Imaging brain-derived neurotrophic factor-mediated calcium signaling and plasticity in developing neurons

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To my beloved parents

Ehrenwörtliche Versicherung:
Ich versichere hiermit ehrenwörtlich, dass ich die Dissertation mit dem Titel "Imaging brain-derived neurotrophic factor-mediated calcium signaling and plasticity in developing neurons" selbständig und ohne unerlaubte Beihilfe angefertigt habe. Ich habe mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Hilfen und Quellen bedient.

Erklärung:
Hiermit erkläre ich, dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe. Die Dissertation wurde in ihrer jetzigen oder ähnlichen Form bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

München, im Februar 2007

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Contents

List of Figures

1 Summary

During the development of the brain, neuronal activity and genetically predefined extracellular molecular cues are known to work hand in hand to set up a functional neuronal network. One important signaling molecule in this context is brain-derived neurotrophic factor (BDNF) since it is involved in many aspects of the formation of functional neuronal networks. BDNF signaling regulates neuronal development not only globally, at the level of entire neurons or networks, but also at a subcellular level with high precision. However, many aspects of intrinsic BDNF signaling like temporal and spatial specificity of its action are still elusive.

The aim of this thesis was to explore the role of the neurotrophic factor BDNF in neuronal signaling by calcium imaging in developing pyramidal neurons of hippocampal slice cultures from postnatal rats. Organotypic hippocampal slice cultures are a well established system to study developmental plasticity processes in a conserved neuronal circuitry. Using calcium imaging of individual electroporated pyramidal neurons, I was able to directly observe intrinsic BDNF signaling. I asked whether spontaneous localized calcium transients are triggered by BDNF and investigated the mechanisms underlying BDNF-mediated local calcium increases.

In the first part of my thesis, I found that blocking intrinsic BDNF signaling reduced the frequency of spontaneously occurring fast and localized calcium rises in dendrites. Conversely, focal application of BDNF evoked fast and local dendritic calcium transients, which required activation of tropomyosin related kinase B (TrkB) receptors, the major receptors for BDNF, as well as activation of voltage gated sodium and calcium channels. To specify the local action of endogenous BDNF in calcium signaling, a virus system for expressing postsynaptic density-95 tagged with cyan fluorescent protein (PSD-95:CFP) was generated to visualize postsynaptic sites along the dendrites of neurons. Calcium imaging in PSD-95:CFP expressing neurons revealed that spontaneous local calcium transients occurred frequently at postsynaptic sites along the dendrite. The frequency of synaptic calcium transients was specifically reduced by blocking intrinsic BDNF signaling, whereas non-synaptic calcium rises were not affected. Furthermore, focal BDNF delivery evoked localized and fast calcium

elevations specifically at postsynaptic sites. Together, the results demonstrate that BDNF-dependent calcium signaling in developing hippocampal neurons is fast and occurs at synapses.
In the second part of this thesis, I followed up the observation of precisely regulated fast BDNF-signaling at synapses and explored possible rapid and sustained plasticity phenomena in developing dendrites upon localized stimulation with BDNF. In principal, two probably related modifications - structural and functional plasticity - can be mediated by neurotrophins. Does BDNF initially regulate the morphology, leading to the formation and stabilization of synaptic sites or does it act directly on (immature) synapses without affecting the structure? I addressed both possibilities. First, I investigated the acute role of BDNF in the motility and growth of dendritic filopodia which are known to be highly motile in a calcium-dependent fashion. Surprisingly, I found no immediate effect of BDNF on morphological plasticity. I next examined whether BDNF induces functional plasticity of dendritic calcium activity during development and asked whether specific sites along the dendrite such as maturating synapses show functional changes, e.g. generate more or fewer local calcium transients, after focal BDNF stimulation. I observed a rapid and long-lasting increase in the frequency of local calcium transients after focal BDNF stimulation which was largely due to the activation of previously ‘silent‘ sites along the dendrite. These results suggest that BDNF induces long-lasting functional plasticity, possibly on the level of individual synapses by promoting - pre- or postsynaptically or both - the maturation and stabilization of developing synapses.
In conclusion, the work presented in this thesis provides further insight into the acute and long-term action of the neurotrophic factor BDNF on neuronal calcium signaling during development. The temporal and spatial characteristics of intrinsic BDNF signaling as well as its relative abundance and possible involvement in long-term changes of synapse formation, maturation and stabilization render BDNF an ideal signaling molecule in the establishment of specific synaptic connectivity and functional neuronal networks.

2 Abbreviations

AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor
ATP	adenosine triphosphate
bp	basepairs
BDNF	brain-derived neurotrophic factor
CA	cornus ammonis
CaMK	calcium-calmodulin dependent kinase
CNS	central nervous system
CPA	cyclopiazinic acid
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DNA	desoxyribonuclein acid
EDTA	ethylendiaminotetraacetat
ENO	early network oscillation
EPSP	excitatory postsynaptic potential
GABA	γ-aminobutyric acid
GDP	giant depolarizing potential
h	hour
Hz	hertz
IgG	immunglobulin G
$InsP_3$ = IP3	inositol 1,4-trisphosphate
kDa	kiloDalton
LTP	long-term potentiation
MAPK	mitogen-activated kinase
min	minute
mEPSC	miniature excitatory postsynaptic current
mm	millimeter
μm	micrometer
MSCC	mechanosensitive calcium channel
NGF	nerve growth factor
NMADR	N-methyl-D-aspartate receptor
NT	neurotrophin
P	postnatal

$p75^{NTR}$	p75 neurotrophin receptor
PB	phosphate buffer
PBS	phosphate buffered saline
PI3K	phosphatidylinositol-3-kinase
PLCγ	phospholipase Cγ
PSD	post synaptic density
ROCC	receptor-operated calcium channel
RT	room temperature
RYR	ryanodine receptor
SKF	SKF 96365 hydrochloride
SDS	sodium dodecylsulfate
SFV	Semliki Forest Virus
SOCC	store-operated calcium channel
TAE	tris-acetate-EDTA
TBS	theta burst stimulation
TBS	tris buffered saline
TEMED	N,N,N‘,N‘-tetramethylendiamin
TeTX	tetanus toxin
Tris	tris-(hydroxymethylen)-aminomethan
Trk	tropomyosine related kinase
TRPC	transient receptor potential channel
TTX	tetrodotoxin
Tween 20	polyoxyethylene sorbitan monolaurate
VOCC	voltage-operated calcium channel

3 Introduction

One of the most exciting questions in neuroscience research is how hundreds of trillions or even more functional connections between neurons are properly formed during the development of the brain. What are the important signals that organize neuronal networks in the different brain regions that later in life determine our perceptions of the external world, control our attention, actions and emotions and are important for learning and memory? Especially during the initial wiring of neuronal circuits the structures of nerve cells are surprisingly plastic and can be remodeled and refined within short time. Among the cellular structures that show this kind of plasticity are axons with growth cones that are steered by guidance molecules along specific pathways to their appropriate targets, and dendrites which are the components of neurons that receive and process information. It is believed that activity-dependent and activity-independent mechanisms control contact formation between axons and dendrites of appropriate neurons (Cline, 2003). To achieve this, neuronal activity and genetically predefined extracellular molecular cues work hand in hand to set up a functional neuronal network. This initial connectivity is not rigid, but is refined by experience-dependent plasticity throughout development and even later during adult life.

3.1 The developing synapse

In a functional neuronal network, information between neurons is transmitted mainly in form of chemical signals. This communication process happens at specialized contact sites between the presynaptic axonal terminals and the postsynaptic sites of the dendrites - the synapses. A major component of the postsynaptic site is the postsynaptic density (PSD) which has been shown to be an electron-dense thickening of the membrane containing neurotransmitter receptors, signaling molecules and scaffolding proteins in high concentrations. Chemical transmitters that are released from synaptic vesicles in the presynapse, diffuse rapidly across the narrow synaptic cleft and bind to their specific recep-

tors in the PSD where the chemical information is then processed, integrated in an electrical signal and propagated. Thus, in the adult brain more than 10^{14} synapses connect neurons with each other to form a highly complex and functionally meaningful network.

It is fundamental for understanding the development of the nervous system to know how synapses are formed and how they mature. During the first two postnatal weeks, the density of synapses gradually increases. At this age, synapses can be observed mainly on dendritic shafts and on filopodia which are thin, highly motile and transient actin-rich protrusions. They can grow and retract within seconds to minutes. Since they - developmentally - precede spines, they may have an exploratory function: their role may be to sculpt the dendritic tree and to contact nearby axons in order to establish early synapses, independently of the eventual formation of spines (spinogenesis vs. synaptogenesis) (Cailliau and Yuste, 2001). Electronmicroscopic studies have shown that filopodia can bear synapses (Dailey and Smith, 1996; Fiala et al., 1998) upon contact with axons. This may help to increase the pool of temporary synaptic contacts from which the final set of synapses will be selected (Fiala et al., 1998; Ziv and Smith, 1996). The cell-cell contact seems to be the important initial step in synapse formation. Trans-synaptic signaling molecules like neuroligins/neurexins (Scheiffele et al., 2000), integrins (Chavis and Westbrook, 2001), cadherins (Togashi et al., 2002) and SynCAMs (Biederer et al., 2002) have shown to participate in this interaction. They are thought to initiate the coordination of presynaptic and postsynaptic assembly. Within only a few hours of the initial contact formation, synaptic proteins have been shown to accumulate on both sites (Friedman et al., 2000). Presynaptic proteins like active zone proteins and synaptic vesicle precursors are recruited in dynamic transport packets to nascent synapses (Ahmari et al., 2000; Zhai et al., 2001). For postsynaptic assembly, PSD-95, an abundant multi-domain scaffolding protein which clusters glutamate receptors and organizes associated signaling complexes, is one of the best studied proteins: rapid recruitment of PSD-95 to the postsynaptic sites of nascent synapses has been reported to occur in a gradual manner from a diffuse cytoplasmic pool rather than from transport packages (Okabe et al., 2001; Marrs et al., 2001). It has also been shown that an exchange of PSD-95 molecules between synapses by lateral diffusion is possible (Gray et al., 2006). Besides multiple binding partners, PSD-95 binds to N-methyl-D-aspartate receptors (NMDARs) and also interacts indirectly with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptors (AMPARs). Both glutamate receptors are essential components of the postsynaptic membrane. It has been demonstrated that NMDAR transport packets

are more rapidly recruited to nascent synapses than AMPAR clusters (Washbourne et al., 2002). Also tropomyosine related kinase (TrkB) receptor clusters have shown to colocalize with NMDAR clusters and become enriched at synapses where they can be activated by brain-derived neurotrophic factor (BDNF), suggesting that they are important in synaptogenesis (Gomes et al., 2006). The existence of non-functional 'silent' synapses during development has been reported (Gasparini et al., 2000): silent synapses are either caused by a deficiency in presynaptic transmitter release (presynaptically 'silent' synapses) (Berninger et al., 1999) or they only contain NMDARs, but not AMPARs (postsynaptically 'silent' synapses) (Malinow and Malenka, 2002) and are under resting physiological conditions nonfunctional due to the Mg^{2+}-block of NMDARs. 'Silent' synapses are converted into fully functional ones through AMPAR trafficking into the PSD of the synapse triggered by NMDAR-dependent activity (Zhu and Malinow, 2002). Maturation and growth of synapses is characterized by activating 'silent' synapses and strengthening of excititory postsynaptic AMAPR-mediated currents (Lee and Sheng, 2000). In conclusion, nascent synapses are characterized by the interaction of trans-synaptic signaling molecules followed by the recruitment of proteins to both synaptic sites within a few hours and their conversion - in the case of glutamatergic synapses - from silent into functional synapses.

3.2 The hippocampal preparation

Many studies investigating developing synapse formation have used hippocampal cultures and hippocampal slice preparations, because the hippocampus is one of the best characterized cortical brain structures. It is named for its resemblance to a sea horse and neuropsychological investigations suggest that this region of the temporal lobes plays a key role in certain aspects of learning and memory and in normal cognitive function. Although the exact function is still unclear, it seems that the hippocampus is essential for the *declarative memory system* (e.g. learning the name of a person) in humans (Squire, 1987). Additionally, interest in the hippocampus derives from the fact that this structure is a site of degenerative disorders, such as Alzheimer´s disease (Robins and Kumar, 1987), and is often involved in temporal lobe epilepsy, an abnormal form of synchronization of neuronal activity (Wong, 1984). Many neurophysiological experiments served the purpose to find the cellular basis for learning and memory. Until now, *long-term potentiation* (LTP) is the major candidate for an adaptive synaptic mechanism of rapid learning in mammals: LTP was first

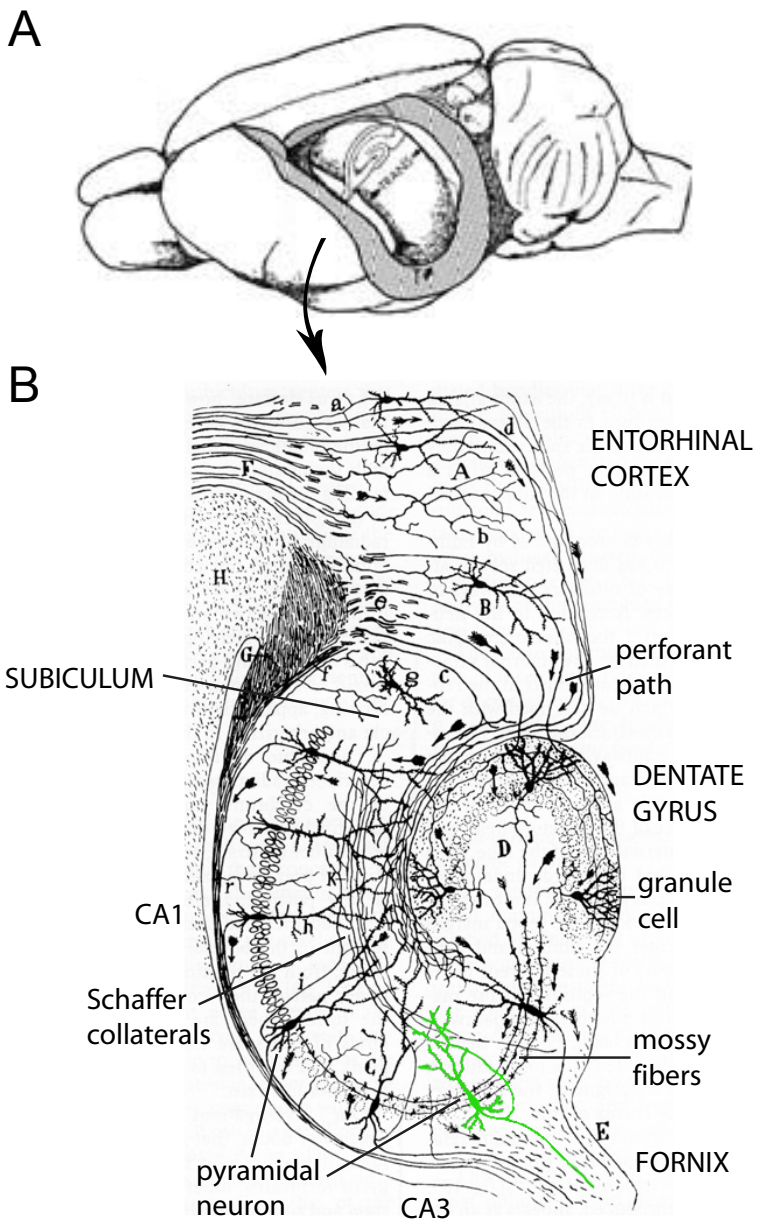

Figure 3.1: Hippocampal preparation. A, Orientation of the hippocampus in the left hemisphere of the rat brain. A transverse section of the hippocampus is indicated. **B**, Neuronal organisation of the hippocampal formation within a single slice. An individual CA3 pyramidal neuron is labeled in green, because those cells were used in this thesis (CA1/CA3 - cornus ammonis; drawing modified from Cajal, 1911).

described in 1973, when Bliss and Lomo (Bliss and Lomo, 1973) showed that the synaptic strength between hippocampal neurons of rabbits was increased following high frequency stimulation ('tetanus') of the presynaptic fibers. Later, in 1986, Gustafsson and Wingström (Gustafsson and Wigström, 1986) demonstrated that simultaneously activating the presynaptic fibers and the postsynaptic neuron resulted in a persistent enhancement of neuronal responsiveness. This supported Hebb's theory postulating that synapses are strengthened when pre- ans postsynaptic neurons are synchronously active (Hebb, 1949). There is evidence that multiple expression mechanisms for LTP exist in the hippocampus and that they are developmentally regulated: it has been reported that, unlike LTP induction in mature hippocampal neurons which is dependent on αCaMKII (Malenka and Nicoll, 1999), LTP in the neonatal hippocampus does not require CaMKII, but cyclic AMP-dependent protein kinase A (PKA) (Yasuda et al., 2003). Furthermore, LTP during development is characterized by an increase in the number of functional release sites and/or an increase in the probability of release (Palmer et al., 2004). These fascinating forms of synaptic plasticity which may be the cellular basis of memory formation have been extensively studied in the hippocampus. Fig. 3.1A shows the orientation of the left hippocampus in a rodent brain. It is a cylindrical structure forming a semicircle around the thalamus. A section of the hippocampus, taken transversely to its longitudinal axis, is indicated. The highly regular organization of the hippocampal network in a single slice is depicted in Fig. 3.1B. This drawing by Ramòn Y Cajal (Cajal, 1911) shows the trisynaptic excitatory pathway which gets input from the entorhinal cortex, the septal region and the contralateral hippocampus. Via the perforant path in the subiculum, the efferent fibers from the entorhinal cortex terminate on dendrites of the granule cells in the dentate gyrus. The granule cells send their axons along the mossy fiber pathway through the hilus to the CA3 (Cornu ammonis 3, due to its resemblance to a ram's horn) region where they terminate at the proximal apical dendrites of the CA3 pyramidal neurons. The axons of the CA3 neurons in turn project via the Schaffer collaterals to the CA1 pyramidal neurons, but also to other CA3 pyramidal cells and to the contralateral hippocampus (commissural pathway). In this study CA3 pyramidal neurons of organotypic hippocampal slice cultures (Stoppini et al., 1991) have been used. Incubating such slices for a few days before using has the advantage that damaged cells at the slice surface are eliminated over time. The principal two-dimensional network structure is preserved after slicing the hippocampus and neuronal activity can still be observed, e.g. by calcium imaging.

3.3 Calcium signaling in the developing hippocampus

Neuronal activity induces marked increases in the cytosolic concentration of calcium which is an ubiquitous intracellular second messenger. Therefore, calcium imaging is a widely used, powerful technique to measure neuronal activity. Changes in the level of intracellular [Ca^{2+}] are essential for the development and function of neurons (Ghosh and Greenberg, 1995; Berridge, 1998; Berridge et al., 2000). Like other cells, neurons use extracellular and intracellular sources of calcium. Changes in cytosolic [Ca^{2+}] can occur by three major routes: 1) elevation of [Ca^{2+}] following binding of transmitters to calcium-permeable transmitter receptors such as NMDARs or nicotinic acetylcholine receptors (receptor-operated calcium channels = ROCCs). 2) calcium-influx from the extracellular space across the cell membrane through voltage-operated calcium channels (L-, N- and P/Q-types of VOCCs), which open upon membrane depolarization through store-operated (SOCC) and mechanosensitive (MSCC) calcium-permeable ion channels. 3) calcium-release from internal stores such as the lumen of the endoplasmatic reticulum (ER) or the mitochondria that can be triggered by calcium influx (calcium-induced calcium release: CICR). The intracellular free calcium concentration of a neuron at rest is ∼100 nM, but upon activity this level raises to ∼500-1000 nM or more. The recovery of the cytoplasmic calcium concentration back to its basal level is an active process involving calcium pumps which transport calcium against its steep electrochemical gradient (extracellular calcium concentration: 2 mM) across the membrane out of the cell. For this purpose, different extrusion mechanisms such as Na^+/Ca^{2+} exchanger or Ca^{2+}-ATPases are used. Additionally, calcium can also be transported back into the intracellular stores via Ca^{2+}-ATPases to refill them.

The versatility of calcium signaling is achieved through a precise regulation of the concentration of calcium, the distribution of calcium in the dimensions of space and time and through interactions with other signaling pathways in the cell. For instance, many of the functions of calcium are accomplished by generating highly localized calcium signals. Upon binding of calcium to calmodulin, a variety of enzymes such as calcium-calmodulin kinases (CaMKII and CaMKIV), Ras/mitogen-activated kinases (Ras/MAPK) and calcium-sensitive adenylate cyclases are activated. These enzymes transduce the calcium signal into innumerable cellular or nuclear responses. These include gene transcription (Gabellini, 2004) and activity-dependent secretion of proteins like BDNF (Shieh

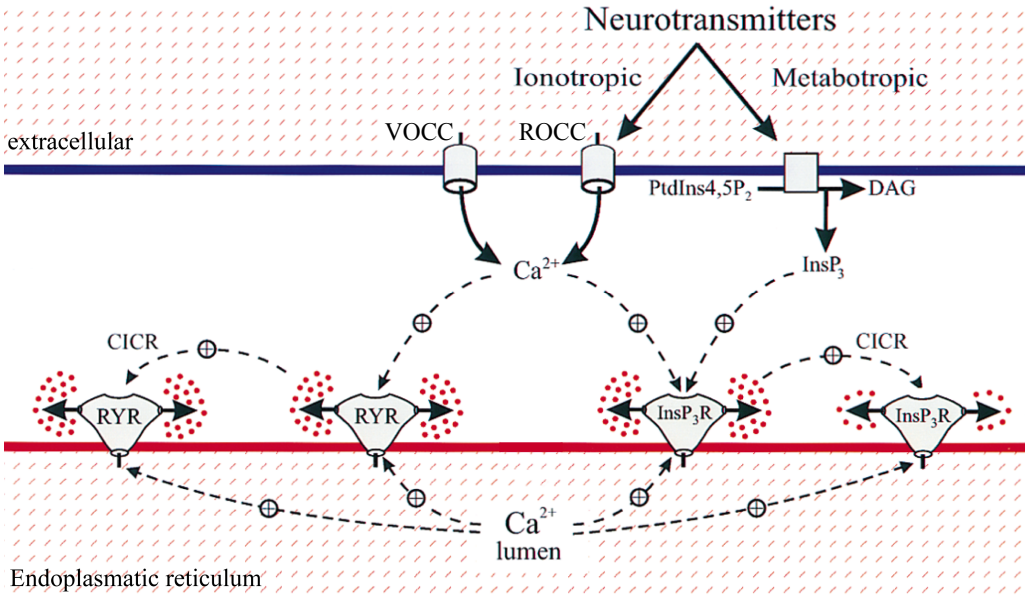

Figure 3.2: Neural calcium signaling. Neurotransmitter-induced calcium entry from the outside of the cell through voltage-operated (VOCCs) and receptor-operated calcium channels (ROCCs) and release of calcium from the endoplasmatic reticulum (ER) through $InsP_3$ receptors ($InsP_3R$) and ryanodine receptors (RYR). Calcium-induced calcium release (CICR) can set up propagated calcium waves (modified from Berridge, 1998).

et al., 1998), ion channel functions (Fucile et al., 2000), cell proliferation, dendritic development (Meberg et al., 1999; Lohmann et al., 2002; Lohmann et al., 2005) and axonal growth cone migration (Gomez et al., 2001).
Since calcium is a critical mediator of development and plasticity, there has been a broad interest in understanding the mechanisms by which changes in intracellular $[Ca^{2+}]$ induce diverse short- and long-lasting cellular effects. During development when synapse and network formation begin, the first 'primitive' pattern of network-driven calcium activity has been described in the hippocampus: mostly in the CA3 region but also in CA1, spontaneous calcium oscillations can be seen that occur simultaneously in groups of neighboring cells and are strictly correlated with large synaptic events, so called *giant depolarizing potentials* (GDPs) (Ben-Ari et al., 1989; Leinekugel et al., 1998). They represent slowly propagating waves of activity (recurring at a frequency of 0.1 per sec) that are generated by calcium-influx through NMDARs and VOCCs. These waves are mainly, but not exclusively, dependent on GABA (Sipilä et al., 2005), which has, unlike in adult neurons, a depolarizing action in immature neurons.

