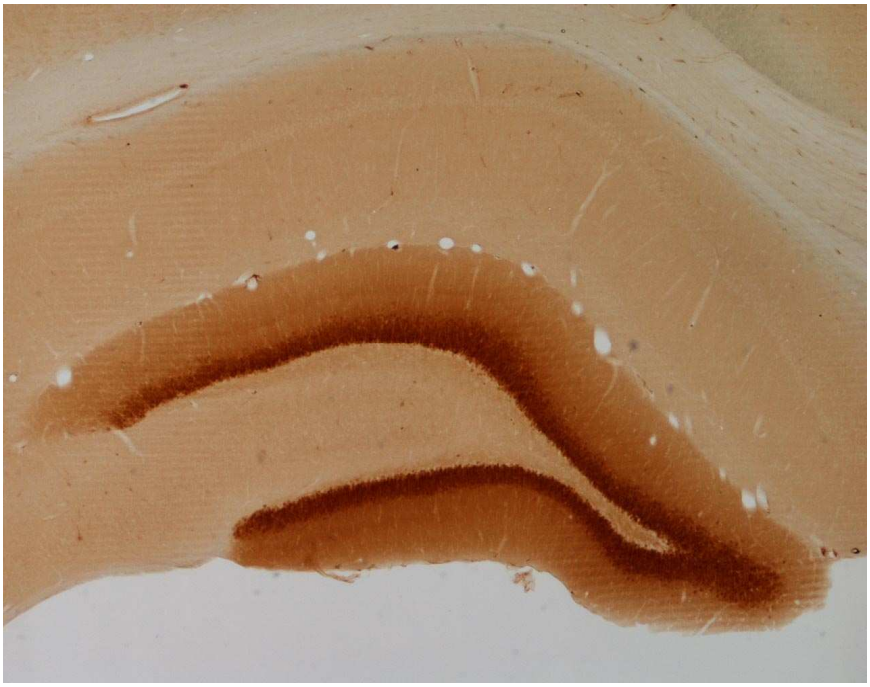
LTP mechanisms in the dentate gyrus in vivo:

BDNF signaling, translation control, and gene function

Tambudzai Kanhema



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Contents

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LIST OF ABBREVIATIONS

ACD	Actinomycin D
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arc	Activity-regulated cytoskeleton-associated protein
BDNF	Brain-derived neurotrophic factor
CA	Cornu Ammonis
CaMKII	Calcium and calmodulin-dependent protein kinase II
CNS	Central nervous system
EC	Entorhinal cortex
eEF2	Eukaryotic elongation factor 2
eIF4E	Eukaryotic initiation factor 4E
ERK	Extracellular signal-regulated protein kinase
GABA	Gamma amino-butyric acid
HFS	High-frequency stimulation
IEG	Immediate early gene
LPP	Lateral perforant path
LTP	Long-term potentiation
LTD	Long-term depression
MAPK	Mitogen-activated protein kinase
MEK	Mitogen and extracellular signal regulated protein kinase
MPP	Medial perforant path
mTOR	Mammalian target of rapamycin
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
PSD	Postsynaptic density
SDs	Synaptodendrosomes
Trk	Tropomyosin-like receptor kinase

LIST OF ORIGINAL PAPERS

This doctoral thesis is based on the following papers, referred to by their roman numerals in the text.

I. Messaoudi, E., Ying, S-W., **Kanhema, T.,** Croll, S.D., and Bramham, CR (2002) Brain-derived neurotrophic factor triggers transcription-dependent, late phase long-term potentiation *in vivo J Neuroscience*.22:7453-7461

II. **Kanhema, T.,** Dagestad, G., Panja, D., Tiron, A., Messaoudi, E., Håvik, B., Ying, S-W., Nairn, A., Sonenberg, N., and Bramham, CR (2006) Dual regulation of translation initiation and peptide chain elongation during BDNF-induced LTP *in vivo*: evidence for compartment-specific translation control. *J.Neurochemistry* 1111: 1471-4149

III. Messaoudi,E, **Kanhema, T**[#], Soulé, J[#], Tiron, A[#], Dagyte, G, da Silva, B, and. Bramham, CR (2006) Sustained Arc synthesis controls LTP consolidation through regulation of local actin polymerization in the dentate gyrus *in vivo* (*Manuscript).*

[#] Dual second authorship.

Introduction

Synaptic plasticity

Neuronal transmission occurs primarily through chemical synaptic transmission. Long-term adaptive brain response such as memory storage are thought to require lasting changes in synaptic strength, a property known as synaptic plasticity. Classical paradigms for studying activity-dependent synaptic plasticity in the mammalian brain are long-term potentiation (LTP) and long-term depression (LTD) (Malenka and Bear, 2004; Bliss and Collingridge, 1993). In 1949, Donald Hebb predicted a form of synaptic plasticity driven by temporal contiguity of pre- and post-synaptic activity. This prediction was verified decades later with the discovery of long-term potentiation (LTP), wherein high-frequency stimulation of afferent fibers produces an enduring increase in synaptic strength (Bliss and Lomo, 1973). Intensive research on LTP since the original description by Bliss and Lomo has given fundamental insights into synapse physiology and the cell biological mechanisms underlying activity-dependent changes in synaptic strength.

Growing evidence implicates LTP-like mechanisms in memory processes in many brain regions including the hippocampal formation (Morris et al., 2003, Pastalkova et al., 2006). The hippocampal formation plays a critical role in memory in rodents and humans, and much of what we know about the mechanisms of LTP has come from studies of rodent hippocampus. This thesis explores LTP mechanisms in the rat dentate gyrus, a critical component of the hippocampal network.

The hippocampal formation

The hippocampus, buried deep in the temporal lobe near the center of the brain stem, is only visible when the overlying cortex is removed. The hippocampus is one of a group of closely interconnected structures within the limbic system called the hippocampal formation (HF). The hippocampal formation in mammals is crucial for spatial learning and memory. The hippocampus is a bilateral limbic structure which resembles two "Cs" leaning together at the top and spread apart at the base. One "C" makes up Ammon's horn or *cornu ammonis* (CA1-CA3), also known as the "hippocampus proper". The principle cell layer of Ammon's Horn is the *stratum pyramidale*, or the pyramidal cell layer. The other "C" is made up of the dentate gyrus, of which the *stratum granulosum*, or granule cell layer is the principle cell layer (Amaral and Witter, 1989). When the hippocampus is cut transverse to its longitudinal (septal-temporal) axis, it shows a set of three pathways known as the trisynaptic circuit or loop (Andersen et al., 1966; Swanson, 1977; Amaral and Witter, 1989). Sensory information enters the hippocampal formation through inputs from the entorhinal cortex (EC). Stellate cells of the entorhinal cortex project to dentate granule cells via the perforant path, granule cells projects to CA3 pyramidal cells via the mossy fiber pathway, and CA3 neurons project to CA1 pyramidal cells via the Schaffer collateral pathway (Amaral and Witter, 1989).

The perforant path input to the dentate gyrus is divided into lateral and medial components arising from the lateral and medial entorhinal cortex, respectively. The lateral and medial perforant path fibers innervate the outer-third and middle-third of the granule cell dendritic tree, respectively, creating discretely segregated laminar inputs. In the present work, synaptic plasticity was studied in the medial perforant path input to the dentate gyrus.

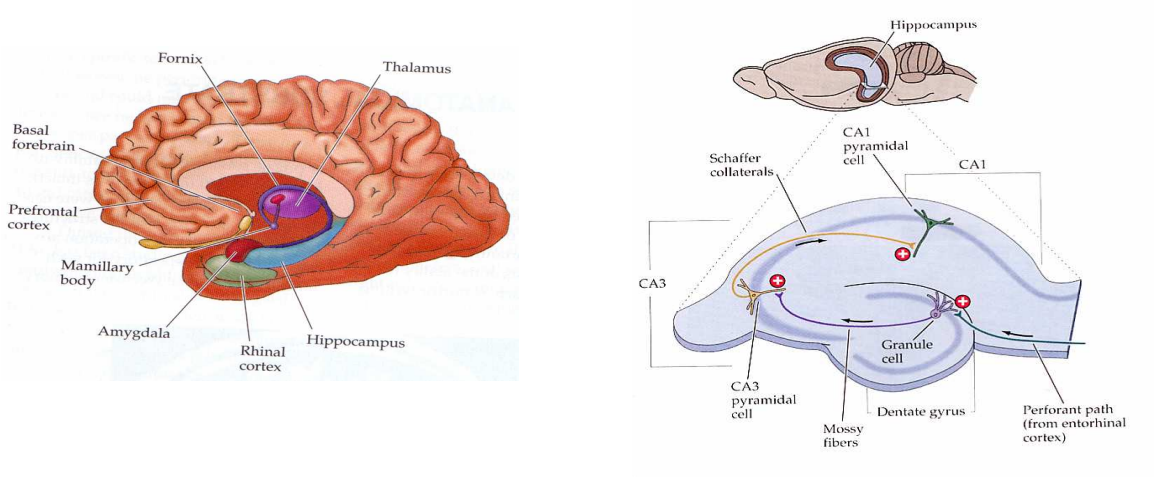

Fig 1.The hippocampal trisynaptic circuit.
Schematic representation of the major synaptic excitatory circuitry in the hippocampus. The tri-synaptic circuit includes three excitatory pathways: fibers from the perforant path innervate granule cell dendrites.Mossy fibers make synapses on CA3 pyramidal cells and the Schaffer collaterals synapses on CA1 pyramidal cell dendrites. Adapted from (Amaral and Witter,1989)

Glutamate is the predominant mediator of fast excitatory synaptic transmission in the hippocampus and elsewhere in the CNS. Although a detailed description of glutamatergic transmission is beyond the scope of this thesis, the most relevant points are to be noted. Excitatory synapses onto projection neurons (pyramidal cells and granule cells in the hippocampus) occur almost exclusively on dendrite spines, often numbering more than 100,000 per neuron. The ionotropic glutamate receptors alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) are located primarily in the plasma membrane on the head of the spine. Glutamate generates an excitatory postsynaptic potential (EPSP) primarily through activation of AMPA receptors. Opening of the AMPA receptors allows Na^{2+} influx, causing depolarization of the postsynaptic neuron. The NMDA receptor has unique properties that come to use in activity-dependent synaptic plasticity. Unlike AMPA receptors, NMDA receptor activation requires membrane depolarization in addition to glutamate binding. The NMDA receptor gates a cation channel with high calcium permeability. The channel is blocked by magnesium ion in a voltage-dependent manner. Strong depolarization, as mediated by AMPA receptors during repetitive or synchronous activation by glutamate synapses,

relieves the magnesium block, allowing calcium to enter the spine. NMDA receptor-gated calcium influx is critical for both LTP and LTD.

Glutamate is typically not the only neurotransmitter found at excitatory synapses. As discussed in detail later, the peptide brain-derived neurotrophic factor (BDNF) appears to have a co-neurotransmitter role at glutamate synapses. Opioid peptides are also co-stored with glutamate in certain hippocampal pathways, where they have important roles in regulating LTP and LTD (Bramham et al., 1996).

Transmission within and between subfields is strongly influenced by inhibitory GABAergic interneurons. This inhibition broadly influences synaptic integration, neuronal excitability and synchronization, within the hippocampus. Specialized interneuron classes have evolved to set the threshold for activation (Andersen et al., 1963), shunt excitatory synaptic inputs (Qian and Sejnowski, 1990), prevent the backpropagation of fast action potentials in the dendrites (Buzsaki et al., 1996; Tsubokawa and Ross, 1996), inhibit dendritic Ca^{2+} electrogenesis (Miles et al., 1996), and synchronize sub- and suprathreshold membrane potential oscillations in spatially distributed principal cells (Cobb et al., 1995) (Whittington et al., 1995). Interneurons are also frequently the targets for subcortical pathways (Buzsaki, 1984) such as those originating in the medial septum (cholinergic and GABAergic), raphe nuclei (serotonergic), and locus coeruleus (noradrenergic) (Freund and Antal, 1988).

The most prominent class of interneurons in the dentate gyrus is the basket cell, located at the junction of the granule cell layer and the polymorphic cell layer (hilus). The basket cells are an important cell type in the dentate gyrus because their axon terminals form a dense plexus within the granule cell layer. The basket cells consist of five morphological types that have different dendritic arborization and localization (Ribak and Seress, 1983; Ribak, 1992). Some interneurons are also present in the molecular layer. One of these interneurons is

the axo-axonic cell, which terminates on the axon initial segments of granule cells (Freund and Buzsaki, 1996).

Long-term potentiation

LTP is the most studied form of activity-dependent synaptic plasticity in the mammalian brain. Although LTP occurs throughout the nervous system, it has been studied most extensively in the hippocampus (Bliss and Collingridge, 1993). As alluded to above, induction of LTP by high-frequency stimulation requires calcium influx through NMDA receptors (Bliss and Richter Levin, 1993; Malenka and Nicoll, 1999). Postsynaptic calcium levels can also be critically modulated by mobilization from intracellular stores and activation of L-type, voltage-dependent calcium channels (Kandel, 2001).

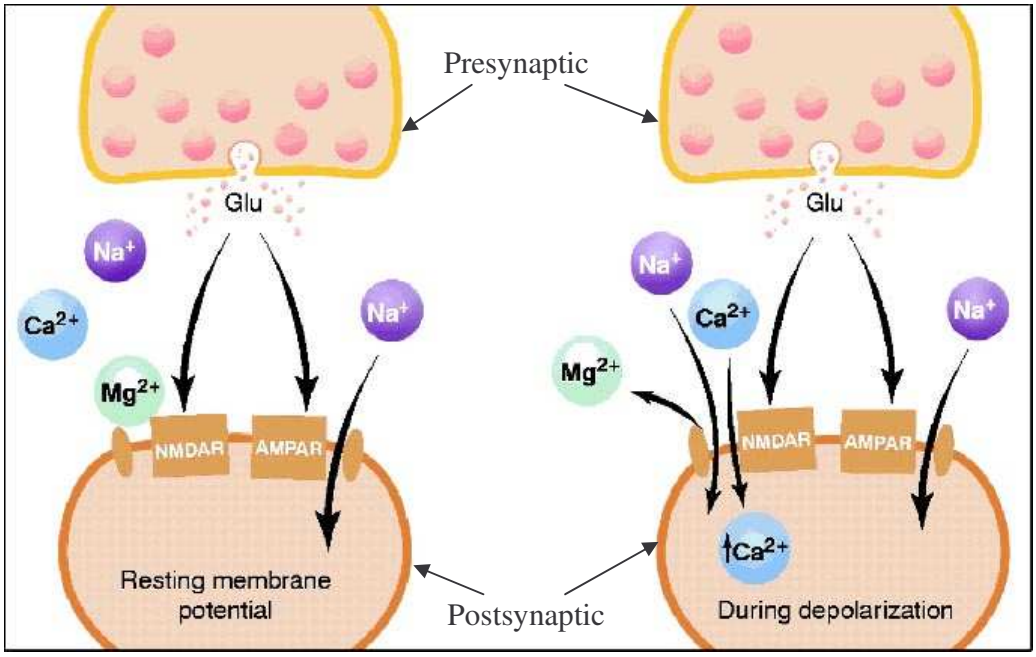

Fig 2. Model of LTP induction (H Sebastian Seung, 1999)

LTP can be separated into at least two temporally distinguished forms that require distinct mechanisms for their maintenance (Malenka and Bear, 2004; Abraham and Williams, 2003) the early-phase LTP (E-LTP) and late-phase LTP (L-LTP). E-LTP can be experimentally induced by a single, high-frequency stimulation (HFS). The early phase of LTP is controlled in large part by activation of calcium-sensitive protein kinases including protein kinase C (PKC), calcium/calmodulin dependent protein kinase II (CaMKII), and protein kinase A (PKA) (Otmakhova et al., 2000). Activation of extracellular-signal regulated protein kinase (ERK) (English and Sweatt, 1997; 2001), through receptor-coupled activation of the Ras signaling cascade is also critical. Thus, early LTP is maintained through rapid phosphorylation of proteins present at the synapse. The preeminent example of this is phosphorylation of the AMPA receptor by CaMKII, leading to enhanced single-channel conductance and thus larger currents in response to glutamate binding (Lisman and Raghavachari, 2006). There is also evidence for lasting increases in glutamate release during LTP. The effect appears to be mediated by retrograde signaling. Nitric oxide, neural cell adhesion molecules, ephrins, and perhaps BDNF, may rapidly convey information from postsynaptic spines to the nerve terminal.

L-LTP, in contrast to E-LTP, requires new gene expression and protein synthesis and can persist for hours or days (Frey et al., 1988; Bourtchuladze et al., 1994; Nguyen et al., 1994). PKA, ERK, CaMKII, CaMKIV act through a range of transcriptional regulators to modulate gene expression in LTP. Members of the transcription factor family cAMP response-element binding protein (CREB) are important components of the switch that converts short-term to long-term synaptic plasticity (Dash et al., 1990; Bourtchuladze et al., 1994; Bartsch et al., 1998). CREB phosphorylation on Ser-133 is necessary for CRE-dependent gene expression. However, CREB modulates genes that contribute to late LTP have not been defined. LTP also induces a variety of early response genes including the zinc-finger transcription factor zif268, and activity-regulated cytoskeleton-associated protein Arc, (also known as Arg3.1). Evidence prior to this thesis

showed the zif268 knockout mice were impaired in late phase LTP and long-term memory (Jones et al., 2001) although the targets for zif268 are unknown. Arc is intriguing because: 1) the mRNA is rapidly transported from granule cell soma into dendrites, and 2) the mRNA appears to be locally translated. (Link et al., 1995), (Lyford et al., 1995; Waltereit et al., 2001). Using antisense oligodeoxynucleotides to block Arc synthesis, (Guzowski et al., 2000) reported that Arc is necessary for long-term memory. Preliminary data suggested a possible effect of Arc antisense treatment on LTP. While interesting, this data was inconclusive in the absence of data from the baseline period prior to HFS.

Recent studies suggest that late phase LTP involves expansion of the synapse and growth of the spine head. This process of spine expansion is linked to polymerization of actin within the spine. However, there are no studies examining the possible link between activity-dependent gene expression, modulation of actin polymerization, and formation of stable LTP.

As mentioned above, evidence suggests that glutamate and BDNF act together at excitatory synapses. BDNF and other neurotrophic factors modulate gene expression to promote neuronal differentiation and outgrowth of neuronal processes during development. The powerful growth-promoting properties of neurotrophic factors, coupled with the discovery of these factors in mature brain, immediately suggested a possible link between neurotrophin signaling and activity-dependent alterations in adult neural networks.

Brain-derived neurotrophic factor and LTP

Neurotrophins are a small family of dimeric secretory proteins in vertebrate neurons with a broad spectrum of functions. They are generated as pro-proteins with a functionality that is distinct from the proteolytically processed form. Neurotrophins are required for cell survival and differentiation during embryonic development and maintain the structure and function of specific neural

systems in the adult brain (Lindsay and Seil, 1994; Lewin and Barde, 1996). The neurotrophin family consists of four members, nerve growth factor (NGF), brain-derived neurotrophin factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). They are produced as precursor proteins which are cleaved to mature proteins of 118-120 amino acids that associate as non-covalent homodimers. These closely related molecules act by binding to two distinct classes of transmembrane receptors. The p75 neurotrophin receptor ($p75^{NTR}$) and the Trk (tropomyosin-like receptor kinase) family of receptor tyrosine kinases, which includes TrkA, TrkB and TrkC (Kaplan and Miller, 2000). The p75 receptor (low-affinity neurotrophin receptor) is common to all four members of the neurotrophin family. The Trk family of receptor tyrosine kinases proteins are the high affinity neurotrophin receptor.

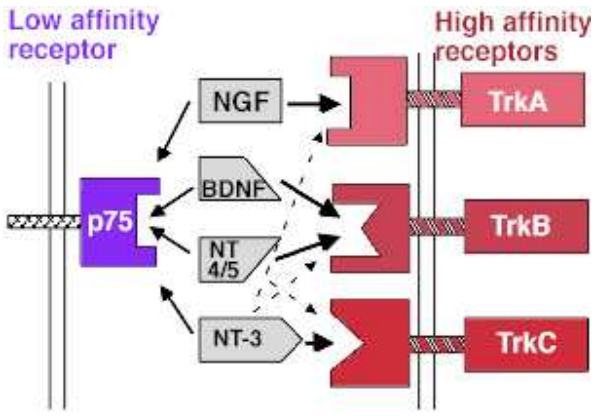

Fig 3 Neurotrophin family peptides bind to distinct Trk receptor tyrosine kinases and a common low-affinity neurotrophin receptor p75. Proneurotrophins bind selectively to p75. (Kirmo Waartiovarra,1998)

The signal transduction pathways known to be activated by Trk receptors are those leading to activation of MAP kinase, PI3 kinase and phospholipase Cγ (Kaplan and Miller, 1997). MAP kinase pathways activated by Trk receptors activate Erk1 and Erk2 at the terminus stimulating axonal growth, and PI3K activates AKT in the terminus as well. Recent work has show that neurotrophins

have effects on synaptic transmission and that they may be involved in the regulation of neuronal plasticity in both developing and mature CNS (Kim et al., 1994; Lessmann et al., 1994; Kang and Schuman, 1995; Levine et al., 1995; Thoenen, 1995; Bonhoeffer, 1996; Lu and Figurov, 1997; Messaoudi et al., 1998). BDNF which is the most widely distributed neurotrophin in the adult brain has been studied extensively, but mostly in cell culture. However, in the mid-1990s evidence began accumulating that BDNF contributes to activity-dependent synaptic plasticity, most notably LTP in the hippocampus (Kang and Schuman, 1995; Messaoudi et al., 1998; Korte et al., 1995; Patterson et al., 1992; Schuman, 1999; Schinder and Poo, 2000; Ying et al., 2002).

BDNF and its cognate receptor TrkB have emerged as a major regulator of synaptic transmission and plasticity at adult synapses in many regions of the CNS (Bramham and Messaoudi, 2005). It has been shown pharmacologically and genetically that the binding of BDNF to its receptor TrkB is essential for LTP maintenance at Schaffer collateral-CA1 synapses (Korte et al., 1995). LTP at these synapses is greatly reduced in BDNF homozygous and heterozygous mutant mice and it can be rescued by infusing exogenous BDNF (Korte et al., 1996; Patterson et al., 1996). Tetanic stimulation enhances the expression of BDNF mRNA in the hippocampus (Patterson et al., 1992; Castren et al., 1993); Bramham et al., 1996) and activity-dependent release of BDNF protein has also been shown by (Goodman et al., 1996). BDNF signaling through TrkB activates several intracellular signaling cascades (Segal and Greenberg, 1996; Gooney et al., 2004) which may directly elicit Ca^{2+} release from intracellular stores via IP_3 production, or indirectly modulate Ca^{2+} influx through voltage-gated Ca^{2+} channels or NMDA receptors (Kovalchuk et al., 2002).

Local intrahippocampal infusion of BDNF induces long-term potentiation (BDNF-LTP) at medial perforant path-granule cell synapses in the adult rat in vivo. Ying et al., (2002) went on to show that BDNF-LTP requires activation of extracellular signal-regulated kinase (ERK), coupled to ERK-dependent phosphorylation of CREB and up regulation of Arc. As observed during HFS-

induced LTP, Arc mRNA is rapidly induced in postsynaptic granule cells and widely transported to dendrites. Local infusion of ERK inhibitors (PD98059, U0126) or the RNA synthesis inhibitor actinomycin D blocked BDNF-LTP and the associated upregulation of Arc. These results coupled BDNF-LTP to upregulation of Arc. However, no causal role for the Arc synthesis in HFS-LTP or BDNF-LTP was established and the function of the protein is unknown. In addition, a series of in vitro studies showed that BDNF can stimulate protein synthesis in isolated dendrites of primary hippocampal neuronal cultures (Aakalu et al., 2001), and evidence from acute hippocampal slices suggests that local protein synthesis is critical for BDNF-LTP at CA3-CA1 synapses (Kang et al., 1996).

Dendritic protein synthesis

A recent surge of studies has demonstrated extrasomatic synthesis of proteins in dendrites and axons (Pierce et al., 2000; Steward and Schuman, 2003; Ju et al., 2004; Feig and Lipton, 1993; Eberwine et al., 2001; Steward and Schuman, 2001, Wu and Baer, 1998; Aakalu et al., 2001; Kacharmina et al., 2000). While axonal synthesis has been seen primarily in developing neurons, dendritic mRNA localization and protein synthesis is a common property of mature neurons (Steward and Schuman, 2003; Wells and Fallon, 2000a; Klann and Dever, 2004). Polyribosomes have been shown to be located near the base of many spines in the hippocampal projection neurons (Steward and Levy, 1982). A variety of mRNAs as well as components of the translational machinery are also present in dendrites (Steward and Schuman, 2001).

At synaptic sites, activity-dependent translation could serve to modulate the structure and function of dendritic branches and spines, perhaps in a synapse-specific manner. While numerous of mRNA species have been localized to dendrites of cultured neurons, less than a dozen different mRNAs have been localized to dendrites of mature neurons (Steward, 1997). Extracellular stimulation of dendrites by BDNF, glutamate, dopamine and other factors has been shown to regulate the translation of mRNAs (Steward et al., 1998; Smith et

al., 2001; Aakalu et al., 2001). Several lines of evidence suggest a critical role for local protein synthesis in LTP consolidation. While this notion is generally accepted, direct evidence is lacking.

The majority of dendritic mRNAs are constitutively stored in dendrites in large ribonucleoprotein particles also known as RNA storage granules. mRNA for the alpha subunit of αCaMKII is the best example of such on mRNA (Steward and Levy, 1982; Wells and Fallon, 2000b; Steward, 1997). Arc has captured particular interest in the context of synaptic plasticity, as the Arc messenger is transported to dendrites during LTP. Arc mRNA can also be localized to specific synaptic inputs in response to intensive synaptic activity.

IEGs are thought to couple changes in synaptic activity to stable changes in synaptic efficacy seen in LTP. Mice harboring a constitutive knockout of the the zinc-finger transcription factor zif268 show impaired LTP consolidation and long-term memory. However, the knockout approach does not reveal the dynamic function of the activity induced mRNA and protein during the process of LTP induction(Ying et al., 2002) showed that BDNF-LTP is associated with induction of Arc but not zif268 mRNA, suggesting the BDNF may selectively stimulate mechanisms associated with Arc. In this thesis examined the dynamic function of Arc using antisense oligodeoxynucleotides to block synthesis of Arc during the induction and maintenance of HFS-LTP and BDNF-LTP

Translation control

L-LTP is blocked by both inhibitors of translation and transcription (Frey et al., 1991; Nguyen and Kandel, 1997; Kelleher et al., 2004a) This shows that L-LTP is involved in transcription and translation process. The protein products required for the establishment of long-term synaptic plasticity are thought to be utilized by activated synapses to stabilize modifications in synaptic strength (Kelleher et al., 2004a). While the basic requirement for protein synthesis is known, the notion that synaptic activity may control and modulate the level of

protein synthesis is new. In recent years, a variety of biochemical mechanisms have emerged whereby neural activity affects both global and transcript-specific translation of mRNA. This is particularly exciting given the discovery of dendritic protein synthesis. We have focused on two key translation factors governing the initiation and elongation steps of protein synthesis.

