Modulation of Synaptic Function by Neurotrophic Factors in the Adult Hippocampus

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To My Parents

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One of the great joys of science lies in the moment of shared discovery. One person's suggestion resonates in the mind of another and suddenly takes on a definite shape. An insightful criticism of one way of thinking about a problem leads to another, better understanding. This thesis grew out of many such moments of interaction with other colleagues in the field.

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ABSTRACT

The neurotrophins including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), are a group of signaling factors that are crucial for neuronal survival and differentiation during development. Previous studies have shown that the hippocampus is a prominent site of expression of the neurotrophins and their Trk receptors in the adult brain. Interestingly, the expression of BDNF, NT-3, and their receptors can be regulated by a variety of neuronal activity, which suggests that the neurotrophins may also participate in adult synaptic plasticity.

The possibility that the neurotrophins directly modulate synaptic strength in the mature brain was investigated at the Schaffer collateral-CA1 synapses in the adult rat hippocampus. Transient application of BDNF or NT-3 but not NGF produced a dramatic and sustained (3 to 4 hours) enhancement of synaptic transmission. Both electrophysiological and immunocytochemical evidence indicated that the penetration and the resulting synaptic potentiation by neurotrophins are influenced by the perfusion rate at which the neurotrophin is applied to hippocampal synapses.

The potentiating effects of BDNF and NT-3 could be completely blocked by inhibiting the function of Trk receptors using either a pharmacological inhibitor of tyrosine kinases or a function-blocking Trk antibody. In addition, blockade of L-type calcium channels or intracellular calcium stores significantly reduced the potentiation induced by either neurotrophin. These data suggest that both the activation of Trk receptors and the subsequent increase in intracellular calcium concentration are essential for the initiation of neurotrophin-induced synaptic enhancement.

The neurotrophin-induced plasticity exhibits an immediate requirement for protein synthesis, which is not somatic in origin. The neurotrophins still produced synaptic potentiation at synapses isolated from their cell bodies; this plasticity displayed a dependence on protein synthesis, raising the possibility that these factors may stimulate local protein synthesis within dendrites and promote site-specific modification of synaptic function.

Finally, I report that the neurotrophins play a functional role in certain forms of hippocampal long-term potentiation (LTP). The maintenance of LTP induced by theta-burst or pairing, but not strong tetanic stimulation, relied on intact neurotrophin signaling. In long-term LTP, the neurotrophins were primarily involved in maintaining the late phases of synaptic enhancement, without significantly affecting an early phase of potentiation. Thus, during adult plasticity, synaptic activity-induced increases in neurotrophin synthesis and release contribute to the late phase of LTP and may eventually lead to structural changes at the synapse.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 1

Introduction

I. Synaptic plasticity as a cellular basis for learning and memory

External events are represented as patterns of neuronal activity in the brain. When an animal is exposed to a new environment, all the information which arrives at the sensory organs is transformed into a series of electrical signals passing along particular nerves to central brain regions where the signals interact with one another and modify the existing spatiotemporal patterns of neuronal activity. To be of survival value, these modifications need to be stored in the brain through the processes of learning and memory. In 1949, Hebb proposed an influential theory for memory storage by synaptic modification based on the rule that synapses linking two cells that are both active at the same time will be strengthened (Hebb, 1949). Experimental representation of such Hebbian or associative synaptic plasticity was first identified in the hippocampus (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973), a brain structure known to be important for the formation of certain kinds of memories.

This fascinating biological phenomenon called long-term potentiation (LTP) has received much attention since it exhibits many properties required for the implementation of the Hebb rule and thus can serve as a potential substrate for learning and memory. LTP is an activity-dependent enhancement of synaptic strength which can persist for many hours in the *in vitro* slice preparation and for days to weeks when produced *in vivo* in anaesthetized or freely moving animals. The associativity and durability of LTP have made this form of synaptic plasticity an attractive candidate for a cellular mechanism of memory formation.

LTP induction

Although LTP occurs in different forms in several areas of the brain, it has been most extensively studied in the hippocampus, especially at synapses between the Schaffer collaterals and the CA1 pyramidal neurons. LTP is induced when presynaptic activity (glutamate release) coincides with postsynaptic depolarization that relieves the Mg^{2+} block of the N-methyl-D-aspartate (NMDA) receptor (Ascher and Nowak, 1988; Collingridge *et al.*, 1983). This condition can be met either by applying high-frequency stimulation to presynaptic fibers (100 Hz; tetanus) or by pairing low frequency presynaptic stimulation with postsynaptic depolarization elicited by current injection into the postsynaptic cell (pairing) (Figure 1). There are several other molecular components which are known to be important for LTP formation. An increase in postsynaptic Ca^{2+} and the subsequent activation of Ca^{2+}-dependent enzymes, including protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and nitric oxide synthase (NOS), have been proven to play a critical role in the generation of LTP (reviewed in Bliss and Collingridge, 1993).

Studies employing either a pharmacological or genetic manipulation of these essential components provide evidence for a link between LTP and memory. Infusion of the NMDA receptor antagonist 2-amino-5-phosphonopropionic acid (AP5) into the rat hippocampus not only completely blocks the induction of LTP but also results in severe learning deficits (Morris *et al.*, 1986). Genetically engineered mice with the deletion of a particular gene, for example, the α subunit of *CaMKII* or nonreceptor tyrosine kinase *fyn*, show both impaired LTP and behavioral deficits in a spatial learning task (Grant *et al.*,

1992; Silva *et al.*, 1992; Silva *et al.*, 1992). Although these results are consistent with the notion that LTP is the cellular mechanism for learning and memory, the interpretation of these studies contains potential problems. Infusion of AP5 is not confined to the hippocampus and therefore the function of NMDA receptors in the surrounding neocortex is also affected. In the case of gene knockout mice, the observed learning deficit could result from the nonspecific developmental effects of gene deletion. The most compelling evidence comes from recent studies by Tsien *et al.* using an exciting new technique employing region-specific gene knockout (Tsien *et al.*, 1997). This technique exploits the *Cre/loxP* system, in which Cre recombinase catalyzes recombination between *loxP* recognition sequences (Sauer and Henderson, 1988). When *loxP* sequences are inserted to flank an exon of a gene of interest (called a "floxed gene") and the *Cre* transgene is under the control of a tissue or cell type-specific promoter, the floxed gene will be deleted by the *Cre/loxP* recombination system specifically in areas where the *Cre*-gene associated promoter is active. Therefore, if an appropriate promoter is available, this technique enables the expression of any gene of interest to be selectively abolished in a specific region. Previously, mice containing a T cell-specific knockout of the DNA polymerase β gene were generated using this technique (Gu *et al.*, 1994). Tsien *et al.* have accomplished both temporal and regional specificity of a gene deletion by choosing a promoter derived from the α*CaMKII* gene to drive *Cre* gene expression. Owing to the lack of activity of the α*CaMKII* promoter during prenatal and perinatal periods, the expression of the gene for NMDAR1 (an NMDA receptor subunit) could be abolished only postnatally and exclusively in the CA1 pyramidal cells of the hippocampus, thus

circumventing the problem of the conventional knockout approach. These mice develop normally but display a significant impairment in CA1 LTP and hippocampal-dependent spatial memory (Tsien *et al.*, 1997), which strongly supports a connection between LTP and certain types of memory.

LTP expression

Although these early steps in LTP induction are relatively well understood, the mechanisms that underlie its expression and maintenance are still the subject of considerable debate. There are in principle two possible mechanisms for enhancing synaptic transmission - an increase in presynaptic transmitter release or an enhanced postsynaptic responsiveness. Attempts to resolve this issue by means of quantal analysis of hippocampal LTP have led to a relatively consistent finding: an increase in quantal content and release probability, implying a presynaptic locus of expression (reviewed in Madison and Schuman, 1991). The postsynaptic induction and at least in part presynaptic expression of LTP necessitate the existence of a retrograde signaling mechanism. Several molecules including nitric oxide (NO), arachidonic acid, and carbon monoxide (CO), have emerged as potential retrograde signaling molecules in LTP (O'Dell *et al.*, 1991; Schuman and Madison, 1991; reviewed in Schuman and Madison, 1994).

The presynaptic interpretation of LTP expression was challenged by recent experiments suggesting that an increase in quantal content may instead reflect a postsynaptic uncovering of clusters of previously silent α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Issac *et al.*, 1995; Liao *et al.*, 1995). Issac *et*

al. and Liao *et al.* have proposed that there exists a subset of synapses with only functional NMDA receptors; minimal stimulation, which fails to elicit AMPA receptor-mediated reponse at a negative holding potential, can often elicit NMDA receptor-mediated response at a positive holding potential. After the induction of LTP, AMPA receptor-mediated responses appear at previously silent synapses; this is thought to be due to unmasking of clusters of AMPA receptors. These results, however, can still be reinterpreted by postulating a presynaptic locus for LTP (Kullmann *et al.*, 1996). NMDA receptors have a much higher affinity for glutamate than AMPA receptors and may detect spillover of glutamate released at neighboring synapses. Therefore, if there are two sets of synapses, one with high probability of release and the other with low or zero probability of release, recording from the cell postsynaptic to the low probability synapse would show only NMDA receptor-mediated signals, generated by spillover of glutamate from the high probability synapse. The appearance of AMPA receptor-mediated responses after LTP induction could result from the increased probability of release at the low probability terminal. Refinements of electrophysiological and imaging methods to assess pre- and postsynaptic events are clearly needed to resolve this controversy. It seems likely that both pre- and postsynaptic mechanisms coexist and their relative contribution to the maintenance of LTP depends on various factors, including the prior history of activity at a synapse.

Long-term plasticity

Memory and its synaptic model LTP have two distinct temporal components; a

short-term phase that involves the covalent modification of preexisting proteins, and a long-term phase that lasts for more than several hours and requires *de novo* gene expression and protein synthesis (Alvarez *et al.*, 1994; Barondes and Cohen, 1966; Flexner *et al.*, 1963; Frey *et al.*, 1988; Nguyen *et al.*, 1994). The long-term phase of LTP might be associated with the growth of new synapses and/or splitting of active zones into two or more synapses (Geinisman *et al.*, 1993). Over the last few years, cyclic AMP (cAMP) and cAMP-responsive element-binding protein (CREB), through their actions on gene expression, have been found to play an important role in various forms of long-term plasticity in systems ranging from *Aplysia* to mammals (Bartsch *et al.*, 1995; Bourtchuladze *et al.*, 1994; Yin *et al.*, 1995; Yin *et al.*, 1994). Mice with a targeted disruption of the CREB gene have normal short-term memory but deficient long-term memory, as revealed in contextual fear conditioning and the spatial version of the water maze. In an interesting parallel, LTP in hippocampal slices from these mice decays back to baseline within 1.5 hours of induction, providing additional support for the involvement of LTP in memory formation.

Although activation of the cAMP pathway and subsequent CREB-induced gene expression is necessary for neuronal changes related to long-term memory, this does not rule out the participation of other second messenger pathways. Moreover, the CREB protein itself is a multifunctional transcriptional factor that can also be activated by CaMKII as well as various neurotrophic factors (Dash *et al.*, 1991; Ginty *et al.*, 1994). An intriguing question is whether the gene expression and protein synthesis induced by CREB or other factors can eventually lead to structural changes which underlie a

persistent modification of synaptic efficacy.

II. Neurotrophins and synaptic plasticity

There is increasing evidence that the nervous system utilizes some of the same molecular players for both developmental and adult plasticity (reviewed in Goodman and Shatz, 1993). For example, during the critical period in the development of visual system, activity-dependent competition among the innervating neurons takes place in a way that follows Hebb rule: the correlated firing of neighboring presynaptic neurons leads to an activation of synaptic NMDA receptors and the influx of calcium (Reiter and Stryker, 1988; Schmidt, 1990; Shatz, 1990). In most cases, developmental plasticity accompanies structural changes, such as neurite outgrowth and synaptic rearrangement. Although changes in synaptic structure have also been correlated with functional alterations linked to long-term memory (reviewed in Bailey and Kandel, 1993), the cellular mechanisms underlying this process remain largely unknown.

In order to induce morphological changes at a synapse, synthesis of new synaptic components is essential. In developing visual cortex, the neurotrophins play a major role in regulating gene expression and altering the morphological properties of cortical neurons. Although it has not been experimentally shown that these factors can induce similar effects in the adult brain, they are clearly attractive candidates for mediators of such structural plasticity. By regulating gene expression and protein synthesis, the neurotrophins could alter neuronal function over a much longer time scale than conventional neuromodulators, and couple short-term functional changes (such as LTP)

to long-term changes involving structural modifications of synapses. Therefore, it is of interest to examine whether developmental molecules, the neurotrophins, modulate synaptic plasticity in the adult and whether this modulation contributes to the morphological changes of synapses following learning.

Neurotrophins and Trk receptors

During development, neuronal survival and differentiation are regulated by a limited amount of a neurotrophic factor present in the target fields of sensitive neurons. The first neurotrophic factor discovered, nerve growth factor (NGF), regulates the survival, neurite growth, and neurotransmitter production of peripheral sensory and sympathetic neurons as well as central cholinergic neurons (Levi-Montalcini, 1987; Thoenen *et al.*, 1987). The purification of a second neurotrophic factor, brain-derived neurotrophic factor (BDNF), has led to the discovery of a whole family of neurotrophic factors, now called the neurotrophins, including neurotrophin-3 (NT-3) and NT-4/5 (Berkemeier *et al.*, 1991; Hohn *et al.*, 1990; Ip *et al.*, 1992; Leibrock *et al.*, 1989; Maisonpierre *et al.*, 1990). They are a group of small (~13 kDa), basic proteins with a remarkable similarity in amino acid sequence including apparent conservation of three cystine disulfide bonds. The neurotrophins exist as dimeric molecules which share approximately 50% sequence identity. The crystal structure of the murine NGF homodimer (McDonald *et al.*, 1991) indicates that the dimer interface corresponds to regions of high sequence conservation throughout the neurotrophin family.The crystal structure of NGF, the prototypic member of this family, contains three pairs of anti-parallel beta-strands connected by beta-hairpin loops, which contain most of the variable residues among the four neurotrophin proteins.

These similarities and differences in overall structural features may account for a distinct yet overlapping spectrum of neurotrophic activities (reviewed in Barde, 1989).

The neurotrophins of the NGF family mediate their biological effects by interacting with specific receptors present on the surface of the responsive neurons. Two different types of receptors have been identified for the neurotrophins; a low-affinity neurotrophin receptor (p75) and the Trk family of receptor tyrosine kinases which has three known members, TrkA, TrkB, and TrkC (reviewed in Chao, 1992). The Trk receptors mediate most of the neurotrophin effects independently of p75 by transducing signals through their tyrosine kinase activity. These receptors share approximately 85% sequence homology and have several significant structural features in common which make them distinct from other receptor tyrosine kinases (Figure 2A). The extracellular domain includes three tandem leucine-rich motifs (LRM) which are bounded by two clusters of cysteine residues, and two immunoglobulin domains (Ig). These domains have been suggested to mediate protein-protein interactions. Truncated, non-signaling Trk receptors have been detected for TrkB and TrkC, predominantly in non-neuronal cells such as astrocytes, ependymal cells, and epithelial cells in choroid plexus (Klein *et al.*, 1990; Valenzuela *et al.*, 1993). These noncatalytic forms may act to maintain a high local concentration of neurotrophins by restricting their diffusion following release from the sites of synthesis.

Cross-talk has been shown to exist between the neurotrophins and various Trk family members in cell culture systems. Although NGF, BDNF, and NT-3 are the principal ligands for TrkA, TrkB, and TrkC, respectively, TrkA and TrkB receptors can respond to

multiple neurotrophins to a varying extent depending on the specific cell culture system used (Figure 2B) (Cordon-Cardo *et al.*, 1991; Ip, *et al.*, 1992; Klein *et al.*, 1991; Squinto *et al.*, 1991). The interactions of TrkB with the neurotrophins are considerably more complicated then those of TrkA and TrkC. TrkB can interact with BDNF and NT-4/5 as primary ligands, but also to a lesser extent with NT-3. Interestingly, the ability of TrkB to interact with NT-3 appears to be influenced by particular neuronal environments; this highlights the importance of appropriate cellular environments for the selective action of neurotrophins. The presence of differences between the phenonypes of a ligand and a receptor knockout suggests that this cross-reactivity may also occur *in vivo* (reviewed in Snider, 1994).

Whereas signal transduction by neurotrophins is mainly achieved by the Trk family receptors, there is evidence to suggest that the p75 receptor plays an important regulatory role by modifying Trk activity. The p75 receptor alters the ligand-binding specificity of TrkA, enhances its binding affinity to NGF, and furthermore increases the NGF-induced Trk tyrosine kinase activity (Benedetti *et al.*, 1993; Mahadeo *et al.*, 1994; Verdi *et al.*, 1994). Recent crosslinking and immunoprecipitation experiments have demonstrated that p75 and Trk receptors form a heterodimeric complex in sensory neurons (Huber and Chao, 1995). This physical interaction between the two NGF receptors can potentially serve as a cellular mechanism for the effects of the p75 receptor on Trk function.

Neurotrophin signaling

The neurotrophins naturally exist as homodimers and initiate their signaling by

activation of Trk receptor tyrosine kinases. Upon binding of the neurotrophin dimers, the Trk receptors form oligomeric complexes and transduce signals through tyrosine phosphorylation of a variety of substrates including enzymes and adaptor proteins as well as themselves. Studies using an inhibitor of tyrosine phosphorylation (K252a) or Trk tyrosine kinase-defective mutants have demonstrated that the intermolecular phosphorylation of Trk receptors is a functional requirement for the biological effects of neurotrophins (Jing *et al.*, 1992; Koizumi *et al.*, 1988).

Characterization of the intracellular signaling pathways that couple Trk receptor activation to the final physiological effects of neurotrophins has mostly been made using cell lines such as rat pheochromocytoma (PC12) cells (reviewed in Greene and Kaplan, 1995). NGF induces neurite outgrowth in PC12 cells by activating a cascade of signaling molecules (Figure 3). Autophosphorylation of Trk provides a binding site for many kinds of src-homology motif 2 (SH2) - containing proteins. These proteins include adaptor protein Shc (SH2-containing sequence), phospholipase Cγ1 (PLC-γ1), PI-3 kinase, and a protein of unknown function called SNT (suc-associated neurotrophic factor-induced tyrosine phosphorylated target). These SH2 domain-containing proteins are themselves phosphorylated at tyrosine residues by the Trk receptor kinase, thus activating a signaling cascade. Studies using mutated receptors or pharmacological inhibitors suggest that these parallel events cooperate to trigger neuritogenesis. Although major advances have been made in the elucidation of the signaling pathway downstream of Shc, the downstream components of the PLC-γ1, PI-3 kinase, and SNT pathways have not yet been identified.

Upon binding to the phosphotyrosine residue of Trk, Shc recruits an SH2/SH3

adaptor protein Grb2 and a guanine nucleotide exchange factor SOS to the membrane, where they form a complex to activate a small G protein, p21 Ras. Once p21 Ras is activated, it induces a chain of phosphorylation events involving a number of kinases, which eventually leads to neurite outgrowth in PC12 cells. Ras appears to be an essential component in NGF-induced neurite outgrowth since inhibition of endogenous Ras by either antibodies or dominant-negative mutants prevents outgrowth (D'Arcangelo and Halegoua, 1993). The kinase cascade starts with the membrane recruitment of the serine/threonine kinase Raf. Activated Raf , in turn, phosphorylates mitogen-activated kinase kinase (MAPKK or MEK) which has the dual specificity of phosphorylating both threonine and tyrosine residues in MAPK (also called extracellular signal-regulated kinase or ERK). PLC-γ1 has also been shown to be involved in regulating the activation of MAPK, demonstrating a partial redundancy of signaling mechanisms (Stephens *et al.*, 1994).

The MAPK eventually induces the transcription of an immediate early gene, *c-fos,* through activation of diverse cellular targets including the cell cycle regulated S6-kinase (RSK) and a transcription factor, Elk (Janknecht *et al.*, 1993). Although MAPK-activated Elk/SRF complex binds to the serum response element (SRE) of the *c-fos* promoter, another regulatory element appears to be essential in confering NGF responsiveness. NGF triggers the activation of a novel Ras-dependent kinase that phosphorylates and activates CREB. The binding of CREB to the cAMP response element (CRE) within the *c-fos* promoter is also indispensable for NGF-induction of *c-fos* transcription (Ginty, *et al.*, 1994). The biological significance of these cooperative mechanisms is not well

understood, but it seems that the multiple branching pathways may lend more elaborate control and specificity to neuronal function.

Upon exposure to NGF, PC12 cells exhibit neuronal differentiation. The process of neuronal differentiation includes not only neurite outgrowth but also the acquisition of appropriate excitability. It is well known that PC12 cells acquire the ability to generate action potentials when grown in the presence of NGF (Dichter *et al.*, 1977). NGF confers the electrical excitability by promoting the expression of essential ion channels. Three types of sodium channel transcripts, the brain type II/IIA (type II/IIA) and peripheral nerve type 1 (PN1), have been shown to be induced through a Ras-independent pathway upon NGF treatment (D'Arcangelo *et al.*, 1993; Toledo-Aral *et al.*, 1995). In addition, NGF-treated PC12 cells manifest a significant increase in Ca^{2+} currents (Furukawa *et al.*, 1993). In PC12 cells, NGF provides most of the necessary elements for neuronal differentiation through the cooperative action of both Ras-dependent and Ras-independent signaling pathways descrived above.

Although neurotrophin signaling for neurite outgrowth in PC12 cells is relatively well established, the mechanism of action of the neurotrophins in CNS neurons is largely unknown. Similar to what has been observed in PC12 cells, tyrosine phosphorylation of Trk receptors, PLC-γ1, MAPK (ERK1), and SNT by BDNF or NT-3 has been described in embryonic brain tissues or primary hippocampal neurons (Knusel *et al.*, 1994; Marsh *et al.*, 1993). In embryonic rat cortical neurons, BDNF and NT-3 induce a rapid phosphorylation of PLC-γ1 followed by phosphatidylinositol (PI) hydrolysis (Widmer *et al.*, 1993). Furthermore, BDNF and NT-3 enhance *c-fos* expression in embryonic

hippocampal neurons (Ip *et al.*, 1993; Marsh, *et al.*, 1993), which suggests that the neurotrophins may use similar signaling mechanisms in PNS and CNS neuronal cells.

Despite this common feature, primary CNS neurons exhibit a great diversity of functional responses to neurotrophins. A subpopulation of cultured hippocampal neurons shows marked increases in the expression of the calcium-binding protein calbindin or choline acetyltransferase (ChAT) upon treatment with BDNF or NT-3 (Collazo *et al.*, 1992; Ip, *et al.*, 1993). BDNF and NT-3 also regulate the synthesis of various neuropeptides which play a modulatory role in synaptic transmission (Carnahan and Nawa, 1995; Nawa *et al.*, 1994). There is further evidence supporting the idea that the neurotrophins affect synaptic activity. The neurotrophins have both transient and long-term effects on calcium conductances in hippocampal and basal forebrain neurons, respectively (Berninger *et al.*, 1993; Levine *et al.*, 1995). As might be expected from the changes in intracellular Ca^{2+}, BDNF induces an enhancement of acetylcholine release from hippocampal synaptosomes (Knipper *et al.*, 1994). Furthermore, BDNF and NGF have been shown to phosphorylate the presynaptic protein synapsin I which have a regulatory function in neurotransmitter release from presynaptic terminal (Jovanovic *et al.*, 1996).

Function of the neurotrophins during development

The neurotrophins support overlapping yet selective neuronal populations in the peripheral and central nervous system. The regulation of survival and maintenance of specific populations of neurons by each neurotrophin is apparent in the PNS, as revealed

by the localization and knockout studies of neurotrophins and their receptors. In contrast, many classes of CNS neurons show more complex patterns of trophic support. The results from gene targeting experiments do not reveal a clear function for individual neurotrophins in a specific population of CNS neurons, except for a few subtle effects on the expression of several neuromodulatory proteins. The phenotypes of knockouts for three of the neurotrophins and their *trk* receptors are summarized in Table 1.

In the PNS, NGF has been demonstrated to support the survival of sympathetic ganglion neurons and a selective population of sensory neurons in the dorsal root ganglion (DRG) and trigeminal ganglia (reviewed in Korsching, 1993). In keeping with this idea, both NGF and *trkA* knockout mice show profound cell loss in sensory and sympathetic ganglia (reviewed in Snider, 1994). Within the DRG, neuronal loss is limited to small peptidergic neurons which mediate nociceptive function, leaving large DRG neurons unaffected. It appears that central cholinergic neurons, despite prominent expression of *trkA,* do not depend on NGF for their survial and differentiation. However, consistent with the results which indicate that NGF increases ChAT expression in embryonic basal forebrain neurons *in vitro*, cholinergic neurons form the NGF knockout mice do exhibit a significant reduction in axonal ChAT activity in the target fields.

The disruption of BDNF and the *trkB* genes results in a severe loss of many sensory neurons, especially the cells of the vestibular and nodose gangla. Although motor neurons have been suggested to require BDNF for survival, homozygous BDNF mutants do not display any significant motor neuron loss. In contrast, many fewer motor neurons are present in homozygous *trkB* mutant mice, which suggests that NT-3 or NT-4/5 may

activate TrkB receptors and compensate for the loss of BDNF in the ligand knockout. As in the case of the NGF knockout, most of the BDNF-responsive CNS neurons, including dopaminergic neurons of the substantia nigra and GABAergic neurons of the forebrain, appear to survive and differentiate normally in the BDNF and *trkB* mutants. There are, however, several interesting phenotypes which suggest a role of BDNF in regulating the function of many CNS neurons. Expression of neuropeptide Y (NPY) and calcium-binding proteins is dramatically reduced in the cerebral cortex and hippocampus of mutant animals. Calcium-binding proteins need to be present in normal quantities for adequate neuronal function since they act as a buffer system for intracellular calcium increases (Baimbridge *et al.*, 1992). Indeed, the level of calcium-binding proteins calbindin and parvalbumin has been correlated with the protection of neurons against activity-induced excitotoxicity. Therefore, the regulation of calcium-binding proteins by neurotrophins may also have important therapeutic implications in several neurodegenerative diseases.

NT-3 has been shown to increase the survival of sympathetic, neural crest- and nodose ganglion-derived sensory neurons *in vitro* (Rosenthal *et al.*, 1990). As predicted, both the NT-3 and *trkC* knockouts are devoid of substantial fractions of their sensory and sympathetic neurons. In particular, proprioceptive DRG neurons and their sensory end organs are largely missing, which results in a striking behavioral phenotype of abnormal movements and posture. Although NT-3 is highly expressed in the developing brain, most CNS neurons appear to be normal in these mutant animals. The only area which seems to be severely affected is the trigeminal mesencephalic nucleus that contains the

proprioceptive neurons of the trigeminal system, although these neurons also show significant loss in BDNF knockout animals as well. Interestingly, peripheral sensory neurons that serve different sensory modalities in maturity, nociceptive DRG neurons, vestibular ganglion cells, and proprioceptive sensory neurons, all depend on different neurotrophins for their survival and development. In the CNS, however, there seems to be a functional redundancy of neurotrophic support. As is obvious from the knockout studies, no single neurotrophin is responsible for the development and maintenance of a specific neuronal population. Several neurotrophins may act in concert to regulate the development of CNS neurons which form a much more complex neuronal circuitry than peripheral neurons.

Plasticity during visual system developement

One of the principal mechanisms for the refinement of neuronal connections during development is activity-dependent competition between profuse presynaptic terminals for indivisual postsynaptic targets. In the visual cortex, these synaptic rearrangements occur during a definite time window called the 'critical period' (reviewed in Goodman and Shatz, 1993). Early in development, LGN (lateral geniculate nucleus) inputs from the left and right eyes are intermixed with each other in the primary visual cortex. However, as development proceeds, the inputs from the two eyes become segregated into alternating, eye-specific stripes within primary visual cortex, forming the ocular dominance columns (ODC). The formation of ODCs can be affected by manipulations of visual input, such as monocular deprivation during the critical period. Monocular deprivation causes a shift in

ocular dominance, so that the stripes of input from LGN axons representing the closed eye are taken over by the input from the open eye. Although it has long been known that the axonal remodeling depends on the electrical activity of competing neurons, the molecular mechanism for this process is not well understood.

In recent years, the neurotrophins have emerged as a promising candidate for cellular mechanisms of such plasticity in the developing visual system (reviewed in Lo, 1995; Thoenen, 1995). Several lines of evidence now suggest that these factors have functions besides conventional trophic support and might play a role in activity-dependent synaptic plasticity during development or adulthood. Based on its expression pattern, one of the most probable neurotrophins to mediate this plasticity is BDNF. Both BDNF and the TrkB receptor are present in high levels in visual system structures and their expression is regulated developmentally in an appropriate fashion, such that it roughly parallels the time course of the critical period (Allendoerfer *et al.*, 1994; Huntley *et al.*, 1992). Moreover, the level of BDNF mRNA is modulated by alterations in visual input such as monocular deprivation or dark rearing followed by exposure to light (Bozzi *et al.*, 1995; Castren *et al.*, 1992).

Cabelli *et al.* have shown that local infusion of exogenous BDNF and NT-4/5, but not NGF, can prevent the formation of ODCs in the developing kitten visual cortex (Cabelli *et al.*, 1995). Further evidence supporting the role of TrkB ligands in visual plasticity comes from an elegant set of experiments using microspheres coated with red or green dye, and NT-4 (Riddle *et al.*, 1995). This new technique allows the identification of specific neurons which have received the exogenous supply of

neurotrophins. In these experiments, NT-4 -treated neurons have been shown to remain unaffected, although control neurons in the closed-eye layer shrink substantially after monocular deprivation. Surprisingly, BDNF which shares the same receptor (TrkB) with NT-4, has failed to prevent this shrinkage. Although these two factors are similarly specific and effective in activating TrkB, previous studies using a mutant TrkB receptor have indicated that BDNF and NT-4/5 display different forms of interaction with TrkB (Ip *et al.*, 1993). This differential interaction could potentially activate distinct sets of signaling cascades which in turn result in different functional responses.

In contrast, experiments by Maffei and co-workers have implicated NGF as a key factor in activity-dependent plasticity in the visual cortex of both rats and kittens (Carmignoto *et al.*, 1993; Maffei *et al.*, 1992). Intraventricular injection of NGF prevents the effects of monocular deprivation, presumably by providing excess NGF and thus alleviating the competition for a limiting amount of neurotrophic factor. The physiological relevance of these findings is supported by the recent demonstration that blocking the function of endogenous NGF by specific antibodies results in an extension of the critical period (Domenici *et al.*, 1994). However, the negligible level of TrkA expression in the developing visual cortex (Martin-Zanca *et al.*, 1990) casts doubt upon the role of endogenous NGF in visual development. It is still possible that NGF may exert its effects through low-affinity p75 receptors which are known to be present in the developing visual system (Allendoerfer *et al.*, 1990). The apparent inconsistency between the *in vivo* experiments on the role of particular neurotrophins could be due to the diverse effects of neurotrophins on different classes of neurons in the developing

visual system.

The cellular and morphological action of different neurotrophins in visual cortical neurons has begun to be assessed recently. Using electrophysiological techniques, Carmignoto *et al.* have reported that the neurotrophins BDNF and NGF rapidly enhance excitatory synaptic transmission in the visual cortex of young (P12-17) rats, which suggests a role of these factors in functional stabilization of weak synapses (Carmignoto *et al.*, 1997). The first demonstration of effects of neurotrophins on the morphology of single neurons was performed using particle-mediated gene transfer. The delivery of BDNF and NT-4/5 induces layer-specific changes in the length and complexity of basal and apical dendrites of visual cortical neurons in an activity-dependent manner (McAllister *et al.*, 1996; McAllister *et al.*, 1995). Interestingly, the two TrkB ligands, BDNF and NT-4, show differential effects on basal and apical dendrites, adding another level of specificity to the action of neurotrophins on cortical neurons. A similar observation has been made in the visual system of *Xenopus laevis*, where BDNF, but not NT-4/5, affects the arborization of optic axon terminals in their target tectum (Cohen-Cory and Fraser, 1995). In the developing CNS, the neurotrophins may mediate activity-dependent remodeling of neuronal circuitry by inducing functional and structural modifications of synaptic elements.

Plasticity in the hippocampus

Over the past few years, it has become clear that the NGF family of neurotrophic factors and their receptors are also present in the adult CNS, where the roles that they

play are less well understood. The hippocampus is a prominent site of expression of all neurotrophins and their receptors. NGF and TrkA mRNAs show very restricted expression only in the regions innervated by cholinergic neurons of the basal forebrain (Korshing *et al.*, 1985). In contrast, mRNAs for BDNF, NT-3 and their cognate receptors are widely distributed in almost all regions of the hippocampus. High levels of BDNF mRNA can be found in CA2, CA3, and the hilus, while NT-3 mRNA has been predominantly detected in CA1 and CA2 (Ernfors *et al.*, 1990) (Figure 4). Immunohistochemistry using specific BDNF antibodies has revealed that BDNF immunoreactivity is present in both the somatic and dendritic compartments of pyramidal cells (Wetmore *et al.*, 1991). A large number of neurons show coexpression of the ligand neurotrophins and their Trk receptors, which suggests an action of neurotrophins as paracrine or autocrine signals.

The expression of neurotrophins in the hippocampus can be regulated by neuronal activity. A variety of strong stimuli, such as elevated potassium concentration (Elliott *et al.*, 1994), pharmacological doses of glutamate agonists (both NMDA and kainate) (Gwag and Springer, 1993; Wetmore *et al.*, 1994), and experimentally induced seizure or injury (Ballarin *et al.*, 1991; Isackson *et al.*, 1991) induce a marked increase in the level of neurotrophin mRNA. Activity-dependent regulation of neurotrophin synthesis also occurs under more physiological conditions. Blockade of glutamate receptors or activation of inhibitory GABAergic systems reduces the synthesis of BDNF and NGF mRNAs (Zafra *et al.*, 1991). In addition, comparatively mild neuronal activity such as LTP-inducing stimuli specifically up-regulates neurotrophin expression in the

hippocampal slice (Castren *et al.*, 1993; Patterson *et al.*, 1992). These characteristics of the regulation of neurotrophin synthesis are consistent with the hypothesis that the neurotrophins, in addition to their classic trophic functions, might act as mediators of neuronal plasticity. Recent reports showing that the neurotrophins can be released in an activity-dependent manner from neuronal dendrites in the hippocampus (Blochl and Thoenen, 1995; Goodman *et al.*, 1996; Griesbeck *et al.*, 1995) lends further support to this idea.