This is due to the fact that the reversal potential for $GABA_A$, which is set by the electrochemical gradient of Cl^-(the main ion for which GABA receptor channels are permeable), is more positive compared to the resting membrane potential (Rivera et al., 1999; Kasyanov et al., 2004): therefore the intracellular concentration of Cl^- is high and $GABA_A$receptor activation causes Cl^- efflux out of the cell and a depolarization (Stein and Nicoll, 2003). However, also glutamate has been shown to participate in the generation of GDPs (Ben-Ari et al., 1989). The patterned, spontaneous activity in the immature hippocampus has also been termed 'giant GABAergic potentials' or 'early network oscillations' (ENOs) (Garaschuk et al., 1998). It seems to be a well preserved feature of the developing neuronal network as it can also be found in many other brain regions, e.g. in the retina ('retinal waves') (Galli and Maffei, 1988; Meister et al., 1991; Wong et al., 1995), the lateral geniculate nucleus (Shatz, 1990) and the neocortex (Yuste et al., 1992; Adelsberger et al., 2005). In the retina for instance, correlated calcium oscillations have been observed in spatially restricted domains of amacrine and ganglion cells (Wong et al., 1995), which are synaptically mediated and dependent on activation of acetylcholine receptors (Feller et al., 1996; Zheng et al., 2006). In the neocortex in contrast, domains of spontaneous co-activation do not require synaptic transmission but propagate through gap junctions (Yuste et al., 1995). They are caused by calcium release from internal stores through inositol 1,4-triphosphate ($InsP_3$) spreading between cells through gap junctions (Kandler and Katz, 1995). This slow synchronous network activity develops shortly after neurogenesis when there is a large amount of silent synapses containing only NMDARs and it disappears before the onset of experience-dependent plasticity. In conclusion, it is likely that these spontaneous increases in $[Ca^{2+}]$, associated with depolarizations, play a major role in driving many aspects of development including changes in synaptic efficacy at yet poorly developed connections between neurons and refinement of local neuronal circuit formation (Kasyanov et al., 2004).

Besides this spontaneous network activity during development, also other activity-mediated global and local changes in calcium dynamics have been described more recently. Advances in imaging and labeling techniques have permitted to observe and characterize cellular and even subcellular calcium events on a single cell level (Koizumi et al., 1999): global increases in $[Ca^{2+}]$ affecting the whole neuron are induced by action potentials and coordinated multiple localized calcium increases, thereby activating effectors throughout the cell. Additionally, spatially restricted local calcium transients occur spontaneously. They often can be observed at branch points and filopodia along the dendrite, can

stimulate processes in the vicinity and may thereby influence dendritic branch stability (Koizumi et al., 1999; Lohmann et al., 2005).
How are these local calcium transients generated? It has been shown that they can arise from calcium influx via VOCCs or ROCCs at dendrites, especially at postsynaptic densities following neuronal input and from calcium release from internal stores upon opening of $InsP_3$ and ryanodine receptors (Koizumi et al., 1999). Furthermore, those synaptically mediated local calcium rises in spines and in dendrites occur fast (rising within msec, decaying within hundreds of msec) and spread only a few μm from the site of stimulation (Finch and Augustine, 1998; Emptage et al., 1999). Local increases in $[Ca^{2+}]$ have also been mapped to synaptic sites using post-hoc immunohistochemical staining of the presynaptic marker synapsin supporting the idea that they are triggered by synaptic signaling (Lohmann et al., 2005). However to date it is unclear which neurotransmitters are involved in eliciting local calcium transients: GABA signaling has been suggested to play a role, but clearly accounts only in part for their generation. Also glutamate might be responsible for the generation of a fraction of local calcium rises (Thomas Kleindienst, personal communication). Since localized calcium transients can still be observed after simultaneous blockade of GABA-ergic and glutamatergic transmission, there must be other signaling molecules as well that are involved in inducing local calcium signaling. Good candidate molecules present in the hippocampus are cell adhesion molecules such as integrins (Schuster et al., 2001) and extracellular guidance molecules such as neurotrophins, especially BDNF (Kang and Schuman, 2000).

3.4 Neurotrophins and their receptors

Neurotrophins (NTs) are a family of structurally related and secreted proteins with a size of 12-13 kDa. They arise from precursors, so-called proneurotrophins (27-35 kDa), which are further processed within the intracellular protein transport pathway (Lessmann et al., 2003). Recently, it has been shown that the proneurotrophins can also be proteolytically cleaved by extracellular proteases to produce mature neurotrophins (Bibel and Barde, 2000; Lee et al., 2001; Chao, 2003). The immature protein form binds with high affinity to $p75^{NTR}$ receptor to mediate cell death by apoptosis whereas the mature NTs form stable, non-covalent dimers and preferentially activate specific tropomyosine related kinase (Trk) receptors to promote survival and differentiation (Lee et al., 2001). To date, five NTs have been identified in the mammalian brain: the first NT was discovered in 1956 and named nerve growth factor (NGF, Fig. 3.3) (Cohen and

Levi-Montalcini, 1956; Levi-Montalcini, 1987). It is still *the* prototypical growth factor. NGF was found to be a protein critical for the survival and maintenance of sympathetic and sensory neurons in the peripheral nervous system (PNS). It is released from target cells, binds to and thereby activates its high affinity receptor TrkA. Subsequently, the NGF/TrkA complex is internalized into the responsive neuron and trafficked retrogradely back to the cell body. Later, NGF has also been also detected in the central nervous system (CNS) (Thoenen et al., 1987). Then, 25 years ago, brain-derived neurotrophic factor (BDNF) was isolated from the pig brain (Barde et al., 1982) and found to be highly homologous in amino acid sequence to NGF (Leibrock et al., 1989). BDNF is the NT showing the most widespread expression in the developing and adult nervous system. Besides being a factor for neuronal survival and differentiation, BDNF has been strongly implicated in mechanisms of neuronal plasticity. It binds with high affinity to the TrkB receptor. Since then, two more NTs have

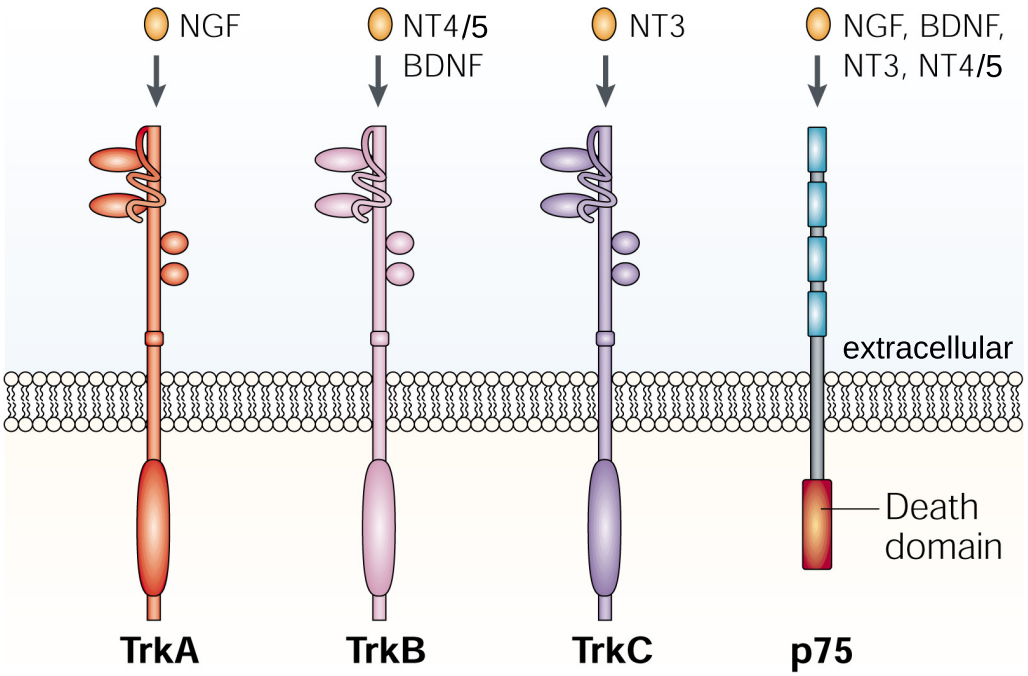

Figure 3.3: Neurotrophins and their respective receptors. The domain structures of the Trk receptors and $p75^{NTR}$ receptor are shown schematically. Neurotrophins can bind to their specific high-affinity receptors and they all can bind to the low-affinity receptor $p75^{NTR}$. NT-3 can also bind with low affinity to TrkA and TrkB (adapted from a review by M.Chao, 2003).

been identified in the mammalian brain: neurotrophin-3 (NT-3) is known to bind to TrkC (Hohn et al., 1990) and is the first NT to be expressed in the PNS during embryogenesis, thereby promoting the survival and differentiation of sensory neurons (Lewin and Barde, 1996). In the CNS, compensatory effects between NT-3 and BDNF on neuron survival have been reported (Minichiello and Klein, 1996). Neurotrophin-4/5 (NT-4/5), like BDNF, signal through TrkB receptors (Berkemeier et al., 1991), but both ligands convey different signals and induce different cellular processes (Minichiello et al., 1998).

Besides the binding to specific high-affinity receptors (TrkA,B,C) through which the NTs are thought to exert their 'positive' biological functions, NTs can also bind to truncated versions of Trk receptors, which lack the intracellular kinase domain, or to non-selective $p75^{NTR}$ receptors, which have a similar low affinity for all NTs and can signal cell death by apoptosis (Dechant and Barde, 1997)(Fig. 3.3). Since Trk and $p75^{NTR}$ receptors are found in close vicinity to each other in the cell membrane, they can interact directly to allow cross talk and fine tuning of responses (Bibel and Barde, 2000).
Since the discovery of NGF, it became now more and more evident that NTs are not simply 'survival factors' but play an important role in synaptogenesis and activity-dependent forms of synaptic plasticity (Huang and Reichardt, 2001). Lack of NTs during development can cause severe phenotypic alterations (Levine et al., 1996), e.g. BDNF-deficient mice suffer developmental defects in the brain, sensory nervous system and cardiovascular system and usually die soon after birth.

3.4.1 BDNF-TrkB-signaling

TrkB receptors are widely expressed in the CNS, in particular in the hippocampus and the cortex. Binding of homodimeric BDNF via the extracellular IgG domain leads to dimerization of TrkB receptors. Upon binding of ATP and stimulation of the intracellular kinase activity, the tyrosine (Y) triplet in the kinase domain needs to be autophosphorylated for further phosphorylation steps (Trk receptor dimers contain 10 tyrosines in their cytoplasmic domains) (Klein et al., 1989; Stephens et al., 1994). Tyrosine residues outside the kinase domain are in turn phosphorylated to induce the activation of three major intracellular signaling cascades by creating docking sites for adaptor proteins like Shc and PLCγ (Fig. 3.4) (Kaplan and Miller, 2000; Huang and Reichardt, 2001; Blum and Konnerth, 2005):

- Activation of phosphatidylinositol-3-kinase (PI3K), which is conveyed by the Shc (src-homology 2 / collagen-related protein) binding site, stimulates the protein kinase Akt. Akt is essential for survival of many types of neurons (Datta et al., 1997; Datta et al., 1999).

- Activation of Ras via Shc stimulates Raf, that subsequently activates MAPK/-Erk kinase (MEK) and mediates via mitogen-activated protein kinase (MAPK) differentiation, survival and neurite outgrowth (Grewal et al., 1999).

- Phosphoslipase Cγ (PLCγ) activation leads via hydrolysation of phosphatidyl inositides to the generation of inositol 1,4-trisphosphate (IP3) and diacylglycerol (DAG). DAG-regulated protein kinase C (PKC) is e.g. required for neurite outgrowth and Erk cascade activation (Corbit et al., 1999). IP3 induces

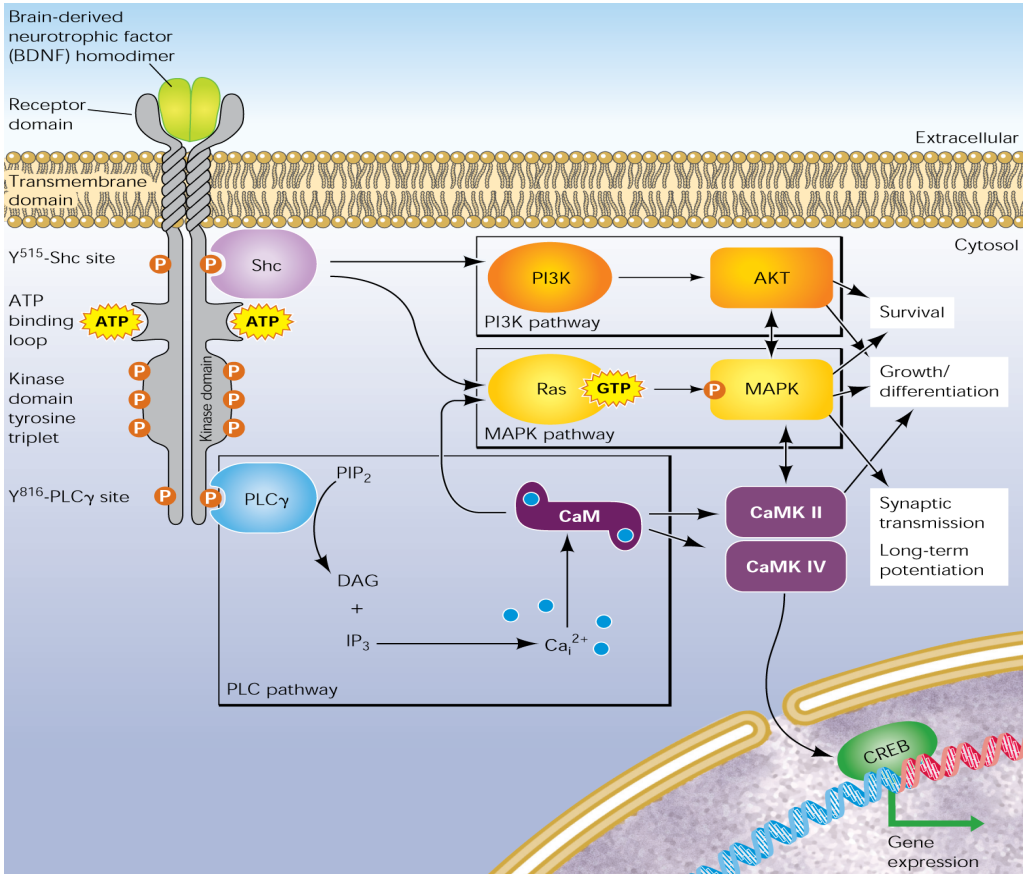

Figure 3.4: BDNF-TrkB-mediated signaling pathways. Downstream signaling pathways of TrkB (adapted from a review by Blum and Konnerth, 2005).

calcium release from internal stores and thereby activates calcium-calmodulin regulated protein kinases (CaMK) (Finkbeiner et al., 1997; Kang et al., 2001). Upon IP3-dependent calcium release also store-operated channels such as the transient receptor potential channel 3 (TRPC3) can be activated and induce a nonselective cation current (Li et al., 1999). The PLCγ pathway is involved in NT-mediated NT release (Canossa et al., 1997) and in synaptic plasticity (Minichiello et al., 2002; Gaertner et al., 2006).

Upon neurotrophin administration, these intracellular signal transduction pathways that occur through TrkB tyrosine kinase activity and further phosphorylation steps, can be triggered within seconds to minutes. For instance the stimulation of PLCγ phosphorylation occurs within 20-30 sec after BDNF addition and lasts for up to 30 min (Widmer et al., 1993). More recent studies have described that TrkB activation by BDNF can also mediate very fast excitatory actions that occur within the range of msec (Kafitz et al., 1999; Kovalchuk et al., 2002) and are independent of the slower signaling pathways described above.
How are these BDNF-mediated rapid effects accomplished? It has been shown that following TrkB-mediated activation of Fyn, a nonreceptor protein kinase, the open probability of postsynaptic NMDA receptor subunits NR1 and NR2B is increased (Levine et al., 1998; Lin et al., 1998), which leads to an influx of Na^+ and Ca^{2+} ions. Moreover, a transmitter-like action of BDNF has been reported (Kafitz et al., 1999): the study revealed that BDNF-TrkB-signaling can depolarize neurons within msec by an immediate activation of a Na^+ conductance (BDNF-evoked I_{Na}). This depolarization results in activation of voltage-operated calcium channels (VOCCs) and thereby produces a calcium influx. Together, these rapid and slow neurotrophin-mediated signaling pathways spanning from fast excitability to induction of gene expression in the nucleus are responsible for many short- and long-term effects of neuronal plasticity (see paragraph below).

3.4.2 BDNF and activity-dependent plasticity

Besides the classical long-lasting effects of neurotrophins on neuronal survival and differentiation during development (Bibel and Barde, 2000; Huang and Reichardt, 2001), recent evidence has revealed acute effects of neurotrophins on neuronal plasticity. Changes in synaptic and in morphological plasticity which are most likely related to each other have been extensively studied. In particular, BDNF - via TrkB receptors - has been reported to affect neuronal morphology in cortical circuitries through the control of dendritic and axonal branching in

an activity-dependent manner (McAllister et al., 1995; McAllister et al., 1999; Gorski et al., 2003). Moreover, activity-dependent synaptic potentiation in the CNS has been proposed to be mediated by pre- and postsynaptic actions of BDNF (Thoenen, 1995; Bonhoeffer, 1996; Katz and Shatz, 1996). BDNF signaling modulates synaptic strength by exerting acute effects on synaptic transmission (Lohof et al., 1993; Knipper et al., 1994; Kang and Schuman, 1995; Levine et al., 1995; Levine et al., 1998; Poo, 2001): addition of exogenous BDNF to developing neuromuscular synapses in culture increased the frequency of spontaneous synaptic currents and the amplitude of evoked synaptic responses (Lohof et al., 1993). Similar effects were observed for glutamatergic synapses in acute hippocampal slices (Kang and Schuman, 1995). Blocking TrkB activation with the tyrosine kinase inhibitor K252-a prevents BDNF-mediated potentiation of excitatory postsynaptic potentials (EPSPs). Furthermore, BNDF-mediated induction and maintenance of hippocampal long-term potentiation (LTP) has been extensively investigated (Korte et al., 1995; Korte et al., 1996; Patterson et al., 1996; Kossel et al., 2001; Kovalchuk et al., 2002): in BDNF knock-out mice (-/-), a marked reduction in LTP and long-lasting LTP has been observed. Interestingly, LTP could almost completely be restored by applying exogenous BDNF to slices or by using Adenoviral vectors to re-express BDNF in slices of the knock-out animals (Korte et al., 1995; Korte et al., 1996; Patterson et al., 1996). Conditional TrkB knock-out mice which lost TrkB receptors only in the forebrain postnatally revealed strongly reduced LTP in correlation with impaired learning (Minichiello, 1999), confirming that BDNF action on synaptic plasticity is indeed meditated by TrkB activation. Moreover, BDNF is involved in the stabilization of synapses (Hu et al., 2005) and in potsynaptic maturation of NMDAR-only synapses into AMPAR transmissible ones (Itami et al., 2003). Since many forms of activity-dependent plasticity occur in a synapse-specific local manner, BDNF must be able to act locally to translate the effect of neuronal activity into synapse-specific structural and functional changes. Indeed, BDNF influences synaptic plasticity not only at a cellular but also at a subcellular level. For instance, apical and basal dendrites of the same pyramidal cells respond differently to the same neurotrophin (McAllister et al., 1995). Furthermore, BDNF release from a single ‘BDNF overexpressing donor neuron‘ has been shown to act as a locally restricted signal that can directly induce local dendritic growth and branching in nearby neurons in a distance-dependent manner within relatively short time (Horch and Katz, 2002). The morphological changes in dendrites including filopodia upon BDNF signaling are due to activation of intracellular signaling pathways which finally regulate the actin cytoskeleton: Cyclic AMPs (cAMP), small G proteins of the Cdc-42/Rac/Rho family and

the MAP kinase pathway have been reported to control the polymerization and turnover of F-actin and thereby can induce rapid cytoskeletal rearrangements (Bishop and Hall, 2000). In addition, it has been reported that local application of BDNF and simultaneous presynaptic activation mediate LTP in mature dentate granule cells (Kovalchuk et al., 2002). The expression and secretion of BDNF is known to be regulated by neuronal activity (Gaertner and Staiger, 2002; Balkowiec and Katz, 2002), and BDNF release occurs at synapses, possibly at pre- or postsynaptic compartments or both (Hartmann et al., 2001; Kohara et al., 2001; Gaertner et al., 2006; Lessmann et al., 2003). The action of BDNF upon secretion is spatially restricted because BDNF is a 'sticky' molecule with limited diffusion capacity and truncated TrkB receptors eventually also limit BDNF diffusion (Biffo et al., 1995). These findings together suggest that BDNF signaling is precisely regulated in space and time.

