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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Brain-Derived Neurotrophic Factor in Cortical Motor Learning

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Neurosciences

by

Mary von dem Bussche

Committee in charge:

Professor Mark Tuszynski, Chair
Professor Armin Blesh
Professor Andrea Chiba
Professor James Conner
Professor Marla Feller
Professor Nicholas Spitzer

2007

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The Dissertation of Mary von dem Bussche is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2007

DEDICATION

This dissertation is dedicated to my best friend and my partner in life, my husband, Nicolas, and to my two most successful experiments, my beautiful sons, Elias and Micah.

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ABSTRACT OF THE DISSERTATION

The Role of Brain-Derived Neurotrophic Factor in Cortical Motor Learning

by

Mary von dem Bussche

Doctor of Philosophy in Neurosciences

University of California, San Diego, 2007

Professor Mark Tusyznksi, Chair

The molecular mechanisms that mediate learning have not yet been fully identified. The neurotrophic factor brain-derived neurotrophic factor (BDNF) exhibits properties that make it a candidate molecule for investigations of learning-related plasticity. BDNF and its high affinity receptor, TrkB, are widely expressed in the normal adult brain. The expression and secretion of BDNF are regulated by neuronal activity, and it has been shown to induce and facilitate synaptic transmission and synaptic plasticity. BDNF and TrkB are upregulated in association

with many learning paradigms, and BDNF has been shown to be involved in and, in some cases, necessary for the formation of certain types of memory.

The experiments described in this dissertation tested the hypothesis that BDNF exerts a role in normal cortical learning and map plasticity in the adult animal. The experimental paradigm used cortical motor learning, in which normal adult rats were trained in a skilled forelimb reaching task. To date, no study has examined the involvement of BDNF in the context of cortical motor learning, and cortical reorganization concomitant with this learning.

The first set of experiments examined the expression of BDNF and TrkB as a function of motor skill learning, to assess whether learning modulates the transcriptional regulation of these molecules. Using quantitative real-time PCR, BDNF was found to be gradually upregulated over the course of learning, in both skilled and unskilled motor learners. TrkB expression was not modulated.

The next set of experiments utilized two methods of attenuating BDNF signaling in the forepaw motor cortex: lentivirus encoding small interfering RNAs (siRNA) directed against BDNF mRNA, and lentivirus encoding a TrkB receptor body which binds BDNF, rendering it unavailable for normal signaling. Both constructs were first tested *in vitro*. siRNA directed against BDNF reduced BDNF protein levels *in vitro* by 81% compared to uninfected cells and 85% compared to

control virus-infected cells, and the TrkB receptor body was found to significantly attenuate BDNF bioactivity in a cell survival/proliferation assay. *In vivo*, the siRNA construct reduced motor cortical BDNF protein levels by 22% compared to uninjected cortices and 34% compared to control virus-injected cortices, and the TrkB receptor body sequestered BDNF protein to an unknown extent. A putative BDNF blocking antibody was also tested *in vitro*, but it was not found to block BDNF bioactivity.

When injected into the forepaw motor cortices of rats trained in the skilled reach task, neither the siRNA nor the TrkB receptor body had an effect on skill learning or on motor map organization.

These combined results lead to the conclusion that, although BDNF expression is modulated by motor learning, reducing the availability of BDNF protein in the motor cortex by 22% using siRNA, or binding extracellular BDNF to an unknown extent using a TrkB receptor body, does not affect motor learning or motor map organization. Techniques that more extensively reduce BDNF signaling in the motor cortex would likely be required to reach definitive conclusions regarding a potential role of BDNF in modulating motor cortical learning and map plasticity.

Chapter 1: Introduction

The ability to use past and present experience to alter future behavior (learning) is one of the essential functions of the brain. Much attention on learning and memory has been focused on the hippocampus, but ablation experiments have demonstrated, and many have asserted (1-7), that for many types and stages of memory, brain loci other than the hippocampus are involved. There is much evidence implicating the cerebral cortex as a locus of, and potentially the ultimate storage site of, various types of memories. Indeed, learning, and in particular, procedural learning has been shown to have cortical underpinnings (reviewed in 8-13). Although the basic neural mechanisms underlying learning and memory are largely unknown, adaptive changes (plasticity) are thought to be a prerequisite. I propose a set of experiments designed to test the hypothesis that molecular, behavioral, and electrophysiological plasticity occur within the cortex during a procedural learning experience, and that the neurotrophic factor brain-derived neurotrophic factor (BDNF) has a role in mediating this plasticity.

Memory Systems

Before narrowing the focus of this discussion to procedural motor learning and the role of BDNF therein, it is worthwhile to devote some attention to broader theories of memory formation.

Declarative Memory

A discussion of memory systems in the brain cannot occur without first addressing the role of medial temporal lobe structures. The hippocampus has been studied largely in the context of its role in spatial and declarative memories. Declarative or explicit memories are memories for facts and events, and they are distinct from procedural or implicit memories (3), which are the focus of the current studies. Despite these differences, much can be learned about the mechanisms associated with hippocampal declarative memory formation, storage, and particularly consolidation, which may be generalized to the study of learning and memory of all types.

One especially relevant phenomenon which has been observed repeatedly over decades in hippocampal declarative memory is that of temporally graded retrograde amnesia (reviewed in 1, 2). In studies of both humans and animals with damage to the hippocampus or related medial temporal lobe structures, it has been observed that more recent memories are disrupted, whereas more remote memories are relatively spared. The more remote a memory, the more preserved it is, indicating that over long periods of time, memories, even declarative ones, become gradually independent of the hippocampal formation. It should be noted that this interpretation is somewhat controversial. For example, there are studies indicating that patients with hippocampal

damage had impaired remote memories (14, 15). However, the patients in these studies may have suffered anatomical damage extending to regions beyond the hippocampus and immediately adjacent structures (16).

The sparing of remote memory after medial temporal lobe damage, and the fact that it is unlikely that the hippocampus has the storage capacity to contain all declarative memories, suggests that it is not the final storage site for these memories. Which other brain regions might store long-term, consolidated declarative memories? The leading hypothesis, for which there is much experimental evidence, is that as time passes after learning, memory is stored in neocortex. Indeed, lesion studies in humans show that cortical areas are necessary for declarative memories. Patients with lesions including the lateral temporal cortex (17, 18), frontal cortex (19, 20), and occipital cortex (21) demonstrated impaired performance on declarative memory tasks, such as autobiographical recall, memory for generally known facts and events, and visual memory for objects.

One hypothesis put forth to explain the relationship between the hippocampus and the cortex is that memories are initially stored in the hippocampus, and then, through a process of consolidation and reciprocal activity with the cortex, the memories become entirely represented in the cortex and entirely independent of the hippocampus (6, 22). An alternative scenario is that even newly formed declarative memories are not stored in the hippocampus; it may be that, at least initially,

hippocampal neurons store only information sufficient to point to and activate the relevant sites in neocortex, rather than storing the details themselves (23-25). Eventually, repetitive interactions between the neocortex and medial temporal lobe co-activate widespread cortical areas, binding neural representations associated with an experience to each other so that they eventually develop long-lasting linkages between detailed memories without hippocampal mediation (23, 26, 27). In either scenario, the hippocampus has a time-limited role, with memory function eventually relegated entirely to the cortex.

Indeed, there is reciprocal connectivity between the hippocampus and adjacent cortical areas, which, in turn, have widespread reciprocal connectivity to neocortical areas, forming the basis of models of memory consolidation describing the gradual shift from hippocampal-dependence to hippocampal-independence (4-7). Several studies have provided convincing evidence for this hypothesis. In one study (28), mice were trained on a hippocampus-dependent spatial learning task. Using 2-deoxyglucose uptake, brain activity was monitored following retrieval of either recent (retested at five days after training) or remote (retested at 25 days after training) memories. Retrieval of recent spatial memories produced more robust hippocampus activation than remote memories. In contrast, several neocortical areas studied, including the prefrontal, frontal, and temporal cortices showed the opposite pattern of activation, with more activation during remote memory tests.

One study (29) showed that hippocampal lesions in rats caused a large deficit in trace conditioning when made early (one day) but not late (four weeks) after training. In contrast, lesions of the medial prefrontal cortex had no effect one day after training but led to impairments when made at the four-week time point, suggesting that the hippocampus was necessary for the formation and/or early processing of the memory, whereas the cortex stored the memory at a later stage. Another study (30) using a hippocampal inhibitory avoidance task elegantly showed the sequential role of the hippocampus and amygdala, followed by the entorhinal and parietal cortices, in both the formation and retention of memory. The first set of experiments was carried out using bilateral, local inactivation with 2-Amino-5-phosphonovalerate (AP5), an N-methyl-D-aspartic acid (NMDA) receptor antagonist, or muscimol, a gamma-aminobutyric acid (GABA) receptor agonist. The authors found that inactivation of the hippocampus and amygdala at 0 minutes after training impaired memory formation, as did inactivation of the entorhinal or parietal cortices at three hours after training, implicating these structures, first the hippocampus and amygdala, followed by the cortices, in short-term memory formation. Another set of experiments involved acute, pre-test inactivation using 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), an alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor blocker which abolishes the majority of glutamatergic synaptic transmission. Twenty-four hours after training, and immediately before retesting, CNQX inactivation of the hippocampus and amygdala led to a retrograde amnesia, whereas acute, pre-test inactivation of these structures at 31 or 60 days after training had no effect. Further,

acute inactivation of the cortical regions caused impaired performance at 24 hours, one month, and, in the case of the parietal cortex, 60 days after training. These data indicate again that the role of the hippocampus is time-limited. They also, however, indicate that the cortex is necessary both early after training, as well as in the long term, providing evidence that the cortex is not only the ultimate storage site of memories, but that it is involved in the establishment of these memories as well.

If the hippocampal-cortical consolidation hypothesis is correct, and memory traces are co-activated repeatedly to mediate this consolidation, when might repetitive interactions between the medial temporal lobe and the neocortex take place? Such events might interfere with moment-to-moment conscious thought processes, but much evidence has accumulated that memory traces are reactivated and thus consolidated during sleep. Patterns of brain activity that are associated with earlier learning have been shown to be selectively replayed during subsequent sleep in humans (31-33), non-human primates (34), rodents (35-41), and songbirds (42). This "offline" processing during sleep has indeed been shown to improve performance on many types of memory tasks.

Non-Declarative Memory

Hypotheses have thus far been discussed describing loci of the formation and storage of declarative memories. Non-declarative memories, including certain

procedural and emotional memories, are thought not to involve medial temporal lobe structures at any point.

