35:567–576. DOI: 10.1002/jnr.490350513.

Brigadski T, Hartmann M, Lessmann V (2005) Differential vesicular targeting and time course of synaptic secretion of the mammalian neurotrophins. J Neurosci 25:7601–7614. DOI: 10.1523/JNEUROSCI.1776-05.2005.

Bruno MA, Cuello AC (2006) Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease cascade. Proc. Natl. Acad. Sci. U.S.A 103:6735–6740. DOI: 10.1073/pnas.0510645103.

Burette A, Zabel U, Weinberg RJ, Schmidt HHHW, Valtschanoff JG (2002) Synaptic localization of nitric oxide synthase and soluble guanylyl cyclase in the hippocampus. J. Neurosci 22:8961–8970.

Burgoyne RD, Morgan A (2003) Secretory granule exocytosis. Physiol. Rev. 83:581–632. DOI: 10.1152/physrev.00031.2002.

Caldeira MV, Melo CV, Pereira DB, Carvalho RF, Carvalho AL, Duarte CB (2007a) BDNF regulates the expression and traffic of NMDA receptors in cultured hippocampal neurons. Mol. Cell. Neurosci. 35:208–219. DOI: 10.1016/j.mcn.2007.02.019.

Caldeira MV, Melo CV, Pereira DB, Carvalho R, Correia SS, Backos DS, Carvalho AL, Esteban JA, Duarte CB (2007b) Brain-derived neurotrophic factor regulates the expression and synaptic delivery of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons. J. Biol. Chem. 282:12619–12628. DOI: 10.1074/jbc.M700607200.

Canossa M, Gärtner A, Campana G, Inagaki N, Thoenen H (2001) Regulated secretion of neurotrophins by metabotropic glutamate group I (mGluRI) and Trk receptor activation is mediated via phospholipase C signalling pathways. EMBO J. 20:1640–1650. DOI: 10.1093/emboj/20.7.1640.

Canossa M, Giordano E, Cappello S, Guarnieri C, Ferri S (2002) Nitric oxide down-regulates brain-derived neurotrophic factor secretion in cultured hippocampal neurons. Proc. Natl. Acad. Sci. U.S.A 99:3282–3287. DOI: 10.1073/pnas.042504299.

Canossa M, Griesbeck O, Berninger B, Campana G, Kolbeck R, Thoenen H (1997) Neurotrophin release by neurotrophins: implications for activity-dependent neuronal plasticity. Proc. Natl. Acad. Sci. U.S.A 94:13279–13286.

Carmignoto G, Pizzorusso T, Tia S, Vicini S (1997) Brain-derived neurotrophic factor and nerve growth factor potentiate excitatory synaptic transmission in the rat visual cortex. J. Physiol. (Lond.) 498 (Pt 1):153–164.

Chanat E, Huttner WB (1991) Milieu-induced, selective aggregation of regulated secretory proteins in the trans-Golgi network. J. Cell Biol. 115:1505–1519. DOI: 10.1083/jcb.115.6.1505.

Chavez-Noriega LE, Stevens CF (1992) Modulation of synaptic efficacy in field CA1 of the rat hippocampus by forskolin. Brain Res. 574:85–92. DOI: 10.1016/0006-8993(92)90803-H.

Chen CA, Okayama H (1988) Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. BioTechniques 6:632–638.

Chen WG, Chang Q, Lin Y, Meissner A, West AE, Griffith EC, Jaenisch R, Greenberg ME (2003) Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. Science 302:885–889. DOI: 10.1126/science.1086446.

Chen Z, Ieraci A, Teng H, Dall H, Meng C, Herrera DG, Nykjaer A, Hempstead BL, Lee FS (2005) Sortilin controls intracellular sorting of brain-derived neurotrophic factor to the regulated secretory pathway. J. Neurosci 25:6156–6166. DOI: 10.1523/JNEUROSCI.1017-05.2005.

Chen Z, Patel PD, Sant G, Meng C, Teng KK, Hempstead BL, Lee FS (2004) Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. J. Neurosci 24:4401–4411. DOI: 10.1523/JNEUROSCI.0348-04.2004.

Cheng P, Song A, Wong Y, Wang S, Zhang X, Poo M (2011) Self-amplifying autocrine actions of BDNF in axon development. Proc. Natl. Acad. Sci. U.S.A. 108:18430–18435. DOI: 10.1073/pnas.1115907108.

Chheda MG, Ashery U, Thakur P, Rettig J, Sheng ZH (2001) Phosphorylation of Snapin by PKA modulates its interaction with the SNARE complex. Nat. Cell Biol. 3:331–338. DOI: 10.1038/35070000.

Christopherson KS, Hillier BJ, Lim WA, Bredt DS (1999) PSD-95 assembles a ternary complex with the N-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. J. Biol. Chem 274:27467–27473. DOI: 10.1074/jbc.274.39.27467.

Cochilla AJ, Angleson JK, Betz WJ (1999) Monitoring secretory membrane with FM1-43 fluorescence. Annu. Rev. Neurosci. 22:1–10. DOI: 10.1146/annurev.neuro.22.1.1.

Collin C, Vicario-Abejon C, Rubio ME, Wenthold RJ, McKay RD, Segal M (2001) Neurotrophins act at presynaptic terminals to activate synapses among cultured hippocampal neurons. Eur. J. Neurosci. 13:1273–1282. DOI: 10.1046/j.0953-816x.2001.01500.x.

Conner JM, Lauterborn JC, Yan Q, Gall CM, Varon S (1997) Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. J. Neurosci 17:2295–2313.

Conover JC, Erickson JT, Katz DM, Bianchi LM, Poueymirou WT, McClain J, Pan L, Helgren M, Ip NY, Boland P (1995) Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. Nature 375:235–238. DOI: 10.1038/375235a0.

Crivellato E, Nico B, Ribatti D (2005) Ultrastructural evidence of piecemeal degranulation in large dense-core vesicles of brain neurons. Anat. Embryol. 210:25–34. DOI: 10.1007/s00429-005-0002-z.

Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, Armanini MP, Ling LH, McMahon SB, Shelton DL, Levinson AD (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76:1001–1011. DOI: 10.1016/0092-8674(94)90378-6.

Crozier RA, Black IB, Plummer MR (1999) Blockade of NR2B-containing NMDA receptors prevents BDNF enhancement of glutamatergic transmission in hippocampal neurons. Learn. Mem. 6:257–266.

Crozier RA, Bi C, Han YR, Plummer MR (2008) BDNF modulation of NMDA receptors is activity dependent. J. Neurophysiol. 100:3264–3274. DOI: 10.1152/jn.90418.2008.

Cui H, Hayashi A, Sun H, Belmares MP, Cobey C, Phan T, Schweizer J, Salter MW, Wang YT, Tasker RA, Garman D, Rabinowitz J, Lu PS, Tymianski M (2007) PDZ protein interactions underlying NMDA receptor-mediated excitotoxicity and neuroprotection by PSD-95 inhibitors. J. Neurosci 27:9901–9915. DOI: 10.1523/JNEUROSCI.1464-07.2007.

Dai S, Hall DD, Hell JW (2009) Supramolecular assemblies and localized regulation of voltage-gated ion channels. Physiol. Rev. 89:411–452. DOI: 10.1152/physrev.00029.2007.

Danzer SC, McNamara JO (2004) Localization of brain-derived neurotrophic factor to distinct terminals of mossy fiber axons implies regulation of both excitation and feedforward inhibition of CA3 pyramidal cells. J. Neurosci. 24:11346–11355. DOI: 10.1523/JNEUROSCI.3846-04.2004.

Davare MA, Avdonin V, Hall DD, Peden EM, Burette A, Weinberg RJ, Horne MC, Hoshi T, Hell JW (2001) A beta2 adrenergic receptor signaling complex assembled with the Ca2+ channel Cav1.2. Science 293:98–101. DOI: 10.1126/science.293.5527.98.

Dean C, Liu H, Dunning F, Chang P, Jackson M, Chapman ER (2009) Synaptotagmin-IV modulates synaptic function and long-term potentiation by regulating BDNF release. Nat. Neurosci 12:767–776. DOI: 10.1038/nn.2315.

Dean C, Liu H, Staudt T, Stahlberg MA, Vingill S, Bückers J, Kamin D, Engelhardt J, Jackson MB, Hell SW, Chapman ER (2012) Distinct subsets of Syt-IV/BDNF vesicles are sorted to axons versus dendrites and recruited to synapses by activity. J. Neurosci. 32:5398–5413. DOI: 10.1523/JNEUROSCI.4515-11.2012.

del Toro D, Canals JM, Ginés S, Kojima M, Egea G, Alberch J (2006) Mutant huntingtin impairs the post-Golgi trafficking of brain-derived neurotrophic factor but not its Val66Met polymorphism. J. Neurosci 26:12748–12757. DOI: 10.1523/JNEUROSCI.3873-06.2006.

Dieni S, Matsumoto T, Dekkers M, Rauskolb S, Ionescu MS, Deogracias R, Gundelfinger ED, Kojima M, Nestel S, Frotscher M, Barde Y (2012) BDNF and its pro-peptide are stored in presynaptic dense core vesicles in brain neurons. J. Cell Biol. 196:775–788. DOI: 10.1083/jcb.201201038.

Drake CT, Milner TA, Patterson SL (1999) Ultrastructural localization of full-length TrkB immunoreactivity in rat hippocampus suggests multiple roles in modulating activity-dependent synaptic plasticity. J. Neurosci. 19:8009–8026.

Dugich-Djordjevic MM, Peterson C, Isono F, Ohsawa F, Widmer HR, Denton TL, Bennett GL, Hefti F (1995) Immunohistochemical visualization of brain-derived neurotrophic factor in the rat brain. Eur. J. Neurosci. 7:1831–1839. DOI: 10.1111/j.1460-9568.1995.tb00703.x.