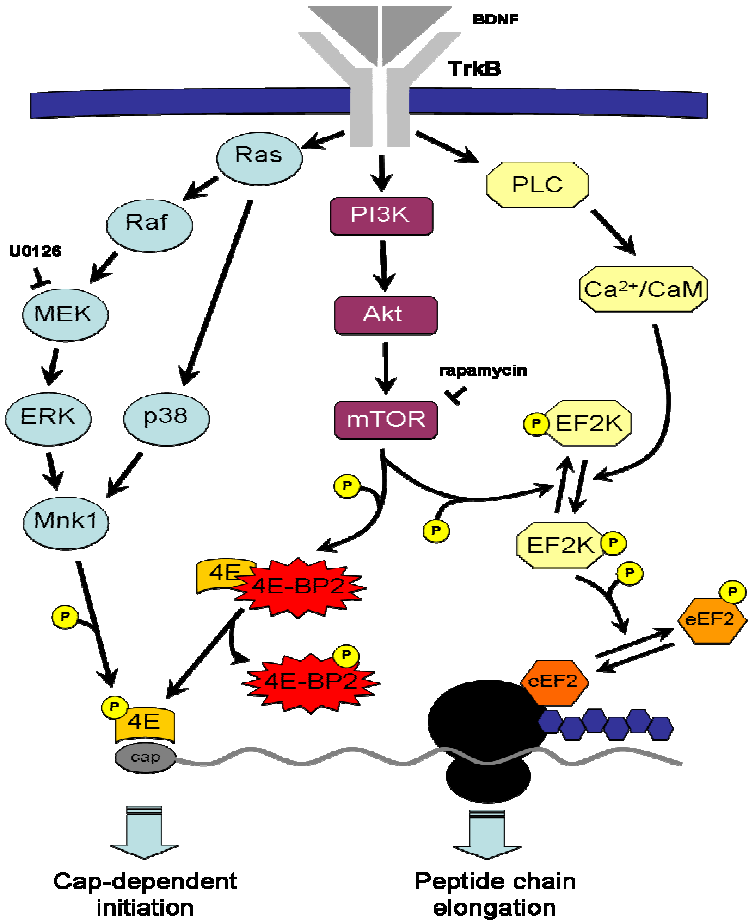

Figure 4. TrkB and translation control in dendritic spines.
The cartoon depicts some of the major signaling pathway coupling TrkB with regulation of eIF4E and eEF2. TrkB activation of PI3K-mTOR and Ras-ERK promotes eIF4E phosphorylation and enhances translation initiation. Phosphorylation of eEF2 stalls ribosomes and arrests peptide chain elongation. BDNF-TrkB signaling has bidirection effects of eEF2 phosphorylation. In isolated synaptodendrosomes, BDNF treatment has no effect on eEF2 phosphorylation state. (Soule et al., 2006)

Initiation factor 4E

In eukaryotic cells the rate of translation is regulated mainly at the initiation phase (Takei et al., 2001). Eukaryotic initiation factor 4E (eIF4E) plays an important role in mRNA translation by binding the 5'-cap structure of the mRNA and facilitating the recruitment to the mRNA of other translation factors and the 40S ribosomal subunit. eIF4E activity is regulated at multiple levels: (1) via modulation of its transcription, (2) by phosphorylation of the eIF4E on Ser^{209} protein and (3) through its interaction with a family of translational repressor proteins (Raught and Gingras, 1999). The mechanisms which regulate transcription of the eIF4E gene are not completely understood, but the eIF4E promoter was shown to contain two bona fide myc binding sites, both of which are required for expression of a heterologous reporter gene (Jones et al., 1996). Phosphorylation of eIF4E at Ser^{209} is induced by two characterized kinases, protein kinase C (PKC) and the MAP kinase-interacting protein 1 (MNK1) (Sonenberg and Gingras, 1998). MNK1 is a substrate for two distinct MAPKs-ERK and p38. eIF4E can interact with the scaffold protein eIF4G or with repressor proteins called eIF4E-binding proteins (4E-BPs) (Scheper and Proud) The activation of mTOR contributes to translational initiation by phosphorylating proteins that bind eIF4E, resulting in the dissociation of 4E-BPs from eIF4E and subsequent initiation of translation (Beretta et al., 1996). mTOR may also regulate translation by direct or indirect phosphorylation of other p70S6K and eIF-4G1 (Tang et al., 2001 Gingras et al., 2001). TrkB stimulates translation through activation of mTOR and ERK (Takei et al., 2001) Importantly, the immunosuppressant drug rapamycin, which inhibits mTOR, blocks both L-LTP and BDNF-LTP at CA3-CA1 synapses (Cammalleri et al., 2003; Tang et al., 2002)

Elongation factor-2

Eukaryotic elongation factor eEF-2 mediates regulatory steps important for the overall regulation of mRNA translation in mammalian cells and is activated by variety of cellular conditions and factors. eEF2 is a GTP-binding protein that mediates the translocation of peptidyl-tRNA from the A site to the P site on the ribosome (Moldave, 1985). Phosphorylation of eEF2 on Thr^{56} by eukaryotic elongation factor 2 kinase (eEF2 kinase), a Ca^{2+}/ calmodulin-dependent enzyme, causes an inhibition of eEF2 activity and a general reduction of peptide elongation (Nairn and Palfrey, 1987; Ryazanov and Davydova, 1989; Redpath and Proud, 1993). eEF2 is the sole substrate of this kinase and does not appear to be phosphorylated by any other kinase (Mitsui et al., 1993; Redpath and Proud, 1993). Three threonin residues located at the NH2 terminus of eEF2 can be phosphorylated by eEF2 kinase in vitro (Ovchinnikov et al., 1990; Price et al., 1991; Redpath and Proud, 1993). Increase in eEF2 phosphorylation on Thr^{56} have been reported in living cell exposed to stimuli known to raise intracellular Ca2+ levels (Palfrey et al., 1987; Mackie et al., 1989; Hincke and Nairn, 1992). Activation of NMDARs is associated with synaptic phosphorylation of eEF2, and, surprisingly, enhanced synaptic synthesis of αCaMKII (Scheetz et al., 1988; Scheetz et al., 1997, 2000).

While initiation and elongation are both highly regulated steps in protein synthesis, these events have never been examined together in the context of synaptic plasticity. Furthermore, little attention has been given to the possible subcellular compartmentalization of translation factor activity. In paper II of this thesis, I examined regulation of eIF4E and eEF2 during BDNF-LTP in the dentate gyrus.

Aims of the study

Aim: 1 To determine whether acute infusion of BDNF mimics late phase LTP.

Aim: 2 To determine the possible effect of BDNF-LTP on translation factors eIF4E and eEF2.

Aim: 3 To investigate a possible function and mechanism for Arc in HFS-LTP and BDNF-LTP.

Methods

The methodological details are given in the individual articles.

Results

A summary of the results from each paper is provided below.

Paper I

As previously reported by our laboratory (Messaoudi et al., 1998; Ying et al., 2002). BDNF infusion led to a slowly developing, stable increase in the medial perforant path-evoked fEPSP slope and population spike amplitude. Here, we investigated a number of basic features regarding BNDF-LTP. Based on tests of the minimal current needed to evoked a fEPSP, we conclude that BDNF-LTP, like HFS-LTP, is not associated with a change in the excitation of presynaptic fibers. Input-output curves were performed to assess changes in excitability (the EPSP-spike function). We report that BDNF-LTP, like HFS-LTP, is associated with an increase in EPSP-spike coupling in addition to the increase in synaptic efficacy. In other experiments, we examined the possibility that BDNF-LTP is

affected by application of test pulses. However, we find that omission of test pulses during and after BDNF infusion has no effect on the amplitude of BDNF-LTP. Next we asked whether NMDA receptor activation contributes to BDNF-LTP induction. Since BDNF is known to acutely increase glutamate release, it's possible that BDNF infusion induces LTP through a conventional NMDA receptor mechanism. However, we find that BDNF-LTP was induced in the presence of the NMDA receptor antagonist CPP, a treatment that blocked HFS-LTP in the same animal. The effect of BDNF on transcription was studied by using RNA synthesis inhibitor actinomycin D (ACD). Our results show that (1) the induction, but not the maintenance of BDNF-LTP, is transcription-dependent, and (2) ACD blocks the upregulation of the immediate early gene product Arc protein in dentate gyrus. Occlusion experiments were performed to test whether HFS-LTP and BDNF-LTP share a common mechanism of expression. Our results shows that BDNF applied during the early phase (1 hour after HFS-LTP) led to normal BDNF-LTP. In contrast BDNF-LTP was completely occluded when BDNF was infused in late phase HFS-LTP (4 hour after HFS-LTP). Thus, BDNF-LTP is occluded by expression of late, but not early, LTP. We conclude that BDNF can trigger a transcription-dependent late phase LTP at MPP synapses of the dentate gyrus in the intact brain.

Paper II

We first examined the possibility that BDNF-LTP involves dual regulation of the initiation and elongation steps of protein synthesis, using phosphorylation of eIF4E and eEF2 as molecular markers. BDNF infusion was carried out as in previous study (paper I) and cytochrome C, which has a similar molecular weight and charge as BDNF was used as control infusion. The brains were collected 15 minutes and 3 hours after the termination of the 25 minute BDNF/control infusion. Translation factors phosphorylation state was determined by Western blot analysis of the homogenized microdissected dentate gyrus. The levels of

phosphorylated eIF4E (ser^{209}) were significantly elevated 15 minutes after the termination of BDNF infusion and the total protein eIF4E was also elevated. At the same time point phosphorylation eEF-2 (Thr^{56}) was significantly elevated, but no change was observed in total protein. No significant increase was seen 3 hours after termination of BDNF infusion on either protein. The effects of local BDNF infusion on translation factors activity were specific to the dentate gyrus as no significant changes were detected in the CA1 and CA3 regions. Next we examined the effect of blocking BDNF-LTP using the MEK inhibitor U0126. We reported that ERK signaling is necessary for rapid phosphorylation of eIF4E and eEF2 following BDNF-LTP induction.

Immunohistochemistry was used to localized p-eIF4E and total eIF4E protein. Significantly enhanced staining was observed within the granule cell layer in the BDNF-treated dentate gyrus, whereas other hippocampal regions showed no change. Cyt C infusion had no effect on p-eIF4E and eIF4E staining. However the immunohistochemical method was not sensitive enough to detect dendritic staining.

In order to isolate possible differential regulation of eIF4E and eEF2 at synaptic sites, we turned to the *in vitro* synaptodendrosomes (SDs) preparation. Synaptodendrosomes are a biochemical fraction highly enriched in pinched off spines attached to pinched-off resealed axon terminals. SDs prepared from homogenized dentate gyrus were treated with BDNF or Cyt C, run in matched-pair design. A significant increase on phospho-eIF4E was detected after 5 minutes of incubation, but no change was observed in phospho-eEF-2. The lack of eEF2 phosphorylation is not due to lack of eEF2 activity in SDs or an anomaly of the method, since eEF2 phosphorylation was readily evoked by potassium depolarization. Thus, BDNF selectively phosphorylates eIF4E at synapses. Furthermore, eIF4E phosphorylation was paralleled by enhanced expression of CaMKII, indicating rapid local synthesis of the protein. These data support demonstrate dynamic ERK-dependent regulation of the eIF4E and eEF2 during BDNF-LTP in vivo. Furthermore, BDNF appears to selectively promote initiation

at synapses, whereas both initiation and elongation are modulated outside of the synaptodendritic compartment.

Paper III

Arc is an immediate early gene known to be rapidly transported to dendrites. Here we ask if Arc has a role in HFS-LTP and BDNF-LTP and begin to characterize that role. Using in situ hybridization and immunohistochemistry on brains collected at various time points (5 min, 30 min, 2 h, 3 h, and 4 h) after application of HFS, we report that HFS-LTP is associated with a rapid (5 min) and sustained elevation of Arc mRNA and protein that reached a plateau at 2-3 hours post-HFS. These results determined our selection of time points for application of Arc AS. LTP was transiently inhibited when Arc AS was applied 5 min before or 15 min after HFS, with peak depression at 1h and recovery by 3 h and Arc protein shows the same pattern of recovery. In contrast, Arc AS application at 2 h, but not 4 h, after HFS permanently reversed LTP. Scrambled AS had no effect at any time point. Importantly, treatment with a second AS sequence targeting a non-overlapping region of the Arc coding region produced a similar striking reversal of LTP. These data suggest early Arc protein has a role in early expression of LTP, while late or sustained Arc protein is necessary for consolidation of LTP.

To evaluate Arc protein knockdown quantitative immunoblot analysis was carried out 2 h after Arc AS infusion which occurred 2 h post HFS. The Arc protein knockdown was about 55 ± 10% when compared to scrambled-treated control. Expression of several relevant control proteins, including calcium/calmodulin-dependent protein kinase II (αCaMKII), the scaffolding protein (PSD-95), β-actin, and a series of actin-binding proteins, were unchanged. We then explored the idea that Arc regulates the local polymerization of F-actin. Using fluorescent phalloidin, we detected enhanced staining of F-actin specifically within the termination zone of the medial perforant

pathway in the middle molecular layer. This nascent band of F-actin was abolished upon treatment with Arc AS. Stabilization of actin by jasplakinolide prevented the reversal of LTP by Arc AS. Finally, 2 h after LTP Arc AS decreased hyperphosphorylation of cofilin, a major regulator of actin dynamics known to sever actin filaments unless phosphorylated.

In the BDNF-LTP paradigm, Arc AS given prior to BDNF blocked induction of BDNF-LTP and the associated upregulation of Arc protein. As seen during HFS-LTP, infusion of Arc AS 2 h, but not 4 h, after BDNF infusion led to a permanent reversal of BDNF-LTP.

In summary these data suggest that dendritic synthesis of Arc supports consolidation of LTP through stabilization of F-actin. Translation must be sustained for~2h to support maintenance of the increase in synaptic strength and actin polymerization. These events appear causally related since the stabilization of actin polymerization prevents Arc AS effects on synaptic plasticity. Furthermore, Arc-dependent consolidation can be directly activated brief BDNF infusion.

Discussion

BDNF-LTP properties

Research in recent years have shown that BDNF is an important modulator of synaptic transmission and plasticity in the adult brain (Bramham and Messaoudi, 2005) The versatility of BDNF is emphasized by its contribution to range of adaptive neuronal responses including LTP and LTD, certain forms of short-term synaptic plasticity, as well as homeostatic regulation of intrinsic neuronal excitability (Asztely et al., 2000; Maffei, 2002; Ikegaya et al., 2002). Application of BDNF in the adult hippocampus can trigger a long-lasting increase in synaptic efficacy dubbed BDNF-induced LTP. This persistent potentiation was

first shown at CA3-CA1 synapses in response to bath perfusion of hippocampal slices with BDNF (Kang and Schuman, 1995; Kang et al., 1996). BDNF-LTP was shown at medial perforant path granule cell synapses of the dentate gyrus *in vivo*, in the insular cortex *in vitro*, and in the visual cortex *in vitro* and *in vivo* (Messaoudi et al., 1998; Jiang et al., 2001; Ying et al., 2002; Escobar et al., 2003). We also discovered that BDNF-LTP is, like HFS-LTP, associated with enhanced EPSP-spike coupling in addition to enhanced synaptic efficacy (Bliss and Lomo, 1973; Lu et al., 2000). The fact that BDNF is capable of acutely increasing glutamate release raises the possibility that BDNF indirectly induces NMDAR-dependent potentiation. We address this issue in paper I where, BDNF was infused into the dentate gyrus following systematic administration of the competitive NMDA receptor antagonist (CPP). While HFS-LTP was abolished, BDNF infusion induced robust potentiation during NMDA receptor blockade. BDNF-LTP at CA3-CA1 synapses in hippocampal slices is similarly NMDA receptor-independent (Kang and Schuman, 1995).

BDNF-LTP is occluded during late phase LTP

Several studies have given evidence that BDNF released during or shortly after HFS-LTP plays an obligatory role in the generation of this L-LTP in the CA1 region of the hippocampal. In this study we examined the effect of BDNF infusion at time points corresponding to early and L-LTP. BDNF applied during early LTP induced robust potentiation indicating a distinct mechanism of expression. In our work we could see a complete occlusion when BDNF was applied during late LTP. Conversely, at CA3-CA1 synapses *in vitro*, (Kang et al., 1997) showed that prior induction of BDNF-LTP occludes expression of late, but not early, HFS-LTP. This time-dependent pattern of occlusion suggests that exogenous BDNF specifically activates mechanisms common to L-LTP. Consistent with previous work (Frey et al., 1995), it also suggests a rapid switch in the mechanism of expression between early and late phase LTP. We also examine this further the distinction between transcription-dependent (late) and transcription-independent

(early) LTP by infusing of the transcription inhibitor actinomycin D (ACD) 1 h before or immediately before BDNF infusion. Since previous work has shown that ACD blocks development of late HFS-LTP, leaving E-LTP intact (Frey et al., 1995); (Nguyen and Kandel, 1996). In contrast, BDNF-LTP is almost completely transcription-dependent, consistent with selective role for BDNF in late phase LTP.

BDNF-LTP induction requires rapid ERK activation and de novo gene expression

ERK signaling leading to CREB activation is required for L-LTP and hippocampal-dependent memory formation. (Ying et al., 2002) has shown that local infusion of the MEK (MAPK or ERK kinase) inhibitors PD98059 and U0126 completely blocks BDNF-LTP induction but had no effect on established BDNF-LTP. Thus, MEK-ERK activation is required for the induction, but not the maintenance of, BDNF-LTP. Furthermore, BDNF-LTP induction is transcription-dependent and associated with ERK-dependent phosphorylation of CREB on serine 133, which is required for CRE-driven gene expression. Taken together this shows that BDNF-LTP in dentate gyrus is transcription-dependent, occludes with L-LTP, and is associated with ERK-dependent upregulation of Arc. Paper III of this thesis extends this line of work to show that Arc synthesis is necessary for the induction and time-dependent consolidation of BDNF-LTP.

Arc function and mechanism in LTP.

The Arc AS study suggests that Arc synthesis defines a critical window in LTP consolidation. Surprisingly, early Arc synthesis contributes to the early expression LTP but is not obligate in LTP consolidation. By contrast, late Arc synthesis (2 hours post-HFS) is required for LTP consolidation. The rapid reversal of LTP combined with the rapid knockdown of Arc mRNA and protein

suggests that LTP consolidation requires sustained synthesis of Arc. Our analysis of Arc function concentrated on the consolidation mechanism 2 h after LTP induction. Rapid knockdown of Arc was associated with dephosphorylation of hyperphosphorylated cofilin and loss of a nascent band of F-actin in the medial perforant pathway, while pharmacological stabilization of F-actin blocked the ability of Arc AS to reverse LTP. Thus Arc function appears to couple two central tenets of LTP consolidation: gene expression and local actin polymerization.

Electron microscopic analysis has revealed upregulation Arc and F-actin specific to spines of medial perforant path synapses during LTP (Moga et al., 2004; Fukazawa et al., 2003; Rodriguez et al., 2005) It is nonetheless evident from many studies that Arc protein is upregulated throughout the dendritic arbor of granule cells. The function of the widespread increase in Arc is enigmatic. However, recent studies performed in cultured hippocampal neurons have revealed a role for Arc in homeostatic synaptic scaling of AMPA receptor transmission via its ability to activate a specific AMPA receptor endocytic pathway (Chowdhury et al., 1998; Rial Verde et al., 2006; Shepherd et al., 2006). Perhaps global increases in Arc serve to scale-down (depress) AMPA receptor transmission as a means of stabilizing overall neuronal excitability.

Guzowski (2000) have previously used Arc AS treatment to reveal a role for Arc in memory consolidation. Although LTP was examined in that paper also, the results were inconclusive. Rats treated with Arc AS prior to HFS exhibited LTP lasting several days, but the potentiation was smaller and decay to baseline sooner than in control-infused rats. However, the results are difficult to interpret in the absence of data from baseline recording period prior to LTP induction. In the present report the specificity and validity of AS effects were corroborated in several ways. First, reversal of LTP maintenance was strikingly dependent on the timing of AS application relative to synaptic activation. Second, similar reversal of LTP was obtained with AS sequences targeting non-overlapping regions of the Arc mRNA. Third, LTP reversal was coupled to rapid knockdown of Arc mRNA and protein. Finally, Arc knockdown was coupled to a biologically compelling mechanism regulation of F-actin.

Previous studies have also shown that BDNF-LTP enhances Arc mRNA and protein as compared to zif268 at the same time points (Ying et al., 2002).The requirement for new transcription indicated that Arc synthesis stems from translation of newly induced, rather than preexisting, mRNA. Here we have shown that treatment with Arc AS prior to BDNF infusion had no effect on baseline synaptic transmission but blocks BDNF-LTP and the associated upregulation of Arc protein, indicating a requirement for Arc induction. Furthermore, Arc AS applied 2 h after BDNF rapidly reversed ongoing BDNF-LTP, while the same treatment 4 h after BDNF infusion had no effect. These results demonstrate that Arc-dependent synaptic strengthening and consolidation is directly activated by local BDNF application.

Arc is likely to interact with other newly synthesized proteins in LTP consolidation. A recent microarray screen identified a panel of genes that are co-upregulated with Arc during BDNF-LTP and HFS-LTP. In situ hybridization shows that these genes are upregulated specifically in dentate granule cells. Interestingly, these genes include neuritin and neuronal activity regulated petraxin (NARP), two proteins implicated in synapse development and AMPA receptor clustering, respectively.

BDNF-LTP and translational control

Paper II demonstrated transient phosphorylation of eIF4E and eEF2 as well as enhancement expression eIF4E protein coincided with the onset of BDNF-LTP. Pharmacological inhibition MEK-ERK signaling blocked all of these changes in parallel with BDNF-LTP induction. Thus, ERK appears to have a central coordinating role in BDNF-LTP, regulating Arc expression and both the initiation and elongation steps of protein synthesis.

BDNF stimulates cap-dependent translation through TrkB-coupling activation of the mTOR and Ras-ERK pathways (Takei et al., 2001, 2004; Schratt et al., 2004). Activation of mTOR leads to phosphorylation of eIF4E binding proteins and release of eIF4E. This process inhibits the association of eIF4E with

eIF4G and thus the formation of the eIF4F complex Pause et al., 1994; (Mader et al., 1995; Haghighat et al., 1995). Phosphorylation modulates eIF4E activity, as the phosphorylated form of eIF4E binds more tightly to the cap structure and has an enhanced affinity for eIF4G (Minich et al., 1994). eIF4E binds to the cap and is phosphorylated by the ERK substrate MAPK integrating kinase (MNK) (Pyronnet et al., 1999). The two pathways which phosphorylate eIF4E are both required for BDNF-induced enhancement of protein synthesis in neurons or tissue slices (Kang et al., 1996; Takei et al., 2001; 2004; Tang et al., 2002; Kelleher et al., 2004b).

Recent work has given evidence that LTP is associated with decreases as well as increases in protein synthesis (Fazeli et al., 1993); (Chotiner et al., 2003). In this study we have reported that BDNF-LTP activates translation initiation in dentate gyrus. The elongation process as well as initiation, is a regulatory step for protein synthesis (Ryazanov et al., 1991; Proud), 2000), and phosphorylation of elongation affects the activities and the overall elongation rate (Chang and Traugh, 1997; Proud, 2000; Traugh, 2001; Browne and Proud 2002). eEF2 phosphorylation observed during LTP may therefore contribute to translation arrest (Chotiner et al., 2003). Peptide chain elongation is highly energy consuming and decreases in ATP levels lead to phosphorylation of eEF2 (Horman et al., 2002; Browne et al., 2004). In this study we discover that *in vivo* BDNF-LTP is associated with a transient ERK- dependent phosphorylation of eEF2 in whole dentate gyrus. Surprisingly, BDNF treatment of synaptodendrosomes does not alter eEF2 phosphorylation state but does lead to rapid eIF4E phosphorylation and enhanced translation of dendritic mRNAs such as Arc, α-CaMKII, and Lim domain kinase 1 (LIMK1) (Kanhema et al., 2006; Yin et al., 2002; Schratt et al., 2004). Thus, BDNF appears to affect translation in a compartment-specific manner, enhancing initiation at synapses while promoting elongation arrest at non-synaptic sites. In contrast, NMDA treatment of synaptoneurosomes enhances eEF2 phosphorylation and suppresses global protein synthesis while increasing α-CaMKII expression (Scheetz et al., 2000).

Further work is therefore needed to resolve how activity-dependent translation of specific transcripts is maintained in the context of synaptic eEF2 phosphorylation.

Previously, (Havik et al., 2003) showed that α-CaMKII mRNA levels are rapidly elevated in synaptodendrosomes following LTP induction in awake rats. No changes in mRNA levels were observed in whole dentate gyrus homogenates, indicating relocalization of pre-existing αCaMKII mRNA into dendritic spines. α-CaMKII protein levels were also increased in synaptodendrosomes, but it could not be determined whether this was due to local synthesis in spines or transport from distant sites. Using a purely in vitro synaptodendrosomes approach, which eliminates protein transport as a mechanism, we show that BDNF application rapidly increases expression of α-CaMKII protein.

Model

A cartoon of Arc-dependent consolidation of LTP is shown in **FIG 5**. In this model, postsynaptic BDNF signaling through TrkB receptors is critical for initial translation activation involving liberation of mRNA from storage granules, fine-positioning of the translation apparatus (mRNA, ribosomes, and translation factors), as well as biochemical activation of translation through hyperphosphorylation of eIF4E and probably through mRNA-specific derepression. Spines or dendritic branches activated in this way may effectively capture and translate local mRNA pools. A sequential mechanism is envisioned whereby translation of pre-existing transcripts such αCaMKII is followed by translation of dendritically transported mRNAs such as Arc. During Arc-dependent consolidation, sustained translation of Arc is necessary for cofilin phosphorylation, local F-actin expansion, and formation of stable LTP.

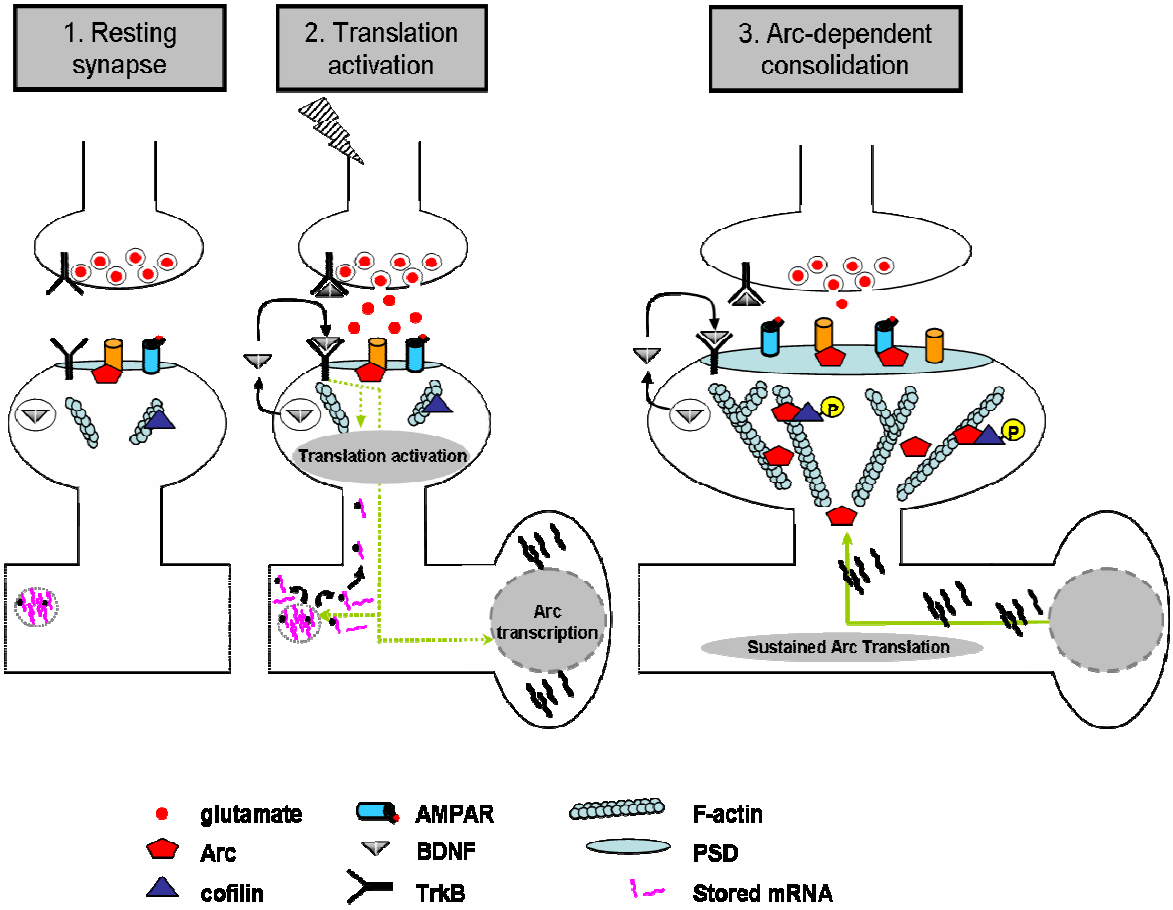

Figure 5. Model of Arc-dependent LTP consolidation in the dentate gyrus. In this model, translation of pre-existing transcripts such αCaMKII is followed by translation of dendritically transported Arc mRNA. Sustained translation of Arc is required for cofilin phosphorylation, local F-actin expansion and spine enlargement underlying stable LTP. Postsynaptic TrkB signaling at glutamate synapses activates local translation and induces upregulation of Arc mRNA. (Soule et al., 2006)

Conclusions and future directions

The work identifies Arc as critical mediator of LTP consolidation and given insight into a novel mechanism involving regulation of actin dynamics. Exogenous BDNF-LTP has proven useful as tool for dissecting mechanisms of synaptic strengthening. Further work is needed in a number of areas. To name only a few issues: 1) The mechanism of Arc coupling to cofilin and F-actin function, 2) The role of endogenous BDNF in these mechanisms, 3) The impact of eIF4E and eEF2 on global and transcript-specific translation. It is hoped that a better understanding of synaptic consolidation will lead to better treatment strategies for conditions associated with impaired synaptic plasticity and dentate gyrus function, including Alzheimer's disease and major depression.