What are the mediators of acute BDNF signaling? Calcium as an important second messenger acts downstream of BDNF and is thought to mediate some of its effects in a specific, fast and localized manner. For example, it has been shown that intracellular calcium signaling conveys the potentiating effect of BDNF at hippocampal synapses (Kang and Schuman, 2000; Kovalchuk et al., 2002). Moreover, the application of exogenous BDNF is known to induce a transient elevation in the intracellular calcium concentration, predominantly due to calcium release from internal stores (Berninger et al., 1993), but also dependent on calcium influx from the extracellular space (Marsh and Palfrey, 1996). In conclusion, BDNF is responsible for many fast and slow signaling processes in neurons which regulate basic cellular phenomena such as survival, but also synaptic transmission and plasticity. How the temporally and spatially precise action of BDNF contributes to the diverse signaling pathways which regulate neuronal function remains partly elusive.

3.5 Objective of this study

Although there is a substantial amount of knowledge about the cellular responses to exogenous BDNF, it has not yet been studied how endogenous BDNF affects neuronal calcium responses. What are the characteristics of BDNF signaling in terms of time and space during development?
The work presented in this thesis aims to explore the role of the neurotrophic factor BDNF in intracellular calcium signaling in developing pyramidal neurons

of hippocampal slice cultures from postnatal rats. To this end, I established the method of single cell electroporation in the laboratory. This technique enabled me to observe calcium dynamics in dendrites of individual CA3 pyramidal neurons. I focused on CA3 neurons mainly for two reasons: the first reason is of technical nature - CA3 pyramidal neurons have larger somata than CA1 neurons and are therefore easier to electroporate. The second reason why to focus on CA3 neurons is because the mossy fiber pathway projecting from the granule cells to the CA3 pyramidal cells contains the highest concentration of endogenous BDNF in the CNS (Conner et al., 1997; Yan et al., 1997; Danzer and McNamara, 2004). I asked whether spontaneous (not experimentally evoked) local calcium transients are triggered by BDNF and investigated the mechanisms underlying BDNF-mediated calcium increases.
To further specify the local action of endogenous BDNF in calcium signaling, a virus system for expressing PSD-95 tagged to CFP in neurons was generated to visualize postsynaptic sites along the dendrites. This allows to investigate whether BDNF triggered calcium signaling occurs at synapses.
Another approach was to investigate the acute role of BDNF in the motility and growth of dendritic filopodia which are known to be highly motile in a calcium-dependent fashion. To resolve a possible BDNF-mediated change in filopodia motility, I used high resolution confocal time-lapse imaging.
Finally, I explored whether BDNF induces functional plasticity in developing dendrites using long-term calcium imaging.

4 Materials and Methods

4.1 Materials

Chemicals and media were purchased from the companies Merck, Sigma, Gibco / Invitrogen / Molecular Probes. Destillated water from Millipore was used to dilute solutions. Restriction enzymes and respective buffers were purchased from Fermentas or New England Biolabs. Special kits are mentioned in the respective method section.

4.1.1 Media and chemicals

Chemical	**Supplier**
BME (basal medium) + Earle's, - L-glutamin	Gibco
DMEM	Gibco
OptiMEM	Gibco
fetal bovine serum	Gibco
horse serum	Gibco
Trypsine/EDTA	Sigma
HBSS (Hanks balanced salt solution 10x) + $MgCl_2$, + $CaCl_2$	Gibco
Glucose ($C_6H_12O_6$)	Merck
Glutamine	Invirtogen
Kynurenic acid	Sigma
Sodiumhydrogencarbonate ($NaHCO_3$)	Merck
Calciumchloride ($CaCl_2$)	Merck
Dimethyl sulfoxide (DMSO)	Sigma
6-Hydroxy-2,5,7,8 tetramethylchroman-2-carbon acid 97% (Trolox)	Sigma

Chemical	Supplier
Agarose	Invitrogen
Bovine albumin powder	Sigma
Lipofectamine 2000	Sigma
α-chemotrypsin	Sigma
Aprotinin	Sigma
Ethidiumbromid	Sigma
DNA-ladder (Ready-load 1kb plus)	Invitrogen
6x loading buffer	Invitrogen
Protease inhibitor cocktail	Sigma
ECL Western blot detection reagent	Amersham Biosciences
Gel/Mount	Biomeda
TEMED	Sigma
Triton X-100	Serva
Tween 20 (Polyoxyethylene sorbitan monolaurate)	Bio-Rad

4.1.2 Equipment

Equipment	Supplier
Puller PC-10	Narishige
Table Centrifuge 5415C	Eppendorf
Centrifuge S2 Omnifuge 2.0 RS	Heraeus
Rotor GSA rotor / type 3	Sorvall
Sonicator	Bransonic
pH meter CG 825	Schott
Electrophoresis power supply E835	Consort
Incubator Shaker Innova 4000	New Brunswick Scientific
CO_2 Incubator	Heraeus
Ultraviolet/visible Spectrophotometer 2100 pro	Ultrospec
Semidry blotting apparatus	Bio-Rad
Bio-Rad Gel System	Bio-Rad
Gene pulser apparatus	Bio-Rad
Epifluorescence microscope Axioplan	Zeiss
Digital Camera VisiCam QE	Visitron Systems
Confocal microscope SP2	Leica
Dissection stereomicroscope	Zeiss
Dissection lamp	Leica
Nanoliter 2000 Injector	World Precision Instruments

4.1.3 Media, buffers and solutions

GBSS	2.5 mM $CaCl_2$ *2 H_2O, 4.96 mM KCl, 0.22 mM KH_2PO_4, 1.03 mM $MgCl_2$ *6 H_2O, 0.28 mM $MgSO_4$ *7 H_2O, 136.89 mM NaCl, 2.7 mM $NaHCO_3$, 0.87 mM Na_2HPO_4, 5.05 mM D-glucose, 1 mM kynurenic acid, pH 7.2, sterile filtered
Culture medium	50% (v/v) BME, 25% (v/v) horse serum, 25% (v/v) HBSS, 1 mM L-glutamin, 5 mg/ml D-glucose, sterile filtered
Perfusion solution	HBSS consisting of 14.61 mM $CaCl_2$, 4.93 mM $MgCl_2$*6 H_2O, 4.07 mM $MgSO_4$*7 H_2O, 53.33 mM KCl, 4.41 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1379.31 mM NaCl, 3.36 mM Na_2HPO_4*7 H_2O, 55.56 mM D-glucose and 0.5 M Trolox. Use within 1 day.
PBS	137 mM NaCl, 2,7 mM KCl, 4.3 mM Na_2HPO_4, 1,4 mM KH_2PO_4, pH 7.4
TBS 10x	0.5 M Tris-HCl, 1.5 M NaCl, pH 7.4
Gradient buffer	0.32 M saccharose, 1 mM $MgCl_2$, pH 7.4
2x sample buffer	0.125 M Tris pH 6.8, 2% SDS, 20% glycerol, 0.001% bromphenolblue
Resolving gel 6%	6.0 ml 30% acrylamide mix, 7.5 ml Tris pH 8.8, 0.3 ml 10% SDS, 0.3 ml 10% APS, 24 μl TEMED, 15.9 ml H_2O
Stacking gel 5%	4.0 ml 30% acrylamide mix, 5.0 ml 0.5 M Tris pH 6.8, 0.2 ml 10% SDS, 200 μl APS, 20 μl TEMED, 10.6 ml H_2O

Running buffer 10x	250 mM Tris, 2.5 M glycin, 1% SDS, pH 8.3
Aminocaproic acid buffer	15.2 g Amino-n-caproic acid, 200 ml methanol
Low Tris	3 g Tris-base, 200 ml methanol
High Tris	36 g Tris-base, 200 ml methanol
Blocking buffer	0.5% gelatine, 0.05 % TWEEN, 5 mM EDTA, 100 ml 10x TBS
Paraformaldehyde 4%	20 g PFA (w/v), 500 ml PBS, 200 μl 5N NaOH, 75 μl HCl (min. 37%)
TAE	10mM Natriumazetat, 1mM Na_2EDTA, 40mM Tris/HCl, pH 8.0
LB–Medium	10g/l Bacto–Tryptone, 5g/l Bacto-Yeast extrakt, 10g/l NaCl, pH 7,2, sterilize by autoclaving, store at RT
LB/Amp–Medium	LB–Medium with 150μg/l Ampicillin
LB–Agar	LB–Medium with 15g/l Agar
LB/Amp–Agar	LB–Agar with 150μg/l Ampicillin

4.1.4 Bacterial strain

The following Escheria coli strain with the relevant genotype was used:

DH5α	$F^-, \varphi 80$ dlacZΔM15, Δ(lacZYA-argF) U169, recA1, endA1, hsdR17, (r_{K^-}, m_{K^+}), supE44, λ^-, thi$^-$1, gyrA, relA1

4.1.5 Vectors

Plasmid	Size (kB)	Resistance	Supplier
pCi-Neo PSD-95:GFP	8.3 kB	AmpR and NeoR	Promega
pECFP N21 PSD-95	5.5 kB	KanR	A.-M. Craig
pSCA	1.1 kB	AmpR	R. Bremner
Helper plasmid			R. Bremner

4.1.6 Dyes

Dye	Concentration	Supplier
Oregon Green 488 BAPTA-1 hexapotassium salt	250 μM (in dH_2O)	Molecular Probes
Oregon Green 488 BAPTA-1 AM cell permeant	50 μg (one vial per experiment)	Molecular Probes
Alexa Fluor 594 Dextran anionic, fixable	0.1 - 0.5 mM (in dH_2O)	Molecular Probes

4.1.7 Pharmacological reagents

Drug	Action	Concentration	Supplier
BDNF	TrkB receptor agonist	200 ng/ml	provided by H. Thoenen
K-252a	tyrosine kinase antagonist	200 nM	Alexis Biochemicals
αBDNF	antibody against BDNF (clone no.9)	4 μg/ml	provided by S.Cambridge
Tetrodo toxin (TTX)	sodium channel blocker	1 μM	Sigma
Cadmium chloride ($CdCl_2$)	calcium channel blocker	5 μM	Sigma
Cyclopiazonic acid (CPA)	Ca^{2+}/ATPases inhibitor	20 μM	Biotrend
SKF 96365 hydrochloride (SKF)	transient receptor potential blocker	3 μM	Tocris
Tetanus toxin (TeTX)	inhibitor of presynaptic transmitter release	20 nM	Sigma

4.1.8 Primary and secondary antibodies

Antibody	**Recognized epitope**	**Concentration**	**Supplier**
αTrkB rabbit polyclonal AB	AA 160-340 of TrkB of origin	1 : 50	Santa Cruz Boitechnology
αactin rabbit polyclonal AB	C-terminal actin fragment	1 : 100	Sigma
αsynapsin rabbit polyclonal AB	Synapsin I (Ia and Ib)	1 : 500	Chemicon
αrabbit AB peroxidase labeled		1 : 10.000	Amersham Biosciences
αrabbit AB FITC labeled		1 : 500	Chemicon

4.2 Methods

4.2.1 Preparation of hippocampal slice culture

Hippocampal organotypic cultures from postnatal day 0-2 (P 0-2) Wistar rats (both sexes) were prepared following the method of Stoppini et al. (Stoppini et al., 1991). After decapitation, the hippocampi from both hemispheres were dissected in ice-cold Gey´s balanced salt solution, containing 0.5 ml kynurenic acid and 0.5 ml of glucose (50%), pH 7.2. Transversal slices were cut (400 μm) using a tissue chopper (Mc Ilwain). Slices were placed back into preparation medium and separated with fine forceps. The sections were stored in the refrigerator for 30 min to allow regeneration and removal of debris. Then, the slices were plated onto Millicell membrane inserts (CM, Millipore) and incubated with BME medium containing 25% horse serum at 37^{o}C, 7% CO_2 for up to 3 days.

4.2.2 Dye loading of developing CA3 pyramidal neurons by single cell electroporation

The recording chamber was temperature controlled at 35^{o}C and perfused with HBSS. The high-affinity calcium indicator Oregon Green BAPTA-1 (250 μM) was introduced into single neurons according to the method for dye electroporation (Haas et al., 2001; Rathenberg et al., 2003). For single cell electroporation, glass electrodes (similar to patch pipettes) with a tip diameter of $\sim$1 μm were pulled. The tip of the electrode was filled with the dye solution ($\sim$10 μl) and the electrode was placed in a holder that contains a silver wire which is connected to a voltage generator, which is in turn controlled by a pulse generator (Fig. 4.1A). The other pole is connected to the medium in the chamber to build a circuitry. The polarity depends on the charge of the dye. Slight air pressure ($\sim$5 mbar) can be applied to the pipette by using a syringe which is connected to the pipette holder by a three-way valve and a tube; this prevents clogging of the tip of the pipette. The discharge of the dye out of the pipette should be checked by positioning the pipette in the perfusion solution under the fluorescence microscope: a visible amount of dye must be released by a single square voltage pulse (10 V, 20 ms duration, Fig. 4.1A). Then, the tip of the pipette is placed close to the cell surface of a CA3 pyramidal neuron under visual control with transmitted light. The cell surface has to be approached from the top until a small dent in the cell membrane becomes visible (Fig. 4.1B). One to three voltage pulses to the pipette are sufficient to fill an individual neuron with the dye. Many neurons can be loaded with one pipette.

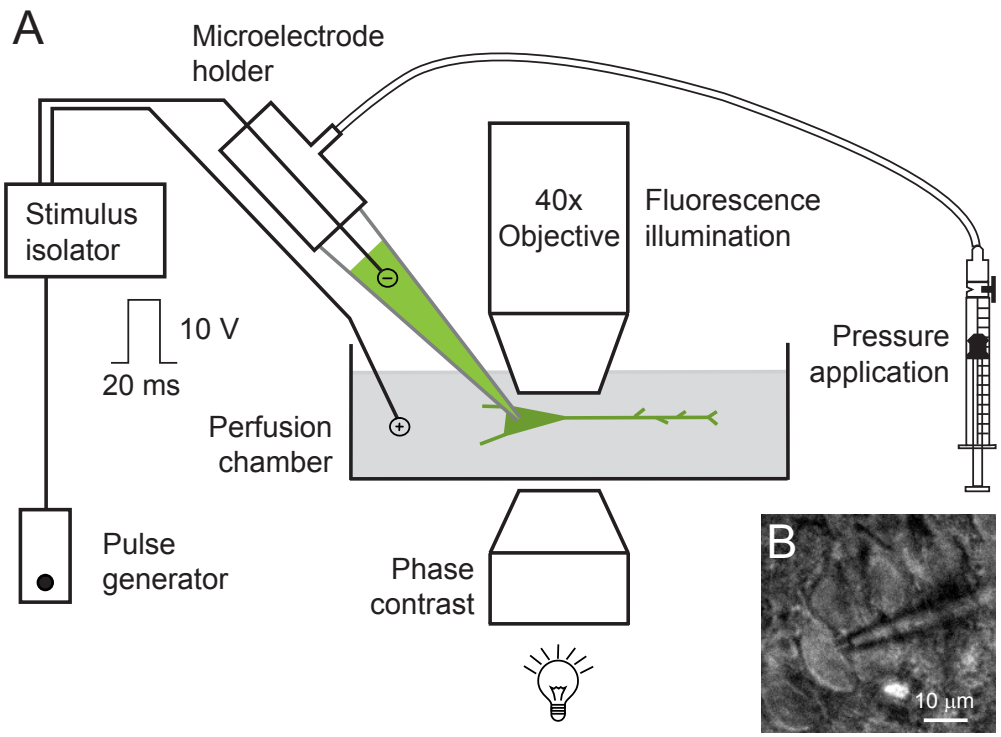

Figure 4.1: Setup for single cell electroporation. **A**, Experimental setup used for dye electroporation of individual neurons of hippocampal slices. One pulse of 10 V and 20 msec duration is sufficient to fill a neuron with the dye of interest. **B**, Under transmitted light, a slight dent in the cell membrane of the neuron is visible at the contact site with the tip of the pipette.

4.2.3 Bolus loading of the CA3 region

For bolus loading (Stosiek et al., 2003), 50 μg (one vial) of Oregon Green 488 BAPTA-1 AM was dissolved in 5 μl DMSO containing 10% of Pluronic acid. After sonicating for 1 min, 50 μl of culture medium were added and the solution was again sonicated. The tip of a patch pipette was filled with the dye solution and the CA3 region of hippocampal slices was loaded by positioning the pipette tip in the stratum radiatum / stratum lacunosum-moleculare and applying short pressure pulses with a picospritzer. After a one-hour incubation, slices were used for experiments.

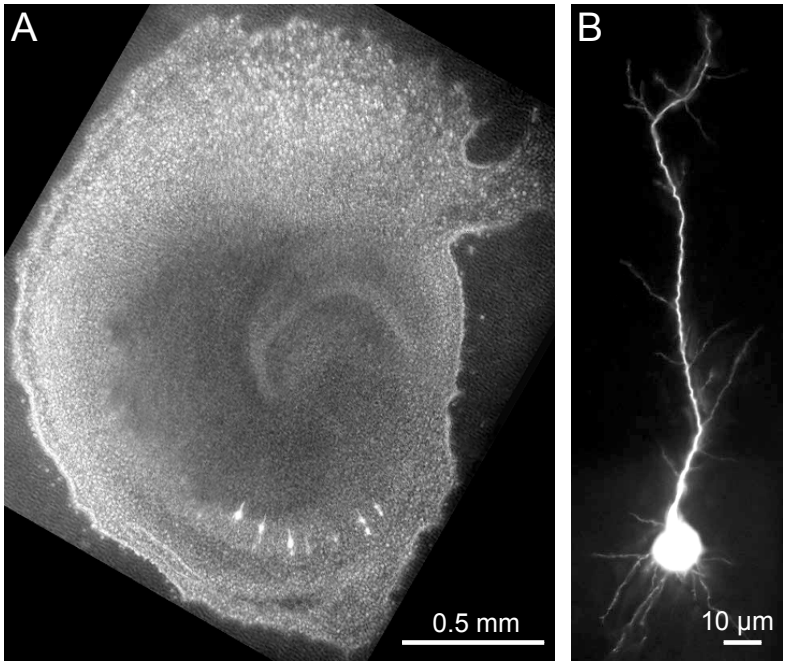

Figure 4.2: Single cell electroporation. **A**, Hippocampal slice praparation in which few CA3 pyramidal cells have been loaded with Oregon Green BAPTA-1 by single cell electroporation. **B**, Electroporated single pyramidal neuron. Even tiny processes are filled with the dye.

4.2.4 Imaging

Recordings were acquired using an Axioplan-2 microscope (Zeiss) and a cooled CCD camera (VisiCam QE, Visitron Systems) controlled with Metamorph software (Universal Imaging). Images were obtained at 0.3-10 Hz with either a Zeiss 63x - 0.95 NA or a Olympus 40x - 0.80 NA water immersion objective. For imaging filopodial motility, an upright Leica SP2 confocal microscope with a 63x - 0.9 NA water immersion objective was used and high-resolution images from dendrites with filopodia were acquired at 0.3 Hz for 5 min. Due to the generation of toxic radicals, fluorescence imaging can generally be deleterious to living neurons. Therefore, as little excitation light as possible was used, the illumination was restricted to an area of interest, mainly the dendrites, and illumination of the somata of neurons was avoided, because it is particularly damaging. For immunohistochemical fixed preparations, an inverted Leica SP2 confocal microscope with a 40x - 1.25 NA oil objective was used.

4.2.5 Application of BDNF and pharmacological agents

The BDNF used in this thesis has been provided by Hans Thoenen and synthesized in its mature, renaturated form in Escherichia coli by the biotech-company Regeneron. BDNF (26 μl, 15 μg/ml in PBS, containing 0.1% BSA to prevent binding to the storage and application ware) was bath-applied using a 100 μl pipette (final concentration 200 ng/ml). Superfusion was interrupted during BDNF bath application; control experiments showed that interruption of superfusion alone did not affect calcium activity for the recording period (not shown). Alternatively, BDNF was applied focally to the apical dendrites with pressure pulses through a micropipette using a picospritzer (pulse duration 40 ms, 1-5 mbar pressure). The BDNF application pipette ($\sim$1 μm tip diameter) was placed at a distance of roughly 10-20 μm from the dendrite and 3 pulses were applied within 1 min. In control experiments, either PBS with 0.1% BSA or heat inactivated BDNF (30 min at 95^{o}C) was pressure applied to the dendrite. The protein kinase antagonist K-252a (200 nM) and the function-blocking mouse monoclonal antibody against BDNF (αBDNF) were bath applied (4μg/ml, clone no.9, IgG), (Kolbeck et al., 1999; Kossel et al., 2001). For αBDNF experiments, tubing was siliconized for 1h, dried, and extensively washed to prevent adhesion of the antibody to tubing surfaces. Additionally, BSA (0.5 mg/ml) was added to the antibody solution. All other pharmacological agents were diluted in HBSS and bath applied: 1 μM tetrodotoxin (TTX), 5 μM cadmium chloride (CdCl2), 20 μM Cyclopiazonic acid (CPA), 3 μM SKF 96365 hydrochloride. 20 nM tetanus toxin (TeTX) was added to the culture medium at least 12 h before the recordings.

4.2.6 Image analysis

Changes in the intracellular [Ca^{2+}] concentration are represented as $\Delta F/F_0$ in percent, where F_0 is the baseline fluorescence, which is typically taken from the first 20 images of a recording ($\Delta F/F_0 = (F - F_0) / F_0$). Global calcium transients were defined as fluorescence increases of at least 10% observed in all structures of the cell within the same focal plane. Local calcium transients were defined as local rises in fluorescence of more than 5% with a spatial extent of 1-30 μm along the dendrite. For the generation of pseudo line scans, image stacks were imported into ImageJ and a ΔF stack was calculated using the respective plugin. There, changes in the intracellular calcium concentration were easily detectable by changes in the fluorescence intensity. A dendrite revealing local calcium transients was choosen and a line was drawn along this dendrite in the original

stack. The plugin 'reslice' was used to generate a pseudo line scan in which the y-axis corresponds to the pixels in the drawn line along the dendrite and the x-axis corresponds to time. Using a Matlab Progam written by Thomas Kleindienst, the pseudo line scans were converted in ΔF-images and local calcium transients were automatically recognized, measured and counted. The major advantages of this approach were the objectiviy and speed of the analysis. For a more comprehensive description of the Matlab Program used please see Kleindienst, 2005 (Diploma thesis).
For analysis of the BDNF application experiments changes caused by dendrite shifts due to the pressure pulse application were discarded.
For statistical analysis, Wilcoxon matched pairs test was used.
For analysis of filopodia motility and growth, 10 high-resolution images from the red channel (spanning 30 s) were averaged to improve the signal to noise ratio. The length of each filopodium was measured at 10 time points of a 5 min recording at 0.3 Hz before, during and after BDNF application respectively using ImageJ.
For comparing the exact positions of local calcium transients along the dendrite before and after focal BDNF application, the observed dendritic stretches were divided into groups of 10 pixels (approximately 7 μm), which turned out to be an adequate range with respect to slight shifting of the dendrite over the recording time and the average distance between synapses at this age ($\sim$16 μm). The frequency of local calcium transients / min / fragment was calculated for each 10 pixel fragment.