Edelmann E, Leßmann V, Brigadski T (2013) Pre- and postsynaptic twists in BDNF secretion and action in synaptic plasticity. Neuropharmacology. DOI: 10.1016/j.neuropharm. 2013.05.043.

Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B, Weinberger DR (2003) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. Cell 112:257–269. DOI: 10.1016/S0092-8674(03)00035-7.

Eipper BA, Milgram SL, Husten EJ, Yun HY, Mains RE (1993) Peptidylglycine alpha-amidating monooxygenase: a multifunctional protein with catalytic, processing, and routing domains. Protein Sci. 2:489–497. DOI: 10.1002/pro.5560020401.

Elhamdani A, Azizi F, Artalejo CR (2006) Double patch clamp reveals that transient fusion (kiss-and-run) is a major mechanism of secretion in calf adrenal chromaffin cells: high calcium shifts the mechanism from kiss-and-run to complete fusion. J. Neurosci. 26:3030–3036. DOI: 10.1523/JNEUROSCI.5275-05.2006.

Ernfors P, Lee KF, Jaenisch R (1994a) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. Nature 368:147–150. DOI: 10.1038/368147a0.

Ernfors P, Lee KF, Kucera J, Jaenisch R (1994b) Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. Cell 77:503–512. DOI: 10.1016/0092-8674(94)90213-5.

Evans S, Irmady K, Ostrow K, Kim T, Nykjaer A, Saftig P, Blobel C, Hempstead BL (2011) Neuronal brain-derived neurotrophic factor is synthesized in excess, with levels regulated by sortilin-mediated trafficking and lysosomal degradation. J. Biol. Chem 286:29556–29567. DOI: 10.1074/jbc.M111.219675.

Farhadi HF, Mowla SJ, Petrecca K, Morris SJ, Seidah NG, Murphy RA (2000) Neurotrophin-3 sorts to the constitutive secretory pathway of hippocampal neurons and is diverted to the regulated secretory pathway by coexpression with brain-derived neurotrophic factor. J. Neurosci 20:4059–4068.

Fariñas I, Jones KR, Backus C, Wang XY, Reichardt LF (1994) Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature 369:658–661. DOI: 10.1038/369658a0.

Fauchais A, Lalloue F, Lise M, Boumediene A, Preud'homme J, Vidal E, Jauberteau M (2008) Role of endogenous brain-derived neurotrophic factor and sortilin in B cell survival. J Immunol 181:3027–3038.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B (1996) Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381:706–709. DOI: 10.1038/381706a0.

Frey U, Huang YY, Kandel ER (1993) Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. U Frey, YY Huang, and ER Kandel. Science 260:1661–1664.

Friedman WJ (2000) Neurotrophins induce death of hippocampal neurons via the p75 receptor. J. Neurosci. 20:6340–6346.

Furutani K, Okubo Y, Kakizawa S, Iino M (2006) Postsynaptic inositol 1,4,5-trisphosphate signaling maintains presynaptic function of parallel fiber-Purkinje cell synapses via BDNF. Proc. Natl. Acad. Sci. U.S.A 103:8528–8533. DOI: 10.1073/pnas.0600497103.

Gao L, Blair LAC, Marshall J (2006b) CaMKII-independent effects of KN93 and its inactive analog KN92: reversible inhibition of L-type calcium channels. Biochem. Biophys. Res. Commun. 345:1606–1610. DOI: 10.1016/j.bbrc.2006.05.066.

Gao L, Blair LAC, Salinas GD, Needleman LA, Marshall J (2006a) Insulin-like growth factor-1 modulation of CaV1.3 calcium channels depends on Ca2+ release from IP3-sensitive stores and calcium/calmodulin kinase II phosphorylation of the alpha1 subunit EF hand. J. Neurosci. 26:6259–6268. DOI: 10.1523/JNEUROSCI.0481-06.2006.

García-Suárez O, Pérez-Pérez M, Germanà A, Esteban I, Germanà G (2003) Involvement of growth factors in thymic involution. Microsc. Res. Tech. 62:514–523. DOI: 10.1002/jemt.10413.

Gärtner A, Polnau DG, Staiger V, Sciarretta C, Minichiello L, Thoenen H, Bonhoeffer T, Korte M (2006) Hippocampal long-term potentiation is supported by presynaptic and postsynaptic tyrosine receptor kinase B-mediated phospholipase Cgamma signaling. J. Neurosci 26:3496–3504. DOI: 10.1523/JNEUROSCI.3792-05.2006.

Gärtner A, Staiger V (2002) Neurotrophin secretion from hippocampal neurons evoked by long-term-potentiation-inducing electrical stimulation patterns. Proc. Natl. Acad. Sci. U.S.A 99:6386–6391. DOI: 10.1073/pnas.092129699.

Gauthier LR, Charrin BC, Borrell-Pages M, Dompierre JP, Rangone H, Cordelieres FP, De MJ, MacDonald ME, Lessmann V, Humbert S, Saudou F (2004) Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. Cell 118:127–138.

Ghosh A, Carnahan J, Greenberg ME (1994) Requirement for BDNF in activity-dependent survival of cortical neurons. Science 263:1618–1623. DOI: 10.1126/science.7907431.

Giese KP, Mizuno K (2013) The roles of protein kinases in learning and memory. Learn. Mem. 20:540–552. DOI: 10.1101/lm.028449.112.

Goodman LJ, Valverde J, Lim F, Geschwind MD, Federoff HJ, Geller AI, Hefti F (1996) Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. Mol. Cell. Neurosci 7:222–238. DOI: 10.1006/mcne.1996.0017.

Gottmann K, Mittmann T, Lessmann V (2009) BDNF signaling in the formation, maturation and plasticity of glutamatergic and GABAergic synapses. Exp Brain Res 199:203–234. DOI: 10.1007/s00221-009-1994-z.

Gottschalk W, Pozzo-Miller LD, Figurov A, Lu B (1998) Presynaptic modulation of synaptic transmission and plasticity by brain-derived neurotrophic factor in the developing hippocampus. J. Neurosci. 18:6830–6839.

Griesbeck O, Canossa M, Campana G, Gärtner A, Hoener MC, Nawa H, Kolbeck R, Thoenen H (1999) Are there differences between the secretion characteristics of NGF and BDNF? Implications for the modulatory role of neurotrophins in activity-dependent neuronal plasticity. Microsc. Res. Tech 45:262–275. DOI: 10.1002/(SICI)1097-0029(19990515/01) 45:4/5<262::AID-JEMT10>3.0.CO;2-K.

Gubellini P, Ben-Ari Y, Gaïarsa J (2005) Endogenous neurotrophins are required for the induction of GABAergic long-term potentiation in the neonatal rat hippocampus. J. Neurosci. 25:5796–5802. DOI: 10.1523/JNEUROSCI.0824-05.2005.

Hallböök F, Ibáñez CF, Persson H (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron 6:845–858. DOI: 10.1016/0896-6273(91)90180-8.

Hallböök F, Wilson K, Thorndyke M, Olinski RP. (2006) Formation and evolution of the chordate neurotrophin and Trk receptor genes. Brain Behav Evol:133–144. DOI: 10.1159/000094083.

Han W, Li D, Stout AK, Takimoto K, Levitan ES (1999) Ca2+-induced deprotonation of peptide hormones inside secretory vesicles in preparation for release. J. Neurosci. 19:900–905.

Harata NC, Choi S, Pyle JL, Aravanis AM, Tsien RW (2006) Frequency-dependent kinetics and prevalence of kiss-and-run and reuse at hippocampal synapses studied with novel quenching methods. Neuron 49:243–256. DOI: 10.1016/j.neuron.2005.12.018.

Hartmann M, Heumann R, Lessmann V (2001) Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. EMBO J. 20:5887–5897. DOI: 10.1093/emboj/20.21.5887.

Haubensak W, Narz F, Heumann R, Lessmann V (1998) BDNF-GFP containing secretory granules are localized in the vicinity of synaptic junctions of cultured cortical neurons. J. Cell. Sci. 111 (Pt 11):1483–1493.

Heck N, Golbs A, Riedemann T, Sun J, Lessmann V, Luhmann HJ (2008) Activity-dependent regulation of neuronal apoptosis in neonatal mouse cerebral cortex. Cereb.Cortex 18:1335–1349. DOI: 10.1093/cercor/bhm165.

Hell JW, Westenbroek RE, Breeze LJ, Wang KKW, Chavkin C, Catterall WA (1996) N-methyl-D-aspartate receptor-induced proteolytic conversion of postsynaptic class C L-type calcium channels in hippocampal neurons. Proc. Natl. Acad. Sci. U.S.A 93:3362–3367.

Hell JW, Westenbroek RE, Warner C, Ahlijanian MK, Prystay W, Gilbert MM, Snutch TP, Catterall WA (1993) Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel alpha 1 subunits. J. Cell Biol 123:949–962. DOI: 10.1083/jcb.123.4.949.

Heymach J, Krüttgen A, Suter U, Shooter EM (1996) The regulated secretion and vectorial targeting of neurotrophins in neuroendocrine and epithelial cells. J. Biol. Chem 271:25430–25437.

Hibbert AP, Morris SJ, Seidah NG, Murphy RA (2003) Neurotrophin-4, alone or heterodimerized with brain-derived neurotrophic factor, is sorted to the constitutive secretory pathway. J. Biol. Chem. 278:48129–48136. DOI: 10.1074/jbc.M300961200.

Hong EJ, McCord AE, Greenberg ME (2008) A biological function for the neuronal activity-dependent component of Bdnf transcription in the development of cortical inhibition. Neuron 60:610–624. DOI: 10.1016/j.neuron.2008.09.024.