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Paper I

Brain-Derived Neurotrophic Factor Triggers Transcription-Dependent, Late Phase Long-Term Potentiation *In Vivo*

Elhoucine Messaoudi,[1] Shui-Wang Ying,[1] Tambudzai Kanhema,[1] Susan D. Croll,[2] and Clive R. Bramham[1]

[1]*Department of Physiology and Locus on Neuroscience, University of Bergen, N-5009 Bergen, Norway, and* [2]*Regeneron Pharmaceuticals, Tarrytown, New York 10591*

Acute intrahippocampal infusion of brain-derived neurotrophic factor (BDNF) leads to long-term potentiation (BDNF-LTP) of synaptic transmission at medial perforant path→granule cell synapses in the rat dentate gyrus. Endogenous BDNF is implicated in the maintenance of high-frequency stimulation-induced LTP (HFS-LTP). However, the relationship between exogenous BDNF-LTP and HFS-LTP is unclear. First, we found that BDNF-LTP, like HFS-LTP, is associated with enhancement in both synaptic strength and granule cell excitability (EPSP–spike coupling). Second, treatment with a competitive NMDA receptor (NMDAR) antagonist blocked HFS-LTP but had no effect on the development or magnitude of BDNF-LTP. Thus, NMDAR activation is not required for the induction or expression of BDNF-LTP. Formation of stable, late phase HFS-LTP requires mRNA synthesis and is coupled to upregulation of the immediate early gene activity-regulated cytoskeleton-associated protein (Arc). Local infusion of the transcription inhibitor actinomycin D (ACD) 1 hr before or immediately before BDNF infusion inhibited BDNF-LTP and upregulation of Arc protein expression. ACD applied 2 hr after BDNF infusion had no effect, defining a critical time window of transcription-dependent synaptic strengthening. Finally, the functional role of BDNF-LTP was assessed in occlusion experiments with HFS-LTP. HFS-LTP was induced, and BDNF was infused at time points corresponding to early phase (1 hr) or late phase (4 hr) HFS-LTP. BDNF applied during the early phase led to normal BDNF-LTP. In contrast, BDNF-LTP was completely occluded during the late phase. The results strongly support a role for BDNF in triggering transcription-dependent, late phase LTP in the intact adult brain.

Key words: long-term potentiation (LTP); synaptic plasticity; neurotrophin; brain-derived neurotrophic factor (BDNF); dentate gyrus; hippocampus; activity-regulated cytoskeleton-associated protein (Arc); gene expression

The neurotrophin family of secretory proteins play a diverse and broad role in regulating neuronal structure and function in the developing and adult nervous system (Thoenen, 1995; Bibel and Barde, 2000). Brain-derived neurotrophic factor (BDNF) is the most widely distributed neurotrophin in the adult brain. Although BDNF actions have been studied extensively in cell culture, the functions of BDNF in the adult brain have not been clearly defined. Growing evidence suggests that BDNF is important in activity-dependent synaptic plasticity, particularly in the context of long-term potentiation induced by high-frequency stimulation (HFS-LTP) (Schuman, 1999; Schinder and Poo, 2000; Binder et al., 2001; Bramham et al., 2002).

The role of endogenous BDNF in HFS-LTP has only been extensively studied at Schaffer collateral→CA1 synapses in the *in vitro* hippocampal slice preparation. Maintenance of HFS-LTP consists of at least two phases: an early, labile phase dependent on covalent modifications of existing proteins; and a late, stable phase requiring new mRNA and protein synthesis (Krug et al., 1984; Matthies et al., 1990; Bliss and Collingridge, 1993; Frey et al., 1996; Nguyen and Kandel, 1996). LTP maintenance is impaired by treatment with antibodies that inhibit activation of the BDNF receptor TrkB, or by deletion of the BDNF or TrkB genes (Figurov et al., 1996; Kang et al., 1997; Korte et al., 1998; Chen et al., 1999; Minichiello et al., 1999; Xu et al., 2000; Kossel et al., 2001; Patterson et al., 2001). Depending on the stimulation parameters used, relatively selective impairment in the ability to generate late, transcription-dependent LTP is seen (Kang et al., 1997; Korte et al., 1998; Minichiello et al., 1999; Patterson et al., 2001).

Exogenously applied BDNF has an impressive range of rapid, short-acting effects, including modulation of axon guidance, synaptic transmission, and membrane depolarization (Song and Poo, 1999; Schinder and Poo, 2000). In addition to these short-term effects, Kang and Schuman (1995, 1996) and Kang et al. (1997) found that bath perfusion of hippocampal slices with BDNF induces a long-lasting enhancement of synaptic strength in the CA1 region. For reasons still unresolved, possibly related to the method of BDNF application (Kang et al., 1996), this finding has not yet been replicated (Patterson et al., 1996; Frerking et al., 1998; Schinder and Poo, 2000). However, when microinfused into the adult dentate gyrus *in vivo*, BDNF induces a lasting strengthening of transmission at perforant path→granule cell synapses (Messaoudi et al., 1998; Ying et al., 2002). We have termed this effect BDNF-induced LTP (BDNF-LTP).

The mechanism of BDNF action in synaptic plasticity is little understood. If BDNF participates in triggering late HFS-LTP, it should regulate new protein synthesis. New proteins could derive from translation of existing mRNA, from new transcription, or both. Kang and Schuman (1996) and Aakalu et al. (2001) have provided evidence that BDNF stimulates protein synthesis in

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Correspondence should be addressed to Dr. Clive Bramham, Department of Physiology, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway. E-mail: clive.bramham@fys.uib.no.

Dr. Ying's present address: Department of Anesthesiology, A-1050, Weill Medical College of Cornell University, 525 East 68th Street, New York, NY 10021.

dendrites from existing mRNA. However, the role of transcription in BDNF-LTP has not been examined. Moreover, the physiological relevance of exogenous BDNF-LTP and its relationship, if any, to mechanisms of HFS-LTP in the intact adult brain is unclear.

Here we provide evidence that (1) induction of BDNF-LTP, in contrast to HFS-LTP, does not require NMDA receptor (NMDAR) activation; (2) BDNF-LTP, like HFS-LTP, is associated with enhanced granule cell excitability [EPSP–spike (E–S) coupling] in addition to enhanced synaptic transmission; (3) BDNF-LTP, like late HFS-LTP, requires rapid new transcription coupled to upregulation of the immediate early gene activity-regulated cytoskeleton-associated protein (Arc); and (4) BDNF-LTP is occluded during late phase but not early phase HFS-LTP. Together these results suggest a functional role for BDNF as a trigger of transcription-dependent, late phase LTP *in vivo*.

MATERIALS AND METHODS

The electrophysiological and infusion methods used are the same as those described previously with minor modifications (Messaoudi et al., 1998; Ying et al., 2002).

Surgery and electrode and cannula placement. Eighty-four Mol: SD rats (Møllegaards Avls-Laboratorium) weighing between 250 and 350 gm were anesthetized with urethane (1.5 gm/kg, i.p.) and placed in a stereotaxic apparatus. Rectal temperature was maintained at 37°C by a servo-heating pad. Electrophysiological methods for obtaining selective stimulation of the medial perforant path have been detailed previously (Bramham et al., 1991). Stereotaxic coordinates relative to Bregma were 7.9 mm posterior, 4.2 mm lateral for stimulation, 3.9 mm posterior, and 2.2 mm lateral for recording. The arrangement of the cannula–electrode assembly is depicted in Figure 1*A*. An outer (guide) cannula (24 gauge; Plastics One, Roanoke, VA) was beveled sharp at the tip to facilitate tissue penetration. A Teflon-coated stainless steel wire-recording electrode (coated diameter, 112 μm) was glued to the cannula shaft and cut so the tip extended 1 mm from the end of the cannula. A bipolar stimulating electrode was lowered into the dorsomedial aspect of the angular bundle for stimulation of the medial perforant path. After a small slit was made in the dura, the guide cannula and attached recording electrode were slowly lowered into the dorsal hippocampus until a positive-going field EPSP (fEPSP) of maximum slope was obtained in the dentate hilus. The final depth of the recording electrode ranged between 200 and 300 μm below the level of the maximum negative-going fEPSP sink recorded in the middle third of the dentate molecular layer. An inner infusion cannula (31 gauge) was then inserted so that it protruded 300 μm below the end of the guide. The tip of the infusion cannula was located in the deep stratum lacunosum-moleculare of field CA1, 700 μm above the hilar recording site and 300–400 μm above the medial perforant synapses.

The infusion cannula was connected via PE50 polyethylene tubing to a 5 μl Hamilton (Reno, NV) syringe. Solutions were delivered by an infusion pump at a rate of 80 nl/min. Two micrograms of BDNF were delivered in a volume of 2 μl over 25 min. Dose–response analysis showed that this was the lowest dose giving maximal BDNF-LTP (Messaoudi et al., 1998).

Electrophysiology. Biphasic rectangular pulses of 150 μsec duration were applied every 30 sec throughout the experiment. The stimulation intensity for test pulses was set to elicit a population spike of one-third of the maximal amplitude. After a 15 min period of stable responses, an input–output curve was collected, followed by baseline recording (20 min), BDNF infusion (25 min), and postinfusion recording (2–7 hr). Input–output curves were constructed from responses (mean of four sweeps) obtained at eight stimulus intensities. The stimulus intensity was increased in 100 μA increments starting 100 μA below the population spike threshold. In some experiments, HFS-LTP was induced using a paradigm that evokes LTP lasting 1–5 d and associated with BDNF and TrkB mRNA upregulation (Bramham et al., 1996). HFS consisted of eight pulses at 400 Hz, repeated four times, at 10 sec intervals. Three session of HFS were given at intervals of 5 min. The stimulus intensity for HFS was the same as for test pulses.

Tissue microdissection and sample preparation. At the end of electrophysiological recording, rats were decapitated, and the brain was rapidly removed and rinsed with oxygenated ice-cold artificial CSF (in mM: NaCl 124.0, $NaHOC_3$ 25.0, D-glucose 10.0, KCl 3.4, KH_2PO_4 1.2, $MgSO_4$ 1.0, and $CaCL_2$ 2.5, pH 7.4). The dentate gyrus and hippocampal CA1 and CA3 regions were rapidly dissected on a glass dish kept on ice. Tissues were hand-homogenized with 15 strokes in 300 μl of freshly made SDS sample buffer containing 10% glycerol, 2.3% SDS, 0.01% bromophenol blue, and 0.5% β-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8 at room temperature. Homogenates were boiled for 5 min, aliquoted into Eppendorf tubes, and stored at −80°C until use.

SDS-PAGE and Arc immunoblotting. Protein levels in homogenate samples were determined using the Lowry method. Equal amounts of protein were loaded onto SDS-PAGE gels (10%) and run overnight at a constant current of 10 mA. Separated proteins were transferred to a nitrocellulose membrane using a constant voltage of 30 V. Membranes were blocked in 3% BSA on a gyro-rocker at 4°C overnight or for 1 hr at room temperature. Primary antibody recognizing the Arc N terminus (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) was diluted in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 3% BSA. Membranes were incubated in primary antibody for 2 hr at room temperature or at 4°C overnight with constant shaking, washed three times in TBST, and incubated for 1 hr with HRP-labeled anti-rabbit IgG (1:10,000 in TBST; Santa Cruz Biotechnology). Blots were washed in TBST and developed using enhanced chemiluminescence. Autoradiographs were scanned on a laser densitometer and quantitated using Phoretics 1D Plus software. Western blots were developed to be linear in the range used for densitometry. Optical density values obtained from the treated hippocampus were normalized relative to values in the nontreated hippocampus for each hippocampal subfield. Statistical analyses were based on paired and unpaired *t* tests for between- and within-group comparisons, respectively.

BDNF immunocytochemistry. BDNF immunocytochemistry was used to the study the distribution and clearance of exogenous BDNF during BDNF-LTP. Cytochrome *c*, which has a molecular weight and charge similar to those of BDNF, was used as a protein control. Cytochrome *c* has no effect on basal synaptic transmission or on several signal transduction pathways that have been studied (Messaoudi et al., 1998; Ying et al., 2002).

Animals were deeply anesthetized with an overdose of chloral hydrate and pentobarbital before killing. They were then transcardially exsanguinated with ~50 ml of heparinized saline containing 1 mM sodium orthovanadate followed by fixation with 4% paraformaldehyde in first acetate, pH 6.5, and then borate, pH 9.5, buffers. All solutions were kept on wet ice throughout the perfusion procedure and were perfused at a rate of ~1 l/hr. After perfusion fixation, brains were removed and placed in 30% sucrose in borate buffer at 4°C until they sank (~5–7 d). Brains were frozen and sectioned coronally on a sliding microtome at 40 μm after sinking. Sections were stored at –20°C in cryoprotectant solution (Watson et al., 1986) until immunostained. Free-floating series of sections (1:12) were immunostained as described previously (Morse et al., 1993) for BDNF with the addition of a pretreatment in 0.1 mM sodium periodate in TBS to reduce endogenous peroxidase activity. Sections were blocked with goat serum and then placed in primary antibody solution (BDNF rabbit polyclonal; Amgen, Thousand Oaks, CA) overnight at 4°C. Staining with BDNF antibody at dilutions of >1:10,000 often reduces the endogenous staining, making it easier to see the distribution of exogenously administered protein. A titer of 1:20,000 was used for BDNF-infused brains, and 1:10,000 was used for cytochrome *c*-infused brains. Sections were washed and incubated with biotinylated secondary antibody (goat anti-rabbit, 1:1500; Vector Laboratories, Burlingame, CA). After washing again, staining was completed using an avidin–biotin–peroxidase complex reaction (Vectastain Elite ABC kit; Vector Laboratories). The peroxidase was visualized using a diaminobenzidine chromagen and nickel sulfate intensification. The specificity of the antibody for BDNF relative to other neurotrophins has been documented previously (Morse et al., 1993).

Drugs. Human recombinant met-BDNF (a gift from Amgen-Regeneron Partners) was obtained as a concentrated stock solution in PBS (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0, and 0.004% Tween 20). (*RS*)-3–2(2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP; Tocris Cookson Ltd., Bristol, UK) was dissolved in saline and injected intraperitoneally at a dose of 10 mg/kg 2 hr before the end of BDNF infusion. Actinomycin D (ACD) and recombinant cytochrome *c* from yeast (Sigma, St. Louis, MO) were dissolved in PBS. All drugs were aliquoted in small volumes and stored at −80°C until use.

Response analysis and statistics. Signals from the dentate hilus were

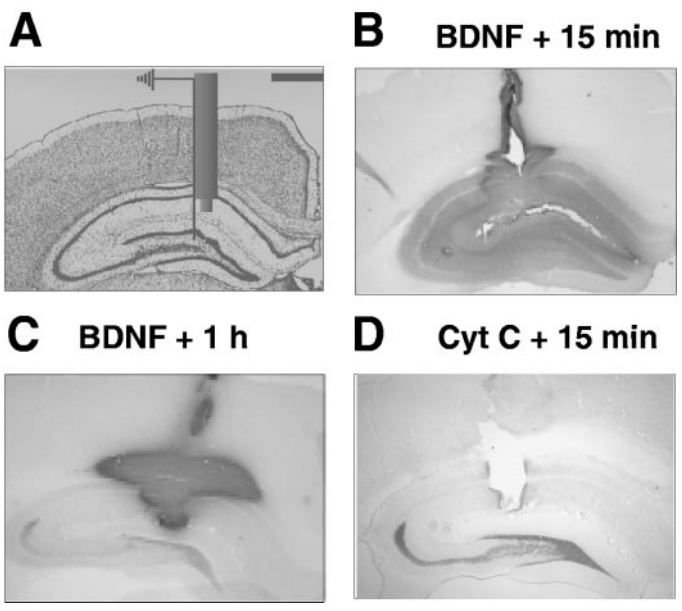

Figure 1. Tissue distribution and clearance of infused BDNF. BDNF (2 μg/2 μl, 25 min) was infused into stratum lacunosum-moleculare CA1, immediately above the hippocampal fissure, ~300 μm above the medial perforant path→granule cell synapses. Cytochrome *c* (*Cyt C*) was infused as a protein control. Brains were obtained at 15 min or 1, 3, 6, or 24 hr after infusion, and coronal sections were immunostained for BDNF. *A*, Schematic depiction of cannula–electrode assembly in the hippocampus. *B, C*, BDNF immunostaining obtained at 15 min and 1 hr after BDNF infusion, respectively. BDNF was rapidly delivered and cleared from the dentate gyrus. *D*, Cyt C 15 min control. *B–D* were taken through the area of the cannula tract. The BDNF antibody titer for the BDNF-infused brains was 1:20,000 (minimizing endogenous staining and facilitating detection of exogenous protein). The antibody titer for Cyt C-infused brains was 1:10,000 (therefore *darker* staining of mossy fibers). Scale bar, 1 mm.

amplified, filtered (1 Hz to 3 kHz), digitized (25 kHz for field potentials), and stored to computer disk. Acquisition and analysis of field potentials were accomplished using DataWave Technologies (Longmont, CO) WorkBench software. The maximum slope of the fEPSP and the amplitude of the population spike measured from its negative-going apex to the tangent line joining the first two positive peaks were measured, and averages of four consecutive responses were obtained. ANOVA for repeated measures followed by a *post hoc* Scheffé test was used for statistical analysis of group effects, and a *t* test for dependent samples was used for analysis of individual effects (Statistica package; StatSoft Inc., Tulsa, OK). Statistics were based on values obtained during baseline and 2 hr after terminating infusion, unless otherwise indicated. $p = 0.05$ was chosen as the level of statistical significance.

RESULTS

BDNF tissue distribution and clearance

Exogenous BDNF diffuses relatively slowly in tissue, and the kinetics of BDNF tissue clearance is not well known (Biffo et al., 1995; Croll et al., 1998). If BDNF is retained in tissue for long periods, this might contribute to the increase in synaptic transmission seen in BDNF-LTP. We therefore sought to determine the spatial distribution and time course of BDNF clearance. Figure 1*A* shows the cannula assembly with the infusion site located in stratum lacunosum-moleculare CA1, immediately above the hippocampal fissure, ~300 μm above the medial perforant path. Rats were transcardially perfused with fixative solution at 15 min or 1, 3, 6, or 24 hr after BDNF (2 μg, 2 μl, 25 min) infusion, and coronal sections were immunostained for BDNF.

At 15 min, BDNF immunostaining was observed in the dentate gyrus in six of six experiments, indicating a rapid delivery of BDNF (Fig. 1*B*). BDNF spread radially from the site of infusion and along the cannula tract in the CA1 region, with variable spread to CA3. Typically, the borders of the BDNF stain were sharply demarcated, indicating an abrupt arrest of BDNF diffusion or detection. At 1 hr, BDNF was cleared from the dentate gyrus in two of two experiments (Fig. 1*C*). At 3 and 6 hr, no BDNF staining could be detected in the dentate gyrus in two of three brains at each time point; the remaining brain showed moderate staining in the dentate gyrus. No staining was observed 24 hr ($n = 2$) after BDNF infusion. Staining of endogenous BDNF, for instance, in mossy fibers, appeared to be identical in ipsilateral (infused) and contralateral hippocampus, although no attempt was made to quantify this relationship. BDNF-LTP lasts for at least 15 hr in anesthetized rats, and induction of the potentiation requires rapid activation of extracellular signal-regulated protein kinase (ERK) (Messaoudi et al., 1998; Ying et al., 2002). The rapid delivery (<15 min) and clearance (<1 hr) of BDNF are consistent with the electrophysiological and biochemical effects and demonstrate that BDNF-LTP does not involve the continuous presence of exogenous BDNF.

BDNF enhances both synaptic transmission and E–S coupling

HFS-LTP is associated with two changes: (1) an increase in synaptic strength and (2) an increased excitability of the postsynaptic neuron for a given excitatory input (Bliss and Lomo, 1973; Abraham et al., 1987; Lu et al., 2000). The latter phenomenon, termed E–S coupling, has not been examined in BDNF-LTP. BDNF infusion resulted in a rapid increase in the fEPSP slope and population spike amplitude (Fig. 2*A*; $n = 6$), corroborating earlier work (Messaoudi et al., 1998). Input–output curves were collected immediately before baseline recording and 2 hr after BDNF infusion (Fig. 2*B*). E–S plots derived from the input–output curves show a marked leftward shift, indicating an increase in granule cell excitability to medial perforant path input (Fig. 2*C*). Thus BDNF-LTP, like HFS-LTP, is associated with a parallel increase in fEPSP strength and granule cell excitability.

Next we asked whether BDNF-LTP is associated with an increase in the excitability of presynaptic fibers. Although presynaptic fiber excitability is not affected during HFS-LTP, this has not been investigated in BDNF-LTP (Bliss and Lomo, 1973). BDNF regulation of presynaptic ion channel function is clearly a possibility, because BDNF has been shown to modulate a voltage-dependent sodium channel (Kafitz et al., 1999) and the potassium-channel Kir3 (Rogalski et al., 2000). We therefore determined the minimum stimulus current needed to consistently (in >90% of trials) evoke an fEPSP. There was no difference in the mean current threshold based on eight rats recorded before (40 ± 12 μA) and 2 hr after (46 ± 13 μA) BDNF infusion. We conclude that BDNF-LTP, like HFS-LTP, does not involve a change in presynaptic excitability.

BDNF-LTP is NMDA receptor-independent

HFS-LTP at medial perforant path→granule cell synapses requires NMDAR activation (Errington et al., 1987; Bramham et al., 1991). BDNF has been shown to facilitate presynaptic transmission in cultured hippocampal neurons (Lessmann et al., 1994) and to increase potassium-evoked glutamate release from synaptosomes prepared from adult dentate gyrus (Gooney and Lynch, 2001) and neocortex (Jovanovic et al., 2000). This raises the possibility that BDNF may induce LTP indirectly by acutely releasing glutamate and activating NMDARs. We therefore ex-

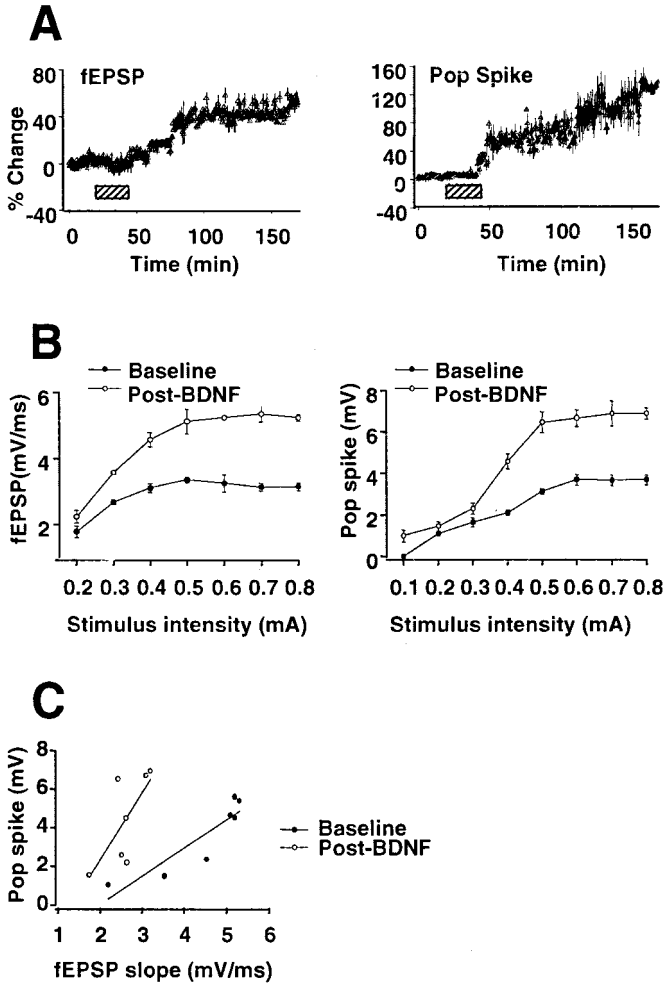

Figure 2. BDNF infusion elicits enhanced synaptic transmission and enhanced E–S coupling at medial perforant path→granule cell synapses. *A*, Time course plots showing BDNF-LTP of the evoked fEPSP and population spike (*Pop Spike*). BDNF was infused during the period indicated by the *hatched bar*. Values are group means ± SEM) expressed as percentage of baseline ($n = 6$). *B*, Representative input–output curves obtained during baseline and 2 hr after BDNF infusion. Values are means of four responses. *C*, E–S plot based on values shown in *B*. The *leftward* shift in the E–S curve indicates an increase in granule cell excitability to synaptic input. The regression coefficient was 0.95 in both plots.

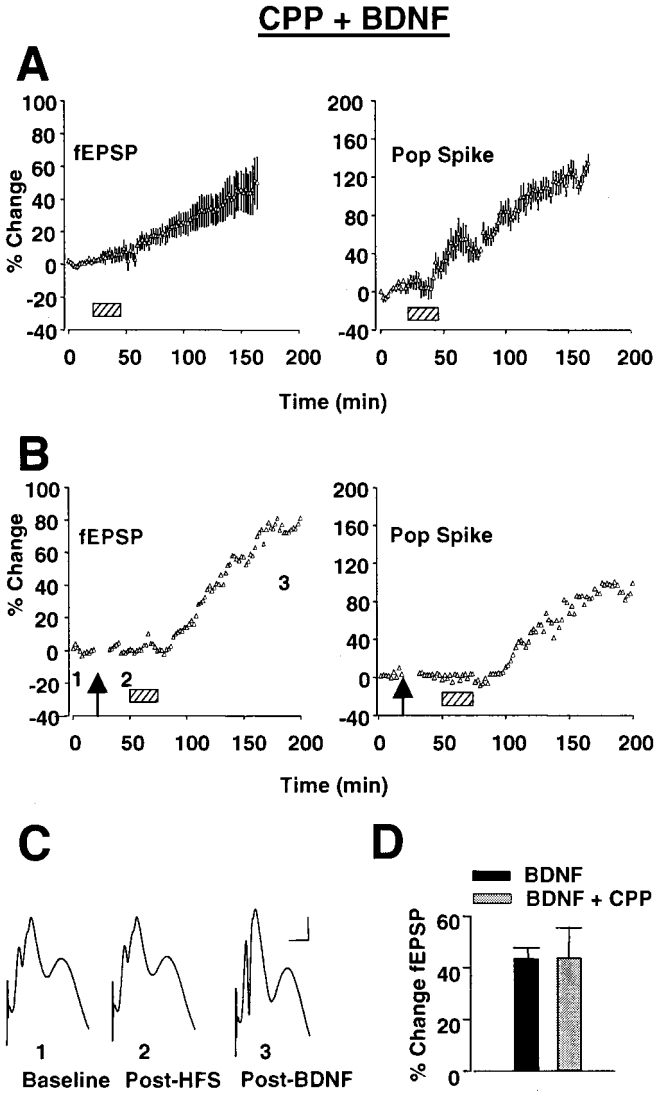

Figure 3. BDNF-LTP does not require NMDAR activation. *A*, Group mean changes in the fEPSP slope and population spike (*Pop Spike*) amplitude. CPP was injected intraperitoneally (10 mg/kg) 2 hr before BDNF infusion (*hatched bar*; $n = 6$). *B*, Representative plot showing the effect of HFS (*arrow*) plus BDNF (*bar*) in the presence of CPP. NMDAR blockade abolished HFS-LTP, but had no effect on BDNF-LTP. *C*, Field potentials (average of four sweeps) obtained at the times indicated in *B*. Calibration: 3 mV, 2 msec. *D*, Mean fEPSP slope obtained 2 hr after BDNF infusion in the CPP-treated specimens and nontreated controls.

amined the induction and expression of BDNF-LTP under conditions of NMDAR blockade. The competitive NMDAR antagonist CPP was injected intraperitoneally at a dose of 10 mg/kg. Under these conditions, NMDAR activation and LTP induction are blocked for at least 8 hr (Abraham and Mason, 1988; Villarreal et al., 2002). As shown in Figure 3*A*, BDNF induced a robust potentiation of the fEPSP slope and population spike amplitude in the CPP-treated animals ($p < 0.05$; $n = 6$). The magnitude and time course of potentiation were not significantly different from those of controls receiving BDNF alone (Fig. 3*D*) ($p < 0.05$). In another series of experiments ($n = 4$), the effect of CPP on HFS- and BDNF-induced LTP was determined in the same animal. Although HFS-LTP was completed abolished, infusion of BDNF 20 min after HFS resulted in full BDNF-LTP (Fig. 3*B,C*). We conclude that NMDAR activation is not required for the induction or expression of BDNF-LTP.

BDNF-LTP does not require low-frequency test stimulation

The protocol for BDNF-LTP includes delivery of low-frequency test stimuli (one per 30 sec) throughout the experiment. We therefore asked whether this test stimulation is required. Low-frequency stimulation may provide an activity-dependent signal (i.e., intracellular calcium rise) acting in concert with stimulation of TrkB receptors. To investigate this issue, test stimulation was omitted during the period of BDNF infusion and for either 2 hr ($n = 5$) or 6 hr ($n = 4$) thereafter, whereupon six test responses were collected. Significant increases in the fEPSP and population spike were seen at both time points, indicating that BDNF-LTP is not dependent on test stimulation (Fig. 4).