Many studies point to an explicit dissociation between hippocampal and non-hippocampal types of memories (43-45). An elegant human case study was done describing a patient who, after contracting viral encephalitis, suffered extensive bilateral damage limited primarily to the medial temporal lobes, with a resulting inability to learn new information about facts and events (46). In this study, the patient was presented with factual pattern information during the course of 12 weeks. While he performed much more poorly than controls, he demonstrated improvement across the training period, but he was not consciously aware of which answers were correct, nor could he respond when the pattern was slightly changed, as could control subjects. The authors' interpretation of this finding is that factual information, which is ordinarily learned as declarative knowledge and with the participation of the medial temporal lobe, can be acquired as nondeclarative memory. The authors suggest that this patient's learning occurred directly within the neocortex.

Other forms of implicit memory include habit learning, which is thought to be mediated by the neostriatum and cerebellum (8, 44), and emotional learning and conditioning, which involve the amygdala (47-49). The subject of the present investigation is a type of procedural learning, which will be discussed in detail in coming sections.

As a final note, if declarative memories initially involve the hippocampus, but then, like procedural memories, are finally stored in the cortex, why might the hippocampus be needed for declarative memories at all? In a review on functional brain imaging and memory (50), it was suggested that skill learning might involve modifications of motor representations confined to a single area, whereas declarative memories might require the binding of many association areas, each storing unique aspects of a single memory. The hippocampus might be a convergence point for such disparate areas, and may therefore be in a unique position to bind the traces and designate them as being part of the same memory.

The Cerebral Cortex and Memory

As has been discussed, the neocortex is likely the storage site for many types of memories. Since many memories involve the representation of past sensory events, it is reasonable to hypothesize that they may be stored, at least in part, within cortical sensory systems. Although sensory systems have traditionally been viewed as stimulus processors, with learning and memory assigned to 'higher' cortical regions, neurophysiological studies have produced evidence for learning-induced plasticity in sensory cortices.

Many studies have shown that sensory regions of the cerebral cortex undergo learning-related plasticity, ranging from synaptic plasticity, such as long-term potentiation and depression, to gross cortical reorganization (reviewed in 51), such that sensory representations are expanded or contracted in response to alterations in input or behavior. This has been shown in a variety of studies in the visual cortex (reviewed in 52, 53), wherein visual receptive fields contract or expand, the somatosensory cortex (reviewed in 53, 54), wherein cortical maps of the skin surface are reorganized, and in the auditory cortex (reviewed in 55), wherein tonotopic representations change with increased or decreased experience of particular sound frequencies. Cortical motor areas also undergo robust learning-related plasticity, which will be discussed in detail in the coming sections.

There is also considerable evidence that plasticity is manifest as structural changes in the cortex, such as synapse formation and elimination, dendritic growth, and axon remodeling (reviewed comprehensively in 56). These structural changes are seen secondary to a variety of manipulations, such as LTP-like synaptic enhancement, enriched environments, simple sensory stimulation, sensory deprivation, such as results from digit and limb amputation or lesions of peripheral sensory organs, and, finally, behavioral paradigms in which learning occurs.

Cortical Motor Learning Paradigm

The mammalian cerebral cortex comprises a combination of discrete and widely distributed networks of neurons capable of a variety of computational tasks. As a result of its heterogeneity of function and connectivity, it is a difficult preparation to study. In this setting, meaningful information can best be gleaned by focusing on a specific cortical area, and by using a tractable learning paradigm. I have chosen to examine learning in the forepaw area of the rat motor cortex in the context of a procedural motor learning task.

As a model of cortical motor learning, I have chosen the Whishaw Forepaw Reach Task, in which rats are trained to reach for and grasp a food pellet through a small slit in a plexiglass cage (57). This task is well-suited to the investigation of cortical plasticity as successful performance on this task has been shown to be dependent upon the integrity of the forelimb area of the rat motor cortex (58, 59). Further, our own and others' studies utilizing this and similar tasks have shown that the forepaw region of the motor cortex undergoes electrophysiological and structural plasticity in association with motor skill learning (57, 60-66). Finally, it has been shown in our laboratory and by others that, after rats have learned this forepaw reach task, the body map in motor cortex contralateral to the trained or preferred paw undergoes reorganization, with the caudal forelimb representation or parts of the caudal forelimb area expanding relative to representations of other body parts (60, 61, 63).

Motor Cortex and Plasticity

Unlike primates, the rat motor cortex is not necessary for movements such as walking, grooming, and manipulating food, but is necessary for learning and performing skilled motor movements which require a high degree of dexterity (59, 67). Perhaps in the rat, the primary role of the motor cortex is in mediating more refined movements, and/or in the establishment of altogether new movements. The motor task with which the subjects of the present studies have been challenged requires that they learn a new motor skill that is not in their natural repertoire.

The architecture of the motor cortex, among other traits, makes it a well-suited substrate for the processing presumably required to learn new motor skills. Layer II/III motor cortical neurons receive input largely from the thalamus or from distant cortical areas, such as sensory cortex. Layer V motor cortical neurons receive input from layer II/III neurons. Some layer V neurons are devoted to local intracortical processing, and others are devoted to output to the spinal cord, striatum, and other areas (68). In the context of skilled reaching, a motor learning arc could exist such that, when an animal tries to reach for a pellet, visual and tactile sensory input is received from sensory cortices by layer II/III neurons, which is then used to calculate the error between the location of the paw and digits relative to the location of the pellet and the current motor output. This "processing" may occur at the level of the input of the signal, within layer II/III, or at the connections between layer II/III and layer V, or intracortically within layer V, or, at some combination of the above. The

error signal calculated could then be used to modify motor output from layer V. The reach can thus be iteratively and gradually corrected.

Indeed, it is known that as rats learn the skilled forelimb reach task, learning modifies motor cortical circuits in the form of morphological and synaptic plasticity. In studies using this paradigm, a subpopulation of superficial layer II/III neurons showed increased complexity of basilar dendrites (66), and input to layer V motor neurons (apical dendrites) showed increased length and number of branches in the trained vs. untrained hemisphere (57). Skilled reaching also caused increased synaptogenesis, which was localized to the caudal forelimb area of the motor cortex (64). Finally, skilled reach training lead to an increase *in vivo* in the size of evoked field potentials in the trained, and not the untrained, hemisphere (65). It is not clear, however, whether these synaptic and structural changes were required for learning, were a byproduct of learning, or were simply coincidental with but unrelated to learning.

Motor Map Plasticity

The idea that the motor cortex is organized such that body movements are represented in an exactly ordered, point-to-point topography, as depicted by the classical homunculous cartoon, is now known to be an over-simplification. Current data show that there is a segregation within the motor cortex of body regions, namely,

the hindlimbs, forelimbs, and head, but that within these regions, there is no precise topography (69-74, reviewed in 75). Motor map organization can be thought of as a fractured somatotopy, where individual movements are represented multiple times and where these representations are interspersed within a cortical area devoted to a body region. Each body part subdivision has a widely distributed network in which control emerges from broad activity patterns. This distributed cortical network allows for large cortical allocation to individual movements, and therefore, provides immense cortical capacity for learning-induced modification and storage.

In fact, the notion of a static motor representation within the cortex is no longer viable. Motor maps are labile, vulnerable to disappearing acutely (76) and able to shift on a moment-to-moment basis (77-79). Motor maps are in a use-dependent state of flux (80, 81), and their expression seems to depend on the constant support of constitutive processes, such as protein synthesis (76).

It is known that the region of the motor cortex devoted to forelimb movements expands when animals learn the skilled reach (60, 61, 63), but it is not clear whether this map reorganization is causative, resultant, or just correlational. Cortical map reorganization secondary to motor skill learning is a much-observed phenomenon, occurring in non-human primates as well as in humans. In one such study using squirrel monkeys (80), learning of a skilled digit manipulation task caused an expansion of digit representations.

In humans, map reorganization has been associated with raquetball playing (expanded hand representation, 82), skilled piano playing (expanded digit representation, 83), motor sequence learning (84, 85), Braille reading (expanded hand representation, 86), and even a skilled tongue protrusion task (87). In one study, the human primary motor cortex topography changed such that muscles involved in a serial reaction time test, wherein subjects were prompted to execute a repeating button press sequence, were over-represented (85). This was not due simply to the motor requirements of the task, as the cortical maps of control subjects performing the task without a repeating sequence did not change over trials.

Synaptic Plasticity

Memory systems at the level of circuits and of brain regions have thus far been discussed. But what are the cellular and synaptic mechanisms that may underlie this system-level plasticity?

Martin, et al (88) articulate a synaptic plasticity and memory hypothesis:

> *Activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed.*

The most thoroughly studied forms of activity-dependent synaptic plasticity are long-term potentiation (LTP; 89) and long-term depression (LTD; 90), in which synaptic potentials increase or decrease in amplitude as a result of brief patterns of high-frequency (in the case of LTP), or low-frequency (in the case of LTD) stimulation. These forms of synaptic plasticity may also be induced in a Hebbian manner, via the pairing of presynaptic activity with postsynaptic depolarization. These changes in synaptic efficacy must persist for some period of time after induction in order to be considered "long-term". The length of this period of time varies across studies, and is dependent upon experimental preparation and induction mechanism, among other experimental parameters.

The synaptic plasticity and memory hypothesis, or some form thereof, is the prevailing assumption under which most, but not all (see 91-93), researchers are currently working as they investigate the mechanisms underlying memory formation.

There is much experimental support, albeit largely indirect, connecting LTP and memory. Evidence comes from studies focusing on synaptic alterations following learning experience, on saturation or occlusion of LTP and LTD following learning, and on pharmacological and genetic manipulations of LTP which affect learning (reviewed in 88, 94-99). Studies reviewed provide evidence that certain forms of learning (such as in a radial-arm maze, 100) lead to LTP-like increases in synaptic efficacy, that learning prevents further modulation of synaptic strength (65), and,

conversely, that experimental enhancement of synaptic strength prior to training impairs learning (such as in a circular maze, 101, or a water maze, 102). Some studies also show that genetic manipulations (such as CamKII knock-ins and knock-outs, 103-105) known to interfere with the induction or expression of LTP also lead to impairments in learning.