To begin to assess the possible function of neuronally released neurotrophins, several labs have examined whether exogenous application of neurotrophins can modulate synaptic activity in the hippocampus. In dissociated cultures of embryonic hippocampal neurons, BDNF and NT-4/5 rapidly potentiate glutamatergic transmission, presumably via postsynaptic TrkB receptors (Lessmann *et al.*, 1994; Levine *et al.*, 1995; Levine *et al.*, 1996). As will be discussed in detail in Chapter 2, I have also demonstrated that BDNF and NT-3 induce a rapid and long-lasting enhancement of excitatory synaptic transmission in the adult hippocampal slice (Kang and Schuman, 1995). The neurotrophin-induced synaptic potentiation may at least in part due to an increase in presynaptic transmitter release since these factors are capable of enhancing the release of acetylcholine and glutamate from hippocampal synaptosomes (Knipper, *et al.*, 1994; Knipper *et al.*, 1994). Modulation of inhibitory synaptic transmission has been studied only in cultured cortical neurons; Kim *et al.* have shown that NT-3 reduces $GABA_A$-mediated inhibitory transmission, resulting in the enhancement of impulse activity (Kim *et al.*, 1994). In contrast, the potentiating effect of BDNF and NT-3 in my studies is not

attributable to a reduction of inhibitory transmission since a $GABA_A$ blocker, bicuculline, does not attenuate the enhancement induced by either neurotrophin (Kang and Schuman, 1996). In the hippocampus, inhibitory interneurons exert an extremely powerful influence on hundreds of excitatory pyramidal cells and are often targets of neuromodulatory peptides (Cohen *et al.*, 1992; Doze *et al.*, 1991). Therefore, it will be of interest to examine whether the neurotrophins might also affect the activity of inhibitory neurons in the hippocampus, in addition to their effects on excitatory transmission.

Given that neuronal activity induces a synthesis and release of neurotrophins, which then in turn can enhance synaptic efficacy, the endogenous neurotrophins could act as mediators of activity-dependent synaptic plasticity such as LTP. Indeed, several recent studies indicate an involvement of neurotrophins, especially BDNF, in the expression of LTP in the hippocampus. Two different lines of BDNF knockout mice have been generated and tested for LTP at synapses between the Schaffer collaterals and CA1 pyramidal neurons (Korte *et al.*, 1995; Patterson *et al.*, 1996). These mutant mice exhibit a slow decline of LTP over time, although a significant potentiation can still be observed at the time of induction. Interestingly, heterozygous mutant mice, in which about 50% of the wild-type level of BDNF is still present, show a similar LTP impairment as homozygotes, suggesting that a threshold level of BDNF is needed for normal LTP expression. The LTP deficits in mutant mice are likely due to the lack of BDNF function rather than other developmental abnormalities since acute treatment with either exogenous BDNF or an adenovirus construct containing the BDNF gene can restore LTP in these mice. Another line of evidence suggesting an involvement of BDNF in

hippocampal LTP is the recent demonstration that exogenous BDNF promotes the induction of LTP by tetanic stimulation in hippocampal slices from young (P12-13) rats (Figurov *et al.*, 1996). Hippocampal synapses in young animals are not capable of following repetitive stimulation, and thus tetanic stimulation gives rise only to short-term potentiation (STP) at this age. Acutely provided BDNF seems to exert its effect by enhancing the ability of synapses in young slices to respond to high-frequency stimulation. Consistent with this idea, blocking the function of endogenous BDNF with TrkB-IgG fusion proteins reduces the synaptic responses to tetanic stimulation in adult slices and thereby greatly attenuates LTP. I have also observed the modulation of various forms of hippocampal LTP by inhibiting TrkB function using either specific TrkB antibodies or TrkB-IgG fusion proteins (chapter 5). Taken together, these studies strongly suggest a functional role of BDNF in the expression of LTP. The cellular mechanisms by which the neurotrophins modulate synaptically-induced LTP in the hippocampus remain to be investigated.

One of the most exciting features of neurotrophin signaling is the regulation of gene expression and neuronal structure. Although it has not been clearly demonstrated in the adult plasticity, most long-term plasticity likely involves changes in neuronal morphology or connectivity. As described earlier, late phases of LTP involve *de novo* gene expression and protein synthesis (Alvarez, *et al.*, 1994; Barondes and Cohen, 1966; Flexner, *et al.*, 1963; Frey, *et al.*, 1988; Nguyen, *et al.*, 1994). Considering the ability of neurotrophins to induce structural modifications during development, it will be worthwhile to examine the potential function of these factors in long-lasting components

of hippocampal LTP. Indeed, the neurotrophins, when applied exogenously, stimulate new protein synthesis. Previously, I have shown that the potentiation induced by BDNF and NT-3 displays a very early dependence on protein synthesis, which presumably occurs locally within dendrites (chapter 4) (Kang and Schuman, 1996). A nice demonstration of neurotrophin-induced local protein synthesis has been recently provided by Crino and Eberwine (Crino and Eberwine, 1996). When a myc-tagged mRNA is transfected into isolated dendritic growth cones of cultured hippocampal neurons and treated with BDNF or NT-3, it can be translated and detected with anti-myc antibody, which indicates that the neurotrophins stimulate protein synthesis in dendrites disconnected from their cell bodies. This mechanism of local protein synthesis can serve as one of the potential ways to promote site-specific modification of synaptic structure and function without involving the problem of selective protein targeting. By inducing protein synthesis at recently activated synapses, the neurotrophins could connect two temporally distinct events, the initial electrical activity and persistent structural modification, during learning-related long-term plasticity in the adult brain.

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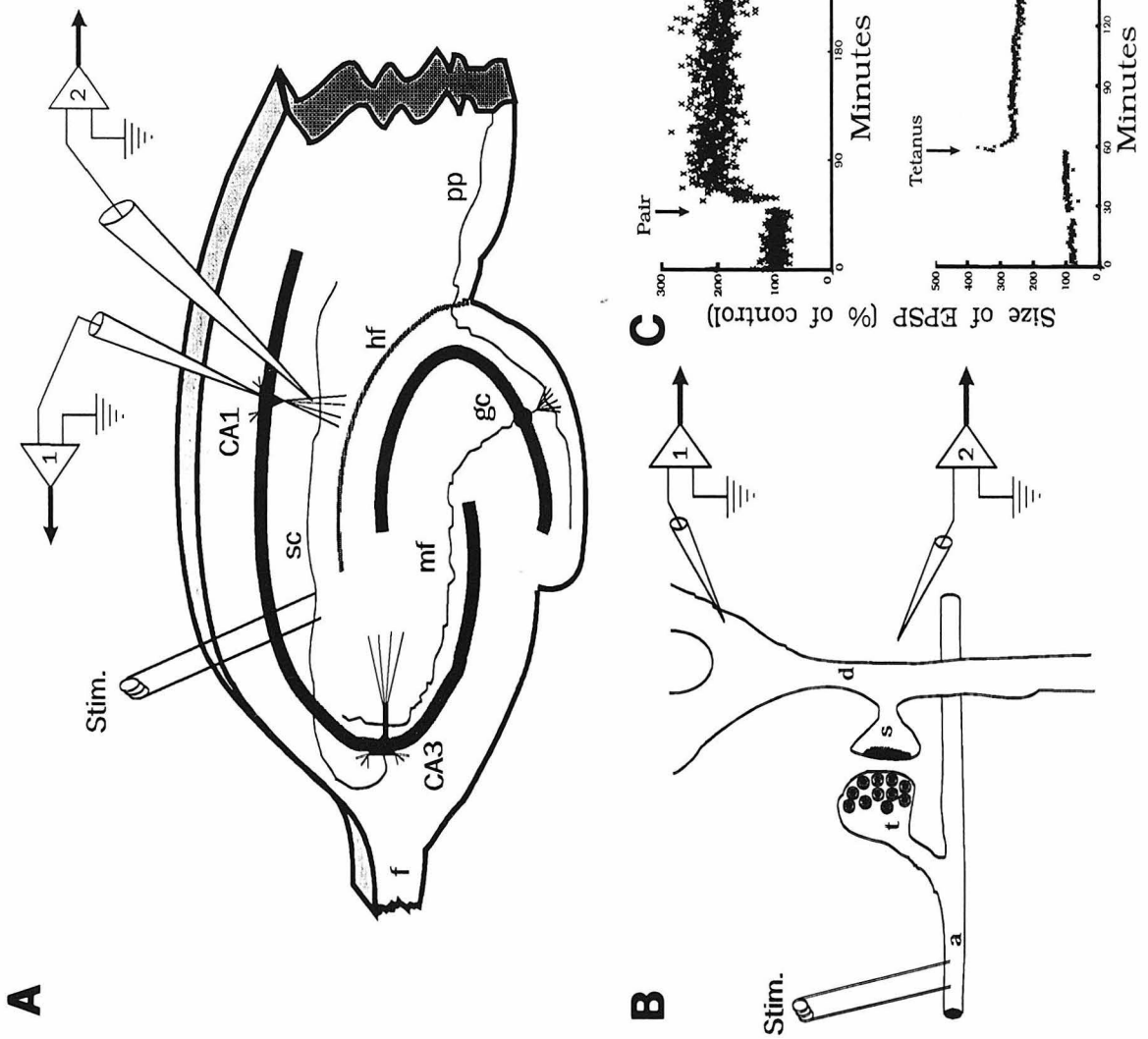
FIGURES AND LEGENDS

Figure 1. Long-term potentiation in the hippocampal slice *in vitro*. (**A**) Schematic diagram of the hippocampal slice showing principal cell types and some major excitatory synaptic connections. Abbreviations: pp, perforant path; hf, hippocampal fissure; gc, dentate granule cell; mf, mossy fibers; f, fimbria; sc, Schaffer collaterals; stim, stimulating electrode; 1 and 2, recording electrodes (1 represents an intracellular recording electrode; 2 represents an extracellular electrode for recording field potentials). (**B**) Schematic diagram representing the parts of a synapse involved in LTP. The parts of the synapse are labeled: a, presynaptic axon; t, presynaptic terminal; s, postsynaptic spine; d, postsynaptic dendrite. (**C**) LTP can be induced by two methods. Upper graph: low frequency stimulation of presynaptic axons paired with artificial depolarization of the postsynaptic cell produces LTP, but no PTP. The cell was artificially depolarized by current passed through the intracellular electrode. Lower graph: tetanic stimulation of presynaptic axons causes short-lasting potentiation (post-tetanic potentiation, or PTP) followed by a persistent potentiation of the EPSP (LTP). (Madison and Schuman, 1991).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1
2
C
Pair
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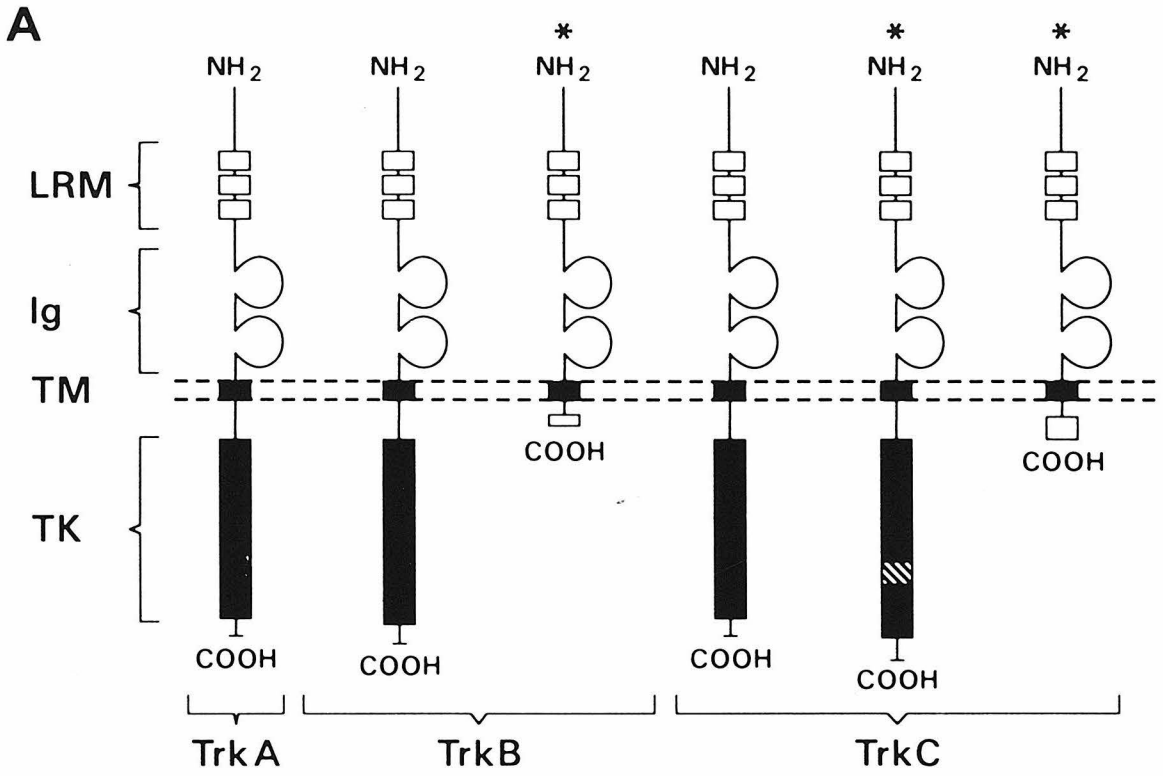
Minutes
Size of EPSP (% of control)

Figure 2. Neurotrophin receptors and binding patterns. (**A**) All members of the Trk family share characteristic structural motifs in the extracellular domain, including immunoglobulin domains (Ig) and leucine-rich motifs (LRM). The receptors are glycosylated to yield gene products of 140, 145, and 145 kDa, respectively. Asterisks indicate TrkB and TrkC isoforms. Both *trkB* and *trkC* loci encode isoforms lacking the tyrosine kinase domain (TK). The *trkC* locus also encodes multiple isoforms with an insert in the tyrosine kinase domain. TM, transmembrane domain. (**B**) The primary ligand is given across from each receptor, as determined on the basis of binding and phosphorylation studies. In vitro, Trk exhibits some capacity to bind neurotrophins other than their primary ligands (indicated by broken arrows). In addition. all neurotrophins bind $p75^{NGFR}$. (Snider, 1994).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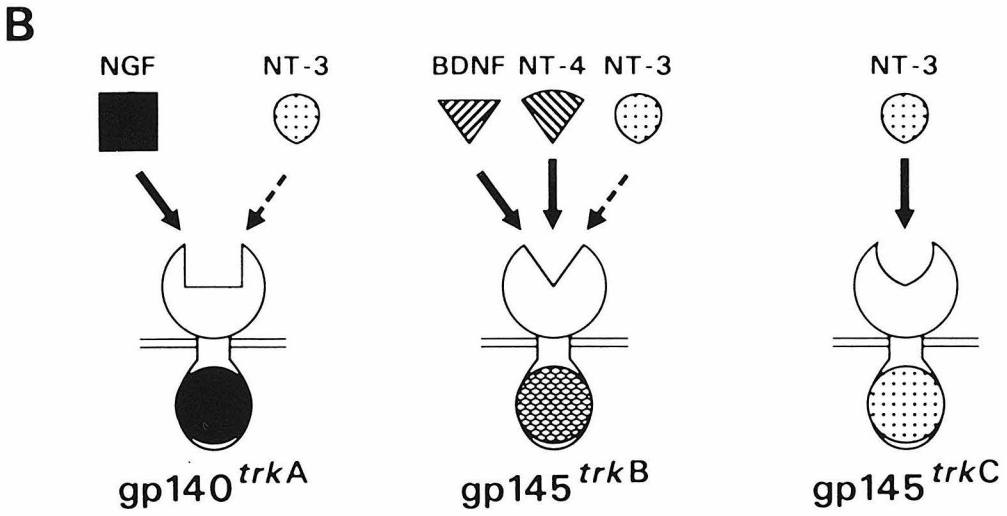
B
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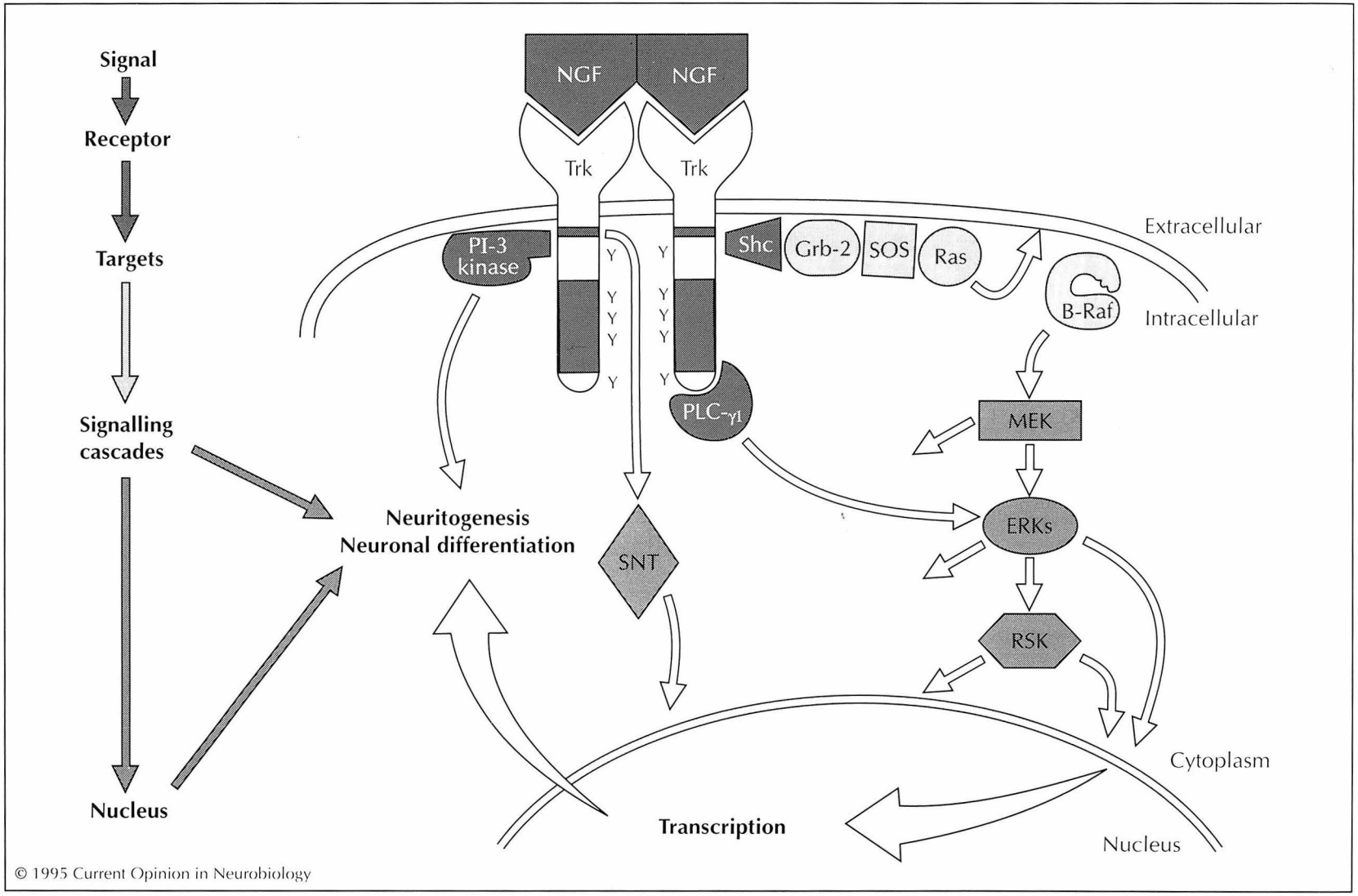
NT-3
BDNF
NT-4
NT-3
NT-3
gp140trkA
gp145trkB
gp145trkC

Figure 3. Some steps in early transmission of NGF signals by Trk. Binding of NGF causes activation of the Trk receptor tyrosine kinase and Trk autophosphorylation on tyrosine residues (Y), both within and outside of the kinase domain (darkened area). Targets such as PI-3 kinase, PLC-γ and Shc bind to specific phosphotyrosine-containing domains of Trk and become phosphorylated. The activated targets then lead to activation of downstream signaling cascades. The best characterized cascade to date is the one in which Shc forms a complex with Grb2 and SOS to trigger Ras activation (i.e. formation of Ras-GTP). B-Raf kinase becomes activated after it is recruited to the plasma membrane by activated Ras. Activated B-Raf, in turn, triggers the MEK, ERK, RSK kinase cascade. The SNT protein also becomes tyrosine phosphorylated in response to NGF by a pathway dependent on the juxtamembrane region of the receptor (hatched area). The signaling cascades lead both to activation of gene transcription and to non-transcriptional events such as phosphorylation/regulation of cytoskeletal components. The transcriptional and non-transcriptional events are integrated to generate cellular responses, such as formation of neurites, and expression of additional neuronal properties, such as excitability. (Greene and Kaplan, 1995).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Grb-2
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Extracellular
Intracellular
PLC-γ1
MEK
ERKs
RSK
SNT
Neuritogenesis
Neuronal differentiation
Cytoplasm
Transcription
Nucleus
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Figure 4. Dark-field micrographs of NT-3, BDNF, and NGF mRNA-expressing cells in the hippocampus. Coronal sections were hybridized to probes specific for NT-3 (**A**), BDNF (**B**), or NGF (**C**) and exposed to photographic emulsion. Note the difference in the distribution of labeled cells between the three factors: the NT-3-specific probe labeled CA1 and CA2, the BDNF-specific probe labeled CA2, CA3, and hilar region, with lower labeling in CA1, and NGF-specific probe labeled scattered neurons in the hilar region, pyramidal layer, and stratum oriens. Arrowheads in (C) indicate some of the labeled cells in the stratum oriens, and arrows indicate labeled cells in the pyramidal layer of the hippocampus. Also note the high labeling in the dentate gyrus for NT-3 mRNA, whereas the BDNF-specific probe appeared to label fewer cells in this region. Low labeling is detected in the granular layer using the NGF-specific probe. Abbreviation: IG, induseum griseum; O, stratum oriens; P, pyramidal cell layer; R, stratum radiatum; Hi, hilar region. Bar in (C), 400 μm; bar applies also to (A) and (B). (Ernfors, *et al.*, 1990).

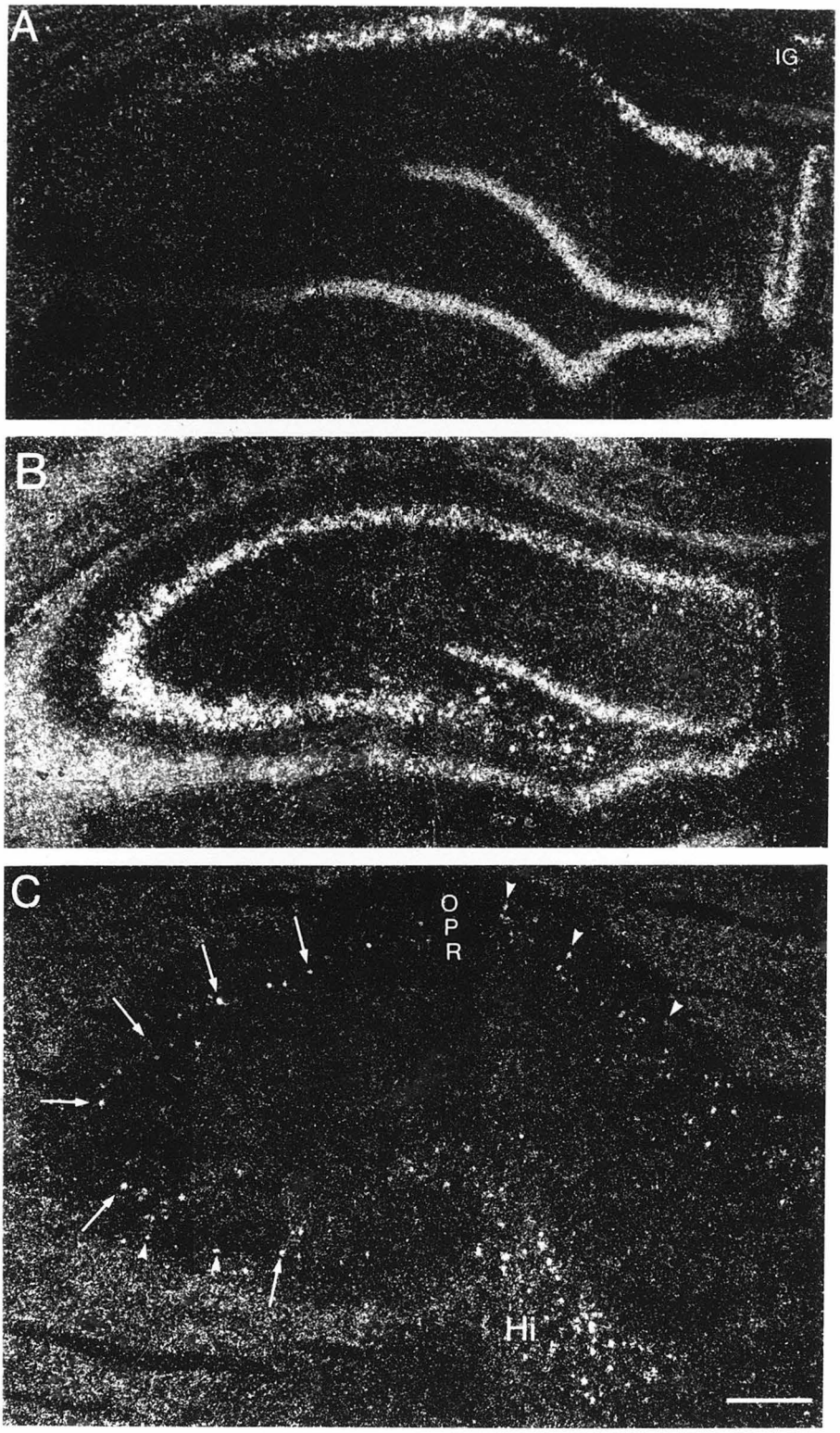
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Table 1. Phenotypes of homozygous neurotrophin and *trk* mutant mice. Percentages are loss of neurons in mutant animals as compared with wild type. Vmes, mesencephalic nucleus of the fifth cranial nerve that contains proprioceptive neurons of the trigeminal system. ChAT, choline acetyltransferase. (Snider, 1994).

Table 1. Phenotypes of Neurotrophin and *trk* mutant mice

mutation	Behavioral	PNS (% Neuron Loss)	CNS (Antibody Staining and % Neuron Loss)
NGF and trkA	↓↓ responses to painful stimuli; most die by 3 weeks	Superior cervical ganglion, 99% DRG, 70%-80% Nodose, no loss of neurons Trigeminal ganglion, 70%	Motor neurons, no loss Basal forebrain, neuronal atrophy, ↓ ChAT, and ↓ axon branching
BDNF and trkB	feed poorly; in trkB, most die in 48 hr; in BDNF, head tuning, spinning in week 2	Superior cervical ganglion, no loss of neurons DRG, 30%-50% Vestibular, > 80% Nodose, 45%-65% Trogeminal ganglion, 25%-55%	Motor neurons trkB, 35% BDNF, no loss Vmes, 40-50% Cortical, ↓ parvalbumin and ↓ NPY
NT-3 and trkC	Strikingly abnormal movements and posture; loss of proprio-ception; some survive 30 days	Superior cervical ganglion; 55% (NT-3) DRG trkC, 20% NT-3, 55% Nodose, 50% Trigeminal ganglion, 65% (NT-3)	Motor neurons trkC, 30% NT-3, no loss Vmes, 50% Cortical, not yet analyzed

CHAPTER 2

Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus

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Long-lasting Neurotrophin-induced Enhancement of Synaptic Transmission in the Adult Hippocampus

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ABSTRACT

The neurotrophins are signalling factors important for the differentiation and survival of distinct neuronal populations during development. To test whether the neurotrophins also function in the mature nervous system, the effects of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3) on the strength of synaptic transmission in hippocampal slices were determined. Application of BDNF or NT-3 produced a dramatic and sustained (2 to 3 hours) enhancement of synaptic strength at the Schaffer collateral-CA1 synapses; NGF was without significant effect. The enhancement was blocked by K252a, an inhibitor of receptor tyrosine kinases. BDNF and NT-3 decreased paired-pulse facilitation, which is consistent with a possible presynaptic modification. Long-term potentiation could still be elicited in slices previously potentiated by exposure to the neurotrophic factors, which implies that these two forms of plasticity may use at least partially independent cellular mechanisms.

The neurotrophins are a group of signalling factors that are essential for the regulation of neuronal survival and differentiation during brain development. In the adult rat central nervous system, the hippocampus is a prominent site of expression of BDNF and NT-3 and their receptors (1). The expression of BDNF, NT-3, and their receptors can be regulated by neuronal activity (2-4), which suggests that the neurotrophins may also participate in synaptic plasticity in the adult central nervous system. Acute exposure to BDNF or NT-3, but not to NGF, rapidly potentiates the frequency of miniature synaptic events at developing neuromuscular synapses in culture (5), prompting us to investigate whether the neurotrophins may regulate synaptic strength in the adult brain.

We applied BDNF, NGF, and NT-3 extracellularly and examined their effects on synaptic transmission at the Schaffer collateral-CA1 neuron synapses in hippocampal slices from young adult rats (6). Field excitatory postsynaptic potentials (EPSPs) were elicited once every 15 sec for the duration of the experiment. BDNF and NT-3 (20 ng/ml) (7) both caused a rapid and dramatic enhancement of the initial slope of the field EPSP (Fig. 1, A and B) [mean percent of baseline: BDNF, 279.7 ± 29.2 ($n = 13$), $P < 0.001$; NT-3, 235.5 ± 26.0 ($n = 11$), $P < 0.05$]. Increases for individual experiments were variable, ranging from 51.3 to 372.6% for BDNF (20 ng/ml) and 32.0 to 344.7% for NT-3 (20 ng/ml). In contrast, NGF (10 to 20 ng/ml) had no significant effect on synaptic transmission (Fig.1C) [mean percent of baseline: NGF, 97.6 ± 5.3 ($n = 8$), not significant (NS)], consistent with the apparent lack of TrkA receptor expression in pyramidal neurons of the hippocampus (8). The potentiating effects of NT-3 and BDNF were concentration-dependent: the lowest effective concentration for both neurotrophins was

10 ng/ml; maximal effects were obtained with 20ng/ml for both BDNF and NT-3 (Fig. 1D). This range of concentrations is similar to that observed for the developmentally significant effects of these factors (9). A single application of BDNF or NT-3 (20ng/ml) produced the maximal increase in synaptic strength, subsequent applications produced no further increase (10). The enhancement produced by BDNF and NT-3 was not accompanied by any significant or consistent change in presynaptic fiber volley, postsynaptic input resistance or excitability (11), which suggests a direct alteration of synaptic transmission.

To identify potential downstream effectors of the neurotrophins, we examined whether two different protein kinase inhibitors, K252a and K252b, were capable of blocking the neurotrophin-induced enhancement. Although structurally similar, these two compounds differ in their potency in inhibiting tyrosine kinases (12); K252a (200 nM) is a potent inhibitor of receptor tyrosine kinases, while K252b (200 nM) is not. Slices exposed to K252a (200 nM) 30 minutes before the introduction of BDNF (20 ng/ml) or NT-3 (20 ng/ml) failed to exhibit synaptic enhancement (Fig. 2, A and B) [mean percent of baseline: BDNF, 116.1 ± 7.7, ($n = 6$), NS; NT-3, 102.4 ± 6.2, ($n = 6$), NS]. In contrast, BDNF and NT-3 still produced a significant increase in synaptic strength in the presence of K252b (Fig. 2, C and D) [mean percent of baseline: BDNF, 194.3 ± 31.5, ($n = 6$), $P < 0.01$; NT-3, 205.2 ± 25.4, ($n = 6$), $P < 0.01$], although this increase was slightly less than that observed in the absence of K252b for both BDNF and NT-3. The inhibition of the BDNF and NT-3 -induced enhancement by K252a suggests the involvement of the Trk family of receptor tyrosine kinases.

The longevity of the synaptic enhancement produced by NT-3 and BDNF (Fig. 1, A and B) could reflect either an enduring modification of the synaptic machinery or the inability to wash out the factors from the slice after re-perfusion with normal artificial cerebral spinal fluid (ACSF). To address this issue, we used a protocol in which neurotrophic factor application was followed by an application of K252a, which we showed in the previous experiment (Fig. 2, A and B) could block the effects of both BDNF and NT-3, presumably at the Trk receptor. If the longevity of the observed enhancement were due to residual neurotrophic factor in the tissue, then the subsequent application of K252a should abbreviate the potentiation. To test this, we elicited an enhancement of synaptic transmission by applying BDNF or NT-3 (20 ng/ml) as before. During the washout of the neurotrophic factor, K252a (200 nM) was introduced into the bath. The application of K252a decreased neither the magnitude nor the duration of the potentiation established by either BDNF or NT-3 (Fig. 2, E and F) [mean percent of baseline: BDNF and $K252a_{pre}$, 155.6 ± 9.1, $K252a_{post}$, 215.3 ± 24.4 (n = 6); NT-3 and $K252a_{pre}$, 173.3 ± 20.4, $K252a_{post}$, 249.9 ± 25.4]. In fact, the enhancement increased further after washout of the factor, as in Fig. 1, which suggests that the long-lasting nature of this enhancement is due to a persistent change initiated by the neurotrophin, rather than to residual factor present in the slice.

During development, neurotrophic factors are thought to be released by postsynaptic target neurons to interact with Trk receptors on axons or growth cones. In the hippocampus, however, BDNF and NT-3 and their receptorsTrkB and TrkC, are located on both pre- and postsynaptic neurons (1), which suggests that these factors could act as

autocrine or paracrine signals. To begin to ascertain the mechanism by which BDNF and NT-3 enhance synaptic strength, we examined whether these factors affect paired-pulse facilitation (PPF). PPF is a form of short-term plasticity in which the size of the postsynaptic response to the second of two closely-spaced (less than 500 ms) stimuli is increased, presumably because of enhanced neurotransmitter release from residual Ca^{2+} in the presynaptic nerve terminal (13). Manipulations that enhance neurotransmitter release usually decrease the magnitude of PPF (14). We thus compared the magnitude of PPF before and after treatment with BDNF and NT-3. Both BDNF and NT-3 significantly decreased PPF (Fig. 3, A and B), which suggests a presynaptic mode of action. The reduction in PPF was not due to neurotrophin-induced saturation of postsynaptic responses because decreasing the stimulus strength to match the size of the postsynaptic response before BDNF and NT-3 treatment did not abolish the attenuation of PPF by the factors (15).