4.2.7 Propagation of human embryonic kidney 293 cells

Human embryonic kidney 293 cells (HEK 293 cells) were used for the transfection of the pSCA PSD-95:CFP plasmid and a helper plasmid to generate Semliki Forest Viruses expressing PSD-95:CFP. Moreover they were used as a control for Western blot analysis. Frozen HEK 293 cells were quickly thawed in DMEM-FBS medium, pelleted and resuspended in 10 ml DMEM-FBS medium. Cells were grown at 37^{o}C with 5% CO_2. Every two to three days, plates with HEK293 cells were washed with PBS and treated with Trypsine/EDTA until cells deattached. Cells were seeded at a 1:4 to 1:8 ratio. They were frozen in 8% DMSO / 92% FBS, stored temporarilly on ice and then transferred to -80^{o}C.

4.2.8 Generation of pSCA PSD-95:CFP plasmid

To generate the pCiNeo PSD-95:CFP construct (Graf et al., 2004) the pCiNeo PSD-95:GFP plasmid was digested with the restriction enzymes XhoI / NotI to obtain the pCiNeo vector (5440 bp) and with the restriction enzymes XhoI / BamHI to obtain the PSD-95 5´fragment (1677 bp). A single restriction digest of the pECFP-N1 PSD-95 plasmid with the restriction enzymes BamHI / NotI leaded to the PSD-95 3'CFP fragment (1229 bp). For this purpose, $\sim$4 μg of DNA was cut in the appropriate units of restriction enzymes and buffer according to the manufacturers protocol for 1-2 h. Fragments were separated on a 1% agarose gel and the three respective DNA bands were excised from the agarose gel with a scalpel and purified using a QIAquick gel extraction kit. The purified vector and the two DNA fragments ('inserts') were ligated in a ratio 1 : 3 : 3 over night using T4-DNA-ligase. The ligation product (pCiNeo PSD-95:CFP) was used to transform competent bacteria (DH5α): electro competent bacteria and 1 μl of the ligation product were mixed on ice and transferred into a cold electroporation cuvette in a Gene pulser apparatus (Bio-Rad). After one pulse at 2.5 kV, bacteria were resuspended in LB medium, incubated at 37^{o}C and then plated on AmpR-LB agar plates and incubated over night. A single bacteria colony was picked from the LB agar plate to inoculate 3ml LB medium. After over night culture the plasmid DNA was purified using Plasmid Mini Purification (Quiagen) and checked by restriction analysis. After re-transformation of the positive clone into competent bacteria, transformed bacteria grew over night in LB medium containing ampicillin in an Erlenmeyer flask at 37^{o}C. The bacterial suspension was pelleted and Midi Preparation of the plasmid DNA was carried out using the Quiagen protocol. A DNA concentration of 0.5 μg/μl was measured in an UV spectrometer at 260 nm.

Subsequently, to produce Semliki Forest Viruses containing PSD-95:CFP, the pCiNeo PSD-95:CFP plasmid was subcloned into the pSCA vector (DiCiommo and Bremner, 1998). The pCiNeo PSD-95:CFP construct was digested using XhoI /NotI to obtain the PSD-95:CPF fragment (2906 bp) and the pSCA plasmid was cut at the multiple cloning site using the restriction enzymes XhoI /NotI (11527 bp). The resulting fragments were again separated on a 1% agarose gel and the respective DNA bands were excised from the agarose gel and purified using a QIAquick gel extraction kit. The purified pSCA vector and the PSD-95:CPF fragment were ligated in a ratio 1 : 3 over night. The pSCA PSD-95:CFP vector was checked by restriction analysis using EcoRI restriction enzyme. Then, using Lipofectamine 2000 HEK293 cells were transiently trans-

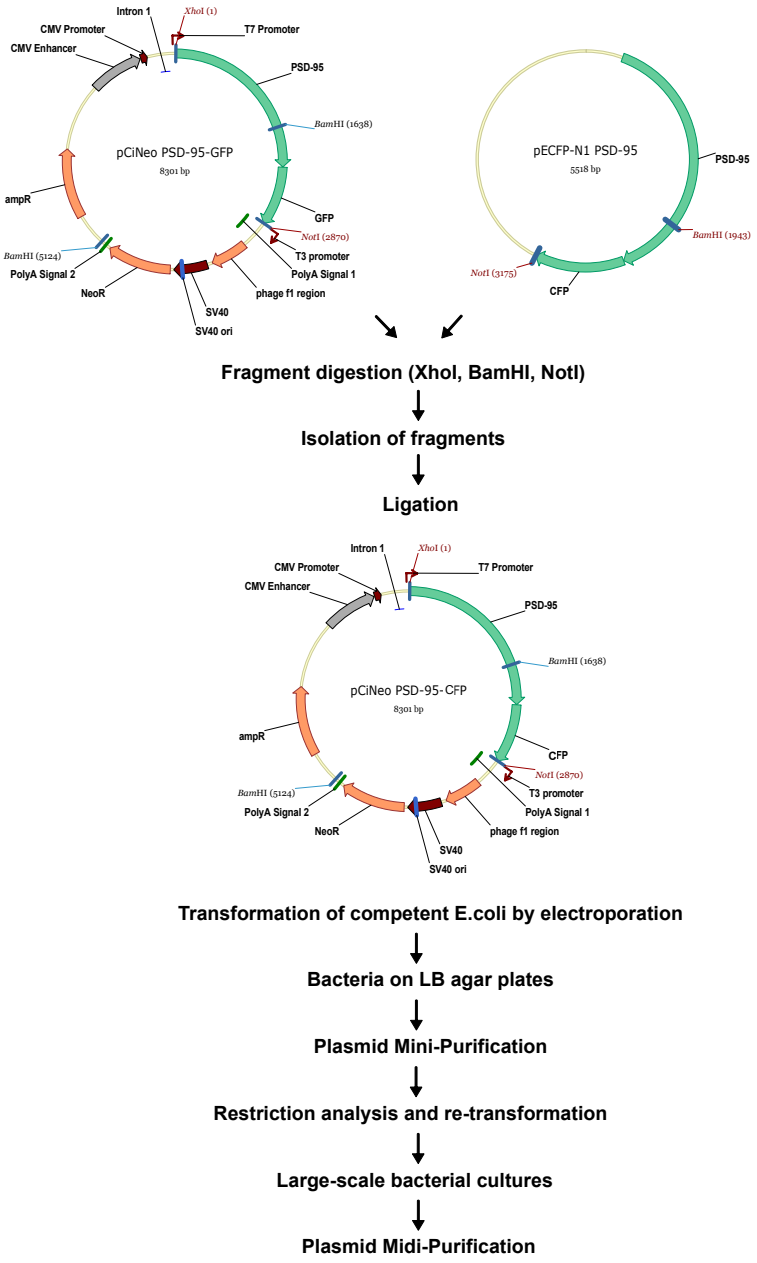

Figure 4.3: Generation of pCiNeo PSD-95:CFP plasmid. The pCiNeo PSD-95:CFP plasmid was generated from the pCiNeo PSD-95:GFP vector and the pECFP-N1 PSD-95 plasmid.

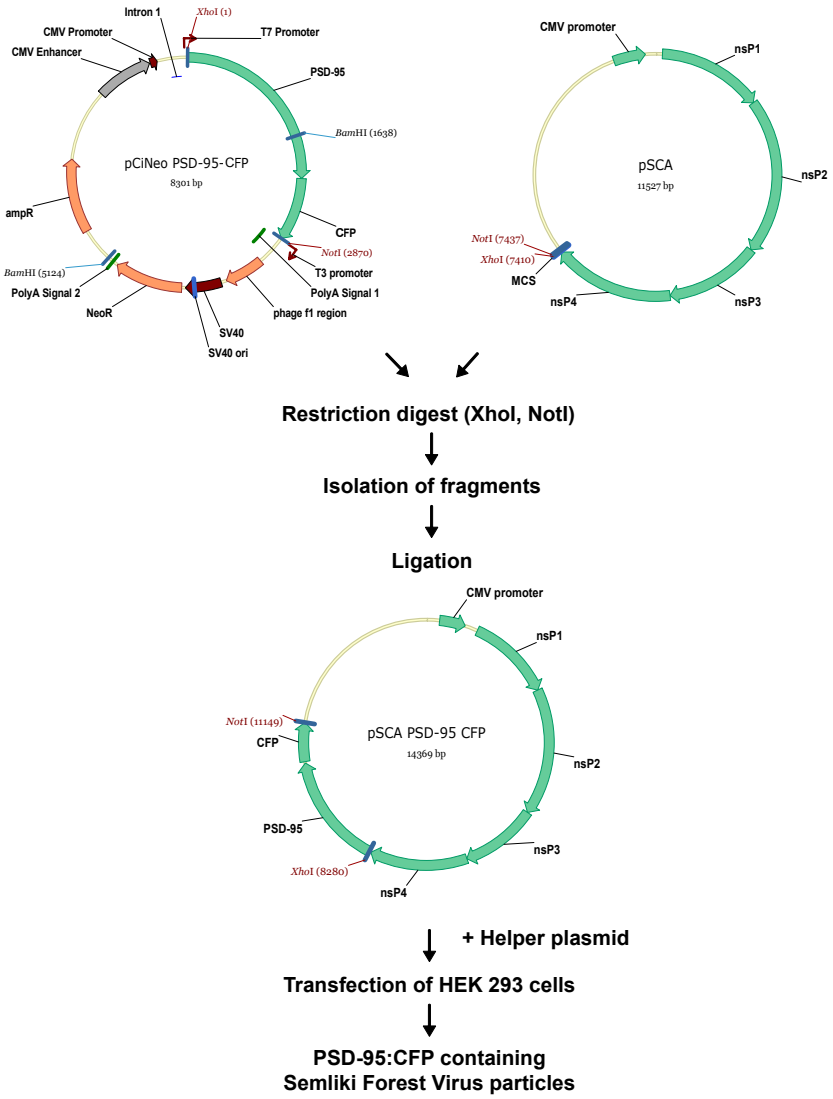

Figure 4.4: Generation of pSCA PSD-95:CFP plasmid. The pCiNeo PSD-95:CFP plasmid was subcloned into the pSCA vector. Semliki Forest Viruses were produced by transfection of pSCA PSD-95:CFP plasmid and Helper plasmid into HEK293 cells.

fected with the pSCA PSD-95:CFP vector and a helper plasmid (ratio 1 : 2), which encodes the packaging proteins of the virus. 48h after transfection the supernatant was harvested, aliquoted and stored at -80°C. The viruses were activated by α-chymotrypsin treatment (1/20, 10 mg/ml in PBS) for 45 min at

RT. The reaction was stopped with 1/15 Aprotinin (10 mg/ml in PBS) and the viruses were injected into the CA3 region using a Nanoliter 2000 Injector. Semliki Forest Virus (SFV) vector system was chosen because it is well studied and established (DiCiommo and Bremner, 1998), neuron-specific and fast expressing.

4.2.9 Western blot

Hippocampal tissue from P3 rats was homogenized in gradient buffer (containing 0.32 M saccharose, 1 mM $MgCl_2$, pH7.4), 2x sample buffer (0.125 M Tris pH 6.8, 2% SDS, 20% Glycerol, 0.001% bromphenolblue), DDT and a protease inhibitor cocktail. HEK 293 cells served as a control. Aliquots of the lysates were then subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE, 6% acrylamide) and transferred to nitrocellulose membranes (transfer membranes, Millipore). The blots were incubated in blocking buffer of 0.05% Tween-20 and 0.5% gelatin in 10x Tris-buffered saline at 4^oC over night. After blocking, the nitrocellulose membrane was incubated either with a rabbit anti-TrkB antibody or with a rabbit anti-actin for 1h at RT. After incubation with the secondary antibody (1h, RT, 1:10.000, peroxidase labeled anti-rabbit antibody), binding of the antibody was detected using an ECL detection kit.

4.2.10 Immunohistochemistry

To test for the presence of TrkB receptors in neonatal rat hippocampus slices and to label presynaptic partners of PSD-95:CFP puncta overexpressed in neurons after viral infection, post-hoc immunohistochemical stainings were performed. Slices were fixed at 4^oC overnight in paraformaldehyde (4% in 0.1 sodium phosphate buffer). After washing for 3h in phosphate buffer (PB), slices were preincubated in a blocking solution (4% Triton X-100, 1.5% Horse Serum, 0.1% BSA in PB, 4^oC, overnight) and then incubated in the primary antibody (rabbit anti-TrkB, Santa Cruz, diluted 1:50 or rabbit anti-Synapsin, Chemicon, diluted 1:500 in 4% Triton X-100, 1.5% Horse Serum, 0.1% BSA in PB) for 7-10 days. Slices were rinsed (3h in PB) and then incubated for 2-3 days with the secondary antibody (anti- rabbit-FITC, 1:50 in PB, 4^oC). Stacks of high-resolution images (voxel size xyz: 0.18 x 0.18 x 0.25 μm) of CA3 neurons electroporated with a fixable red dye and anti-TrkB labeled structures or of PSD-95:CFP infected dendrites and anti-Synapsin labeled structures were obtained with a confocal microscope (Leica SP2, sequential acquisition mode, 1.25/40x oil immersion objective). Quantification of colocalisation of PSD-95:CFP and synapsin was per-

formed using ImageJ software. Puncta were considered colocalized when pixels of PSD-95:CFP puncta (‘red channel‘) and synapsin puncta (‘green‘ channel,) overlapped in high magnification x-y and orthogonal (y-z) views (‘yellow‘ pixels). The specificity of colocalisation was tested using the reverse order of the synapsin stack rotated by 180^o.

5 Results

5.1 Endogenous BDNF induces fast calcium rises

5.1.1 Endogenous BDNF induces spontaneous local calcium transients

In my study, I focused on the first postnatal week when dendrites and axons undergo dynamic structural remodeling and form new synapses in vivo and in organotypic slice cultures (Dailey and Smith, 1996; Fiala et al., 1998). In this early phase of development, spontaneous local rises of intracellular [Ca^{2+}] concentration could be observed in CA3 pyramidal neurons, which were labeled with a [Ca^{2+}] indicator by single cell electroporation (Fig.5.1). These local cal-

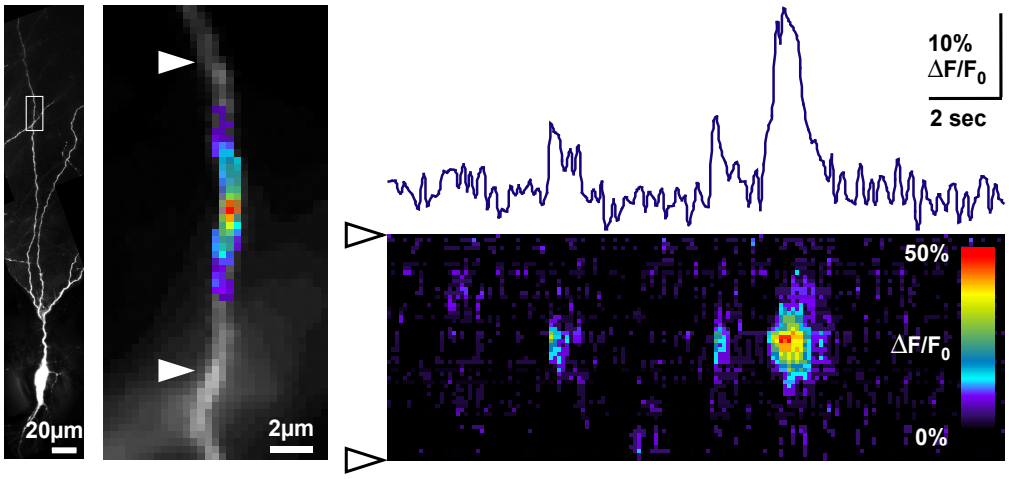

Figure 5.1: Spontaneously occurring local calcium transients in dendrites of developing hippocampal neurons. An electroporated single CA3 pyramidal neuron (P 2 + DIV 3) filled with Oregon Green BAPTA-1 (OGB-1). Enlarged part of the dendrite shows spontaneous local increases in the intracellular [Ca^{2+}] concentration, represented as $\Delta F/F_0$ in pseudocolor. Arrowheads indicate the extent of the measured dendritic segment used for the pseudo line scan and the trace.

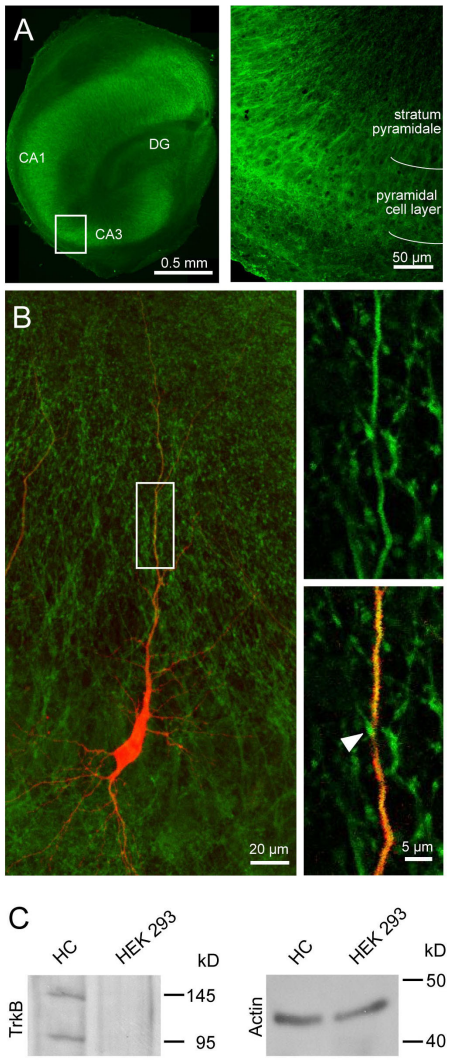

Figure 5.2: Expression of TrkB receptors in developing hippocampal CA3 pyramidal cells. **A**, Hippocampal slice culture in low magnification (left) and part of the CA3 region in higher magnification (right) shows a homogeneous TrkB antibody staining along the dendrites. **B**, Confocal reconstruction of a CA3 pyramidal neuron (red) and TrkB antibody staining (green). Enlarged region with and without the red channel reveals homogeneous TrkB expression in the apical dendrite. Also structures such as presynaptic terminals show TrkB expression (arrowhead). **C**, Western blot analysis of TrkB receptor and actin in hippocampal lysates from developing rats (P 2). Both full-length TrkB and truncated TrkB (molecular weights 145 and 95 kDa, respectively) is present in the hippocampus, but absent in the control (lysate of HEK 293 cells). Actin served as loading control.

cium transients occurred at a mean frequency of 73.8 ± 10.8 per min and mm dendrite. The average duration of such calcium rises was 680 ± 120 msec and they were restricted to small stretches of 9.0 ± 0.6 μm of the dendrite. The amplitude of the transients (measured as $\Delta F/F_0$) was 17.3 ± 0.6 % and their rise time was 0-4 frames of 100 ms (median 2 frames). Besides local calcium transients, hippocampal neurons also generated global calcium transients occurring in the soma, dendrite and axon, presumably representing network activity (Garaschuk et al., 1998).

The aim of this study was to investigate the role of endogenous BDNF for spontaneous local calcium signaling. Many studies have shown that BDNF signaling is mediated by TrkB receptors (Berninger and Poo, 1996; McAllister et al., 1999; Kafitz et al., 1999; Rutherford et al., 1998) and that BDNF is expressed during early development in the hippocampus with increasing concentrations from P0 - P120 (Katoh-Semba et al., 1997). Before interfering with the BDNF-TrkB signaling pathway, the presence of TrkB, the principal receptor for BDNF, in the developing hippocampus was tested by immunohistochemical antibody staining against TrkB and by Western blot analysis (Fig. 5.2). In the immunohistochemical approach, an homogeneous TrkB antibody staining along the dendrites of pyramidal neurons could be observed. Also presynaptic terminals showed TrkB expression (Fig. 5.2B). Since there were concerns about the specificity of the used TrkB antibody (Santa Cruz) for immunohistochem-

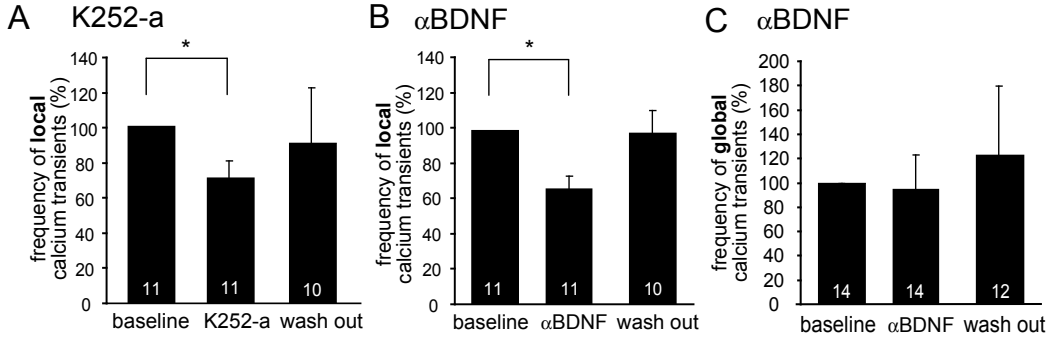

Figure 5.3: **A**, Frequency of spontaneous local calcium transients before, during, and after bath application of K252-a normalized to baseline frequency. Calcium activity is significantly reduced during K252-a application (n = 11 neurons; error bars s.e.m., p $\leq$ 0.05). **B**, Similarly, bath application of αBDNF causes a significant and reversible decrease in the frequency of spontaneous local calcium transients (n = 11 neurons, p $\leq$ 0.05). **C**, The frequency of spontaneous global calcium transients is unchanged during BDNF antibody application.

istry and since TrkB knockout mice for controls were not available, Western blot analysis was performed additionally. Both full-length TrkB and truncated TrkB (molecular weights 145 and 95 kDa, respectively) were present in the hippocampus, but absent in the control (lysate of HEK 293 cells, Fig. 5.2C). After having confirmed the presence of TrkB receptors in the developing hippocampus, I focused on the TrkB-pathway. Endogenous BDNF-TrkB-signaling was interrupted and the effect on local calcium signaling was tested. I used either K252-a, which blocks tyrosine phosphorylation of Trk receptors, including TrkB, or function blocking BDNF antibodies to inactivate endogenous BDNF. During bath application of 200 nM K252-a, a significant and reversible reduction in the frequency of spontaneous local calcium transients (-26.2% ± 9.4%, n = 11, p≤ 0.05; Fig. 5.3A) was observed. Temporal and spatial characteristics of local calcium transients were not changed. The frequency of global calcium transients remained stable (data not shown). Similarly, bath application of BDNF antibodies (4 μg/ml) diminished the frequency of spontaneously occurring local calcium transients significantly (-33.8% ± 7.4%, n = 11, p≤ 0.05; Fig. 5.3B). In contrast, the frequency of the global signals did not change significantly (-5.4% ± 28.5%; Fig. 5.3C). These results show that interfering with the BDNF-TrkB-signaling pathway reduces the frequency of spontaneous local calcium transients indicating that endogenous BDNF generates intracellular calcium signals via TrkB receptor activation.