LTP/LTD in the Motor Cortex

If these cellular mechanisms account for motor cortical plasticity at the cellular level, it must first be demonstrated that they do in fact occur in the motor cortex. Indeed, LTP and LTD (106-127) have been induced in motor cortical slice preparations as well as *in vivo*. Stimulation of sensory pathways (118, 119, 122) can elicit motor cortical LTP, as can tetanization of vertical pathways paired with horizontal activation (119, 120), and stimulation of horizontal pathways with reduction of inhibition (120). LTP can also be induced between superficial and deep layers within the motor cortex (121). LTD can be elicited by low-frequency stimulation alone (114). It is also known that induction of LTP in the motor cortex *in vivo* leads to increased spine density and altered dendritic morphology (123-127). Finally, skilled reach training leads to an occlusion of LTP and a facilitation of LTD *in vivo* in the trained versus untrained hemisphere (65).

Introduction to BDNF

While LTP and LTD are working models of synaptic plasticity, the molecular systems in the brain that mediate plasticity have yet to be fully defined. A number of proteins have been implicated in processes leading to short or long lasting changes. Synaptic proteins, such as neurotransmitter receptor subunits, neurotransmitter vesicle components, and synaptic scaffolding proteins are obvious and proven targets of, and mediators of, modification (128-136). Components of intracellular signaling cascades, such as kinases and phosphatases, leading from the synapse to the nucleus, are also mediators of cellular change (137-147). Within the nucleus, transcription factors (147-151) and, subsequently, genes involved in all levels of cellular plasticity have been shown to exert a role in processes resulting in change.

Another logical candidate class of molecules for modulating neural plasticity in the context of learning and memory is the neurotrophins. Neurotrophins are a family of structurally related, secreted proteins that regulate the survival, differentiation, and maintenance of function of different populations of peripheral and central neurons (152-155). The neurotrophin family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Each neurotrophin binds to one or more of the tropomyosin-related kinase (trk) receptors, members of a receptor tyrosine kinase family (reviewed in 156-158). BDNF was the second neurotrophin to be

characterized, after NGF. It was first purified in 1982 from pig brain, when it was shown to promote survival of a population of dorsal root ganglion neurons (159).

Among the neurotrophins, BDNF is of particular interest, for reasons that will be further detailed further in the coming sections. As previously noted, activity-dependent changes in synaptic strength appear to underlie cellular and synaptic plasticity. BDNF stands out among the neurotrophins in that both its expression and its secretion are tightly regulated by neuronal activity (160-166). In contrast to BDNF, a clear correlation between neuronal activity and expression and secretion of NGF, NT-3 or NT-4/5 has not yet been established (167-170). Even NT-4/5, which, like BDNF, is a ligand for TrkB, is not well-suited to play as large a role in the adult brain as is BDNF. In fact, it has been shown using a two site enzyme-linked immunosorbent assay sensitive to NT-4/5 at the 1 pg/ml level that NT-4/5 protein is barely detectable in the post-natal rat brain (171).

BDNF binds with highest affinity to TrkB (156-158, 172-175). TrkB exists in full-length (TrkB.FL) and truncated (TrkB.T1, TrkB.T2) forms lacking a kinase domain (176, 177). Despite their lack of enzymatic activity, truncated TrkB receptors have been shown to have physiological effects, such as an increase in the release of acidic metabolites (178), outgrowth of filopodia and neurites in neuroblastoma cells *in vitro* (179), and elongation of distal dendrites in ferret visual cortex slices (180). Notably, the truncated TrkB.T1 appears to act as a dominant negative inhibitor of

normal TrkB signaling, leading to effects such as interference with BDNF intracellular signaling (181) and synaptic potentiation (182), an increased susceptibility to cortical injury (183), inhibition of axon regeneration (184), decreased cell survival (185, 186), decreased cell surface expression of full-length TrkB (187), and impaired spatial memory (188).

When BDNF binds full-length TrkB, the receptor dimerizes, resulting in kinase activation. The receptor is then autophosphorylated on multiple tyrosine residues, creating binding sites for various intracellular target proteins. These, in turn, initiate several signal transduction cascades such as the mitogen-activated protein kinase (MAPK) pathways, the phosphatidylinositol 3-kinase (PI3K) pathway, and the phospholipase C-γ (PLC-γ) pathway. These pathways promote neuronal survival, differentiation, and plasticity, and act differentially and in combination in the nervous system to mediate functions as diverse as neuronal development and programmed neuronal cell death. The actions of one of these pathways, the Ras-MEK-ERK1/2 cascade, has been implicated in mechanisms of plasticity, and so will be discussed in further detail in a separate section (see Chapter 5).

In addition to high-affinity binding to TrkB, BDNF also binds a non-tyrosine kinase receptor, $p75^{NTR}$ (189). BDNF signaling through $p75^{NTR}$ receptor binding mediates functions distinct from those of TrkB activation, most notably, the initiation of programmed cell death (189). It has also been suggested that $p75^{NTR}$ may

determine neurotrophin binding specificity to Trk (190-192). Because the work described here is focused on BDNF's plasticity-related roles, BDNF-mediated TrkB activation will be the focus.

BDNF and its high affinity receptor, TrkB, are widely distributed throughout the central nervous system (193-203) and BDNF protein is actively transported anterogradely by the cells which produce it (195, 204-207). BDNF/TrkB complexes are also thought to be transported within the cell in order to mediate retrograde signaling from the axon to the cell body (208).

Endogenous BDNF expression and BDNF release are not static. In fact, BDNF levels are upregulated by a variety of processes. BDNF mRNA and/or BDNF protein have been shown to be increased by manipulations such as direct neuronal stimulation, kainic acid infusion, GABA receptor antagonists, seizures, stimulation of sensory receptors, and, in the case of the visual system, both spontaneous activity and light (164, 170, 209-220, reviewed in 160, 163, 166, 221). In addition, BDNF signaling itself upregulates BDNF levels (222). Activity-dependent release of BDNF protein has also been observed (161, 162, 165). In fact, activity-dependent transfer of BDNF from a pre- to a post-synaptic cell has been directly demonstrated using GFP-tagged BDNF (161, 162).

Transcription of BDNF in response to neuronal activity is regulated by calcium. A cAMP response element (CRE) is located upstream of the BDNF coding sequence, which CREB may bind to initiate BDNF transcription (223). In fact, BDNF has been referred to as an immediate early gene (IEG) because of its transcriptional responsiveness to calcium. This has implications for BDNF's role in translating synaptic signals resulting in acute and local shifts in calcium concentration to processes which may aid in the establishment of short- and long-lasting synaptic and neuronal modification.

It is often proposed that mechanisms of plasticity in the adult nervous system may recapitulate mechanisms of development in the immature nervous system. Though the focus of the investigation described here is the role of BDNF signaling in the adult brain, its role during the development of the nervous system has been extensively studied and is essential to the development of the animal, and is therefore worthy of mention. BDNF homozygous knock-out mice fail to survive beyond three weeks after birth, and, among other neuronal phenotypes, lack specific populations of peripheral sensory neurons (224, 225). In addition to being essential for the survival of those neurons, BDNF exerts its role in nervous system development by promoting the survival and growth of many other types of neurons, such as hippocampal and cortical neurons (211, 226), cerebellar granule cells (227), mesencephalic dopaminergic neurons (228-230), cholinergic neurons (228, 231, 232), and GABAergic neurons (reviewed below). BDNF also has a role in the development of

certain brain circuitry, such as normal patterning of ocular dominance columns during the critical period of visual cortex development (233, 234). The patterning deficits that occur when BDNF signaling is disrupted in this system may be related to axon pathfinding dysfunction.

The role of BDNF in the developing motor cortex is unclear. It has been shown that BDNF, unlike NT-4/5, glial cell line-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF), does not promote the survival of neonatal rat corticospinal neurons *in vitro* (235), nor does BDNF, in contrast to NT-3, promote sprouting of early post-natal corticospinal neurons *in vivo* (236). However, BDNF was shown in one study to promote axon branching and arborization, an effect eliminated by the addition of TrkB antibodies, though it had no effect on axon outgrowth in cultures of early postnatal corticospinal motor neurons (237). In this study, BDNF had no effect on the size of neuronal somata, further suggesting that it did not exert an effect on neuronal viability. In yet another study using a pure population of callosal projection neurons (CPN) *in vitro*, BDNF was shown to promote cell survival (238), in contrast to the earlier finding. Finally, the same group found that BDNF led to an increase CPN dendritic branching and soma size, but that it did not alter axonal or dendritic length (239). Taken together, these data suggest that BDNF may have effects on developing motor cortical neurons, though there are some contradictory findings as to what those effects are.

BDNF and Neuronal Structure

The role of BDNF in affecting structural neuronal changes has been best explored in the developing nervous system. BDNF regulates axonal growth (240-242), and also plays a role in establishing spine and dendrite numbers and morphology during development (243-251, but see also 252). Forebrain restricted BDNF or TrkB knock-out mice were shown to have reduced cortical and hippocampal volumes (244, 253, 254). In these studies, no initial changes in neuronal number were observed, but rather a reduction of cell soma size and dendritic complexity. In the human, a valine to methionine substitution in the BDNF gene (BDNFMet) was shown to lead to anatomical reductions in the volume of the hippocampus (255, 256), cortical regions, and subcortical structures (255), with associated memory impairments. Though these anatomical abnormalities were studied using structural magnetic resonance imaging (MRI), which is not of sufficient resolution to assess underpinnings of the volume reductions at the cellular level, given BDNF's role in regulating cell survival and dendritic structure, and given the findings in genetically altered mice, these changes may have resulted from a number of processes, including decreased volume of dendritic arborization, increased cell death or decreased neurogenesis, or a combination of the above during embryological development or over the lifespan of the brain.

Though to a lesser extent, BDNF has also been associated with structural modification of adult neurons. It has been shown, for example, to regulate the maintenance of spines in adult visual cortex (257). The authors of this study used transgenic mice expressing a dominant negative TrkB-EGFP fusion protein in sparse pyramidal neurons of the visual cortex and CA1 field of the hippocampus, producing a Golgi-staining-like pattern. They found that in the visual cortex, attenuated BDNF signaling resulted in a reduced number and reduced persistence of the more mature and stable mushroom spines, accompanied by an increase in the number of the presumptive immature long and thin spines and filopodia. The authors found that this was not true in the hippocampus, in agreement with an earlier study using conditional TrkB knock out mice, which showed that attenuation of BDNF signaling did not affect the number of adult hippocampal Schaffer collateral synapses (258). Another study of the role of BDNF in the adult utilized BDNF heterozygous knock-out mice (259). Though levels of BDNF were reduced in these mice from development, electron microscopy in the adult barrel cortex revealed no effect of BDNF reduction on synapse density, spine morphology, and synaptic vesicle distribution. However, the structural plasticity that wild type mice undergo after intense whisker stimulation was disrupted, implicating BDNF in mechanisms of structural plasticity in the adult.