Hoogland TM, Saggau P (2004) Facilitation of L-type Ca2+ channels in dendritic spines by activation of beta2 adrenergic receptors. J. Neurosci 24:8416–8427. DOI: 10.1523/JNEUROSCI.1677-04.2004.

Horch HW, Krüttgen A, Portbury SD, Katz LC (1999) Destabilization of cortical dendrites and spines by BDNF. Neuron 23:353–364. DOI: 10.1016/S0896-6273(00)80785-0.

Horch HW, Katz LC (2002) BDNF release from single cells elicits local dendritic growth in nearby neurons. Nat. Neurosci. 5:1177–1184. DOI: 10.1038/nn927.

Huang EJ, Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. 24:677–736. DOI: 10.1146/annurev.neuro.24.1.677.

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annu. Rev. Biochem 72:609–642. DOI: 10.1146/annurev.biochem.72.121801.161629.

Huang YY, Kandel ER (1994) Recruitment of long-lasting and protein kinase A-dependent long-term potentiation in the CA1 region of hippocampus requires repeated tetanization. Learn. Mem. 1:74–82.

Husi H, Ward MA, Choudhary JS, Blackstock WP, Grant SG (2000) Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. Nat. Neurosci. 3:661–669. DOI: 10.1038/76615.

Isaac JT, Nicoll RA, Malenka RC (1995) Evidence for silent synapses: implications for the expression of LTP. Neuron 15:427–434. DOI: 10.1016/0896-6273(95)90046-2.

Itami C, Kimura F, Kohno T, Matsuoka M, Ichikawa M, Tsumoto T, Nakamura S (2003) Brain-derived neurotrophic factor-dependent unmasking of "silent" synapses in the developing mouse barrel cortex. Proc. Natl. Acad. Sci. U.S.A 100:13069–13074. DOI: 10.1073/pnas.2131948100.

Jia Y, Zhou J, Tai Y, Wang Y (2007) TRPC channels promote cerebellar granule neuron survival. Nat. Neurosci. 10:559–567. DOI: 10.1038/nn1870.

Jia Y, Gall CM, Lynch G (2010) Presynaptic BDNF promotes postsynaptic long-term potentiation in the dorsal striatum. J. Neurosci. 30:14440–14445. DOI: 10.1523/JNEUROSCI.3310-10.2010.

Jian K, Chen M, Cao X, Zhu X, Fung M, Gao T (2007) Nitric oxide modulation of voltage-gated calcium current by S-nitrosylation and cGMP pathway in cultured rat hippocampal neurons. Biochem. Biophys. Res. Commun. 359:481–485. DOI: 10.1016/j.bbrc.2007.05.113.

Jiang B, Akaneya Y, Ohshima M, Ichisaka S, Hata Y, Tsumoto T (2001) Brain-derived neurotrophic factor induces long-lasting potentiation of synaptic transmission in visual cortex in vivo in young rats, but not in the adult. Eur. J. Neurosci. 14:1219–1228. DOI: 10.1046/j.0953-816x.2001.01751.x.

Jiang X, Zhou J, Mash DC, Marini AM, Lipsky RH (2009) Human BDNF isoforms are differentially expressed in cocaine addicts and are sorted to the regulated secretory pathway independent of the Met66 substitution. Neuromolecular Med 11:1–12. DOI: 10.1007/s12017-008-8051-0.

Jones KR, Fariñas I, Backus C, Reichardt LF (1994) Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell 76:989–999. DOI: 10.1016/0092-8674(94)90377-8.

Jourdi H, Hsu Y, Zhou M, Qin Q, Bi X, Baudry M (2009) Positive AMPA receptor modulation rapidly stimulates BDNF release and increases dendritic mRNA translation. J Neurosci 29:8688–8697. DOI: 10.1523/JNEUROSCI.6078-08.2009.

Jovanovic JN, Czernik AJ, Fienberg AA, Greengard P, Sihra TS (2000) Synapsins as mediators of BDNF-enhanced neurotransmitter release. Nat. Neurosci 3:323–329. DOI: 10.1038/73888.

Kang H, Schuman EM (1996) A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. Science 273:1402–1406. DOI: 10.1126/science.273. 5280.1402.

Kang H, Schuman EM (1995) Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science 267:1658–1662. DOI: 10.1126/ science.7886457.

Kang H, Welcher AA, Shelton D, Schuman EM (1997) Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation. Neuron 19:653–664. DOI: 10.1016/S0896-6273(00)80378-5.

Katoh-Semba R, Kaisho Y, Shintani A, Nagahama M, Kato K (1996) Tissue distribution and immunocytochemical localization of neurotrophin-3 in the brain and peripheral tissues of rats. J. Neurochem. 66:330–337. DOI: 10.1046/j.1471-4159.1996.66010330.x.

Kerschensteiner M, Gallmeier E, Behrens L, Leal VV, Misgeld T, Klinkert WE, Kolbeck R, Hoppe E, Oropeza-Wekerle RL, Bartke I, Stadelmann C, Lassmann H, Wekerle H, Hohlfeld R (1999) Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation? J. Exp. Med. 189:865–870. DOI: 10.1084/jem.189.5.865.

Klein R, Silos-Santiago I, Smeyne RJ, Lira SA, Brambilla R, Bryant S, Zhang L, Snider WD, Barbacid M (1994) Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. Nature 368:249–251. DOI: 10.1038/368249a0.

Klyachko VA, Jackson MB (2002) Capacitance steps and fusion pores of small and large-dense-core vesicles in nerve terminals. Nature 418:89–92. DOI: 10.1038/nature00852.

Knowles RG, Palacios M, Palmer RM, Moncada S (1989) Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. Proc. Natl. Acad. Sci. U.S.A 86:5159–5162.

Kohara K, Kitamura A, Morishima M, Tsumoto T (2001) Activity-dependent transfer of brain-derived neurotrophic factor to postsynaptic neurons. Science 291:2419–2423. DOI: 10.1126/science.1057415.

Kohara K, Yasuda H, Huang Y, Adachi N, Sohya K, Tsumoto T (2007) A local reduction in cortical GABAergic synapses after a loss of endogenous brain-derived neurotrophic factor, as revealed by single-cell gene knock-out method. J. Neurosci. 27:7234–7244. DOI: 10.1523/JNEUROSCI.1943-07.2007.

Kohrmann M, Haubensak W, Hemraj I, Kaether C, Lessmann V, Kiebler MA, Köhrmann M (1999) Fast, convenient, and effective method to transiently transfect primary hippocampal neurons. J. Neurosci. Res 58:831–835. DOI: 10.1002/(SICI)1097-4547(19991215) 58:6<831::AID-JNR10>3.0.CO;2-M.

Kojima M, Takei N, Numakawa T, Ishikawa Y, Suzuki S, Matsumoto T, Katoh-Semba R, Nawa H, Hatanaka H (2001) Biological characterization and optical imaging of brain-derived neurotrophic factor-green fluorescent protein suggest an activity-dependent local release of brain-derived neurotrophic factor in neurites of cultured hippocampal neurons. J. Neurosci. Res 64:1–10. DOI: 10.1002/jnr.1080.

Kolarow R, Brigadski T, Lessmann V (2007) Postsynaptic secretion of BDNF and NT-3 from hippocampal neurons depends on calcium calmodulin kinase II signaling and proceeds via delayed fusion pore opening. J Neurosci 27:10350–10364. DOI: 10.1523/ JNEUROSCI.0692-07.2007.

Kolarow R, Kuhlmann CRW, Munsch T, Zehendner C, Brigadski T, Luhmann HJ, Lessmann V (2014) BDNF-induced nitric oxide signals in cultured rat hippocampal neurons: time course, mechanism of generation, and effect on neurotrophin secretion. Front Cell Neurosci 8:323. DOI: 10.3389/fncel.2014.00323.

Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T (1995) Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. Proc. Natl. Acad. Sci. U.S.A 92:8856–8860.

Korte M, Griesbeck O, Gravel C, Carroll P, Staiger V, Thoenen H, Bonhoeffer T (1996) Virus-mediated gene transfer into hippocampal CA1 region restores long-term potentiation in brain-derived neurotrophic factor mutant mice. Proc. Natl. Acad. Sci. U.S.A 93:12547–12552.

Kossel AH, Cambridge SB, Wagner U, Bonhoeffer T (2001) A caged Ab reveals an immediate/instructive effect of BDNF during hippocampal synaptic potentiation. Proc. Natl. Acad. Sci. U.S.A. 98:14702–14707. DOI: 10.1073/pnas.251326998.

Kovalchuk Y, Eilers J, Lisman J, Konnerth A (2000) NMDA receptor-mediated subthreshold Ca(2+) signals in spines of hippocampal neurons. J. Neurosci. 20:1791–1799.

Kovalchuk Y, Hanse E, Kafitz KW, Konnerth A (2002) Postsynaptic Induction of BDNF-Mediated Long-Term Potentiation. Science 295:1729–1734. DOI: 10.1126/ science.1067766.

Krüttgen A, Möller JC, Heymach JV, Shooter EM (1998) Neurotrophins induce release of neurotrophins by the regulated secretory pathway. Proc. Natl. Acad. Sci. U.S.A 95:9614–9619.

Kucharz K, Krogh M, Ng AN, Toresson H (2009) NMDA receptor stimulation induces reversible fission of the neuronal endoplasmic reticulum. PLoS ONE 4:e5250. DOI: 10.1371/journal.pone.0005250.

Kucharz K, Wieloch T, Toresson H (2011) Potassium-induced structural changes of the endoplasmic reticulum in pyramidal neurons in murine organotypic hippocampal slices. J Neurosci Res 89:1150–1159. DOI: 10.1002/jnr.22646.