The rapid onset and longevity of the neurotrophin-induced enhancement are similar to those observed during long-term potentiation (LTP) induced by high-frequency stimulation. Accordingly, we tested whether the neurotrophin-induced potentiation might share common cellular mechanisms with synaptically induced LTP by testing the ability of each form of potentiation to occlude subsequent induction of the other form. We first applied either tetanic stimulation or a neurotrophic factor and allowed the potentiation to develop and stabilize for 1 hour. At this time, the stimulus intensity was adjusted to match the size of the field potential to the pre-potentiation level. Potentiation was then induced by the second mechanism. We found that prior enhancement of synaptic

transmission by BDNF or NT-3 did not occlude synaptically induced LTP (Fig. 4, A,C and E) [mean percent of baseline: $LTP_{control}$ = 165.3 ± 7.8, (n = 8); $LTP_{post\text{-}BDNF}$ = 152.4 ± 8.6 (n = 8), NS; $LTP_{control}$ = 175.8 ± 15.3, (n = 8); $LTP_{post\text{-}NT\text{-}3}$ = 164.5 ± 9.1 (n = 8), NS]. Prior induction of LTP slightly, although not significantly, attenuated the subsequent potentiation by BDNF or NT-3 (Fig. 4, B, D and E) [mean percent of baseline: $BDNF_{control}$ = 248.9 ± 33.5 (n = 8) ; $BDNF_{post\text{-}LTP}$ = 184.8 ± 5.5, (n = 8) NS; $NT\text{-}3_{control}$ = 230.1 ± 35.9 (n = 8); $NT\text{-}3_{post\text{-}LTP}$ = 175.2 ± 14.8 (n = 8), NS]. Delivery of repeated trains of tetanic stimulation to maximally induce LTP (16) further reduced the amount of potentiation elicited by BDNF or NT-3, although each factor still significantly enhanced synaptic transmission [mean percent of baseline: $BDNF_{post\text{-}LTP}$ = 139.7 ± 16.3, (n = 5) $P < 0.05$; $NT\text{-}3_{post\text{-}LTP}$ = 133.8 ± 12.2 (n = 5), $P < 0.05$]. As shown in Fig. 4, on average the LTP developed with a fairly slow time course (~10 min). In a separate set of experiments, we confirmed that the LTP induced by the above stimulation parameters relies on the activation of N-methyl-D aspartate (NMDA) receptors by conducting experiments in the presence of the NMDA receptor antagonist AP5 [mean percent of baseline: AP5 (50 μM) = 98.1 +/- 3.7 (n = 4), (10)]. In contrast, we found that both BDNF and NT-3 (20 ng/ml) could still enhance synaptic transmission in the presence of AP5 (50 μM), (Fig. 4 E) [mean percent of baseline: BDNF, 239.2 ± 45.6 (n = 6), $P < 0.05$; NT-3, 284.1 ± 70.3 (n = 6), $P < 0.05$]. Taken together, these data suggest that these two forms of synaptic enhancement may involve at least partially independent cellular mechanisms. Although synaptic potentiation elicited by BDNF or NT-3 did not prevent subsequent LTP measured 1 hour after induction, it is still possible that the neurotrophin-induced enhancement may interact with later phases of LTP that appear to rely on protein

synthesis (17,18).

These experiments demonstrate that BDNF and NT-3 produce rapid changes in synaptic transmission at mature synapses. Both BDNF and NT-3 decrease PPF but do not interact significantly with the early (less than 1 hour) phase of LTP, which is consistent with previous observations that have failed to detect any interaction of PPF with LTP in CA1(14). Therefore, there are likely to be at least two independent mechanisms by which synaptic strength can be enhanced at these synapses. The mRNA levels for BDNF and NT-3 are enhanced after tetanic stimulation (3), however, which suggests that the synaptic enhancement documented here may contribute to later phases of LTP. Taken together, these data and a previous study (5) suggest that one action of neurotrophins may be to alter synaptic strength acutely in the period of time preceding the long-term structural changes that underlie developmental and adult plasticity.

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6. Hippocampal slices were prepared from young adult male Sprague-Dawley rats (mean age = 46.8 ± 1.4 days). Slices were submerged in a stream of ACSF (flow rate 250 ml/hr) (119 mM NaCl. 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11.0 mM glucose) maintained at room temperature (22 to 25° C) and gassed with 95% O_2 and 5% CO_2. Field EPSPs measured in stratum radiatum, were evoked by stimulation of the Schaffer collateral-commissural afferents (once every 15 sec); the initial (1 to 2 ms) slope was measured. Percent baseline values are reported for 1 hour after neurotrophin addition or LTP induction. Input resistance was monitored before and after neurotrophin application by current injection through intracellular recording

electrodes placed in stratum pyramidale of CA1. Ensemble average plots represent group means of each EPSP, for all experiments, aligned with respect to the time of neurotrophin application or LTP induction (four individual 100 Hz trains delivered for 1 second each at the test intensity; inter-train interval = 15 s). To assess statistical significance, paired *t* tests were done on nonnormalized data, comparing mean EPSP slope values for the 10 min preceding the application of the neurotrophin to values 50 to 60 min after application. *P* values greater than 0.05 are designated as NS.

7. Great care was taken in the application and storage of the neurotrophic factors. BDNF and NT-3 were kept at -70°C; phosphate buffer stock solutions were made every 1 to -3 days and kept at 4°C. NGF (R&D Systems, Minneapolis, MN) was kept at 4°C. New supplies of NT-3 and BDNF were obtained on a regular basis (every 2 to 4 months), as it was observed that individual stocks became less potent over time. BDNF was prepared in phosphate-buffered saline (PBS) and NT-3 was prepared in 0.5% sucrose and 4.5% mannitol. Application of vehicle alone at the appropriate dilutions (10^{-5} to 10^{-6}) had no effect on synaptic transmission. The perfusion apparatus was modified to include chemically inert materials: silicon tubing and a Teflon beaker. Bovine serum albumin was not used as a carrier, as previous work (19) has suggested that it has independent effects on synaptic transmission and LTP in the hippocampus. The K252 compounds (Kamiya Biochemical, Thousand Oaks, CA) were kept as 10^4 stock solutions in dimethyl sulfoxide (DMSO) at 4°C. The final concentration of DMSO in our experiments

was 0.01%, which has no detectable effect on synaptic transmission.

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11. The amplitude of the presynaptic fiber volley did not change significantly after treatment with BDNF (20 ng/ml) (mean percent of baseline: 106.2 ± 3.6; n = 12) or NT-3 (20 ng/ml) (mean percent of baseline: 107.0 ± 4.1; n = 11). The input resistance of CA1 neurons did not change significantly after treatment with BDNF (50 ng/ml) (mean percent of baseline: 94.0 ± 7.2; n = 6) or NT-3 (50 ng/ml) (mean percent of baseline: 87.6 ± 11.9; n = 5). Although simultaneous measurements of the population spike in the CA1 pyramidal cell layer revealed significant increase in amplitude on exposure to either BDNF or NT-3 [mean percent of baseline: BDNF, 253.0 ± 60.5 % (n = 3); NT-3, 230.7 ± 47.0 % (n = 3)], multiple population spikes were never observed as would be expected if the slice were exhibiting epileptic activity or large increase in neuronal excitability.

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15. PPF was examined at three interstimulus intervals: 100, 50, and 25 ms. BDNF decreased PPF to 92.0, 77.4, and 66.9% of control levels (n = 7), respectively, without reducing the stimulus strength, and 93.7, 78.0 and 67.8% of control levels (n = 3), when the stimulus strength was reduced to match the size of the field EPSP to pre-BDNF levels. PPF after NT-3 was 103.0, 90.0, and 67.7% of control levels (n = 6) without stimulus readjustment, and 108.6, 89.4 and 74.9% of control levels (n = 2) with stimulus readjustment. PPF measurements were made from 30 to 90 min after neurotrophin washout. PPF after LTP was 108.1, 104.4, and 100.3% of control levels (n = 4) without stimulus readjustment.
16. To maximally induce LTP we delivered two to four sets of tetanic stimulation (one set = four trains of 100 Hz stimulation delivered for 1 s, inter-train interval = 15 s, inter-set interval = 5 to 20 min) until the field EPSP had reached its apparent maximum value and no further potentiation could be elicited. This stimulation protocol resulted in LTP of the following magnitude: mean percent of baseline ± S.E.M. 183.6 ± 23.4% (n = 10). The magnitude of this potentiation was not significantly different from the potentiation obtained with the use of normal induction protocols (one set of tetanic stimulation)[170.5 ± 8.4% (n = 16)].
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20. We thank D. Anderson, M. Kennedy, G. Laurent, P. Patterson and members of the

Schuman lab for helpful discussions and comments and C. Hung for writing data acquisition and analysis software. We thank Amgen for supplying BDNF and NT-3. E.S. is a John Merck Scholar and an Alfred P. Sloan Fellow.

FIGURES AND LEGENDS

Fig. 1. BDNF and NT-3 enhance excitatory synaptic transmission. **(A)** Ensemble average for all experiments in which BDNF (20 ng/ml) was applied extracellularly. Mean field EPSP slope before BDNF was 0.13 ± 0.01 mV/ms (mean ± SEM) and 0.34 ± .03 mV/ms after BDNF. In (A) through (C), two representative field EPSPs are shown for the time points indicated. **(B)** Ensemble average for all experiments in which NT-3 (20 ng/ml) was applied extracellularly. Mean field EPSP slope before NT-3 was 0.11 ± 0.01 mV/ms and 0.34 ± 0.09 mV/ms after NT-3. **(C)** Ensemble average for all experiments in which NGF (10 to 20 ng/ml) was applied extracellularly. (The data for 10 and 20 ng/ml were combined, because they were not significantly different from one another.) Mean field EPSP slope before NGF was 0.14 ± 0.01 mV/ms (mean ± SEM) and 0.13 ± 0.01 mV/ms after NGF. **(D)** Summary graph depicting concentration-response relation for each neurotrophic factor. Asterisks indicate statistical significance at the $P < 0.05$ level.

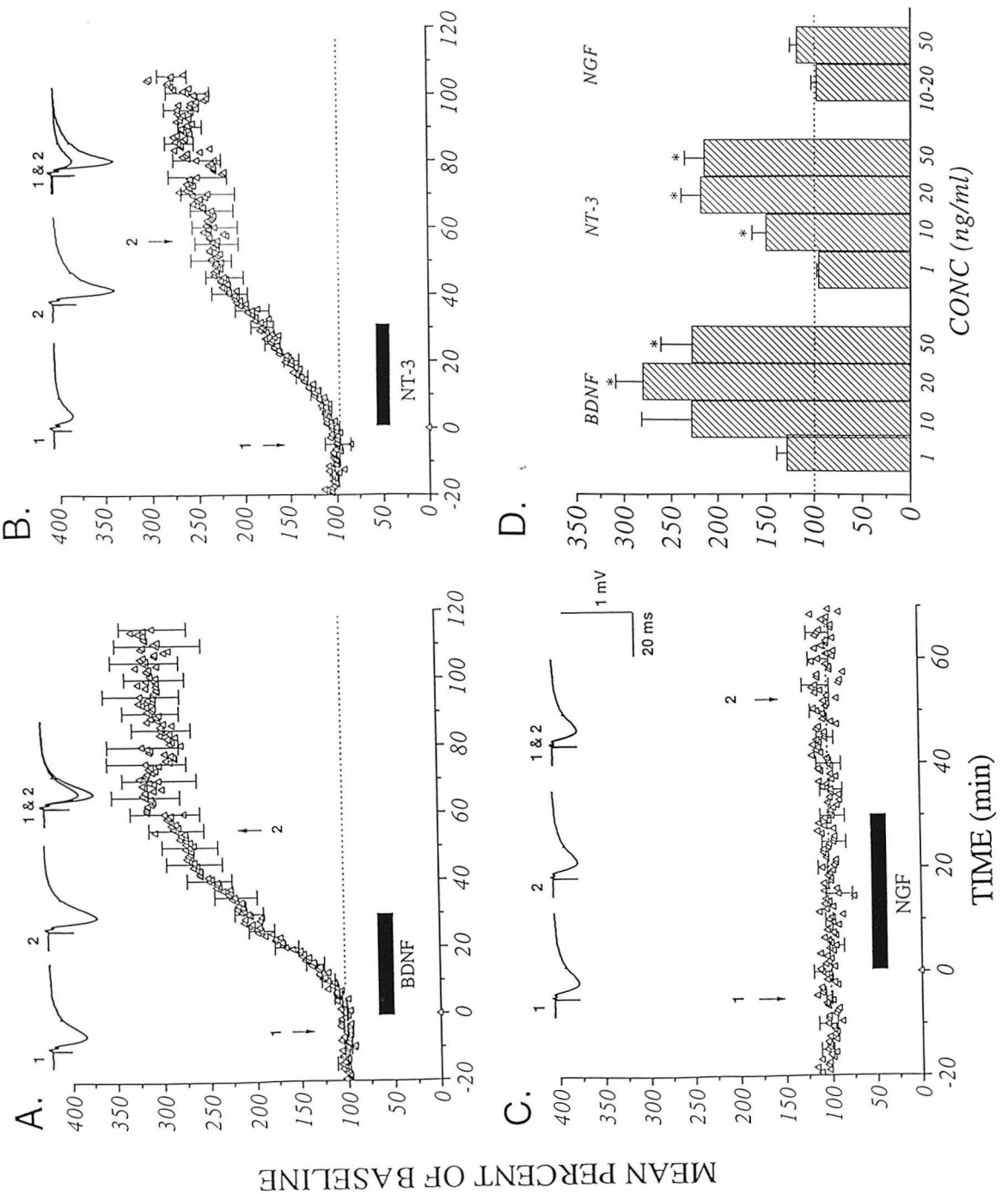

A.
B.
C.
D.
MEAN PERCENT OF BASELINE
TIME (min)
BDNF
NT-3
NGF
1 mV
20 ms
CONC (ng/ml)
1 10 20 50 1 10 20 50 10-20 50

Fig. 2. The effects of BDNF and NT-3 are attenuated by prior treatment with K252a, but not by subsequent treatment. **(A)** Ensemble average for all experiments in which BDNF (20 ng/ml) was applied extracellularly in the presence of K252a. Mean field EPSP slope before BDNF was 0.13 ± 0.03 mV/ms (mean ± SEM) and 0.15 ± 0.02 mV/ms after BDNF. In (A) through (F) two representative field EPSPs are shown for the time points indicated. **(B)** Ensemble average for all experiments in which NT-3 (20 ng/ml) was applied extracellularly in the presence of K252a. Mean field EPSP slope before NT-3 was 0.15 ± 0.02 mV/ms and 0.16 ± 0.02 mV/ms after NT-3. **(C)** Ensemble average for all experiments in which BDNF (20 ng/ml) was applied extracellularly in the presence of K252b. Mean field EPSP slope before BDNF was 0.10 ± 0.02 mV/ms and 0.18 ± 0.02 mV/ms after BDNF. **(D)** Ensemble average for all experiments in which NT-3 (20 ng/ml) was applied extracellularly in the presence of K252b. Mean field EPSP slope before NT-3 was 0.14 ± 0.03 mV/ms and 0.26 ± 0.05 mV/ms after NT-3. **(E)** Ensemble average for all experiments in which BDNF (20 ng/ml) was applied extracellularly and then followed by application of K252a. Mean field EPSP slope after 20 to 30 min in the presence of BDNF was 0.18 ± 0.02 mV/ms and 0.24 ± 0.03 mV/ms after 50 to 60 min in the presence of K252a. **(F)** Ensemble average for all experiments in which NT-3 (20 ng/ml) was applied extracellularly and then chased by application of K252a. Mean field EPSP slope after 20 to 30 min in the presence of NT-3 was 0.19 ± 0.01 mV/ms and 0.29 ± 0.03 mV/ms after 50 to 60 min in the presence of K252a.

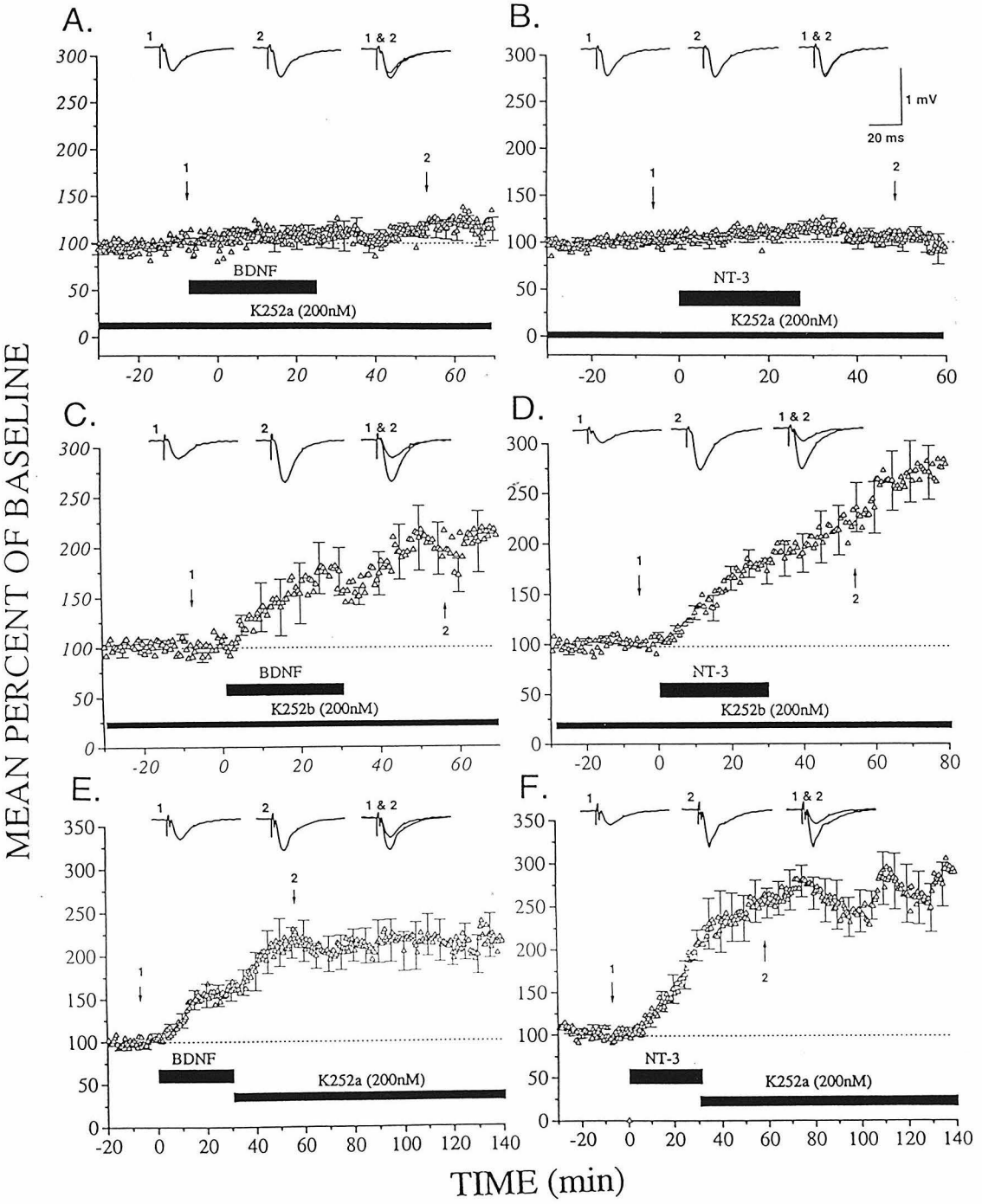

A.
B.
C.
D.
E.
F.
1
2
1 & 2
1 mV
20 ms
BDNF
NT-3
K252a (200nM)
K252b (200nM)
MEAN PERCENT OF BASELINE
TIME (min)

Fig. 3. BDNF and NT-3 decrease PPF. Both plots show the facilitation ratios [slope of second EPSP / slope of first EPSP] for three different interstimulus intervals: 100, 50, and 25 ms before (solid triangle) and after (inverted triangle) the addition of the factor. BDNF **(A)** and NT-3 **(B)** significantly decreased PPF at the 50- and 25- ms interpulse intervals.

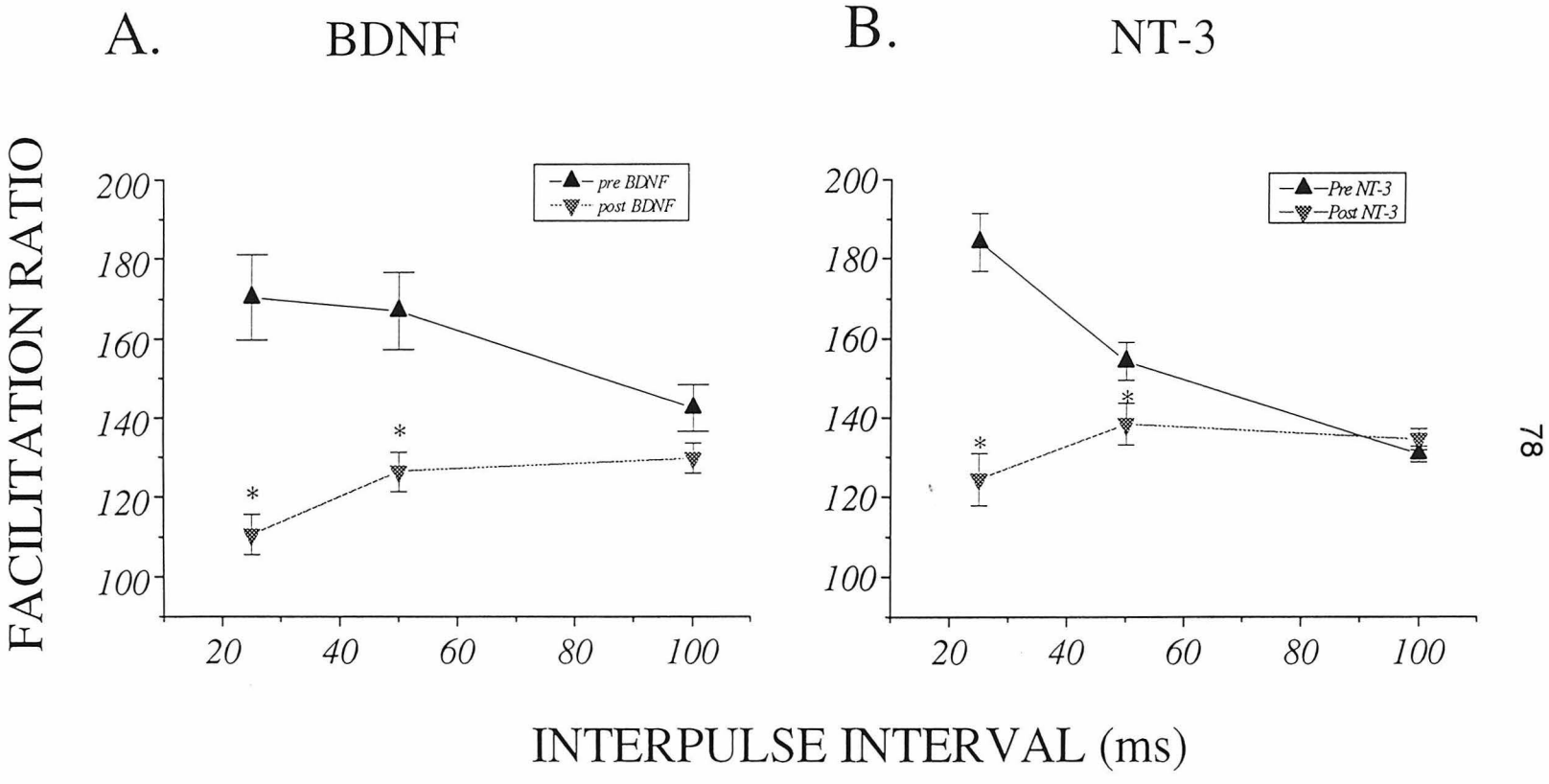
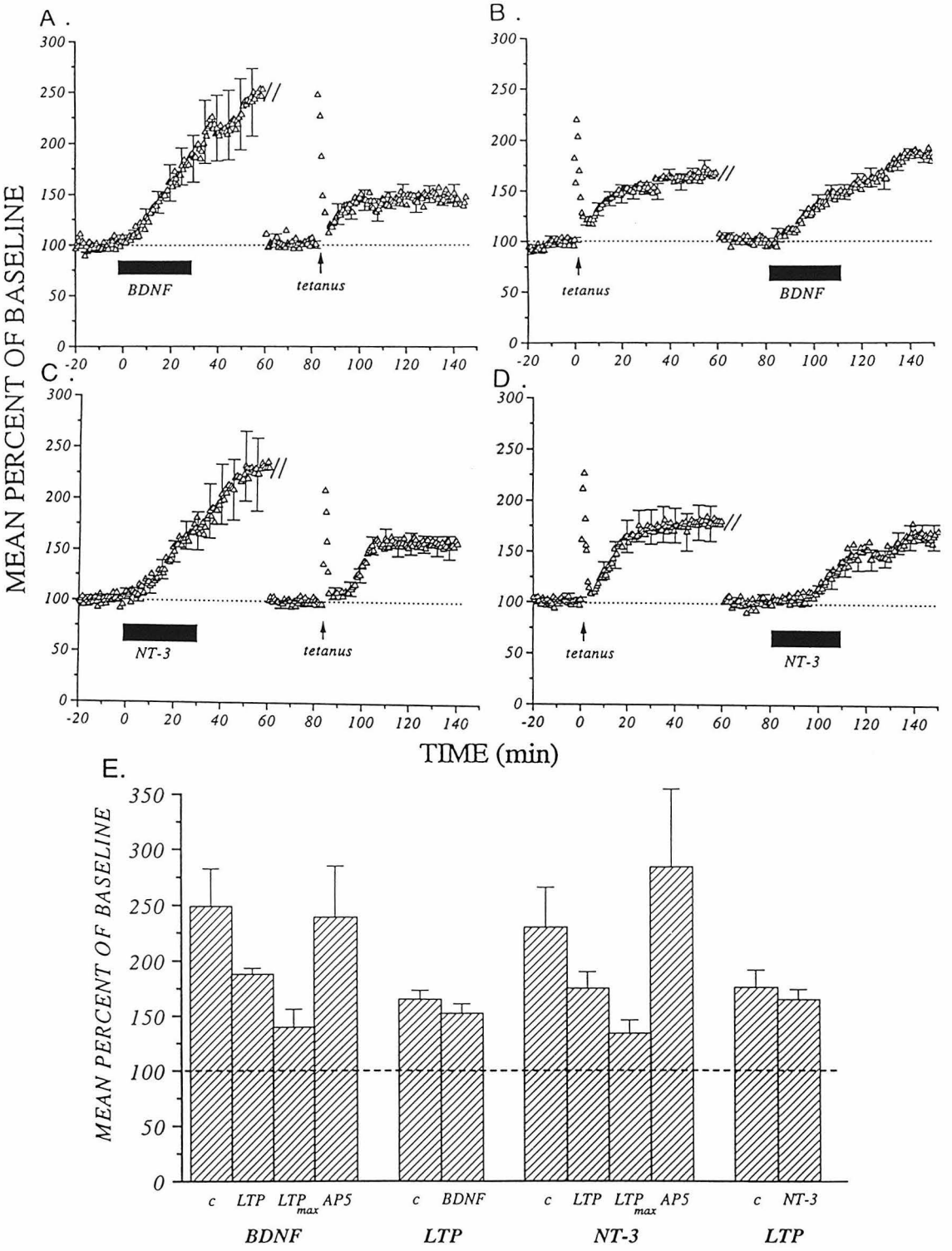

A.
BDNF
B.
NT-3
FACILITATION RATIO
200
180
160
140
120
100
20
40
60
80
100
pre BDNF
post BDNF
Pre NT-3
Post NT-3
*
INTERPULSE INTERVAL (ms)

Fig. 4. Neurotrophin-induced potentiation and LTP do not significantly occlude one another. In (A) through (D) the stimulus intensity was reduced to match the size of the field EPSP to control levels at the time indicated by the two slanted bars. **(A)** Ensemble average showing experiments in which BDNF induced potentiation was followed by tetanic stimulation to induce LTP. LTP could still be elicited at synapses previously potentiated by BDNF. **(B)** Ensemble average showing experiments in which LTP was followed by BDNF (20 ng/ml) application. BDNF was still capable of enhancing transmission at potentiated synapses, although the magnitude of the enhancement was slightly less than that observed in control pathways. **(C)** Ensemble average showing experiments in which NT-3 -induced potentiation was followed by tetanic stimulation to induce LTP. LTP could still be elicited at synapses previously potentiated by NT-3. **(D)** Ensemble average showing experiments in which LTP was followed by NT-3 (20 ng/ml) application. NT-3 was still capable of enhancing transmission at potentiated synapses, although the magnitude of the enhancement was slightly less than that observed in control pathways. (E) Summary plot showing the magnitude of potentiation produced at LTP-induced or neurotrophin-treated synapses in naive (control, C) slices or slices previously exposed to either a neurotrophic factor, normal LTP induction (LTP), maximal LTP induction (LTP_{max}), or an NMDA receptor antagonist (AP5).


A .
B .
C .
D .
E.
MEAN PERCENT OF BASELINE
TIME (min)
BDNF
tetanus
NT-3
MEAN PERCENT OF BASELINE
c
LTP
LTP max
AP5
BDNF
NT-3
BDNF
LTP
NT-3
LTP

CHAPTER 3

Determinants of BDNF-induced hippocampal synaptic plasticity: role of the TrkB receptor and the kinetics of neurotrophin delivery.

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Determinants of BDNF-induced Hippocampal Synaptic Plasticity: role of the TrkB receptor and the kinetics of neurotrophin delivery.

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ABSTACT

The neurotrophins are a class of signaling molecules known for their growth and survival-promoting activities during neuronal development. Recent studies suggest that the neurotrophins, including brain-derived neurotrophic factor (BDNF), can also dramatically influence synaptic transmission in the adult hippocampus. The experiments described in this paper indicate that ability of BDNF to potentiate synaptic transmission in the hippocampus relies on functional TrkB receptors. Moreover, the rate at which BDNF is applied to hippocampal synapses is also a potent determinant of whether synaptic potentiation will result. Hippocampal slices perfused with BDNF at a very slow flow rate (e.g , ≤ 25 ml/hr) did not show synaptic potentiation. Increasing the rate of BDNF application resulted in synaptic potentiation in which the magnitude and onset kinetics of the potentiation were determined by the rate of BDNF delivery. Immunocytochemical analysis of BDNF detected with confocal microscopy confirmed these electrophysiological observations, indicating that the penetration of BDNF into hippocampal slices is influenced dramatically by the perfusion rate.

INTRODUCTION

The ability of the brain to alter information processing by changing the structure and strength of synaptic connections is essential for the successful development and survival of organisms. There is increasing evidence that the central nervous system (CNS) uses some of the same molecular mechanisms during both developmental and adult plasticity (Goodman and Shatz 1993). The neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and NT-3, are proteins that promote the survival and differentiation of some neurons during development. Although the neurotrophins are known primarily for their growth promoting activities during neuronal development, recent studies suggest that they can also influence synaptic transmission and plasticity (for review, see Lo 1995; Thoenen 1995).

Lohof *et al.*(1993) were the first to demonstrate an acute effect of neurotrophins on synaptic transmission. They applied neurotrophins to developing frog nerve-muscle synapses in culture and showed that BDNF and NT-3 can enhance both evoked synaptic responses as well as the frequency of miniature synaptic events (Lohof *et al.* 1993). Subsequent studies in hippocampal cell cultures have documented similar findings (Lessmann *et al.* 1994; Levine *et al.* 1995).

In previous studies, we demonstrated an enhancement of synaptic transmission in hippocampal slices produced by the application of BDNF or NT-3 in the extracellular bathing medium (Kang and Schuman 1995b; 1996). The synaptic potentiation produced by the neurotrophins was very rapid in onset (10-20 min) and long-lasting (hours). In this previous study, the rate of perfusion of the slice was ~200-250 ml/hr. BDNF is not very

diffusible owing to its basic nature (Leibrock *et al.* 1989), and perhaps, the abundance of both full-length and truncated Trk receptors (Zhou *et al.* 1993; Escandon *et al.* 1994). In this study, we have examined the effects of perfusion rate on both the kinetics and the magnitude of the enhancement produced by BDNF. Using immunocytochemical techniques and confocal microscopy, we have also examined the penetration of BDNF into hippocampal slices as a function of perfusion rate.

Does exogenously-applied BDNF work through the same signal transduction mechanisms used by BDNF to bring about changes in survival during neuronal development? In situ hybridization studies have shown that TrkB, the primary receptor for BDNF, is expressed in all the CA fields, in dentate granule cells, as well as in interneurons (Merlio *et al.* 1992; Ip *et al.* 1993). Our previous study (Kang and Schuman 1995b) demonstrated that the receptor tyrosine kinase inhibitor K252a blocked the synaptic potentiation produced by BDNF. However, K252a has also been reported to block other classes of protein kinases (Kase *et al.* 1987). To ascertain specifically the role of TrkB, we examined whether pretreatment of hippocampal slices with a function-blocking TrkB antibody can prevent BDNF-induced potentiation.

MATERIALS AND METHODS

Slice preparation, electrophysiology and analysis.

Hippocampal slices were prepared using a Stoelting tissue chopper from young (6-8 weeks) adult male Sprague-Dawley rats. Before electrophysiological recording, slices were stored for at least 1.5 hr on a Millipore membrane (#1) placed over a tissue culture dish containing oxygenated Ringer's solution. The top of the slice was exposed to 95% O_2, 5% CO_2 circulating in an enclosed chamber. For electrophysiological recordings, slices were submerged in a stream of ACSF (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11.0 mM glucose) maintained at room temperature (22-25°C) and gassed with 95% O_2, 5% CO_2. In different sets of experiments, the solution flow rate was varied between 25 and 250 ml/hour. Field excitatory postsynaptic potentials (EPSPs) measured in stratum radiatum, were evoked by stimulation of the Schaffer collateral-commissural afferents (1 stimulation every 15 sec); the initial slope was measured. The maximal potentiation, time to a 25% increase, and slope of potentiation onset were calculated for each individual experiment, relative to baseline measurements before BDNF application. The slope of potentiation onset was calculated for each experiment by linear regression analysis of all data points between the time of BDNF application and the maximal potentiation. To assess statistical significance, paired t-tests, comparing the average slope size for 10 min before BDNF to either 50-60 or 170-180 min after BDNF application, were performed on nonnormalized data. In addition, a one-way analysis of variance (ANOVA) was conducted between perfusion groups for the maximum potentiation, time to 25% increase and the slope of

potentiation onset. [Significance levels are as stated in the text; P values > 0.05 are designated as not significant, (ns)].

Blocking antibody experiments.

The function blocking TrkB antibody, which blocks BDNF-induced phosphorylation of TrkB receptors in 3T3 cells, was kindly provided by Andy Welcher (Amgen). Before electrophysiological recording, slices were incubated individually for 2-3 hr. in single wells of a 24-well tissue culture plate in 200 µl of Ringer's solution containing either a TrkB antibody (1:10) or an equivalent dilution of pre-immune serum. The culture plate was placed in an oxygenated chamber. Slices were then transferred to a recording chamber, submerged, and perfused with normal Ringer's solution for ~30 min before the addition of BDNF.

Neurotrophin application and handling.

Great care was taken in the application and storage of the BDNF. BDNF (generous gift from Amgen) was kept at 4°C. Phosphate buffer stock solutions were made every 1-3 days and kept at 4°C. New supplies of BDNF were obtained on a regular basis (every 2-4 months) as it was occasionally observed that individual stocks became less potent over time. The neurotrophins were added directly to the perfusion ACSF. The perfusion apparatus was modified to include chemically inert materials- silicon tubing (OD 0.183 inches; ID 0.132 inches) and a Teflon beaker.

Immunocytochemistry and confocal microscopy.