BDNF and Synaptic Transmission

This review has thus far examined the endogenous expression and induced expression of BDNF in the adult brain and its role in modulating neuronal structure. Perhaps the most striking aspect of BDNF, among other neurotrophins and among neuromodulators in general, is its robust effects on the basic function of a neuron – synaptic transmission.

BDNF enhances glutamatergic synaptic transmission (reviewed in 260-262) in cultured cortical neurons (263-268), cultured cerebellar neurons (269, 270), cultured hippocampal neurons (268, 271-278), and in visual cortical slices (279, 280). A BDNF-mediated increase in both evoked and mini (spontaneous) excitatory synaptic potentials is accomplished both pre-synaptically, by stimulating glutamate release, but also at the post-synapse, by changing the phosphorylation states of post-synaptic glutamate receptor channels (268, 272, 276). These BDNF-induced channel modifications have been shown to result in an increased open channel probability and an increased opening frequency (273).

BDNF has not only been shown to increase sub-threshold post-synaptic potentials, but also to bring hippocampal, cortical, and cerebellar neurons to threshold and cause action potentials (281). This stimulation was powerful, occurring just as rapidly with BDNF as with glutamate, and at a more than thousand-fold lower concentration.

Finally, BDNF knock-out mice exhibit basal synaptic transmission deficits which are partially rescued by BDNF (282). Using extracellular field potential recordings of the Shaffer Collateral-CA1 pathway in hippocampal slices, the authors found that basal synaptic transmission was reduced in the BDNF mutant mice and that this reduction reflected gene dosage; homozygotes were more severely compromised than heterozygotes, which were compromised relative to wild-type littermates. Incubation of the slices with BDNF increased basal synaptic transmission, though not to the level of wild-type slices.

BDNF and LTP/LTD

As has been discussed, LTP and LTD have been proposed as a cellular mechanism underlying learning and memory. This is still a matter of active investigation (283). Nonetheless, there is much evidence of an association between these forms of synaptic plasticity and BDNF.

As was mentioned previously, BDNF transcription is regulated by neuronal activity. It is also upregulated by particular patterns of activity that elicit LTP. Protocols which elicit LTP have been shown to upregulate BDNF mRNA in the hippocampus, both *in vitro* and *in vivo* (284-287). In one of these studies in which LTP was induced in the dentate gyrus of awake rats (284), TrkB (and TrkC) mRNA were up-regulated, followed by a delayed upregulation of BDNF (and NT-3, but see

285) mRNA. This indicates a possible mechanism whereby shorter-term LTP first establishes a means (in the form of increases in the availability of BDNF receptors) by which subsequent BDNF upregulation can act to stabilize and possibly potentiate the increase in synaptic efficacy for longer-term periods.

Interestingly, and relevant to the discussion in the next section of BDNF's association with both excitatory and inhibitory systems, BDNF expression was also found to be up-regulated by a protocol which caused long-term desensitization of AMPA receptors, a mechanism of LTD (288). This upregulation was assayed using RT-PCR in cultured cerebellar slices, and was found to peak four hours after the induction protocol.

LTP and LTD are able to regulate BDNF expression, and, conversely, BDNF is able to regulate LTP and LTD. In hippocampal slices, BDNF enhanced LTP, whereas blocking it reduced LTP (289, 290). In these studies, BDNF's action seemed to occur via enhancement of the ability of hippocampal synapses to respond to LTP induction, rather than a direct modulation of the LTP induction mechanism. In other studies, BDNF has been shown to not only enhance but induce LTP in adult hippocampal slices (290, 291), and in hippocampal cultures (292). Further, blocking BDNF using a TrkB fusion protein or a BDNF blocking antibody was found to reduce LTP in hippocampal slices (293). Notably, this effect was found to be specific to BDNF. NT-3 blocking antibodies did not affect LTP. Also, because the attenuation

of LTP by the TrkB fusion protein, which would affect both BDNF and NT-4/5 signaling, reduced LTP to a similar degree as did the BDNF antibody, which specifically blocks only BDNF, an inference can be made that NT-4/5, though also a ligand for TrkB, did not modulate this effect. In developing visual cortical slices, BDNF was shown to enhance LTP induced by a 20 Hz tetanus (294), and to be necessary for long term maintenance (for periods greater than 30 minutes) of LTP (295). *In vivo*, BDNF was shown to induce LTP in the insular cortex (296) and in the hippocampus (297), and to enhance LTP in the intact visual cortex (298). Finally, rats injected with BDNF antisense oligonucleotides into the dentate gyrus exhibit reduced LTP (299).

As BDNF enhances or elicits LTP, it has also been shown to block LTD in hippocampal cultures and slices (292, 300), as well as in cultured visual cortical neurons (301). One study found that BDNF had no effect on LTD in developing visual cortical slices *in vitro* (295), but other studies have shown that it can, in fact, attenuate LTD in visual cortical slices (294, 302, 303), and that BDNF (but not NT-4/5) can also block LTD in the developing visual cortex *in vivo* (304). Notably, and contradictory to the results in visual cortical slices, this study showed that low frequency stimulation of the visual cortex *in vivo* was not able to induce LTD, an effect that was attributed to endogenous BDNF after infusion of a TrkB fusion protein was shown to permit induction of LTD. The authors of this study tested the hypotheses that temperature differences, or the presence of spontaneous activity from

the retina could account for the differences between slice and *in vivo* preparations, but they did not.

Several studies have been described, all done in the rat visual cortex, investigating the role of BDNF in LTD induction, with contradictory results. The *in vitro* studies (294, 295, 301-303) disagree as to the effects of BDNF modulation of LTD, and in the *in vivo* study (304), low frequency stimulation could not induce LTD in the endogenous environment. The authors of the *in vivo* study found that levels of TrkB and phosphorylated Trks decrease in slices with time, confounding any study of LTD, and, in particular, the role of BDNF therein. This finding might account for the discrepancies in these studies, and would argue that visual cortical slice preparations may not be ideal for the study of low frequency stimulation-induced LTD.

In addition to the reciprocal relationship of BDNF and LTP/LTD discussed above, studies using genetically altered mice also show that BDNF is necessary for full expression of LTP. BDNF and TrkB knock-out mice show deficits in hippocampal LTP (305, 306) which can be restored by reintroducing BDNF into the preparation (282, 307, 308). Further, BDNF +/- mice have impaired LTP in the visual cortex in slice (280) and *in vivo* (309).

Though this section focuses on the relationships between LTP/LTD and BDNF, it should be noted that BDNF potentiates neuronal responses non-synaptically

as well. A change in the excitability of neurons, in addition to LTP/LTD, is a model of cellular plasticity that may underlie learning and memory (310). Neurons are able to increase intrinsic ionic conductances, which serves to increase the excitability of these neurons in response to periods of low activity (311). Activity was shown to regulate the intrinsic excitability of cultured cortical neurons through changes in the release of BDNF (312). The increase in firing rates produced by activity blockade was prevented by bath application of BDNF, whereas application of a TrkB fusion protein mimicked the effects of activity blockade. Thus, the role of BDNF in modulating neuronal plasticity can be both synaptic and non-synaptic.

BDNF and GABA

Though the role of BDNF in development has been discussed, as has its expression and function in the adult, particular attention should be drawn to its role in inhibitory signaling. During CNS development and in the adult, BDNF plays an important role in the survival, differentiation, and function of GABAergic neurons, which may have profound effects on the overall function of neuronal signaling and neuronal plasticity in the adult.

BDNF has been shown to increase the number of neurons which differentiate to a GABAergic phenotype (230, 313-318), and/or the survival rate of GABAergic neurons (230, 314, 315, 317, 319). BDNF can elicit the GABAergic phenotype in a

number of ways. It can lead to increased expression of GABA receptor subunits (320-324), and to increased levels of glutamate decarboxylase (GAD), the enzyme which synthesizes GABA (324-328). BDNF has also been shown to affect the morphology of developing GABAergic neurons (313, 315, 317), including the size of their somata (316, 324, 329, 330), the morphology of their dendrites (329, 331-333), and the number or size of their synapses (275, 329, 333, 334).

As has been noted in earlier sections, BDNF can upregulate glutamatergic excitation, but it has been shown that this is not only true in glutamatergic cells. BDNF has also been shown to upregulate AMPA-mediated currents in cultured neocortical GABAergic neurons, effectively rendering the GABAergic cells more sensitive to input and therefore facilitating their inhibitory functions. In the same study, and in agreement with that result, GABAergic neurons from BDNF heterozygous knock-out mice exhibit a reduction in EPSP amplitude (335).

BDNF can also increase GABAergic signaling in a variety of ways. It can increase GABA receptor clustering at the synapse (336), and it can facilitate release of GABA from the presynapse (324). BDNF can increase GABAergic synaptic transmission, as measured by the frequency and/or amplitude of evoked and/or spontaneous inhibitory post-synaptic potentials (IPSPs) (275, 320, 322, 329, 337).

In some preparations, BDNF actually suppresses GABAergic inhibition. In the developing superior olive, BDNF mediates long-lasting inhibitory synaptic depression (338). In hippocampal slices, BDNF decreases IPSCs (339), and in the developing mouse superior colliculus and in hippocampal cultures, BDNF has the same effect, mediated by acute downregulation of GABA(A) receptors (340, 341). In mouse cerebellar neurons, BDNF decreased short-term GABAergic activity by changing receptor kinetics, and then longer-term inhibition by downregulating surface GABA receptors (342).

There are many ways in which the same molecule, BDNF, signaling via the same receptor, TrkB, may exert different actions. For example, BDNF can elicit nonspecific neurotransmitter vesicle exocytosis. Indeed, in visual cortical synaptosomes, BDNF was shown to enhance the depolarization-evoked release of acetylcholine, GABA, and glutamate (265).

Other mechanisms may account for seemingly opposing actions of BDNF on synaptic activity. For example, it may act by activating different combinations of intracellular signaling cascades, as in (343). In that study, BDNF was found to potentiate excitatory and inhibitory systems separately, increasing GABAergic signaling via MAPK activation, and increasing glutamatergic signaling via MAPK and PLC-gamma activation. In another study, glutamate release was potentiated and

GABA release attenuated via Ca2-dependent and Ca2-independent mechanisms, respectively (344).