Kuczewski N, Porcher C, Ferrand N, Fiorentino H, Pellegrino C, Kolarow R, Lessmann V, Medina I, Gaiarsa JL (2008) Backpropagating action potentials trigger dendritic release of BDNF during spontaneous network activity. J Neurosci 28:7013–7023. DOI: 10.1523/JNEUROSCI.1673-08.2008.

Lander HM, Milbank AJ, Tauras JM, Hajjar DP, Hempstead BL, Schwartz GD, Kraemer RT, Mirza UA, Chait BT, Burk SC, Quilliam LA (1996) Redox regulation of cell signalling. Nature 381:380–381. DOI: 10.1038/381380a0.

Lang SB, Stein V, Bonhoeffer T, Lohmann C (2007) Endogenous brain-derived neurotrophic factor triggers fast calcium transients at synapses in developing dendrites. J. Neurosci. 27:1097–1105. DOI: 10.1523/JNEUROSCI.3590-06.2007.

Lanner JT, Georgiou DK, Joshi AD, Hamilton SL (2010) Ryanodine receptors: structure, expression, molecular details, and function in calcium release. Cold Spring Harb Perspect Biol 2:a003996. DOI: 10.1101/cshperspect.a003996.

Lau AG, Irier HA, Gu J, Tian D, Ku L, Liu G, Xia M, Fritsch B, Zheng JQ, Dingledine R, Xu B, Lu B, Feng Y (2010) Distinct 3'UTRs differentially regulate activity-dependent translation of brain-derived neurotrophic factor (BDNF). Proc. Natl. Acad. Sci. U.S.A. 107:15945–15950. DOI: 10.1073/pnas.1002929107.

Le AP, Friedman WJ (2012) Matrix metalloproteinase-7 regulates cleavage of pro-nerve growth factor and is neuroprotective following kainic acid-induced seizures. J. Neurosci. 32:703–712. DOI: 10.1523/JNEUROSCI.4128-11.2012.

Lee R, Kermani P, Teng KK, Hempstead BL (2001) Regulation of cell survival by secreted proneurotrophins. Science 294:1945–1948. DOI: 10.1126/science.1065057.

Lessmann V (1998) Neurotrophin-dependent modulation of glutamatergic synaptic transmission in the mammalian CNS. Gen.Pharmacol. 31:667–674. DOI: 10.1016/S0306-3623(98) 00190-6.

Lessmann V, Brigadski T (2009) Mechanisms, locations, and kinetics of synaptic BDNF secretion: an update. Neurosci Res 65:11–22. DOI: 10.1016/j.neures.2009.06.004.

Lessmann V, Gottmann K, Heumann R (1994) BDNF and NT-4/5 enhance glutamatergic synaptic transmission in cultured hippocampal neurones. Neuroreport 6:21–25.

Lessmann V, Gottmann K, Malcangio M (2003) Neurotrophin secretion: current facts and future prospects. Prog. Neurobiol 69:341–374. DOI: 10.1016/S0301-0082(03)00019-4.

Lessmann V, Heumann R (1997) Cyclic AMP endogenously enhances synaptic strength of developing glutamatergic synapses in serum-free microcultures of rat hippocampal neurons. Brain Res. 763:111–122. DOI: 10.1016/S0006-8993(97)00406-X.

Lessmann V, Heumann R (1998) Modulation of unitary glutamatergic synapses by neurotrophin-4/5 or brain-derived neurotrophic factor in hippocampal microcultures: presynaptic enhancement depends on pre-established paired-pulse facilitation. Neuroscience 86:399–413. DOI: 10.1016/S0306-4522(98)00035-9.

Lessmann V, Stroh-Kaffei S, Steinbrecher V, Edelmann E, Brigadski T, Kilb W, Luhmann HJ (2011) The expression mechanism of the residual LTP in the CA1 region of BDNF k.o. mice is insensitive to NO synthase inhibition. Brain Res. 1391:14–23. DOI: 10.1016/j.brainres.2011.03.061.

Levi-Montalcini R (1987) The nerve growth factor: thirty-five years later. Nobel Lecture, 8 december 1986. EMBO J. 6:1145–1154.

Levine ES, Dreyfus CF, Black IB, Plummer MR (1995) Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. Proc. Natl. Acad. Sci. U.S.A 92:8074–8077.

Li HS, Xu XZ, Montell C, Li H, Xu XS, Montell C (1999) Activation of a TRPC3-Dependent Cation Current through the Neurotrophin BDNF. Neuron 24:261–273. DOI: 10.1016/S0896-6273(00)80838-7.

Li YX, Xu Y, Ju D, Lester HA, Davidson N, Schuman EM (1998a) Expression of a dominant negative TrkB receptor, T1, reveals a requirement for presynaptic signaling in BDNF-induced synaptic potentiation in cultured hippocampal neurons. Proc. Natl. Acad. Sci. U.S.A 95:10884–10889.

Li YX, Zhang Y, Lester HA, Schuman EM, Davidson N (1998b) Enhancement of neurotransmitter release induced by brain-derived neurotrophic factor in cultured hippocampal neurons. J. Neurosci 18:10231–10240.

Li Y, Jia Y, Cui K, Li N, Zheng Z, Wang Y, Yuan X (2005) Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. Nature 434:894–898. DOI: 10.1038/nature03477.

Li Z, Zhang G, Marjanovic JA, Ruan C, Du X (2004) A platelet secretion pathway mediated by cGMP-dependent protein kinase. J. Biol. Chem 279:42469–42475. DOI: 10.1074/jbc.M401532200.

Liao D, Hessler NA, Malinow R (1995) Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. Nature 375:400–404. DOI: 10.1038/375400a0.

Lin SY, Wu K, Len GW, Xu JL, Levine ES, Suen PC, Mount HT, Black IB (1999) Brain-derived neurotrophic factor enhances association of protein tyrosine phosphatase PTP1D with the NMDA receptor subunit NR2B in the cortical postsynaptic density. Brain Res. Mol. Brain Res 70:18–25. DOI: 10.1016/S0169-328X(99)00122-9.

Lin SY, Wu K, Levine ES, Mount HT, Suen PC, Black IB (1998) BDNF acutely increases tyrosine phosphorylation of the NMDA receptor subunit 2B in cortical and hippocampal postsynaptic densities. Brain Res. Mol. Brain Res. 55:20–27. DOI: 10.1016/S0169-328X(97)00349-5.

Liu Q, Walther D, Drgon T, Polesskaya O, Lesnick TG, Strain KJ, Andrade M de, Bower JH, Maraganore DM, Uhl GR (2005) Human brain derived neurotrophic factor (BDNF) genes, splicing patterns, and assessments of associations with substance abuse and Parkinson's Disease. Am. J. Med. Genet. B Neuropsychiatr. Genet. 134:93–103. DOI: 10.1002/ajmg.b.30109.

Lochner JE, Honigman LS, Grant WF, Gessford SK, Hansen AB, Silverman MA, Scalettar BA (2006) Activity-dependent release of tissue plasminogen activator from the dendritic spines of hippocampal neurons revealed by live-cell imaging. J. Neurobiol. 66:564–577. DOI: 10.1002/neu.20250.

Lohof AM, Ip NY, Poo M (1993) Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. Nature 363:350–353. DOI: 10.1038/363350a0.

Lommatzsch M, Quarcoo D, Schulte-Herbrüggen O, Weber H, Virchow JC, Renz H, Braun A (2005) Neurotrophins in murine viscera: a dynamic pattern from birth to adulthood. Int. J. Dev. Neurosci. 23:495–500. DOI: 10.1016/j.ijdevneu.2005.05.009.

Lonart G, Südhof TC (1998) Region-specific phosphorylation of rabphilin in mossy fiber nerve terminals of the hippocampus. J. Neurosci. 18:634–640.

Longo FM, Massa SM (2013) Small-molecule modulation of neurotrophin receptors: a strategy for the treatment of neurological disease. Nat Rev Drug Discov 12:507–525. DOI: 10.1038/nrd4024.

Lou H, Kim S, Zaitsev E, Snell CR, Lu B, Loh YP (2005) Sorting and activity-dependent secretion of BDNF require interaction of a specific motif with the sorting receptor carboxypeptidase e. Neuron 45:245–255. DOI: 10.1016/j.neuron.2004.12.037.

Lubin FD, Roth TL, Sweatt JD (2008) Epigenetic regulation of BDNF gene transcription in the consolidation of fear memory. J. Neurosci. 28:10576–10586. DOI: 10.1523/JNEUROSCI.1786-08.2008.

Lucini C, Maruccio L, Girolamo P de, Castaldo L (2003) Brain-derived neurotrophic factor in higher vertebrate pancreas: immunolocalization in glucagon cells. Anat. Embryol. 206:311–318. DOI: 10.1007/s00429-002-0304-3.

Lyons M, West A (2011) Mechanisms of specificity in neuronal activity-regulated gene transcription. Prog. Neurobiol. 94:259–295. DOI: 10.1016/j.pneurobio.2011.05.003.

Lyons MR, Schwarz CM, West AE (2012) Members of the myocyte enhancer factor 2 transcription factor family differentially regulate Bdnf transcription in response to neuronal depolarization. J. Neurosci. 32:12780–12785. DOI: 10.1523/JNEUROSCI.0534-12.2012.

Magby J, Bi C, Chen Z, Lee FS, Plummer MR (2006) Single-cell characterization of retrograde signaling by brain-derived neurotrophic factor. J Neurosci 26:13531–13536. DOI: 10.1523/JNEUROSCI.4576-06.2006.

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247:1446–1451. DOI: 10.1126/science.2321006.

Maletic-Savatic M, Koothan T, Malinow R (1998) Calcium-evoked dendritic exocytosis in cultured hippocampal neurons. Part II: mediation by calcium/calmodulin-dependent protein kinase II. J. Neurosci. 18:6814–6821.