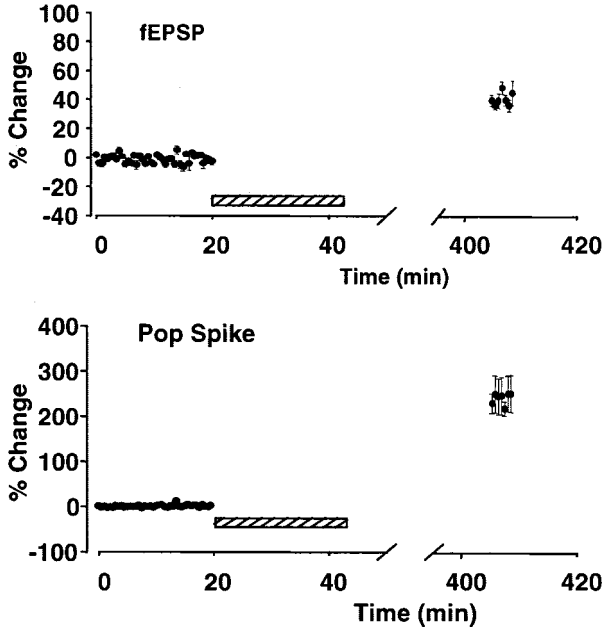

Figure 4. BDNF-LTP does not require low-frequency test stimulation. The normal paradigm for monitoring responses involves delivery of low-frequency test responses throughout the experiment at a rate of one per 30 sec. Here, test stimulation was omitted during BDNF infusion (*hatched bar*) and for 6 hr after infusion. At the end of this time, six responses were collected. Changes in the fEPSP and population spike (*Pop Spike*) are shown. Values are means ± SEM of four experiments expressed as percentage of baseline. Note that potentiation is seen in response to the first stimulus applied after infusion. The magnitude of the fEPSP increase (41.6 ± 4.1%) was not significantly different from that of controls (47.6 ± 5%; $n = 5$) receiving continuous test stimulation.

BDNF-LTP is transcription-dependent

The development of late HFS-LTP requires new gene transcription. A possible role for transcription in BDNF-LTP was investigated using local infusion of the RNA synthesis inhibitor ACD. Figure 5 shows the effect of ACD application at various time points relative to BDNF infusion. Infusion of BDNF alone induced stable potentiation of the fEPSP and population spike (Fig. 5*A*; only the fEPSP is shown). Infusion of ACD (4 μg in 1 μl; $n = 6$) 1 hr before BDNF completely abolished the BDNF-LTP. ACD alone had no effect on baseline synaptic transmission in the interval before BDNF infusion (Fig. 5*C*) or in a separate group of animals receiving ACD alone and recorded for >7 hr (Fig. 5*B*) ($n = 5$).

However, it is possible that ACD blocks an ongoing transcriptional process (housekeeping function) that, although not critical for basal synaptic transmission, allows synapses to respond to BDNF. To obviate this issue, ACD was applied immediately before BDNF. A rapid potentiation was observed during the first hour after BDNF infusion, followed by a sharp decline to a stable plateau level (Fig. 5*D*) ($n = 6$). In this paradigm, the initial response to BDNF is intact, but the sustained response is strongly inhibited. The mean increases of the fEPSP slope and population spike amplitude measured 2 hr after BDNF infusion were 12.6 and 17.27%, respectively. Although inhibited relative to control, the residual potentiation remained significantly elevated above baseline ($p < 0.05$). Finally, ACD was applied 2 hr after BDNF infusion during established BDNF-LTP. As shown in Figure 5*E*, the increase in the fEPSP slope and population spike amplitude observed 2 hr after ACD infusion was not significantly different

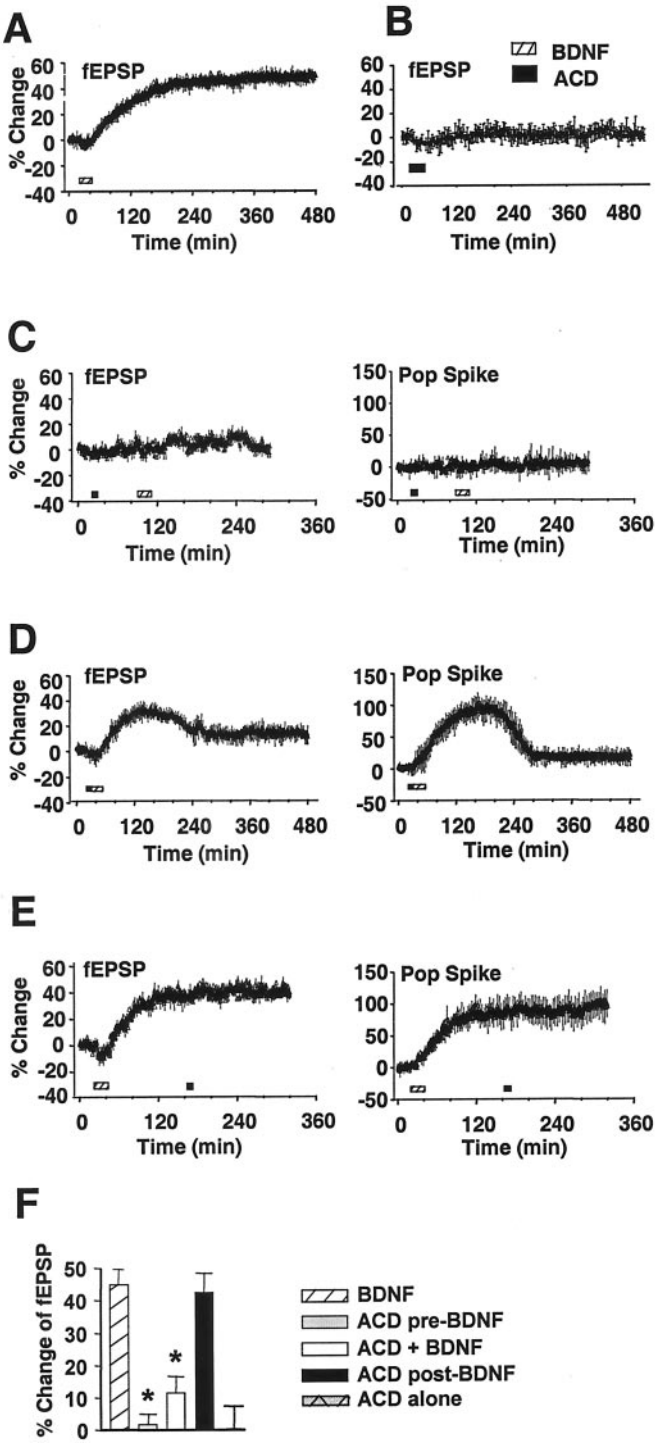

Figure 5. BDNF-LTP requires rapid transcription. The RNA synthesis inhibitor ACD was applied at various time points relative to infusion of BDNF. *A–E*, Group time course plots. *A*, BDNF alone ($n = 5$). *B*, ACD alone ($n = 5$). ACD was given 1 hr before BDNF (*C*; $n = 6$), immediately before BDNF (*D*; $n = 6$), or 2 hr after BDNF (*E*; $n = 5$). The periods of ACD infusion (4 μg, 1 μl; *black bar*) and BDNF infusion (*hatched bar*) are indicated. *F*, Summary bar graph of fEPSP changes. All values are group means ± SEM expressed as percentage of baseline. Values for the *bar graph* were obtained 2 hr after BDNF infusion in the ACD pretreatment group and 4 hr after BDNF (or ACD alone) infusion in the other groups. *Significantly different from BDNF group. The residual potentiation in the *ACD + BDNF* group was significantly elevated above baseline. *Pop Spike*, Population spike.

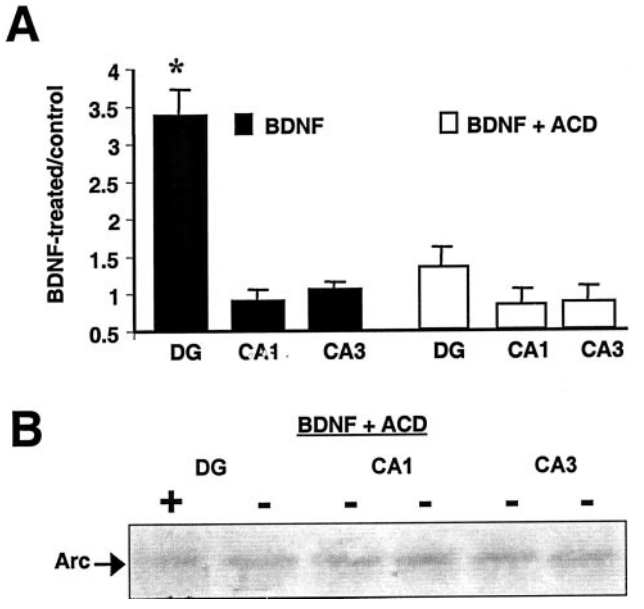

Figure 6. ACD blocks Arc upregulation associated with BDNF-LTP. Western blot assays of Arc were run on aliquoted samples from microdissected dentate gyrus (*DG*) and hippocampal regions CA1 and CA3 after BDNF-LTP *in vivo*. *A*, Group mean + SEM changes in Arc immunoreactivity levels based on densitometric analysis. Optical density values are expressed as a ratio between the treated and nontreated (control) side for each region. BDNF-LTP is associated with enhanced Arc expression at 3 hr ($n = 7$) but not 15 min ($n = 8$; data not shown). ACD infusion 1 hr before BDNF blocked BDNF-LTP and the associated increase in Arc expression ($n = 5$). No changes in Arc expression were detected in the CA1 or CA3 regions. *B*, Representative immunoblot from the ACD-pretreated group. Infusions were made into the left hippocampus.

from those of time-matched controls receiving BDNF alone ($n = 5$) ($p < 0.05$). Together the results suggest that the induction but not the maintenance of BDNF-LTP requires new transcription. The effects are summarized in Figure 5*F*.

The immediately early gene Arc is required for generation of late HFS-LTP and long-term memory (Guzowski et al., 2000). We have shown recently that BDNF-LTP is accompanied by upregulation of *Arc* mRNA and protein in the dentate gyrus (Ying et al., 2002). Here we assessed the effect of ACD on Arc upregulation (Fig. 6) ($n = 5$). Arc protein levels were measured by Western blot analysis of homogenates obtained from microdissected dentate gyrus and CA1 and CA3 regions. ACD blocked a threefold upregulation of Arc protein expression in dentate gyrus in parallel with blockade of BDNF-LTP. ACD had no effect on basal levels of Arc expression in CA1 and CA3. These results strengthen the correlation between Arc and BDNF-LTP and demonstrate that Arc protein derives predominantly from transcription of new *Arc* mRNA rather than from translation of preexisting transcripts.

BDNF-LTP is occluded during late phase but not early phase HFS-LTP

A major outstanding issue is whether BDNF-LTP is physiologically relevant. Does exogenous BDNF reflect the actions of endogenous BDNF? If BDNF-LTP and HFS-LTP have a common mechanism, the expression of one should occlude expression of the other. To be able to draw conclusions from occlusion experiments, it is critical to establish saturation of the phenomenon on which the occlusion test is based. HFS-LTP is suitable for this purpose, because saturation is rapidly achieved and easily assessed, whereas BDNF-LTP is unsuitable in our view because of the slow time course of the potentiation.

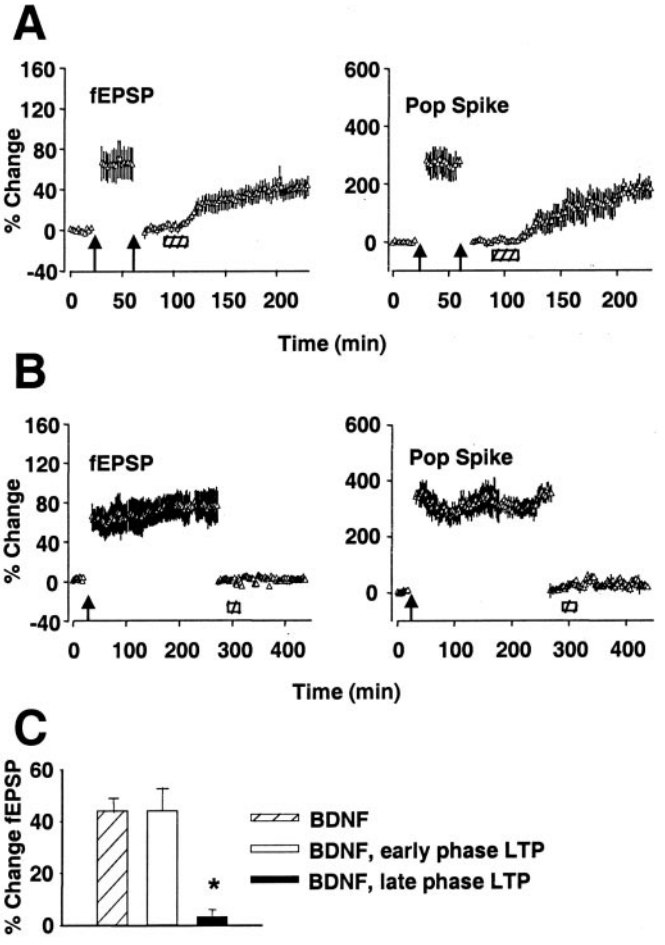

Figure 7. BDNF-LTP is occluded by late phase but not early phase HFS-LTP. *A*, LTP was induced by three sessions of HFS (400 Hz) and recorded for 30 min. The stimulus intensity was then lowered to reset the fEPSP slope to baseline. A second session of HFS produced no further increase, demonstrating saturation of HFS-LTP. BDNF infusion (*hatched bar*) 60 min after the first HFS led to increased synaptic transmission ($n = 6$). *B*, HFS-LTP was induced and recorded for 240 min and then reset to baseline as in *A*. BDNF infusion 260 min after HFS had no effect on fEPSP slope or population spike (*Pop Spike*) amplitude for the duration of recording ($n = 6$). Values are group means ± SEM expressed as percentage of baseline. *C*, Summary of fEPSP slope increases obtained in group receiving BDNF after baseline recording (*BDNF*), 60 min after HFS (*BDNF, early phase LTP*), or 260 min after HFS (*BDNF, late phase LTP*). *Significantly different from *BDNF* group. The magnitude of LTP in the *BDNF* group and *BDNF, early phase LTP* group was not statistically different ($p < 0.05$).

HFS-LTP was induced using parameters (three sessions of 400 Hz stimulation) that generate LTP lasting days (Bramham et al., 1996). After 30 min of recording, the stimulus intensity was lowered to reset the fEPSP slope to baseline, keeping the response on the rising phase of the input–output curve. As shown in Figure 7*A* ($n = 6$), a second round of HFS produced no further increase, demonstrating saturation of HFS-LTP. By contrast, infusion of BDNF-LTP at this time (1 hr after the first HFS) led to an increase in the fEPSP slope and population spike. The kinetics of the increase and the magnitude of the increase were not significantly different from those of the control (Fig. 7*C*). The lack of occlusion indicates that BDNF-LTP does not contribute to expression of early HFS-LTP.

BDNF may nonetheless take part in late LTP based on a transcription-dependent mechanism. Occlusion experiments were performed 260 min after HFS, which by all accounts falls within the period of mRNA synthesis-dependent LTP. As shown in Figure 7*B* ($n = 6$), BDNF-LTP was completely occluded under these conditions. These results show that exogenous BDNF-LTP selectively occludes with late HFS-LTP.

DISCUSSION

The present *in vivo* study demonstrates that exogenous BDNF triggers a long-term increase in synaptic strength (BDNF-LTP), which requires rapid transcriptional activation and which occludes with late but not early HFS-LTP. Endogenous BDNF has multiple actions in the regulation of synapse function and plasticity (Schinder and Poo, 2000; Bramham et al., 2002). Endogenous BDNF has been shown to contribute to the generation of stable HFS-LTP (Kang et al., 1997; Korte et al., 1998; Chen et al., 1999; Minichiello et al., 1999; Xu et al., 2000; Patterson et al., 2001). Late HFS-LTP and long-term memory both depend on activation of ERK and induction of the immediate early gene Arc (Atkins et al., 1998; Impey et al., 1998; Davis et al., 2000; Guzowski et al., 2000; Rosenblum et al., 2002). We have shown recently that BDNF-LTP similarly requires ERK activation coupled to ERK-dependent activation of the nuclear transcription factor calcium/cAMP responsive-element binding protein and upregulation of Arc mRNA and protein (Ying et al., 2002). As seen with HFS-LTP (Lyford et al., 1995; Link et al., 1995; Valentine et al., 2000), *Arc* mRNA is selectively induced in dentate granule cells and delivered to dendritic processes. In the present work, local inhibition of transcription blocked BDNF-LTP and the attendant increase in Arc protein expression. By applying ACD at different time points relative to BDNF infusion, we have identified a window of critical transcription, which parallels the window of ERK activation. Taken together, these data strongly support a role for BDNF as a trigger for transcription-dependent synaptic strengthening. Signal-transducing TrkB receptors are located on axon terminals and in the postsynaptic density of glutamatergic synapses (Drake et al., 1999; Aoki et al., 2000). Without excluding presynaptic effects, these data clearly point to a role for postsynaptic modifications in dentate granule cells.

In these experiments, BDNF is infused immediately above the dentate gyrus, some 300 μm above the medial perforant path synapses. BDNF infusion at this site results in selective activation of signal transduction pathways in the dentate gyrus; no biochemical effects have so far been observed in microdissected CA1 or CA3 tissue (Kanhema et al., 2001; Ying et al., 2002). Using BDNF immunostaining, we show that exogenous BDNF is rapidly delivered and cleared from the dentate gyrus. BDNF is clearly being delivered to the CA1 region, and the lack of effect on signal transduction is interesting in this regard. It could be related to differences in activity-dependent gene expression between the CA1 region and dentate gyrus (French et al., 2001). However, there are salient methodological points to this issue. Because the cannula is placed into deep-field CA1 (stratum lacunosum-moleculare), differences can be expected with regard to the concentration and kinetics of BDNF as it travels upward along the cannula tract as well as possible trauma and pressure effects. Experiments combining recording of Schaffer collateral–CA1 responses with infusion of BDNF above the synaptic region are needed to resolve this issue.

The physiological relevance of exogenous BDNF application may be questioned. The fact that BDNF-LTP occludes selectively with late phase LTP and activates a common set of critical intracellular events in the intact adult brain attests to the physiological relevance of the phenomenon. The similarity between HFS-LTP and BDNF-LTP is further evidenced by the fact that both are associated with a parallel increase in synaptic strength and E–S coupling.

ACD has commonly been used to draw the distinction between transcription-dependent (late) and transcription-independent (early) LTP. Previous work has shown that ACD blocks development of late HFS-LTP, leaving early LTP intact (Frey et al., 1996; Nguyen and Kandel, 1996). In contrast, BDNF-LTP is almost completely transcription-dependent, consistent with a selective role for BDNF in late phase LTP. When ACD is applied immediately before BDNF, a normal initial response to BDNF is followed by a drop to a small but stable plateau level. There are two plausible explanations for the residual potentiation. First, it could reflect an mRNA synthesis-independent process, such as post-transcriptional regulation of protein synthesis or protein phosphorylation, and evidence for both actions exists (discussed below). Alternatively, the residual potentiation could reflect incomplete inhibition of transcription by ACD. For example, if ACD takes effect after BDNF, this would allow time for initiation of a small, transcription-dependent potentiation. We cannot rule out this scenario, although ACD was shown to act rapidly and potently (1 hr pretreatment blocked BDNF-LTP).

In the CA1 region of the hippocampus, BDNF-LTP appears to involve protein synthesis from dendritically localized mRNA (Kang and Schuman, 1996). Using slices in which the synaptic neuropil was isolated (connections with the CA3 and CA1 cell bodies were severed), Kang and Schuman (1996) showed that BDNF induces LTP that was blocked by protein synthesis inhibitors. However, a role for transcription has not been investigated in CA1. In this context, it should be pointed out that activation of the translation machinery in dendrites may go hand in hand with the arrival of *Arc* transcripts, effectively driving Arc protein synthesis at synaptic sites (Ying et al., 2002). Supporting this view, BDNF has been shown recently to stimulate post-transcriptional synthesis of Arc in isolated synaptoneurosomes (Yin et al., 2002). This mechanism may well be accentuated in the context of elevated *Arc* mRNA. The present work using the transcription inhibitor ACD shows that Arc protein expression in BDNF-LTP derives predominantly from new mRNA synthesis rather than from translation of preexisting mRNA. BDNF can modulate translation through multiple signaling cascades, and the contribution of these pathways to dendritic protein synthesis and synaptic plasticity are only beginning to be resolved (Steward and Schuman, 2001; Takei et al., 2001). BDNF-LTP and late HFS-LTP in the CA1 field are both blocked by inhibition of mammalian target of rapamycin, a key regulator of translation initiation (Tang et al., 2002). In the dentate gyrus, BDNF-LTP is coupled to ERK-dependent activation of elongation factor-2, an important regulator of peptide chain elongation (Kanhema et al., 2001).

The selective occlusion of the BDNF response during late LTP suggests a rapid transition (switch) in the mechanism of LTP expression taking place between 1 and 4 hr after HFS. Frey et al. (1995) reached a similar conclusion based on occlusion experiments using two sessions of HFS. They found that early LTP was occluded at 1 hr after HFS (as would be expected) but could again be induced at 4 hr. Our results are consistent with these findings and implicate BDNF as a trigger for the conversion between early and late LTP.

The mechanism of occlusion remains to be identified. Although Arc synthesis is required in late HFS-LTP, there is no evidence that it is sufficient. Work by Chowdhury et al. (1998) suggests that Arc acts as a physical tether for calcium/calmodulin-dependent protein kinase II (CaMKII), anchoring it to the cytoskeleton within or in close proximity to the postsynaptic density. Arc and CaMKII are both synthesized from dendritically localized mRNA (Lyford et al., 1995; Steward and Schuman, 2001), and recent work shows that BDNF-LTP is associated with CaMKII activation (Kanhema et al., 2001). The exact functional relationship between Arc and CaMKII will be important to define. Taken together, the data suggest that BDNF stimulates synaptic consolidation through transcription-dependent and -independent mechanisms in which Arc may play a key role.

The critical period of BDNF release and TrkB receptor activation in LTP remains to be defined. Hartmann et al. (2001) found that HFS triggers immediate release of BDNF–green fluorescent protein from postsynaptic sites in hippocampal cell cultures. Evidence for rapid release has also been obtained after LTP-inducing stimuli in hippocampal slices (Kossel et al., 2001). Other evidence suggests an important function for more sustained or delayed events. TrkB activation in the dentate gyrus is enhanced 40 min after HFS (Gooney and Lynch, 2001), and late LTP is impaired by application of a BDNF scavenger, TrkB-Fc, 30–60 min after HFS (Kang et al., 1997). A sustained or delayed activation of TrkB by endogenous BDNF is most consistent with our data, because BDNF-LTP was not occluded 1 hr after HFS.

BDNF has been shown to act presynaptically to enhance glutamate release, raising the possibility that BDNF might, by releasing glutamate, trigger classic NMDAR-dependent LTP (Jovanovic et al., 2000; Gooney and Lynch, 2001). This is not the case, however, because CPP abolished HFS-LTP but had no effect on BDNF-LTP. Our results confirm a previous study by Kang and Schuman (1995) in the CA1 region *in vitro* and extend it to the dentate gyrus *in vivo*. This result does not rule out a contribution of other glutamate receptor types in BDNF-LTP induction. The role of glutamatergic transmission in BDNF release is another issue. Release of endogenous BDNF during HFS depends on activation of postsynaptic ionotropic glutamate receptors (Hartmann et al., 2001). Using exogenous application, we are bypassing this initial release event.

In cultured hippocampal neurons, BDNF elicits a transient potentiation of excitatory synaptic transmission (Gottschalk et al., 1998; Lessmann and Heumann, 1998; Li et al., 1998; Crozier et al., 1999). This effect is inhibited by NMDAR antagonists and is mediated at least in part by enhanced conductance of postsynaptic NMDAR-2B-containing receptors (Levine et al., 1998; Crozier et al., 1999). Although the NMDAR-2B mechanism is important in the developing hippocampus, the fact that BDNF-LTP is undiminished during NMDAR blockade argues against this mechanism occurring in the adult dentate gyrus. On the other hand, there is evidence that NMDAR-2B is upregulated later (2 d) in the process of HFS-LTP (Williams et al., 1998).

BDNF is a versatile molecule acting in the short and long term to regulate a diverse range of functions in the developing and adult nervous systems. In the context of synaptic plasticity, the question has been asked of whether BDNF serves a housekeeping function, maintaining the machinery that makes synapses modifiable, or an active, instructive role in triggering synaptic change (Schinder and Poo, 2000). The present study supports a direct, instructive role for BDNF in transcription-dependent, late phase LTP. Furthermore, the phenomenon of exogenous BDNF-LTP provides a valuable tool for elucidating the molecular basis of BDNF action specific to the consolidation process.

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Paper II

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Dual regulation of translation initiation and peptide chain elongation during BDNF-induced LTP *in vivo*: evidence for compartment-specific translation control

Tambudzai Kanhema,* Grethe Dagestad,* Debabrata Panja,* Adrian Tiron,* Elhoucine Messaoudi,* Bjarte Håvik,* Shui-Wang Ying,* Angus C. Nairn,† Nahum Sonenberg‡ and Clive R. Bramham*

**Department of Biomedicine and Bergen Mental Health Research Center, University of Bergen, Bergen, Norway*

†*Department of Psychiatry, Yale University School of Medicine, New Haven, USA*

‡*Department of Biochemistry, McGill University, Montreal, QC, Canada*

Abstract

Protein synthesis underlying activity-dependent synaptic plasticity is controlled at the level of mRNA translation. We examined the dynamics and spatial regulation of two key translation factors, eukaryotic initiation factor 4E (eIF4E) and elongation factor-2 (eEF2), during long-term potentiation (LTP) induced by local infusion of brain-derived neurotrophic factor (BDNF) into the dentate gyrus of anesthetized rats. BDNF-induced LTP led to rapid, transient phosphorylation of eIF4E and eEF2, and enhanced expression of eIF4E protein in dentate gyrus homogenates. Infusion of the extracellular signal-regulated kinase (ERK) inhibitor U0126 blocked BDNF-LTP and modulation of the translation factor activity and expression. Quantitative immunohistochemical analysis revealed enhanced staining of phospho-eIF4E and total eIF4E in dentate granule cells. The *in vitro* synaptodendrosome preparation was used to isolate the synaptic effects of BDNF in the dentate gyrus. BDNF treatment of synaptodendrosomes elicited rapid, transient phosphorylation of eIF4E paralleled by enhanced expression of α-calcium/calmodulin-dependent protein kinase II. In contrast, BDNF had no effect on eEF2 phosphorylation state in synaptodendrosomes. The results demonstrate rapid ERK-dependent regulation of the initiation and elongation steps of protein synthesis during BDNF-LTP *in vivo*. Furthermore, the results suggest a compartment-specific regulation in which initiation is selectively enhanced by BDNF at synapses, while both initiation and elongation are modulated at non-synaptic sites.

Keywords: dentate gyrus, hippocampus, neurotrophic factors, synaptic plasticity, translation control.

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Stable activity-dependent changes in synaptic strength, as observed in long-term potentiation (LTP) and depression (LTD), are thought to underlie memory storage and other adaptive mechanisms such as mood stability and drug addiction (Bliss and Collingridge 1993; Hyman *et al*. 2006; Kuipers and Bramham 2006). Activity-dependent changes in synaptic strength require one or more period of new protein synthesis. Recent work has shown that protein synthesis underlying synaptic plasticity is critically controlled at the level of mRNA translation (for a review see Klann and Dever 2004; Richter and Sonenberg 2005; Soule *et al.* 2006). In combination with mRNA transport and localization, control of mRNA translation determines the timing and subcellular location of protein synthesis. In neurons this has been exquisitely exemplified by local translation of mRNA in dendrites (Wu *et al*. 1998; Aakalu *et al*. 2001; Steward and Schuman 2003; Tsokas *et al*. 2005).

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Address correspondence and reprint requests to Clive Bramham, Department of Physiology, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway. E-mail: clive.bramham@biomed.uib.no

Abbreviations used: Arc, activity-regulated cytoskeletal-associated protein; BDNF, brain-derived neurotrophic factor; CaMKII, calcium and calmodulin-dependent protein kinase II; Cyt C, cytochrome C; DMSO, dimethylsulfoxide; eEF2, eukaryotic elongation factor-2; eIF4E, eukaryotic initiation factor 4E; ERK, extracellular signal-regulated protein kinase; HFS, high-frequency stimulation; LTP, long-term potentiation; mTOR, mammalian target of rapamycin; PSD, postsynaptic density; SD, synaptodendrosome; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel.; TrKB, tropomyosin-related receptor kinase B.