Temporally regulated control may also contribute to BDNF's varied actions. In one study, BDNF was found to promote clustering of both NMDA receptors and GABA receptors, and to increase the proportion of receptor clusters apposed to presynaptic terminals. In this hippocampal culture preparation, the BDNF-mediated GABAR clustering preceded the NMDAR clustering, and, in fact, GABAergic activity was found to be necessary for the upregulation of NMDAR clusters (336). In another study, BDNF first mediated an enhancement in IPSC amplitude, followed by a prolonged inhibitory current depression in cultured rat hippocampal and cortical neurons. Again, this is thought to occur via different intracellular mechanisms – enhancing GABAergic signaling on a fast time-scale by GABA receptor phosphorylation and then more chronically attenuating it by decreasing GABAR cell-surface stability (345).

Another mechanism by which BDNF can exert differential effects on GABAergic function is related to developmental stage. One study found that the action of BDNF on GABA(A) currents changed gradually from potentiating to suppressing as rat hippocampal CA1 pyramidal neurons matured (346) from post-natal day 6, to 10, to 14. This change is concurrent with the shift, at the end of the first postnatal week, from depolarizing to hyperpolarizing GABA currents (347, 348).

Thus, both the potentiation of GABA(A) currents by BDNF observed at P6 and the attenuation observed at P14 may lead to increased activity in the developing hippocampus. The mechanism of the shift of BDNF-mediated potentiation to inhibition may be related to the finding that the ability of BDNF to occlude GABA(B) receptor-mediated inhibition of GABA release develops between P7 and P14 (349).

Finally, BDNF may have distinct presynaptic actions depending on the phenotype of the postsynaptic cell. In hippocampal culture, BDNF was shown to induce rapid and persistent potentiation of glutamate release when the postsynaptic neuron was glutamatergic, or excitatory (E-->E), but not when it was GABAergic, or inhibitory (E-->I) (350). Interestingly, the post-synaptic target-specific action of BDNF was found at different outputs of a single presynaptic neuron innervating both glutamatergic and GABAergic postsynaptic cells, suggesting that individual presynaptic terminals can be independently modified. The likely mechanism is a locally generated and locally read-out signal from the postsynapse. A similar effect is seen in visual cortical slices (351).

As is evident from the findings described above, there are many ways whereby BDNF signaling can affect GABAergic activity. GABA-mediated signaling can also influence BDNF, suggesting the existence of feed-back or feed-forward mechanisms. Activation of GABA-A receptors has been shown to upregulate levels of BDNF (352). GABAergic activity can also have differential effects on BDNF synthesis. In the

developing hippocampus *in vitro*, GABA switches from enhancing to suppressing BDNF expression as the hippocampal neurons mature (353, 354). As in the cortex, hippocampal inhibitory cells do not themselves produce BDNF, but BDNF from neighboring pyramidal cells enhances their differentiation. As the GABAergic neurons differentiate and mature, they, via paracrine negative feedback, begin to suppress instead of enhance BDNF expression. Other studies corroborating this found that in more mature hippocampal cultures, stimulation of the GABAergic system reduces BDNF mRNAs (163, 218).

The role of BDNF in GABAergic modulation has probably been most elegantly investigated in the developing rat visual cortex, specifically, in terms of the role of activity in the development of normal visual patterning. In the first in a series of studies on the topic, it was shown that activity blockade in visual cortical cultures (mimicking dark rearing in the whole animal) reduced the percentage of GABA-positive neurons (by about 30%) and the magnitude and frequency of inhibitory currents. Exogenous BDNF (and not NT-3 or NGF) prevented this reduction, indicating that the activity blockade –mediated reduction in GABAergic inhibition was a result of reduced endogenous production of BDNF (355). The same group later reported that BDNF had opposite effects on quantal amplitude at inhibitory versus excitatory synapses. When activity levels were low and BDNF levels were reduced, synaptic strength was scaled up to promote excitation onto excitatory neurons, and, when activity levels increased and BDNF became more available, synaptic strength

was scaled up to promote excitation onto inhibitory neurons, which should in turn have resulted in increased inhibition onto pyramidal neurons (356). In another set of studies, overexpression of BDNF in transgenic mice was shown to reverse many of the effects of dark-rearing, i.e., activity deprivation. In the visual cortex in the context of visual deprivation, BDNF accelerated the maturation of GABAergic innervation and inhibition and consequently the age-dependent decline in white-matter stimulation-induced LTP (357). It also restored visual acuity, the receptive field size of visual cortical neurons, and the critical period for ocular dominance plasticity, such that dark-reared transgenic mice were indistinguishable from light-reared littermates (357, 358).

Animals reared in the dark also show a reduction in the expression of synaptic proteins. Though BDNF application in normally reared animals was not able to induce expression of these proteins, it was able to restore GRIP and GAD to normal or super-normal levels in dark-reared animals (359). GRIP may be involved in AMPA receptor clustering, but it has also been found in GABAergic synapses, indicating that it might regulate both excitatory and inhibitory synaptic function (360).

Another related study on the effects of activity-deprivation also found BDNF to be ameliorative. In this case, blocking neural activity in hippocampal cultures reduced the amplitude of IPSCs, corresponding with diminished sizes of GAD puncta. BDNF was able to reverse these activity-dependent effects (361).

The studies described thus far were carried out in developing neurons, such as in embryonic or postnatal animals, or in cell culture of immature neurons. But evidence of BDNF's role in mediating GABAergic function exists in the adult as well. And, as in development, BDNF can have opposing effects on inhibitory signaling, in some contexts enhancing it, and in others attenuating it. Examples of the latter are seen in adult rat hippocampal slices (362), and in adult hypothalamus (363), where BDNF reduced GABA-mediated IPSCs, In the hypothalamus, this is thought to occur via reduction of the surface expression of the GABA(A) receptor. Examples in the adult where BDNF enhances GABAergic signaling are seen in rat striatal slices, where it increased evoked GABA release (364), and in the substantia nigra *in vivo*, where BDNF infusions increased the size of GABAergic cells and their expression of GAD (365).

As described above, BDNF has been shown in many different neurons, and at stages from development through adulthood, to be able to up- *and/or* downregulate GABAergic activity. In some preparations, it has been shown to simultaneously do both. Though at first this may seem like the makings of a controversy on BDNF's role in GABAergic modulation, it can also be viewed as a fine example the biological principle that context determines function.

BDNF and Learning

As has been discussed above, BDNF has been shown to enhance, facilitate, or induce LTP, and it has also been shown to up- and downregulate inhibition and GABAergic function. Whether LTP and LTD are the synaptic correlates of learning or not, the most relevant evidence for my hypothesis are the many lines of data indicating that BDNF is upregulated with, and in some cases necessary for, certain types of memory formation.

Learning and Memory Upregulate BDNF Expression

The studies described above have shown that BDNF expression is upregulated by neuronal activity and by protocols which modulate synaptic efficacy. It is desirable, however, to relate BDNF function more directly to behavior at the whole animal level, and not only at the synaptic and cellular levels. Indeed, numerous studies show that BDNF can be upregulated by learning.

Using RT-PCR, BDNF was shown to be increased in the hippocampus subsequent to a one-trial fear-motivated learning task in rats, called inhibitory avoidance training (366). In rat hippocampal-dependent contextual learning, *in situ* hybridization revealed a rapid and selective increase in BDNF expression (367).

Nuclease protection assays showed an increase in TrkB mRNA after spatial learning in the water maze (368). In this latter study, message for synapsin 1 was also upregulated, in parallel with TrkB. Synapsin I is a synaptic vesicle-associated protein thought to facilitate neurotransmitter release downstream of BDNF/TrkB-mediated phosphorylation (369). In another study using a nuclease protection assay and *in situ* hybridization, BDNF mRNA was shown to be elevated in the rat hippocampus, but not in the cerebellum, striatum, or neocortex after 3 and 6 days of training in a water maze task (370). Both BDNF and TrkB mRNAs were upregulated in the cortex of monkeys during (but interestingly, not after) tool use learning (371, 372), and BDNF and TrkB protein levels were upregulated in the cerebellar and motor cortices after acrobatic motor learning (373).

Pavlovian fear-conditioning has also been shown to upregulate BDNF mRNA in the amygdala (374, 375). As shown using *in situ* hybridization in one study and BDNF enzyme-linked immunosorbent assay in another, BDNF mRNA and protein levels were found to be elevated temporarily in the amygdala during the period following fear conditioning, peaking at one to two hours after training, and returning to baseline by four hours after training. In comparison, levels of BDNF protein did not change in the hippocampus or cortex with this manipulation (375), nor did mRNA levels of three other neurotrophins (NGF, NT-4/5, and NT-3) change significantly in the amygdala (374). Further, the authors of both studies found by using Western blotting or immunoprecipitation that levels of the activated, or phosphorylated form of

TrkB rise in the amygdala following fear conditioning, whereas levels of total TrkB protein do not change.

This upregulation of BDNF signaling occurs not only in different brain regions, but across learning task and species. For example, it was also shown in the context of social recognition memory formation in cortical and limbic regions in sheep (376). Sheep form a selective recognition memory for their lambs within two hours of birth, which is initially exclusively based on olfactory cues, but which subsequently extends to incorporate visual cues. By 2-3 hours following the initial formation of olfactory recognition memory, BDNF and Trk-B expression are upregulated in brain areas involved in the consolidation of olfactory memory, as well as in areas involved in visual memory, even before behavioral recognition is made using visual cues. This suggests that in visual areas, BDNF and/or TrkB upregulation may have a permissive role in the establishment of visual memory.

Modulation of BDNF expression by learning has been shown to be temporally specific and restricted to animals that exhibited good performance in an inhibitory avoidance task (299). In animals which exhibited what the authors call “good memory”, BDNF mRNA was not increased at 0.5 hours after training, but was increased at one hour, three hours, and six hours after training as compared to in animals who exhibited “poor memory”. At 24 hours, BDNF levels in both groups were comparable. BDNF levels in animals with poor memory were not modulated at

any time point. These data indicate that a short-term consolidation process occurred shortly after learning and was associated with increased BDNF transcription.

BDNF protein and mRNA were assessed via enzyme immunoassay (EIA) and RT-PCR, respectively, and were shown to be increased at 15 minutes and 30 minutes after the final trial in an eight-arm radial maze task (377). This upregulation was specific to the hippocampus; there was no learning-related BDNF modulation in the frontal cortices of trained rats.