Marini AM, Rabin SJ, Lipsky RH, Mocchetti I (1998) Activity-dependent release of brain-derived neurotrophic factor underlies the neuroprotective effect of N-methyl-D-aspartate. J. Biol. Chem. 273:29394–29399.

Martínez A, Alcántara S, Borrell V, Del Río JA, Blasi J, Otal R, Campos N, Boronat A, Barbacid M, Silos-Santiago I, Soriano E (1998) TrkB and TrkC signaling are required for maturation and synaptogenesis of hippocampal connections. J. Neurosci. 18:7336–7350.

Matsuda N, Lu H, Fukata Y, Noritake J, Gao H, Mukherjee S, Nemoto T, Fukata M, Poo M (2009) Differential activity-dependent secretion of brain-derived neurotrophic factor from axon and dendrite. J. Neurosci 29:14185–14198. DOI: 10.1523/JNEUROSCI.1863-09.2009.

Matsumoto T, Rauskolb S, Polack M, Klose J, Kolbeck R, Korte M, Barde Y (2008) Biosynthesis and processing of endogenous BDNF: CNS neurons store and secrete BDNF, not pro-BDNF. Nat. Neurosci 11:131–133. DOI: 10.1038/nn2038.

McAllister AK, Katz LC, Lo DC (1996) Neurotrophin regulation of cortical dendritic growth requires activity. Neuron 17:1057–1064. DOI: 10.1016/S0896-6273(00)80239-1.

McAllister AK, Katz LC, Lo DC (1997) Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth. Neuron 18:767–778. DOI: 10.1016/S0896-6273(00)80316-5.

Meis S, Endres T, Lessmann V (2012) Postsynaptic BDNF signalling regulates long-term potentiation at thalamo-amygdala afferents. J. Physiol. (Lond.) 590:193–208. DOI: 10.1113/jphysiol.2011.220434.

Messaoudi E, Bârdsen K, Srebro B, Bramham CR (1998) Acute intrahippocampal infusion of BDNF induces lasting potentiation of synaptic transmission in the rat dentate gyrus. J. Neurophysiol. 79:496–499.

Meyer-Franke A, Kaplan MR, Pfrieger FW, Barres BA (1995) Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. Neuron 15:805–819. DOI: 10.1016/0896-6273(95)90172-8.

Minichiello L, Calella AM, Medina DL, Bonhoeffer T, Klein R, Korte M (2002) Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron 36:121–137. DOI: 10.1016/S0896-6273(02)00942-X.

Mohajerani MH, Sivakumaran S, Zacchi P, Aguilera P, Cherubini E (2007) Correlated network activity enhances synaptic efficacy via BDNF and the ERK pathway at immature CA3 CA1 connections in the hippocampus. Proc. Natl. Acad. Sci. U.S.A. 104:13176–13181. DOI: 10.1073/pnas.0704533104.

Moremen KW, Tiemeyer M, Nairn AV (2012) Vertebrate protein glycosylation: diversity, synthesis and function. Nat. Rev. Mol. Cell Biol. 13:448–462. DOI: 10.1038/nrm3383.

Morris JF, Nordmann JJ, Dyball RE (1978) Structure-function correlation in mammalian neurosecretion. Int Rev Exp Pathol 18:1–95.

Mowla SJ, Farhadi HF, Pareek S, Atwal JK, Morris SJ, Seidah NG, Murphy RA (2001) Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. J. Biol. Chem 276:12660–12666. DOI: 10.1074/jbc.M008104200.

Müller D, Davidoff MS, Bargheer O, Paust H, Pusch W, Koeva Y, Jezek D, Holstein AF, Middendorff R (2006) The expression of neurotrophins and their receptors in the prenatal and adult human testis: evidence for functions in Leydig cells. Histochem. Cell Biol. 126:199–211. DOI: 10.1007/s00418-006-0155-8.

Musumeci G, Minichiello L (2011) BDNF-TrkB signalling in fear learning: from genetics to neural networks. Rev Neurosci 22:303–315. DOI: 10.1515/RNS.2011.031.

Nagy G, Reim K, Matti U, Brose N, Binz T, Rettig J, Neher E, Sørensen JB (2004) Regulation of releasable vesicle pool sizes by protein kinase A-dependent phosphorylation of SNAP-25. Neuron 41:417–429.

Nanamori M, Chen J, Du X, Ye RD (2007) Regulation of leukocyte degranulation by cGMP-dependent protein kinase and phosphoinositide 3-kinase: potential roles in phosphorylation of target membrane SNARE complex proteins in rat mast cells. J Immunol 178:416–427.

North RA, Jarvis MF (2013) P2X receptors as drug targets. Mol. Pharmacol. 83:759–769. DOI: 10.1124/mol.112.083758.

Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Jacobsen C, Kliemannel M, Schwarz E, Willnow TE, Hempstead BL, Petersen CM (2004) Sortilin is essential for proNGF-induced neuronal cell death. Nature 427:843–848. DOI: 10.1038/nature02319.

Obermair GJ, Szabo Z, Bourinet E, Flucher BE (2004) Differential targeting of the L-type Ca2+ channel alpha 1C (CaV1.2) to synaptic and extrasynaptic compartments in hippocampal neurons. Eur. J. Neurosci 19:2109–2122. DOI: 10.1111/j.0953-816X.2004.03272.x.

Oheim M, Kirchhoff F, Stühmer W (2006) Calcium microdomains in regulated exocytosis. Cell Calcium 40:423–439. DOI: 10.1016/j.ceca.2006.08.007.

Otmakhov N, Tao-Cheng J, Carpenter S, Asrican B, Dosemeci A, Reese TS, Lisman J (2004) Persistent accumulation of calcium/calmodulin-dependent protein kinase II in dendritic spines after induction of NMDA receptor-dependent chemical long-term potentiation. J. Neurosci. 24:9324–9331. DOI: 10.1523/JNEUROSCI.2350-04.2004.

Palmada M, Kanwal S, Rutkoski NJ, Gustafson-Brown C, Johnson RS, Wisdom R, Carter BD, Gufstafson-Brown C (2002) c-jun is essential for sympathetic neuronal death induced by NGF withdrawal but not by p75 activation. J. Cell Biol. 158:453–461. DOI: 10.1083/jcb.200112129.

Pang PT, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung W, Hempstead BL, Lu B (2004) Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. Science 306:487–491. DOI: 10.1126/science.1100135.

Paoletti F, Malerba F, Kelly G, Noinville S, Lamba D, Cattaneo A, Pastore A (2011) Conformational plasticity of proNGF. PLoS ONE 6:e22615. DOI: 10.1371/ journal.pone.0022615.

Paredes A, Romero C, Dissen GA, DeChiara TM, Reichardt L, Cornea A, Ojeda SR, Xu B (2004) TrkB receptors are required for follicular growth and oocyte survival in the mammalian ovary. Dev. Biol. 267:430–449. DOI: 10.1016/j.ydbio.2003.12.001.

Park H, Poo M (2013) Neurotrophin regulation of neural circuit development and function. Nat. Rev. Neurosci. 14:7–23. DOI: 10.1038/nrn3379.

Park JJ, Cawley NX, Loh YP (2008) A bi-directional carboxypeptidase E-driven transport mechanism controls BDNF vesicle homeostasis in hippocampal neurons. Mol. Cell. Neurosci 39:63–73. DOI: 10.1016/j.mcn.2008.05.016.

Patterson SL, Abel T, Deuel TAS, Martin KC, Rose JC, Kandel ER (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron 16:1137–1145. DOI: 10.1016/S0896-6273(00)80140-3.

Patterson SL, Pittenger C, Morozov A, Martin KC, Scanlin H, Drake C, Kandel ER (2001) Some forms of cAMP-mediated long-lasting potentiation are associated with release of BDNF and nuclear translocation of phospho-MAP kinase. Neuron 32:123–140. DOI: 10.1016/S0896-6273(01)00443-3.

Paul J, Gottmann K, Lessmann V (2001) NT-3 regulates BDNF-induced modulation of synaptic transmission in cultured hippocampal neurons. Neuroreport 12:2635–2639.

Perrais D, Kleppe IC, Taraska JW, Almers W (2004) Recapture after exocytosis causes differential retention of protein in granules of bovine chromaffin cells. J. Physiol. (Lond.) 560:413–428. DOI: 10.1113/jphysiol.2004.064410.

Perrard M, Vigier M, Damestoy A, Chapat C, Silandre D, Rudkin BB, Durand P (2007) beta-Nerve growth factor participates in an auto/paracrine pathway of regulation of the meiotic differentiation of rat spermatocytes. J. Cell. Physiol. 210:51–62. DOI: 10.1002/jcp.20805.

Petoukhov E, Fernando S, Mills F, Shivji F, Hunter D, Krieger C, Silverman MA, Bamji SX (2013) Activity-dependent secretion of progranulin from synapses. J. Cell. Sci. 126:5412–5421. DOI: 10.1242/jcs.132076.

Petzold GC, Scheibe F, Braun JS, Freyer D, Priller J, Dirnagl U, Dreier JP (2005) Nitric oxide modulates calcium entry through P/Q-type calcium channels and N-methyl-d-aspartate receptors in rat cortical neurons. Brain Res. 1063:9–14. DOI: 10.1016/j.brainres.2005.09.048.

Polleux F, Whitford KL, Dijkhuizen PA, Vitalis T, Ghosh A (2002) Control of cortical interneuron migration by neurotrophins and PI3-kinase signaling. Development 129:3147–3160.

Pozzo-Miller LD, Gottschalk W, Zhang L, McDermott K, Du J, Gopalakrishnan R, Oho C, Sheng ZH, Lu B (1999) Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. J. Neurosci. 19:4972–4983.