The initiation and elongation steps of protein synthesis are both highly regulated events. Two of the key translation factors involved are eukaryotic initiation factor 4E (eIF4E) and eukaryotic elongation factor 2 (eEF2). eIF4E binds to the 7-methyl-guanosine residue that caps the 5′ end of nuclear–encoded RNAs. Interaction of eIF4E with ribosomal-associated translation factors is required for translation intiation. Phosphorylation of eIF4E on Ser209 is correlated with enhanced rates of translation, whereas hypophosphorylation is associated with decreased translation (Gingras *et al*. 2004). eEF2 is a GTP-binding protein that mediates translocation of peptidyl-tRNAs from the A-site to the P site on the ribosome. Phosphorylation of eEF2 on Thr56 inhibits eEF2-ribosome binding and arrests elongation (Nairn and Palfrey 1987; Ryazanov *et al*. 1988). While EF2 phosphorylation is associated with reduction in global protein synthesis, some transcripts appear to be selectively translated under these conditions (Scheetz *et al*. 2000; Chotiner *et al*. 2003). Recent studies have established a critical role for cap-dependent translation initiation in synaptic plasticity (Kelleher *et al*. 2004; Banko *et al*. 2005). However, the coordination and spatial control of translation initiation and elongation during synaptic plasticity are little understood.

The secretory peptide brain-derived neurotrophic factor (BDNF) has emerged as major regulator of synaptic plasticity in the hippocampus of adult animals (Blum and Konnerth 2005; Bramham and Messaoudi 2005). In the dentate gyrus of intact rats, local infusion of BDNF triggers a long-term potentiation (BDNF-LTP) that requires new gene expression and protein synthesis (Messaoudi *et al*. 2002; Ying *et al*. 2002; Messaoudi *et al*. 2006). Here, we examined the dynamics and spatial regulation of eIF4E and eEF2 following BDNF-LTP induction in the dentate gyrus *in vivo*. Phosphorylation of eIF4E on Ser209 and phosphorylation of eEF2 on Thr56 was examined by quantitative immunoblotting and immunohistochemistry following *in vivo* treatment, while the isolated synaptic actions of BDNF were investigated *in vitro* using synaptodendrosomes (SDs), a subcellular fraction enriched in pinched-off re-sealed terminals attached to re-sealed dendritic spine structures (Rao and Steward 1991; Weiler and Greenough 1993; Havik *et al*. 2003). The results demonstrate rapid, parallel extracellular signal-regulated protein kinase (ERK), ERK-dependent phosphorylation of eIF4E and eEF2 during BDNF-LTP *in vivo*. In synaptodendrosomes, BDNF selectively stimulates eIF4E phosphorylation, without affecting net eEF2 phosphorylation. Thus, BDNF regulates the translation initiation and elongation steps of protein synthesis in a compartment-specific manner.

Materials and methods

Electrophysiology and intrahippocampal infusion

The methods have been detailed elsewhere (Messaoudi *et al*. 2002; Yin *et al*. 2002). Briefly, adult male Sprague-Dawley rats were anesthetized with urethane and electrodes were positioned for seletive unilateral stimulation of the medial perforant path and recording of evoked field potentials from the dentate gyrus. Test pulses were applied every 30 s. BDNF was infused immediately above the dorsal dentate gyrus, into deep stratum-lacunosum moleculare of field cornu ammonis (CA) 1, approximately 300 μm from the nearest medial perforant path-granule synapses.

Tissue microdissection and sample preparation

At the end of electrophysiological recording rats were decapitated and the dentate gyrus and hippocampal CA1 and CA3 regions were rapidly dissected on ice. Tissues were hand-homogenized in sodium dodecyl sulfate (SDS) sample buffer. Homogenates were boiled for 5 min, aliquoted, and stored at − 80°C until use.

Preparation and stimulation of synaptodendrosomes (SDs)

Dentate gyrus from urethane anesthetized rats was frozen and stored at − 80°C. Sets of six dentate gyri were thawed 5 min on ice and 5 min at room temperature prior to homogenization in ice-cold homogenization buffer (HB) containing (in mM): sucrose (320), Tris-HCl pH 7.4 (20), EGTA (2), $MgCl_2$ (2), dithiothreitol (DTT) 2.5 and NaF (0.25 μM). Homogenization was performed manually with 10–12 gentle strokes in a tissue grinder with a clearance of 0.1–0.15 mm (Thomas Scientific, Swedesboro, NJ, USA). Calcium influx and changes in magnesium levels during preparation of synaptic fractions is a concern as it may affect basal phosphorylation. In attempt to obtain more physiological conditions we used buffer containing 2 mM EGTA and 2 mM $MgCl_2$. Significantly lower basal levels of CaMKII phosphorylation were obtained compared to buffer containing EGTA and EDTA.

The homogenate was passed through a nylon filter (30 μm pore-size MACS filter) and mixed with a 50% OptiPrep solution to make a 35% solution. This was placed in the bottom of a Beckman centrifugation tube, onto which OptiPrep solutions of (25%, 15%, 12.5% and 9%) were layered. After centrifugation for 24 min at 10 000 *g* in an ultracentrifuge (Beckman LE-80K Ultracentrifuge, SW41Ti rotor), the SD containing fraction in the 9–12.5% interface was collected, diluted 2–4 times in 1 x PBS and loaded on top of a Sucrose-Percoll step gradient (6, 10, 15, 20 and 23). The gradients were made by diluting Percoll in HB. After centrifugation for 9 min at 32 000 *g*, the SDs were collected from the 15/20% interface and from the 20% fraction (often a double band). The synaptodendrosome fraction was then diluted in HB and centrifuged at 6000 *g* for 1–2 min. The resulting pellet was washed once by resuspension in HEPES-buffered artificial cerebrospinal fluid (ACSF), centrifuged at 6000 *g* for 1–2 min, and stored at −80°C.

The synaptodendrosomes pellet was thawed on ice for 30 min and diluted in pre-warmed (37°C) oxygenated ACSF to give a protein concentration between 2 and 9 mg/mL (approximately 1 : 3 dilution). SD samples were continuously oxygenated in an eppendorf tube and treated with BDNF or cytochrome C, the latter serving as a time-matched control. The reactions were stopped (5 x SDS buffer) and samples were boiled for 5 min. A concentration of 200 ng/mL BDNF was selected for the main body of experiments based on dose–response effects on phosphorylation events (present work), Tropomyosin-related receptor kinase B (TrkB) activation, and protein synthesis (Takei *et al*. 2001).

Drugs and antibodies

Recombinant BDNF and cytochrome C from yeast (Sigma, St Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS). The ERK kinase inhibitor U0126 (gift of James Trzaskos, DuPont Pharmaceuticals, Wilmington, DE, USA), was dissolved in dimethylsulfoxide (DMSO) and diluted in PBS to a final concentration of 30 μM inhibitor containing 0.3% DMSO. Primary antibodies used for immunoblotting were as follows: total eIF4E (1 : 1000, Cell Signaling polyclonal 5853), Ser209 phosphorylated eIF4E (1 : 1000, Cell Signaling polyclonal), total eEF2 (G118, 1 : 500), Thr56 phospho-eEF2 (CC81, 1 : 300), total αCaMKII (1: 2000, Affinity BioReagents mouse monoclonal 6G9), Thr286 phospho-αCaMKII (1 : 5000, Promega rabbit polyclonal V111A), and β-actin (1 : 5000 Sigma mouse monoclonal AC-15).

SDS–PAGE and Western Blotting

Protein levels in homogenate samples were determined using the Lowry method. Equal amounts of protein were separated on sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) gels (peEF2 and total eEF2 on 8%, pCaMKII and total αCaKII on 10%, and peIF4E and total eIF4E on 12%) and run overnight at constant 10 mA. Separated proteins were transferred to nitrocellulose membranes (Hybond-C, Biorad, Rjukan, Norway) using a constant voltage of 30 V overnight or 100 V for 1 h. Membranes were blocked on a gyro-rocker for 1 h at room temperature (RT) (21–23°C). Blocking buffer (BB) consisted of TBST (Tris-buffered saline/0.1% Tween-20) and 5% Bovine Serum Albumin (BSA). For pCaMKII and total αCaMKII, membranes were blocked 1 h at 37°C. eIF4E, eEF-2 and αCaMKII were analyzed by sequential immunoblotting with primary antibodies specifically recognizing the phosphorylated (P) or total proteins. The primary antibodies were dissolved in BB containing 3% BSA and the blots were incubated for 2 h at RT (for total proteins) or at 4°C overnight (phospho-proteins) with constant shaking. Following three washes with TBST, blots were incubated for 1 h in horseradish peroxidase-conjugated secondary antibody dissolved in TBST. The blots were then washed again and visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Norway). Blots treated with phospho-specific antibody were stripped with 100 mM 2-mercapto-ethanol, 2% SDS and 62.5 mM Tris-HCL, pH 6.7 at 60°C for 1 h and reprobed. with antibody recognizing total protein. Autoradiographs were quantitated using Phoretics ID plus software. Western blots were developed to be linear in the range used for densitometry. In the *in vivo* studies, optical density values obtained from the treated hippocampus were normalized relative to values in the contralateral hippocampal subfield. In the *in vitro* studies, the BDNF and cytochrome C-treated samples were analyzed as matched pairs. Statistical analyses were based on paired *t*-tests.

Immunohistochemical staining and analysis

At the end of the experiments, rats were deeply anesthetized and then perfused intracardially with saline followed by ice cold 4% paraformaldehyde in 1 × PBS, pH 7.4. Brains were removed, postfixed overnight in 4% paraformaldehyde/PBS at 4°C, and equilibrated in 25% sucrose in PBS until the brains sank. Brains were sectioned coronally at a thickness of 30 μm and stored in 1 × PBS at 4°C. For antigen retrieval, free-floating sections were heat treated at 95°C for 45 min in citric acid solution (10 mM, pH = 6.0). After antigen retrieval, sections were blocked for 1 h at room temperate in blocking buffer (3% normal goat serum + 0.5% BSA + 0.1% TritonX-100 in PBS). Primary antibodies recognizing eIF4E (1 : 500) and p-eIF4E (1 : 25) were diluted in blocking buffer and incubated overnight at 4°C. Negative control incubations were performed by substituting non-immune serum for the primary antibody. After primary antibody incubation the sections were washed with 1 × PBS several times and incubated in biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1 : 200 in blocking buffer for 1 h at RT. The sections were washed and incubated in Vector ABC kit (Vector Laboratories) for 1 h and final color was developed using 3,3′-diaminobenzidine with nickel as the chromogen. Sections were mounted on poly-L-lysine slides, dehydrated through alcohols to xylene, and coverslipped with DPX.

For quantitative immunostaining, seven sets of 12 serial coronal sections were collected along the dorsal hippocampus in the region 1.15 mm rostral and caudal to the infusion site. Phospho-eIF4E and total eIF4E staining and analysis were performed on 7 sections (one from each set). Images (10 × magnification) were captured on a Nikon DS-OM digital camera using a DS-L1 camera control unit (Inter Instruments AS, Hovik, Norway) and analyzed using NIH IMAGE J software (Scion, Frederick, MD, USA). Mean optical density values corrected for background were determined across the entire granule cell layer from each section. Differences between treated and contralateral control values were determined by one-way ANOVA and a post hoc Bonferroni test.

Results

Rapid and transient phosphorylation of eIF4E and eEF2 during BDNF-LTP *in vivo*

BDNF-LTP was induced by infusion of BDNF (2 μg in 2 μL) into stratum lacunosum-moleculare of CA1, approximately 300 μm above the medial perforant path synapses. Increases in the medial perforant path-evoked field excitatory post-synaptic potentials (fEPSPs) reached statistical significance at 15 min and climbed gradually to a stable plateau at 2–3 h after BDNF infusion (Fig. 1a). Accordingly, experiments were terminated at 15 min or 3 h after BDNF infusion in order to monitor the induction and maintenance phases of BDNF-LTP (Fig. 1b). Infusion of cytochrome C, which has a similar molecular weight and charge as BDNF, had no significant effect on synaptic efficacy during 3 h of recording (Fig. 1b). Homogenates from microdissected dentate gyrus and hippocampal regions CA1 and CA3 were subjected to quantitative immunoblotting and comparisons were made between the treated and non-treated serine contralateral control regions. Levels of 209 phosphorylated eIF4E were significantly elevated 2.2-fold in the BDNF-infused dentate gyrus 15 min after infusion, but returned to control levels at 3 h (Fig. 1c). Interestingly, this early increase in phosphorylation was paralleled by a significant 2.3-fold increase in total eIF4E levels (Fig. 1d). Changes in phospho-eIF4E were normalized to total eIF4E levels and thus reflect a genuine state of hyperphosphorylation.

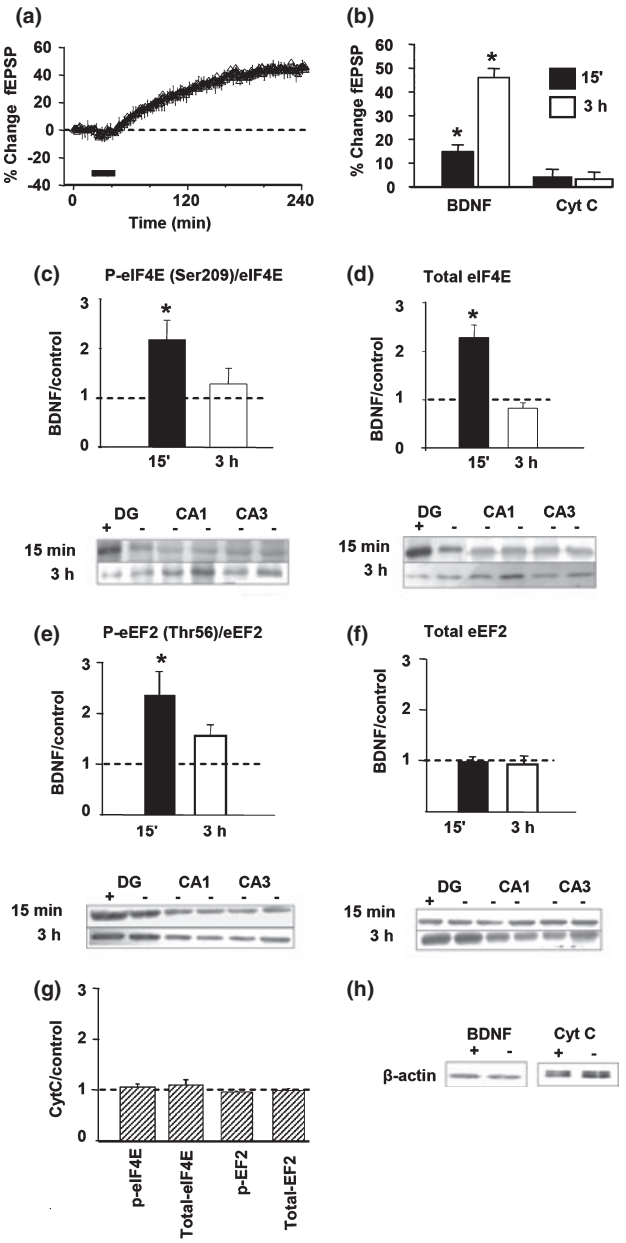

Fig. 1 Rapid and transient phosphorylation of eIF4E and eEF2 during BDNF-LTP in the dentate gyrus of anesthetized rats. (a) Time course plot showing changes in medial perforant path-granule cell-evoked fEPSPs expressed in percent of baseline. Values are group means ± SEM ($n = 5$). Test pulses were applied at a rate of 1 every 30 s. BDNF (2 μg/2 μL) was unilaterally infused 300 μm above the medial perforant-granule cell synapses, during the period indicated by the solid bar. (b) Magnitude of fEPSP change in groups of rats killed at 15 min ($n = 8$) and 3 h ($n = 7$) after BDNF or Cytochrome C (Cyt C) infusion. *Significant difference from baseline ($p < 0.05$). Western blot assays were performed on aliquoted samples from microdissected dentate gyrus (DG), and hippocampal regions CA1 and CA3. Bar graphs show group mean (± SEM) changes in p-eIF4E (c) and total eIF4E levels (d) based on densitometric analysis. Optical density values are expressed as a ratio between the treated and non-treated (control) side for each hippocampal subfield. Changes in phosphorylation are normalized relative to total phosphoprotein. Significant increases in eIF4E phosphorylation and eIF4E expression specific to the dentate gyrus were detected 15 min after BDNF infusion. *Significant difference from control ($p < 0.05$). Representative immunoblots are shown in the lower panels. (e) Significant increases in eEF2 phosphorylation were detected 15 min ($n = 8$), but not 3 h ($n = 7$) after BDNF infusion. (f) No change in total eEF2 levels. (g) Cytochrome C (Cyt C) infusion had no significant effect on translation factor phosphorylation or expression. Bar graphs shows effects 15 min after Cyt C infusion. (h) Immunoblots of β-actin expression 15 min after BDNF or Cyt C infusion.

The effects of BDNF infusion on eEF2-threonine56 phosphorylation and total eEF2 levels are shown in Figs 1(e and f). Phospho-eEF2 levels were significantly elevated 2.3 fold at 15 min after BDNF infusion. Phospho-eEF2 levels remained elevated at 3 h, although the 1.5- fold increase was not significant. No changes in total eEF2 levels were seen at either time point. The effects of local BDNF infusion on translation factor activity were specific to the dentate gyrus as no significant changes were detected in the CA1 and CA3 regions (sample blots are shown in Fig. 1). Infusion of the cytochrome C had no effect on phosphorylation or expression of eIF4E or eEF2 levels (Fig. 1g). BDNF and Cyt C infusion also had no effect on β -actin expression, which served as a loading control (Fig. 1h).

Rapid modulation of eIF4E and eEF2 requires ERK signaling

We have previously reported that BDNF-LTP requires rapid ERK signaling coupled to calcium/cyclic AMP response-element binding protein (CREB) activation and up-regulation of the immediate early gene (Messaoudi *et al*. 2002; Ying *et al*. 2002). BDNF can also signal through TrkB-ERK to regulate phosphorylation of eIF4E (Takei *et al*. 2001; Kelleher *et al*. 2004). Here, we examined a possible role of ERK activation in regulating both eIF4E and eEF2 during BDNF-induced synaptic plasticity *in vivo*. As shown in Fig. 2, intrahippocampal coinfusion of the ERK kinase inhibitor U0126 (30 μM, $n = 8$) with BDNF blocked BDNF-LTP induction and the associated increases in phospho-eIF4E, phospho-eEF2, and total eFI4E observed 15 min post-infusion. At 3 h post-infusion, no differences were detected between rats receiving BDNF alone or BDNF in the presence of U0126.

Immunohistochemical localization of phospho-eIF4E and total eIF4E in the dentate gyrus following BDNF-LTP *in vivo*

Next we sought to anatomically localize the changes in translation factor activity and expression 15 min after BDNF infusion. The analysis focused on phosphorylated and total eIF4E as these antibodies yielded clear and specific immunostaining. Immunohistochemical staining was performed on seven coronal sections collected in the region 1.15 mm

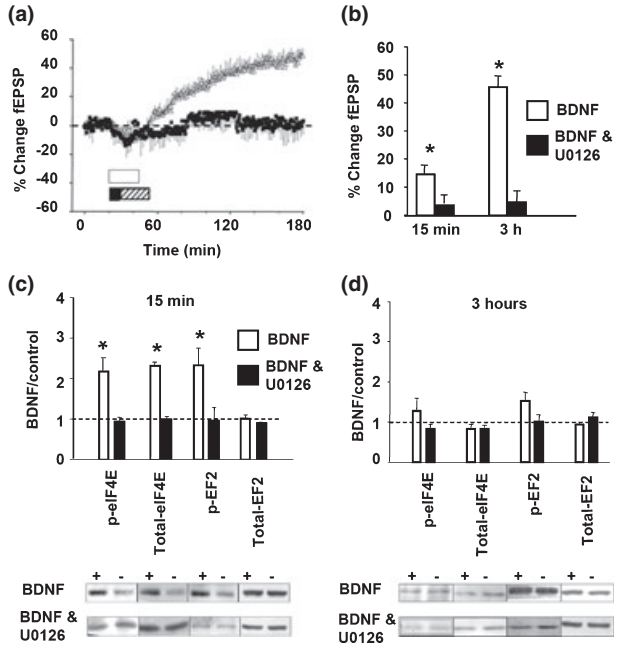

Fig. 2 Regulation of eIF4E and eEF2 activity during BDNF-LTP is ERK-dependent. (a) Time course plot showing changes in medial perforant path-granule cell evoked fEPSPs expressed in percent of baseline. Values are group means ± SEM (n = 5). Test pulses were applied at a rate of 1 every 30 s. BDNF (2 μg/2 μL) was infused 300 μm above the medial perforant-granule cell synapses, during the period indicated by the open bar. Infusion of 1 μL U0126 (30 μm; filled bar) was followed immediately by infusion of 2 μg BDNF in 2 μL U0126 (hatched bar). Values are means ± SEM expressed in percent of baseline. (b) Bar graph showing changes in fEPSP slope 15 min and 3 h after infusion. *Significant difference from baseline ($p < 0.05$). Western blot assays were performed on aliquoted samples from microdissected dentate gyrus. Bar graphs show group mean (± SEM) changes 15 min (c) and 3 h (d) after infusion. Optical density values are expressed as a ratio between the treated and non-treated (control) dentate gyrus. Representative immunoblots are shown in the lower panels. BDNF-LTP induction and the associated increases in p-eIF4E, total eIF4E, and p-eEF2 were abolished in U0126-infused rats. N = 4–8. β-actin levels were unchanged following BDNF-LTP induction.

rostral and caudal to the infusion site. Increases in phospho-eIF4E and total eIF4E were observed in BDNF-infused dentate gyrus, but not cytochrome C-treated dentate gyrus, in 4 of 4 rats (Fig. 3a and c). No changes in immunostaining were observed outside of the dentate gyrus. Within the dentate gyrus enhanced staining was evident in the cytoplasmic margins of granule cell somata (Fig. 3b and d). Enhanced phospho-eIF4E and total eIF4E staining was also inconsistently observed across the dentate molecular layer up to the hippocampal fissure, but the expression appeared to be near the detection threshold of the method. Similar results were obtained at a range of antibody titers, and no staining was observed in control experiments in which primary antibody was substituted with non-immune serum. Quantitative densitometric analysis of the immunostaining demonstrated statistically significant increases in phosho-eIF4E and total eIF4E in the granule cell layer of the BDNF-infused dentate gyrus compared to contralateral control (Fig. 3e and f). No significant differences between sides were detected following cytochrome C infusion.

BDNF elicits a rapid and transient phosphorylation eIF4E, but not eEF2, in isolated synaptodendrosomes

Translation factors and other components of the translational machinery are found in somata, dendrites, and spines, but not in axon terminals of adult synapses (Tiedge and Brosius 1996; Pierce *et al*. 2000). The simultaneous phosphorylation of eIF4E and eEF2 suggests a coordinate process of enhanced initiation and reduced peptide chain elongation. We considered that these seemingly opposing biochemical mechanisms might be spatially segregated in neurons. Specifically, we hypothesized that BDNF signaling selectively promotes translation initiation in dendritic spines, while initiation and elongation are both modulated at non- synaptic sites. Whole dentate gyrus homogenates and light microscopic immunocytochemistry is incapable of resolving mechanisms at the level of dendritic spines. In order to isolate the direct effects of BDNF at the level of the dendritic spines, we turned to the *in vitro* synaptodendrosome (SD) preparation. The SD preparation is a subcellular fraction containing axon terminals attached to pinched-off spine-like elements containing a postsynaptic density (PSD) characteristic of excitatory synapses. SDs are highly enriched in PSD-95 protein and depleted of cell body and nuclear markers (Havik *et al*. 2003). As observed during BDNF-LTP *in vivo*, BDNF-treatment of SDs resulted in rapid (5 min) and transient phosphorylation of eIF4E compared to the cytochrome C-treated control (Fig. 4a). However, no significant changes in total eIF4E were detected in BDNF-treated SDs (Fig. 4b). Furthermore, in contrast to the western blot and immunohistochemical data from the *in vivo* studies, phospho-eEF2 levels in SDs were unchanged following BDNF treatment (Fig. 4c). Potassium depolarization of SDs was used as a positive control for eEF2 modulation. As shown in Fig. 4(d), high-potassium (50 mM) treatment evoked a significant 1.6-fold increase in phospho-eEF2 in synaptodendrosomes (Fig. 4d). A small but significant increase in total eEF2 protein was also detected 5 min after BDNF incubation (Fig. 4e).

BDNF enhances synaptic synthesis and activation of CaMKII

What impact does compartmental regulation of the translational machinery have on the expression of proteins controlling synaptic strength and structure? To begin to address this issue we examined expression of α-calcium/calmodulin-

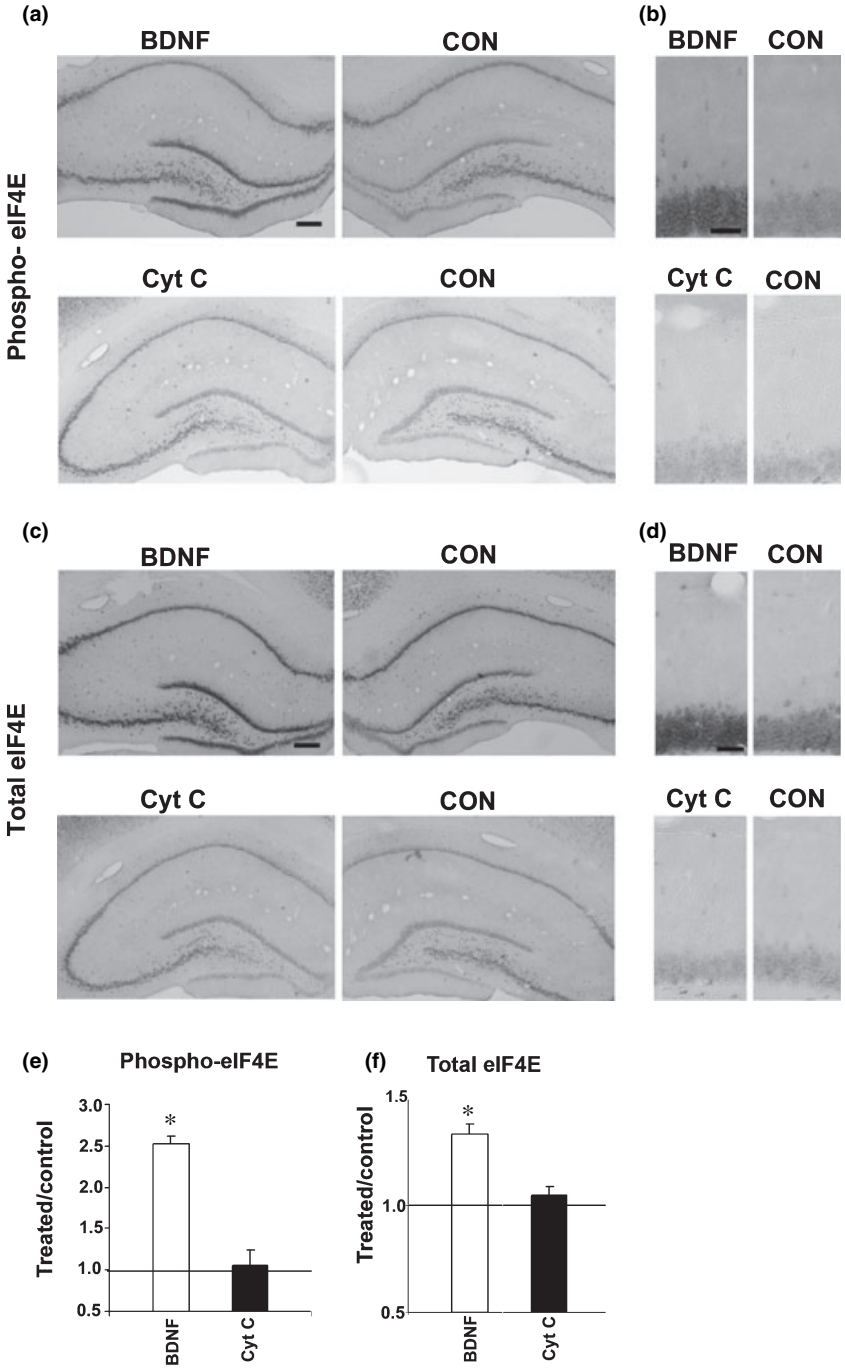

Fig. 3 Immunohistochemical localization of phosphorylated and total eIF4E in the dentate gyrus following *in vivo* BDNF-LTP. Coronal sections were collected 15 min after BDNF infusion or Cyt C infusion. Panels (a) and (c) show phospho-eIF4E and total eIF4E immunostaining of treated and contralateral, control (CON) hippocampus. Panels (b) and (d) show corresponding high magnification (20 ×) images of the inner blade of the dentate gyrus in the region extending from the granule cell layer to the hippocampal fissure (the region indicated by a white box). Scale bar is 50 μm in (a) and 100 μm in (b). Enhanced p-IF4E and total eIF4E labeling was observed in the granule cell layer. Quantitative analysis of the immunostaining in the granule cell layer is shown in panels (e) and (f). Bar graphs shows densitometric values in treated dentate gyrus normalized relative to contralateral control. *Significantly different from contralateral control ($n = 4$; $p < 0.05$).

dependent protein kinase II (αCaMKII) using an antibody that recognizes both phosphorylated and unphosphorylated forms of the enzyme. mRNA encoding αCaMKII is stored in dendrites and local translation of this message affects the size of the PSD and development of late LTP (Miller *et al*. 2002; Havik *et al*. 2003). Havik *et al*. previously reported that synapse-specific increases in αCaMKII mRNA and protein are masked in homogenates from whole dentate gyrus. We therefore compared the effects of BDNF infusion *in vivo* with treatment of isolated SDs *in vitro*. Levels of total CaMKII were unchanged in dentate gyrus, CA1 and CA3 tissue samples collected 15 min and 3 h after BDNF infusion *in vivo* (Fig. 5a, left panel). In contrast, BDNF-treatment of dentate gyrus SDs led to a significant 45% increase in CaMKII expression paralleling the rapid phosphorylation of eIF4E (Fig. 5a, right panel). Finally, CaMKII autophosphorylation was assessed using an antibody that detects phosphorylation of α-CaMKII at threonine 286. CaMKII was

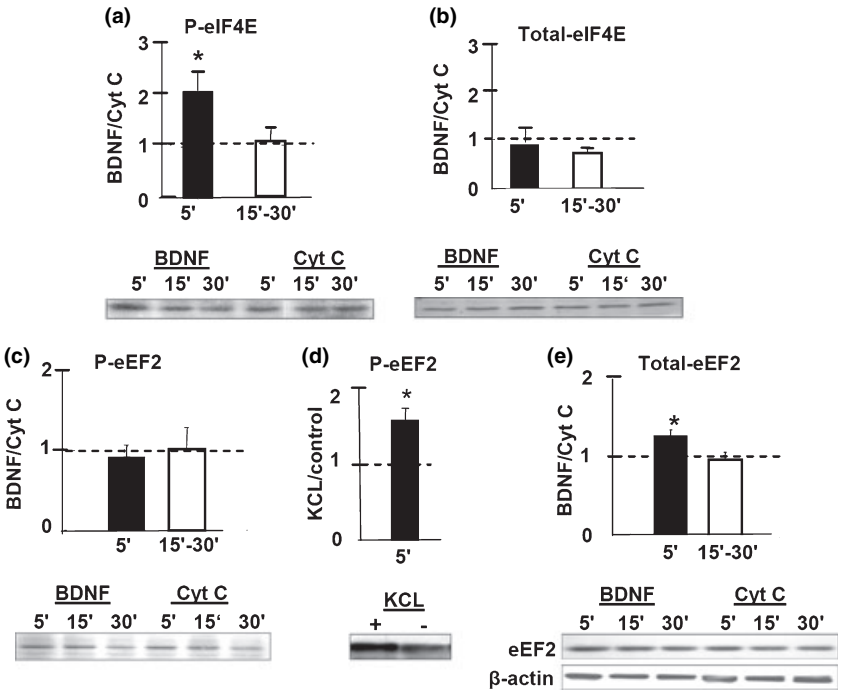

Fig. 4 BDNF elicits rapid and transient phosphorylation of eIF4E, but not eEF2, in isolated synaptodendrosomes. Bar graphs show changes in p-eIF4E (a) and total eIF4E (b) expression following BDNF treatment of synaptodendrosomes obtained by subcellular fractionation of microdissected dentate gyrus. Western blots were performed on matched pairs of BDNF and cytochrome C-treated samples. Optical density values are group means (± SEM) expressed as the ratio of BDNF/Cyt C ($n = 5$). Significant increases in p-eIF4E were detected at 5 min ($p < 0.05$). No significant changes were detected at the 15 min or 30 min time points (pooled data shown). Changes in phosphorylation are normalized relative to total phosphoprotein. Representative immunoblots are shown below. (c) *p*-eEF2 levels were unchanged following BDNF treatment. (d) High-potassium (50 mM) treatment of synaptodendrosomes elicited significant increases in p-eEF2 levels relative to time-matched, non-treated synaptodendrosomes ($n = 4$; $p < 0.05$). (e) Total eEF2 levels were weakly but significantly elevated at 5 min ($p < 0.05$). $n = 13$ at 5 min; $n = 10$ at 15–30 min. Representative eEF2 and β-actin immunoblots are shown below.

transiently activated during BDNF-LTP *in vivo* and following BDNF incubation of SDs *in vitro* (Fig. 5b).