Finally, in another study, monkeys were trained on a visual pair-association memory task and on a control visual discrimination task, and various regions of their brains assayed for expression of BDNF, TrkB, nerve growth factor (NGF), and neurotrophin-3 (NT-3). BDNF, specifically, was found to be upregulated in the association task by approximately 30% in a cortical visual association area (378).

BDNF Effects on Learning and Memory

In addition to regulation by learning, BDNF has been shown to be able to modulate learning, a persistent finding which is the primary impetus for the experiments described in the upcoming chapters.

As was mentioned previously, BDNF homozygous knock-out mice do not live long past the postnatal period, but partial genetic ablations of BDNF have yielded insights into BDNF's causal role in memory formation and learning.

Studies in BDNF heterozygous mice (BDNF +/-) are controversial. One study showed deficits in contextual fear conditioning that were partially rescued by infusing BDNF protein into the hippocampus (379), whereas another study showed no deficits relative to controls (380). One group found that BDNF +/- mice also showed deficits in spatial learning in the Morris Water Maze, but not in memory retention (381). A different group (382), however, found no impairments in BDNF +/- mice in water maze or elevated plus maze learning. Of note, BDNF heterozygous knock-out mice have been shown to exhibit abnormal eating behavior and locomotor activity (383), making interpretation of their learning deficits problematic.

Given these contradictory findings in BDNF +/- mice, which are confounded by background strain differences and unknown lasting effects in the adult of BDNF reductions during the critical period of development, methods to genetically manipulate BDNF signaling in a more limited way, and methods to attenuate BDNF signaling using other means have yielded more interpretable findings. For example, forebrain-restricted BDNF knock-out mice exhibited impairments in water maze spatial learning and pattern discrimination (384). Of note, in these mice, cued-contextual fear conditioning was enhanced. This might have been due to increased

anxiety in these mice, leading to increased freezing behavior. However, when they were tested in other tasks that could indicate a high level of anxiety, such as an open-field task or an elevated plus maze task, these mice did not differ from controls. A postnatally activated, forebrain restricted TrkB knock-out also led to impaired learning in a water maze task and to partial impairments in the eight-arm radial maze, but did not impair simple passive avoidance learning (306), suggesting a role for BDNF signaling in more complex learning. In an inducible BDNF knock-out, removing BDNF in adulthood from broad forebrain areas, including the cortex, hippocampus, amygdala, and striatum, lead to impairments in contextual but not cured fear conditioning (385). It should be noted that in this study, removal of BDNF earlier in life lead to a hyperactive phenotype, which might explain decreased freezing in studies where BDNF is reduced earlier in life.

Eye-blink conditioning, a form of associative learning for which the site of memory formation and storage is thought to be the cerebellum, is impaired in a spontaneous mutant mouse with cerebellar BDNF deficiency (386, 387). Both alleles of this mutation, generating the stargazer and waggler mice, exhibit a selective deficit in cerebellar granule cell BDNF expression without apparent abnormality in cerebellar cytoarchitecture, and the eye-blink conditioning learning impairment was shown to occur in the absence of sensory or performance deficits. In humans, a valine to methionine polymorphism in the BDNF gene (BDNFMet) was found to be associated with impaired episodic memory (388, 389), which *in vitro* studies have shown might

be related to disruptions in BDNFMet subcellular sorting and, ultimately, secretion from the cell (388, 390). Carriers of the polymorphism were shown to perform worse on hippocampal-dependent memory tasks, with concomitant anatomical abnormalities of their hippocampi. As discussed earlier, in studies of brain morphometry using structural MRI scans, BDNFMet individuals were shown to have a smaller hippocampal volume relative to controls who don't carry the polymorphism (255, 256). BDNF is widely distributed in the cortex as well, and, given decreased volumes in cortical regions seen by MRI (255), it is likely that other cognitive processes might also be affected, but that tasks currently employed are not sufficiently sensitive to detect a behavioral deficit.

In the context of genetic alterations in BDNF levels, even temporally- or spatially-restricted disruptions affect multiple developmental time periods and/or brain regions, and may thus lead to confounded results. For that reason, many studies have employed acute pharmacological, rather than genetic BDNF blocking techniques, to assess the role of endogenous BDNF in learning and memory. One recent study elegantly combined a genetic manipulation with local, lentiviral infection to block BDNF function (391). Lentivirus expressing Cre recombinase was injected into the hippocampus of adult mice floxed at the BDNF locus, producing learning deficits in object recognition, spatial memory, and extinction of fear conditioning.

Most acute BDNF blocking techniques have employed function-blocking antibodies, antisense oligonucleotides directed against BDNF, and forms of dominant negative TrkB. A number of these studies investigated the role of BDNF in various temporal phases of memory formation, consolidation, and retention. For the purposes of this review, I will refer to three roughly defined epochs of memory establishment: short term (immediate up to 3 hours), intermediate term (24 hours), and long term (several weeks).

One study found that infusion of BDNF blocking antibodies into the parietal cortex (0.5 μg/side) led to impaired performance in a one-trial inhibitory avoidance task in rats (392). These authors had previously shown that pharmacological inactivation of neurotransmitters in the parietal cortex as early as three hours (using AP5 or muscimol), and as late as 60 days (using CNQX), but not immediately after task acquisition, impaired performance on an inhibitory avoidance task in rats (30), implicating the parietal cortex in early memory consolidation and long-term storage. In the BDNF antibody experiment, they found that blocking BDNF in the parietal cortex had no effect on short-term memory formation (tested at 1.5 hours after training), but did impair intermediate-term performance (24 hours after training). In the same study, the authors found that infusion of recombinant BDNF protein into the parietal cortex (0.25μg/side) facilitated intermediate-term, but not short-term memory. These findings parallel their earlier findings describing the time course of parietal

cortex involvement in the establishment of this memory, and implicate BDNF as a candidate molecule underlying the mechanism of memory consolidation and storage.

The authors of the studies described above had also investigated BDNF's role in establishing the memory for the inhibitory avoidance task in the hippocampus. In a study discussed earlier, the hippocampus was found to be involved in this type of learning earlier than was the cortex (immediately after, and one day after, training), and its role was time-limited; at 30 days after training, inactivation of the hippocampus no longer affected task performance (30). In another study using infusion of BDNF blocking antibodies, this time into the CA fields of the hippocampus (366), BDNF was found to be important for both short- and intermediate-term memory formation. Conversely, recombinant human BDNF administration facilitated both phases of learning (366, 393). Once again, the time course of the effects of blocking BDNF in the hippocampus are consistent with the time course of hippocampal involvement in this task, further suggesting that BDNF may have a mechanistic role in this memory formation.

In another oft-cited study (394) of BDNF function in hippocampal-dependent learning, antibodies to BDNF were infused intraventricularly and led to learning impairments in the Morris Water Maze. It should be mentioned, however, that this lab is the same source from which we received the BDNF antibodies, which, in our hands, did not block or even bind BDNF.

In a study using antisense BDNF oligonucleotides to block BDNF signaling, reference and working memory in an eight-arm radial arm maze were shown to be impaired (377). Deficits were seen in memory formation, as indicated by experiments in which infusions of BDNF antisense began 3 days before training. Notably, memory retention was also impaired in this study; infusions of BDNF beginning 29 days after training led to a greater number of reference and working memory errors in this hippocampal-dependent spatial task. This latter finding would seem to contradict the studies indicating the hippocampus' time limited role in memory storage. However, the BDNF antisense in this study was infused intraventricularly, making the blocking oligonucleotides available throughout the brain parenchyma. Though this was not tested directly, BDNF antisense in structures other than the hippocampus, such as cortical structures, might underlie the memory retention deficits seen here, which would be consistent with the other studies.

In yet another study using BDNF antisense to investigate its role in hippocampal memory, contextual fear conditioning was found to be impaired after infusion of the blocking oligonucleotides into the dorsal hippocampus (395). In this study the infusion occurred 90 minutes before training, and impaired only intermediate term (retested at 24 hours) memory, and not shorter term memory (retested at three hours). This is in contrast to the findings using BDNF antibodies in the hippocampus in an inhibitory avoidance task (366), in which blocking BDNF signaling did affect

short term memory. The disparity between these two studies may be due to the different memory tasks employed, or the different methods used to block BDNF. Indeed, the BDNF antibody acts by blocking the function of existing BDNF protein, whereas the BDNF blocking oligonucleotides only exert their effect in the later, protein synthesis-dependent phase of learning.

Antisense BDNF injected directly into the dentate gyrus of the hippocampus was again shown to lead to an impairment in an inhibitory avoidance learning task (299). In this study, BDNF antisense treatment impaired learning when administered before training and during hippocampal memory consolidation (from two days before training, to 12 hours after training), but not when administration began only after consolidation had presumably taken place, at six and 12 hours after training. As in the BDNF blocking antibody studies described above, this study attenuating BDNF signaling in a different way again implicates BDNF in the molecular events underlying the hippocampus' time-limited role in memory formation and early memory consolidation.

Finally, two studies of fear conditioning in the amygdala found that both K252a (a pan-Trk receptor blocker) and, more specifically, a dominant negative form of TrkB were able to disrupt the acquisition of the learning task (374, 375). Intra-amygdala infusion of K252a, as well as infusion of TrkB-IgG (a non-signaling receptor body fusion protein, 375) or lentiviral expression of a dominant negative

truncated TrkB receptor, TrkB.T1 (374), resulted in an impairment of fear conditioning, as assessed by the fear-potentiated startle response. The dominant negative TrkB constructs did not, in these studies, impair basal amygdala function, performance, or expression of fear.

The studies described above have shown some effects of attenuation of BDNF signaling. Conversely, overexpression of TrkB postnatally leads to improved learning in water maze, contextual fear conditioning, and conditioned taste aversion tasks (396) in unimpaired mice. BDNF infusion into the amygdala increases fear-potentiated startle (375), and one recent study found that BDNF infusion into the insular cortex of normal adult rats enhances retention in a conditioned taste aversion task (397).

Though some controversy exists regarding the role of BDNF as examined in genetically altered animals, it is clear from partial knock-out experiments and from acute blocking experiments that BDNF is involved in the establishment and/or storage of several types of memories in a variety of brain structures. To date, no study has examined BDNF in the context of cortical motor learning, and in the characteristic cortical reorganization concomitant with this learning. The experiments described in this dissertation focus on the time course of BDNF expression and role of BDNF signaling in this specialized form of plasticity.

Hypothesis

The experiments undertaken were aimed at testing the hypothesis that BDNF exerts a significant role in mediating behavioral and cortical map plasticity in the context of normal learning.