Following electrophysiology and BDNF perfusion, slices were fixed in 4.0% paraformaldehyde, 0.2% glutaraldehyde for 4 hr and then transferred to PBS. Individual slices were then cut into 50 μM sections, permeabilized with 0.7% Triton X-100 for 1 hr, and incubated in primary antibody [1:100 in 5% rabbit serum in h-PBS + 0.5 % Triton X-100 (preblock)] overnight at 4°C. Sections were washed in preblock solution and then incubated in secondary antibody for 1 hr at room temperature (1:100 in preblock solution) and then rinsed and mounted on glass slides. The primary antibody used was a chicken anti-human BDNF (Promega); the secondary antibody was a fluorescein-conjugated rabbit anti-chicken (Jackson Labs). For immunostaining for function-blocking TrkB antibody, slices were fixed and sectioned as indicated above at 0, 30, or 60 min following perfusion with normal Ringer's solution in the electrophysiology recording chamber. The protocol for immunostaining with the secondary antibody was the same as above. Following immunostaining, confocal images were obtained with a Zeiss 310 confocal laser-scanning microscope. The 488 nm line of an argon-ion laser attenuated to 1/10 of the maximal intensity with a neutral density filter was used to excite fluorescein. Images were visualized with either a 10X objective (Plan-NeoFluor, n.a. 0.30, Zeiss) or a 63X oil immersion objective (Plan-Apochromat, n.a. 1.40 Zeiss). Brightness and contrast settings were kept constant for all images taken within a given set of experiments. For BDNF visualization the following settings were used: contrast, 420; brightness, 9800; pinhole, 40. For TrkB antibody staining the following settings were used: contrast, 320; brightness, 9800; pinhole, 40.

RESULTS

Function-blocking Trk antibodies.

The dependence of BDNF-induced potentiation on TrkB function was assessed using function-blocking TrkB antibodies. We confirmed the penetration of the TrkB antibody into the depth of the slice using fluorescent secondary antibodies and confocal microscopy. After one hr of washing slices with antibody-free Ringer's solution there was still a substantial amount of TrkB antibody detected throughout the slice (Fig. 1A,B). Slices exposed to the TrkB antibody before BDNF treatment (50 ng/ml- 165 ml/hr) exhibited greatly diminished BDNF-induced potentiation relative to control slices incubated in pre-immune serum [mean percent of baseline ± S.E.M.: TrkB: 113.3 ± 6.3 (n =9); control: 181.4 ± 7.9 (n=9)] (Fig. 1C). Slices pre-treated with the TrkB antibody failed to exhibit significant potentiation, whereas the BDNF-induced enhancement in control slices was statistically significant ($P < 0.001$). These results indicate that BDNF requires functional TrkB receptors to potentiate synaptic transmission in the hippocampal slice.

Rate of BDNF delivery: effects on synaptic plasticity.

The ability of BDNF to potentiate synaptic transmission was examined at six different perfusion rates: 25, 50, 80-95, 110-140, 160-180, and 220-250 ml/hr. In individual experiments, the rate of perfusion was constant and was not changed before, during, or after the application of BDNF. Control experiments, in which no BDNF was applied, were also conducted for each perfusion rate. For each experiment, three

variables were analyzed: the maximum potentiation achieved following BDNF application, the time to a 25% increase in synaptic strength, and the slope of potentiation onset. The onset slope was calculated for all data points beginning with the initial BDNF application and ending with the maximum potentiation value for a given experiment.

There was a significant effect of the perfusion rate on all of the indices of BDNF-induced enhancement examined. Ensemble averages of experiments conducted at four different perfusion rates and their associated controls are shown in Figure 2. At slowest flow rate examined, 25 ml/hr, BDNF did not produce a consistent enhancement of synaptic transmission (Fig. 2A). A modest potentiation of synaptic transmission was often observed in experiments with perfusion rates of 50 (Fig. 2B) and 80 ml/hr; the enhancement for these groups, however, failed to reach statistical significance, likely due to the small sample size. At the 110-140 (Fig. 2C), 160-180 (Fig. 2D), and 220-240 perfusion rates, BDNF induced a significant increase in synaptic strength ($P < 0.05$ for all groups). In comparison, control experiments conducted at identical flow rates did not show any significant enhancement (Fig. 2E-H). Only 1 of 13 control experiments (conducted at different perfusion rates) exhibited an increase of synaptic transmission > 10% [mean percent of baseline (all controls) at 80-90 min: 96.0 ± 2.5%].

As shown in Figure 3, increasing the perfusion rate increased both the onset kinetics and the maximum potentiation produced by BDNF. The maximum potentiation (Fig. 3A) increased as a function of perfusion rate; the enhancement was the smallest (nonsignificant) for the slowest (25 ml/hr) flow rate and largest for the fastest (220-240 ml/hr) flow rate (mean percent of baseline: 25 ml/hr: 114.0 ± 4.8%; 220-240 ml/hr: 281.2

± 32.8%). Intermediate perfusion rates (e.g., 50-140 ml/hr) yielded BDNF-induced potentiation values between those observed for the slowest and fastest rates. An ANOVA comparing the maximum potentiation between groups indicated a significant effect of perfusion rate ($P < 0.01$).

The time to a 25% increase in synaptic strength (Fig. 3B) decreased as a function of perfusion rate. None of the experiments conducted at 25 ml/hr exhibited a 25% increase in synaptic strength; for graphical purposes each of these experiments is assigned a maximum value of 180 minutes in Figure 3B. As the perfusion rate increased across groups, the time required to achieve a 25% enhancement of synaptic strength decreased; for the slowest group (50 ml/hr) that exhibited a 25% increase the mean time was 80.0 ± 27.0 min and for the fastest (220-240 ml/hr) group the mean time was 12.2 ± 2.1 min. Increasing the flow rate also decreased the variability between individual experiments within a given group, perhaps reflecting a more reliable delivery of BDNF at faster perfusion rates. An ANOVA comparing the time to 25% increase between the groups indicated a significant effect of perfusion rate ($P < 0.01$).

The slope of the potentiation onset was also affected by the perfusion rate: The slower the perfusion, the slower the onset of the synaptic enhancement. The mean slope value (mean percent of baseline / min) for the slowest perfusion group (25 ml/hr) was 0.08 ± 0.03 and for the fastest (220-240 ml/hr) perfusion group was 2.98 ± 0.97. An ANOVA comparing the onset slope between groups indicated a significant effect of perfusion rate ($P < 0.01$). These data indicate that the rate at which BDNF is introduced into the slice influences how quickly synaptic potentiation reaches its maximal value.

BDNF slice penetration: immunostaining and confocal microscopy.

Do the observed effects of perfusion rate on BDNF-induced synaptic enhancement reflect differences in the ability of BDNF to penetrate the slice? To address this issue, we examined the penetration of BDNF into hippocampal slices as a function of the perfusion rate using immunocytochemistry and confocal microscopy. The first issue we addressed is whether the perfusion rate also influences significantly the rate at which BDNF is introduced to the slice. This was explored by examining BDNF immunostaining at different time points following its addition using either the slowest (25 ml/hr) or the fastest (220 ml/hr) perfusion rates used in the electrophysiology studies. We focused our analysis on sections 150-300 μm into the depth of the slice. As shown in Fig. 4A and D, there was a small amount of endogenous BDNF detected in control slices placed in the recording chamber but not exposed to exogenous BDNF. The perfusion rate significantly affected the kinetics of BDNF penetration into the slice at all time points examined. In slices fixed 15 min after the addition of BDNF, there was a clear increase in the amount of BDNF in the slice perfused at the fast flow rate (Fig. 4E), whereas there was no obvious increase in BDNF detected in the slice perfused at the slow rate (Fig. 4B). This observation is consistent with the increase in synaptic strength evident within 15 min in the fast flow rate electrophysiology experiments (e.g., Fig. 2D; Kang and Schuman 1995b) and the absence of such an increase in the slow rate electrophysiology experiments (Fig. 2A). Slices fixed 45 min after BDNF addition (15 min into the washout period) showed no further increase in immunoreactivity for the fast flow rate (Fig. 4E vs. F) and, perhaps, a very small increase for the slower flow rate (Fig. 4B vs.

C). Therefore, increasing the perfusion rate increases the rate at which BDNF penetrates the interior of the slice.

In the second set of experiments, all slices which had been exposed previously to BDNF were fixed at the termination of electrophysiological recording. Confocal images were taken of slices perfused at several different rates. Slices treated with a fluorescent secondary antibody in the absence of a primary antibody showed negligible background staining (Fig. 5A). (Note that the bright horizontally oriented blobs, evident in Fig. 5 as well as Fig. 4, are blood vessels which react nonspecifically with the secondary antibody.) As observed above, there was a small amount of endogenous BDNF detected in control slices placed in the recording chamber but not exposed to exogenous BDNF (cf. Fig. 5A and B). Control experiments revealed that the level of endogenous BDNF observed over time did not appear to change as a function of perfusion rate (data not shown). As shown in Figure 5C-F, there was a dramatic effect of flow rate on the incorporation of exogenous BDNF into the slice. Increasing the perfusion rate increased the absolute quantity of BDNF which penetrated the tissue successfully. This is indicated by the increase in the punctate clusters of staining as well as the increase in the overall number of white pixels evident in the background across the different panels.

DISCUSSION

The electrophysiological data shown above indicate that functional TrkB receptors are required for BDNF-induced synaptic potentiation. In addition, our examination of several different perfusion rates indicates that the rate of BDNF delivery is an important determinant of both the magnitude and the kinetics of BDNF-induced synaptic potentiation. At the slowest perfusion rate examined, no significant synaptic potentiation was observed. Increasing the rate of perfusion to higher rates, however, resulted in a BDNF-induced potentiation whose magnitude and steepness of onset was related significantly to the speed of application.

Our immunohistochemical data indicate that the perfusion rate potently influences the amount of exogenous BDNF detected in the slice. These data suggest that faster perfusion rates increase the levels of BDNF that reach the slice interior. Given the volume of our recording chamber, we estimate that BDNF reaches an equilibrium concentration in the chamber in ~1 min and 10-15 min, respectively, for the fast and slow perfusion rates. If this initial difference accounts for the observed difference in BDNF penetration, then we would expect that the levels of BDNF observed at 45 min at 25 ml/hr (Fig. 4C) should be at least as high as those observed at 15 min at 220 ml/hr (Fig. 4E). Clearly, this is not the case. Therefore, the flow rate itself appears to influence the ease with which BDNF penetrates the slice.

The flow rate also influences the maximal amount of BDNF that enters the tissue. Given that BDNF is only applied for 30 min, this observation may be explained by the fact that BDNF enters the tissue more quickly at faster perfusion rates. The data shown

in Figure 5 were taken ~2 hr after BDNF washout. It is important to note that the immunostaining procedure we used involves membrane permeabilization before treatment with the primary antibody to BDNF. As such, we cannot distinguish between extracellularly versus intracellularly localized BDNF in these experiments. Moreover, we also cannot rule out the possibility that exogenous BDNF can increase the synthesis of endogenous BDNF. Can the persistence of BDNF staining in Figure 5 account for the persistence of the BDNF-induced potentiation? Our earlier experiments suggest that this is not the case. The application of the receptor tyrosine kinase inhibitor K252a after the initiation of BDNF-induced enhancement did not result in a return to basal levels of synaptic transmission, suggesting that new BDNF-Trk interactions are not responsible for the maintenance phase of the synaptic enhancement. We are currently addressing this issue biochemically by monitoring the extent of BDNF-induced TrkB phosphorylation during early and late aspects of the synaptic enhancement.

Does the maximal values of BDNF delivered to the tissue determine whether potentiation will occur or is it the rate itself that is important? At the slowest flow rate examined here (25 ml/hr) we did not observe any significant potentiation of synaptic transmission, although our confocal studies indicate that some BDNF penetrated the slice at this slow flow rate (Fig. 4). We observed that amount of BDNF immunostaining observed in these slices at later time points (data not shown) was sometimes within the range of levels observed at other flow rates that were effective in enhancing synaptic strength. This suggests that the rate at which BDNF is presented to the synaptic regions is perhaps more important than the absolute concentration in determining whether

synaptic transmission will be enhanced.

The effects of BDNF on synaptic transmission and plasticity have been examined by several other groups. In cell cultures studies of nerve-muscle and hippocampal synapses (Lessmann *et al.*, 1994; Levine *et al.*, 1995; Lohof *et al.*, 1993), BDNF has been shown to potentiate synaptic transmission within minutes of its application. Clearly, BDNF's access to the relevant synaptic sites is not a problem for culture studies which use cell monolayers to study synaptic transmission. This may explain why rapid effects have been readily observed in cell culture.

Two recent studies have shown that both homozygous and heterozygous BDNF knockout mice exhibit compromised long-term potentiation (LTP) (Korte *et al.* 1995; Patterson *et al.* 1996). Patterson *et al.* (1996) attempted to rescue the deficit in both basal synaptic transmission and LTP by treating slices from mutant mice with BDNF. They reported that prolonged treatments with BDNF (5- 8 hr) were required to rescue LTP in slices from the knockout mice and noted that these BDNF treatments did not enhance synaptic transmission. Another recently published study (Figurov *et al.* 1996) has reported that BDNF application facilitates the induction of LTP in young animals, but does not enhance synaptic transmission independently. In the latter study, 2.5 to 3.0 hr BDNF incubations were required to produce an effect on LTP.

There are two important variables worth noting. Both of these studies used interface type chambers in which only one surface of the slice was exposed directly to the BDNF-containing solution. The perfusion rates used were very slow (10-25 ml/hr). Immunocytochemical analysis in one of these studies (Patterson *et al.* 1996) indicated

that BDNF did not penetrate the tissue substantially until after several hours of incubation. The combination of the interface chamber and the slow perfusion rate clearly can reduce dramatically the rate of BDNF delivery relative to the fast perfusion rate conditions described here and in our previous study (Kang and Schuman 1995b). Moreover, the parametric manipulation of perfusion rate described in this paper further highlights the importance of the rate of BDNF delivery in determining whether synaptic potentiation will result. As indicated above, the slowest flow rate we examined (25 ml/hr), perhaps most similar to the slow delivery conditions employed by others, did not produce any potentiation of synaptic transmission, although our confocal studies indicated that a small amount BDNF penetrated the slice at this slow flow rate (Fig. 4C). In contrast, we showed that at a fast flow rate (210 ml/hr) a substantial quantity of BDNF reaches the interior of the slice within 15 min (Fig. 4E). This underscores again the importance of rate of BDNF delivery as a key variable in determining whether synaptic potentiation will result.

What is the molecular underpinning of the demonstrated dependence of the BDNF-induced potentiation on perfusion rate? BDNF application to cultured hippocampal cells (Berninger *et al.* 1993) and nerve-muscle cultures (Stoop and Poo 1996) has been shown to dramatically increase intracellular Ca^{2+}. Our own studies indicate that Ca^{2+} influx through voltage-gated Ca^{2+} channels and intracellular Ca^{2+} stores is obligatory for the induction of the BDNF-induced potentiation (Kang and Schuman 1995a). It is therefore possible that a threshold concentration of intracellular Ca^{2+} must be reached to initiate the neurotrophin-induced enhancement. Moreover, the magnitude of the Ca^{2+} increase may

determine the degree to which Ca^{2+} activates downstream effectors, and consequently influence the overall magnitude of the synaptic potentiation. Therefore, small quantities of BDNF that reach the synapse slowly may bind available TrkB receptors but fail to raise Ca^{2+} to sufficient levels to initiate synaptic enhancement. Trk receptors are known to be internalized following the binding of a neurotrophin (Hendry *et al.* 1974). As such, an additional effect of slow BDNF delivery may be to decrease the availability of TrkB receptors for subsequent BDNF that reaches the synapse. Taken together, it appears imperative that future attempts to observe BDNF-induced potentiation confirm that the experimental methods employed achieve a rapid (e.g., ≤30 min) and substantial delivery of BDNF to the depths of the slice preparation.

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FIGURES AND LEGENDS

Figure 1: Function-blocking TrkB antibodies penetrate hippocampal slices and prevent BDNF-induced synaptic potentiation. *(A)* A 63X confocal image to secondary antibody in the absence of primary antibody. Bar, 25 μm. *(B)* A 63 X confocal image of the CA1 region from a slice exposed to a function-blocking TrkB antibody for 2 hr and then perfused with normal Ringer's solution in the recording chamber for 60 min. Positive antibody staining is indicated by the color white. This image was taken from a section 150 μm from the surface of the slice. *(C)* Ensemble averages for experiments in which slices were preincubated in either a function-blocking TrkB antibody or an equivalent dilution of preimmune serum before electrophysiological recording. BDNF (50 ng/ml) was applied for time indicated by the solid bar. In the TrkB antibody treated slices the mean field EPSP slope before BDNF was 0.11 ± 0.01 mV/msec (Mean ± SEM) and 0.12 ± 0.01 mV/msec 50-60 min after BDNF. In the preimmune serum-treated slices the mean field EPSP slope before BDNF was 0.11 ± 0.01 mV/msec and 0.19 ± 0.03 mV/msec after BDNF. Two superimposed representative field EPSPs are shown for each group, 5 min before and 60 min after BDNF application. Calibration bar, 1 mV, 20 msec.

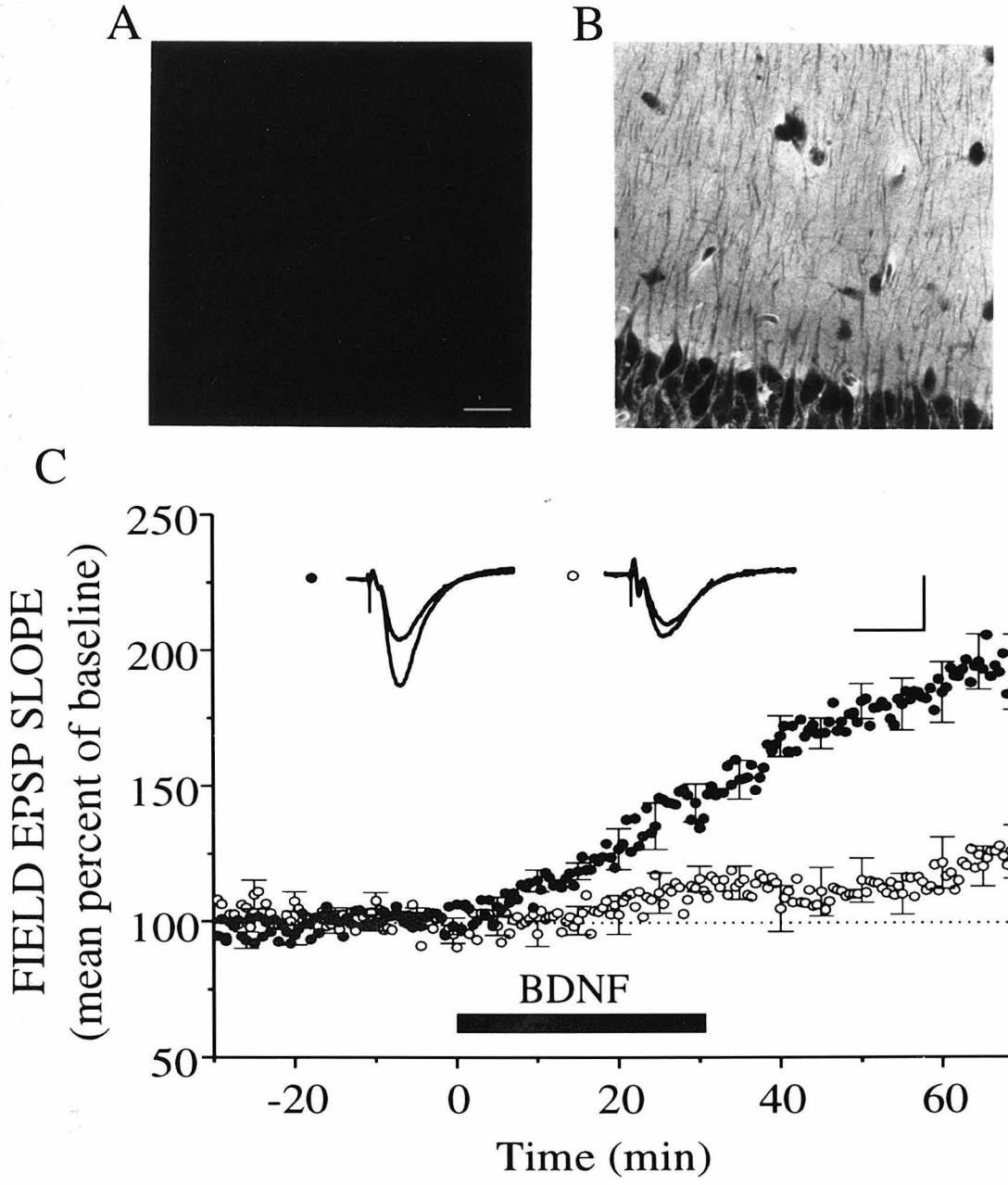

A
B
C
FIELD EPSP SLOPE
(mean percent of baseline)
250
200
150
100
50
BDNF
-20
0
20
40
60
Time (min)

Figure 2: The influence of rate of perfusion on BDNF-induced synaptic potentiation. Ensemble averages for sets of experiments conducted at different perfusion rates are shown. *(A-D)* Ensemble averages for experiments in which BDNF (50 ng/ml) was applied for 30 min, as indicated by the solid bar. *(E-H)* Control experiments conducted at identical perfusion rates, in which a sham solution addition of PBS was performed in lieu of BDNF addition. Perfusion rates are indicated in the upper right-hand corner of each graph. The sample sizes for the graphs shown in *A-H* are as follows: n = 4, 5, 4, 6, 3, 3, 4, and 3, respectively. In all panels two superimposed representative field EPSPs are shown, 5 min before and 160 min after BDNF application. EPSPs shown in control experiments are taken from equivalent time points. Calibration bar, 1 mV, 20 msec.

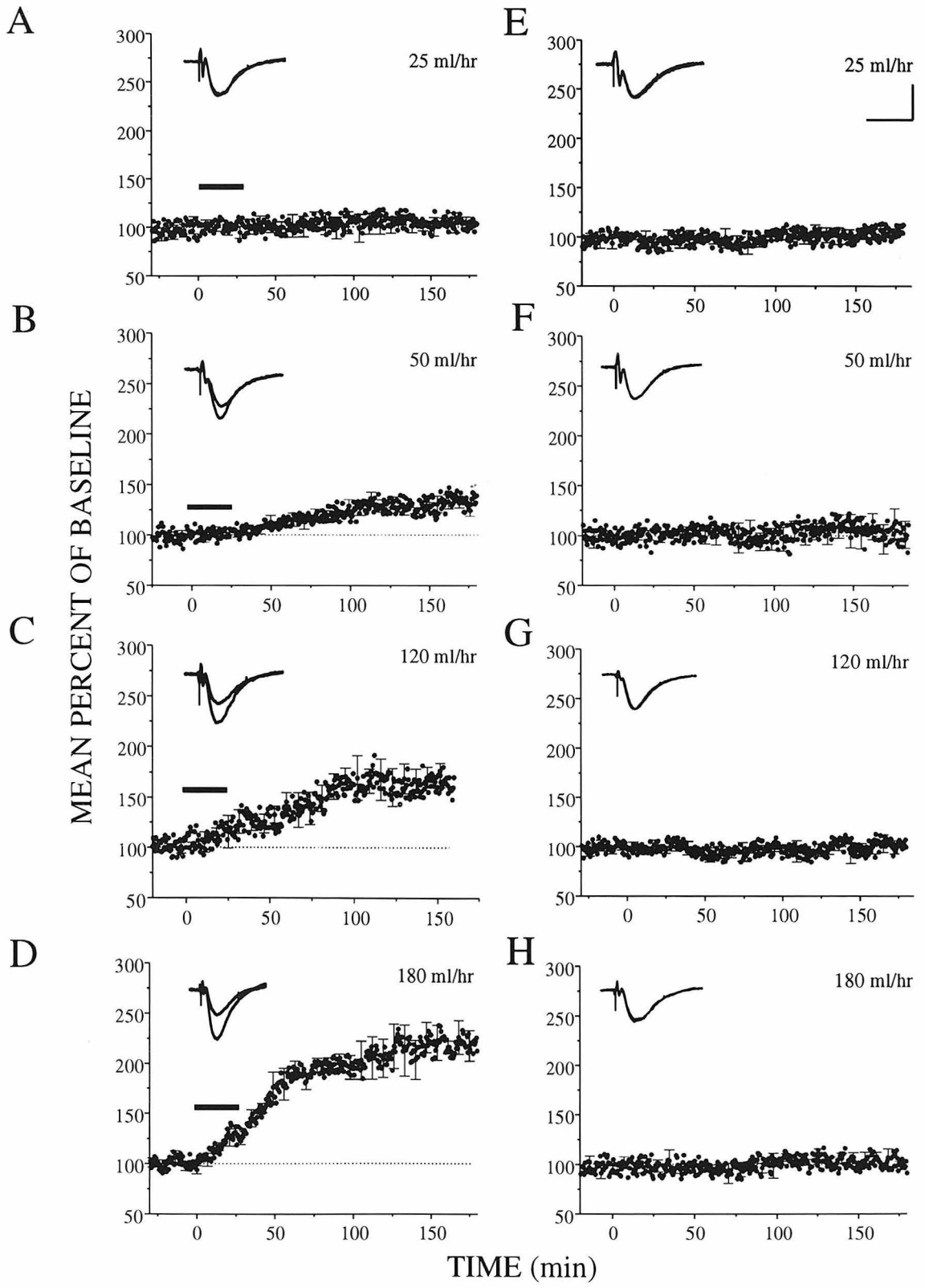
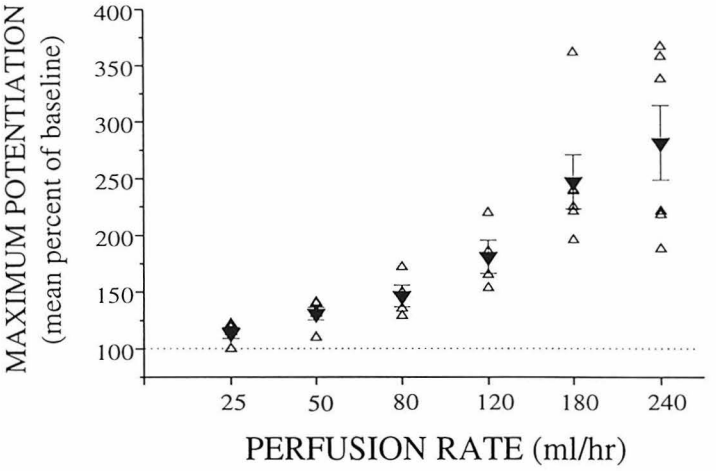
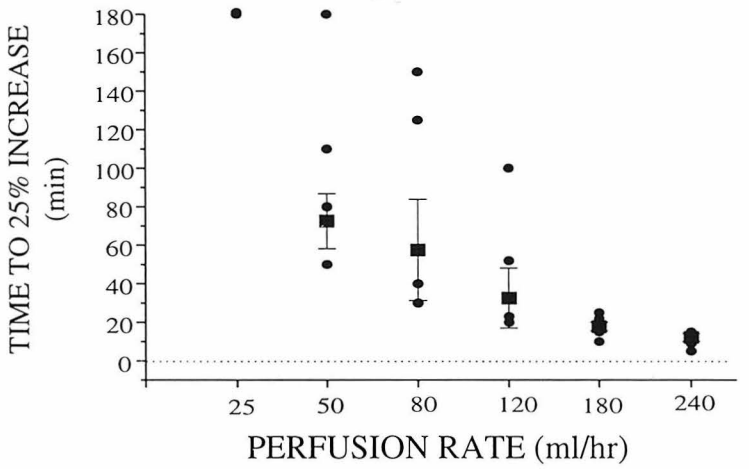

A
B
C
D
E
F
G
H
25 ml/hr
50 ml/hr
120 ml/hr
180 ml/hr
MEAN PERCENT OF BASELINE
TIME (min)
300
250
200
150
100
50
0
50
100
150

Figure 3: The perfusion rate affects the maximum potentiation, time to 25% increase, and slope of potentiation onset. Shown are scatter plots of individual experiments conducted at different perfusion rates. For each perfusion rate the mean ± SEM is indicated by the large symbol with the error bars. *(A)* Plot depicting the relationship between maximum potentiation and perfusion rate. *(B)* Plot depicting the relationship between time to 25% increase and perfusion rate. *(C)* Plot depicting the relationship between the potentiation onset slope (see Materials and Methods) and perfusion rate.

A


MAXIMUM POTENTIATION
(mean percent of baseline)
400
350
300
250
200
150
100
25
50
80
120
180
240
PERFUSION RATE (ml/hr)

B


TIME TO 25% INCREASE
(min)
180
160
140
120
100
80
60
40
20
0
25
50
80
120
180
240
PERFUSION RATE (ml/hr)

C

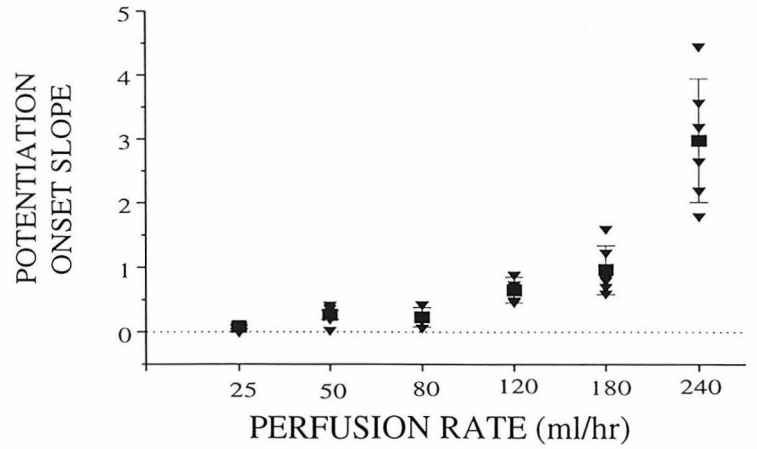

POTENTIATION
ONSET SLOPE
5
4
3
2
1
0
25
50
80
120
180
240
PERFUSION RATE (ml/hr)

Figure 4: Fast perfusion rates increase the rate of BDNF penetration into hippocampal slices. Slices exposed to a slow (25 ml/hr) or fast (220 ml/hr) flow rate were examined for BDNF immunoreactivity before BDNF addition (endogenous BDNF staining only), and 15 min or 45 min after BDNF addition. Each panel shows a 63 X confocal image of the CA1 region of a hippocampal slice stained with an antibody to BDNF. The cell bodies (stratum pyrimidale) are oriented horizontally across the bottom of each image with the apical dendrites of the CA1 neurons extending up, vertically in the stratum radiatum. The images shown are on a black-white scale, where black indicates absence of immunoreactivity and changes from black (e.g., gray to white) indicate the presence of immunoreactivity. Thus, BDNF immunoreactivity is indicated by both the brightest spots and the overall brightness of the image. *(A-C)* BDNF immunoreactivity in slices perfused at 25 ml/hr. *(A)* Control immunostaining for endogenous BDNF, staining is most evident in the stratum radiatum. Bar, 25 μm. *(B)* Immunostaining in a slice exposed to BDNF for 15 min. *(C)* Immunostaining in a slice exposed to BDNF for 45 min. *(D-F)* BDNF immunoreactivity in slices perfused at 220 ml/hr. *(D)* Control immunostaining for endogenous BDNF. *(E)* Immunostaining in a slice exposed to BDNF for 15 min. *(F)* Immunostaining in a slice exposed to BDNF for 45 min.

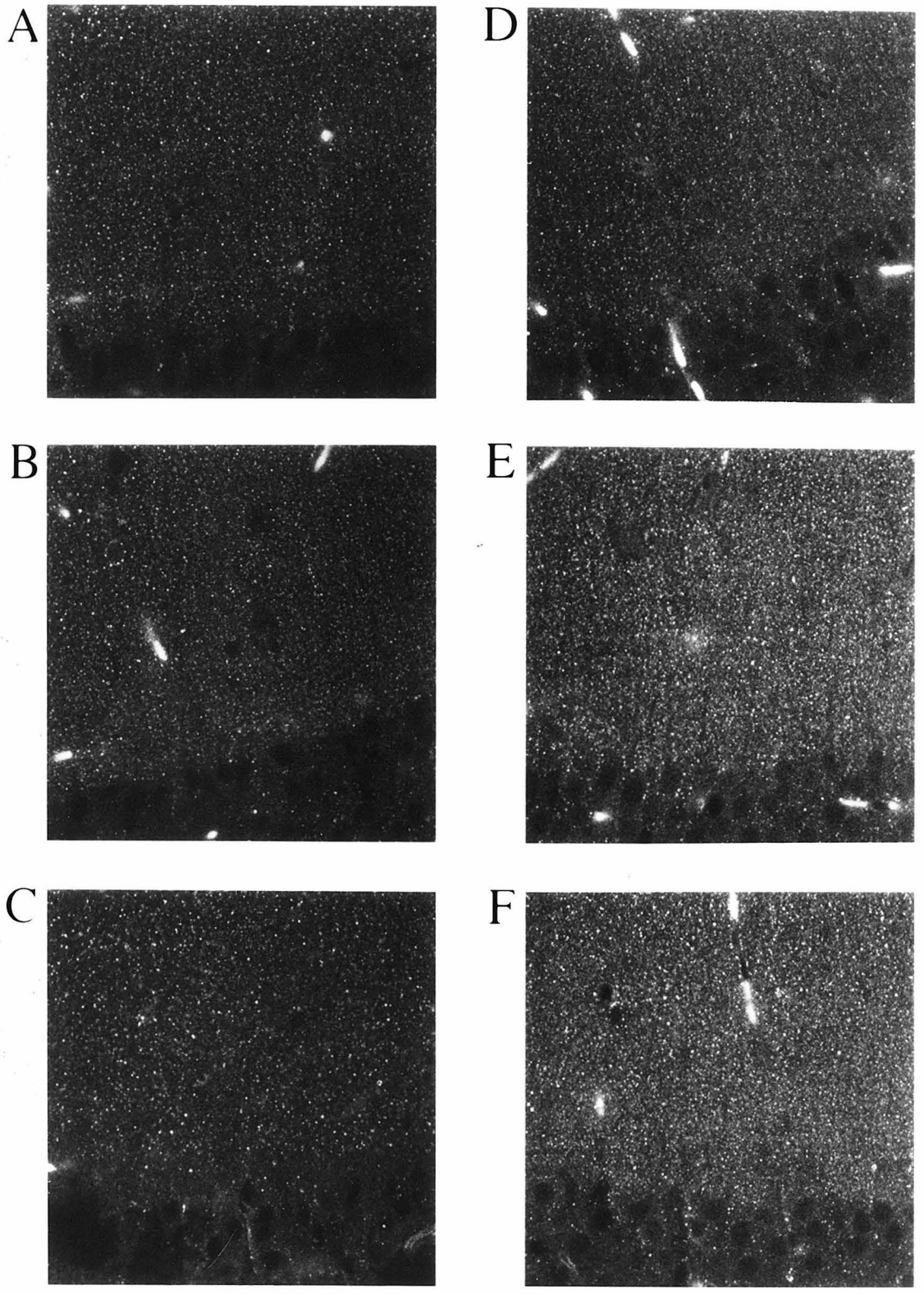
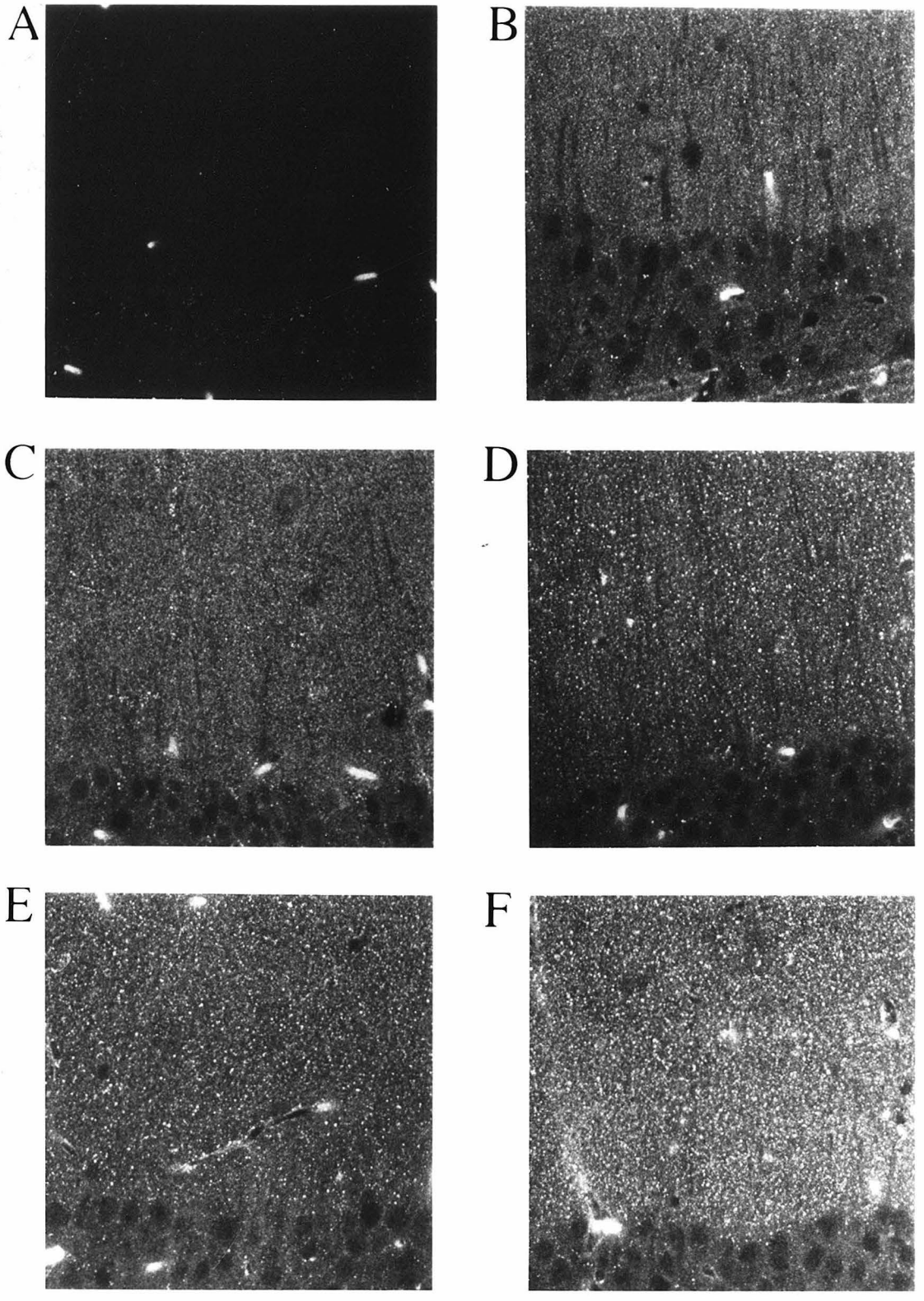

A
D
B
E
C
F

Figure 5: Faster perfusion rates increase the absolute penetration of BDNF into hippocampal slices. Images are from slices used in electrophysiological experiments exposed to BDNF for 30 min at the perfusion rates indicated below. All slices shown here were fixed 170 min following BDNF application. *(A)* Control experiment in which the slice was not exposed to primary antibody but was exposed to the secondary antibody. Bar, 25 μm. *(B)* Control immunostaining for endogenous BDNF in a slice, staining is most evident in the stratum. *(C)* Slice exposed to BDNF at a perfusion rate of 50 ml/hr. A small increase in the magnitude of staining over control *(B)* is evident, particularly in the dendritic region. This slice exhibited a maximum potentiation of 140.0% of baseline following exposure to BDNF. *(D)* Slice exposed to BDNF at a perfusion rate of 90 ml/hr. A clear increase in staining relative to the previous panels is evident. This slice exhibited a maximum potentiation of 156.0% of baseline following exposure to BDNF. *(E)* Slice exposed to BDNF at a perfusion rate of 145 ml/hr. This slice exhibited a maximum potentiation of 165.0% of baseline following exposure to BDNF. *(F)* Slice exposed to BDNF at a perfusion rate of 210 ml/hr. Note that this slice exhibits the most abundant staining for BDNF. This slice exhibited a maximum potentiation of 360.0% of baseline following exposure to BDNF.