Discussion

In the present study transient phosphorylation of eIF4E and eEF2 as well as enhanced expression eIF4E protein coincided with the onset of BDNF-LTP. Like BDNF-LTP induction, regulation of eIF4E and eEF2 requires ERK signalling. Quantitative immunohistochemical staining confirmed the Western blot data on phosphorylated and total eIF4E expression and revealed sharply enhanced staining in granule cell somata. The present work thus demonstrates dynamic, ERK-dependent regulation of both the initiation and elongation steps of protein synthesis during long-term synaptic plasticity *in vivo*. The synaptodendrosome preparation gave important insight into the synaptic actions of BDNF which could not be resolved by light microscopy. In synaptodendrosomes, BDNF elicited rapid and transient phosphorylation of eIF4E and this was associated with enhanced expression and activation of CaMKII. However, in contrast to observations in whole dentate gyrus, BDNF treatment of synaptodendrosomes had no effect on eEF2 phosphorylation state. As a positive control, we show that eEF2 is phosphorylated in response to potassium depolarization. Thus, BDNF appears to selectively facilitate initiation at synapses, while both initiation and elongation are modulated at non-synaptic sites.

BDNF stimulates cap-dependent translation through TrkB-coupled activation of the mammalian target of rapamycin (mTOR) and Ras-ERK pathways (Takei *et al.* 2001, 2004; Schratt *et al*., 2004). Activation of mTOR leads to phosphorylation of eIF4E binding protein and release of eIF4E. eIF4E binds to the cap structure and is phosphorylated by the ERK substrate mitogen-activated protein kinase integrating kinase (MNK) (Flynn *et al*. 1997; Pyronnet *et al*. 1999). Both of these pathways are required for BDNF-induced enhancement of protein synthesis in neurons or isolated dendrites in vitro (Kang and Schuman 1996; Takei *et al.* 2001, 2004; Tang *et al.* 2002; Kelleher *et al.* 2004). The present data extends these findings to ERK-dependent

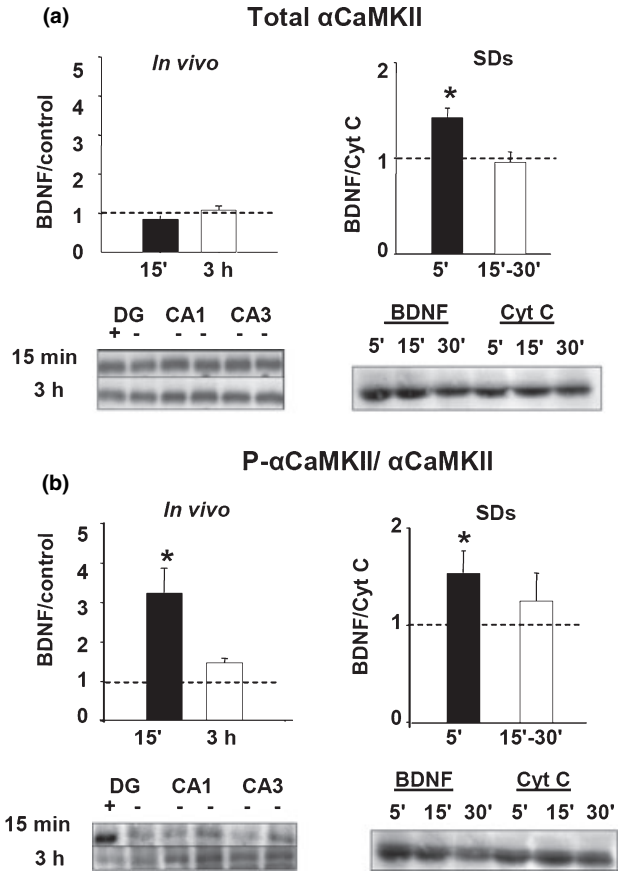

Fig. 5 BDNF stimulates local expression and activation of αCaMKII. (a) Changes in total αCaMKII expression following *in vivo* BDNF-LTP (left panel) or BDNF treatment of synaptodendrosomes (SDs) (right panel). In the *in vivo* experiments Western blot assays were performed using homogenates from microdissected dentate gyrus and CA subfields. Bar graphs show group mean (± *SEM*) changes based on densitometric analysis. Optical density values are expressed as a ratio between the treated and non-treated, contralateral tissue. In the SD experiments, Western blots were performed on matched pairs of BDNF and cytochrome C-treated samples. Optical density values are group means (± SEM) expressed as the ratio BDNF-treated/control. BDNF led to rapid increase in total αCaMKII expression 5 min after incubation. *Significant difference from control ($n = 17$; $p < 0.05$). (b) Corresponding analysis of phospho-CaMKII. Significant increases in CaMKII phosphorylation were detected 15 min ($n = 8$), but not 3 h ($n = 7$), after BDNF infusion *in vivo*. In SDs, phospho-CaMKII levels were elevated at 5 min ($n = 17$), returning to control levels at 15 min (15 min and 30 min values were pooled; $n = 12$). Changes in phosphorylation were normalized relative to total phosphoprotein.

regulation of eIF4E phosphorylation and expression during BDNF-induced synaptic plasticity *in vivo*.

LTP is associated with decreases as well as increases in protein synthesis (Fazeli *et al*. 1993; Chotiner *et al*. 2003). eEF2 phosphorylation observed during LTP may therefore contribute to translation arrest (Chotiner *et al*. 2003). Peptide chain elongation is highly energy consuming and decreases in ATP levels lead to phosphorylation of eEF2 (Horman *et al*. 2002; Browne *et al*. 2004). Transient arrest of elongation at non-synaptic sites might serve to conserve metabolic energy or inhibit translation of mRNA during transport. Paradoxically, however, certain transcripts undergo maintained or enhanced translation under conditions of reduced global protein synthesis and eEF2 phosphorylation. This is the case for Arc and αCaMKII mRNA, both of which are critical for stable LTP formation (Scheetz *et al*. 2000; Chotiner *et al*. 2003; Soule *et al*. 2006). Interestingly, consolidation of taste memory is associated with enhanced phosphorylation of eEF2 in conjunction with enhanced synaptic expression of αCaMKII in the taste cortex (Belelovsky *et al*. 2005). Thus, BDNF-LTP and memory consolidation are both coupled to transient eEF2 phosphorylation. In the taste learning paradigm increases in eEF2 phosphorylation are found in synaptoneurosomes as well as cortex tissue homogenates. Synaptic phosphorylation of eEF2 may be elicited by NMDA receptor activation (Scheetz *et al*. 2000), which is not involved in BDNF-LTP induction (Messaoudi *et al*. 2002). In primary cortical neurons, BDNF induces dephosphorylation of eEF2 and increases elongation rates (Inamura *et al*. 2005). Serotonin similarly reduces eEF2 phosphorylation in Aplysia synaptosomes. In this case, serotonin appears to offset an increase in eEF2 phosphorylation triggered by calcium influx (Carroll *et al*. 2004). Thus, the direction of eEF2 phosphorylation is controlled by multiple transmitters and is highly context-dependent. Taken together, this suggests an important role for eEF2 in transcript-specific and compartment-specific control of protein synthesis.

Previously we reported increases in α-CaMKII protein expression in synaptodendrosomes, but not in whole homogenates, following high-frequency stimulation (HFS)-induced LTP in awake rats (Havik *et al*. 2003). Because the synaptodendrosomes were obtained after *in vivo* LTP, transport of protein from non-synaptic sites could not be ruled out. The increase in αCaMKII expression observed in the present study following *in vitro* treatment of synaptodendrosomes with BDNF cannot be due to protein transport. As α-CaMKII mRNA is enriched in synaptodendrosomes and absent from axon terminals, glia, and interneurons (Jones *et al*. 1994; Sik *et al*. 1998; Zhang *et al*. 1999; Havik *et al*. 2003), any *de novo* synthesis of αCaMKII must be occuring in or near dendritic spines. αCaMKII mRNA and ribosomes are thought to translocate from dendritic shafts into spines during LTP (Ostroff *et al*. 2002; Havik *et al*. 2003). In cell cultures, BDNF treatment increases the spine content of eIF4E and induces the association of this translation factor with the granule-rich cytoskeletal fraction (Smart *et al*. 2003). Local regulation of αCaMKII expression by BDNF is therefore likely to involve activity-regulated positioning of the translation apparatus in addition to biochemical regulation of translation factors.

Current data suggests a model in which BDNF controls synaptic consolidation through dual regulation of transcription and translation (Bramham and Messaoudi 2005; Soule *et al*. 2006; Wibrand *et al*. 2006). Development of stable LTP in response to high-frequency stimulation (HFS-LTP) requires a period of sustained BDNF release and TrkB activation (Kang *et al*. 1997; Aicardi *et al*. 2004; Gooney *et al*. 2004). BDNF-LTP and stable HFS-LTP require ERK activation, transcription, and translation of Arc mRNA (Messaoudi *et al*. 2002; Ying *et al*. 2002; Soule *et al*. 2006). BDNF induces phosphorylation of eIF4E and enhances translation of a dendritic mRNA pool that includes áCaMKII and Arc (Aakalu *et al*. 2001; Yin *et al*. 2002; Schratt *et al*. 2004). Selective enhancement of translation initiation could therefore serve to capture mRNA liberated from RNA storage granules (i.e., αCaMKII) as well as newly induced Arc mRNA in transit along dendrites.

It will be important in future studies to determine the impact of eIF4E and eEF2 phosphorylation on translation of subpopulations of mRNA found in spines, dendrites, and somata. Activation of eIF4E is expected to increase the translation of mRNAs containing strong secondary structure in their untranslated region. Many of these mRNAs encode proteins involved in cellular transformation and growth (Richter and Sonenberg 2005). The increase in eIF4E expression may also be important for understanding translation control mechanisms in synaptic plasticity. Because the availability of eIF4E protein can be rate-limiting for translation, increases in eIF4E expression during BDNF-LTP may serve to maintain translational capacity during periods of intensive cap-dependent translation.

Finally, in view of the BDNF hypothesis of major depression (Castren 2004; Kuipers and Bramham 2006), it interesting to note that the same pattern of enhanced eIF4E and eEF2 phosphorylation observed here during BDNF-LTP is also observed in the dentate gyrus following chronic antidepressant drug treatment.

Acknowledgements

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Paper III

Sustained Arc synthesis controls LTP consolidation through regulation of local actin polymerization in the dentate gyrus in vivo

Elhoucine Messaoudi, Tambudzai Kanhema[#], Jonathan Soulé[#], Adrian Tiron[#], Girstaute Dagyte, Bruno da Silva, and Clive R. Bramham
Department of Biomedicine and Bergen Mental Health Research Center, University of Bergen, Jonas Lies vei 91, N-5009 Bergen, Norway.

Running title: Arc function and mechanism in LTP

7 Figures
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41 pages

Address correspondence to:
Clive Bramham, MD, PhD.
Department of Biomedicine
University of Bergen
Jonas Lies vei 91
N-5009 Bergen, NORWAY
Phone: 47 55 58 60 32
Fax: 47 55 58 64 10
Email: clive.bramham@biomed.uib.no

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# equal contribution.

Abstract

Although gene expression is considered necessary for LTP consolidation, causal roles for specific activity-induced mRNAs have not been defined. Here we probed the dynamic function of activity-induced Arc/Arg3.1 mRNA using brief, local infusions of antisense (AS) oligodeoxynucleotides at multiple time points during dentate gyrus LTP in vivo. Surprisingly, early Arc synthesis is necessary for early expression of LTP, while sustained synthesis is required to generate stably modified synapses. Late inhibition of Arc synthesis, two hours after LTP induction, rapidly reverses hyperphosphorylation of actin depolymerization factor/cofilin and abolishes local expansion of F-actin at synaptic sites. Infusion of the F-actin stabilizing drug, jasplakinolide, during LTP maintenance blocks the ability of AS to reverse LTP. Furthermore, this process of Arc-dependent synaptic consolidation is directly activated by BDNF infusion, thus identifying a molecular effector for BDNF in LTP consolidation. These results couple activity-induced expression of Arc to expansion of the actin cytoskeleton underlying enduring LTP.

Introduction

Activity-dependent changes in synaptic strength are implicated in a range of adaptive brain responses including memory formation, mood stability, and drug addiction (Hyman et al., 2006;Nestler and Carlezon, Jr., 2006;Morris, 2006;Kuipers and Bramham, 2006). The formation of long-term changes in synaptic strength and behavior depends on at least one period of new gene expression. New gene expression and protein synthesis is required to convert transient early-phase LTP to stable late-phase LTP (Bliss and Collingridge, 1993;Nguyen and Kandel, 1996;Frey et al., 1996). However, these traditional phases are operationally defined based on the sensitivity of LTP to broad spectrum inhibitors of gene expression and protein synthesis. Causal roles for activity-induced genes in LTP consolidation have not been defined. Alterations in gene expression in response to changes in neuronal activity are common, and, as emphasized by several authors (Sanes and Lichtman, 1999;Routtenberg and Rekart, 2005), it is likely that many regulated genes have no role or only a subsidiary role in generating LTP. Although gene deletion studies give insight into overall gene function, these methods cannot resolve the dynamic actions of induced mRNAs during LTP.

LTP is associated with the induction of a variety of immediate early genes. Knockouts of several of these genes, including zinc-finger transcription factor zif268/egr1, tissue plasminogen activator, and activity-regulated cytoskeleton-associated protein (Arc; aka Arg3.1) have defects in LTP maintenance and/or memory consolidation (Huang et al., 1996;Jones et al., 2001;Plath et al., 2006). A specialized role of Arc in synaptic plasticity is implied by the fact that Arc mRNA is rapidly

transported to dendritic processes where it undergoes local translation (Link et al., 1995;Lyford et al., 1995;Steward and Worley, 2001).

The present study investigated the dynamic function of activity-induced Arc using brief, local infusions of antisense oligodeoxynucleotides (ODNs) to inhibit Arc synthesis during LTP in the dentate gyrus of anesthetized rats. We show that Arc synthesis defines a novel time-window in LTP maintenance, during which early Arc synthesis is necessary for expression of LTP, while sustained synthesis is required to generate stably modified synapses. Arc is known to co-sediment with crude F-actin and localize to the postsynaptic density (PSD) of excitatory synapses (Lyford et al., 1995;Husi et al., 2000;Donai et al., 2003;Plath et al., 2006). Recent work suggests that development of stable LTP involves actin polymerization-dependent expansion of the PSD and dendritic spine (Fukazawa et al., 2003;Matsuzaki et al., 2004;Zito et al., 2004). Here, we report that late inhibition of Arc synthesis, 2 hours after LTP induction, rapidly reverses expansion of F-actin at synaptic sites and reverses hyperphosphorylation of cofilin, a major regulator of actin dynamics. These results couple activity-induced expression of a single gene, Arc, to expansion of the actin cytoskeleton underlying LTP consolidation. Several lines of evidence suggest that stable LTP is critically regulated by brain-derived neurotrophic factor (BDNF) (Bramham and Messaoudi, 2005). We find that Arc synthesis is necessary for the induction and time-dependent consolidation of LTP elicited by BDNF application, thus identifying a molecular effector for BDNF in LTP consolidation

Materials and Methods

Electrophysiology and Intrahippocampal Infusion

Data were obtained from 179 male Sprague-Dawley urethane-anesthetized (1.4-1.8 g/kg i.p.) rats weighing 250-320 g. Stereotaxic coordinates relative to Bregma were 7.9 mm posterior, 4.2 mm lateral for stimulation, 3.9 mm posterior, and 2.2 mm lateral for recording. An outer (guide) cannula (24 ga, PlasticsOne, Roanoke, Virginia) was beveled sharp at the tip to facilitate tissue penetration. A Teflon-coated stainless steel wire-recording electrode (coated diameter=112 µm) was glued (cyanoacrylate, Mega-G base, Mega Metal, Oslo) to the shaft of the outer cannula. The electrode was then cut so that it extended 900 µm from the end of the cannula. A concentric bipolar stimulating electrode (tip separation 500 µm; SNEX 100, Rhodes Medical Instruments) was lowered into the dorsomedial aspect of the angular bundle for stimulation of the medial perforant path. After making a small slit in the dura, the guide cannula and attached recording electrode was slowly lowered into the dorsal hippocampus until a positive-going fEPSP of maximum slope was obtained in the dentate hilus. The final depth of the recording electrode ranged between 200-300 µm below the level of the maximum negative-going fEPSP sink recorded in the middle-third of the dentate molecular layer. An inner infusion cannula (31 ga) was then inserted so that it protruded 300 µm below the end of the guide. The tip of the infusion cannula was located in deep stratum lacunosum-moleculare of field CA1, 700 µm above the hilar recording site and 300-400 µm above the medial perforant synapses

Biphasic rectangular pulses of 150 µs duration were applied every 30 s throughout the experiment. The stimulation intensity for test pulses was set to elicit a

population spike amplitude of 30 % of the maximal response. The infusion cannula was connected via PE50 polyethylene tubing to a 5-µl Hamilton syringe. Solutions were delivered by an infusion pump at a rate of 80 nl/min. BDNF-LTP was induced by infusing BDNF (2 µg in 2 µl PBS) for 25 min. The paradigm for HFS-LTP induction consisted of eight pulses at 400 Hz, repeated four times, at 10s sec intervals. Three sessions of HFS were given at intervals of 5 min.

Signals from the dentate hilus were amplified, filtered (1 Hz-10 kHz), and digitized (25 kHz). Acquisition and analysis of field potentials were accomplished using DataWave Technologies WorkBench software (Longmont, CO). The maximum slope of the fEPSP and the amplitude of the population spike measured from its negative going apex to the tangent line joining the first two positive peaks were measured, and averages of four consecutive responses were obtained. Analysis of variance (ANOVA) for repeated measures followed by a post hoc Scheffé test was used for statistical analysis of group effects. Statistics were based on values obtained during the 5 minutes at end of baseline and at the end of post-infusion recording.

Oligodeoxynucleotides

Chimeric ODNs containing phosphorothioate linkages between the three bases on the 5' and 3' ends and phosphodiester internal linkages were synthesized, HPLC purified, ultrafiltrated, and sterilized (Biognostik, Gottingen, Germany). The main Arc AS ODN used was directed against a 20-mer sequence (bases 209-228) covering the Arc startsite. Scrambled Arc ODN containing the same base composition in randomized order served as control. A second AS ODN targeting a non-overlapping region of the Arc coding region (bases 943-960) was designed and manufactured by Biognostik®, Germany. ODNs did not contain motifs such as G-quartets, kinase domains, or zinc-

fingers and search of the EMBL databases revealed no potential off-target genes (with significant homology and open secondary structure).

Drugs and antibodies.

Human recombinant met-BDNF (a gift from Amgen-Regeneron Partners) was obtained as a concentrated stock solution (1.0 mg/ml) in phosphate-buffered saline (PBS: 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0, 0.004% Tween-20). Jasplakinolide and Y27632 (Calbiochem) were dissolved in DMSO and 0.9% saline respectively and stored frozen. Cycloheximide and anisomycin (Sigma) were dissolved in saline. The anisomycin solution was adjusted to pH 7.4

Primary antibodies used for immunoblotting were as follows: N-terminal domain of Arc (E-19: sc 6382 goat polyclonal IgG, 1:100 Santa Cruz), β-actin (clone AC-15 mouse monoclonal 1:5000 Sigma), α-CaMKII (clone 6G9, mouse monoclonal, 1:2000, Affinity BioReagents), PSD-95 (mouse IgG1, 1:500 BD Transduction Laboratories), phoshpho-cofilin (Ser 3) (sc-12912, 1:1000, Santa Cruz), gelsolin (C-20, 1:200, Santa Cruz), profilin-1 (N-20, 1:200 Santa Cruz), α-spectrin (AB992, 1:200, Chemicon International) and cofilin

Tissue Microdissection and Sample Preparation

At the end of electrophysiological recording rats were decapitated and the brain was removed and rinsed with oxygenated ice-cold artificial cerebrospinal fluid (ACSF in mM: NaCl 124.0, $NaHOC_3$ 25.0, D-glucose 10.0, KCl 3.4, KH_2PO_4 1.2, $MgSO_4$ 1.0, $CaCL_2$ 2.5, pH 7.4). The hippocampus was then removed and the dentate gyrus was resected on ice. The entire procedure took less than 5 minutes. Tissues were hand-homogenized with 15 strokes in 300 µl of Dynal lysis/binding buffer.

SDS-PAG and Western Blotting

Protein levels in homogenate samples were determined using the Lowry method. Equal amounts of protein were loaded onto SDS-PAGE gels (10%) and run overnight at constant 10 mA. Separated proteins were transferred to a nitrocellulose membrane (Hybond-C, Amersham) at a constant voltage of 30 V overnight or 100 V for one hour. Membranes were blocked on a gyro-rocker for 1 hour at room temperature (RT). Blocking buffer (BB) consisted of TBST (Tris-buffered saline/0.1% Tween-20) and 5% BSA. For CaMKII, membranes were blocked 1 hour at 37°C. The primary antibodies were dissolved in BB containing 3% BSA and the blots incubated for 2 hours at RT or 4°C overnight with constant shaking. Following three washes with TBST, blots were incubated for 1 hour in horseradish peroxidase-conjugated secondary antibody dissolved in TBST. The blots were washed three times with TBST and proteins were visualized using enhanced chemiluminescence (ECL Western Blotting Analysis System, Amersham pharmacia biotech, Norway). Blots were stripped with 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCL, pH 6.7 at 60°C for 1 hour and reprobed with another antibody detecting the protein of interest. Optical density values obtained from the AS-treated dentate gyrus were normalized relative to values in the scrambled-treated or contralateral dentate gyrus Statistical analyses were based on unpaired or paired t-tests as appropriate.

Preparation of sections

Animals were transcardiacally perfused with 4% paraformaldehyde in 0.1M phosphate buffer (PB). Brains were dissected and post-fixed in the fixative overnight at 4°C and subsequently washed in PB containing 8% dextrose and 0.1% sodium azide. Coronal vibratome sections (50 µm-thick) were stored in 0.1% sodium azide in PB at 4°C. The same sections were used for *in situ* hybridization and immunohistochemistry.

***In Situ* Hybridization**

RNA probes were prepared from a cDNA insert matching the first 2975 nucleotides of the Arc mRNA (GenBank accession number NM_019361) cloned into the pCR®II-TOPO® vector (Invitrogen). Antisense and sense probes were transcribed from linearized plasmids using T7 and SP6 polymerase in the presence of DIG labeling mix according to the manufacturer's instructions (Roche).

Floating sections were placed in PBS for 5 minutes, permeabilized with proteinase K (10ug/ml) for 5 min at 37°C, and post-fixed (5 min with 4% PFA/PBS). After fixation sections were treated with 0.25% acetic anhydride in 0.1M TEA (pH 8) for 10 min, washed twice in 2 x SSC, and placed for 10 minutes in a prehybridization buffer. Probes were applied to the sections and hybridization was performed in a humidified chamber at 60°C for at least 16 hours. Sections were washed twice with 2 x SSC at RT for 30 min, once with 50% formamide in 2xSSC at 65°C, rinsed in 2 x SSC at 37°C, incubated with 20µg/ml RNase A at 37°C for 30 min and incubated in RNase A buffer for at 65°C for 30 min. After blocking in 2% blocking reagent for one hour at RT, alkaline coupled anti-DIG antibody (1:2000, Roche) was applied. Visualization was done with the chromogenic substrates NBT and BCIP (Roche).

Pictures were taken on a Nikon Eclipse 80i microscope coupled to a Nikon DS-5M camera.

Immunohistochemistry

Sections were first treated with PB containing 100 mM glycine (Sigma), then washed in PBT (0.1% Tween 20 in PB), incubated in 0,3% H2O2 diluted in PBT, permeabilized for 20 minutes with 0.5% Triton X-100 diluted in PBT, rinsed and immersed for 30 minutes in blocking buffer (4% BSA, 5% horse serum in PBT). They were then incubated overnight at 4°C with the primary antibody diluted in blocking buffer. After three washes in PBT, biotinylated secondary antibody was applied for 1 hour at RT. Sections were then washed in PBT, incubated in Streptavidin-HRP diluted in PBT, washed in TBS and finally processed for DAB staining.

Primary antibodies were Arc E19 (sc-6382, Santa Cruz) and Arc H300 (sc-15325, Santa Cruz). Secondary antibodies were biotin-conjugated donkey anti-goat or anti-rabbit Ig (Amersham Biosciences). Streptavidin-conjugated HRP was obtained from Amersham Biosciences; diaminobenzidine (Sigma, 1mg/ml) was diluted in 0.1M Tris-buffer saline containing approximately 0.03% H_2O_2.