5.1.2 Exogenous BDNF strongly increases the frequency of calcium transients

The observation of a reduced frequency of local calcium transients during inhibition of the BDNF-TrkB-signaling pathway led me to ask whether additional exogenous BDNF would enhance calcium activity. To address this question, exogenous BDNF (final concentration: 200 ng/ml) was applied into the bath solution of the recording chamber and a strong and reversible increase in the frequency of global calcium transients could be observed (+239.4% ± 88.9%, n = 8, p ≤ 0.05; Fig. 5.4A and B). In contrast to global calcium rises, the frequency of local calcium transients did not increase significantly during exogenous BDNF application (+41.9% ± 19.9%, n = 12; Fig. 5.4C).

The increase in global activity might be a direct cellular response to BDNF. Alternatively, BDNF may modulate the network activity of the hippocampal circuit, resulting in an increase in global calcium activity (Sakai et al., 1997). To test whether the strong increase in the frequency of global calcium transients

was a direct effect or due to enhanced network activity, I used bolus loading (Stosiek et al., 2003) of Oregon Green BAPTA-1 AM to visualize network activity in CA3 pyramidal neurons. After fast BDNF application, a sustained increase in the frequency of calcium transients in many neurons could be observed (before: 0.8 ± 0.3 , after: 4.9 ± 0.8 global transients / min, $p \leq 0.05$,

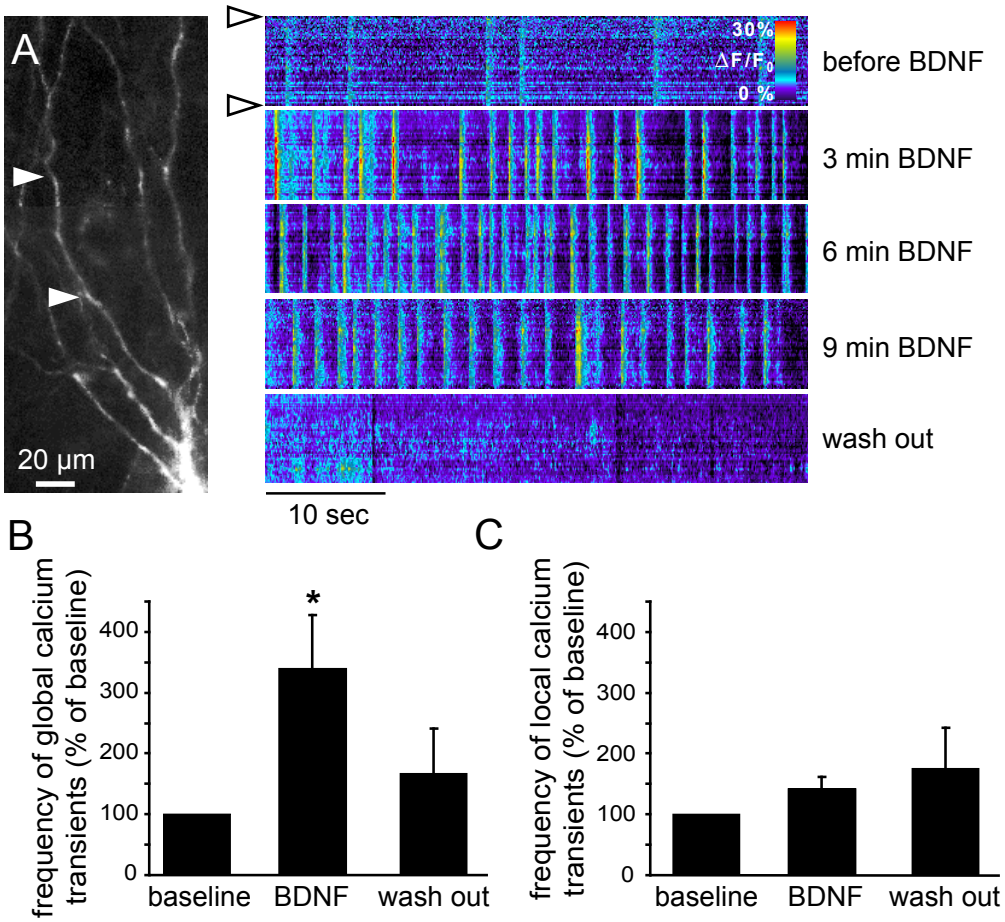

Figure 5.4: BDNF bath application causes a strong increase in the frequency of global calcium transients. **A**, Spontaneous changes in the intracellular $[Ca^{2+}]$ concentration in the dendritic segment marked with the arrowheads are shown in pseudo line scans as $\Delta F/F_0$. Line scans are shown before and during BDNF bath application (at 3 different time points) and after wash out. **B**, The frequency of spontaneously occurring global calcium transients is significantly ($p \leq 0.05$) increased during bath application of BDNF (n = 8 neurons, values normalized to baseline frequency). **C**, Frequency of spontaneous local calcium transients is not significantly increased during BDNF bath application (n = 12 neurons).

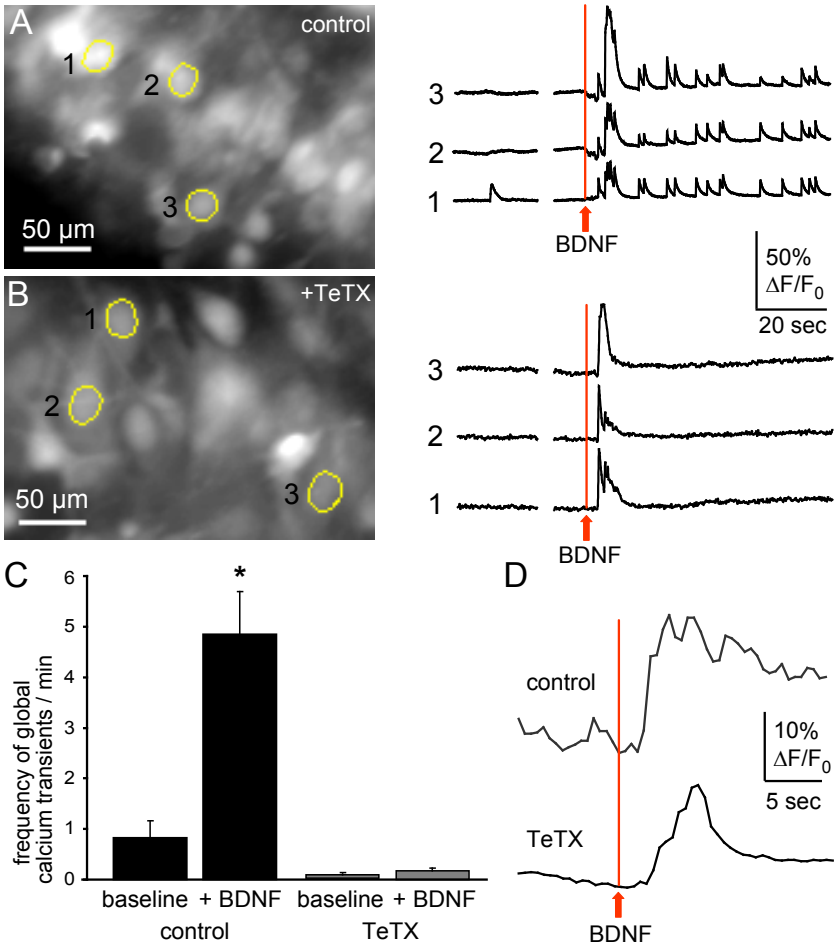

Figure 5.5: Exogenous BDNF directly triggers a transient rise in the intracellular calcium concentration and causes an increase in network activity. **A**, Bolus loading of CA3 neurons in control slices shows an acute increase in the intracellular $[Ca^{2+}]$ concentration upon BDNF bath application and a delayed increase in correlated network activity (traces correspond to the encircled neurons in the left image). **B**, Neurons in slices incubated for 12 hs in tetanus toxin (TeTX) also reveal an immediate increase in the intracellular Ca^{2+}concentration to BDNF bath application, but otherwise remain silenced. **C**, Frequency of global calcium transients in control- and in TeTX-incubated slices before and after BDNF bath application (80 neurons from 8 slices; control slices: before 0.8 ± 0.3, after 4.9 ± 0.8 global calcium transients / min; TeTX- incubated slices: before 0.07 ± 0.02, after 0.17 ± 0.06 global calcium transients / min; $p \leq 0.05$). **D**, Averaged acute calcium responses to fast BDNF bath application in control slices and in TeTX-incubated slices (80 neurons from 8 slices).

n = 8 slices, 80 cells, Fig. 5.5A and C). The calcium transients where highly correlated across neurons, indicating that they reflected an increase in network activity. Averaging the responses from the entire set of cells showed that there was also an immediate - but transient - calcium response upon BDNF application (Fig. 5.5D). When the experiments were repeated in slices preincubated (12h) in tetanus toxin (TeTX) to block network activity, BDNF elicited only the immediate calcium response (Fig. 5.5B and D), demonstrating that the increase in frequency that was observed in control slices after BDNF application was a consequence of a BDNF-induced modulation of network activity. Fast application of control solution (PBS) caused neither an acute rise in the calcium concentration nor an increase in the frequency of calcium transients (data not shown). Together, these observations show that exogenous BDNF has two effects on CA3 neurons: it 1) directly triggers a transient rise in the intracellular $[Ca^{2+}]$ concentration and 2) causes an increase in network activity, probably by modulating synaptic function (Walz et al., 2006; Shen et al., 2006; Tyler et al., 2006).

5.1.3 Focal BDNF application triggers fast and local calcium transients

To further characterize calcium transients evoked directly by BDNF, I tested its specific action on dendrites of neurons by focally applying BDNF via a micropipette using a picospritzer. A pipette filled with BDNF was placed 10-20 μm away from the dendrite (Fig. 5.6A) and a single BDNF pulse (40 ms, 200 ng/ml) was applied to the dendrite of neurons exhibiting spontaneous calcium activity. In about 60% of those neurons, single BDNF pulses elicited dendritic calcium rises (Fig. 5.6A). I recorded twice for 1 min at 10 Hz with an interval of 3 min between acquisitions. During these two recordings, a total of six BDNF pulses was applied to the dendrite. The calcium responses to the six BDNF pulses were averaged for each cell as shown in the upper trace in Fig. 5.6B. I could repetitively evoke fast (rise time $\leq$100 ms) and spatially restricted calcium transients. Only cells that responded to at least two of the six BDNF pulses with a calcium rise and that showed an average response greater than 5% $\Delta F/F_0$ were analyzed (n = 48 neurons). I performed control experiments to verify that the observed calcium response was specific for the application of

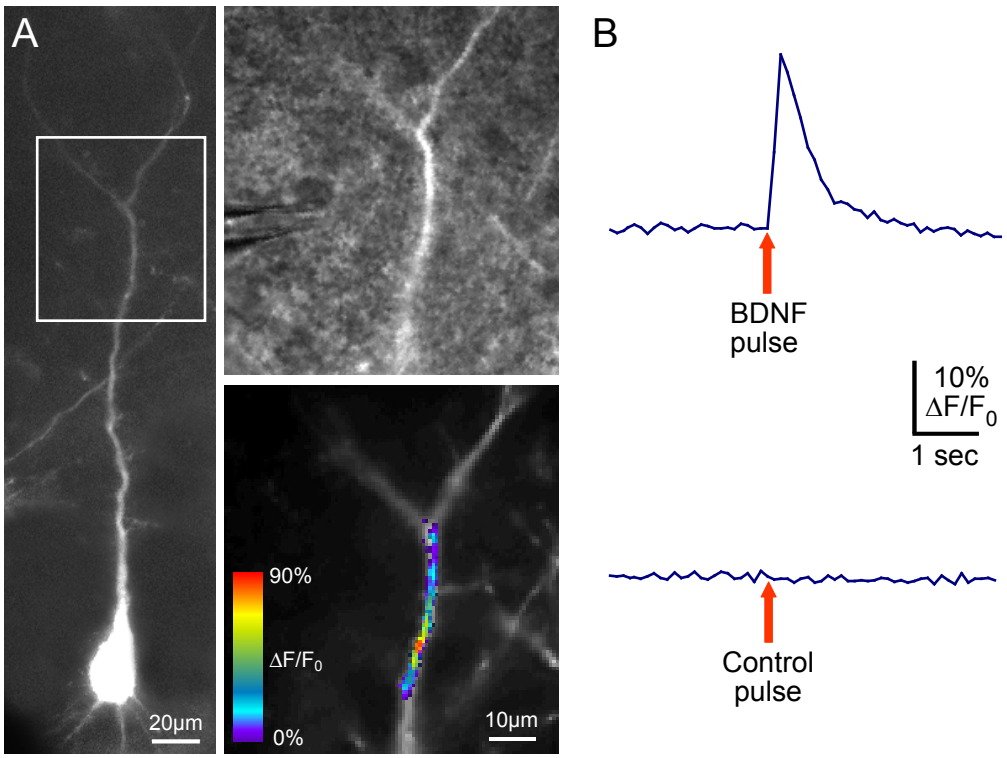

Figure 5.6: Dendritic calcium transients can be elicited by focal BDNF application. **A**, An electroporated CA3 neuron with the BDNF application pipette, placed ~20 μm from the dendrite (top right), responds to a single 40 ms BDNF pulse with a strong local increase in the intracellular [Ca^{2+}] concentration (bottom right). **B**, Upper trace shows an average of calcium responses to 6 BDNF pulses applied at the indicated dendritic region in A. Lower trace shows averaged responses to 6 pulses of a control solution (heat-inactivated BDNF) in another neuron.

BDNF : neither PBS + 0.1% BSA alone (n = 5 neurons) nor heat-inactivated BDNF (n = 10 neurons) elicited calcium transients in the dendrite (lower trace in Fig. 5.6B). Moreover, focal PBS + 0.1% BSA application did not evoke calcium transients in those dendrites that responded to BDNF pulses before and after PBS application (n = 3 neurons; not shown).

Next, the mechanisms of fast BDNF-TrkB-mediated calcium signaling were determined by pharmacologically characterizing exogenously BDNF-evoked calcium transients. The maximal amplitude of the calcium response within 1s after the BDNF pulse was measured before, during and after bath applying drugs. K252 a (200 nM) as well as tetrodotoxin (TTX; 1 μM) and cadmium chloride

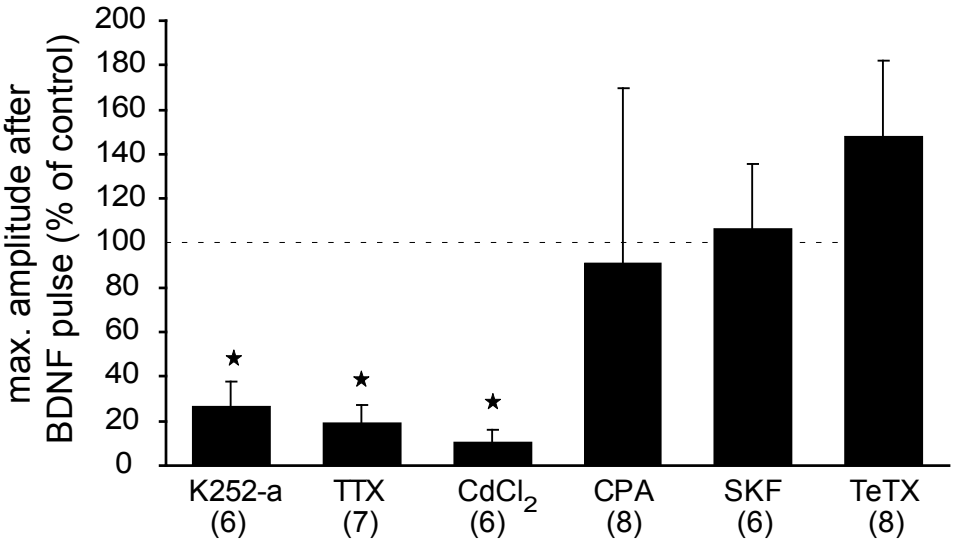

Figure 5.7: Pharmacology of BDNF-evoked calcium transients. The amplitudes of BDNF-evoked calcium transients are shown as percentage of baseline. Bath application of K252-a, tetrodotoxin (TTX) and cadmium chloride ($CdCl_2$) reduced the amplitude of the calcium transients evoked by local BDNF application significantly ($p \leq 0.05$). In contrast, no reduction in the amplitude of the BDNF-induced calcium transients was observed during bath application of cyclopiazonic acid (CPA), SKF 96365 hydrochloride, or in tetanus toxin (TeTX) incubated slices.

($CdCl_2$; 5 μM), which block TrkB receptors, voltage-gated sodium and calcium channels respectively, reduced the maximal amplitude of the calcium response after the BDNF pulse significantly (Fig. 5.7). In contrast, neither Cyclopiazonic acid (CPA; 20 μM), an agent that interferes with calcium release from internal stores, nor SKF 96365 hydrochloride (3 μM), an unspecific inhibitor of store-operated calcium-entry and transient receptor potential channels (TRPC) (Merritt et al., 1990; Li et al., 1999), nor tetanus toxin (TeTX; 20 nM), which blocks presynaptic transmitter release, reduced the amplitude of the calcium response after the BDNF pulse (Fig. 5.7). Taken together, these pharmacological experiments indicate that activation of the TrkB receptor and voltage-gated sodium as well as calcium channels are required for BDNF-evoked calcium transients.

5.2 Intrinsic BDNF signaling is localized to synapses

5.2.1 BDNF-mediated local calcium transients occur at synaptic sites

The observation that spontaneous BDNF-dependent calcium transients were highly localized along the dendrite (see Fig. 5.6) led me to ask whether these transients occur at specific sites, possibly synapses. Using viral infection (Semliki forest virus) (Ehrengruber et al., 1999), the postsynaptic marker PSD-95:CFP (Graf et al., 2004) was expressed in hippocampal neurons. PSD-95:CFP labeling was apparent in the soma and as puncta along the dendrites. Presynaptic counterparts of the dendritic PSD-95:CFP puncta were identified with immunohistochemical staining for synapsin. High-magnification confocal reconstruction in three dimensions allowed to visualize synaptic structures by colocalization analysis. 74% of 122 putative PDS-95:CFP puncta were opposed by at least one positive synapsin punctum, whereas 26% did not show a clear colocalization (Fig. 5.8). The specificity of colocalization was tested using the reverse order of the synapsin stack rotated by 180°. Here, significantly fewer of the PSD-95 clusters colocalized with presynaptic synapsin puncta (16% colocaliza-

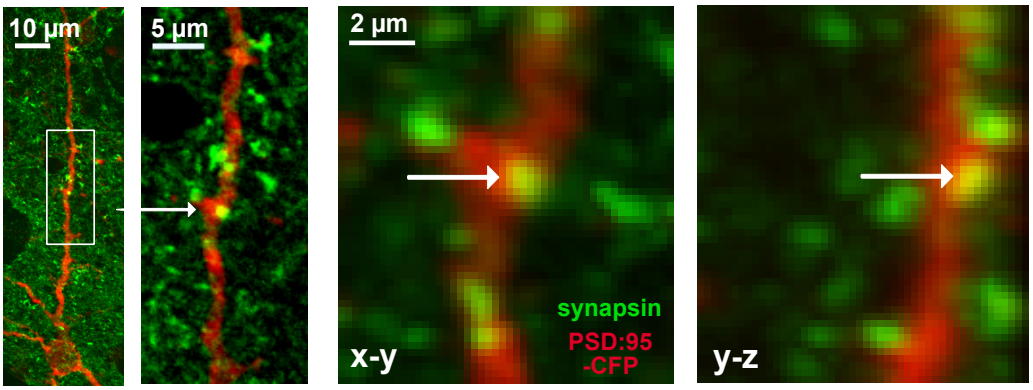

Figure 5.8: PSD-95:CFP colocalisation with synapsin. Neuron expressing PSD-95:CFP (shown in red) by virus-mediated gene transfer after immunolabeling with anti-synapsin antibodies and high-resolution confocal reconstruction (left, maximum intensity projection of the neuron in red, single plane of synapsin staining in green). Arrow indicates a synapse as PSD-95:CFP and synapsin overlap. High-magnification (x-y) and orthogonal (y-z) views of the marked synapse (yellow pixels in both views).

tion, 84% non-colocalization, n = 8 neurons, $p \leq 0.05$). This indicates that the majority of the PSD-95:CFP puncta are indeed postsynaptic sites.