A
B
C
D
E
F

CHAPTER 4

A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity

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A Requirement for Local Protein Synthesis in Neurotrophin-induced Hippocampal Synaptic Plasticity

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ABSTRACT

Two neurotrophic factors, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), are able to produce a long-lasting enhancement of synaptic transmission in the hippocampus. Unlike other forms of plasticity, neurotrophin-induced plasticity exhibited an immediate requirement for protein synthesis. Plasticity in rat hippocampal slices in which the synaptic neuropil was isolated from the principal cell bodies also required early protein synthesis. Thus, the neurotrophins may stimulate the synthesis of proteins in either axonal or dendritic compartments, allowing synapses to exert local control over the complement of proteins expressed at individual synaptic sites.

The cellular changes that underlie both synaptic and behavioral plasticity are usually classified as either (I) short-term because they are based on the modification of preexisting proteins, or (ii) long-term because they require protein synthesis. For example, studies of synaptic plasticity in the hippocampus and in *Aplysia* have shown that whereas the short-term phase (0 to 1 hour) of synaptic enhancement is not blocked by inhibitors of protein translation, the long-term phase (> 1 hour) is [(1), but see (2)]. These cellular studies are paralleled by many studies of behavioral plasticity that also indicate that short-term memories are insensitive to inhibitors of protein synthesis (3). Two neurotrophic factors BDNF and NT-3 can enhance synaptic efficacy (4), and we have examined the temporal sensitivity of the neurotrophin-induced synaptic enhancement to inhibitors of protein synthesis.

Synaptic transmission was examined at the Schaffer collateral-CA1 pyramidal neuron synapse in the adult rat hippocampal slice with the use of conventional extracellular recording techniques (5). In control experiments, extracellular application of BDNF (50 ng/ml) or NT-3 (50 ng/ml) elicited a robust enhancement of synaptic transmission (Fig. 1, A and B) (4) [mean percent of baseline: BDNF, 221.4 ± 16.4 (mean ± SEM, n = 7), $P < 0.005$; NT-3, 231.1 ± 19.5 (n = 8), $P < 0.005$]. Pretreatment with one of two protein synthesis inhibitors (6), either anisomycin (40 μM) or cycloheximide (40 μM), markedly attenuated the synaptic enhancement induced by either neurotrophin (Fig. 1, C through F and H) [mean percent of baseline: BDNF plus anisomycin, 134.2 ± 8.4 (n = 9), $P < 0.05$, BDNF plus cycloheximide, 138.7 ± 13.2 (n = 7), $P < 0.05$; NT-3, plus anisomycin, 130.1 ± 7.6 (n =9), $P < 0.05$, NT-3 plus cycloheximide, 118.5 ± 14.0 (n = 7),

not significant (NS)]. In contrast to previous studies of synaptic plasticity, the sensitivity to inhibitors of protein synthesis was evident within minutes of neurotrophin application (Fig. 1, C through F). Similar pretreatment of hippocampal slices with an inhibitor of prokaryotic protein synthesis, chloramphenicol (80 μM), did not significantly reduce the synaptic enhancement induced by either BDNF or NT-3 (Fig. 1H) [mean percent of baseline: BDNF plud chloramphenicol, 216.2 ± 6.7 ($n = 5$), $P < 0.005$; NT-3 plus chloramphenicol, 250.8 ± 32.6 ($n = 5$), $P < 0.005$].

This early requirement for new protein synthesis is temporally inconsistent with the time necessary for somati synthesis and transport of proteins to synaptic sites in pyramidal neurons (7). A potential source of protein synthesis closer to synaptic sites has been described in hippocampal pyramidal cells (8). To investigate whether the the early requirement for protein synthesis resulted from synthesis independent of somatic protein translation machinery, we isolated the synaptic regions from either CA3 (Fig. 2, A and B) or CA1 (Fig.3, A and B) cell bodies with the use of a microlesion (9). Previous studies have indicated that synaptic transmission and short-term plasticity can be recorded in similarly lesioned hippocampal slices (10). To control for general damage associated with the lesion, we conducted experiments in which a sham lesion was made in the dentate gyrus region of the hippocampal slice (9). The extracellular application of BDNF or NT-3 potentiated synaptic transmission in the sham-lesioned slices to a similar extent to that observed in unlesioned slices (Fig. 2, C and D)[mean percent of baseline: BDNF, 237.6 ± 35.5 ($n = 7$), $P < 0.005$; NT-3, 216.3 ± 27.0 ($n = 6$), $P < 0.01$].

To address the possibility that the protein synthesis inhibitor-sensitive compartment

resides in the presynaptic cell bodies, we isolated the CA3 cell bodies from the stratum radiatum (Fig. 2, A and B). In CA3-isolated slices, both neurotrophins continued to enhance synaptic strength (Fig. 2, C and D) [mean percent of baseline: BDNF, 230.7 ± 26.2 (n = 7), $P < 0.001$; NT-3, 211.33 ± 22.3 (n = 6), $P < 0.005$]. In CA3-isolated slices pretreated with anisomycin, however, the enhancement produced by BDNF or NT-3 was markedly reduced (Fig. 2, C and D)[mean percent of baseline: BDNF plus anisomycin, 109.5 ± 8.3 (n = 5), NS; NT-3 plus anisomycin, 115.9 ± 15.7 (n = 5), NS]. This continued sensitivity to a protein synthesis inhibitor in the absence of CA3 somata indicated that the relevant protein translation machinery did not reside in the presynaptic cell bodies.

We next addressed the potential contribution of postsynaptic somatic protein synthesis by dissociating CA1 somata from the synaptic region in the stratum radiatum. The CA1 lesion was placed just beyond the apical boundary of the pyramidal cell layer (Fig. 3, A and B). We confirmed the complete dissociation of CA1 cell bodies from their dendrites by cresyl violet staining (Fig. 3B) and by placing a recording electrode in the cell body region and attempting to record a synaptic response to stimulation of the Schaffer collaterals. In no such case did we see any synaptic response. The remaining isolated neuronal cell bodies present in the synaptic neuropil were likely interneurons, which have been shown with immunocytochemistry and in situ hybridization to contain glutamic acid decarboxylase (11).

Excitatory synaptic responses recorded in slices with isolated CA1 somata still exhibited significant potentiation on exposure to either BDNF or NT-3 (Fig. 3, C and D)

[mean percent of baseline: BDNF, 182.1 ± 12.7 (n = 8), P < 0.005; NT-3, 182.3 ± 9.5, (n =6) P < 0.005]. The enhancement observed in these slices, however,was slightly, but significantly, smaller than that observed in sham-lesioned slices (P <0.05). Preexposure of the CA1-isolated slices to anisomycin again attenuated the enhancement induced by either BDNF or NT-3 (Fig. 3, C and D)[mean percent of baseline: BDNF plus anisomycin, 119.7 ± 8.0 (n = 6), NS; NT-3 plus anisomycin, 119.6 ± 9.5 (n = 6), NS].

The results of the CA3 and CA1 isolation experiments indicate that neurotrophin-induced synaptic potentiation required protein synthesis at sites distinct from the pyramidal neuron cell bodies (12). To rule out the possibility that the site of protein synthesis changed systematically as a result of the lesioned area, we simultaneously isolated both pre- and postsynaptic cell bodies (Fig. 4A). In such slices, application of BDNF or NT-3 enhanced synaptic strength (Fig. 4, B and C) [mean percent of baseline: BDNF, 193.5 ± 6.7 (n = 8), P < 0.001; NT-3, 192.8 ± 12.4 (n = 6), P < 0.001]. Moreover, pretreatment of slices with anisomycin prevented the neurotrophin-induced enhancement (Fig. 4, B and C) [mean percent of baseline: BDNF plus anisomycin, 122.0 ± 7.0, (n = 6) NS; NT-3 plus anisomycin, 116.4 ± 9.1 (n = 5), NS]. The persistence of anisomycin sensitivity in slices isolated from both pre- and postsynaptic cell bodies indicates that the neurotrophins made use of a protein synthesis machinery that resides in the synaptic neuropil of the hippocampal slice.

The presumptive local site of protein synthesis could reside in axons or dendrites of pyramidal neurons, or in neighboring interneurons or glia. Although hippocampal interneurons may express Trk receptors (13), the synaptic enhancement induced by the

neurotrophins does not require inhibitory transmission mediated by γ-aminobutyric acid type A ($GABA_A$) receptors (14). Thus, proteins synthesized in interneurons would presumably have to influence excitatory synaptic transmission by non-$GABA_A$ receptor-mediated diffusible signalling. Likewise, the potential involvement of glial protein synthesis would also require a diffusible signal. Astrocytes in the CA1 region, however, do not appear to express full-length TrkB or TrkC receptors (15), making glial participation unlikely. These data are most consistent with the hypothesis that the neurotrophins stimulate local protein synthesis within the pyramidal neurons themselves. The mRNAs encoding both TrkB and TrkC have been detected in both the presynaptic CA3 and the postsynaptic CA1 neurons (16). Although some mRNA species have been detected in axons (17), no protein synthesis has been detected in hippocampal axons (18). Thus, the most likely site of neurotrophin-induced protein synthesis is the dendrites of CA1 pyramidal neurons. Ultrastructural, in situ hybridization, and single-cell polymerase chain reaction techniques have revealed that both polyribosomes (8) and mRNAs (19) are present in CA1 dendrites, often associated with individual postsynaptic spines. Moreover, protein synthesis has been detected in synaptosomes (20), isolated axonal and dendritic fractions (18), and hippocampal slices exposed to synaptic stimulation and carbachol (7) or N-methyl-D-aspartate or nitric oxide (21).

Application of anisomycin has no detectable effect on basal synaptic transmission (Fig. 1G) or short-term synaptic plasticity (22). Basal levels of protein synthesis in hippocampal dendrites are low and completely blocked by chloramphenicol, but are not affected by anisomycin or cycloheximide (7). Moreover, anisomycin did not affect the

abundance of TrkB protein or its phosphorylation induced by BDNF (23). These observations argue against the interpretation that anisomycin reduces the amount of locally synthesized proteins that are necessary for signal transduction by BDNF or NT-3.

Our data suggest that BDNF and NT-3 stimulate the local synthesis of proteins that are required for the induction of synaptic enhancement (24). In the hippocampus, neurotrophins stimulate Trk phosphorylation (25) and increase intracellular Ca^{2+} concentration (26); these same signalling events may be coupled to protein kinase activities (27) to stimulate protein synthesis. The newly synthesized proteins may act locally to enhance postsynaptic responsiveness or may communicate with the presynaptic terminal to increase neurotransmitter release (4, 28). During developmental and adult plasticity, the regulated release (29) of neurotrophins and consequent stimulation of local protein synthesis may permit the site-specific modification of synaptic transmission (4) and structure (30).

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enhancement

5. Hippocampal slices (thickness, 500 μm) were prepared from young adult male Sprague-Dawley rats. Slices were submerged in a stream (flow-rate, 210 ml/hour) of artificial cerebral spinal fluid (ACSF) (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 11.0 mM glucose) maintained at room temperature (22 to 25°C) and gassed with 95% O_2, 5% CO_2. The initial slope (1 to 2 ms) of field excitatory postsynaptic potentials (EPSPs) evoked by stimulation of the Schaffer collateral-commissural afferents (once every 15 s) was measured in stratum radiatum at a depth of 100 to 150 μm below the slice surface. The application and storage of the neurotrophic factors were as previously described (4). Anisomycin and cycloheximide were maintained as 40 mM stock solutions in ethanol at 4°C. The final concentration (0.1 %) of ethanol to which the slices were exposed had no detectable effect on synaptic transmission. All experiments with protein synthesis inhibitors were paired with a same-day control experiment in which BDNF or NT-3 potentiated synaptic transmission. Inhibitors were applied at least 30 min before the addition of neurotrophin. BDNF and NT-3 potentiated synaptic strength at least 30% in 84.4 and 83.7% of control experiments, respectively. The percent of baseline measurements indicated in the text were obtained 170 to 180 min after the application of neurotrophin, unless otherwise noted. Ensemble average plots represent group means of each EPSP slope, for all experiments, aligned with respect to the time of neurotrophin application. Statistical significance was

assessed by paired *t* tests or one-way analysis of variance; a P value of < 0.05 was considered statistically significant.

6. In vitro assays of protein synthesis inhibition in hippocampal slices were performed basically as previously described (2). Slices were individually maintained in 200 µl of ACSF in a 24-well tissue culture dish for 1 hour at room temperature. Either anisomycin (40 µM) or cycloheximide (40 µM) was added to each well. After 30 min, [^{3}H]Leucine (20 µCi/ml) was introduced and the slice was incubated for an additional hour. The inhibitor and [^{3}H]Leucine were washed out with ice-cold ACSF and the slices were homogenized. Protein synthesis was measured by incorporation of [^{3}H]Leucine into trichloroacetic acid-precipitable material. When compared to controls, anisomycin and cycloheximide inhibited protein synthesis by 71.9 ± 5.2 and $71.2 \pm 6.0\%$, respectively ($n = 4$ for each inhibitor).

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9. Pyramidal neurons were disconnected from the stratum radiatum using a small microdissection knife visualized under a dissecting microscope. Sham lesions were introduced as a single vertical cut through both the upper and lower blades of the dentate gyrus. The microlesions were performed 1 hour after preparation of the

hippocampal slices. Lesioned slices were allowed to recover for at least 2 hours before electrophysiological recording. Only those lesioned slices that required stimuli of < 220 μA to produce a field EPSP slope of 0.1 mV/ms were used. The average stimulus size in control and lesion experiments was 90.1 and 123.1 μA, respectively. Complete dissociation of the cell bodies from the neuropil was confirmed by cresyl violet staining. Sections were visualized with a Zeiss Axioplan microscope (2.5X objective).

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12. The isolation of either CA3 or CA1 cell bodies also allowed the assessment of the relative importance of somatic gene transcription and translation in the pre- and postsynaptic neurons, respectively. In the CA3 isolation experiments, the synaptic enhancement induced by either BDNF or NT-3 did not differ in magnitude or duration from that observed in the sham-lesioned slices (Fig. 2C and D). However, in the CA1 isolation experiments, the late phase of the neurotrophin-induced potentiation was significantly reduced relative to that apparent in controls ($P < 0.05$), although the duration was unchanged (Fig. 3 C and D). On the basis of these observations, postsynaptic somatic transcription or translation, or both, appear to be required at times greater than 1 hour after neurotrophin application.

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14. Both BDNF and NT-3 enhanced synaptic transmission in the presence of the $GABA_A$ antagonist bicuculline [mean percent of baseline at 80 to 90 min: BDNF plus bicuculline, 192.5 ± 13.7 (n = 6), $P < 0.001$; NT-3 plus bicuculline, 188.2 ± 16.0 (n = 6), $P < 0.001$]. However, another group [H. G. Kim, T. Wang, P. Olafsson, B. Lu, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12341 (1994)] has shown that NT-3 can modulate GABAergic transmission in cultured cortical neurons.

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22. Anisomycin had no effect on basal synaptic transmission [mean percent of baseline: 105.7 ± 10.9 (n = 6), NS], the fiber volley amplitude [mean percent of baseline 170 to180 min after anisomycin, 98.5 ± 5.7 (n = 6), NS], or post-tetanic potentiation after 100 Hz stimulation [mean percent of baseline 1 min after tetanus: 251.9 ± 13.9 before and 254.2 ± 5.8,after anisomycin (n = 4), NS].

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24. A small amount of neurotrophin-stimulated synaptic enhancement persisted in the presence of anisomycin. Because our in vitro assays indicate that anisomycin inhibited protein synthesis by 72% (6), we cannot determine whether the residual enhancement was truly independent of protein-synthesis or simply reflected the incomplete inhibition of protein synthesis by anisomycin.

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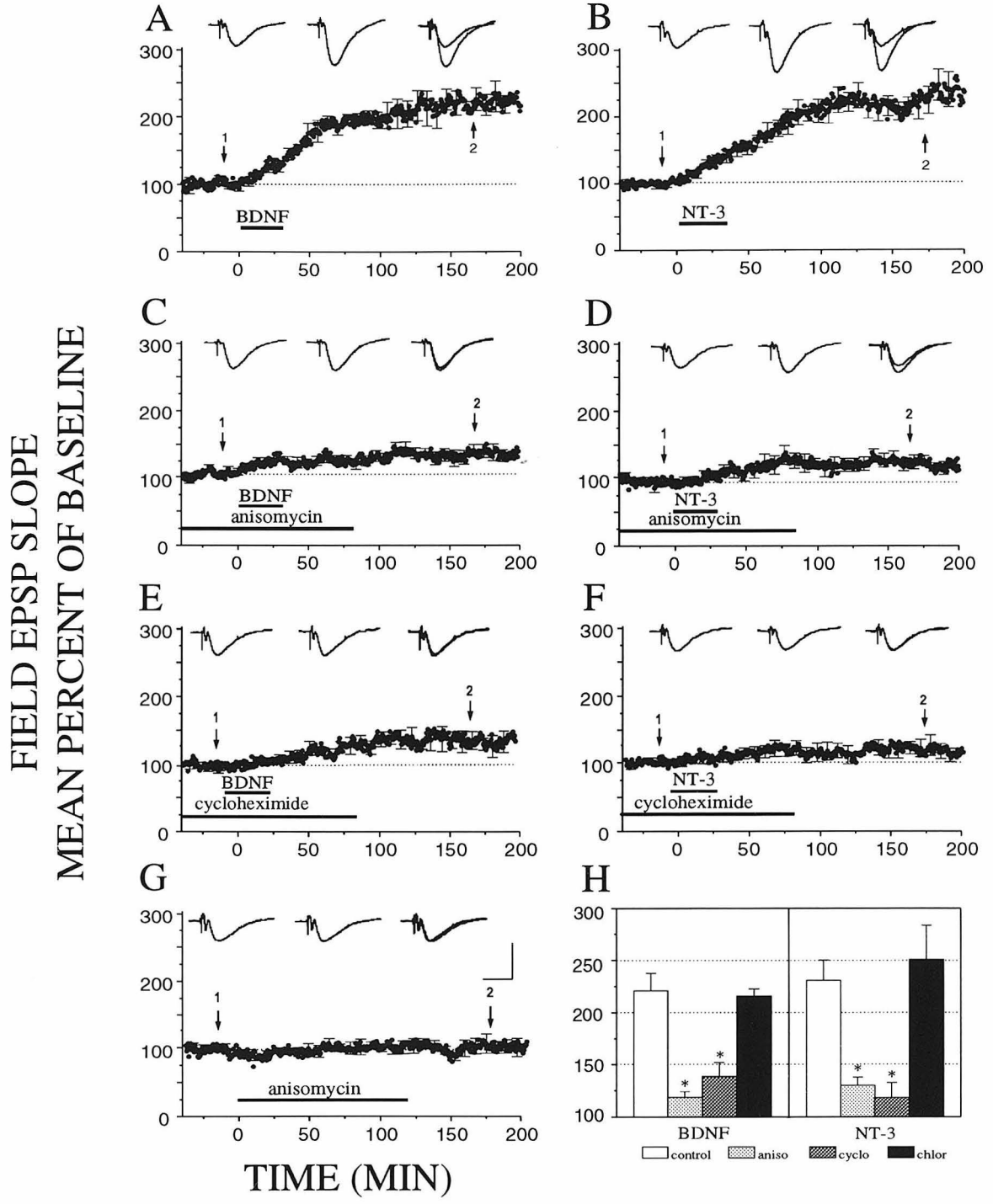
FIGURES AND LEGENDS

Fig. 1. Attenuation of neurotrophin-induced synaptic plasticity by inhibitors of protein synthesis. (**A** and **B**) Ensemble averages for control experiments in which application of BDNF (A) or NT-3 (B) (50 ng/ml) induced a rapid and persistent enhancement of synaptic strength. Mean field excitatory postsynaptic potential (EPSP) slope was 0.11 ± 0.01 mV/ms (mean ± SEM) before and 0.24 ± 0.02 mV/ms after BDNF, and 0.12 ± 0.01 mV/ms before and 0.27 ± 0.02 mV/ms after NT-3. Two representative field EPSPs and their superimposition are shown for the time points arrows labeled 1 and 2) indicated. (**C** and **D**) Pretreatment of hippocampal slices with anisomycin attenuated the synaptic enhancement induced by BDNF (C) or NT-3 (D). Mean field EPSP slope was 0.13 ± 0.01 mV/ms before and 0.17 ± 0.02 mV/ms after BDNF, and 0.12 ± 0.01 mV/ms before and 0.16 ± 0.01 mV/ms after NT-3. (**E** and **F**) Pretreatment of hippocampal slices with cycloheximide attenuated the synaptic enhancement induced by BDNF (E) or NT-3 (F). Mean field EPSP slope was 0.13 ± 0.01 mV/ms before and 0.18 ± 0.02 mV/ms after BDNF, and 0.14 ± 0.01 mV/ms before and 0.16 ± 0.03 mV/ms after NT-3. (**G**) Control experiment demonstrating that anisomycin had no effect on basal synaptic strength. Similar results were obtained with cycloheximide. Mean field EPSP slope was 0.12 ± 0.01 mV/ms before and 0.13 ± 0.02 mV/ms after anisomycin, and 0.18 ± 0.01 mV/ms before and 0.20 ± 0.02 mV/ms after cycloheximide. Calibration bars [for (A) through (G)], 1 mV and 20 ms. (**H**) Summary of the percent enhancement of mean field EPSP slopes shown in (A) through (F) for control, anisomycin (Aniso), and cycloheximide (Cyclo), or chloramphenicol (Chlor). $*P < 0.05$ versus control group.


A
B
C
D
E
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G
H
BDNF
NT-3
BDNF
anisomycin
NT-3
anisomycin
BDNF
cycloheximide
NT-3
cycloheximide
anisomycin
FIELD EPSP SLOPE
MEAN PERCENT OF BASELINE
TIME (MIN)
control
aniso
cyclo
chlor

Fig. 2. Requirement of protein synthesis for neurotrophin-induced enhancement at synapses isolated from the presynaptic pyramidal cell somata. (**A**) Schematic representation of a hippocampal slice showing the placement of a microlesion to isolate CA3 cell bodies from their axons and CA1 dendritic area, where electrophysiological recordings were made. DG, dentate gyrus. (**B**) Representative cresyl violet-stained hippocampal slice after the isolation of the CA3 cell somata from the synaptic region. Arrowheads indicate the site of the lesion. Scale bar, 550 μm. (**C** and **D**) Filled circles indicate the control enhancement obtained in sham-lesioned slices after the application of BDNF (C) or NT-3 (D). Mean field EPSP slope was 0.12 ± 0.01 mV/ms (Mean ± SEM) before and 0.28 ± 0.04 mV/ms after BDNF, and 0.14 ± 0.01 mV/ms before and 0.29 ± 0.03 mV/ms after NT-3. Open circles indicate the enhancement obtained in CA3 somata-isolated slices. BDNF (C) and NT-3 (D) enhanced synaptic transmission to a similar extent as that observed in sham-lesioned slices. Mean field EPSP slope was 0.11 ± 0.01 mV/ms before and 0.25 ± 0.03 mV/ms after BDNF, and 0.13 ± 0.01 mV/ms before and 0.28 ± 0.02 mV/ms after NT-3. Open triangles show that pretreatment of the slices with anisomycin prevented the neurotrophin-induced plasticity at CA3 somata-isolated synapses. Mean field EPSP slope was 0.13 ± 0.02 mV/ms before and 0.14 ± 0.03 mV/ms after BDNF, and 0.16 ± 0.01 mV/ms before and 0.18 ± 0.03 mV/ms after NT-3. Superimposed representative EPSPs were recorded 5 min before and 3 hours after the application of neurotrophin. Calibration bars, 1 mV and 20 ms.

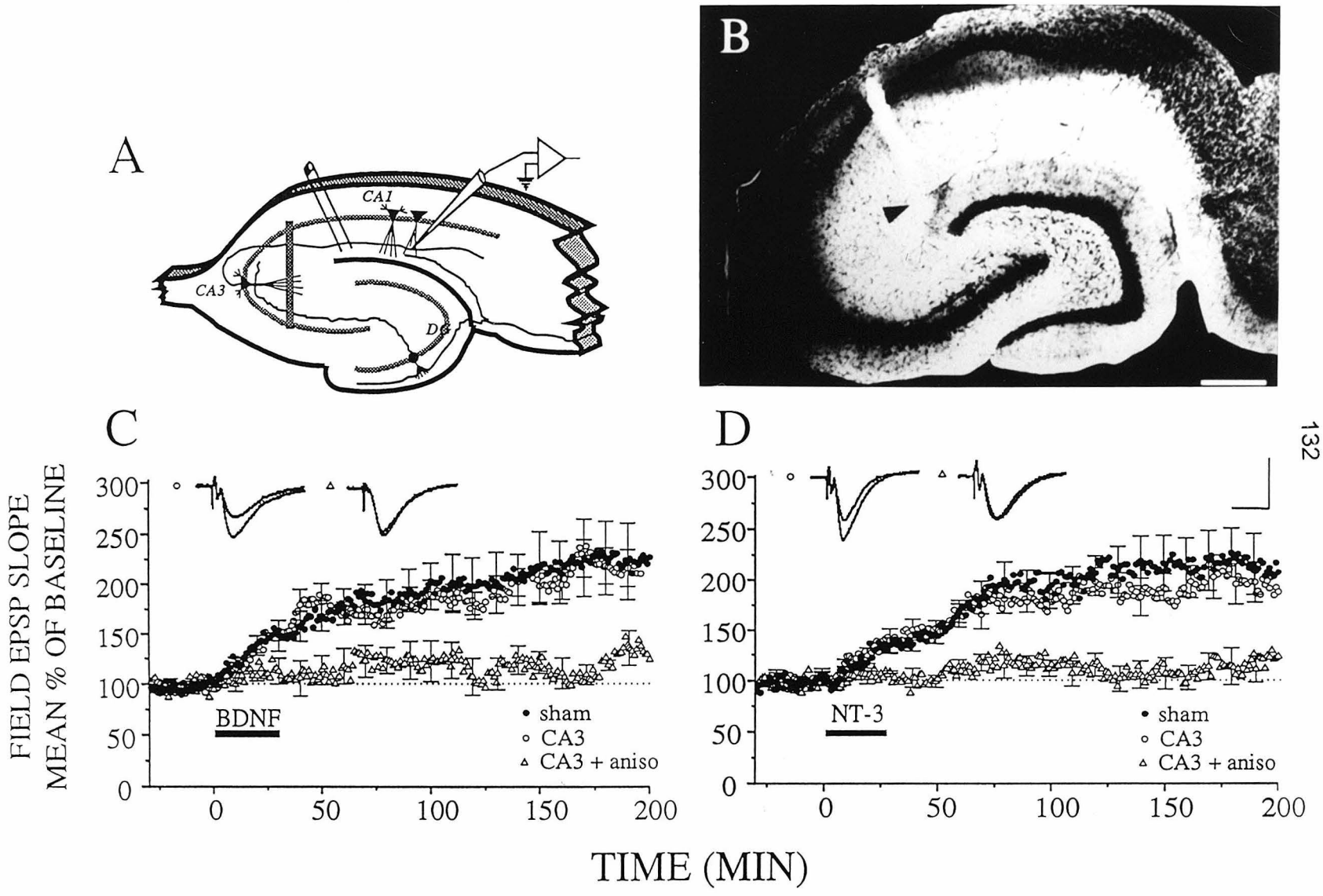

A
CA1
CA3
DG
B
C
D
FIELD EPSP SLOPE
MEAN % OF BASELINE
300
250
200
150
100
50
0
BDNF
NT-3
• sham
◦ CA3
▵ CA3 + aniso
0
50
100
150
200
TIME (MIN)

Fig. 3. Requirement of protein synthesis for neurotrophin-induced enhancement at synapses isolated from the postsynaptic pyramidal cell somata. (**A**) Schematic representation of a hippocampal slice showing the placement of a microlesion to isolate CA1 cell bodies from their dendrites, where electrophysiological recordings were made. (**B**) Representative cresyl violet-stained hippocampal slice after the isolation of the CA1 cell somata from the synaptic region. Arrowheads indicate the site of the lesion. Scale bar, 550 μm. (**C** and **D**) Filled circles indicate the control enhancement obtained in sham-lesioned slices after the application of BDNF (C) or NT-3 (D). Mean field EPSP slope was 0.11 ± 0.01 mV/ms (Mean ± SEM) before and 0.26 ± 0.02 mV/ms after BDNF, and 0.13 ± 0.01 mV/ms before and 0.29 ± 0.01 mV/ms after NT-3. Open circles indicate the enhancement obtained in CA1 somata-isolated slices (CA1). BDNF (C) and NT-3 (D) enhanced synaptic transmission at synapses isolated from the postsynaptic cell bodies, although the magnitude of enhancement was slightly less than that observed in sham-lesioned slices. Mean field EPSP slope was 0.10 ± 0.01 mV/ms before and 0.17 ± 0.02 mV/ms after BDNF, and 0.10 ± 0.02 mV/ms before and 0.18 ± 0.03 mV/ms after NT-3. Open triangles show that pretreatment of the slices with anisomycin prevented the neurotrophin-induced plasticity at CA1 somata-isolated synapses. Mean field EPSP slope was 0.09 ± 0.01 mV/ms before and 0.11 ± 0.01 mV/ms after BDNF, and 0.10 ± 0.01 mV/ms before and 0.12 ± 0.01 mV/ms after NT-3. Superimposed representative EPSPs were recorded 5 min before and 3 hours after the application of neurotrophin. Calibration bars, 1 mV and 20 ms.