Phalloidin staining

Sections were incubated in PBT containing 100 mM glycine (Sigma), washed with PBT, permeabilized in PBT containing 0.5% Triton X-100 and rinsed three times for 10 minutes. They were then incubated for at least 30 minutes with phalloidin-FITC (0.1 µg/ml, Sigma) diluted in PBT containing 4% BSA and 2% horse serum. After several washes, sections were mounted in Vectashield. Pictures were acquired on a Zeiss AxioImager microscope. Fluorescence intensity was measured in 15 µm steps

across the molecular layer using ImageJ software (NIH). Optical density values from 10 neighboring pixels were averaged and normalized relative to a reference area.

Results

Arc antisense ODN reverses ongoing LTP

Arc AS ODN targeting the Arc startsite (bases 209-228) was used to inhibit Arc synthesis during LTP induction at medial perforant path-granule cell synapses in anesthetized rats. In order to select optimal time points for AS delivery we first examined expression of Arc mRNA and protein (Fig. 1). LTP was induced by spaced stimulation consisting of three sessions of high-frequency stimulation (HFS; 400 Hz, 8-pulses) with 5 minutes between sessions. LTP was associated with a rapid and sustained elevation of Arc mRNA and protein, as assessed by in situ hybridization histochemistry and immunohistochemistry performed at various time points (5 min, 30 min, 2 h, 3 h, and 4 h) after application of HFS (Fig. 1). At 5 and 30 min post-HFS, Arc mRNA and protein staining were already increased in the granule cell layer and into the dendritic field (molecular layer) of the dentate gyrus. At 2 hours post-HFS, Arc mRNA and protein expression reached a plateau where it remained at 3 and 4 hours, the intense staining filling the entire molecular layer.

Based on the large increases in Arc mRNA and protein at 2 h post-HFS, we selected this time point for application of Arc AS. Infusions (1 µl, 12.5 min) were made into deep stratum lacunosum-moleculare of CA1, approximately 300 microns from the nearest medial perforant path synapses in the upper blade of the dentate gyrus. As shown in Figure 2A, Arc AS applied 2 hours after HFS led to a rapid and

profound reversal of ongoing LTP. Potentiated field excitatory postsynaptic potentials (fEPSPs) declined to a stable baseline level within 60 minutes where they remained for the duration of recording (at least 2 hours). In striking contrast, infusion of Arc AS 4 hours after HFS (Fig. 2B) had no effect on LTP maintenance, demonstrating the time-sensitivity of the AS effect. Infusion of control, scrambled Arc ODN at the critical 2 hour time point was also without effect (Fig. 2C). In addition, Arc AS treatment in the absence of HFS had no effect on basal synaptic efficacy (Fig. 2D). In order to rule out non-specific effects and possible specific off-target effects of the AS sequence, we examined the effects of a second AS sequence targeting a non-overlapping 18 nucleotide region of the Arc coding region (bases 943-960). Infusion of this AS sequence two hours after HFS similarly resulted in rapid inhibition of LTP (Fig. 2E). The convergent physiological effects of non-overlapping AS sequences indicated inhibition of Arc synthesis as the mechanism of action.

The efficacy and specificity and of the Arc protein knockdown was assessed by quantitative immunoblot analysis. Arc AS was infused 2 hours post-HFS and homogenates of microdissected dentate gyrus were collected 2 hours later. Arc protein expression in the AS-treated group was significantly reduced to 55$\pm$10% of SC-treated control (Fig. 2G). A panel of proteins was chosen to evaluate the specificity of the Arc knockdown. mRNA encoding the alpha-subunit of calcium/calmodulin-dependent protein kinase II (α-CaMKII) is stored in dendrites and local translation of this message affects the size of the postsynaptic density (PSD) and development of late LTP (Miller et al., 2002;Havik et al., 2003). The scaffolding protein PSD-95 is part of the core PSD complex of excitatory synapses, where it colocalizes with Arc and CaMKII (Lyford et al., 1995;Husi et al., 2000;Moga et al., 2004). β-actin serves as a loading control, but is also of interest because Arc co-sediments with crude F-

actin (Lyford et al., 1995). AS treatment 2 hours post-HFS had no effect on the expression of these proteins relative to SC-treated control (Fig. 2G). Thus, reversal of LTP by Arc AS is coupled to specific knockdown of Arc protein expression.

These results indicate that Arc synthesis at two hours, but not four hours, post-HFS is required for consolidation of LTP. Next we sought to determine the onset and possible early contribution of Arc synthesis to LTP. When Arc AS was applied 90 or 15 min before HFS, non-decremental LTP of fEPSPs was obtained (Fig. 3A and 3B). The lack of effect of AS infusion at these time points suggested that the concentration of AS ODN dropped below a critical level needed to target the massive LTP-induced increases in Arc mRNA. Corroborating this notion, LTP was rapidly and transiently inhibited when AS was applied 5 minutes before or 15 minutes after HFS (Fig. 3C and 3D). In rats receiving AS 5 min pre-HFS, fEPSP slope values started to decline within 5 minutes of HFS, reached a maximum trough at about 1 hour, and returned to the original level of enhancement by 3 hours post-HFS (Fig. 3C and 3F). Arc protein expression was similarly elevated at 3 hours, but not 1 hour, post-HFS (Fig. 3F). SC-treated rats had non-decremental LTP and the size of the fEPSP increase recorded immediately after HFS and 3 hours post-HFS was not significant differently from the AS group (Fig. 3C and 3E; $p>0.05$). Infusion of Arc AS 15 min after HFS similarly produced a rapid reversal of LTP expression followed by return to the original level of potentiation (Fig. 3D). Taken together the results suggest that early Arc synthesis is necessary for early expression, but not for consolidation of LTP. Sustained or late synthesis of Arc is necessary for LTP consolidation.

LTP reversal is coupled to rapid knockdown of induced Arc mRNA and protein in dentate granule cells.

In view of the critical role for late Arc synthesis we focused our further analysis on the 2 hour time point after HFS. *In situ* hybridization and immunohistochemistry were performed to further evaluate the speed, cellular localization, and mechanism of the Arc knockdown. Figure 4 shows changes in Arc mRNA and protein expression in experiments in which Arc AS or scrambled Arc ODN was infused 2 hours post-HFS and brains were collected 1 hour later. This time was selected because LTP in all rats had reversed to a steady level by 1 hour. In SC-infused rats, HFS resulted in robust induction of Arc mRNA and protein in the granule cell layer and throughout the molecular layer of the dentate gyrus. As shown in Figure 4A and 4B, the LTP-associated increase in Arc mRNA and protein expression was strongly diminished in time-matched, AS-treated rats. The knockdown occurred in both blades of fascia dentate, but was generally stronger in the molecular layer of the upper blade near the infusion site. The loss of Arc mRNA indicated effective AS-mediated degradation of newly induced Arc transcripts in granule cell dendrites. In contrast, expression of dendritically stored α-CaMKII mRNA was unaffected by Arc AS treatment (Fig. 4C). We conclude that AS treatment rapidly inhibits Arc synthesis from upregulated mRNA on a time course corresponding to the reversal of LTP.

Western blotting was used to compare changes in Arc expression from the earliest time of LTP reversal (30 min post-AS) to the end of recording (2 h post-AS). The Arc knockdown was smaller at 30 minutes (83%) than it was at two hours (55%), indicating a progressive reduction in Arc protein levels (Fig. 4E). However, the amount of Arc knockdown is expected to be underestimated in Western analysis

performed in homogenates of whole dentate gyrus, particularly at early time points after infusion. This is because Arc is elevated throughout the dorsal dentate gyrus following LTP induction, whereas AS concentrations are maximal near the site of infusion. Indeed, in situ hybridization showed that Arc mRNA expression one hour after AS infusion was not reduced at distances greater than 500 μm rostral to the recording site in the mid-dorsal dentate gyrus.

Late Arc synthesis is required for stabilization of F-actin during LTP.

Recent work suggests that development of stable LTP involves actin polymerization-dependent remodeling of dendritic spines (Fukazawa et al., 2003;Matsuzaki et al., 2004;Zito et al., 2004). We explored a role for late Arc synthesis in this process using fluorescently labeled phalloidin to visualize changes in F-actin content. In agreement with Fukazawa et al., (2003), LTP was accompanied by enhanced phalloidin staining within a narrow band corresponding to the termination zone of medial perforant path synapses in the middle molecular layer of the dentate gyrus (Fig. 5A). This band of phalloidin staining appeared with 5 minutes of HFS and remained constant during 4 hours of recording (time course shown in Fig. S1). Rats receiving SC-infusions 2 hours post-HFS exhibited a clear band of phalloidin staining that was absent in time-matched AS-treated rats (Fig. 5A; lower right panels). Densitometric analysis of the staining confirmed a peak corresponding to the middle molecular layer in SC-treated controls that was lacking in AS-treated animals (Fig. 5B). This suggested that late Arc synthesis is necessary for consolidation of LTP and stabilization of F-actin at synaptic sites.

We next asked whether F-actin stabilization mediates Arc-dependent consolidation. We predicted that drug-induced stabilization of F-actin during LTP

maintenance would substitute for Arc and therefore occlude (block) the effect of Arc AS treatment. Fig. 5D shows the results of experiments in which the F-actin stabilizer, jasplakinolide, was briefly infused during LTP maintenance 1 hour before Arc AS treatment. Remarkably, jasplakinolide had no effect on LTP maintenance yet completely abolished the ability of AS to reverse LTP. Jasplakinolide also had no effect on LTP maintenance during a 2.5 hour recording period in the absence of AS application (not shown).

Arc synthesis maintains cofilin phosphorylation during LTP.

Next we began to examine mechanisms that may couple Arc synthesis to regulation of F-actin. Cofilin is a member of a highly conserved family of actin-associated proteins that enhance actin filament severing and increase the off-rate of actin monomers (Sarmiere and Bamburg, 2004). Phosphorylation of cofilin on Ser-3 inhibits cofilin activity and promotes F-actin formation. Previous work showed that cofilin phosphorylation is critical for F-actin formation underlying late LTP (Fukazawa et al., 2003). Here, we examined the role of ongoing Arc synthesis in the regulation of cofilin activity. LTP was associated with marked hyperphosphorylation of cofilin, while infusion of Arc AS 2 hours after LTP induction resulted in rapid dephosphorlyation of cofilin (Fig. 6A and 6B). Expression of total cofilin and other actin-binding proteins connected to actin function in spines, including, gelsolin, α-spectrin, and profilin, did not change significantly following LTP induction or AS-induced reversal of LTP (Fig. 6B).

BDNF triggers Arc-dependent synaptic consolidation

BDNF has emerged as major regulator of excitatory synaptic transmission and plasticity in the CNS (Blum and Konnerth, 2005;Bramham and Messaoudi, 2005). Development of transcription-dependent late phase LTP following spaced HFS requires activation of TrkB receptor tyrosine kinases and is associated with a period of sustained BDNF release (Kang et al., 1997;Aicardi et al., 2004). However, the molecular effector mechanisms by which BDNF regulates LTP consolidation are unknown. Exogenous application of BDNF induces a long-term potentiation (BDNF-LTP) that mimics many features of late phase LTP. In the dentate gyrus, BDNF is transcription-dependent and associated with induction, dendritic transport, and translation of Arc mRNA (Messaoudi et al., 2002;Ying et al., 2002). Here, we used Arc antisense infusion to explore a possible causal role for Arc in BDNF-LTP.

Rats infused with scrambled Arc-ODN prior to BDNF infusion exhibited a stable fEPSP increase equivalent in magnitude to that seen in rats receiving BDNF alone (Fig. 7A). Pretreatment with Arc AS completely blocked BDNF-LTP induction, while having no effect on baseline synaptic efficacy (Fig. 7B). Inhibitors of RNA synthesis or extracellular signal-regulated kinase (ERK) block BDNF-LTP induction, but have no effect when applied during the maintenance phase of the potentiation (Messaoudi et al., 2002;Ying et al., 2002) In striking contrast, application of Arc AS 2 hours after BDNF infusion led to a rapid reversal of ongoing BDNF-LTP (Fig. 7C). As seen during HFS-LTP maintenance, fEPSP slope values returned completely to baseline levels within 1 hour of AS treatment and remained at baseline for the duration of recording. Local infusion of scrambled Arc ODN 2 hours after BDNF infusion (Fig. 7D, or treatment with Arc AS 4 hours after BDNF infusion (Fig. 7E),

had no effect on the magnitude of potentiation during 2 hours of subsequent recording. The effects of Arc AS on BDNF-LTP are summarized in (Fig. 7F).

The blockade of BDNF-LTP induction was associated with specific knockdown of Arc expression on immunoblots (Fig. 7G). Arc protein expression and cofilin phosphorylation was then examined in tissue obtained after reversal of BDNF-LTP by Arc AS infusion. Arc AS treatment two hours after BDNF infusion significantly reduced Arc expression and phospho-cofilin levels relative to SC-treated control (Fig. 7H). Expression of cofilin (Fig. 7H) and other actin binding proteins (gelsolin, α-spectrin and profilin), was unchanged. The results suggest that Arc synthesis is required for both the induction and time-dependent consolidation of BDNF-LTP.

Broad spectrum protein synthesis inhibitors have been used to define the traditional phases of LTP. In general, treatment with protein synthesis inhibitors before HFS cause a slowing decaying LTP, while treatment during LTP maintenance has no effect. We have replicated these effect on LTP in the dentate gyrus using systemic injection of the protein synthesis inhibitors cycloheximide and anisomycin (Fig. S2). In contrast to the sharp reversal of LTP obtained following specific inhibition of Arc synthesis, protein synthesis inhibitors injected 30 min or 90 min post-HFS had no effect on LTP maintenance. Among myriad pitfalls associated with the use of broad spectrum inhibitors is the fact that translation of some mRNAs is maintained or even enhanced (Hughes et al., 1997;Klann and Dever, 2004;Routtenberg and Rekart, 2005). Arc may be one such mRNA. Several studies of synaptic plasticity have reported maintained or enhanced synthesis of dendritically localized mRNAs (Arc, α-CaMKII, EF1A) in the presence of protein synthesis inhibitors (Steward and Halpain, 1999;Huang et al., 2005) or during global translation

arrest (Scheetz et al., 2000;Chotiner et al., 2003). As shown in Fig. S2, injection of anisomycin early in the window or Arc synthesis (30 min post-HFS) failed to block the LTP-associated increase in Arc protein expression.

Discussion

This study demonstrates a dynamic, causal role for activity-induced Arc synthesis in LTP maintenance in the dentate gyrus. The time-window of Arc function, commencing immediately after HFS and lasting between 2 and 4 hours, is unexpectedly protracted and sharply defined. Surprisingly, early Arc synthesis is necessary for expression of LTP, while late synthesis is required for LTP consolidation. Importantly, the study provides a casual link between two central tenets of LTP consolidation: gene expression and local actin polymerization.

Evidence indicates that persistent LTP occurs when small stubby dendritic spines are converted into large mushroom-shaped spines through a mechanism dependent on local actin polymerization (Weeks et al., 2001;Fukazawa et al., 2003;Harris et al., 2003;Matsuzaki et al., 2004). Expansion of the PSD and spine head may depend on a number of F-actin functions including tethering of receptors and signaling complexes, trafficking of receptors, and positioning of organelles such as polyribosomes (Kim and Lisman, 1999;Halpain, 2000;Zhou et al., 2001;Matsuzaki et al., 2004;Zito et al., 2004;Carlisle and Kennedy, 2005). Our analysis of Arc function concentrated on the consolidation mechanism 2 hours after LTP induction, at which time Arc AS infusion stably reversed LTP and knocked down newly induced Arc mRNA and protein. Infusion of AS induced dephosphorylation of hyperphosphorylated cofilin while

abolishing the nascent band of F-actin at medial perforant path synapses. Furthermore, application of the F-actin-stabilizer jasplakinolide blocked the AS-induced reversal of LTP. These findings indicate that Arc synthesis controls LTP consolidation through expansion or stabilization of F-actin.

LTP maintenance has been previously divided into early and late phases based on the effects of general protein synthesis inhibitors. Such inhibitors perturb cell metabolism and may fail to block translation of certain transcripts including several dendritically localized mRNAs (Steward and Halpain, 1999;Huang et al., 2005;Routtenberg and Rekart, 2005). The present study shows that LTP can be rapidly reversed by blocking the ongoing synthesis of a single protein, Arc, 2 hours after LTP induction. This finding underscores the importance of evaluating the function of single mRNA species in LTP consolidation and suggests that protein synthesis inhibitors do not reveal the true kinetics of the process.

Several lines of evidence suggests that late LTP expression is mediated by sustained activation of the atypical protein kinase C (PKC) isoform PKCζ (Ling et al., 2006). It was recently found that pharmacological inhibition of PKCζ rapidly reverses LTP 1 day after its induction (Pastalkova et al., 2006). Interestingly, treatment with the F-actin destabilizing agent latrunculin B blocks new synthesis of PKMζ and attenuates LTP maintenance (Kelly et al., 2006). Taken together with the present findings, this suggests a sequential mechanism of LTP maintenance in the dentate gyrus in which Arc-dependent consolidation couples to PKCζ-dependent expression at the level of actin polymerization.

In an earlier study of Guzowski et al (2000) intrahippocampal injection of AS 90 min before HFS was reported to decrease the amplitude and stability of LTP over a 5 day period. This contrasts with the robust, rapid function of Arc reported here.

However, the effect of AS in the Guzowski et al. (2000) study is difficult to assess in the absence of data from baseline electrophysiological recordings prior to HFS. In the present report the specificity and validity of AS effects were corroborated in several ways. First, reversal of LTP maintenance was strikingly dependent on the timing of AS application relative to synaptic activation. Second, similar reversal of LTP was obtained with AS sequences targeting non-overlapping regions of the Arc mRNA. Third, LTP reversal was coupled to rapid knockdown of Arc mRNA and protein. Finally, Arc knockdown was coupled to a biologically compelling mechanism—regulation of F-actin.

Electron microscopic analysis has revealed upregulation Arc and F-actin specific to spines of medial perforant path synapses during LTP (Fukazawa et al., 2003;Moga et al., 2004;Rodriguez et al., 2005). It is nonetheless evident from many studies that Arc protein is upregulated throughout the dendritic arbor of granule cells. The function of the widespread increase in Arc is enigmatic. However, recent studies performed in cultured hippocampal neurons have revealed a role for Arc in homeostatic synaptic scaling of AMPA-type glutamate receptors (AMPAR) via its ability to activate a specific AMPAR endocytic pathway (Rial Verde et al., 2006;Chowdhury et al., 2006;Shepherd et al., 2006). The global increase in Arc may therefore serve to scale down (depress) AMPAR transmission in non-potentiated synapses as a means of stabilizing overall excitability.

The mechanisms coupling Arc synthesis to phosphorylation of cofilin remain to be determined. Arc and cofilin are both PSD proteins. Arc also co-immunoprecipates with PSD-95, co-sediments with crude (but not pure) F-actin, and contains spectrin homology repeats suggestive of a structural role (Lyford et al., 1995;Husi et al., 2000;Donai et al., 2003). Phosphorylation of cofilin on serine 3 is

regulated by multiple kinases and phosphatases (Arber et al., 1998;Yang et al., 1998;Meng et al., 2002). One of the major cofilin kinases in neurons, Lim domain kinase (LIMK), is regulated by the Rho GTPase effectors Rho kinase (ROCK) and p21-activated kinase (PAK). The fact that LTP maintenance is not inhibited by local infusion of the ROCK inhibitor Y27632 (A.Tiron and C.R.Bramham, unpublished) places emphasis on PAK, which both activates LIMK1 and inhibits activity of the cofilin phosphatase slingshot (Sarmiere and Bamburg, 2004).

In Arc knockout mice LTP is initially enhanced and then falls quickly to baseline (Plath et al., 2006). The loss of stable LTP resembles the effect of Arc antisense, while the superinduction of LTP resembles observations in LIMK1 knockout mice which exhibit large numbers of small, actin-poor spines. Given the role of Arc in regulation of actin polymerization we predict Arc knockouts also have small spines that cannot undergo actin-dependent enlargement to mushroom-shaped spines.

Endogenous BDNF signaling at glutamate synapses is critical for formation of late phase LTP (Bramham and Messaoudi, 2005). However, the molecular effector mechanisms by which BDNF promotes LTP consolidation are unknown. Exogenous BDNF-LTP provides a way to isolate these effect. BDNF-LTP in the dentate gyrus is transcription-dependent, it occludes with late LTP, and it is associated with ERK-dependent upregulation of Arc. In the present study we show that BDNF-LTP is completely abolished by Arc AS treatment prior to BDNF infusion, and rapidly reversed by AS treatment at 2 hours (but not 4 hours) after BDNF infusion. As with HFS-LTP, Arc AS treatment selectively inhibited Arc expression and suppressed phosphorylation of cofilin. We therefore conclude that Arc is necessary for the

induction and time-dependent consolidation of BDNF-LTP. These results identify Arc as a molecular effector for BDNF in LTP consolidation.

Rather than acting alone, Arc is likely to be part of a coordinated transcriptional and translational response. A panel of genes that are co-upregulated with Arc during BDNF-LTP and HFS-LTP was recently identified (Wibrand et al., 2006). Several of these genes (Narp, neuritin, Nedd4 WW-binding protein-4) have functions in AMPAR clustering, excitatory synaptogenesis, and axonal guidance. BDNF signaling also modulates the local dendritic translation of Arc, α-CaMKII, LIMKI and other plasticity-associated mRNAs (Aakalu et al., 2001;Yin et al., 2002;Schratt et al., 2004;Ju et al., 2004). Although light microscopic in situ hybridization shows that Arc mRNA is transported to proximal dendrites within 5 minutes of HFS (15 minutes from first session of HFS), the possible contribution of pre-existing Arc mRNA in early LTP expression warrants attention. Arc and α-CaMKII mRNA are stored in the same Pur-α containing RNA transport granules in dendrites (Kanai et al., 2004), and BDNF treatment facilitates synthesis of both proteins in isolated synaptic preparations derived from adult rats (Yin et al., 2002;Kanhema et al., 2006).

Arc is expressed in many cortical and limbic structures during behavioral training and is necessary for long-term memory in a variety of hippocampus-dependent and hippocampus-independent memory tasks (Guzowski et al., 1999;Guzowski et al., 2000;Plath et al., 2006). Arc mRNA levels are elevated for hours following LTP induction or exploration of a novel environment, whereas Arc mRNA increases in the hippocampal region CA1 and many other brain areas is only short-lived (minutes) (Kelly and Deadwyler, 2003;Ramirez-Amaya et al., 2005;Guzowski et al., 2006). Recent work suggests that the dentate gyrus allows fine

spatiotemporal separation of novel and complex cues, thereby disambiguating stimuli to allow sparse encoding of information (Kesner et al., 2004;Lee et al., 2005). It is tempting to speculate that the protracted phase of Arc-dependent consolidation reflects the time-dependent function of the dentate gyrus in disambiguating and encoding information in the entorhinal-hippocampal circuitry.

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Figure legends

Figure 1. Time course of changes in Arc mRNA and protein expression following LTP induction in the dentate gyrus. Arc in situ hybridization histochemistry (ISH) and immunohistochemistry (IHC) were performed at five time points (in minutes) after high-frequency stimulation of the medial perforant pathway. The high-frequency stimulation consisted of 3 sessions of 400 Hz bursts separated by 5 minutes (10 minutes total duration). Control (CON) shows staining in contralateral, unstimulated dentate gyrus. Representative images from 4-5 experiments at each time point.

Figure 2. Arc antisense infusion two hours after high-frequency stimulation blocks LTP consolidation. Time course plots show changes in the medial perforant path-evoked fEPSP slope expressed in percent of baseline. Values are means $\pm$ SEM. HFS is indicated by the arrow. Infusion of Arc antisense and scrambled ODN are indicated by solid and open bars, respectively.

(A) LTP maintenance was rapidly and persistently inhibited following local infusion (1 µl, 0.5 mM, 12.5 min) of Arc AS ODN at 2 hours post-HFS.

(B) Arc AS ODN infusion 4 hours post-HFS had no effect on LTP maintenance.

(C) Infusion of scrambled (SC) Arc ODN 2 hours post-HFS had no effect on LTP maintenance.

(D) Arc AS infusion had no effect on basal synaptic efficacy.

(E) Infusion of a second AS sequence (AS2) targeting a non-overlapping region of the Arc mRNA similarly reversed ongoing LTP (n=5).

(F) Magnitude of fEPSP slope changes. n=5-8 in all groups. *Significantly different from baseline $P<0.05$.

(G) LTP reversal is coupled to specific knockdown of Arc protein. Quantification of Western blots from dentate gyrus homogenates. Tissue was collected at the end of the experiments shown in (A) and (C). Expression of Arc, but not β-actin, αCaMKII, or PSD-95, were significantly reduced in AS-treated rats (*$P<0.05$). Representative immunoblots. + (infused dentate gyrus); - (contralateral, non-infused dentate gyrus).

Figure. 3. Arc antisense transiently inhibits early LTP expression.

(A-B) Nondecremental LTP of fEPSPs was induced when Arc AS was infusion 90 min (A) or 15 min (B) pre-HFS.

(C-D) Infusion of Arc AS 5 min pre-HFS (C) or 15 min after HFS (D) resulted in transient suppression of LTP.

(E) Nondecremental LTP was observed in SC-treated controls.

(F) Bar graphs show changes in fEPSP and Arc protein expression in rats infused with Arc AS 5 minutes pre-HFS. Values for both were unchanged at 1 hour (reversal) and significantly elevated at 3 hours (recovery) post-HFS ($P<0.05$). fEPSP is expressed in percent of baseline. Arc protein immunoreactivity on Western blots is expressed as percent of contralateral control. N=6-8 in all groups. Representative Western blots shown in right panel.

Figure 4. LTP reversal is coupled to rapid knockdown of upregulated Arc mRNA and protein expression. Arc antisense (AS) or scrambled (SC) ODN was infused into the dentate gyrus 2 hours after HFS and the brain was fixed by transcardial perfusion 1 hour later.

(A) Coronal sections processed for *in situ* hybridization using a digoxigenin-labeled Arc riboprobe show robust upregulation of Arc mRNA in granule cell somata and dendrites in SC-treated controls. *Arc* mRNA expression was strongly inhibited following Arc AS treatment. The lower right panels show high magnification images of AS-treated and SC-treated dentate gyrus. CON=contralateral non-treated dentate gyrus. These are representative images based on 5 experiments in each treatment group. Images were obtained from the mid-dorsal dentate gyrus within approximately 300 μm of the recording site.

(B) Immunohistochemical staining similarly shows enhanced expression of Arc protein in SC-treated dentate gyrus and rapid knockdown following AS treatment.

(C) α*CaMKII* mRNA expression in Arc AS-treated and contralateral dentate gyrus.

(D) Changes in fEPSP slope in rats receiving AS or SC infusion 2 hours post-HFS followed by recordings of 30 minute or 120 minutes at time of tissue collection.

(E) Left panel: Bar graph shows changes in Arc protein expression relative to the time-matched SC-treated control group. Inhibition of Arc expression was rapid and progressive. *Significantly different from control ($P<0.05$). Right panel: Corresponding Western blots.

Figure 5. Late Arc synthesis is required to stabilize F-actin during LTP. Arc antisense (AS) or scrambled (SC) ODN was locally infused 2 hours after HFS and the brain was fixed by transcardial perfusion 1 hour later. Coronal sections were stained with phalloidin-FITC.

(A) Upper left panel: A band of phalloidin staining specific to the termination zone of the medial perforant path was observed in the dentate gyrus SC-treated rats. Upper right panel: CON=contralateral, unstimulated dentate gyrus. Lower left panel: Arc AS treatment abolished the band of enhanced phalloidin staining. Lower right panels: Comparison of phalloidin staining in infusion of scrambled or Arc AS ODN. OML, outer molecular layer; MML, middle molecular layer; IML, inner molecular layer; GCL, granule cell layer. Arrowheads mark phalloidin staining in the MML. The white bar marks the hippocampal fissure. Representative images based on four AS-treated and five SC-treated rats. The time course of phallodin labelleing is shown in Supplemental Fig. 1.

(B) Left panel: Profile of phallodin-FITC fluorescence intensity ratio (ipsilateral/contralateral) in rats receiving SC (gray) or AS (black) infusion. Fluorescence intensity of the phalloidin-FITC signal was measured in 15 μm steps along the shortest line (rectangle in panel A) extending from 20 μm above the granule cell layer border to the hippocampal fissure. Optical density values from 10 neighboring pixels were averaged. Florescence values at each pixel were normalized relative to a reference area in CA1 (small box in panel A). Values obtained at 3 sites along the dentate gyrus inner blade were averaged. All sections were from within 300 μm of the recording site. Right panel: Mean (+SEM) changes in fluorescence intensity in the MML.

(C) Palloidin staining following 2 hours of 400 Hz, 8-pulse bursts applied at 10 second intervals. A bright, sharply demarcated band appears in the middle molecular layer of the dentate gyrus.