After electroporation of the calcium indicator into PSD-95:CFP expressing cells, I was able to visualize postsynaptic sites along the dendrite and spontaneous calcium transients simultaneously. Cellular morphology and the frequency of spontaneously occurring global and local calcium transients in these transfected neurons were similar to uninfected neurons. Frequently, spontaneous local calcium transients could be observed at PSD-95:CFP expressing sites along the dendrite (Fig. 5.9A). I identified the positions of putative synapses and local calcium transients along the dendrites and calculated the distance between the center of the nearest putative synaptic site and the center of each calcium transient. Almost half of the local calcium rises occurred within a distance of 2 μm of a putative synapse (41 of 99 from 8 neurons; (Fig. 5.9B), which is consistent with previous observations (Lohmann et al., 2005). The mean distance between visualized PSD-95:CFP sites was 17.3 $\pm$ 1.9 μm, which is in good agreement with numbers that can be deduced from electron microscopy studies of CA1 tissue in the hippocampus for these ages (Steward and Falk, 1991). To test whether BDNF triggers local calcium transients specifically at PSD-95 sites, function blocking antibodies against BDNF were again used to interfere with the BDNF-TrkB-signaling pathway. I observed that during BDNF antibody bath application, the frequency of local calcium transients that occurred within a distance of less than 2 μm from a putative synaptic site was significantly and reversibly reduced to 65.9% $\pm$ 16.8% (black bars in Fig. 5.9C, n = 51) compared to baseline. At those synapses which exhibited calcium activity during baseline recordings (21/51), calcium transients were completely blocked in most cases (17) or were reduced (2) or remained stable (2) during BDNF application. Intrinsic BDNF signaling occurred at excitatory synapses at a mean rate of 0.22 transients per minute and synapse. In contrast to the reduced frequency of calcium transients at synapses, no change in the frequency of local transients that occurred more than 2 μm away from the nearest synapse during BDNF antibody application (grey bars in Fig. 5.9C) could be found. Furthermore, there was no significant change in the frequency of global calcium rises during BDNF antibody application (1.44 global transients $\pm$ 0.51 / min) compared to baseline conditions (1.94 global transients $\pm$ 0.82 / min; data not shown). Together with the experiments described above, this finding indicates that endogenous BDNF directly triggers local calcium transients, but not global calcium rises and that these BDNF-TrkB dependent local calcium transients occur specifically at synaptic sites.

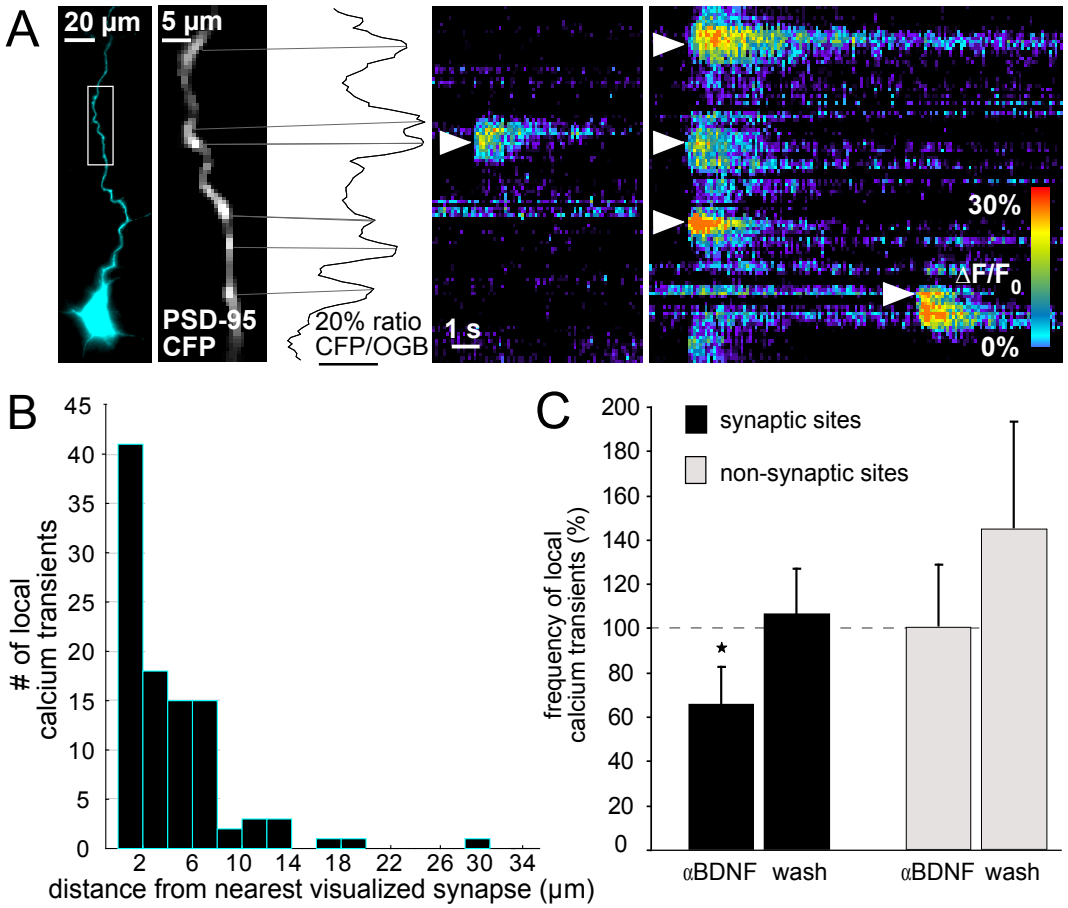

Figure 5.9: BDNF-mediated local calcium transients occur preferentially at synaptic sites. **A**, PSD-95:CFP (blue) expressing hippocampal neuron electroporated with the calcium indicator OGB-1 (green). Enlarged part of the dendrite shows PSD-95:CFP puncta; the puncta are also revealed in a ratio plot of CFP/OGB pixel intensity along the dendrite. Line scan of the enlarged part of the dendrite shows spontaneously occurring local calcium transients at four of the PSD-95:CFP sites (right). **B**, Distribution of spontaneous local calcium transients with respect to putative synaptic sites (99 local calcium signals from 8 neurons). Most transients occur at or very close to the nearest synapse (the mean intersynapse distance of 17.3 ± 1.9 μm agrees with numbers observed in CA1 neurons in the first postnatal week (Steward and Falk, 1991). **C**, Frequency of spontaneous local calcium transients occurring at synapses ($\leq$ 2 μm apart) or at non-synaptic sites ($\leq$ 2 μm apart from the nearest synapse) before, during and after bath application of BDNF antibodies. The frequency of local calcium transients at synaptic sites, but not at non-synaptic sites, is significantly reduced during bath application of BDNF antibodies (n = 8 neurons; % of baseline; $p \leq 0.05$).

5.2.2 Local calcium transients elicited at synaptic sites by focal BDNF delivery

Focal BDNF application demonstrated a dendritic mechanism of the BDNF-evoked calcium responses (see Fig. 5.6). Additionally, I showed that spontaneous local calcium transients occur at PSD-95:CFP sites along the dendrite (see Fig. 5.9). Therefore, it was interesting to test whether dendrites would respond to exogenous BDNF only at postsynaptic sites. Neurons with spontaneous calcium activity were used for these experiments. The tip of a BDNF-filled glass pipette was placed 10-20 μm away from the PSD-95 site of the dendrite (Fig. 5.10A). A single BDNF pulse evoked a strong and localized calcium transient exactly at the PSD-95:CFP sites. The respective line scan indicates that the BDNF-mediated calcium rise started precisely at the two PSD-95 puncta on the dendrite and reaches the maximum change in fluorescence there (Fig. 5.10A). Another example of a BDNF pulse - followed immediately by a localized calcium rise at a synaptic site - is shown in Fig. 5.10B. Here, BDNF evoked a larger signal at the PSD-95:CFP punctum (blue region) than at the adjacent non-synaptic dendrite (grey region; Fig. 5.10C). On average, the amplitude of the $\Delta F/F_0$ signal after the BDNF pulse was significantly higher at the PSD-95:CFP puncta than at the adjacent non-synaptic dendritic sites (Fig. 5.10D). These findings show that focal, pulse-like delivery of BDNF evokes localized calcium rises predominantly at synaptic sites.

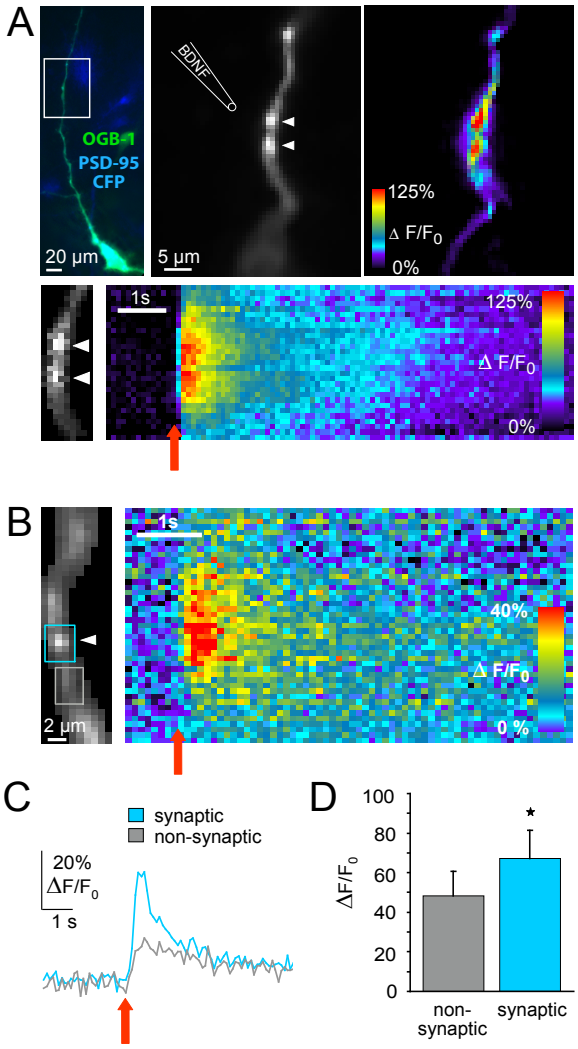

Figure 5.10: BDNF-evoked local calcium transients can be induced at synaptic sites. **A**, PSD-95:CFP expressing pyramidal neuron, electroporated with OGB-1 (left); arrowheads mark PSD-95:CFP puncta along the apical dendrite. A BDNF pulse caused a strong calcium rise (represented as $\Delta F/F_0$ values) exactly at the sites of two putative synapses (right). Bottom, line scan reveals that the calcium transient induced by the BDNF pulse (red arrow) starts at the two PSD-95:CFP sites and shows exactly there the highest fluorescence increase. **B**, Another example of a BDNF-evoked calcium transient at a PSD-95:CFP site. **C**, BDNF-evoked calcium transients at a PSD-95:CFP punctum (blue trace) and at an adjacent region (grey trace) indicated in B. **D**, Maximal amplitudes of $\Delta F/F_0$ within 1 s after the BDNF pulse at non-synaptic dendritic sites and at PSD-95:CFP puncta (n = 9 neurons).

5.3 Investigating the acute effect of BDNF on morphological changes

Since I observed that endogenous BDNF mediates calcium signaling which is precisely regulated in space and time, the question arises whether BDNF-evoked calcium signaling can result in changes in the motility and growth rate of dendritic filopodial structures. Previous work had already shown that dendritic filopodia are highly motile during synapse formation (Wong and Wong, 2001; Portera-Cailliau et al., 2003; Jontes and Smith, 2000) and that local calcium dynamics strongly correlate with filopodia motility (Lohmann et al., 2005).

I set out to investigate the acute effect of BDNF in the regulation of dendritic filopodia growth. Hippocampal CA3 pyramidal neurons were electroporated with Alexa-594 dextran to visualize fine morphological structures such as filopodia. Using a confocal microscope (Leica SP2), time-lapse imaging revealed that filopodia can grow out of dendrites, extend and retract within minutes (neurons of that early stage of development do not carry spines yet). An example of this motility is shown in Fig. 5.11A, where two filopodia (1 and 2) along a stretch of dendrite grow out within a 5 min-recording and one filopodium (3) stays stable. In Fig. 5.11B, the growth curve of these three filopodia is plotted over time. The averaged baseline motility of filopodia was ∼0.3 μm / min. These values fit to previously observed values (Lohmann et al., 2005). After recording the baseline motility of filopodia for 5 min at 0.33 Hz, exogenous BDNF was either bath applied for three minutes to the solution of the recording chamber (superfusion was interrupted until wash out) or BDNF was focally applied with three pressure pulses (pulse duration 40 ms) through a micropipette using a picospritzer. The overall motility of filopodia in the subsequent 5 min-recording was not altered compared to baseline, neither in the BDNF bath application experiments (Fig.5.12A, n = 8 neurons, 65 filopodia; 0.33 ± 0.04 μm / min compared to 0.32 ± 0.03 μm / min baseline) nor in the experiments of focal BDNF delivery (n = 4 neurons, 26 filopodia; 0.27 ± 0.03 μm / min compared to 0.26 ± 0.03 μm / min baseline). There was also no change after BDNF had been washed out (BDNF bath application: 0.34 ± 0.06 μm / min; focal BDNF application: 0.28 ± 0.01 μm / min). Also regarding filopodial net growth in μm / min, no change could be observed, neither during global BDNF application (Fig. 5.12B; 0.01 ± 0.02 μm / min compared to -0.01 ± 0.01 μm / min baseline) nor during focal BDNF delivery (-0.03 ± 0.02 μm / min compared to -0.04 ± 0.02 μm / min baseline). Additionally, the same filopodia were again analyzed by a 'blind observer'. Also in this analysis, no change in filopodia motility and

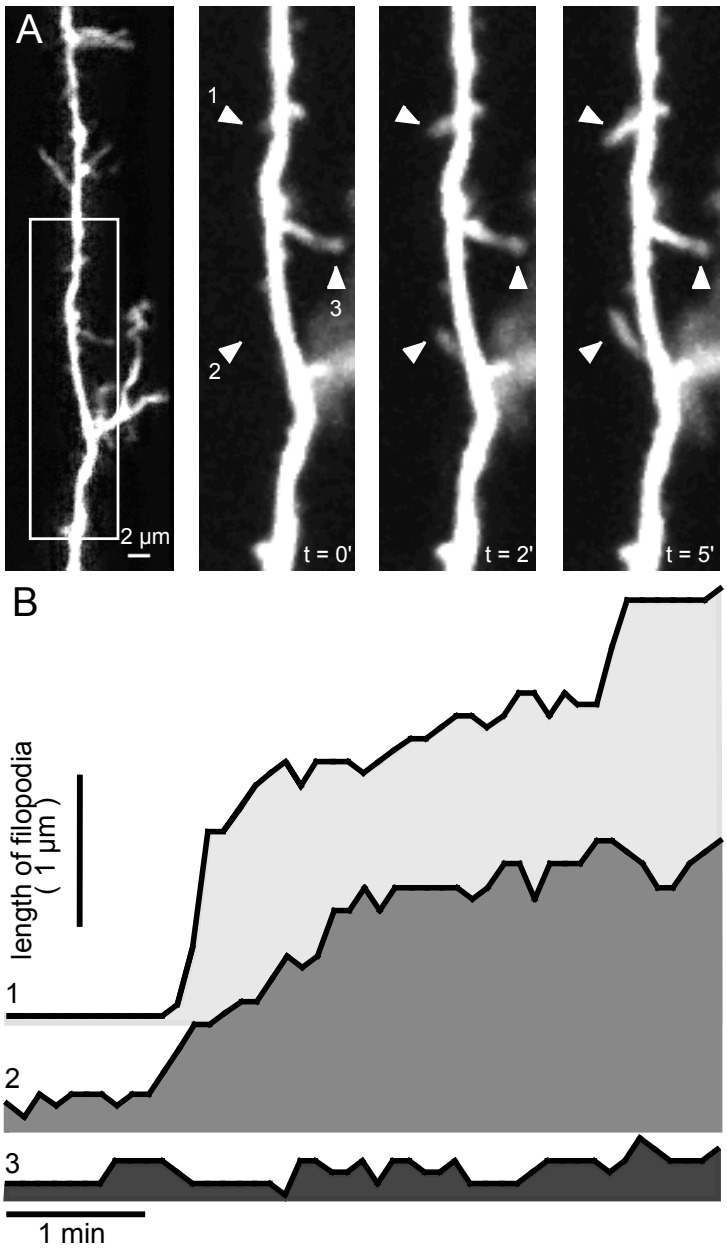

Figure 5.11: **A**, Time-laps series (t = 0, 2 and 5 min) of a stretch of dendrite (enlargement from the left image) that shows two filopodia (1 and 2) growing out of this dendrite and one (3) that does not change its length over the recording time. **B**, Growth curves of the three filopodia shown in A.

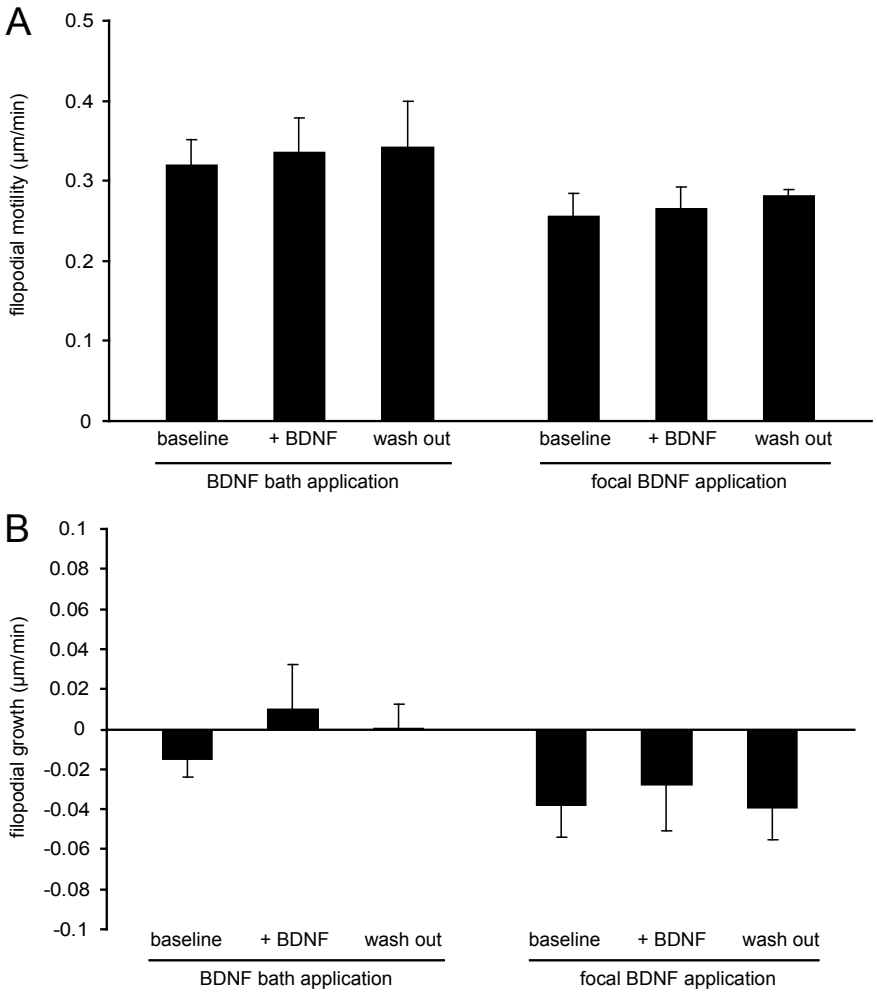

Figure 5.12: **A**, Average motility of filopodia in μm / min before, during and after BDNF bath application and focal BDNF application respectively. **B**, Average filopodial growth in μm / min before, during and after BDNF bath application and focal BDNF application respectively. No significant change in filopodia motility and growth could be observed, neither with BDNF bath application nor with focal BDFN delivery.

growth was found after BDNF application compared to baseline and wash out. Taken together, there seems to be no acute effect of BDNF on filopodial motility and growth. It is possible that filopodial growth is either independent of BDNF signaling or that it takes longer than a few minutes to induce BDNF-mediated changes in filopodia length (see discussion).

5.4 Plasticity effect of BDNF on local calcium transients

5.4.1 Long-lasting increase in the frequency of local calcium transients after focal BDNF pulses

I showed that BDNF has an acute effect on calcium signaling by triggering fast and localized calcium transients, which preferentially occur at synapses. However, since BDNF is well known to modulate neuronal function not only acutely (Lohof et al., 1993; Kang and Schuman, 1995; Levine et al., 1998), but also by exerting a long-lasting effect (e.g. maintenance of hippocampal long-term potentiation (Korte et al., 1995; Korte et al., 1996; Patterson et al., 1996; Kovalchuk et al., 2002) and stabilisation of synapses (Hu et al., 2005)), I decided to investigate calcium signaling that occurred long-term after focal BDNF application. The baseline calcium activity of a dendrite was acquired in two or four 1 min recordings at 10 Hz. The interval between acquisitions was always 5 min. Then, a total of six BNDF pulses were applied to the dendrite within two 1 min recordings, as described in 5.1.3. Subsequently, the calcium activity was recorded every 5 min for up to 120 min. In 15 out of 23 cases, an increase in the frequency of local calcium transients after BDNF pulses was observed compared to baseline. An example is shown in Fig. 5.13B, where the calcium activity of a dendrite before, during and 95 min after focal BDNF application is shown. The frequency of local calcium rises was much higher after BDNF addition compared to baseline. This increase occurred mostly within 10 min after the BDNF pulses application and was long-lasting (up to 140 min).

Comparing the average frequency of local calcium transients during the two recordings before and the two after focal BDNF delivery revealed that the frequency of local calcium transients was greater after BDNF application than before (Fig. 5.14A, n = 23 cells, average: before 49 $\pm$ 5, after 67 $\pm$ 7 local calcium transients/mm dendrite/min, $p \leq 0.05$). Some of the neurons that exhibited an above average frequency of local calcium transients during baseline recordings showed no increase or a reduction in the frequency of transients after BDNF application. The mean increase in the frequency of local calcium transients after focal BDNF delivery was + 83% ($\pm$ 28%, $p \leq 0.05$, Fig. 5.14B). This increase was observed consistently until 30 min after BDNF application (Fig. 5.14C) and - in those cases of longer recordings - was maintained for up to 2h (in 5 cases). This effect was specific for BDNF, because pulses with a control solution (PBS + 0.1% BSA) to the dendrite did not induce a change in the

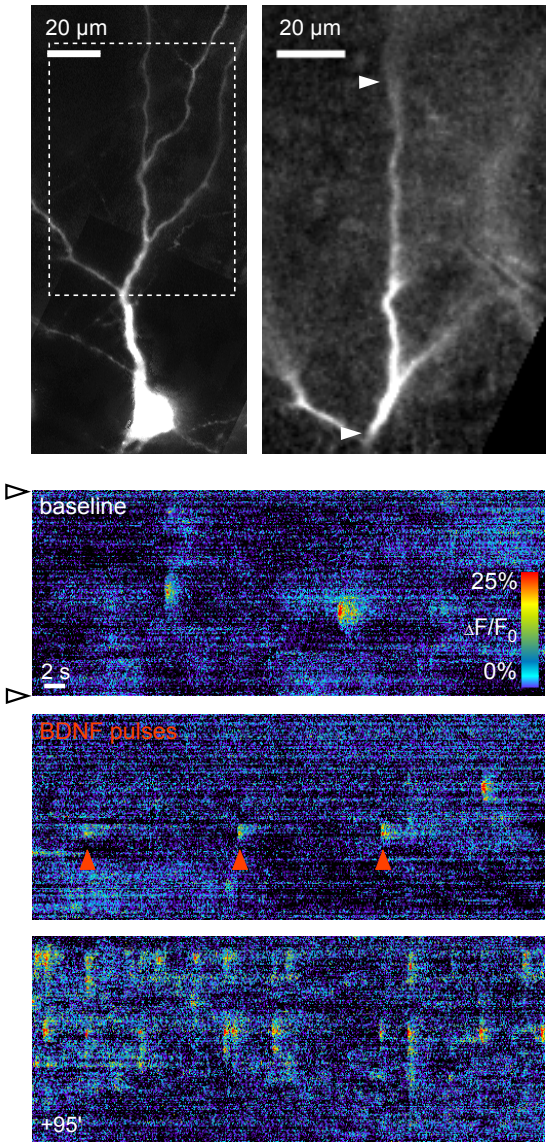

Figure 5.13: Increased frequency of local calcium transients after focal BDNF pulse application Electroporated CA3 neuron (left) and high magnification of the recorded dendrite (right). The pipette filled with BDNF is placed $\sim$ 20 μm away from the dendrite. In the pseudo line scans, few spontaneously occurring local calcium transients were recorded during baseline. Calcium responses upon three BDNF pulses are indicated with red arrowheads. Even 95 min after BDNF pulse application the frequency of local calcium rises is still be increased.