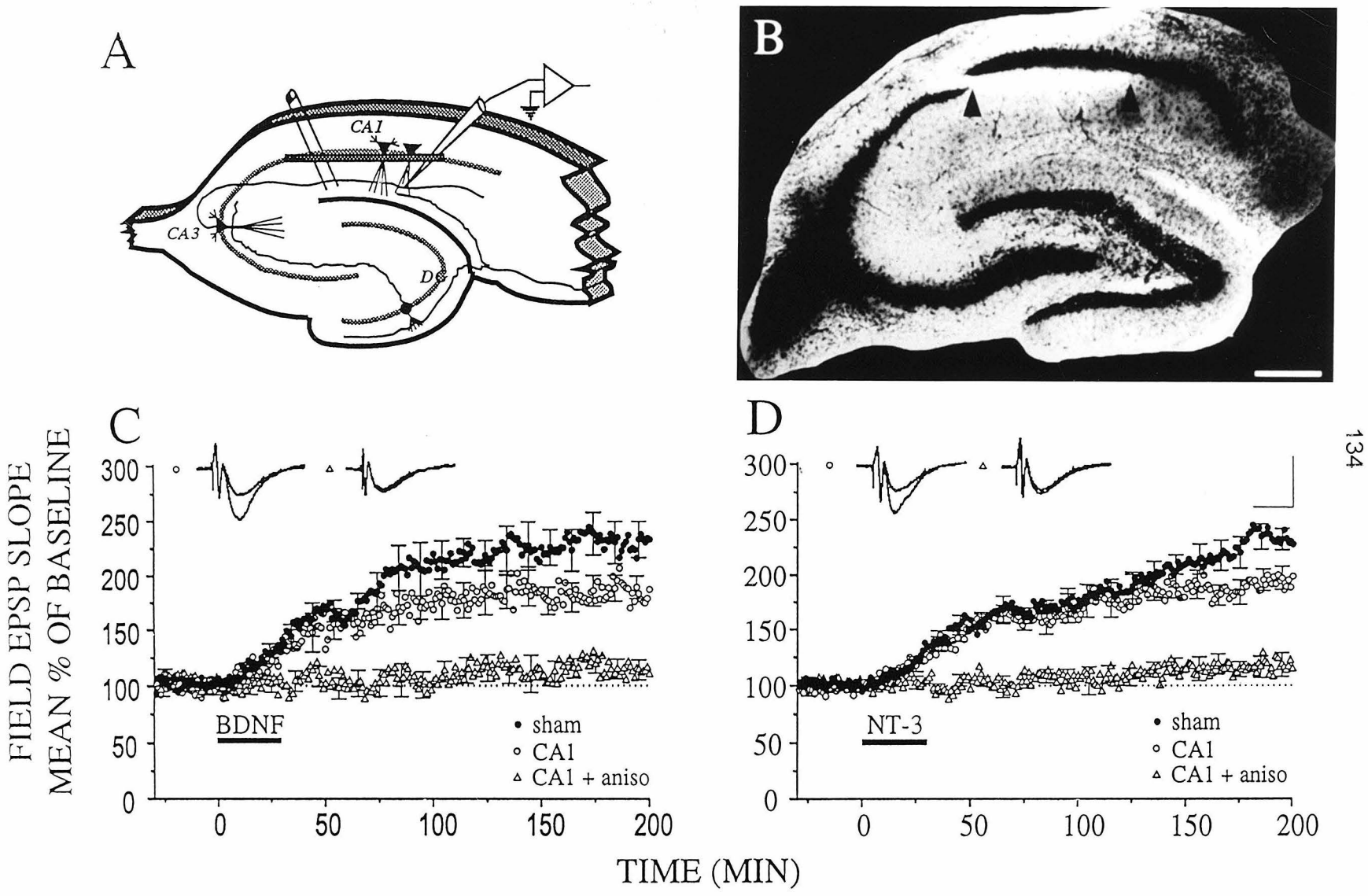
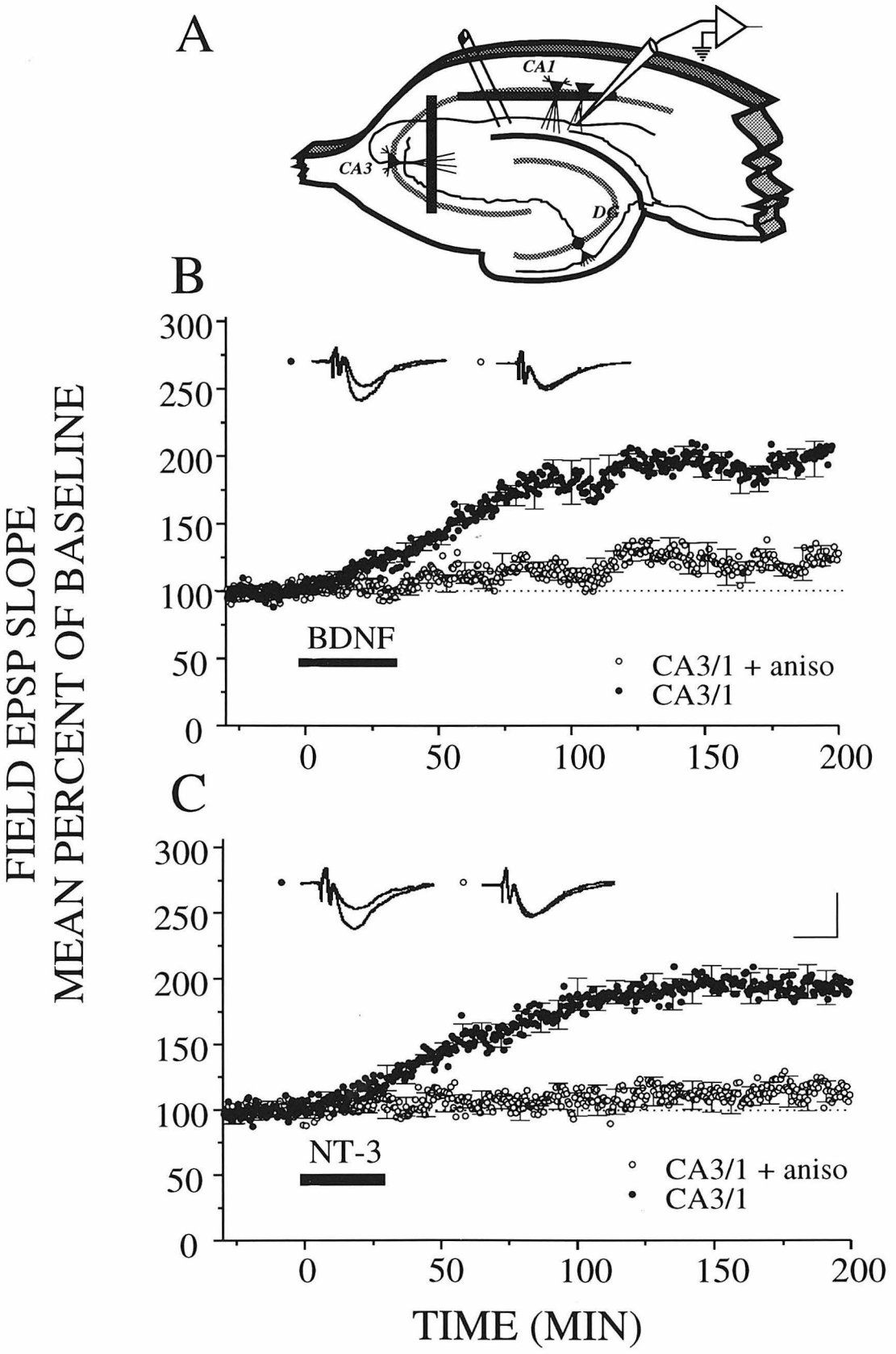

A
CA1
CA3
D
B
C
D
FIELD EPSP SLOPE
MEAN % OF BASELINE
300
250
200
150
100
50
0
BDNF
NT-3
• sham
◦ CA1
△ CA1 + aniso
0
50
100
150
200
TIME (MIN)

Fig. 4. Requirement of protein synthesis for neurotrophin-induced enhancement at synapses isolated from both pre- and postsynaptic pyramidal cell somata. (**A**) Schematic representation of a hippocampal slice showing the placement of two microlesions to isolate the pre- and postsynaptic cell bodies from the synaptic region of the slice, where electrophysiological recordings were made. (**B** and **C**) Filled circles indicate enhancement of synaptic transmission by BDNF (B) or NT-3 (C) at synapses isolated from both pre- and postsynaptic cell bodies. Mean field EPSP slope was 0.09 ± 0.01 mV/ms (Mean ± SEM) before and 0.17 ± 0.01mV/ms after BDNF, and 0.11 ± 0.01 mV/ms before and 0.19 ± 0.01mV/ms after NT-3. Open circles show that the neurotrophin-induced plasticity in synaptically isolated slices require protein synthesis. Mean field EPSP slope was 0.13 ± 0.01 mV/ms before and 0.14 ± 0.01 mV/ms after BDNF, and 0.11 ± 0.01 mV/ms before and 0.12 ± 0.01 mV/ms after NT-3. Superimposed representative EPSP traces were recorded from control and anisomycin-treated slices 5 min before and 3 hours after the application of neurotrophin. Calibration bars, 1 mV and 20 ms.


A
CA1
CA3
DG
B
300
250
200
150
100
50
0
BDNF
CA3/1 + aniso
CA3/1
0
50
100
150
200
C
300
250
200
150
100
50
0
NT-3
CA3/1 + aniso
CA3/1
0
50
100
150
200
TIME (MIN)
FIELD EPSP SLOPE
MEAN PERCENT OF BASELINE

CHAPTER 5

Different roles for TrkB signaling in long-term potentiation in the adult rat hippocampus

In preparation for submision to *Neuron* (1997)

Different Roles for TrkB Signaling in Long-Term Potentiation in the Adult Rat Hippocampus

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Running Title: Roles of TrkB in Hippocampal LTP

SUMMARY

Several recent studies have demonstrated the importance of neurotrophic factors in synaptic plasticity in the hippocampus. We examined the role of TrkB ligands in hippocampal long-term potentiation (LTP) using function-blocking TrkB antiserum and Trk-IgG fusion proteins. Incubation of hippocampal slices with TrkB antiserum did not have any effect on basal synaptic transmission, short-term synaptic plasticity or LTP induced by several trains of high frequency stimulation. The anti-TrkB treated-slices, however, showed significant deficits in the expression of LTP induced by either theta-burst stimulation (TBS) or pairing postsynaptic depolarization with low frequency stimulation. Accordingly, prior treatment of slices with the TrkB ligand brain-derived neurotrophic factor (BDNF) significantly reduced the amount of potentiation subsequently elicited by TBS. The late-phase of LTP (2-3 hrs) was also significantly impaired in antiserum-treated slices, slices treated with TrkB-IgG before LTP induction, or slices pre-exposed to BDNF, indicating the involvement of TrkB in maintaining long-term synaptic enhancement. To determine the time window during which TrkB ligands are required for LTP, we applied a TrkB-IgG 30 minutes after LTP induction. The application of a TrkB-IgG caused previously potentiated synapses to return to baseline levels of synaptic transmission without exerting any effect on the control, non-potentiated synapses within the same slice. Delaying the application of TrkB-IgG to over an hour after induction, however, had no effect on the level of potentiated transmission. Thus, TrkB ligands are required to maintain LTP for up to an hour after the initiating

stimuli. Taken together, these results suggest that the activation of TrkB by its endogenous ligands such as BDNF and NT-4/5, plays a role both in initiating and maintaining LTP in the hippocampus.

INTRODUCTION

The neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) have recently gained attention as signaling molecules in both developmental and adult synaptic plasticity (reviewed in Lo, 1995; Thoenen, 1995; Bonhoeffer, 1996; Berninger and Poo, 1996). The neurotrophins and the Trk receptors are prominently expressed in the adult hippocampus (Ernfors *et al.*, 1990b; Klein *et al.*, 1990; Lamballe *et al.*, 1991). In addition, there is substantial evidence that the neurotrophins are directly involved in activity-dependent neuronal plasticity in the adult hippocampus. Neuronal activity can regulate neurotrophin expression: activation of excitatory or inhibitory receptors up-regulate or down-regulate, respectively, the synthesis of neurotrophins (Gwag and Springer, 1993; Knipper *et al.*, 1994; Wetmore *et al.*, 1994; Zafra *et al.*, 1991). Experimentally-induced seizure or injury can also induce a marked increase in neurotrophin mRNA levels (Ballarin *et al.*, 1991; Isackson *et al.*, 1991).

In addition, activity-dependent release of NGF and BDNF from hippocampal neurons has recently been reported (Bloch and Thoenen, 1995; Griesbeck *et al.*, 1995; Goodman *et al.*, 1996), suggesting that neurotrophins may modulate synaptic function. Indeed, the extracellular application of BDNF or NT-3 can enhance excitatory synaptic transmission (Lessmann *et al.*, 1994; Kang and Schuman, 1995a; Levine *et al.*, 1995; Carmignoto *et al.*, 1997) and inhibit GABAergic transmission (Kim *et al.*, 1994). Although it is likely that interaction of neurotrophins with the Trk receptor tyrosine kinases is the first signal transduction event (Kang *et al.*, 1996), the downstream cellular

mechanisms (Greene and Kaplan, 1995) by which neurotrophins function to modify synaptic strength remain to be elucidated. In cultures of embryonic neurons, the neurotrophins have been reported to induce a transient increase in intracellular Ca^{2+} (Berninger *et al.*, 1993; Stoop and Poo, 1996), activate the mitogen-associated-protein (MAP) kinases (Marsh *et al.*, 1993) and stimulate the phosphorylation of the presynaptic protein synapsin I (Jovanovic *et al.*, 1996). Furthermore, the neurotrophins have been shown to increase the expression of c-Fos, calbindin, and neurotransmitter-synthesizing enzymes (Ip *et al.*, 1993).

Several recent studies have begun to address whether endogenous TrkB ligands play a role in long-term potentiation (LTP), a widely-studied model of synaptic plasticity. The earliest data suggesting a link between neurotrophic factors and hippocampal LTP was the demonstration that both BDNF and NT-3 mRNA are up-regulated by high frequency stimulation used to induce LTP (Patterson *et al.*, 1992). Recently, two groups independently generated mutant mice with a targeted disruption of the BDNF gene (Ernfors *et al.*, 1994; Korte *et al.*, 1995). Both lines of BDNF knock-out mice exhibit a significant impairment in hippocampal LTP (Korte *et al.*, 1995; Patterson *et al.*, 1996), although only one line displays deficits in basal synaptic transmission (Patterson *et al.*, 1996). Importantly, the deficits in LTP in BDNF knock-out mice can be rescued by acute treatment with either exogenous BDNF (Patterson *et al.*, 1996) or an adenovirus construct containing the BDNF gene (Korte *et al.*, 1996), excluding the possibility that the observed LTP deficits were due to developmental abnormalities. Figurov *et al.* (1996) have also recently demonstrated that exogenous BDNF promotes the induction of LTP in

young hippocampal slices by tetanic stimulation which normally gives rise to only short-term potentiation. In addition, preventing TrkB activation with TrkB-IgG fusion proteins greatly reduces the magnitude of LTP induced by theta burst stimulation in slices from adult hippocampus (Figurov *et al.*, 1996). Taken together, these data support the view that BDNF or other TrkB ligand(s) play an active role in the expression of hippocampal LTP.

In this report, we examine the functional involvement of TrkB ligands in both early (1 hr.) and late-phase (2-3 hr.) LTP, using antisera that recognize and block TrkB function (Kang *et al.*, 1996; Yan *et al.*, 1997) as well as Trk-IgG fusion proteins that bind and inhibit endogenous TrkB ligands (Shelton *et al.*, 1995). We find a differential effect of inhibiting TrkB function on early LTP, which depends on the LTP-inducing stimulation paradigm used, suggesting that different patterns of stimulation used to induce LTP may invoke, at least in part, distinct cellular signaling mechanisms. For late-phase LTP, TrkB function may play a role in the late-, protein synthesis-dependent, phase of synaptic enhancement, which is consistent with previous reports demonstrating neurotrophin-induced protein synthesis and gene expression in brain (Ginty *et al.*, 1994; Ip *et al.*, 1993; Kang and Schuman, 1996; Nawa *et al.*, 1994).

RESULTS

Trk B antisera prevent Trk B signaling

We previously described a rabbit polyclonal antiserum which specifically recognizes the TrkB receptor (Yan *et al.*, 1997). As this antiserum was generated against a non-denatured antigen produced in a mammalian cell line, it should recognize correctly folded, glycosylated TrkB protein. It was of interest to determine if this antiserum would functionally block the BDNF-induced phosphorylation of the TrkB receptor. This was first tested using recombinant NIH3T3 cells, which express the full-length TrkB protein. The cells were co-incubated with antiserum in the presence or absence of 50 ng/ml BDNF and phosphorylation of the TrkB receptor was examined. As shown in the upper panel of Figure 1, BDNF induced a strong phosphorylation of the TrkB receptor in the cells when co-incubated with either media alone or media containing 10 % preimmune seum. When cells were treated with a mixture of BDNF and media containing 10 % anti-TrkB antisera, however, TrkB phosphorylation was completely blocked.

In order to investigate the mechanism of action of the anti-TrkB antiserum, we examined the effect of antiserum treatment on the levels of TrkB protein by probing similar Wester blots with the anti-Trk antiserum. As seen in the lower panel of Figure 1, treatment of NIH3T3 cells with the anti-TrkB antiserum for 1 hour resulted in a profound decrease in the amount of TrkB protein to levels below detection. Similar results were observed regardless with or without co-treatment of BDNF with the antiserum. In contrast, treatment without antiserum or treatment with preimmune serum had no effect on the levels of TrkB protein in the cells (Figure 1). The slightly reduced levels of

protein seen in the BDNF-treated samples likely result from ligand-induced internalization and degradation, a phenomenon commonly observed with ligand treatment of many receptor protein tyrosine kinases. Thus, it appears that the antiserum rapidly and efficiently reduces TrkB receptor levels. Therefore the net effect of treatment with the anti-TrkB antiserum is to render the cells incapable of responding to ligands through the TrkB receptor. We previously demonstrated that treatment of hippocampal slices with this anti-TrkB antisera completely prevents the synaptic enhancement produced by exogenous application of BDNF (Kang *et al.*, 1996). Using the same protocol to introduce the antibody into hippocampal slices, we have now examined the effects of blocking TrkB function on basal synaptic transmission and short- and long-term synaptic plasticity.

Function-Blocking TrkB Antiserum Does Not Affect Basic Synaptic Function

To ascertain the specificity of the function-blocking TrkB antiserum (Ab) on LTP, we first examined several aspects of basic synaptic function at Schaffer collateral-CA1 synapses in the hippocampal slice. We confirmed both the successful penetration of the Ab into hippocampal slices and the persistence of the antibody following perfusion with normal ACSF (Figure 2). Basal synaptic transmission was examined in anti-TrkB treated slices by generating input-output curves of synaptic strength. We plotted stimulus strength against the slope of the field excitatory postsynaptic potential (field EPSP) to compare the size of the response for a given intensity of stimulus in preimmune serum or TrkB Ab-incubated slices (Figure 3A). For all of the stimulus strengths tested, equivalent

synaptic responses were evoked for both the control and the anti-TrkB-treated groups. Dividing the slope of field EPSP by the size of the presynaptic fiber volley (PSFV) provides a measure of the synaptic response size produced by stimulation of a given number of presynaptic axons. We found no significant difference in either the mean or the distribution of these values between preimmune and TrkB Ab- incubated slices (Figure 3B). Similar measurements were obtained from slices treated with Ab for over 3 hours (data not shown). These data suggest that basal synaptic transmission is not modified by acute manipulation of TrkB function.

We next examined two forms of short-term synaptic plasticity, paired-pulse facilitation (PPF) and post-tetanic potentiation (PTP). Both PPF and PTP are thought to represent presynaptic plasticity, the larger synaptic responses observed following stimulation being due to enhanced neurotransmitter release via an accumulation of Ca^{2+} in the presynaptic nerve terminal (Delaney *et al.*, 1989; Katz and Miledi, 1968). The TrkB Ab had no distinguishable effect on the size of PTP (Figure 4) [preimmune, 345.8 ± 48.7; TrkB Ab, 314.3 ± 13.6 (n = 9)] or PPF (Figure 3C) [100 ms delay: preimmune, 129.3 ± 4.2; TrkB Ab, 124.9 ± 3.1 (n =16)]. Similar measurements were obtained in slices treated with Ab for over 3 hours (data not shown). These results suggest that presynaptic release processes are unperturbed by acute manipulation of TrkB function.

The Differential Effect of Inhibiting TrkB function on early LTP

The early-phase of LTP, which is usually measured one hour after induction, can be induced by various stimulation protocols such as high-frequency stimulation (tetanus),

theta-burst stimulation (TBS), or pairing postsynaptic depolarization with low frequency stimulation (pairing) (Bliss and Lomo, 1973; Gustafsson *et al.*, 1987; Larson and Lynch, 1986). Potentiation induced by each of these methods requires the activation of N-methyl-D-aspartate (NMDA) receptors and an increase in postsynaptic Ca^{2+} during LTP induction. We examined whether TrkB receptor signaling plays a role in the induction or expression of LTP induced by these different stimulation patterns. At the highest frequency stimulation protocol examined, 4 trains of 100 Hz (1 sec) tetani, treatment with the TrkB Ab did not affect either the induction or the maintenance of LTP (Figure 4A) [mean percent of baseline at 50 to 60 min: preimmune, 186.0 ± 11.9 (n = 9), $P < 0.001$; TrkB Ab, 170.2 ± 11.9 (n = 9), $P < 0.005$]. (This lack of inhibition is consistent with our previous observation, Kang and Schuman, 1995a, that pre-exposure of hippocampal slices to BDNF does not occlude subsequent attempts to induce LTP by 4 trains of 100 Hz stimulation). When we induced LTP by patterned or low frequency stimulation protocols, however, such as TBS or pairing, TrkB Ab-incubated slices showed significant deficits in LTP (Figure 4B, C) [mean percent of baseline at 50 to 60 min: TBS: preimmune, 137.7 ± 6.0 (n = 9), $P < 0.001$; TrkB Ab, 112.0 ± 5.5 (n = 9), $P < 0.05$, Pairing: preimmune, 167.3 ± 15.9 (n = 9), $P < 0.001$; TrkB Ab, 108.0 ± 9.1 (n = 9), not significant (NS)]. A 10-fold lower dilution of TrkB Ab was ineffective in preventing TBS-induced LTP, whereas a ~1.5 fold higher dilution produced no greater inhibition [mean percent of baseline at 50-60 min: TrkB Ab 1:100 dilution, 137.3 ± 7.2 (n = 3), $P \leq 0.05$; TrkB Ab 1:7 dilution, 102.4 ± 6.7 (n = 3), NS].

If TrkB signaling is important for LTP induced by TBS, then prior exposure of

hippocampal slices to a TrkB ligand might be expected to attenuate the amount of LTP elicited by subsequent TBS. Indeed, we found that prior potentiation of synaptic transmission by BDNF application significantly reduced the amount of LTP subsequently elicited by TBS (Figure 5) [mean percent of baseline at 50-60 min: 108.7 ± 10.2 (n=6), NS]. Taken together, these data suggest that the TrkB receptor, activated by BDNF or other TrkB ligands, plays a critical role in LTP induced by TBS or by pairing. Potentiation induced by several trains of high frequency stimulation, however, does not appear to require TrkB function, suggesting that neurotrophin-mediated signaling pathways may not be activated during this particular protocol or that compensatory mechanisms exist that obviate the need for TrkB function.

In the above experiments, there are several differences in the stimulation protocols used including the frequency of stimulation, the pattern of stimulation, and the number of times a given stimulation protocol was applied (e.g. 1 epoch of TBS vs. 4 epochs of 100 Hz stimulation). Any one or combination of these variables could determine the TrkB-dependence or independence of the synaptic enhancement. It is possible, for example, that increasing the number of times a TBS protocol is applied to induce LTP may confer TrkB independence to the synaptic enhancement. Accordingly, we tested this in TrkB Ab-treated slices and found that LTP was still sensitive to TrkB inhibition when multiple (3) TBS epochs were used as inducing stimuli (Figure 6) [mean percent of baseline at 50 to 60 min: preimmune, 139.1 ± 11.5 (n = 7), $P \leq 0.005$; TrkB Ab, 110.4 ± 8.8 (n = 7), NS]. Moreover, the inhibition of LTP observed in TrkB Ab-treated slices exposed to 3 epochs of TBS was not significantly different from that observed in slices exposed to a

single epoch of TBS. These results indicate that in the case of TBS, increasing the number of exposures to TBS does not alter the dependence of LTP on TrkB function.

We next investigated whether the particular pattern of stimulation used to induce LTP is a relevant variable. To do this, we applied a stimulation protocol that included the same number of pulses as 3 epochs of TBS (120 pulses total) delivered in a different pattern (100 Hz for 1.2 sec). Interestingly, the TrkB Ab-treated slices exposed to this LTP induction protocol did not show diminished LTP when compared to preimmune controls (Figure 6) [mean percent of baseline at 50 to 60 min: preimmune, 153.9 ± 9.4 (n = 8), $P \leq 0.005$; TrkB Ab, 155.4 ± 5.8 (n = 7), $P \leq 0.005$]. Thus, an equivalent number of pulses, delivered either as a single 100 Hz epoch or several bursts of stimuli delivered at the theta frequency, can produce LTP that is either independent or dependent on TrkB function. In addition, it should be noted that the inter-stimulus interval within an individual theta burst is the same as that in the 100 Hz paradigm (100 Hz; Figure 6); this indicates the importance of the macroscopic, rather than microscopic, temporal pattern in determining the TrkB dependence of the potentiation.

The Involvement of TrkB Signaling in Late-phase LTP

Long-lasting forms of synaptic plasticity induced by high-frequency stimulation require new protein synthesis (Frey *et al.*, 1988; Nguyen *et al.*, 1994; Otani *et al.*, 1989). Since neurotrophins induce new protein synthesis and gene expression in a variety of systems (Ip et al, 1993; Ginty et al, 1994; Nawa et al, 1994), we examined the possible involvement of TrkB function in late-phase LTP. Long-term or late-phase LTP (L-LTP)

was induced in the slice by 3 trains of high-frequency stimulation, separated by 5 min intervals. In contrast with control slices (incubated with preimmune serum) in which LTP persisted for as long as 4 hours, TrkB Ab-incubated slices exhibited a slow decay of LTP over time, completely returning to baseline within 3 hours of induction (Figure 7A, B) [mean percent of baseline at 170 to 180 min: preimmune, 149.0 ± 8.3 (n = 8), $P < 0.005$; TrkB Ab, 98.7 ± 12.0 (n = 8), NS]. The difference in the magnitude of LTP observed in TrkB-treated and preimmune control groups became significant approximately 1 hour after induction ($P < 0.05$), indicating that TrkB function is required to maintain the late-phase of LTP. Similar results were obtained when an alternative method of blocking TrkB function was employed, a TrkB-IgG. The Trk-IgGs, composed of the extracellular portion of Trk receptors fused to the Fc domain of human IgG, are readily diffusible and serve as fast-acting competitive antagonists of functional Trk receptors by binding endogenous ligands (Shelton *et al.*, 1995). Extracellular application of a TrkB-IgG to hippocampal slices 15 minutes prior to LTP induction resulted in a decaying potentiation that returned to baseline values within 150 minutes, without exerting any effect on synaptic transmission in a second control pathway within the same slice (Figure 7B) [mean percent of baseline at 170 to 180 min: control pathway, 97.7 ± 5.6 (n = 3), NS; LTP pathway, 103.0 ± 5.5 (n = 3), NS]. In contrast, pretreatment of slices with a Trk- IgG,which scavenges TrkA ligands such as NGF, had no effect on late-phase LTP [mean percent of baseline at 170 to 180 min: TrkA-IgG: 156.0 ± 10.3, (n = 3), $P \leq 0.05$]. Thus, hippocampal slices exposed to either a function-blocking TrkB Ab or a TrkB IgG prior to high frequency stimulation failed to exhibit L-LTP. Consistent with

these observations, prior exposure of slices to BDNF also significantly attenuated both the magnitude and duration of late-phase LTP (Figure 8) [mean percent of baseline at 170-180 min: 117.5 ± 11.3, (n = 6), NS].

The pattern of decaying potentiation evident in the TrkB-disabled slices is reminiscent of the potentiation kinetics observed when slices are tetanized in the presence of protein synthesis inhibitors (Frey *et al.*, 1988). To test whether this truncation of LTP is due in part to the lack of neurotrophin-induced new protein synthesis (Kang and Schuman, 1996), we attempted to induce L-LTP in the presence of protein synthesis inhibitor, anisomycin (40 μM) alone and in combination with the function-blocking TrkB Ab. Treatment with anisomycin alone or anisomycin in combination with the TrkB Ab produced no further reduction in L-LTP when compared to TrkB Ab-only treated slices (Figure 8A, B) [mean percent of baseline at 170 to 180 min: anisomycin alone, 115.5 ± 14.9 (n = 7), NS; TrkB Ab + anisomycin, 107.1 ± 10.7 (n = 8), NS], suggesting that TrkB signaling may contribute to the protein synthesis dependence of L-LTP.

What is the temporal window during which TrkB signaling is required for L-LTP? The slight reduction of LTP almost immediately after the tetanus in the above experiments indicates that neurotrophin signaling may be required around the time of induction. We addressed whether neurotrophins signal only during the induction of LTP by introducing Trk-IgG fusion proteins well after the time associated with inductive signal transduction events. We conducted two-pathway experiments in which L-LTP was induced in one pathway while the other pathway, which received only the test stimuli, served as a control. TrkA- or TrkB-IgG was introduced into the superfusate for 30

minutes, 30 minutes following LTP induction. The introduction of TrkA-IgG (1 μg ml^{-1}) was without effect on either the potentiated (Figure 9A) or control pathway (Figure 9B) [mean percent of baseline at 170 to 180 min: potentiated pathway, 166.8 ± 14.4 (n = 6), $P < 0.01$; control pathway, 90.1 ± 3.4 (n = 5), NS]. Application of TrkB-IgG (1 μg ml^{-1}) 30 minutes after LTP induction, however, produced a reversal of LTP in the potentiated pathway (Figure 9C) without affecting synaptic transmission in the control pathway (Figure 9D) [mean percent of baseline at 170 to 180 min: potentiated pathway, 107.4 ± 8.6 (n = 7), NS; control pathway, 104.5 ± 5.7 (n = 6), NS]. These data suggest that the interaction of the TrkB receptor with its endogenous ligands continues to occur at least 30 minutes after L-LTP induction and is required to maintain potentiated synaptic transmission. Does continuous signaling by TrkB ligands underlie LTP maintenance for the entire duration of the synaptic enhancement? To address this, the TrkB-IgG was applied beginning 70 minutes after LTP induction. This delayed application of the TrkB-IgG had no effect on the previously established potentiation (Figure 9) [mean percent of baseline 170-180 min: 171.5 ± 18.2 (n = 8), $P \leq 0.005$]. These data suggest that the critical temporal window during which TrkB signaling is required for L-LTP begins with induction and extends 30-60 minutes following the initiating synaptic stimuli.

DISCUSSION

Several recent studies have supported the hypothesis that BDNF is involved in hippocampal LTP (Korte *et al.*, 1995; Korte *et al.*, 1996; Patterson *et al.*, 1996). We used two different specific inhibitors of TrkB function, a function-blocking TrkB antiserum and a TrkB-IgG fusion protein, to identify more specifically the role of TrkB and its ligands in synaptic plasticity induced by a variety of stimulation paradigms.

.Hippocampal slices treated with the TrkB antiserum exhibited normal basal synaptic transmission, paired-pulse facilitation, and post-tetanic potentiation, consistent with results from one study of BDNF mutant mice (Korte *et al.*, 1995; but see Patterson *et al.*, 1996). The observation that synaptic transmission and short-term plasticity are intact in the absence of TrkB signaling suggests that the deficits in LTP in TrkB antisera-treated slices are not due to disruption of normal synaptic processes. Moreover, two observations argue against the idea that the perturbations of TrkB function disable the synapse's ability to produce and detect the inducing stimuli: i) at least one form of NMDA receptor-dependent LTP (4 X 100 Hz) can be elicited in the presence of the Ab (Figure 4), indicating that NMDA-dependent signaling is intact; ii) pairing-induced LTP is inhibited, indicating that the blockers cannot be working by preventing depolarization, as both control and Ab-treated slices were depolarized to an equivalent extent by intracellular current injection. A previous report indicated that TrkB-IgG reduced the postsynaptic response to repetitive stimulation (Figurov *et al.*, 1996); we have not observed this under the experimental conditions used in this study [mean percent of first EPSP in a TBS burst: control, 30.3 ± 8.3% (n = 3); TrkB IgG, 31.8 ± 4.9% (n = 3)].

Our data indicate that BDNF and/or other TrkB ligands play a critical role in the both early and late-phase of synaptic potentiation in the adult rat hippocampus. Synaptic potentiation induced by bursts of stimuli delivered at the theta frequency or by pairing depolarization with low frequency stimulation was significantly attenuated in TrkB Ab-treated slices. LTP induced by 4 trains of 100 Hz stimulation, however, was relatively insensitive to TrkB blockade. Moreover, occlusion experiments in this study and in a previous study (Kang and Schuman, 1995a) are consistent with the idea that different induction protocols invoke different requirements for TrkB signaling. Prior treatment of hippocampal slices with BDNF significantly attenuates subsequent LTP induced by TBS, but not by 4 trains of 100 Hz stimulation (Kang and Schuman, 1995a).

What particular feature of the various LTP induction protocols renders them more or less vulnerable to inhibition of BDNF-TrkB signaling? The different stimulation patterns used by us and others vary both in the absolute number of pulses given and the pattern and/or frequency of synaptic stimulation. It appears that one crucial variable is the pattern of stimulation used to initiate the synaptic enhancement. A stimulation protocol consisting of 120 pulses delivered at a theta-like frequency revealed a dependence on TrkB function whereas the same number of pulses delivered continuously at 100 Hz produced TrkB-independent LTP. There are at least two explanations to account for this observation: i) different temporal patterns of stimulation can differentially promote the release of TrkB ligands; ii) TrkB ligands are released under all stimulation protocols but continuous, rather than burst-like, stimuli confer TrkB independence by recruiting additional biochemical signaling pathways. Regardless of the particular molecular

explanation, these data indicate that hippocampal synapses are sensitive to the temporal pattern of their inputs and invoke different signaling pathways to produce synaptic enhancement, potentially reflecting the nature of the plasticity-inducing stimuli.

Thus, unlike many signal transduction events, including activation of NMDA receptors, rises in postsynaptic Ca^{2+}, and activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Collingridge *et al.*, 1983; Lynch *et al.*, 1983; Malinow *et al.*, 1989), which are essential for the induction of most forms of LTP in area CA1, the BDNF-TrkB signaling pathway does not appear to be necessary for LTP induced by all protocols. Continuous stimulation protocols, such as repeated trains of 100 Hz stimulation, may not favor release of TrkB ligands; alternatively, TrkB ligands may be released but not required for LTP, presumably due to compensation by alternative signaling pathways. The observed variable requirement for TrkB signaling as a function of stimulation pattern, is true of other signaling molecules like nitric oxide (e.g. Schuman and Madison, 1994; Haley *et al.*, 1993; Williams *et al.*, 1993), and reinforces the notion that different LTP induction protocols involve different, although potentially overlapping, biochemically processes. Whether the recruitment of distinct biochemical induction pathways results in different mechanisms of LTP expression (e.g. pre- vs. postsynaptic) remains to be determined. As such, it is becoming increasingly clear that the term LTP describes enhanced synaptic transmission at the phenomenological level, but cannot necessarily be taken to represent a singularity of signaling mechanisms.

The cellular mechanisms by which neurotrophins modulate synaptic plasticity are not well understood. BDNF application in cultured hippocampal neurons (Berninger *et*

al., 1993) and nerve-muscle synapses (Stoop and Poo, 1996) dramatically increases intracellular Ca^{2+}. Our own studies indicate that Ca^{2+} influx through voltage-gated Ca^{2+} channels and intracellular Ca^{2+} stores is obligatory for the induction of BDNF-induced potentiation (Kang and Schuman, 1995b). It is therefore possible that ligands of the TrkB receptor act together with other signaling pathways to regulate the level of intracellular Ca^{2+} upon the neuronal stimulation. The magnitude of the Ca^{2+} rise may determine the degree to which Ca^{2+} activates downstream effectors (Gallin and Greenberg, 1995), and consequently influence the overall probability or magnitude of synaptic potentiation.