(D) Infusion of the F-actin stabilizing agent jasplakinolide blocks the inhibitory effect of Arc AS on LTP maintenance. Jasplakinolide (Jasp; 1 μM) and Arc AS were infused at the times indicated following HFS. Pre-treatment with Jasp did not affect ongoing LTP abolished the effect of Arc AS on LTP maintenance. fEPSP slope measurements of 6 consecutive responses obtained immediately before and 10 minutes after Jasp or Arc infusion were not significantly different ($P<0.05$).

Figure 6. Arc synthesis is necessary to maintain hyperhosphorylation of cofilin during LTP.

(A) Western blots were performed in dentate gyrus homogenates prepared 2 hours after HFS, and 2 hours after infusion of AS or SC Arc ODN during LTP. Cofilin phosphorylation was significantly enhanced during LTP and this increase was inhibited by Arc AS treatment (*$P<0.05$; n=6-7 in all groups). Expression of total cofilin and other actin-associated proteins was unchanged.

(B) Representative immunoblots. + (ODN infused dentate gyrus); - (contralateral, non-infused dentate gyrus).

Figure 7. BDNF induces Arc-dependent synaptic strengthening.

Time course plots show changes (means ± SEM) in the medial perforant path-evoked fEPSP slope expressed in percent of baseline. Arc AS oligodeoxynucleotide or scrambled (SC) Arc sequence were infused 90 minutes before BDNF.

(A) Robust BDNF-LTP was induced in SC-treated rats (n=5; $P>0.05$).

(B) Arc AS pre-treatment abolished BDNF-LTP (n=6; $P<0.05$). There was no significant difference in fEPSP slope values obtained immediately before and 2 hours after BDNF infusion ($P>0.05$).

(C) Arc AS infusion at 2 hours rapidly reverses ongoing BDNF-LTP (n=5; $P<0.05$).

(D) Infusion of scrambled (SC) Arc ODN at 2 hours has no significant effect on ongoing BDNF-LTP (n=5; $P>0.05$).

(E) Arc AS infusion at 4 hours had no significant effect on BDNF-LTP maintenance (n=6; $P>0.05$).

(F) Magnitude of fEPSP slope and population spike changes. N=5-7 in all groups. *$P<0.05$.

(G) Quantification of Western blots from dentate gyrus homogenates. Tissue was collected at the end of the experiments shown in A and B. Expression of Arc, but not β-actin, α-CaMKII, or PSD-95, were significantly reduced in AS-treated rats (*$P<0.05$). Representative immunoblots below. + (infused dentate gyrus); - (contralateral, non-infused dentate gyrus).

(H) Dentate gyrus was microdissected at the end experiments shown in panels (C) and (D) and homogenate samples were analyzed by quantitative Western blot. Mean (+SEM) changes are expressed as AS-treated versus SC-treated dentate gyrus.

(*P<0.05; n=6-7 in all groups). Representative immunoblots are shown below. + (infused dentate gyrus); - (contralateral, non-infused dentate gyrus).

Supplemental Material

Supplemental Figure 1. Time course of phallodin-FITC staining during LTP in the rat dentate gyrus. CON= Unstimulated, contralateral dentate gyrus. Images show phalloidin staining at various time points (in minutes) after HFS of the medial perforant pathway. Arrows mark borders of phalloidin band in the middle molecular layer.

Supplemental Figure 2. Effect of broad spectrum protein synthesis inhibitors on LTP maintenance.

(A) Systemic (i.p.) injection of cycloheximide (CHX; 25 mg/kg) 90 min prior to HFS resulted in LTP that decayed to baseline within 3-4 hours (n=3).

(B) Systemic injection of CHX 90 min post-HFS had no effect on LTP maintenance (n=4).

(C-D) Systemic injection of CHX or anisomycin (ANI; 100 mg/kg) 30 min after HFS had no effect on LTP maintenance (n=3 for each inhibitor).

(E) Injection of anisomycin 30 minutes post-HFS failed to block the increase in Arc protein immunostaining during LTP maintenance.

Figure 1

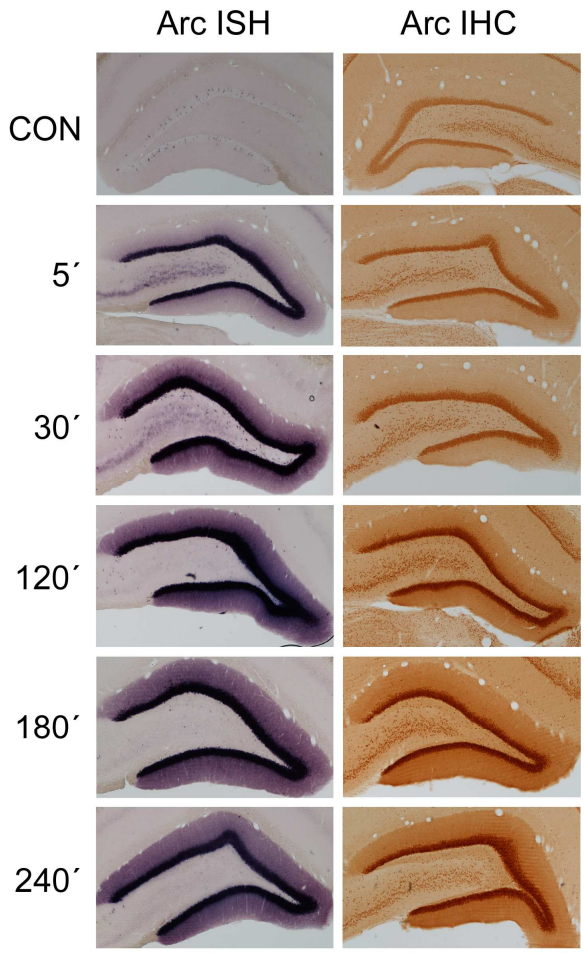


Figure 2

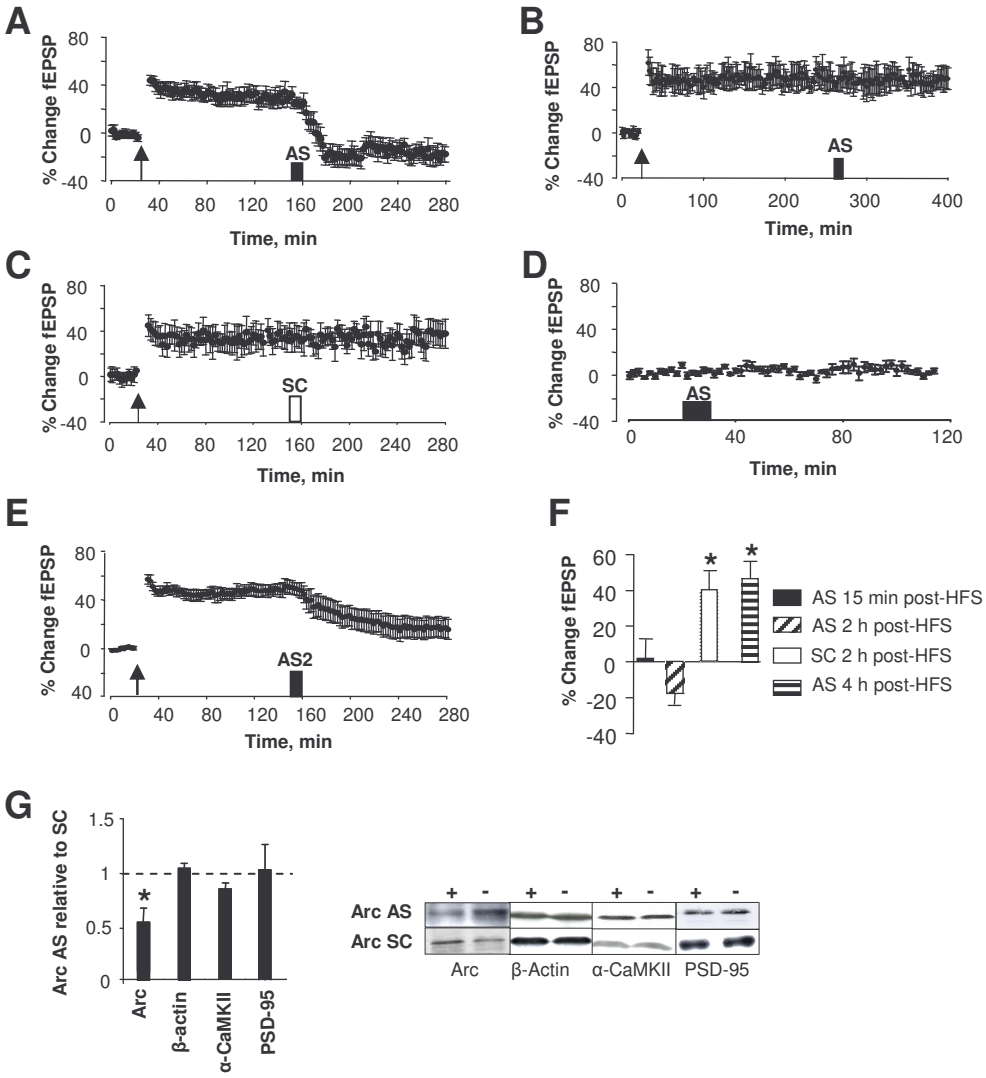

A
% Change fEPSP
80
40
0
-40
AS
0 40 80 120 160 200 240 280
Time, min
B
% Change fEPSP
80
40
0
-40
AS
0 100 200 300 400
Time, min
C
% Change fEPSP
80
40
0
-40
SC
0 40 80 120 160 200 240 280
Time, min
D
% Change fEPSP
80
40
0
-40
AS
0 40 80 120
Time, min
E
% Change fEPSP
80
40
0
40
AS2
0 40 80 120 160 200 240 280
Time, min
F
% Change fEPSP
60
40
20
0
-20
-40
*
*
AS 15 min post-HFS
AS 2 h post-HFS
SC 2 h post-HFS
AS 4 h post-HFS
G
Arc AS relative to SC
1.5
1
0.5
0
*
Arc
β-actin
α-CaMKII
PSD-95
+ - + - + - + -
Arc AS
Arc SC
Arc
β-Actin
α-CaMKII
PSD-95

Figure 3

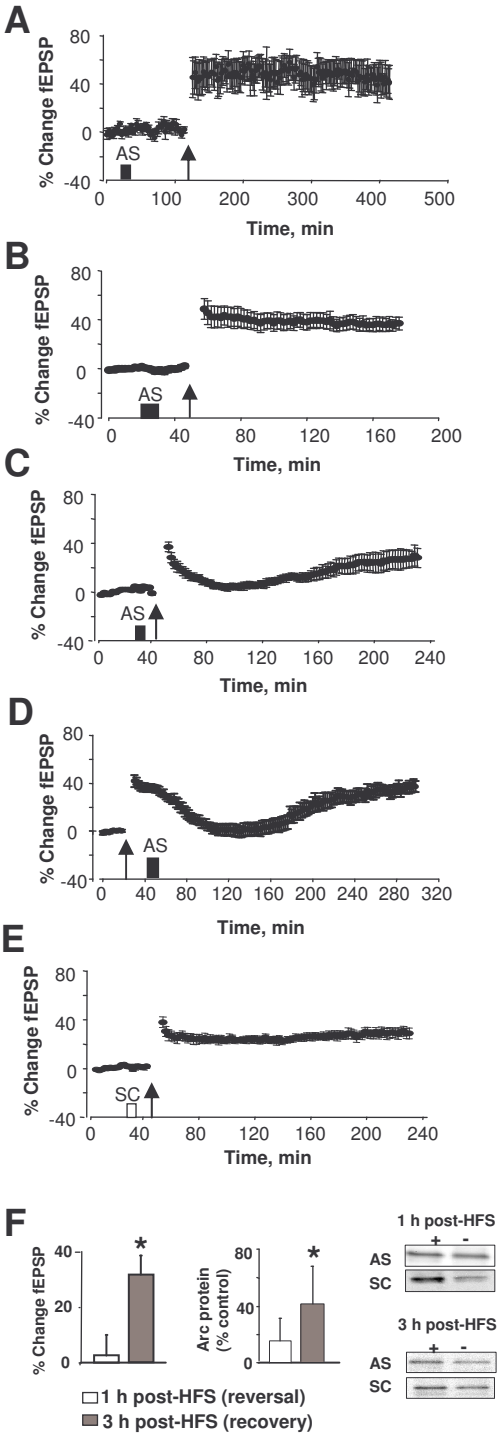

A
% Change fEPSP
80
40
0
-40
AS
0
100
200
300
400
500
Time, min
B
% Change fEPSP
80
40
0
-40
AS
0
40
80
120
160
200
Time, min
C
% Change fEPSP
80
40
0
-40
AS
0
40
80
120
160
200
240
Time, min
D
% Change fEPSP
80
40
0
-40
AS
0
40
80
120
160
200
240
280
320
Time, min
E
% Change fEPSP
80
40
0
-40
SC
0
40
80
120
160
200
240
Time, min
F
% Change fEPSP
40
20
0
*
Arc protein (% control)
80
40
0
*
1 h post-HFS (reversal)
3 h post-HFS (recovery)
1 h post-HFS
+
-
AS
SC
3 h post-HFS
+
-
AS
SC

Figure 4

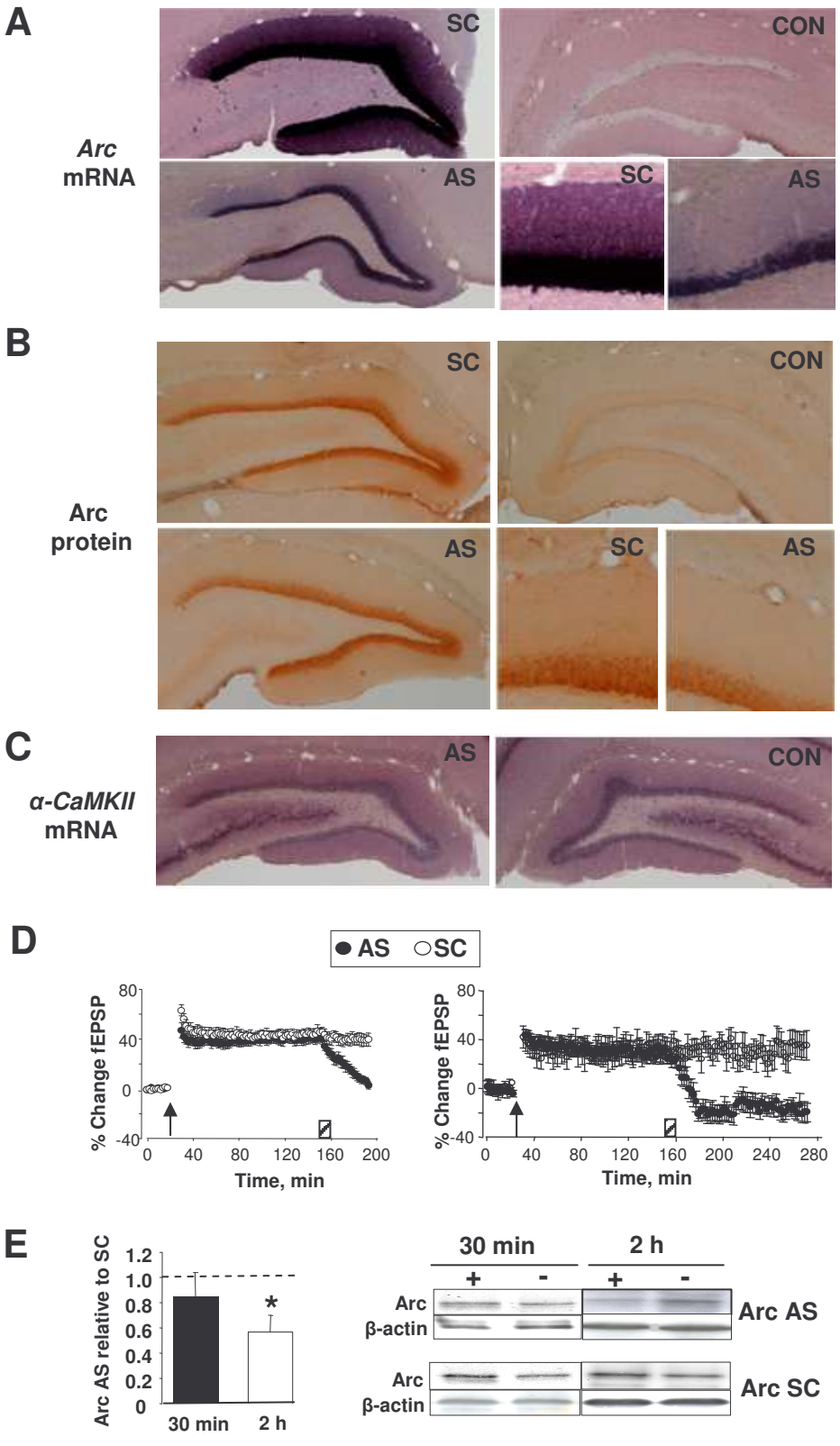

A
Arc mRNA
SC
CON
AS
SC
AS
B
Arc protein
SC
CON
AS
SC
AS
C
α-CaMKII mRNA
AS
CON
D
AS
SC
% Change fEPSP
80
40
0
-40
0
40
80
120
160
200
Time, min
% Change fEPSP
80
40
0
-40
0
40
80
120
160
200
240
280
Time, min
E
Arc AS relative to SC
1.2
1.0
0.8
0.6
0.4
0.2
0
*
30 min
2 h
30 min
2 h
+
-
+
-
Arc
β-actin
Arc AS
Arc
β-actin
Arc SC

Figure 5

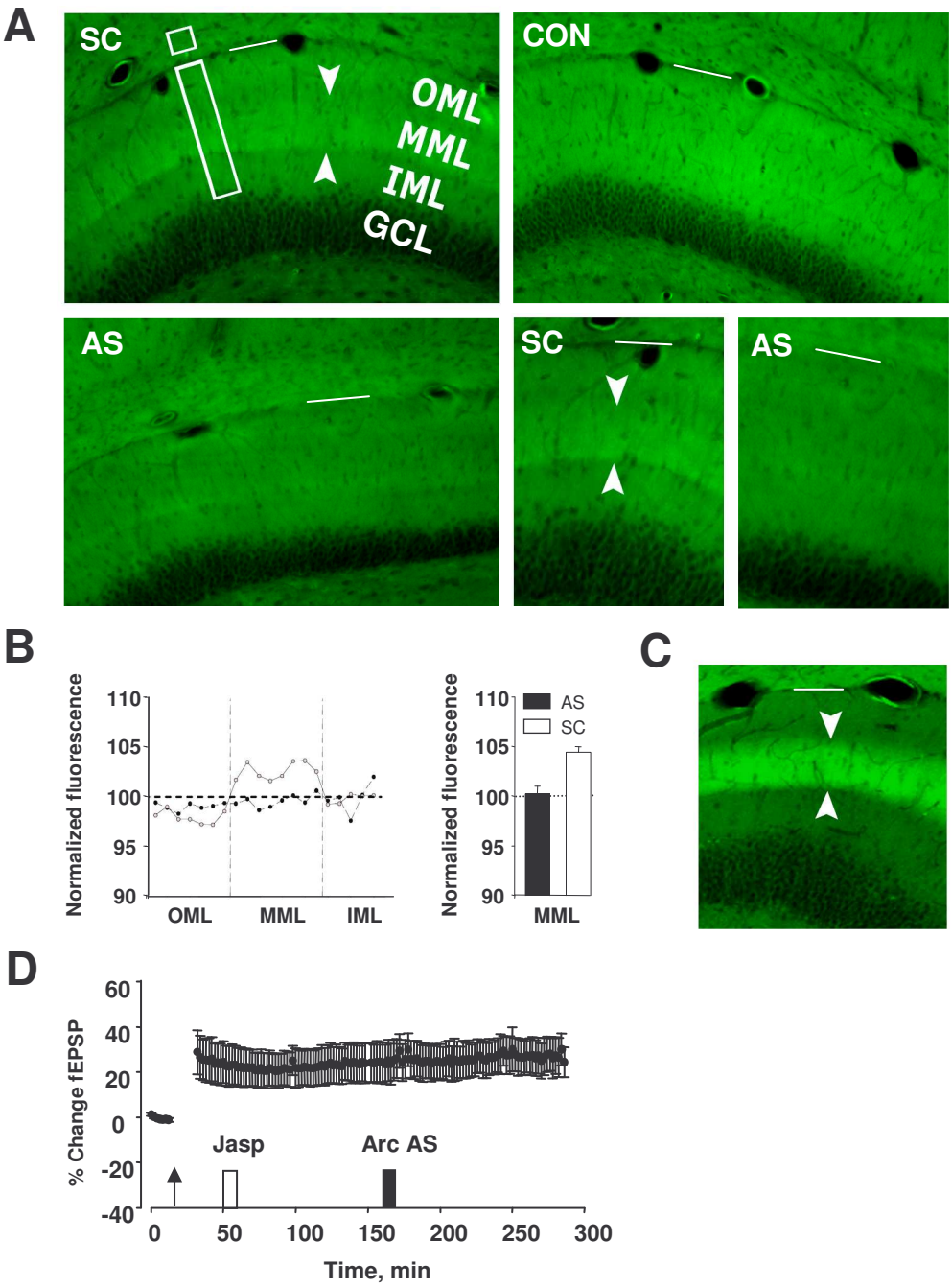

A
SC
OML
MML
IML
GCL
CON
AS
SC
AS
B
Normalized fluorescence
110
105
100
95
90
OML
MML
IML
Normalized fluorescence
AS
SC
MML
C
D
% Change fEPSP
60
40
20
0
-20
-40
Jasp
Arc AS
0
50
100
150
200
250
300
Time, min

Figure 6

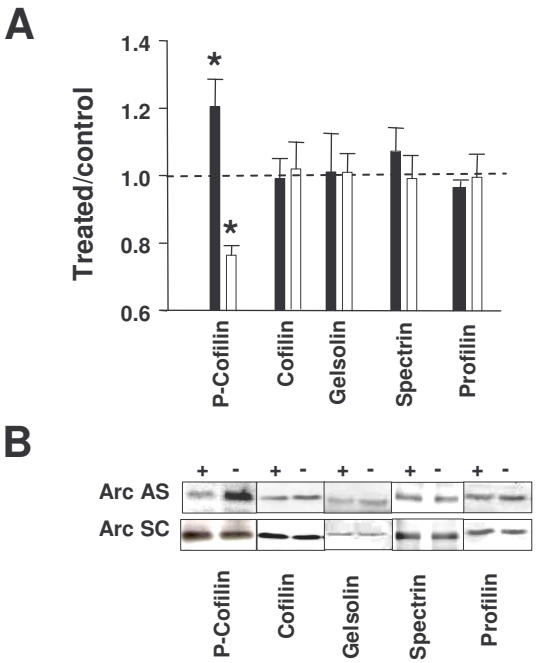
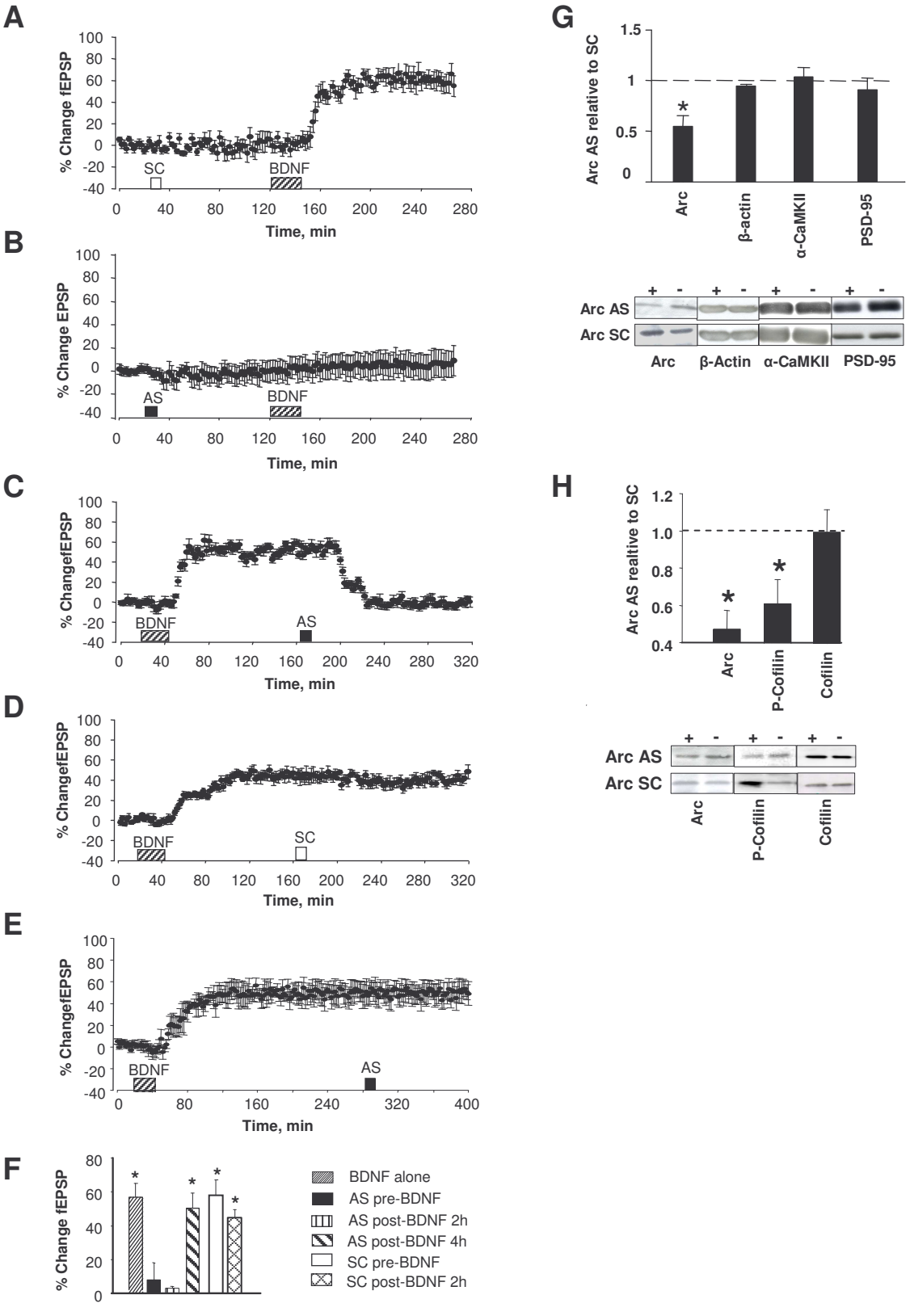
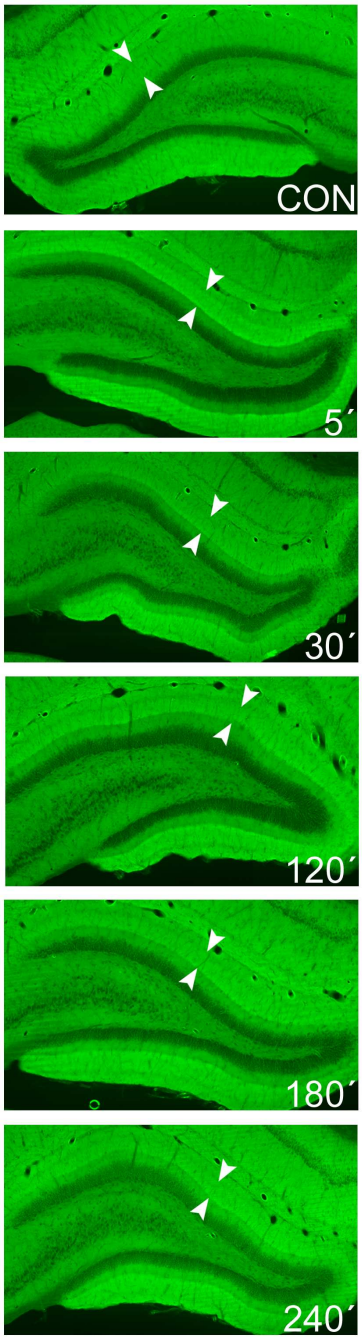

A
1.4
1.2
1.0
0.8
0.6
Treated/control
*
*
P-Cofilin
Cofilin
Gelsolin
Spectrin
Profilin
B
+ - + - + - + - + -
Arc AS
Arc SC
P-Cofilin
Cofilin
Gelsolin
Spectrin
Profilin

Figure 7


A
% Change fEPSP
SC
BDNF
Time, min
B
% Change EPSP
AS
BDNF
Time, min
C
% ChangefEPSP
BDNF
AS
Time, min
D
% ChangefEPSP
BDNF
SC
Time, min
E
% ChangefEPSP
BDNF
AS
Time, min
F
% Change fEPSP
BDNF alone
AS pre-BDNF
AS post-BDNF 2h
AS post-BDNF 4h
SC pre-BDNF
SC post-BDNF 2h
G
Arc AS relative to SC
Arc
β-actin
α-CaMKII
PSD-95
Arc AS
Arc SC
H
Arc AS realtive to SC
Arc
P-Cofilin
Cofilin
Arc AS
Arc SC

Supplemental Figure 1



Supplemental Figure 2