Pruunsild P, Kazantseva A, Aid T, Palm K, Timmusk T (2007) Dissecting the human BDNF locus: bidirectional transcription, complex splicing, and multiple promoters. Genomics 90:397–406. DOI: 10.1016/j.ygeno.2007.05.004.

Pruunsild P, Sepp M, Orav E, Koppel I, Timmusk T (2011) Identification of cis-elements and transcription factors regulating neuronal activity-dependent transcription of human BDNF gene. J. Neurosci 31:3295–3308. DOI: 10.1523/JNEUROSCI.4540-10.2011.

Rex CS, Lin C, Kramár EA, Chen LY, Gall CM, Lynch G (2007) Brain-derived neurotrophic factor promotes long-term potentiation-related cytoskeletal changes in adult hippocampus. J. Neurosci. 27:3017–3029. DOI: 10.1523/JNEUROSCI.4037-06.2007.

Riccio A, Ahn S, Davenport CM, Blendy JA, Ginty DD (1999) Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science 286:2358–2361. DOI: 10.1126/science.286.5448.2358.

Rizzuto R, Pozzan T (2006) Microdomains of intracellular Ca2+: molecular determinants and functional consequences. Physiol. Rev. 86:369–408. DOI: 10.1152/physrev.00004.2005.

Robinson RC, Radziejewski C, Spraggon G, Greenwald J, Kostura MR, Burtnick LD, Stuart DI, Choe S, Jones EY (1999) The structures of the neurotrophin 4 homodimer and the brain-derived neurotrophic factor/neurotrophin 4 heterodimer reveal a common Trk-binding site. The structures of the neurotrophin 4 homodimer and the brain-derived neurotrophic factor/neurotrophin 4 heterodimer reveal a common Trk binding site. Protein Sci. 8:2589–2597. DOI: 10.1110/ps.8.12.2589.

Rösch H, Schweigreiter R, Bonhoeffer T, Barde Y, Korte M (2005) The neurotrophin receptor p75NTR modulates long-term depression and regulates the expression of AMPA receptor subunits in the hippocampus. Proc. Natl. Acad. Sci. U.S.A. 102:7362–7367. DOI: 10.1073/pnas.0502460102.

Rosenbaum T, Vidaltamayo R, Sánchez-Soto MC, Zentella A, Hiriart M (1998) Pancreatic beta cells synthesize and secrete nerve growth factor. Proc. Natl. Acad. Sci. U.S.A. 95:7784–7788.

Rusznák Z, Harasztosi C, Stanfield PR, Kovács L, Szûcs G (2000) Potassium-depolarization-induced cytoplasmic [Ca2+] transient in freshly dissociated pyramidal neurones of the rat dorsal cochlear nucleus. Pflugers Arch. 440:462–466. DOI: 10.1007/s004240000314.

Sabatini BL, Oertner TG, Svoboda K (2002) The life cycle of Ca(2+) ions in dendritic spines. Neuron 33:439–452. DOI: 10.1016/S0896-6273(02)00573-1.

Samuels BA, Hsueh Y, Shu T, Liang H, Tseng H, Hong C, Su SC, Volker J, Neve RL, Yue DT, Tsai L (2007) Cdk5 promotes synaptogenesis by regulating the subcellular distribution of the MAGUK family member CASK. Neuron 56:823–837. DOI: 10.1016/j.neuron.2007.09.035.

Santi S, Cappello S, Riccio M, Bergami M, Aicardi G, Schenk U, Matteoli M, Canossa M (2006) Hippocampal neurons recycle BDNF for activity-dependent secretion and LTP maintenance. EMBO J. 25:4372–4380. DOI: 10.1038/sj.emboj.7601303.

Scharfman HE (1997) Hyperexcitability in combined entorhinal/hippocampal slices of adult rat after exposure to brain-derived neurotrophic factor. J. Neurophysiol. 78:1082–1095.

Schildt S, Endres T, Lessmann V, Edelmann E (2013) Acute and chronic interference with BDNF/TrkB-signaling impair LTP selectively at mossy fiber synapses in the CA3 region of mouse hippocampus. Neuropharmacology. DOI: 10.1016/j.neuropharm.2013.03.041.

Schinder AF, Berninger B, Poo M (2000) Postsynaptic target specificity of neurotrophin-induced presynaptic potentiation. Neuron 25:151–163. DOI: 10.1016/S0896-6273(00)80879-X.

Schweigreiter R (2006) The dual nature of neurotrophins. Bioessays 28:583–594. DOI: 10.1002/bies.20419.

Schweitzer ES, Sanderson MJ, Wasterlain CG (1995) Inhibition of regulated catecholamine secretion from PC12 cells by the Ca2+/calmodulin kinase II inhibitor KN-62. J. Cell. Sci. 108 (Pt 7):2619–2628.

Segal RA (2003) Selectivity in neurotrophin signaling: theme and variations. Annu. Rev. Neurosci. 26:299–330. DOI: 10.1146/annurev.neuro.26.041002.131421.

Seidah NG, Prat A (2012) The biology and therapeutic targeting of the proprotein convertases. Nat Rev Drug Discov 11:367–383. DOI: 10.1038/nrd3699.

Sharp AH, McPherson PS, Dawson TM, Aoki C, Campbell KP, Snyder SH (1993) Differential immunohistochemical localization of inositol 1,4,5-trisphosphate- and ryanodine-sensitive Ca2+ release channels in rat brain. J. Neurosci. 13:3051–3063.

Shelton DL, Sutherland J, Gripp J, Camerato T, Armanini MP, Phillips HS, Carroll K, Spencer SD, Levinson AD (1995) Human trks: molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. J. Neurosci. 15:477–491.

Shen W, Wu B, Zhang Z, Dou Y, Rao Z, Chen Y, Duan S (2006) Activity-induced rapid synaptic maturation mediated by presynaptic cdc42 signaling. Neuron 50:401–414. DOI: 10.1016/j.neuron.2006.03.017.

Shi SH, Hayashi Y, Petralia RS, Zaman SH, Wenthold RJ, Svoboda K, Malinow R (1999) Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. Science 284:1811–1816. DOI: 10.1126/science.284.5421.1811.

Shieh PB, Hu SC, Bobb K, Timmusk T, Ghosh A (1998) Identification of a signaling pathway involved in calcium regulation of BDNF expression. Neuron 20:727–740. DOI: 10.1016/S0896-6273(00)81011-9.

Silva AJ, Stevens CF, Tonegawa S, Wang Y (1992) Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science 257:201–206. DOI: 10.1126/science.1378648.

Silverman MA, Johnson S, Gurkins D, Farmer M, Lochner JE, Rosa P, Scalettar BA (2005) Mechanisms of transport and exocytosis of dense-core granules containing tissue plasminogen activator in developing hippocampal neurons. J. Neurosci. 25:3095–3106. DOI: 10.1523/JNEUROSCI.4694-04.2005.

Smeyne RJ, Klein R, Schnapp A, Long LK, Bryant S, Lewin A, Lira SA, Barbacid M (1994) Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature 368:246–249. DOI: 10.1038/368246a0.

Snapyan M, Lemasson M, Brill MS, Blais M, Massouh M, Ninkovic J, Gravel C, Berthod F, Götz M, Barker PA, Parent A, Saghatelyan A (2009) Vasculature guides migrating neuronal precursors in the adult mammalian forebrain via brain-derived neurotrophic factor signaling. J. Neurosci. 29:4172–4188. DOI: 10.1523/JNEUROSCI.4956-08.2009.

Son H, Hawkins RD, Martin K, Kiebler M, Huang PL, Fishman MC, Kandel ER (1996) Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. Cell 87:1015–1023. DOI: 10.1016/S0092-8674(00)81796-1.

Song W, Volosin M, Cragnolini AB, Hempstead BL, Friedman WJ (2010) ProNGF induces PTEN via p75NTR to suppress Trk-mediated survival signaling in brain neurons. J. Neurosci. 30:15608–15615. DOI: 10.1523/JNEUROSCI.2581-10.2010.

Spacek J, Harris KM (1997) Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat. J. Neurosci. 17:190–203.

Squire LR (2008) Fundamental neuroscience. Amsterdam; Boston: Elsevier / Academic Press.

Staal RGW, Mosharov EV, Sulzer D (2004) Dopamine neurons release transmitter via a flickering fusion pore. Nat. Neurosci. 7:341–346. DOI: 10.1038/nn1205.

Stephens RM, Loeb DM, Copeland TD, Pawson T, Greene LA, Kaplan DR (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. Neuron 12:691–705. DOI: 10.1016/0896-6273(94)90223-2.

Steyer JA, Almers W (2001) A real-time view of life within 100 nm of the plasma membrane. Nat. Rev. Mol. Cell Biol. 2:268–275. DOI: 10.1038/35067069.

Sun PD, Davies DR (1995) The cystine-knot growth-factor superfamily. Annu Rev Biophys Biomol Struct 24:269–291. DOI: 10.1146/annurev.bb.24.060195.001413.

Sutcliffe B, Forero MG, Zhu B, Robinson IM, Hidalgo A (2013) Neuron-type specific functions of DNT1, DNT2 and Spz at the Drosophila neuromuscular junction. PLoS ONE 8:e75902. DOI: 10.1371/journal.pone.0075902.

Swanwick CC, Harrison MB, Kapur J (2004) Synaptic and extrasynaptic localization of brain-derived neurotrophic factor and the tyrosine kinase B receptor in cultured hippocampal neurons. J. Comp. Neurol. 478:405–417. DOI: 10.1002/cne.20295.

Tabuchi A, Sakaya H, Kisukeda T, Fushiki H, Tsuda M (2002) Involvement of an upstream stimulatory factor as well as cAMP-responsive element-binding protein in the activation of brain-derived neurotrophic factor gene promoter I. J. Biol. Chem. 277:35920–35931. DOI: 10.1074/jbc.M204784200.