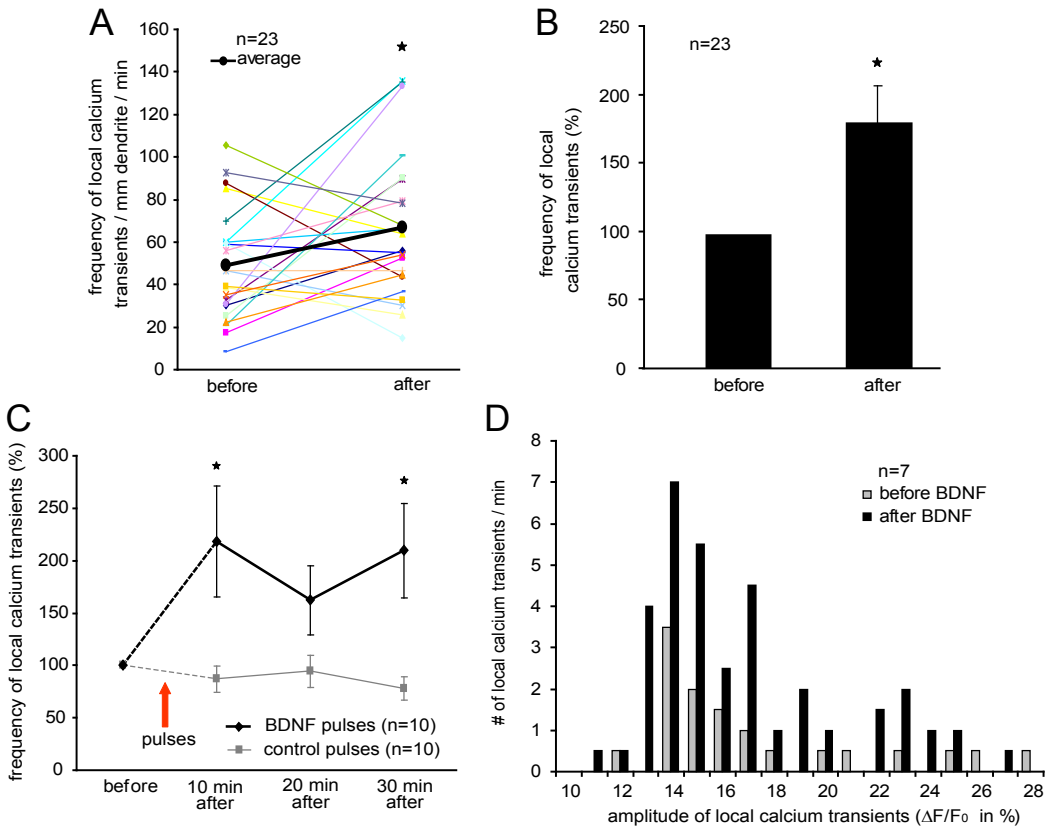

Figure 5.14: BDNF-mediated plasticity effect on the frequency of local calcium transients. A, Absolute frequency of local calcium transients / mm dendrite / min before and after focal BDNF application. Values are averages from two recordings respectively (5 and 10 min before/after BDNF application, n = 23 neurons, $p \leq 0.05$). **B**, Frequency of local calcium transients after BDNF pulses normalized to the baseline level (same 23 neurons as in A, $p \leq 0.05$) **C**, Sustained increase in the frequency of local calcium rises after focal BDNF pulses, but not after PBS control pulses to the neuron. Pulse application is indicated by the red arrow (n = 10 neurons each, $p \leq 0.05$). **D**, Only the frequency, not the amplitude of the local calcium transients is increased after BDNF has been focally applied to the dendrites (n = 7 neurons).

frequency of local calcium transients (Fig. 5.14C, n = 10 neurons respectively, $p \leq 0.05$). Interestingly, while calcium transients occurred more frequently after the BDNF pulses, their amplitude range was unchanged compared to baseline (Fig. 5.14D). This finding rules out the possibility that the observed increase in frequency after BDNF application was due to an increase of amplitudes above the detection level.

5.4.2 Local calcium transients occur at previously 'inactive' sites along the dendrite after BDNF delivery

The observation of a sustained increase in the frequency of local calcium transients after focal BDNF pulses raised the question where along the dendrite those transients occurred. Were they generated more frequently at dendritic positions that revealed local calcium transients already at baseline conditions? Or did they occur at sites along the dendrite that did not show local calcium rises before BDNF application? To address this question, the levels of calcium activity along a dendrite were calculated before and after BDNF application. The respective part of a dendrite was divided into sections of 10 pixels (approximately 7 μm). For each 10 pixel segment, the frequency of local calcium transients / min / group was calculated for baseline conditions and after BDNF application. An example is shown in 5.15B. Local calcium transients occurred at five positions along this dendrite at baseline (grey bars in Fig. 5.15B). After BDNF application however, many more sites along the dendrite showed local calcium rises (black bars in Fig. 5.15B). Interestingly, the frequency of local calcium transients was mostly not increased at dendritic sites that revealed calcium activity at baseline conditions, but rather previously 'silent' positions along the dendrite that did not show calcium rises before, generated local transients after BDNF application. Generally, the number of active sites / mm dendrite that showed local calcium transients was significantly increased after focal BDNF application compared to baseline (Fig. 5.15C, n = 10 neurons, 32 $\pm$ 5 before vs. 58 $\pm$ 6 active dendritic sites / mm after BDNF addition, $p \leq 0.05$). In the next step, the frequency of local calcium transients / min was normalized to baseline and the occurrence of calcium transients after focal BDNF and control pulses respectively was classified into two groups: transients that were generated at positions along the dendrite which revealed calcium activity already at baseline ('at previously active positions') and transients that were generated at dendritic positions which did not show calcium transients before pulse application ('at new positions'). In dendrites of neurons that have experienced control pulses (PBS + 0.1 % BSA), the frequency of local calcium rises afterwards was almost uniformly distributed between previously active (64% $\pm$ 17%) and new positions (52% $\pm$ 18%, grey bars in Fig. 5.15D, n = 10 cells). Compared to baseline, there was only a little increase in the frequency of calcium transients (+16%). In contrast, dendrites that underwent focal BDNF pulses showed a more than 2.5-fold increase in the frequency of calcium rises (+156%) compared to baseline. Remarkably, the distribution of transients between previously active positions and new positions was shifted towards new positions (174 % $\pm$

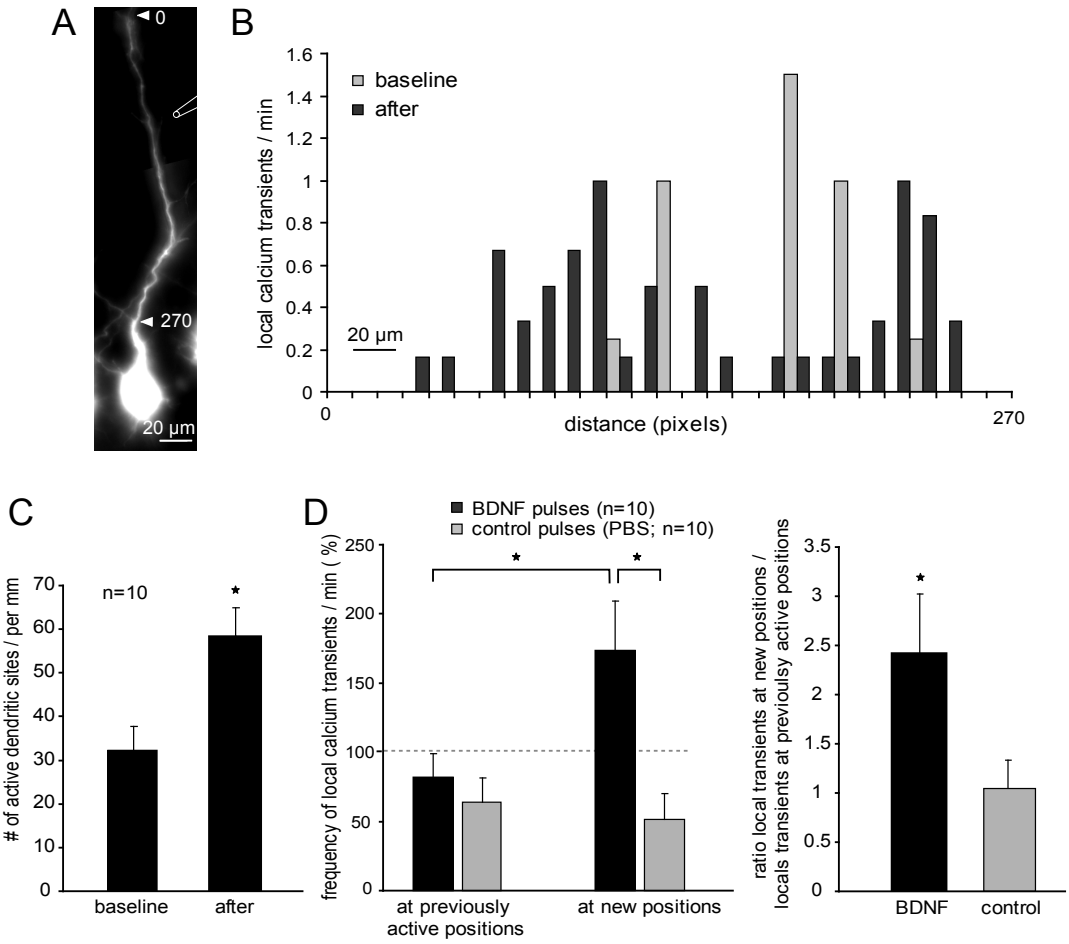

Figure 5.15: BDNF-mediated increase in the frequency of local calcium transients is mainly due to newly activated sites along the dendrite. **A**, Arrowheads indicate the extend (pixels) of the dendrite used for the analysis in B. The position of the tip of the BDNF filled pipette is shown. **B**, Frequency of local calcium transients along the dendritic length shown in A (bin size: 10 pixels, average of four recordings before and six recordings after focal BDNF application). More positions along the dendrite reveal local calcium transients after BDNF application (black bars) than at baseline (grey bars). **C**, The number of active sites / mm dendrite that generate local calcium transients is increased after BDNF pulse application ($n = 10$ neurons, $p \leq 0.05$). **D**, Frequency of local calcium transients / min at previously active positions and at newly activated positions along the dendrite after BDNF and control pulses respectively, normalized to baseline ($n = 10$ neurons each, $p \leq 0.05$). The ratio of local calcium transients at new positions / local calcium transients at previously active positions is more than 2-fold increased after BDNF pulses compared to control pulses ($n = 10$ neurons, $p \leq 0.05$).

35% at new positions versus 82% ± 17% at previously active positions, black bars in Fig.5.15D, n = 10 cells, $p \leq 0.05$). The frequency of local calcium rises occurring at new positions after BDNF pulses was significantly larger compared to the control group at new positions and compared to the frequency of local calcium transients after BDNF pulses at previously active sites (Fig. 5.15D). Plotting the ratio of local transients at new positions / local calcium transients at previously active positions for the BDNF-treated neurons (2.4 ± 0.6) and the control group (1.0 ± 0.3) also clearly showed that the significant increase in the frequency of calcium rises at new posititons was dependent on BDNF (Fig. 5.15D right, n = 10 cells, $p \leq 0.05$).

5.4.3 BDNF-mediated activation of silent synapses?

I showed that previously silent sites along the dendrite are activated through focal BDNF stimulation (Fig.5.15D). Are these sites synapses? To investigate whether calcium activity at PSD-95:CFP puncta is modulated by BDNF in the long run and whether possibly individual synapses are activated by BDNF, focal application of BDNF in PSD-95:CFP expressing CA3 neurons was used. After electroporation of the calcium indicator into PSD-95:CFP expressing cells, postsynaptic sites along the dendrite and spontaneous calcium transients could be visualized simultaneously. One example is shown in Fig. 5.16A, where BDNF was focally applied to a PSD-95:CFP punctum. At baseline, one calcium rise was recorded close by, but not exactly at the PSD-95:CFP site. After 30 min, many local calcium transients occurred exactly at this PSD-95:CFP site (the maximum of fluorescence change of the calcium rise at time point -10 min is at a different position in the pseudo line scan than the maxima of the following transients which correspond to the PSD-95:CFP punctum). In this preliminary dataset of 3 neurons, 18 PSD-95:CFP puncta were silent during baseline and only one was active. 9/19 PSD-95 puncta that did not reveal calcium transients at baseline, showed calcium activity up to 30 min after BDNF had been applied to the dendrite close to a PSD-95:CFP site (Fig. 5.16B). However, also 9/19 puncta remained silent after BDNF application and in one case, even a reduction was noted (Fig. 5.16B, average: before 0.03, after 0.2 local calcium transients / PSD-95:CFP punctum / min). These preliminary data may be a hint suggesting that BDNF can activate pre- or postsynaptically silent synapses.

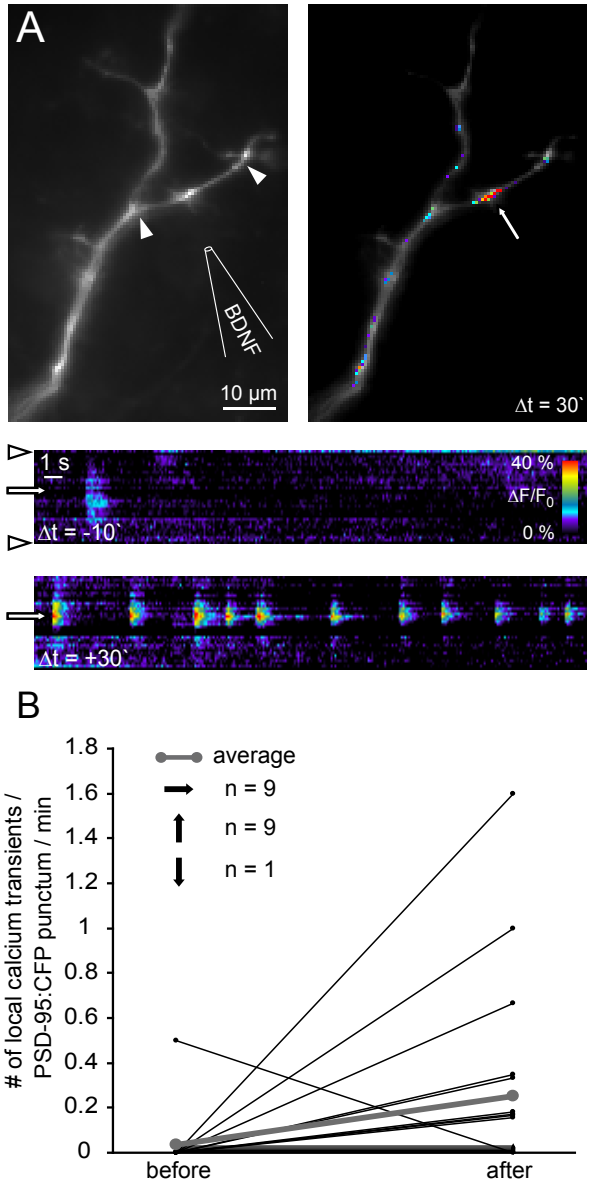

Figure 5.16: Activation of silent synapses? **A**, PSD-95:CFP expressing dendrite of a pyramidal neuron, electroporated with OGB-1 (left); arrowheads mark the extent of the dendrite used for the pseudo line scans. BDNF was puffed to a PSD-95:CFP punctum (arrow) in that region. At this punctum, local calcium transients occur 30 min after the BDNF pulses (right, arrow). Respective line scans before and 30 min after focal BDNF application (represented as $\Delta F/F_0$ values). **B**, 9 out of 19 PSD-95:CFP puncta of 3 neurons show local calcium transients after focal BDNF application, but not before. 9 puncta do neither reveal local calcium rises before nor after BDNF pulses. At 1 punctum, local calcium transients occur before, but not after BDNF application.

6 Discussion

I have used calcium imaging to investigate directly intrinsic BDNF signaling in developing hippocampal neurons during synapse formation. The results of this thesis provide strong evidence that intrinsic BDNF signaling is highly specific in space and time: endogenous BDNF induces fast calcium transients at synapses in developing dendrites. Furthermore, by quantifying the characteristics of BDNF triggered calcium transients, constraints for the regulation of developmental processes by fast BDNF signaling were determined. The temporal (< 1 sec) and spatial characteristics (< 10 μm) of intrinsic BDNF signaling as well as its relative abundance (> 10 transients/hour/synapse) render BDNF an ideal signaling molecule for establishing specific synaptic connectivity and functional neuronal networks. Moreover, the observed BDNF-mediated long-term effect on local calcium activity mainly at previously 'silent' sites along the dendrite points toward an important role of this neurotrophin in synapse development and maturation.

6.1 Intrinsic BDNF signaling is fast

Blocking intrinsic BDNF signaling in developing hippocampal slices reduced the frequency of spontaneous fast and locally restricted calcium transients. This was shown using the unspecific tyrosine kinase blocker K252-a as well as specific BDNF antibodies which scavenge soluble BDNF. Both ways of blocking BDNF-TrkB-signaling caused a similar reduction in the frequency of local calcium transients, but not of global transients. Conversely, focal application of exogenous BDNF elicited fast local calcium transients. The specificity of BDNF in mediating local calcium rises was probed by focally applying control solutions or heat-inactivated BDNF. Together, these results show that the neurotrophin BDNF is an important mediator of fast local calcium transients in developing dendrites. What makes BDNF signaling so fast? I investigated the mechanism of BDNF triggered dendritic calcium transients in developing dendrites of hippocampal neurons (see Fig. 5.7). Blocking TrkB receptors as well as voltage-gated sodium and calcium channels significantly reduced the maximal amplitude of the cal-

cium response to BDNF pulses indicating that the BDNF-evoked calcium rises depend on the activation of TrkB receptors and fast opening of voltage-gated sodium and calcium channels. They are not dependent on release of calcium from internal stores (blocked with CPA). Cation influx via TRPC3, a member of the transient receptor potential channel family, is not involved in the generation of fast BDNF-evoked calcium transients. In line with this observation, it has been reported that the TrkB/PCLγ-mediated, IP3-dependent calcium release from internal stores which activates ion influx through TRPC3 channels (Li et al., 1999), occurs within 20 - 30 sec after BDNF addition (Widmer et al., 1993; Rose et al., 2004). The tetanus toxin (TeTX) experiments exclude the possibility that focal BDNF application stimulated presynaptic transmitter release, which could in turn induce dendritic calcium transients. Together the results show that BDNF directly triggers dendritic calcium transients via TrkB and voltage gated currents (Fig. 6.1). Such a mechanism is reminiscent of the effects of exogenously applied BDNF, which have been described previously. BDNF can cause depolarizations in cultured neurons within a few milliseconds (Kafitz et al., 1999) that require the activation of voltage dependent sodium channels (Blum et al., 2002) as well as fast calcium transients through the opening of voltage gated calcium channels (Berninger et al., 1993; Kovalchuk et al., 2002). The results presented in this thesis extend these findings by demonstrating that developing hippocampal neurons intrinsically generate fast BDNF signaling.

The onset kinetics of BDNF-evoked calcium transients are faster than 100 ms, the temporal resolution of the recordings, suggesting that they are in the range of several tens of milliseconds or less. This is within the temporal requirements for spike timing dependent plasticity in the mature nervous system (Dan and Poo, 2004). There is evidence, however, for a reduced requirement for temporal precision in activity-dependent plasticity during development (0.1-1 sec) (Kasyanov et al., 2004; Lee et al., 2002). Therefore, the temporal characteristics of BDNF-induced calcium transients (rise time $<$ 100 ms, duration $\sim$700 ms) accurately reflect the temporal requirements for activity-dependent synaptic plasticity during development. Furthermore, my results show that this phenomenon occurs under natural conditions frequently enough to play a pivotal role in synapse development.

In principle, two scenarios could explain fast intrinsic BDNF signaling: 1) Rapid release of BDNF at specific sites, most likely at synapses (see below), triggers a direct response in the dendrite. 2) Tonic BDNF release establishes a diffuse distribution of BDNF throughout the tissue and stochastic activation

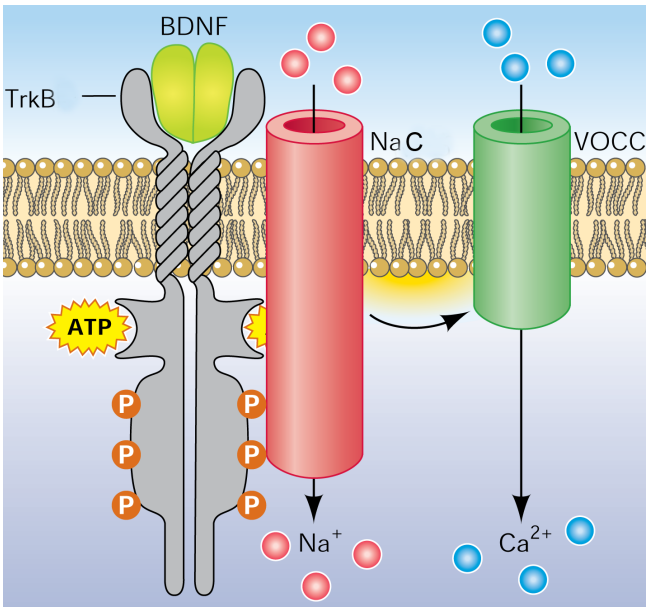

Figure 6.1: Fast action of BDNF signaling. BDNF binding to TrkB receptors results in activation of sodium channels (NaC) and the following depolarization leads to a calcium influx via activation of VOCCs (modified from a review by Blum and Konnerth, 2005).

of TrkB receptors generates fast dendritic calcium transients. My experiments support the first scenario, because fast and localized application of BDNF triggers immediate calcium rises (Fig. 5.5D, 5.6, 5.9). In contrast, the data are not consistent with the second scenario, because the persistent and diffuse presence of BDNF is not an appropriate stimulus for the direct activation of fast calcium rises (Fig. 5.4C, 5.5B). Desensitization of TrkB receptors might prevent a sustained response during long-term BDNF exposure. From these findings, I infer that the induction of fast calcium transients requires the rapid release of BDNF in close vicinity to the dendrite. In addition, these results support the idea that both endogenous BDNF release and dendritic response are very precisely regulated in time.