Our data demonstrating that treatment of slices with function-blocking TrkB antiserum or TrkB-IgG reduces late-phase LTP indicate a direct involvement of TrkB signaling in the maintenance of L-LTP. The kinetics and extent of LTP decline under these conditions are similar to the decay of LTP observed in the presence of protein synthesis inhibitors (Frey *et al.*, 1988). Moreover, there was no additive inhibitory effect on LTP when slices were treated with both the TrkB antisera and the protein synthesis inhibitor anisomycin. This finding suggests that TrkB ligands released during LTP may participate in stimulating the protein synthesis that is required for the late-phase of LTP. Indeed, we recently demonstrated that the synaptic enhancement induced by BDNF in hippocampal slices requires the synthesis of new proteins, perhaps in dendritic compartments (Kang and Schuman, 1996), and neurotrophin-stimulated protein synthesis has been observed in isolated cultured hippocampal neurites (Crino and Eberwine, 1996).

At what time are neurotrophins released during LTP? Our experiments with the function- blocking TrkB antiserum indicate a noticeable difference between antiserum-

treated and control slices as early as 15 minutes following LTP induction by either pairing or theta-burst stimulation, suggesting a release at or around the time of induction. In addition, TrkB antisera-treated slices also showed slightly, although not significantly, diminished LTP within 10-20 minutes following the late-phase induction protocol. Thus, these data would suggest that the time of induction is an important period of neurotrophin release. The experiments using the TrkB-IgG fusion proteins, however, indicate that TrkB signaling is required for up to an hour following L-LTP induction. Taken together, these observations suggest that the induction of L-LTP either results in the sustained release of TrkB ligands or continuous signaling by ligands released at earlier times. Since the neurotrophins are secreted in both a regulated and constitutive manner (Bloch and Thoenen, 1995), it is possible that the particular patterns of synaptic activity associated with TrkB-dependent LTP result in the regulated release of BDNF or other TrkB ligands. The continued dependence of LTP on TrkB ligands during the enduring phase of the synaptic enhancement, which is not typically associated with increased neuronal activity, suggests the possibility that the constitutive release of neurotrophic factors participates in the maintenance of L-LTP. Current studies examining the spatial and temporal patterns of neurotrophin release will indicate whether such a scenario is possible.

What is the molecular basis of the demonstrated dependence of late-phase LTP on intact TrkB function? The maintenance of L-LTP involves new protein synthesis and perhaps cAMP-mediated gene expression (Frey *et al.*, 1988, 1993; Impey *et al.*, 1996; Nguyen *et al.*, 1994). Although it has not been clearly demonstrated in vertebrate models

of long-term plasticity, these protein-synthesis-dependent processes result in morphological changes and growth of new synaptic connection in *Aplysia* (Bailey and Chen, 1983; Nazif *et al.*, 1991). Since the TrkB ligand BDNF requires local protein synthesis to enhance synaptic strength in the adult hippocampus (Kang and Schuman, 1996), it is possible that local protein synthesis involving TrkB ligands may contribute to L-LTP. Whether this same signaling pathway that results in clear changes in synaptic strength also alters synaptic structure, as has been observed in the developing nervous system (e.g. Cohen-Cory and Fraser, 1995; McAllister *et al.*, 1995), remains to be determined.

EXPERIMENTAL PROCEDURES

Antisera preparation and immunodetection

Rabbit polyclonal antisera were prepared according to standard immunological techniques using as an antigen the previously described extracellular domain of the TrkB receptor (Philo *et al.*, 1994). This antiserum was shown to be specific for the TrkB receptor (Yan *et al.*, 1997). The ability of this antisera to functionally block the BDNF-induced phosphorylation of the TrkB receptor was tested in recombinant NIH3T3 cells which express the full-length Trk B receptor. Cell were treated for 1 hour at 37°C with media containing either 10 % preimmune serum, 10 % anti-Trk B antiserum, or no antiserum in the absence or presence of 50 ng/ml BDNF. After treatment the cells were lysed, and Trk B receptors were immunoprecipitated and examined by western blot analysis using either anti-phospho Tyr antiserum or anti-Trk antiserum, as previously described (Barnea *et al.*, 1996). For immunodetection of TrkB Ab, hippocampal slices were fixed and sectioned (50 μm) and then treated with a Cy3-conjugated goat anti-rabbit secondary antibody. Immunostained specimens were viewed with a Zeiss LSM 310 laser-scan confocal microscope through either a 10x or a 63x oil-immersion lens. Cy3 was excited at 543 nm. Images were recorded through standard emission filters with the same contrast and brightness settings for all experimental and control sections.

Slice Preparation

Hippocampal slices were prepared using a Stoelting tissue chopper from young (6-8 weeks) adult male Sprague-Dawley rats. Prior to electrophysiological recording, slices

were stored for at least 1.5 hrs. on a Millipore membrane (#1) placed over a tissue culture dish containing oxygenated Ringer's solution. The slice was exposed to 95% O_2, 5% CO_2 circulating in an enclosed chamber. For electrophysiological recordings, slices were submerged in a stream of ACSF (119 mM NaCl; 2.5 mM KCl; 1.3 mM $MgSO_4$; 2.5 mM $CaCl_2$; 1.0 mM NaH_2PO_4; 26.2 mM $NaHCO_3$; 11.0 mM glucose) maintained at room temperature (22-25°C) and gassed with 95% O_2, 5% CO_2. The remaining slices were stored in an interface chamber at room temperature.

Electrophysiology

Field or intracellular excitatory postsynaptic potentials (EPSPs) measured in stratum radiatum or in CA1 pyramidal cells, respectively, were evoked by stimulation of the Schaffer collateral-commissural afferents (1 stim. every 30 sec). Extracellular recording electrodes were filled with 3 M NaCl; intracellular recording electrodes were filled with 2 M cesium acetate. Tetanic stimulation was delivered at the test intensity in 1-s trains at 100 Hz, with four trains 30 s apart or 3 trains 5 min apart, respectively for short-term and long-term LTP. Theta-burst stimulation (TBS) consisted of 10 bursts of stimuli, each of 4 pulses at 100 Hz; interburst interval, 200 ms. Pairing was accomplished by sustained depolarization (to 0 mV) of the intracellularly recorded neuron by dc current injection in conjunction with low-frequency (1 Hz) stimulation of the test pathway for 30 s. We analyzed the initial slope of the field EPSP and the slope and amplitude of the intracellular EPSP. Ensemble averages were constructed using all data points, aligned with respect to the time of LTP induction or BDNF application. In occlusion

experiments, in some experiments the stimulus strength was reduced following BDNF-induced potentiation in order to keep fEPSP in a dynamic range; This had no affect on the subsequent ability to induce LTP. Error bars are standard error of the mean calculated for the entire data set for a given time point. To assess statistical significance, paired t-tests, comparing the average slope size for 10 minutes prior to LTP induction to either 50-60 or 170-180 minutes after LTP induction, were performed on non-normalized data. Significance levels are as stated in the text; p values greater than 0.05 are designated as not significant, (ns).

Blocking Antibody and Trk-IgG Experiments

Prior to electrophysiological recording, slices were individually incubated in single wells of a 24-well tissue culture plate in 200 µL of Ringer's solution containing either a TrkB antiserum (1:7, 1:10, or 1:100) or an equivalent dilution of preimmune serum. Slices were incubated in the antiserum or preimmune serum for 1.5-2 hours prior to recording. Slices were then transferred to a recording chamber and perfused with normal Ringer's solution for approximately 30 minutes prior to LTP induction (Kang *et al.*, 1996).

The Trk-IgG fusion proteins were kindly provided by Dr. D.L. Shelton (Genentech) and stored at 4 °C. Two independent pathways were stimulated in a single slice. Following LTP induction in a single pathway, Trk-IgG molecules were introduced into the ACSF superfusate at an appropriate dilution (1 µg ml^{-1}) for 30 minutes.

ACKNOWLEDGMENTS

We thank David Shelton for the kind gift of the Trk-IgG molecules and Hilary Chute for technical assistance. We also thank several members of the Caltech faculty for their comments on the manuscript. This work was supported by the PEW Charitable Trusts (EMS) and the John Merck Fund (EMS).

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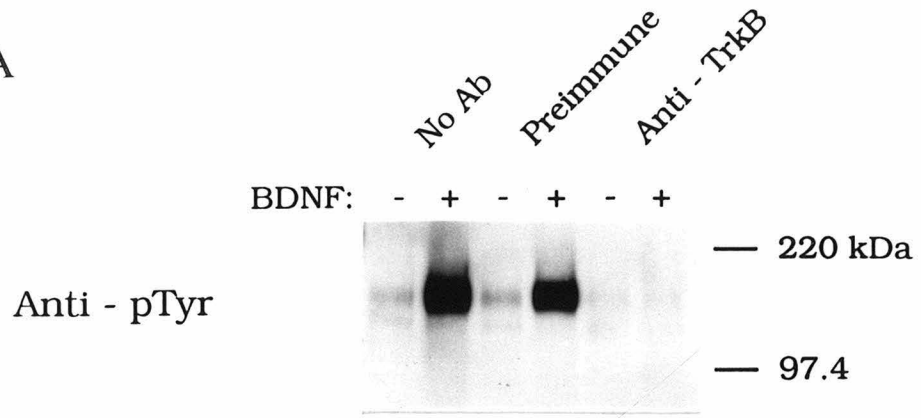
FIGURES AND LEGENDS

Figure 1. Anti-TrkB antisera block BDNF-induced TrkB phosphorylation in NIH3T3 cells.

(**A**) Western blot of phosphotyrosine immunoreactivity in naive, preimmune, or anti-TrkB antisera-treated NIH3T3 cells in the presence (+) or absence of (-) of BDNF.

(**B**) Western blot of TrkB immunoreactivity in naive, preimmune, or anti-TrkB antisera-treated NIH3T3 cells in the presence (+) or absence of (-) of BDNF.

A


No Ab
Preimmune
Anti - TrkB
BDNF: - + - + - +
Anti - pTyr
220 kDa
97.4

B

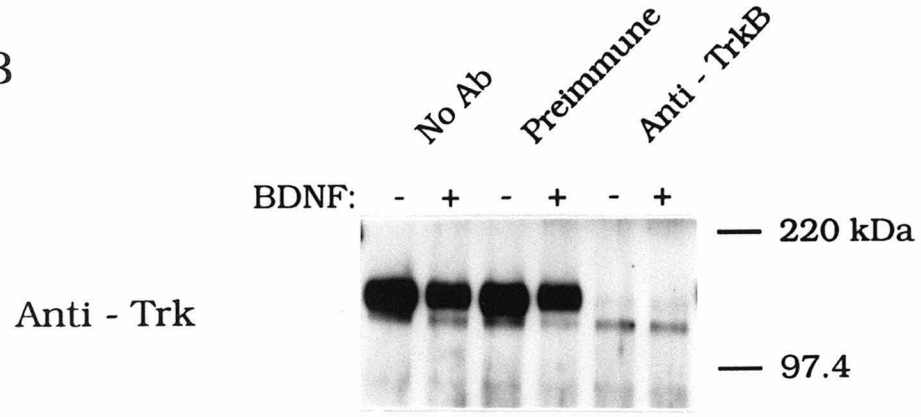
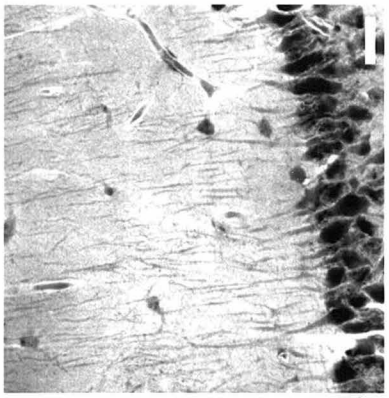
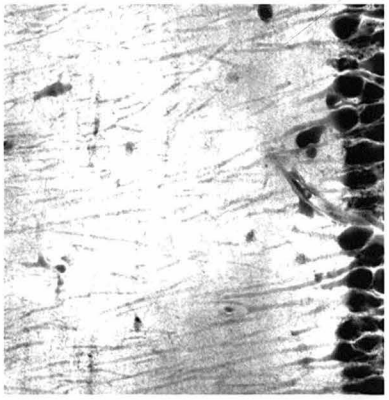
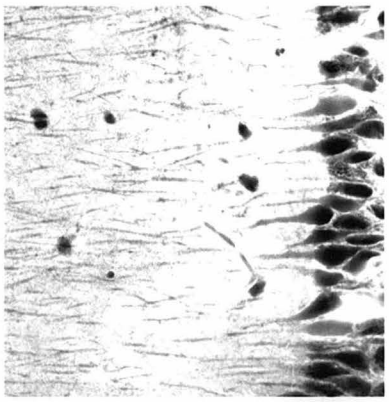

No Ab
Preimmune
Anti - TrkB
BDNF: - + - + - +
Anti - Trk
220 kDa
97.4

Figure 2. TrkB Ab can penetrate hippocampal slices and persist following perfusion with ACSF.

Shown are 63X confocal images taken from the middle 150-200 μm of a hippocampal slice treated with a secondary antibody to recognize the TrkB Ab. From left to right are sections from slices treated with no primary antibody (left), or a TrkB Ab, perfused with normal ACSF for 0, 30, or 60 minutes. Scale bar is 25 μm.







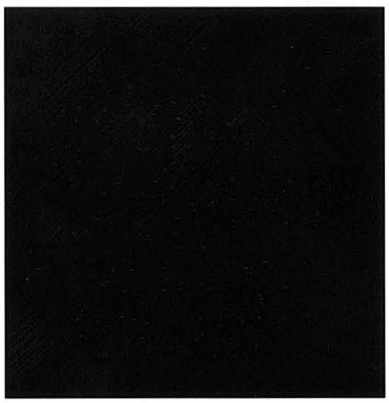


Figure 3. Basal synaptic transmission and paired-pulse facilitation (PPF) are normal in slices pretreated with function-blocking TrkB antisera.

In A-C, the control and TrkB antisera groups are not significantly different from one another.

(**A**) Input-output curves plotting stimulus strength against the initial slope of the field EPSP in preimmune serum (squares; solid line) - and TrkB antisera (circles; dotted line) - treated slices (n = 7).

(**B**) Scatter plot depicting the ratio of the field EPSP slope to the presynaptic fiber volley (PSFV) amplitude over a range of stimulus strengths (n = 7). For each group the mean ± S.E.M. is indicated by the large symbol with the error bars.

(**C**) Scatter plot depicting the facilitation ratios (slope of second EPSP / slope of first EPSP) obtained with an interstimulus interval of 100 ms. The mean ± S.E.M. was 129 ± 7.0 % for preimmune serum-treated slices and 124 ± 0.8 % for TrkB ab-treated slices (n = 16).

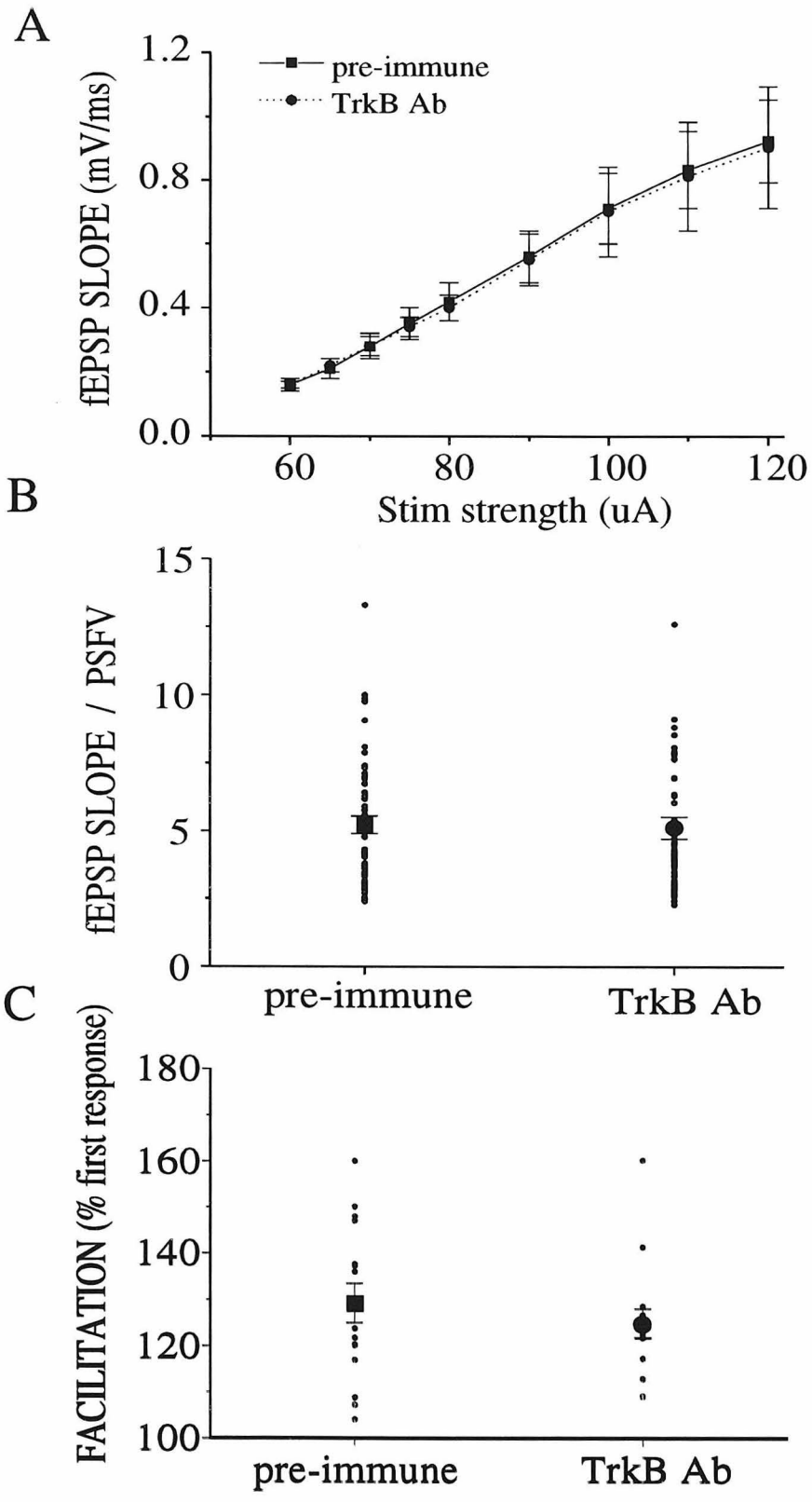

A
1.2
0.8
0.4
0.0
fEPSP SLOPE (mV/ms)
pre-immune
TrkB Ab
60
80
100
120
Stim strength (uA)
B
15
10
5
0
fEPSP SLOPE / PSFV
pre-immune
TrkB Ab
C
180
160
140
120
100
FACILITATION (% first response)
pre-immune
TrkB Ab

Figure 4. Effects of function-blocking TrkB antisera on LTP induced by different protocols.

Ensemble averages showing mean percent of baseline values of field EPSP initial slopes (**A** and **B**) or intracellular EPSP amplitudes (**C**) before and after LTP induction in slices pretreated with either preimmune serum (filled circles) or TrkB Ab (open circles). In each panel superimposed representative EPSPs shown were recorded 5 min before and 1 hour after LTP induction. N = 9 for each control and experimental group below. Scale bars, 1 mV and 20 ms (**A** and **B**); 5 mV and 20 ms (**C**).

(**A**) LTP induced by 4 trains of tetanic stimulation (1 train = 100 Hz for 1sec). Mean field EPSP slope was 0.12 ± 0.02 mV/ms (mean ± S.E.M.) before and 0.21 ± 0.02 mV/ms after LTP induction in preimmune serum-treated slices and 0.15 ± 0.01 mV/ms before and 0.25 ± 0.03 mV/ms after LTP induction in TrkB Ab-treated slices.

(**B**) LTP induced by theta-burst stimulation (TBS-see methods). Mean field EPSP slope was 0.20 ± 0.01 mV/ms before and 0.27 ± 0.02 mV/ms after LTP induction in preimmune serum-treated slices, and 0.18 ± 0.01 mV/ms before and 0.20 ± 0.01 mV/ms after LTP induction in TrkB Ab-treated slices.

(**C**) LTP induced by pairing postsynaptic depolarization with low frequency stimulation (see methods). Mean EPSP amplitude was 5.26 ± 0.38 mV before and 8.54 ± 0.55 mV after LTP induction in preimmune serum-treated slices, and 6.71 ± 0.50 mV before and 7.17 ± 0.67 mV after LTP induction in TrkB antibody-treated slices.

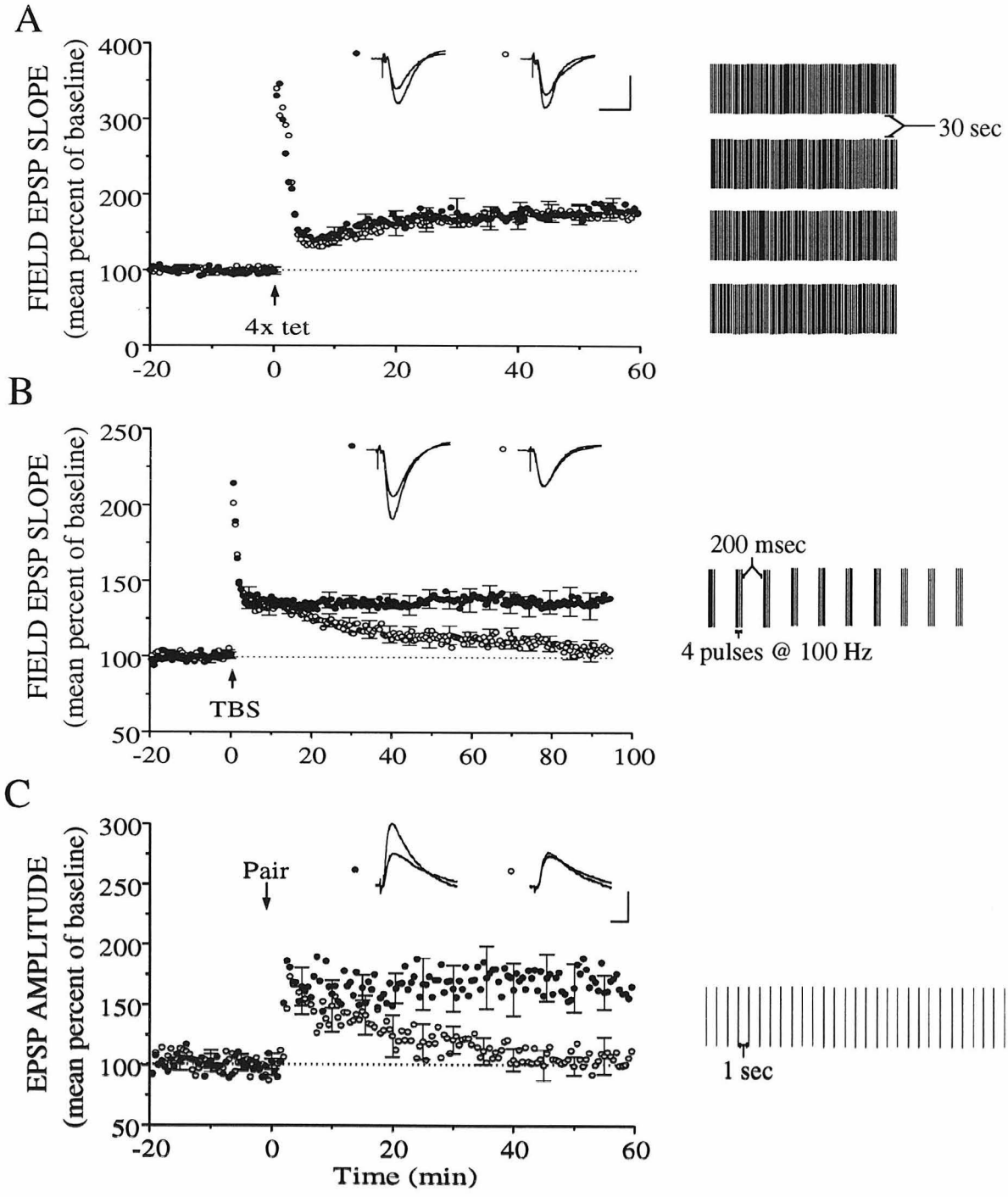

A
FIELD EPSP SLOPE
(mean percent of baseline)
400
300
200
100
0
4x tet
-20
0
20
40
60
30 sec
B
FIELD EPSP SLOPE
(mean percent of baseline)
250
200
150
100
50
TBS
-20
0
20
40
60
80
100
200 msec
4 pulses @ 100 Hz
C
EPSP AMPLITUDE
(mean percent of baseline)
300
250
200
150
100
50
Pair
-20
0
20
40
60
Time (min)
1 sec

Figure 5. Prior exposure to BDNF reduces subsequent TBS-induced potentiation. Ensemble average of 6 experiments showing mean percentage of baseline values of field EPSP slopes before and after application of BDNF (50 ng/ml) and before and after application of TBS. Superimposed traces shown are from 10 min before and 50-60 min after either BDNF application (right) or TBS (left). Scale bar is 1 mV/20 ms. The amount of potentiation elicited by TBS in these experiments was significantly less than that observed in control slices ($P \leq 0.05$; see Figure 4, indicated by dashed line), but not significantly different from TrkB Ab-treated slices (Figure 4). Mean field EPSP slope was 0.10 ± 0.02 mV/msec before and 0.17 ± 0.01 mV/msec 50-60 min after BDNF application. Mean field EPSP slope was 0.16 ± 0.01 mV/msec before and 0.18 ± 0.02 mV/msec 50-60 min after TBS.

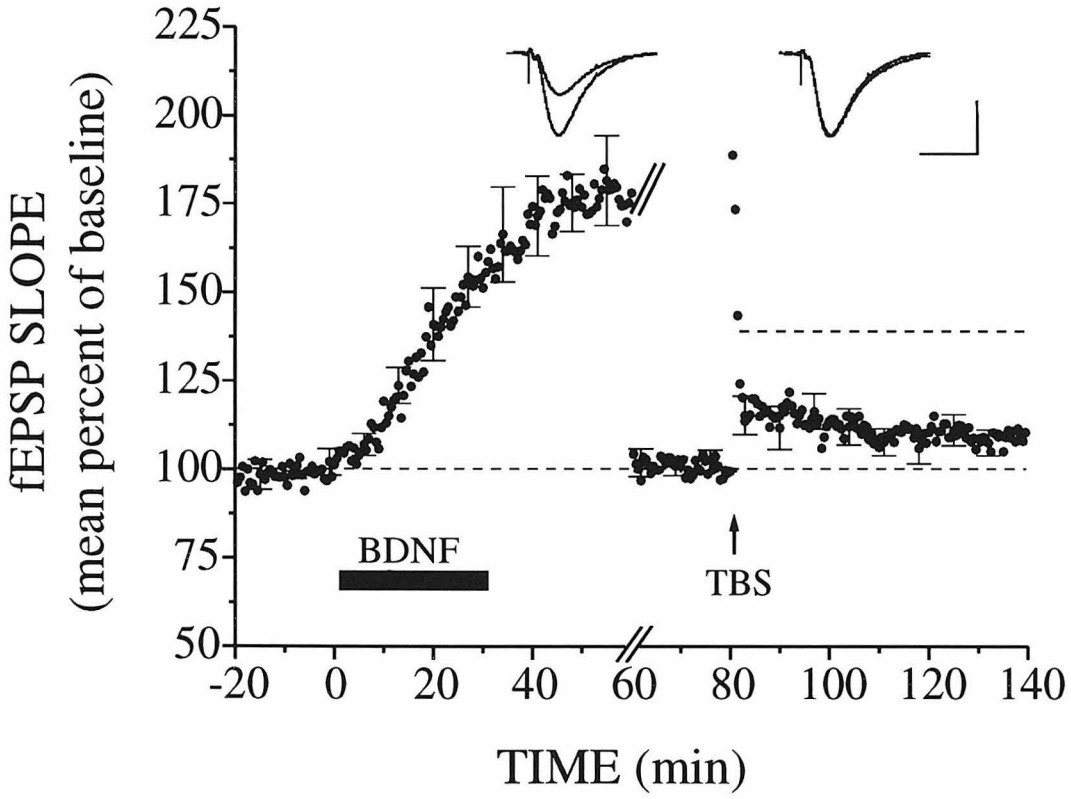

225
200
175
150
125
100
75
50
fEPSP SLOPE
(mean percent of baseline)
BDNF
TBS
-20
0
20
40
60
80
100
120
140
TIME (min)

Figure 6. The pattern of stimulation, not the number of pulses, determines the TrkB dependence of LTP.

Superimposed traces shown are from 10 min before and 50-60 afterLTP induction. Scale bar is 1 mV/20 ms.

(**A**) Slices treated with TrkB Ab (open circles) show significantly diminished LTP in response to 3 epochs of TBS, relative to preimmune controls (closed circles) (n = 7 for each). In control slices, the mean field EPSP slope was 0.17 ± 0.01 and 0.25 ± 0.03, before and after TBS stimulation, respectively. In TrkB Ab-treated slices, the mean field EPSP slope was 0.20 ± 0.01 and 0.22 ± 0.03, before and after TBS stimulation, respectively.

(**B**) Slices treated with TrkB Ab (open circles) show unimpaired LTP in response to a single epoch of 100 Hz stimulation, relative to preimmune controls (closed circles) (n = 7 for TrkB Ab, 8 for controls). In control slices, the mean field EPSP slope was 0.16 ± 0.01 and 0.25 ± 0.01, before and after 100 Hz stimulation, respectively. In TrkB Ab-treated slices, the mean field EPSP slope was 0.16 ± 0.01 and 0.25 ± 0.02, before and after 100 Hz stimulation, respectively.

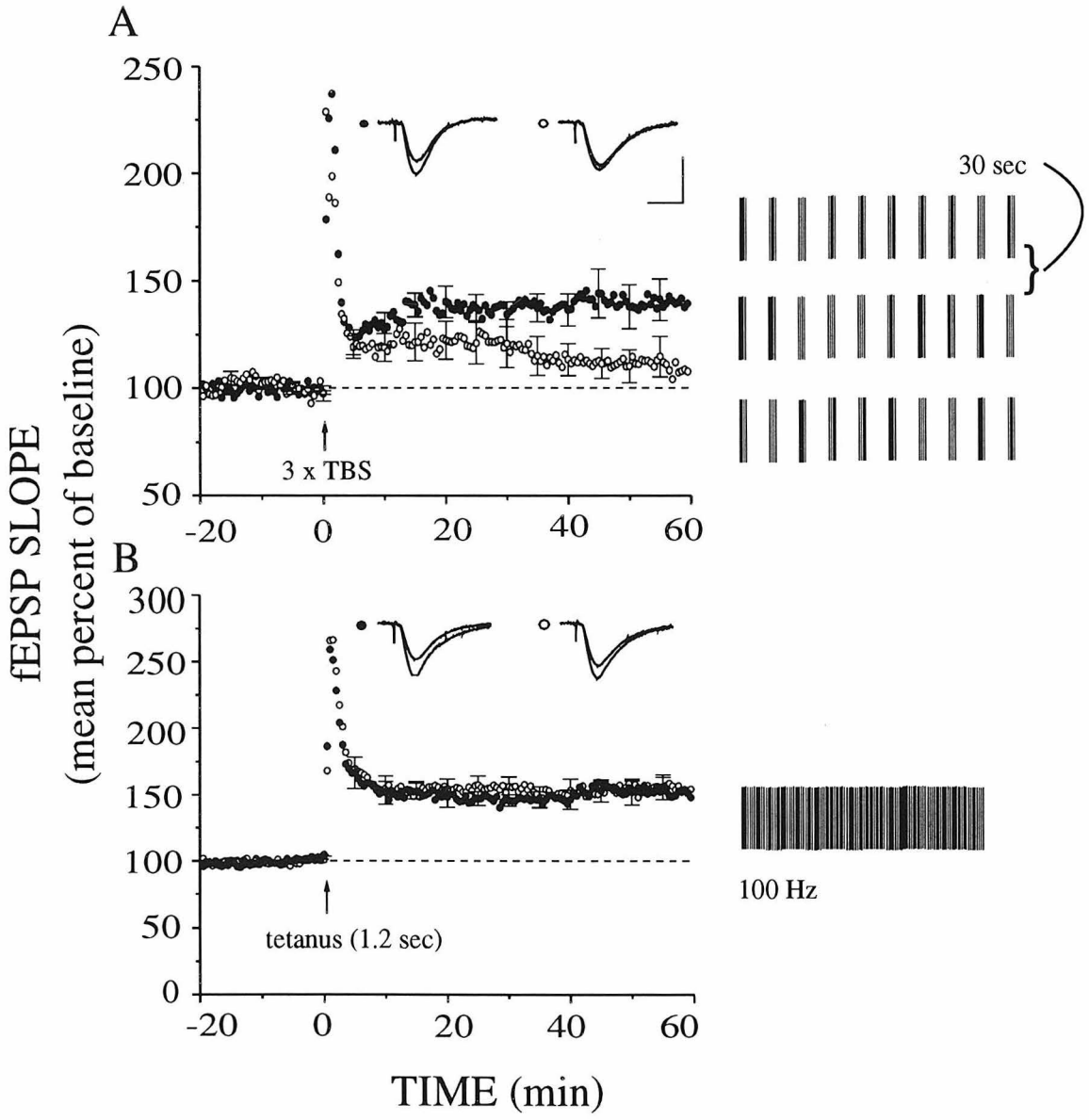

A
250
200
150
100
50
3 x TBS
-20
0
20
40
60
B
300
250
200
150
100
50
0
tetanus (1.2 sec)
fEPSP SLOPE
(mean percent of baseline)
TIME (min)
30 sec
100 Hz

Figure 7. Attenuation of late-phase LTP by function-blocking TrkB antisera and TrkB-IgG.

(**A**) Filled circles indicate the control LTP obtained in preimmune serum-treated slices (n = 8). Mean field EPSP slope was 0.16 ± 0.01 mV/ms (mean ± S.E.M.) before, 0.28 ± 0.02 mV/ms 1 hour after, and 0.23 ± 0.02 mV/ms 3 hours after LTP induction. Filled triangles indicate LTP obtained in TrkB antibody-treated slices (n = 8). Mean field EPSP slope was 0.19 ± 0.01 mV/ms before, 0.28 ± 0.02 mV/ms 1 hour after, and 0.18 ± 0.02 mV/ms 3 hours after LTP induction. Open circles indicate LTP obtained in the presence of TrkB Ab plus anisomycin (n = 8). Mean field EPSP slope was 0.18 ± 0.02 mV/ms before, 0.28 ± 0.03 mV/ms 1 hour after, and 0.19 ± 0.02 mV/ms 3 hours after LTP induction. Superimposed representative EPSPs were recorded 5 min before and 3 hours after L-LTP induction. Calibration bars, 1 mV and 20 ms.

(**B**) Summary of the percent potentiation of mean field EPSP slopes 170-180 min (3 hours) after LTP induction for various groups, as shown. *P < 0.05 versus control (preimmune) group.