Specific Aims

I investigated the contribution of BDNF to cortical motor learning and plasticity by pursuing the following aims:

Aim 1. Determine whether motor skill learning induces changes in BDNF and trkB gene transcription in the forepaw motor cortex of the rat. Based upon studies demonstrating that a) molecular changes occur as a result of experience, b) the motor cortex is a site of representation of motor skill learning, and c) transcription of BDNF and its receptor trkB are upregulated in the context of plasticity, learning, and memory in other systems, I predict that changes in the expression of the genes encoding these proteins occur in the motor cortex during motor learning. I examined this by training rats in a forepaw reach task and determining alterations in the expression of these genes in the forepaw motor cortex.

Aim 2. Determine whether blockade of BDNF alters motor learning. It has been shown by many investigators in a variety of settings that BDNF has a role in learning and memory, but the role of BDNF in cortical motor learning has not yet been examined. I have blocked BDNF availability within the forepaw motor cortex using a lenti-virus expressing siRNA directed against BDNF, or a lenti-virus expressing a non-signaling TrkB receptor body, and have examined subsequent effects on performance in a skilled motor learning task.

Aim 3. Determine whether blockade of BDNF alters electrophysiological correlates of motor learning. It has been shown in our laboratory and others that in forepaw reach trained animals, the cortical area representing the forelimb is expanded relative to other representations within the motor cortex. For Aim 3, I have blocked BDNF availability within the forepaw motor cortex using a lenti-virus expressing siRNA directed against BDNF, or a lenti-virus expressing a non-signaling TrkB receptor body, and I have examined subsequent effects on cortical map plasticity.

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Chapter 2: BDNF and TrkB Expression During Cortical Motor Learning

Abstract

The expression of brain-derived neurotrophic factor (BDNF) and its high-affinity receptor, TrkB, have been shown in many contexts to be modulated by learning. As the first specific aim of this dissertation, I sought to determine whether BDNF and/or TrkB are dynamically regulated in the motor cortex as a function of acquisition of motor learning and the motor map plasticity with which it is associated. The time-course of alterations in BDNF and TrkB gene expression were examined in adult male rats as a function of skilled forelimb learning compared to unskilled motor activity in the same task. A preliminary study using Affymetrix Gene Chips was conducted to assess expression at time points from one to 14 days after initiation of motor training. Based on the findings from the array study, quantitative real-time PCR was used for a larger study with a focus on particular time points. Using real-time PCR, a significant training-mediated upregulation of BDNF expression, but not of TrkB expression, was observed. This modulation was specific to the forepaw motor cortex and occurred in animals that learned the skilled reach task (82% BDNF upregulation) as well as in animals which learned to extend their forelimbs without grasping the reward (77% BDNF upregulation). These findings indicate that both skilled and unskilled motor experience is associated with a training-dependent

upregulation of BDNF.

Introduction

Learning-related plasticity occurs in various forms, including behavioral, electrophysiological, structural, and molecular changes. The molecules and molecular mechanisms underlying the establishment of memories, however, are not fully characterized.

The neurotrophic factor brain-derived neurotrophic factor (BDNF) has drawn considerable interest among proteins identified as mediating various forms of plasticity. BDNF is highly expressed in areas of adult synaptic plasticity, such as the hippocampus and cortex (2-5), and the expression and secretion of BDNF are tightly regulated by neuronal activity in response to experimental manipulations, such as the application of electrical or pharmacological neuronal stimuli, and natural stimuli, such as light (6-12). Notably, BDNF exerts acute effects on synaptic transmission (reviewed in 13-15) and can cause or facilitate changes in synaptic efficacy (16-29). Another intriguing aspect of BDNF is that it is can modulate or be modulated by the expression, translation, and/or function of many other plasticity-related molecules, such as synaptic proteins (30), neurotransmitter receptors (30-32), intracellular signaling proteins (33-36), and transcription factors (34, 37). Finally, the expression of BDNF and of its high-affinity receptor, TrkB, have been shown to be upregulated

by learning in many different paradigms (35, 38-49), such as fear conditioning, spatial learning, declarative memory formation, tool use learning, and notably, motor learning. These findings suggest that BDNF signaling may be involved in mediating behavioral and other manifestations of plasticity associated with learning.

Though several studies investigated the transcriptional regulation of BDNF and TrkB with learning, none has tracked changes in BDNF and TrkB expression serially over time in a learning model. This could provide particularly useful insight into the dynamic regulation of these molecules through the varied stages of motor learning that might be missed by an examination of expression only after learning has occurred. Indeed, BDNF and TrkB are both expressed in the motor cortex (Figure 2-1) and may be involved in mediating motor learning-related plasticity.

One prototypical model of motor learning is the Whishaw Forepaw Reach Task (50), in which animals are trained to reach for and grasp a food reward (Figure 2-2). At the beginning of training, successful grasp is rare, but by the end of the training period, rats become skilled at reaching for the reward, grasping it, and bringing it to their mouths. Successful performance of this task is dependent on the integrity of the motor cortex (51, 52). Further, our own and others' studies utilizing this and similar tasks have shown that the forepaw region of the motor cortex undergoes electrophysiological (53-55, 56) and structural (50, 57-60) plasticity in association with this motor skill learning. When rats learn this forepaw reach task, the body map

in motor cortex contralateral to the trained or preferred paw undergoes reorganization, with the caudal forelimb representation or parts of the caudal forelimb area expanding relative to representations of other body parts (53-55). It is also known that this motor learning effects morphological changes in the motor cortex. A subpopulation of superficial layer II/III neurons shows increased complexity of basilar dendrites (60). Input to layer V motor neurons (apical dendrites) show increased length and number of branches in the trained vs. untrained hemisphere (50). Finally, skilled reaching also causes increased synaptogenesis, specifically in the caudal forelimb area of the motor cortex (57).

Time course studies of behavioral, electrophysiological, and structural plasticity associated with motor learning (56, 58) have indicated that: 1) behavioral performance on this task significantly improves from baseline at three days into training; 2) synaptic/structural modifications occur after seven and eight days of training; the number of synapses per neuron and the size of evoked potentials become significantly increased at this time point; and 3) significant expansion of the distal movement representation in the caudal forelimb area occurs at 10 days into training. The studies described in this chapter examined in the forepaw motor cortex the learning-related BDNF and TrkB expression changes concomitant with some of these other forms of plasticity. Rats trained in the skilled motor task were compared to rats trained in a similar task, but in the absence of skill learning, and to rats which were not trained at all.

The expression of BDNF and TrkB were examined using Affymetrix gene array methods and real-time PCR. BDNF expression has been shown in many studies employing microarray technology to be modulated in various experimental paradigms.

Gene expression profiling has revealed that BDNF is upregulated after the induction of long term potentiation (LTP), a form of synaptic plasticity thought to be among the mechanistic underpinnings of memory formation. In one study using microarray techniques, BDNF was upregulated secondary to LTP of the mossyfiber-CA3 pathway of the adult rat hippocampus (61). BDNF was also found to be modulated in a study of genes differentially expressed secondary to other forms of synaptic plasticity in the rat hippocampus (62). In this study, the authors found that BDNF was upregulated following a stimulation protocol which elicited LTP, and downregulated following stimulation which induces LTD or stimulation of the post-synaptic cell uncorrelated with pre-synaptic activity.

In another study (63), the authors studied genes involved in generating late-phase LTP (L-LTP) using transgenic mice expressing facilitated L-LTP. Microarrays were used to identify mRNA transcripts differentially expressed in the hippocampus of transgenic versus wild-type mice. Using strict selection criteria, BDNF was among only four genes which emerged from the array analysis, and its expression was found to be increased in the transgenic mice. In subsequent experiments, in which BDNF

signaling was pharmacologically blocked (with the BDNF scavenger TrkB receptor body, TrkB-Fc) or genetically attenuated (using BDNF +/- mice), BDNF was found to contribute significantly to the L-LTP phenotype.

Based on the preceding data indicating that BDNF may exert a role in cortical plasticity associated with learning and memory in various cortical regions, I sought to test the hypothesis that its expression, as well as that of TrkB, are dynamically modulated as a function of cortical motor skill learning and the motor map reorganization associated therewith.

Materials and Methods

Behavior

All animal procedures were performed in compliance with AAALAC and institutional guidelines and regulations for the humane treatment of animals. Adult male Fischer-344 rats (aged 3-5 months) were trained in the Whishaw Forepaw Reach Task, a procedural learning paradigm wherein animals reach through a small slot and grasp a food reward (50). A 31 cm (length) x 21 cm (width) x 19 cm (height) clear Plexiglass chamber was constructed with a 1.5 cm-wide slot on the front wall. Outside the slot was an adjustable tray whose distance from the slot could range from 1-2 cm. The tray was fitted with an indented well where the reward, a 45 mg sucrose

pellet (Research Diets, New Brunswick, NJ), was placed. The training paradigm included one week of handling and habituation before training, followed by up to 14 days of skilled reach or active control training, as outlined below.

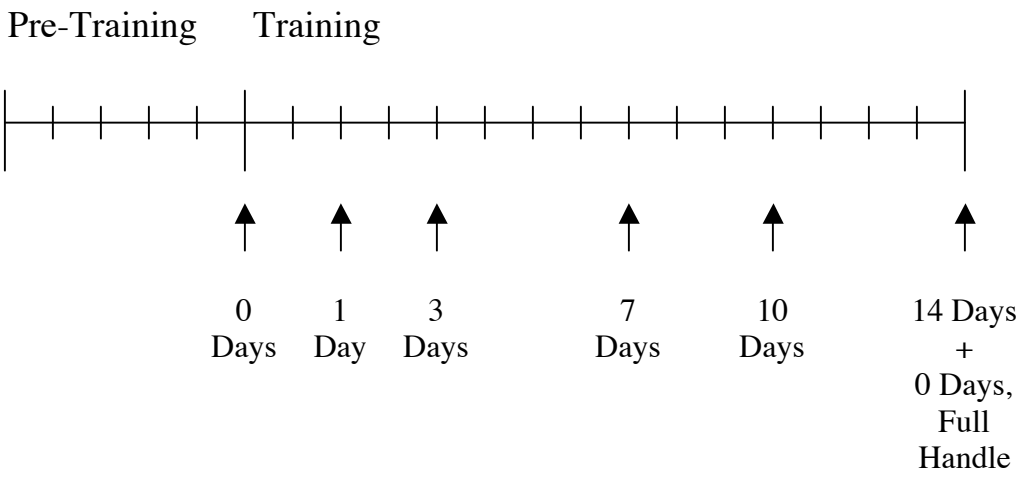
For one week prior to the beginning of training, rats were handled daily and placed in groups of three into the training chamber to be habituated to the new environment. During this "pre-training" week, sucrose pellets were introduced to the rats in their home cages, and regular diets were restricted. For the duration of the training period, animals were maintained at 85%-95% of their free-feeding weights to motivate them to reach for sugar pellets.