Tanaka J, Horiike Y, Matsuzaki M, Miyazaki T, Ellis-Davies GCR, Kasai H (2008) Protein synthesis and neurotrophin-dependent structural plasticity of single dendritic spines. Science 319:1683–1687. DOI: 10.1126/science.1152864.

Tao X, West AE, Chen WG, Corfas G, Greenberg ME (2002) A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. Neuron 33:383–395. DOI: 10.1016/S0896-6273(01)00561-X.

Taraska JW, Perrais D, Ohara-Imaizumi M, Nagamatsu S, Almers W (2003) Secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells. Proc. Natl. Acad. Sci. U.S.A. 100:2070–2075. DOI: 10.1073/pnas.0337526100.

Teng HK, Teng KK, Lee R, Wright S, Tevar S, Almeida RD, Kermani P, Torkin R, Chen Z, Lee FS, Kraemer RT, Nykjaer A, Hempstead BL (2005) ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J. Neurosci 25:5455–5463. DOI: 10.1523/JNEUROSCI.5123-04.2005.

Thakker-Varia S, Alder J, Crozier RA, Plummer MR, Black IB (2001) Rab3A is required for brain-derived neurotrophic factor-induced synaptic plasticity: transcriptional analysis at the population and single-cell levels. J. Neurosci. 21:6782–6790.

Thomas P, Surprenant A, Almers W (1990) Cytosolic Ca2+, exocytosis, and endocytosis in single melanotrophs of the rat pituitary. Neuron 5:723–733.

Timmusk T, Palm K, Metsis M, Reintam T, Paalme V, Saarma M, Persson H (1993) Multiple promoters direct tissue-specific expression of the rat BDNF gene. Neuron 10:475–489. DOI: 10.1016/0896-6273(93)90335-O.

Timmusk T, Lendahl U, Funakoshi H, Arenas E, Persson H, Metsis M (1995) Identification of brain-derived neurotrophic factor promoter regions mediating tissue-specific, axotomy-, and neuronal activity-induced expression in transgenic mice. J. Cell Biol 128:185–199. DOI: 10.1083/jcb.128.1.185.

Timmusk T, Palm K, Lendahl U, Metsis M (1999) Brain-derived neurotrophic factor expression in vivo is under the control of neuron-restrictive silencer element. J. Biol. Chem 274:1078–1084.

Tongiorgi E, Righi M, Cattaneo A (1997) Activity-dependent dendritic targeting of BDNF and TrkB mRNAs in hippocampal neurons. J. Neurosci. 17:9492–9505.

Tongiorgi E (2008) Activity-dependent expression of brain-derived neurotrophic factor in dendrites: facts and open questions. Neurosci. Res. 61:335–346. DOI: 10.1016/ j.neures.2008.04.013.

Tozer AJB, Forsythe ID, Steinert JR (2012) Nitric oxide signalling augments neuronal voltage-gated L-type (Ca(v)1) and P/q-type (Ca(v)2.1) channels in the mouse medial nucleus of the trapezoid body. PLoS ONE 7:e32256. DOI: 10.1371/journal.pone.0032256

Tucker KL, Meyer M, Barde YA (2001) Neurotrophins are required for nerve growth during development. Nat. Neurosci. 4:29–37. DOI: 10.1038/82868.

Tyler WJ, Pozzo-Miller LD (2001) BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. J. Neurosci. 21:4249–4258.

Tyler WJ, Zhang X, Hartman K, Winterer J, Muller W, Stanton PK, Pozzo-Miller LD (2006) BDNF increases release probability and the size of a rapidly recycling vesicle pool within rat hippocampal excitatory synapses. J. Physiol. (Lond.) 574:787–803. DOI: 10.1113/jphysiol.2006.111310.

Ulmann L, Hatcher J, Hughes J, Chaumont S, Green P, Conquet F, Buell G, Reeve A, Chessell I, Rassendren F (2008) Up-regulation of P2X4 receptors in spinal microglia after peripheral nerve injury mediates BDNF release and neuropathic pain. J Neurosci 28:11263–11268. DOI: 10.1523/JNEUROSCI.2308-08.2008.

Valente P, Casagrande S, Nieus T, Verstegen AMJ, Valtorta F, Benfenati F, Baldelli P (2012) Site-specific synapsin I phosphorylation participates in the expression of post-tetanic potentiation and its enhancement by BDNF. J. Neurosci. 32:5868–5879. DOI: 10.1523/JNEUROSCI.5275-11.2012.

Varshavsky A (2011) The N-end rule pathway and regulation by proteolysis. Protein Sci. DOI: 10.1002/pro.666.

Verderio C, Bianco F, Blanchard MP, Bergami M, Canossa M, Scarfone E, Matteoli M (2006) Cross talk between vestibular neurons and Schwann cells mediates BDNF release and neuronal regeneration. Brain Cell Biol 35:187–201. DOI: 10.1007/s11068-007-9011-6.

Verhage M, Sørensen JB (2008) Vesicle docking in regulated exocytosis. Traffic 9:1414–1424. DOI: 10.1111/j.1600-0854.2008.00759.x

Vicario-Abejón C, Collin C, McKay RD, Segal M (1998) Neurotrophins induce formation of functional excitatory and inhibitory synapses between cultured hippocampal neurons. J. Neurosci. 18:7256–7271.

Vicario-Abejón C, Owens D, McKay R, Segal M (2002) Role of neurotrophins in central synapse formation and stabilization. Nat. Rev. Neurosci. 3:965–974. DOI: 10.1038/nrn988.

Walz C, Jüngling K, Lessmann V, Gottmann K (2006) Presynaptic plasticity in an immature neocortical network requires NMDA receptor activation and BDNF release. J. Neurophysiol 96:3512–3516. DOI: 10.1152/jn.00018.2006.

Wang X, Butowt R, Vasko MR, Bartheld CS von (2002) Mechanisms of the release of anterogradely transported neurotrophin-3 from axon terminals. J. Neurosci 22:931–945.

Wayman GA, Lee Y, Tokumitsu H, Silva AJ, Soderling TR (2008) Calmodulin-kinases: modulators of neuronal development and plasticity. Neuron 59:914–931. DOI: 10.1016/j.neuron.2008.08.021

West A (2011) Biological functions and transcriptional targets of CaRF in neurons. Cell Calcium 49:290–295. DOI: 10.1016/j.ceca.2011.04.003.

Westenbroek RE, Hell JW, Warner C, Dubel SJ, Snutch TP, Catterall WA (1992) Biochemical properties and subcellular distribution of an N-type calcium channel alpha 1 subunit. Neuron 9:1099–1115. DOI: 10.1016/0896-6273(92)90069-P.

Wetmore C, Cao YH, Pettersson RF, Olson L (1991) Brain-derived neurotrophic factor: subcellular compartmentalization and interneuronal transfer as visualized with anti-peptide antibodies. Proc. Natl. Acad. Sci. U.S.A. 88:9843–9847.

Wilson KHS (2009) The genome sequence of the protostome Daphnia pulex encodes respective orthologues of a neurotrophin, a Trk and a p75NTR: evolution of neurotrophin signaling components and related proteins in the bilateria. BMC Evol. Biol. 9:243. DOI: 10.1186/1471-2148-9-243.

Woo NH, Teng HK, Siao C, Chiaruttini C, Pang PT, Milner TA, Hempstead BL, Lu B (2005) Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. Nat. Neurosci. 8:1069–1077. DOI: 10.1038/nn1510.

Wu LL, Fan Y, Li S, Li X, Zhou X (2010) Huntingtin-associated protein-1 interacts with pro-brain-derived neurotrophic factor and mediates its transport and release. J. Biol. Chem. 285:5614–5623. DOI: 10.1074/jbc.M109.073197.

Wu YJ, Krüttgen A, Möller JC, Shine D, Chan JR, Shooter EM, Cosgaya JM (2004) Nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 are sorted to dense-core vesicles and released via the regulated pathway in primary rat cortical neurons. J. Neurosci. Res 75:825–834. DOI: 10.1002/jnr.20048.

Xia X, Lessmann V, Martin TFJ (2009) Imaging of evoked dense-core-vesicle exocytosis in hippocampal neurons reveals long latencies and kiss-and-run fusion events. J. Cell. Sci. 122:75–82. DOI: 10.1242/jcs.034603.

Xu B, Gottschalk W, Chow A, Wilson RI, Schnell E, Zang K, Wang D, Nicoll RA, Lu B, Reichardt LF (2000) The role of brain-derived neurotrophic factor receptors in the mature hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB. J. Neurosci. 20:6888–6897.

Xu F, Plummer MR, Len G, Nakazawa T, Yamamoto T, Black IB, Wu K (2006) Brain-derived neurotrophic factor rapidly increases NMDA receptor channel activity through Fyn-mediated phosphorylation. Brain Res. 1121:22–34. DOI: 10.1016/j.brainres.2006.08.129.

Yan Q, Rosenfeld RD, Matheson CR, Hawkins N, Lopez OT, Bennett L, Welcher AA (1997) Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. Neuroscience 78:431–448. DOI: 10.1016/S0306-4522(96)00613-6.

Yang J, Siao C, Nagappan G, Marinic T, Jing D, McGrath K, Chen Z, Mark W, Tessarollo L, Lee FS, Lu B, Hempstead BL (2009) Neuronal release of proBDNF. Nat. Neurosci 12:113–115. DOI: 10.1038/nn.2244.

Yang M, Lim Y, Li X, Zhong J, Zhou X (2011) Precursor of brain-derived neurotrophic factor (proBDNF) forms a complex with Huntingtin-associated protein-1 (HAP1) and sortilin that modulates proBDNF trafficking, degradation, and processing. J. Biol. Chem. 286:16272–16284. DOI: 10.1074/jbc.M110.195347.