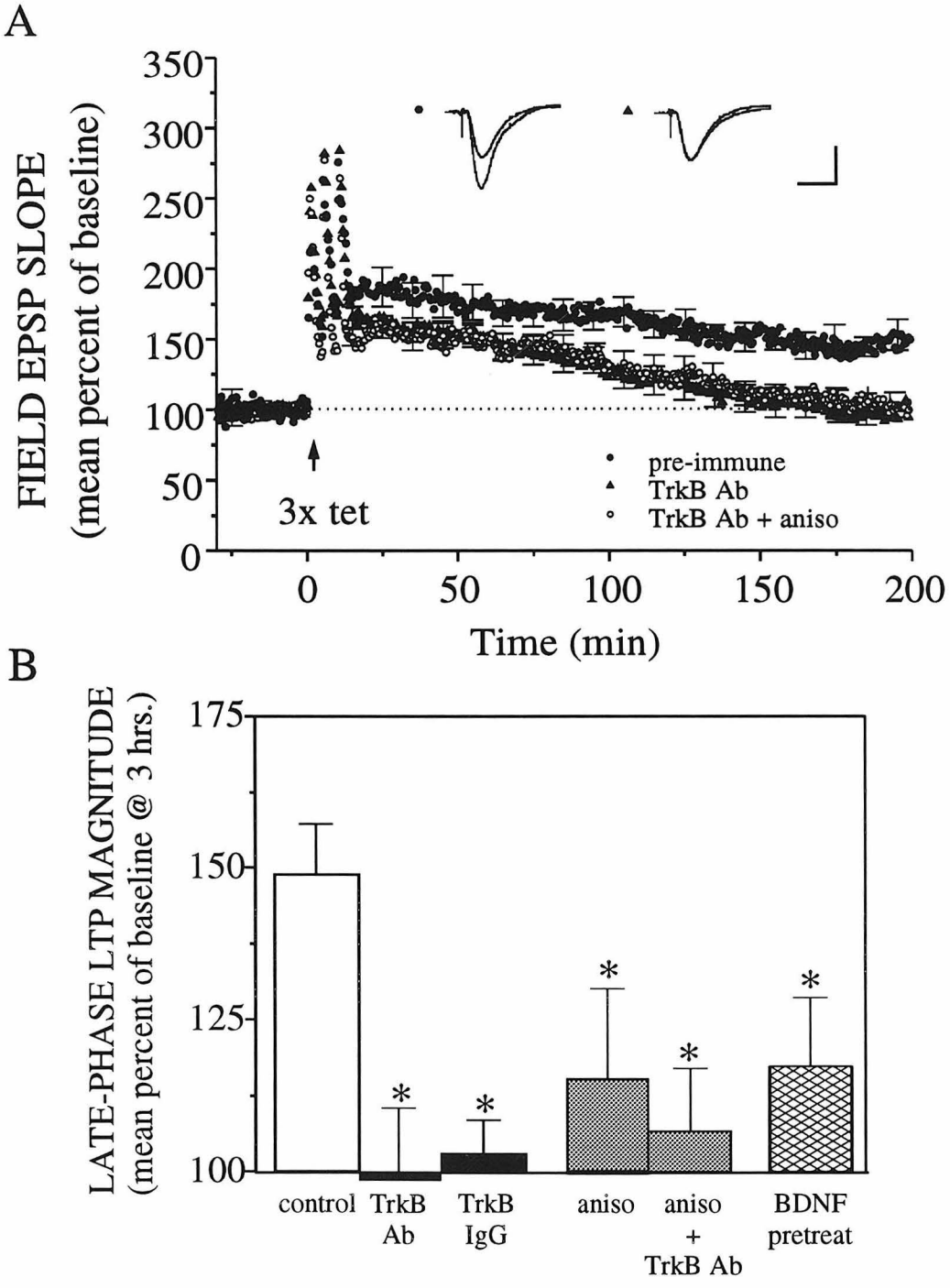

A
FIELD EPSP SLOPE
(mean percent of baseline)
350
300
250
200
150
100
50
0
3x tet
pre-immune
TrkB Ab
TrkB Ab + aniso
0
50
100
150
200
Time (min)
B
LATE-PHASE LTP MAGNITUDE
(mean percent of baseline @ 3 hrs.)
175
150
125
100
control
TrkB Ab
TrkB IgG
aniso
aniso + TrkB Ab
BDNF pretreat

Figure 8. Prior potentiation by BDNF reduces late-phase LTP.

Ensemble average of 6 experiments showing mean percentage of baseline values of field EPSP slopes before and after application of BDNF (50 ng/ml) and before and after application of 3 trains of 100 Hz stimulation to induce late-phase LTP. The amount of potentiation elicited by 100 Hz stimulation in these experiments was significantly less than that observed in control slices ($P < 0.05$; see Figure 7), but not significantly different from TrkB Ab-treated slices (Figure 7). Mean field EPSP slope was 0.11 ± 0.01 and 0.19 ± 0.03 mV/msec, before and 80-90 min after BDNF application, respectively. Mean field EPSP slope was 0.15 ± 0.01 and 0.18 ± 0.02 mV/msec, before and 170-180 min after LTP induction, respectively.

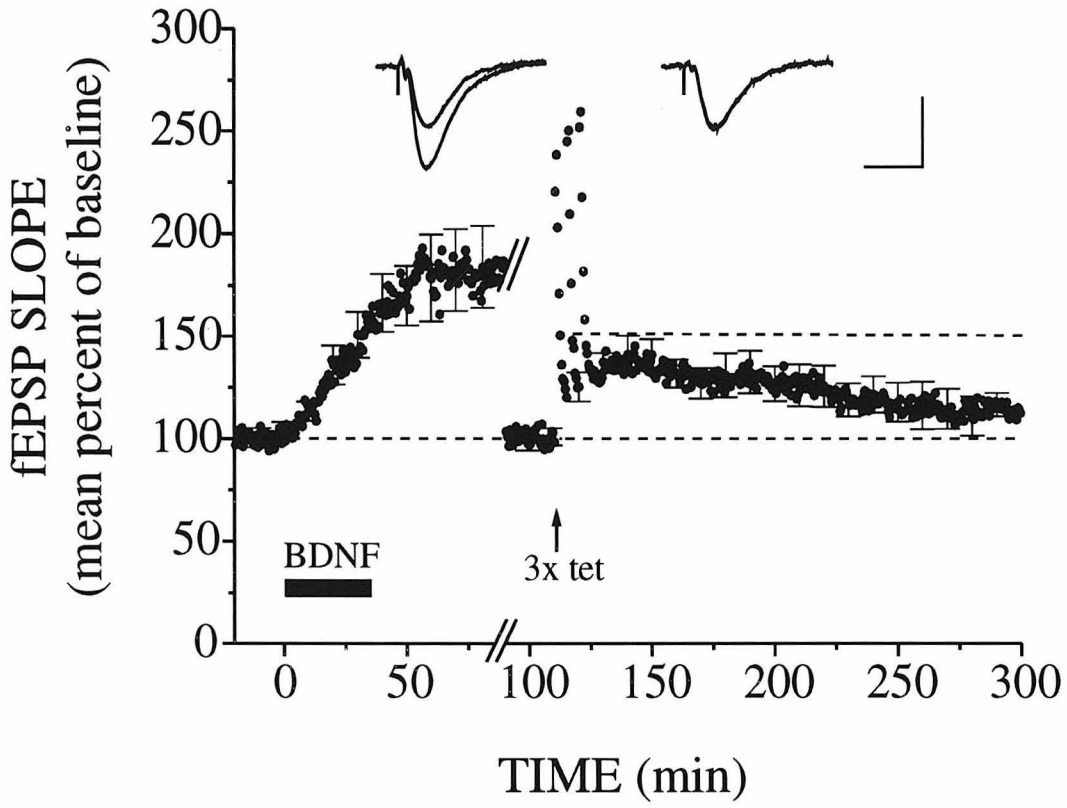

fEPSP SLOPE
(mean percent of baseline)
300
250
200
150
100
50
0
BDNF
3x tet
0
50
100
150
200
250
300
TIME (min)

Figure 9. Effects of TrkA-IgG and TrkB-IgG on late-phase LTP.

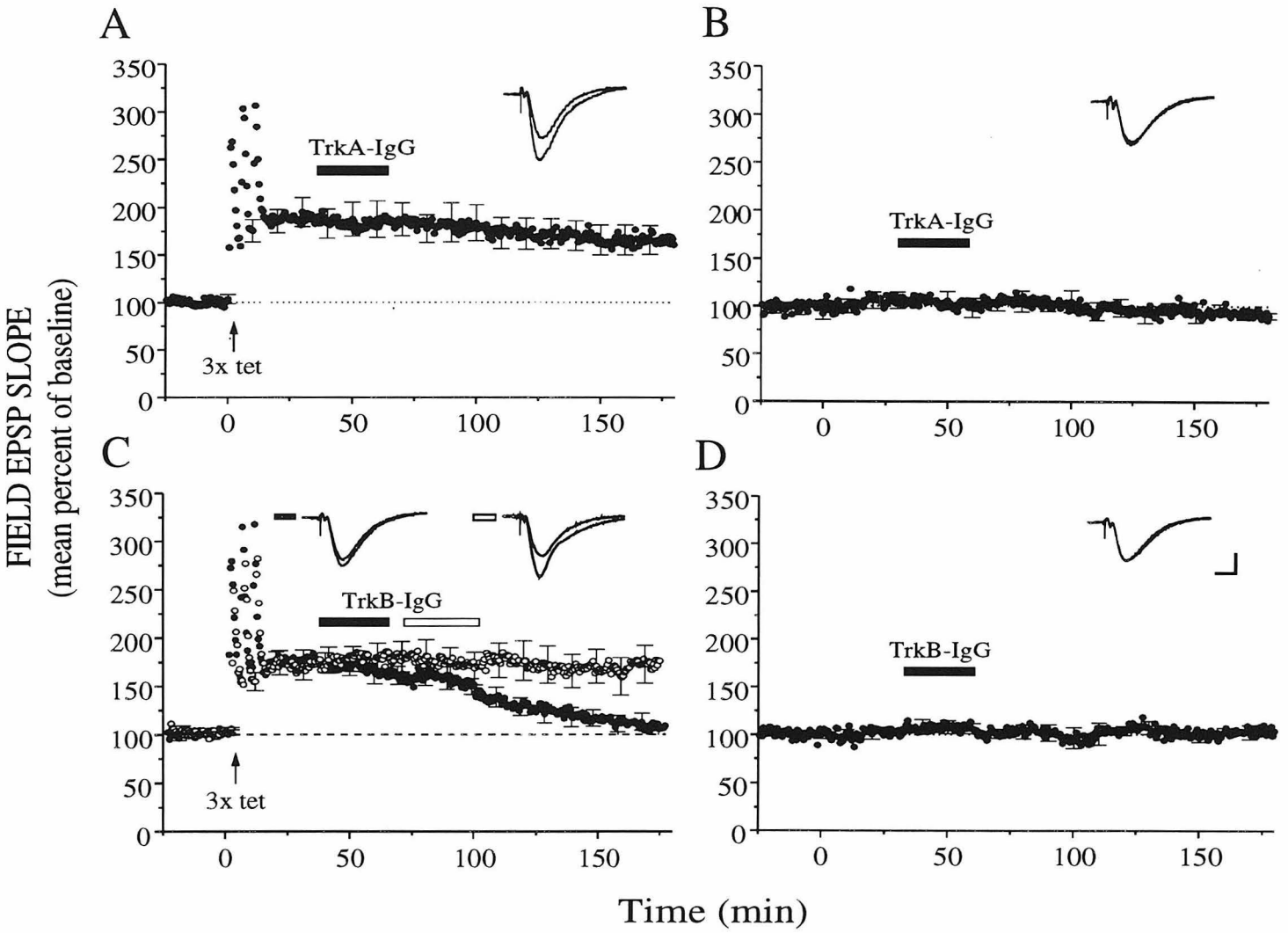
Ensemble averages showing mean percentage of baseline values of field EPSP slopes before and after L-LTP induction in slices treated for 30 min with either TrkA-IgG (**A** and **B**) or TrkB-IgG(**C** and **D**). Superimposed representative EPSPs were recorded 5 min before and 3 hours after LTP induction. Calibration bars, 1 mV and 20 ms.

(**A**) Effects of TrkA-IgG fusion proteins on L-LTP (n = 6). Mean field EPSP slope was 0.17 ± 0.01 mV/ms (mean ± S.E.M.) before and 0.27 ± 0.02 mV/ms 3 hours after LTP induction.

(**B**) Effects of TrkA-IgG fusion proteins on basal synaptic transmission in non-tetanized pathway (n =5). Mean field EPSP slope was 0.18 ± 0.01 mV/ms before and 0.16 ± 0.02 mV/ms 3 hours after LTP induction of pathway 1 (tetanized).

(**C**) Effects of TrkB-IgG fusion protein, applied either 30-60 (closed bar; n = 7) or 70-100 (open bar; n = 8) following induction, on L-LTP. For the 30-60 min application, the mean field EPSP slope was 0.17 ± 0.01 before and 0.18 ± 0.01 mV/ms before and 3 hours after LTP induction, respectively. For the 70-100 min application, the mean field EPSP slope was 0.15 ± 0.01 before and 0.25 ± 0.02 mV/ms before and 3 hours after LTP induction, respectively.

(**D**) Effects of TrkB-IgG fusion proteins on basal synaptic transmission in non-tetanized pathway. Mean field EPSP slope was 0.16 ± 0.01 mV/ms and 0.16 ± 0.01 mV/ms 3 hours after LTP induction of pathway 1 (tetanized).


A
B
C
D
TrkA-IgG
TrkB-IgG
3x tet
FIELD EPSP SLOPE
(mean percent of baseline)
Time (min)
350
300
250
200
150
100
50
0
0
50
100
150

APPENDIX

Role of intracellular calcium elevations in neurotrophin-induced potentiation

The calcium ion (Ca^{2+}) is an important regulatory element in neuronal plasticity and survival. The influx of Ca^{2+} through Ca^{2+}-permeable channels or the release from intracellular stores leads to a local increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) which is coupled to diverse biochemical and electrical events, such as membrane excitability, transmitter release, enzyme activation, and gene expression (reviewed in Mattson, 1992; Tsien *et al.*, 1988). Synaptic transmission between hippocampal CA3 and CA1 neurons is mainly mediated by N-type and P-type, but not L-type, Ca^{2+} channels (Wu and Saggau, 1994, but see Wheeler *et al.*, 1994), although all three types of voltage-dependent Ca^{2+} channels are expressed in the membrane of pyramidal cell bodies and dendrites (Hillman *et al.*, 1991; Mills *et al.*, 1994; Westenbroek *et al.*, 1990). In addition to its role in normal synaptic transmission, an increase in $[Ca^{2+}]_i$ has been implicated in a variety of short-term and long-term plasticity in the hippocampus. The induction of both long-term potentiation (LTP) and long-term depression (LTD) in area CA1 is dependent on the Ca^{2+} influx through NMDA receptor channels (Dudek and Bear, 1992; Malenka *et al.*, 1988). Thapsigargin, which depletes intracellular Ca^{2+} stores by inhibiting ATP-dependent Ca^{2+} uptake, has also been shown to block the induction of LTP (Harvey and Collingridge, 1992), indicating that the NMDA receptors are not the only source of Ca^{2+} necessary for LTP induction.

Since intracellular Ca^{2+} serves as a key regulatory element for many kinds of synaptic plasticity, it was of interest to examine whether the potentiating effects of neurotrophins in the hippocampus may also be mediated by altering $[Ca^{2+}]_i$. The neurotrophins have been found to modulate Ca^{2+} channels and $[Ca^{2+}]_i$ in various central

and peripheral neurons (see introduction). Short application of BDNF and NT-3 to cultured hippocampal neurons results in a rapid rise in $[Ca^{2+}]_i$ (Berninger *et al.*, 1993). Moreover, BDNF-induced synaptic potentiation in developing neuromuscular junctions is accompanied by an increase in presynaptic $[Ca^{2+}]_i$ and can be abolished by removing external Ca^{2+} (Stoop and Poo, 1996). I have examined whether the rise in hippocampal Ca^{2+} concentration can serve as a trigger for the synaptic enhancement induced by BDNF or NT-3.

Synaptic transmission was examined at the Schaffer collateral-CA1 pyramidal neuron synapses in adult rat hippocampal slices with the use of conventional extracellular recording techniques as previously described (Kang and Schuman, 1995; Kang and Schuman, 1996). First, I tested the potential contribution of one class of voltage-dependent Ca^{2+} channels using a specific pharmacological blocker. Pretreatment with L-type Ca^{2+} channel antagonist nifedipine (10 μM), without affecting basal synaptic transmission, almost completely blocked both BDNF (20 ng/ml) and NT-3 (20 ng/ml) - induced potentiation (Figure 1 and 3) [mean percent of baseline at 50-60 min: BDNF, 189.7 ± 16.1 (mean ± SEM, n = 7), $P < 0.005$; BDNF plus nifedipine, 119.9 ± 8.7 (n = 11), not significant (NS), NT-3, 180.0 ± 8.4 (n = 7), $P < 0.005$; NT-3 plus nifedipine, 113.6 ± 5.7 (n = 11), NS]. However, when nifedipine was introduced following the neurotrophin application, it affected neither the magnitude nor the duration of the potentiation established by either BDNF or NT-3 [mean percent of baseline at 50-60 min: BDNF plus delayed nifedipine, 177.9 ± 12.1 (n = 7), $P < 0.005$; NT-3 plus delayed nifedipine, 178.1 ± 11.1 (n = 7), $P < 0.005$]. In fact, the enhancement induced by the

neurotrophin continued to increase in the presence of nifedipine [mean percent of baseline at 80-90 min: BDNF plus delayed nifedipine, 240.4 ± 17.4 (n = 7), $P < 0.005$; NT-3 plus delayed nifedipine, 261.6 ± 10.7 (n = 7), $P < 0.005$], which suggests that Ca^{2+} influx through L-type Ca^{2+} channels is only required for the initiation of the neurotrophin-induced enhancement. It is an interesting question how the neurotrophins exert their effects through L-type Ca^{2+} channels which are known to be activated only at a high voltage (positive to -10 mV), without altering postsynaptic membrane potential or input resistance (Kang and Schuman, 1995). One possibility is that the neurotrophins may shift the voltage-dependence of L-type channel activation as, for example, the L-type agonist Bay K 8644 or phorbol ester do, such that these factors can open L-type channels at voltages close to the resting membrane potential (Parfitt and Madison, 1993). Alternatively, there may exist a novel type of dihydropyridine-sensitive Ca^{2+} channel which has an activation range near the resting membrane potential. Indeed, Johnston *et al.* have recently reported the presence of a low voltage-activated dihydropyridine-sensitive Ca^{2+} current in the dendrites of hippocampal CA1 pyramidal neurons (Johnston *et al.*, 1996).

Since Ca^{2+} influx itself or a signaling factor such as IP_3 can trigger Ca^{2+} release from intracellular stores, I next examined the potential role of intracellular Ca^{2+} stores in neurotrophin-induced synaptic enhancement. To perturb intracellular stores, I pretreated hippocampal slices with thapsigargin (5 μM) for at least 30 min before the addition of a neurotrophin. Application of thapsigargin had no effect on basal synaptic transmission, but greatly reduced the potentiation induced by BDNF or NT-3 (Figure 2 and 3) [mean

percent of baseline at 50-60 min: BDNF, 190.4 ± 11.4 (mean ± SEM, n = 7), $P < 0.005$; BDNF plus thapsigargin, 107.6 ± 8.3 (n = 7), NS, NT-3, 180.0 ± 7.7 (n = 7), $P < 0.005$; NT-3 plus thapsigargin, 108.0 ± 7.9 (n = 7), NS]. To determine if intracellular Ca^{2+} stores contribute to the sustained phase of the enhancement, I performed similar experiments in which thapsigargin was applied after the neurotrophic factor. Slices exposed to thapsigargin 30 min after the introduction of BDNF or NT-3 did not show any significant difference from the control [mean percent of baseline at 50-60 min: BDNF plus delayed thapsigargin, 168.3 ± 8.2 (n = 6), $P < 0.005$; NT-3 plus delayed thapsigargin, 209.3 ± 35.6 (n = 6), $P < 0.05$], suggesting an involvement of intracellular Ca^{2+} stores during the induction phase of potentiation. The complete blockade of neurotrophin effects by inhibiting either of the two different Ca^{2+} sources may indicate that both mechanisms contribute to the threshold Ca^{2+} level needed in order to activate essential downstream signaling components.

The signaling mechanism by which the neurotrophins induce an increase in $[Ca^{2+}]_i$ is yet clear. One potential candidate is the signaling pathway which involves the activation of PLC-γ1. The Trk receptors can trigger activation of PLC-γ1 which hydrolyzes phosphatidylinositol into two important second messengers, diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3). IP_3 may act through IP_3 receptors in the membrane of intracellular Ca^{2+} stores and induce the release of Ca^{2+} (Allbritton *et al.*, 1992). It is promising that hippocampal CA1 pyramidal cells express a high level of IP_3 receptor proteins (Sharp *et al.*, 1993). The other product DG activates protein kinase C (PKC) which has been shown to exert modulatory effects on both N- and L-type Ca^{2+} channels in

the hippocampus (Parfitt and Madison, 1993; Swartz *et al.*, 1993). Since the activation of PKC by phorbol esters enhance synaptic transmission, and this effect is sensitive to nifedipine, it is possible that the neurotrophins may utilize this signaling mechanism together with IP_3-mediated Ca^{2+} signaling to induce potentiation in the hippocampus.

In conclusion, the data presented here suggest that the intracellular Ca^{2+} increase mediated by L-type Ca^{2+} channels and intracellular stores is essential for the induction of potentiation by BDNF and NT-3. The increase in intracellular Ca^{2+} may play a critical role in neurotrophin-induced synaptic enhancement by initiating a variety of Ca^{2+}-dependent second messenger cascades that modify the activity of existing synaptic proteins and furthermore induce a synthesis of new proteins which may be important for synaptic structure or function.

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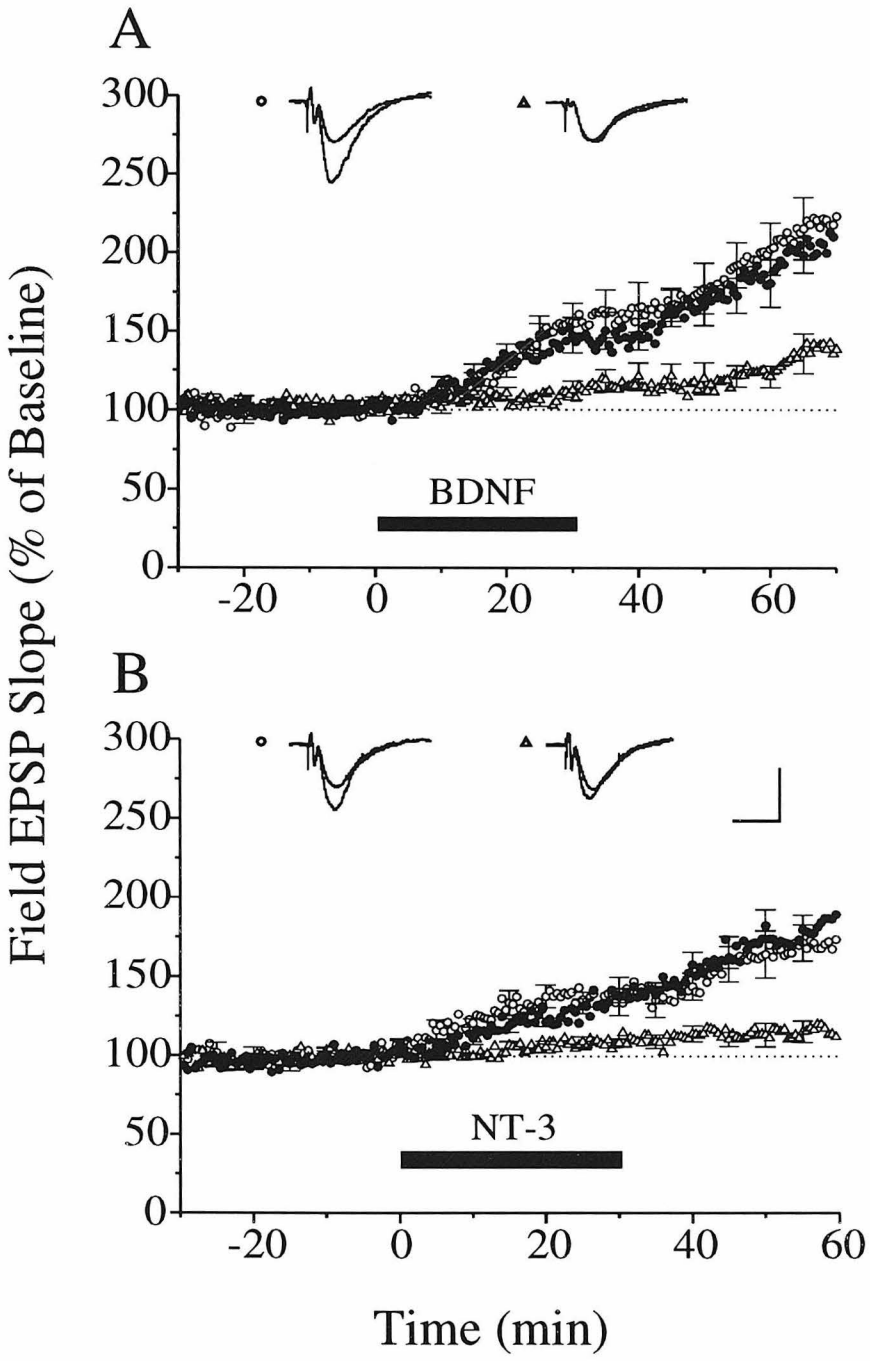
presynaptic voltage-dependent calcium channels at CA3-CA1 synapses of the hippocampus. J. Neurosci. *14(9)*, 5613-5622.

FIGURES AND LEGENDS

Figure 1. The potentiating effects of BDNF and NT-3 are inhibited by prior treatment with nifedipine but not by subsequent treatment. Ensemble average plots for experiments in which nifedipine (10 μM) was applied either 30 min before or 30 min after the addition of BDNF (**A**) or NT-3 (**B**). BDNF or NT-3 (20 ng/ml) was applied for time indicated by a solid bar. Mean value of field EPSP slopes indicated in the figure legends were obtained -10 to 0 min before and 50 to 60 min after the application of neurotrophin. Open circles indicate the control enhancement. Mean field EPSP slope was 0.14 ± 0.01 mV/ms (mean ± SEM) before and 0.27 ± 0.02 mV/ms after BDNF, and 0.12 ± 0.01 mV/ms before and 0.22 ± 0.02 after NT-3. Open triangles indicate the enhancement obtained in slices pretreated with nifedipine. Mean field EPSP slope was 0.13 ± 0.01 mV/ms before and 0.14 ± 0.01 mV/ms after BDNF, and 0.13 ± 0.01 mV/ms before and 0.15 ± 0.01 after NT-3. Filled circles show the experiments in which BDNF or NT-3 was applied for 30 min and then followed by application of nifedipine. Mean field EPSP slope was 0.12 ± 0.01 mV/ms before and 0.21 ± 0.01 mV/ms after BDNF, and 0.12 ± 0.01 mV/ms before and 0.22 ± 0.01 after NT-3. Superimposed representative EPSPs were recorded 5 min before and 60 min after the application of neurotrophin. Calibration bars, 1 mV and 20 ms.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-20
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20
40
60
BDNF
NT-3
Field EPSP Slope (% of Baseline)
Time (min)

Figure 2. The potentiating effects of BDNF and NT-3 are inhibited by prior treatment with thapsigargin but not by subsequent treatment. Ensemble average plots for experiments in which thapsigargin (5 μM) was applied either 30 min before or 30 min after the addition of BDNF (**A**) or NT-3 (**B**). BDNF or NT-3 (20 ng/ml) was applied for time indicated by a solid bar. Open circles indicate the control enhancement. Mean field EPSP slope was 0.10 ± 0.01 mV/ms (mean ± SEM) before and 0.19 ± 0.03 mV/ms after BDNF, and 0.11 ± 0.01 mV/ms before and 0.19 ± 0.01 after NT-3.

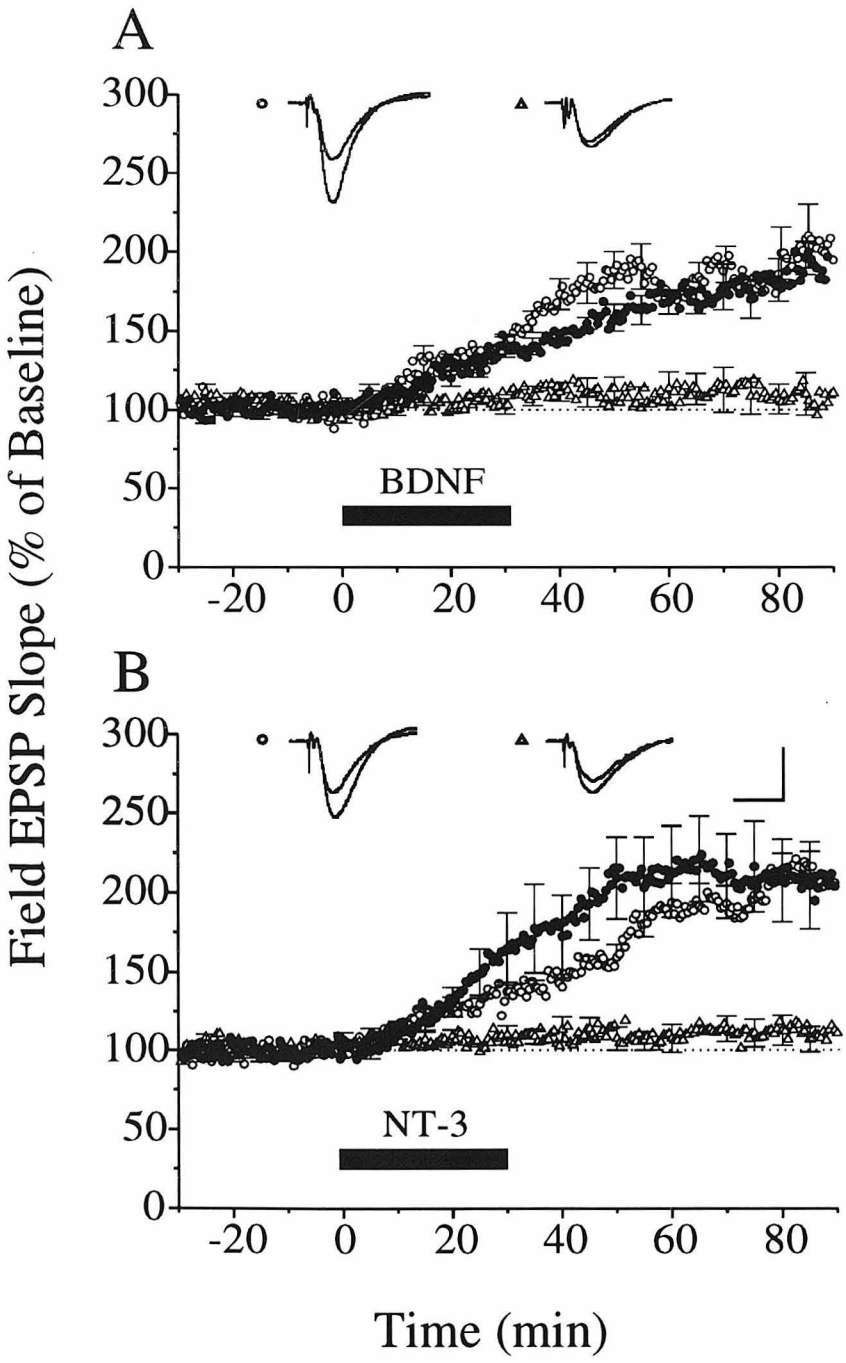
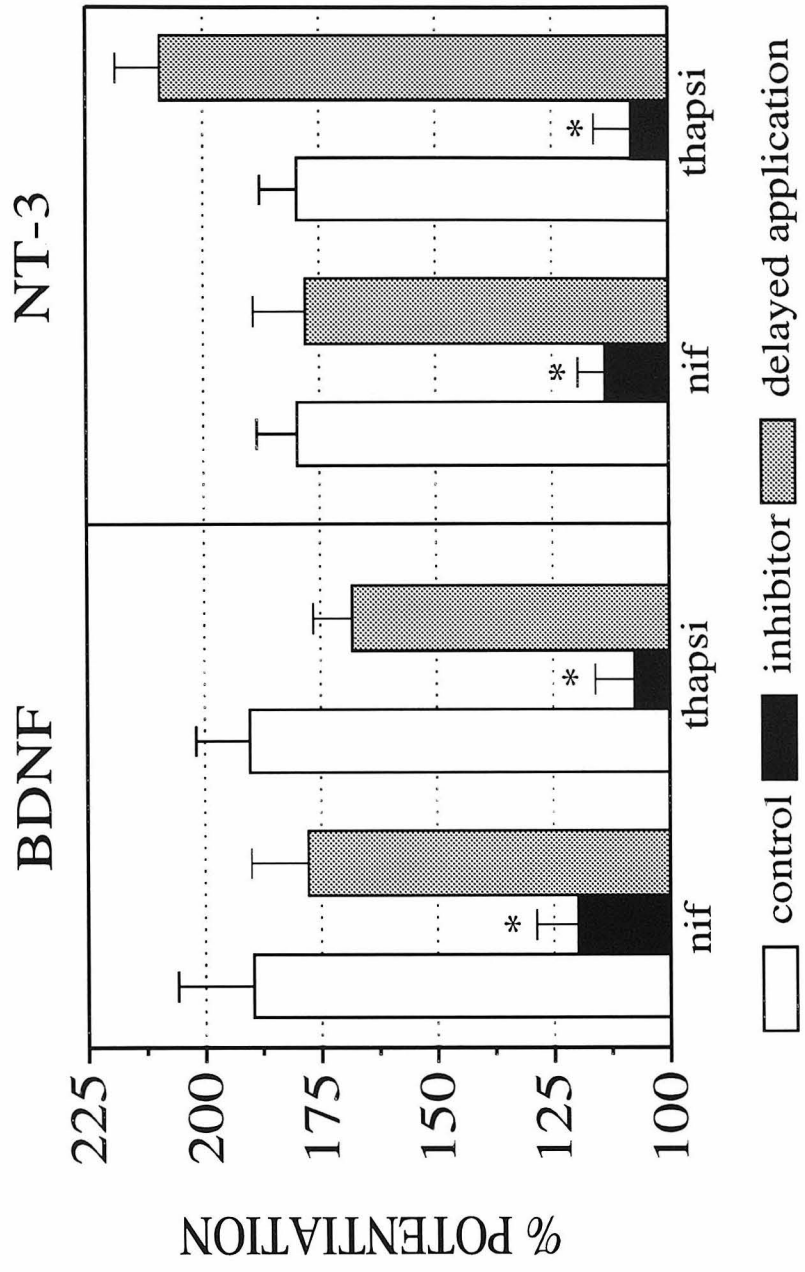

A
B
300
250
200
150
100
50
0
-20
0
20
40
60
80
BDNF
NT-3
Field EPSP Slope (% of Baseline)
Time (min)

Figure 3. Summary graph showing the magnitude of BDNF or NT-3 -induced potentiation in control slices (control), slices pretreated with the inhibitor (inhibitor) either nifedipine (nif) or thapsigargin (thapsi), and slices treated with neurotrophin and then chased with the inhibitor (delayed application). *$P < 0.05$ versus control group.


BDNF
NT-3
% POTENTIATION
225
200
175
150
125
100
nif
thapsi
nif
thapsi
control
inhibitor
delayed application
*
*
*
*