6.2 Intrinsic BDNF signaling is localized to synapses

I observed that BDNF-evoked calcium transients are localized to specific dendritic sites. Interestingly, simultaneous imaging of putative postsynaptic structures, labeled with PSD-95:CFP by viral transfection, and local calcium transients indicated that BDNF-triggered transients occur primarily or exclusively at synapses (Fig. 5.9B). One potential caveat of virally over-expressing synaptic PSD-95 is that it may affect health of infected neurons. Additionally, it might change signaling as it increases the number of synaptic AMPA receptors as shown previously (Schnell et al., 2002). The increase in synaptic AMPA receptors will most likely not influence the localization of TrkB as no direct interaction between TrkB and AMPARs or PSD-95 has been shown. Furthermore, no difference in the structure or function between transfected and wild type neurons was observed, indicating that over-expression did not affect viability or signaling: 1) the cellular morphology of transfected pyramidal neurons was not different from non-transfected neurons. 2) The frequencies of spontaneously occurring global and local calcium transients were similar in normal and over-expressing neurons. 3) The proportion of local calcium transients occurring at synapses was similar to that found in a previous study using post fixation immunohistochemistry instead of PSD-95:CFP for labeling synapses (Lohmann et al., 2005). 4) The proportion of local calcium transients that was blocked by interfering with BDNF signaling was similar in normal and transfected cells (Fig. 5.3A and B, Fig. 5.9C). Together with the finding that most PSD-95:CFP puncta were opposed by synapsin positive structures (Fig. 5.8), these observations confirm that virally over-expressed PSD95:CFP is an appropriate label for identifying postsynaptic signaling.

BDNF-induced calcium transients were found at synaptic sites during intrinsic BDNF signaling (Fig. 5.9A and C) and after focal delivery of BDNF (Fig. 5.10). The second observation implicates that dendrites are particularly sensitive to BDNF at synapses. The most straightforward explanation would be the specific presence of TrkB at the synapse. This fits well with an immuno-electronmicroscopical study which reported TrkB immunoreactivity predominantly at synapses within the dendrites of hippocampal pyramidal neurons (Drake et al., 1999). TrkB, which resides at non-synaptic sites along dendrites, is mostly confined to intracellular membranes (Drake et al., 1999) suggesting that functional receptors are enriched at synaptic sites (Gomes et al.,

2006). This predominantly synaptic distribution of TrkB likely underlies the local specificity of BDNF-evoked calcium transients observed here. While my results imply that endogenous BDNF is released near the synapse and directly triggers calcium transients at the postsynapse, it is not clear whether BDNF originates from the pre- or postsynapse. Evidence for both modes of release has been obtained (Kohara et al., 2001; Hartmann et al., 2001; Lessmann et al., 2003; Gaertner et al., 2006). Independent of the site of release, it has previously been shown that BDNF regulates neuronal development on a local as well as on a global level. The intrinsic form of fast BDNF signaling identified here occurs at synaptic sites and extends only a few micrometers along the dendrite, much less than the average distance between excitatory synapses at these early stages of synapse development ($\sim$16μm). This observation implicates intrinsic BDNF signaling in regulating the development of single synapses, possibly by mediating synaptic maturation (Rutherford et al., 1998; Walz et al., 2006; Itami et al., 2003). The spatial specificity in the action of BDNF signaling can be achieved by pre- or postsynaptic restricted release sites, synaptic localization of the TrkB receptors and limited extracellular diffusion. In addition, since TrkB activates diverse intracellular signaling pathways, the intriguing possibility arises that BDNF-TrkB signaling activates parallel pathways to regulate local and global as well as rapid and slower aspects of neuronal development differentially.

Taken together, the results so far also indicate that BDNF is one of several factors, which contribute to local dendritic calcium activity, since only a fraction of local dendritic calcium transients is blocked by interfering with intrinsic BDNF signaling. In conclusion, developing neurons are able to respond to a number of different extracellular cues during development and they integrate those by generating very specific patterns of local and global calcium transients. Such multi-factorial calcium transients may regulate structural development and synapse formation and eventually help establishing the complex neuronal networks of the brain.

6.3 Role of BDNF in structural and functional modifications during development

To follow up the phenomenon of precisely regulated fast BDNF-signaling at synapses, the next aim of this thesis was to explore its function by investigating possible rapid plasticity effects in developing dendrites upon localized BDNF stimulation. Generally two, probably related, modifications are known to be mediated by neurotrophins: structural and functional plasticity. If structural and functional changes elicited by BDNF act at developing synaptic sites, what happens first? Does BDNF initially regulate the morphology, which in turn leads to the formation and stabilization of synaptic sites or does it act directly on (immature) synapses without affecting the structure? I first addressed the question whether BDNF can acutely induce morphological changes such as growth of highly motile filopodia along the dendrites. Next, I examined a possible short- and long-term effect of BDNF on the level of dendritic calcium activity. Here, I asked whether specific sites along the dendrite such as maturing synapses show functional changes, e.g. generate more or fewer local calcium transients, after focal BDNF stimulation.

6.3.1 BDNF shows no rapid effect on fine neuronal morphology

The shape and extent of neuronal arborization undergo major alterations during development and thereby also influence the functional properties of neurons. For instance, interactions between axonal and dendritic filopodia, which are highly motile and grow and retract within seconds to minutes, induce new branching and synaptogenesis (Jontes and Smith, 2000). These processes can be regulated by many factors such as the genetic program of the cell, neuronal activity levels, but also extracellular guidance molecules. It has been shown that different forms of neurotransmission influence dendritic branching and filopodia motility and growth in a developmentally regulated manner (Wong and Wong, 2001; Portera-Cailliau et al., 2003). Yet, besides neurotransmitters there are also additional extracellular molecular cues which act in an activity-dependent manner in order to sculpt a synaptic contact.

BDNF is an attractive candidate molecule mediating the structural properties of neurons. How fast does BDNF mediate morphological changes in dendritic arborization? Most of the reported BDNF-mediated structural changes on den-

dritic branching and complexity have not been investigated earlier than 16-72 h of BDNF addition, overexpression or signaling inhibition (McAllister et al., 1995; McAllister et al., 1997; Horch and Katz, 2002; Gorski et al., 2003) although more rapid actions of BDNF on the morphology and motility of developing neuronal processes may occur (Berninger and Poo, 1996). Theoretically, neurotrophin-induced rapid cytoskeletal rearrangements must be possible. They involve small G proteins of the Cdc-42/Rac/Rho family, which are regulated by Trk receptor signaling: upon tyrosine phosphorylation, PI-3 kinase is activated and stimulates the MAP kinase pathway which controls the polymerization and turnover of F-actin (Bishop and Hall, 2000). Since I observed rapid BDNF-evoked calcium signaling, I wondered whether this is correlated with morphological modifications and asked whether it is possible to visualize an acute effect of BDNF on small morphological changes such as dendritic filopodia.

As described in 5.3, surprisingly no acute effect of BDNF on filopodial motility and length was observed, neither by bath-, nor by focal BDNF application (Fig. 5.12). The rate of motility and growth was constant over the time of the experiments, comparable between BDNF bath- and focal application and similar to what others have shown before (Lohmann et al., 2005). It is unlikely that the electroporation or the fluorescence imaging procedure were deleterious to the neurons: reduced spontaneous motility of filopodia is one of the first signs of deteriorating health, however this has not been observed in the experiments. From these results, I infer that BDNF does not induce rapid morphological changes in filopodia length within few minutes. It is more likely that BDNF-mediated structural changes occur within several hours to days.
Support for this suggestion also exists for spines, which are thought to replace filopodia during development to form mature and more persistent synaptic connections. Influencing BDNF signaling for several hours up to days and weeks has shown to promote changes in dendritic spine density and structure (Murphy et al., 1998; Shimada et al., 1998; Horch et al., 1999; Horch and Katz, 2002; Tyler and Pozzo-Miller, 2001; Tyler and Pozzo-Miller, 2003; Ji et al., 2005): continuous treatment with exogenous BDNF stimulates the formation and maturation of spines and increases spine density in Purkinje neurons (Shimada et al., 1998) and in apical dendrites of CA1 pyramidal neurons of hippocampal slice cultures (Tyler and Pozzo-Miller, 2001). Furthermore, overexpression of BDNF in pyramidal neurons of slices from visual cortex causes local destabilization of spines and an increased turnover rate suggesting that this local dendritic instability facilitates activity-dependent morphological changes and synaptic remodeling (Horch et al., 1999). Later, Horch and Katz reported that focal

BDNF release from 'BDNF overexpressing donor neurons' elicits local growth of dendritic branches of neighboring neurons, but does surprisingly not alter the density, length and motility of dendritic spines and filopodia (Horch and Katz, 2002). Moreover, BDNF binding to $p75^{NTR}$ has been shown to negatively modulate dendritic complexity and spine density in mature hippocampal neurons and to act antagonistically to TrkB receptor signaling. This suggests that BDNF can modulate neuronal morphogenesis by a bidirectional - growth-promoting as well as growth-inhibiting - mode (Zagrebelsky et al., 2005). Recently, different roles of BDNF-TrkB-signaling in structural plasticity in distinct brain areas have been proposed *in vivo*: whereas in the adult visual cortex expression of dominant-negative TrkB-EGFP caused a reduced maintenance of mushroom-like spines accompanied by an increase of long and thin spines and filopodia, the maintenance of spines in the CA1 region of the hippocampus was unaffected (Chakravarthy et al., 2006). Together, these findings indicate that BDNF is a signaling molecule that acts instructively to modulate particular patterns of dendritic arborization and effects spine density within hours to days. However, BDNF-mediated structural changes e.g. in spine density or filopodia growth within shorter periods of time (in the range of minutes) have not yet been reported. I conclude that morphological changes in filopodia motility and growth upon BDNF addition occur on longer time-scales, probably in the range of hours. One possible explanation for this could be that BDNF first stimulates the modulation of functional properties of neurons before morphological effects are visible.

6.3.2 Brief stimulation with BDNF induces plasticity of calcium signaling

Next, I investigated a possible BDNF-TrkB-mediated effect on functional plasticity that might occur on the level of dendritic calcium activity. Do particular sites along the dendrite such as developing synapses show functional changes after focal BDNF stimulation, e.g. generate more or fewer local calcium transients in comparison to before? Generally, I observed an increase in the frequency of local calcium transients after focal BDNF pulses to the dendrite (Fig. 5.14A and B). This effect was already evident 5-10 min after brief stimulation with BDNF and stable over time (5 min - 2h, rapid and long-lasting) (Fig. 5.14C). Such an effect is reminiscent of the mechanisms of BDNF in acute modulation of synaptic efficacy and long-term changes in synaptic connectivity (Lohof et al., 1993; Knipper et al., 1994; Kang and Schuman, 1995; Levine et al., 1995; Thoenen,

1995; Bonhoeffer, 1996; Levine et al., 1998; Poo, 2001; Korte et al., 1995; Korte et al., 1996; Patterson et al., 1996; Kossel et al., 2001; Kovalchuk et al., 2002). During continuous presence of the neurotrophin BDNF, a fast increase in the frequency of mEPSCs has been reported which, however, went back to baseline after 5-10 min (Lessmann et al., 1994). In contrast, I did not observe any reduction of the BDNF-mediated increase in local calcium activity suggesting that the spatially and temporally restricted application of BDNF is the appropriate stimulus for inducing a rapid and long-lasting change in plasticity. It can be assumed that this kind of stimulus also mimics the natural conditions, because, as argued above, also intrinsic BDNF signaling is fast and localized. Interestingly, this effect was evident exclusively as an increase in the frequency of local calcium transients, not in the amplitude. This excludes the possibility that the observed increase in frequency after focal BDNF application was due to an increase of amplitudes above the detection level. The question arises whether the same sites along the dendrite are more active after BDNF stimulation than before or new sites which were 'silent' before became activated by focal BDNF application. Both scenarios are conceivable and can be easily distinguished from each other. I observed that active sites along the dendrite sometimes showed increased activity in local calcium signaling after brief BDNF stimulation. The probability of activating previously 'silent' sites, however, was significantly higher (Fig. 5.15B and D). This indicates that stimulation with BDNF preferentially activates sites along the dendrite that did not generate local calcium activity before. Together with the preliminary data of the PSD-95:CFP expressing neurons, in which 9 out of 9 PSD-95 puncta that revealed an increase of local calcium transients after focal BDNF stimulation were silent before BDNF application (Fig. 5.16), these results suggest that BDNF can induce long-lasting functional plasticity, possibly on the level of individual synapses.

There is substantial evidence that BDNF is one of the key mediators for synapse maturation. BDNF can act at three levels to regulate synapse function in an activity-dependent manner during development: it can promote synaptogenesis, it can influence the maturation of developing synapses and, finally, it can stabilize and potentiate existing synapses (Vicario-Abejón et al., 2002). The BDNF-mediated conversion of pre- as well as postsynaptically silent synapses to functional synapses has been reported in a number of studies: at the presynaptic site, BDNF facilitates transmitter release from presynaptic terminals by increasing the total number and the number of docked synaptic vesicles by modulating the rapidly recycling pool at hippocampal excitatory synapses (Fig. 6.2) (Pozzo-Miller et al., 1999; Tyler and Pozzo-Miller, 2001; Collin et al., 2001;

Tyler et al., 2006). Moreover, the frequency, but not the amplitude of mEPSCs is increased after BDNF treatment which is generally attributed to a change in the probability of neurotransmitter release (Lohof et al., 1993; Kang and Schuman, 1995; Figurov et al., 1996; Lessmann and Heumann, 1998). Additionally, in hippocampal cultures, the application of BDNF potentiates preferentially immature synapses with lower release probability without affecting the nearby mature synapses (Lessmann et al., 1994; Lessmann and Heumann, 1998; Berninger et al., 1999). In the context of unsilencing of presynaptically silent synapses, presynaptic activation of TrkB receptors by BDNF followed by activation of the Cdc42 signaling pathway is involved; this leads to the remodeling of the actin cyotskeleton which may be required for presynaptic maturation (Shen et al., 2006). On the postsynaptic site, BDNF has been shown to affect NMDAR function in hippocampal cultures by increasing the open probability of their channels (Levine et al., 1998) and enhancing NMDAR transmission through activation of NR2B subunits (Crozier et al., 1999). Additionally, BDNF is known to be involved in the maturation of postsynaptically silent synapses: it regulates AMPAR trafficking into postsynaptic sites, which involves a transient calcium increase, and thereby converts NMDAR-only synapses into AMPAR-transmissible synapses (Itami et al., 2003). Moreover, it has been reported that BDNF strengthens excitation primarily by augmenting the amplitude of AMPAR-mediated mEPSCs consistent with a postsynaptic mechanism (Bolton et al., 2000). It is likely that BDNF exerts its action not only pre- or postsynaptically, but perisynaptically: BDNF might act as a retrograde messenger which is released from the postsynaptic neuron in an activity-dependent manner and induces presynaptic long-term plasticity. Presynaptic plasticity, especially the appearance of new functional release sites has been shown to be strictly dependent on postsynaptic NMDAR activation and requires the retrograde messenger BDNF (Walz et al., 2006). Similarly, subthreshold postsynaptic depolarisation has been reported to elicit calcium-dependent release of BDNF that diffuses retrogradely and thereby enhances presynaptic transmitter release (Magby et al., 2006).

In conclusion, a presynaptic locus of BDNF action seems to be crucial for the promotion of excitatory synaptic development, but also a postsynaptic mechanism is involved. Interestingly, in the bolus loading experiments of slices preincubated with tetanus toxin (TeTX) for 12 h (Fig. 5.5) I observed a BDNF-mediated effect that suggests a presynaptic mechanism of its long-term action: if the slices after preincubation with TeTX were not kept continuously in a TeTX-containing solution, but in normal HBSS solution (after bolus loading for $\geq$1 h before using them for the experiments) I observed no spontaneous calcium

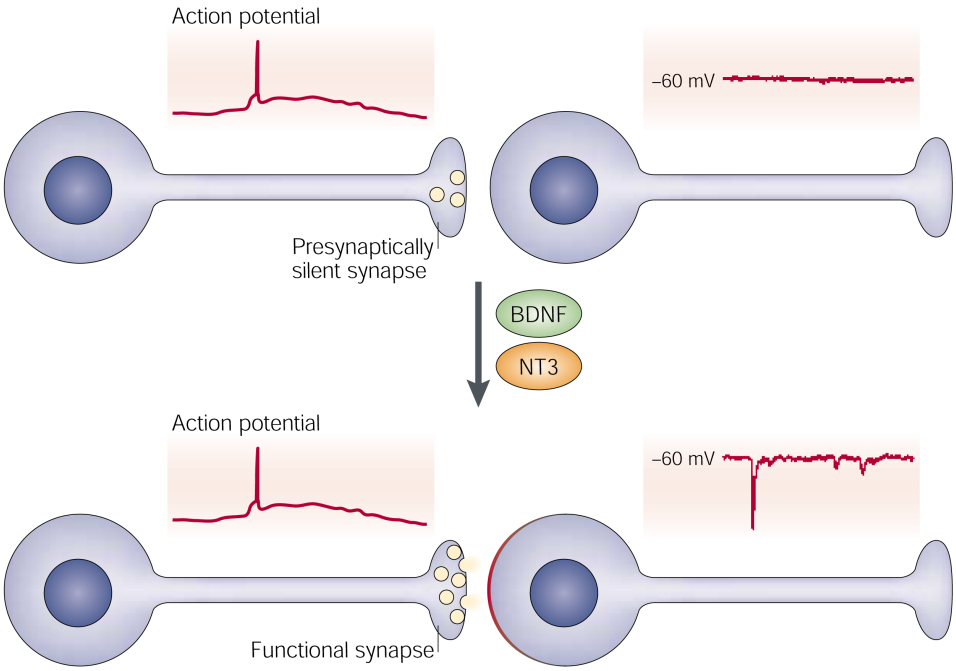

Figure 6.2: Establishment of presynaptically functional synapses through the action of neurotrophins., In the absence of neurotrophins, developing neurons are functional silent (upper panel). BDNF and Neurotrophin 3 increase the probability of neurotransmitter release at the presynaptic terminal and induce the establishment of functional synapses (taken from a review by Vicario-Abejon, 2002).

activity in the CA3 region, but upon BDNF bath application a sustained and strong increase in the network activity like in the control slices. This effect was not visible in slices kept continuously in TeTX-containing solution. Here, the BDNF-mediated modulation of network activity was blocked due to a dysfunctional presynaptic release machinery. This observation suggests that within one hour in TeTX-free solution, the presynaptic release machinery in the active zone of axonal terminals recovered partly and could be activated by BDNF. Independently of the functionality of the postsynapse, the potentiating effect of BDNF on the network activity seemed to be a consequence of the activation of presynaptic function. Although the two possibilities of pre- and postsynaptic action of BDNF are not mutually exclusive, the majority of publications and also my observation point toward a crucial role of the presynapse in BDNF-regulated maturation of excitatory synapses. Possibly the maturation of the

presynaptic transmitter-release apparatus is the first step to further promote the maturation of the postsynaptic site. The following picture might emerge for the long-term action of BDNF and its function in synapse formation: developing neurons respond to extracellular signaling molecules like BDNF by generating local calcium transients which may regulate the maturation and stabilization of synapses and control neuronal circuit formation - how exactly this process is accomplished still remains to be determined.

6.4 Concluding remarks and Outlook

It would be interesting now to examine the relationship between the two phenomena described in this thesis. Is the fast BDNF-evoked local calcium signaling which takes place primarily at synaptic sites a requirement for the long-lasting functional plasticity change? Alternatively, these two observations could also be completely independent of each other. To address this question, one needs to characterize the modulatory effect of temporally and spatially restricted BDNF stimulation on long-term functional plasticity in calcium signaling: is the functional plasticity effect conserved if, for instance, the fast BDNF-mediated dendritic calcium response is inhibited by sodium- or calcium channel antagonists? Such a finding would argue for the involvement of other, probably slower signaling cascades in the long-term functional change of calcium signaling. Furthermore, uncovering the role of enhanced presynaptic transmitter release in the sustained increase of local calcium activity could be accomplished by blocking specifically TrkB receptors of the postsynaptic neuron during focal BDNF stimulation. Revealing those and related questions is particularly interesting and requires further study.
Although there is increasing evidence that intrinsic BDNF-TrkB-signaling promotes the formation, maturation and stabilization of synapses in the CNS, the underlying cellular and molecular mechanisms that mediate the action of BDNF at synapses are still partly unknown. Since BDNF is known to be responsible for innumerable neuronal signaling processes, it will be challenging to complete the picture and bring together fast and slow signaling processes which regulate different cellular phenomena. It will be important to visualize how temporally and spatially restricted availability of BDNF contributes to different signaling pathways and how constitutive and activity-dependent secretion of BDNF regulate the formation and stabilization of new and existing synapses. Solving these kinds of questions will help to further understand synaptic development and plasticity.

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8 Publications

Publications based on the work in this thesis:

Lang SB, Stein V, Bonhoeffer T and Lohmann C (2007)

Endogenous Brain-Derived Neurotrophic Factor Triggers Fast Calcium Transients at Synapses in Developing Dendrites

Journal of Neuroscience, 27 (5): 1097-1105

Lang SB, Bonhoeffer T and Lohmann C (2006)

Simultaneous imaging of morphological plasticity and calcium dynamics in dendrites

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10 Curriculum vitae

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Publications

Lang SB, Stein V, Bonhoeffer T and Lohmann C (2007)

Endogenous Brain-Derived Neurotrophic Factor Triggers Fast Calcium Transients at Synapses in Developing Dendrites

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