Yano H, Ninan I, Zhang H, Milner TA, Arancio O, Chao MV (2006) BDNF-mediated neurotransmission relies upon a myosin VI motor complex. Nat. Neurosci. 9:1009–1018. DOI: 10.1038/nn1730.

Yasuda R, Sabatini BL, Svoboda K (2003) Plasticity of calcium channels in dendritic spines. Nat. Neurosci 6:948–955. DOI: 10.1038/nn1112.

Yuen EC, Gunther EC, Bothwell M (2000) Nitric oxide activation of TrkB through peroxynitrite. Neuroreport 11:3593–3597.

Yuste R, Denk W (1995) Dendritic spines as basic functional units of neuronal integration. Nature 375:682–684. DOI: 10.1038/375682a0.

Yuste R, Majewska A, Cash SS, Denk W (1999) Mechanisms of calcium influx into hippocampal spines: heterogeneity among spines, coincidence detection by NMDA receptors, and optical quantal analysis. J. Neurosci. 19:1976–1987.

Zafra F, Hengerer B, Leibrock J, Thoenen H, Lindholm D (1990) Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. EMBO J. 9:3545–3550.

Zafra F, Lindholm D, Castrén E, Hartikka J, Thoenen H (1992) Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. J. Neurosci 12:4793–4799.

Zakharenko SS, Patterson SL, Dragatsis I, Zeitlin SO, Siegelbaum SA, Kandel ER, Morozov A (2003) Presynaptic BDNF required for a presynaptic but not postsynaptic component of LTP at hippocampal CA1-CA3 synapses. Neuron 39:975–990. DOI: 10.1016/S0896-6273(03)00543-9.

Zhou Z, Hong EJ, Cohen S, Zhao W, Ho HH, Schmidt L, Chen WG, Lin Y, Savner E, Griffith EC, Hu L, Steen JAJ, Weitz CJ, Greenberg ME (2006) Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation. Neuron 52:255–269. DOI: 10.1016/j.neuron.2006.09.037.

Zhu B, Pennack J, McQuilton P, Forero M, Mizuguchi K, Sutcliffe B, Gu C, Fenton J, Hidalgo A (2008) Drosophila neurotrophins reveal a common mechanism for nervous system formation. PLoS Biol 6:e284. DOI: 10.1371/journal.pbio.0060284.

Zuccato C, Tartari M, Crotti A, Goffredo D, Valenza M, Conti L, Cataudella T, Leavitt BR, Hayden MR, Timmusk T, Rigamonti D, Cattaneo E (2003) Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. Nat. Genet. 35:76–83. DOI: 10.1038/ng1219.

8. APPENDIX

8.1. Abbreviations

APV or AP-5	2-amino-5-phosphopentanoic acid
bAPs	backpropagating action potentials
BBS	BES-buffered saline
BDNF	Brain derived neurotrophic factor
BDNF	human BDNF gene
Bdnf	rodent BDNF gene
BES	N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
BME	Basal medium Eagle
BPB	Bromophenol blue
BSA	Bovine serum albumin
CaMKII	Calcium calmoduline protein kinase II
CPA	Cyclopiazonic acid
DCV	Dense core vesicle (LDCV: large dense core vesicle)
DIV	Days *in vitro*
DMEM	Dubelcco's modified Eagle's medium
DNQX	6,7-Dinitroquinoxaline-2,3-dione
dNTPs	Deoxyribonucleotides
DsRed	*Discosoma sp* red fluorescent protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPSP	Excitatory postsynaptic potential
ER	Endoplasmic reticulum (SER: smooth endoplasmic reticulum)
FCS	Fetal calf serum
GABA	γ amino butyric acid
GBZ	Gabazine or SR-95531
GFP	Green fluorescent protein (EGFP: enhanced green fluorescent protein)
HBS	HEPES-buffered saline
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
KN-62	4-[(2S)-2-[(5-isoquinolinylsulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl]phenyl isoquinolinesulfonic acid ester

KN-92	2-[N-(4_-methoxybenzenesulfonyl)]amino-N-(4_-chlorophenyl)-2-propenyl-N-methylbenzylamine phosphate
KN-93	N-[2-[[[3-(4-chlorophenyl)-2-propenyl]methylamino]methyl] phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide
LTD	Long-term depression
LTP	Long-term potentiation
MEM	Modified Eagle medium
mGluR	metabotropic glutamate receptor
NA	Numeric aperture
NB	Neurobasal medium
NGF	Nerve growth factor
Nif	Nifedipine
NMDA	N-methyl-D-aspartate (NMDAR: NMDA receptor)
NOS	Nitric oxide synthase (nNOS/eNOS: neuronal/endothelial NOS)
NT-3, NT-4	Neurotrophin-3, neurotrophin-4 (NT[s]: neurotrophin[s])
OD	Optic density
PBS	Phosphate-buffered saline
PKA	Protein kinase A
PO	Polyornithine
PSD-95	Postsynaptic density protein 95
PTP1D	Protein tyrosine phosphatase 1D
ROI	Region of interest
rpm	Rotations per minute
RT	Room temperature
SEM	Standard error of the mean
SERCA	Sarcoplasmic/Endoplasmic reticulum calcium ATPase
SNAP-25	Synaptosome-associated protein of 25,000 daltons
SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment receptor
TBE	Tris-borate-EDTA
TGN	Trans-Golgi network
tPA	transplasminogen activator
Trk	Tropomyosin receptor kinase
TTX	Tetrodotoxin
VGCC	Voltage-gated calcium channel

8.2. Publications

Kolarow R, Kuhlmann CRW, Munsch T, Zehendner C, Brigadski T, Luhmann HJ, Lessmann V (2014) BDNF-induced nitric oxide signals in cultured rat hippocampal neurons: time course, mechanism of generation, and effect on neurotrophin secretion. Front Cell Neurosci 8:323. DOI: 10.3389/fncel.2014.00323.

Wang X, Hu B, Zieba A, Neumann NG, Kasper-Sonnenberg M, Honsbein A, Hultqvist G, Conze T, Witt W, Limbach C, Geitmann M, Danielson H, Kolarow R, Niemann G, Lessmann V, Kilimann MW (2009) A protein interaction node at the neurotransmitter release site: domains of Aczonin/Piccolo, Bassoon, CAST, and rim converge on the N-terminal domain of Munc 13-1. J Neurosci 29:12584-12596. DOI: 10.1523/ JNEUROSCI.1255-09.2009.

Kuczewski N, Porcher C, Ferrand N, Fiorentino H, Pellegrino C, Kolarow R, Lessmann V, Medina I, Gaiarsa JL (2008) Backpropagating action potentials trigger dendritic release of BDNF during spontaneous network activity. J Neurosci 28:7013–7023. DOI: 10.1523/JNEUROSCI.1673-08.2008.

Kolarow R, Brigadski T, Lessmann V (2007) Postsynaptic secretion of BDNF and NT-3 from hippocampal neurons depends on calcium calmodulin kinase II signaling and proceeds via delayed fusion pore opening. J Neurosci 27:10350–10364. DOI: 10.1523/ JNEUROSCI.0692-07.2007.

8.3. Poster contributions

Kolarow R., Brigadski T., Lessmann V.: Intracellular signaling cascades involved in activity-dependent synaptic secretion of neurotrophins. Nitric oxide symposium, Mainz, 2007.

Kolarow R., Brigadski T., Lessmann V.: L-type Ca^{2+}-channels, CAMKII, and cAMP cooperate in mediating postsynaptic secretion of BDNF and NT-3. 31st Göttingen Neurobiology Conference, 2007.

Kolarow R., Brigadski T., Lessmann V.: Intracellular targeting of neurotrophins and signaling cascades involved in synaptic secretion of BDNF and NT-3. 6th annual meeting of the interdisciplinary science network 'molecular and cellular neurobiology', Mainz, 2006.

Lessmann V., Brigadski T., Kolarow R.; Intracellular targeting of neurotrophins and signalling cascades involved in synaptic secretion of BDNF and NT-3. Society for Neuroscience 36th Annual Meeting, Atlanta, 2006.

Kolarow R., Brigadski T., Hartmann M., Kuhlmann Ch., Luhmann H., Leßmann V.: Intracellular signaling cascades involved in activity-dependent synaptic secretion of neurotrophins. FENS Forum , Vienna, 2006.

Kolarow R., Brigadski T., Hartmann M., Kuhlmann Ch., Luhmann H., Leßmann V.. Intracellular signalling cascades involved in activity-dependent synaptic secretion of neurotrophins. 5rd annual meeting of the interdisciplinary science network 'molecular and cellular neurobiology', Mainz, 2005.

Brigadski T., Hartmann M., Kolarow R. and Leßmann V. Time course of synaptic secretion of neurotrophins from hippocampal neurons. 84th Annual Meeting DPG, Göttingen, 2005.

Brigadski T., Hartmann M., Kolarow R. & Leßmann V. Truncated TrkB receptor induced outgrowth of dendritic filopodia involves the p75 neurotrophin receptor. 30th Göttingen Neurobiology Conference, 2005.

Brigadski T., Hartmann M., Kolarow R. & Leßmann V. Synaptic targeting and time course of secretion of neurotrophins from hippocampal neurons. 30th Göttingen Neurobiology Conference, 2005.

Brigadski T., Hartmann M., Kolarow R. and Leßmann V. Differential synaptic targeting and secretion of the mammalian neurotrophins. 4rd annual meeting of the interdisciplinary science network 'molecular and cellular neurobiology', Mainz, 2004.

Kolarow et al. (2007) is accessible at http://dx.doi.org/10.1523/JNEUROSCI.0692-07.2007 (see references).

Kolarow et al. (2014) is accessible at http://dx.doi.org/10.3389/fncel.2014.00323 (see references).