The first day of the following week, the subjects were placed individually into the chamber. This was not considered a training day, as the rats were still habituating to the training conditions and just beginning to reach. The tray was placed at 1 cm from the slot and rats were oriented to the slot and fed sugar pellets through it. They were rewarded for attempting to reach for the pellets, and remained in the chamber until they extended their forepaws through the slot 5-10 times.

The second day in the chamber, training "Day 1", the tray was still at 1 cm from the slot, and rats were encouraged to reach for and grasp the pellets. At the beginning of the session, peanut butter was placed on each sugar pellet so that the pellet stuck to the animals' paws to encourage them to notice it and eat it. Once rats

successfully reached for, grasped, and ate two peanut butter-covered sugar pellets, all peanut butter was removed, and reach counting began. Each rat completed 30 reaches on this day. On "Day 2", the tray was moved to 1.5 cm away and each rat completed 50 reaches, and beginning on "Day 3" and for the duration of the training, the tray was at 2 cm from the slot and each rat completed 60 reaches per day. A reach was counted if the rat extended his forepaw through the slot and retracted it. (On occasion, rats would extend their forepaws multiple times without retracting them. These events were not considered reaches.) A reach was considered a "hit" if the rat successfully grasped and brought the pellet to his mouth and ate it. Otherwise, the reach was considered a "miss". If the rat dropped the pellet en route to his mouth, this was considered a "miss". Rarely, a rat would bring a pellet to his mouth and not eat it, but this was still considered a "hit". The percentage of reaches that were "hits" were reported as "percent correct reaches". The order in which the rats were trained was randomized each day, and the paw preference of each rat was recorded.

For the array experiment (Experiment I), at each of five training time points (one day/30 reaches, three days/140 reaches, seven days/380 reaches, 10 days/560 reaches, and 14 days/800 reaches), eight rats were sacrificed – four that learned to reach and grasp (trained) and four that were rewarded semi-randomly for simply extending their paws through the slot (active controls). Eight additional rats were handled and habituated to the training chamber (four for the pre-training period only and four for the entire training period) but had 0 days of training (see timeline below).



For PCR confirmation (Experiment I and Experiment II), an additional 40 rats were trained (Experiment II), with eight animals in each of five groups. One group was handled for the pre-training period only (0 days), one group was trained for three days (3 days trained), and one group for 14 days (14 days trained). At the three day and 14 day time points, eight rats each were also trained as active controls.

RNA extraction

Within two hours of the last handling or training session for each animal, subjects were anesthetized using isofluorane gas anesthesia or a cocktail (2ml/kg) of ketamine (25 mg/ml), xylazine (1.3 gm/ml), and acepromazine (0.25 mg/ml), and decapitated. For each rat, the 2mm x 2.5mm section corresponding to the forepaw motor cortex contralateral to the trained paw was dissected. The forepaw motor cortex

was defined as the approximate area of cortex which, in other animals studied in our laboratory (54), routinely elicited forelimb movements in response to intracortical microstimulaiton. The mediolateral borders of this area are from 2 mm lateral to midline to 4.5 mm lateral to midline, and the rostrocaudal borders are from bregma to 2 mm rostral to bregma. The entire volume of grey matter (all VI cortical layers) corresponding to this area was dissected. Two animals in the study reached with both paws, in which cases, the paw which performed the highest number of reaches was considered the trained paw. In addition to the forepaw motor cortex dissection, a section of grey matter from the parieto-occipital cortex was dissected from animals in Experiment II in order to assess the motor cortex specificity of learning-mediated transcriptional changes.

The cortical sections were immediately placed in RNAlater (Ambion, Austin, TX) and, according to the manufacturer's recommendation, kept at 4˚ overnight. They were then transferred to dry eppendorfs, weighed, and frozen until RNA extraction.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). Briefly, the RNAlater-preserved tissue was lysed and homogenized, and debris was spun down. Ethanol was added to the lysate supernatant, and then poured over the RNA-binding column. The column was then washed, and the RNA was eluted in water.

The concentration and purity of RNA was examined by measuring optical density at 260 nm and 280 nm using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). RNA quality was confirmed by gel electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA), including inspection for any degradation. For all samples, the 28S:18S ribosomal band ratio was greater than 1.4. Table 2-1 summarizes the spectrophotometer and bioanalyzer readings for the RNA samples from Experiment I prior to hybridization on the array, and Table 2-2 summarizes the spectrophotometer and bioanalyzer readings for the motor cortical RNA samples run using PCR (from Experiment I and II).

Microchip hybridization and analysis

Samples from the first experiment were sent to Berlex, Germany for hybridization to Affymetirix high-density oligonucleotide GeneChip® Rat Genome 230 2.0 Arrays (Affymetrix, Santa Clara, CA), comprised of more than 30,000 probe sets corresponding to the rat genome. RNA from each forepaw motor cortex sample was reverse transcribed, amplified, and then labeled with a fluorescent dye, and hybridized to one chip. Each gene on the chip is represented by a number of probe sets. Each probe set contains thousands of short, identical, 25-mer sequences corresponding to a specific sequence of the gene represented by the probe.

Forty-eight rats were used for this experiment (Experiment I), four rats per group per time point. Two rats were pooled to create each RNA sample, yielding 24 RNA samples to be hybridized. One RNA sample (corresponding to two of the rats in the trained group at the 10 day time point) was found to be of low quality and was discarded, leaving one remaining sample for this group and time point. Another sample (corresponding to two of the rats in the trained group at the one day time point) failed hybridization due to low cRNA yields even after repeated attempts, also leaving one sample for this group and time point. Thus, a total of 22 chips were analyzed.

Raw signal intensity values from the chips were sent from Berlex as CEL files. The values were then normalized using two different methods, with generous assistance from Dr. Nicholas Webster (University of California, San Diego) and Dr. Giovanni Coppola (University of California, Los Angeles). The Corgon method (64) was developed as a microarray data analysis tool specifically for experiments in which there are a series of samples. It is model-based, assumes multiplicative noise, and employs elimination of statistically significant outliers. This is accomplished by first determining which probes are considered to be background probes. Perfect match probes (PM probes; those which are perfectly complementary to a region of the target gene) which are within a certain intensity value from mismatch probes (MM probes; those which contain a mismatch base) are considered to be background probes. The log2 intensities of background probes are plotted, and if the distribution is found to be significantly non-Gaussian, the probe which contributes most is eliminated. This

process is repeated until the resulting distribution is not significantly different from Gaussian or until the number of retained probes drops below a certain threshold. The mode of the background probe intensities is then subtracted from the intensity of each PM probe on the array. In this way, probes whose intensity becomes negative as a result of background subtraction are eliminated. For each remaining probe set, instead of an Absent/Present call, which is used by the Affymetrix software to indicate whether the intensity of a probe set is above the level of background, a p-value is generated by Corgon which can be used to estimate confidence in a gene being expressed above background level. A p-value of 0.1 was used as the cut-off, as it yields approximately the same number of genes as a present call in the Affymetrix software, Microarray Suite 5 (64).

Array data analysis was also done in the R computing environment (http://www.r-project.org) using Bioconductor, an open source software project for the analysis and comprehension of genomic data (http://www.bioconductor.org, 65). Raw data were processed using robust multi-array average (RMA; 66), which performs: 1) background correction under the exponential-normal convolution model. This assumes that the PM intensities result from a convolution of the true signal, assumed to be exponentially distributed, and a background noise component, assumed to have a normal distribution. Estimates of the mean and variance of the normal distribution and the rate parameter of the exponential distribution are made to estimate the signal; 2) normalization using the 'quantile' method (67), which matches intensity values

across the entire group of arrays so that the smallest value on each array is identical, the second smallest is identical, and so forth. Each intensity value is therefore replaced by its quantile value on a hypothetical reference chip, to which each chip is then normalized; and 3) probe summarization, which collapses expression data from several probes querying a single transcript. Quality control analysis was performed using several indices, most notably, RNA degradation plots (Figure 2-3) to aid in the assessment of RNA, cDNA, and cRNA quality. The process of reverse transcription of RNA begins with reverse transcriptase binding to a primer oligonucleotide annealing at the 3' end of an mRNA transcript and processing toward the 5' end. However, reverse transcription generally does not continue to completion because it typically drops off before reaching the 5' end. Affymetrix gene chips include probe sets that interrogate both the 3' and 5' ends of selected transcripts. Averages of the 3' to 5' signal intensities of probes are calculated for each internal control gene as a quality assessment demonstrating the degree to which the gene was transcribed, An inappropriate relationship between 3' and 5' intensities indicates a problem with RNA degradation, and/or incomplete cDNA synthesis from mRNA, and/or a transcription and labeling reaction where cDNA was not properly converted to labeled cRNA. Linear, upward sloping curves (from 5' to 3') that are consistent among all of the chips run indicate good quality RNA and homogeneity of quality across samples. All of the chips in this study were found to be of high quality.

The results using the Corgon-normalized values were similar to those using the

RMA-normalized values. Because the RMA analysis method is more widely used, and for clarity, only those results have been reported and illustrated.

The Affymetrix GeneChip Rat 230.2 expression array includes two different probe sets corresponding to the BDNF gene and seven different probe sets corresponding to the TrkB gene. One of the BDNF probe sets is only a partial match to all but a particular splice variant of BDNF mRNA which is expressed mostly in peripheral tissues, and not in the brain (68), making it an unreliable probe set for the purposes of this study. Three of the TrkB probe sets yielded signal intensity values which did not meet the Corgon cut-off criterion of being significantly expressed beyond the level of background ($p \leq 0.1$) and which were in the range of background noise (average log2 values of less than six) as assessed by BioConductor. Of the remaining four TrkB probe sets, only two matched full length TrkB mRNA. The truncated TrkB receptors function differently than does the full length receptor, in some cases as dominant negative regulators of BDNF signaling (69-78). The two probe sets that matched the truncated isoforms of TrkB were therefore not used. For the final analysis, RMA-normalized values for the reliable BDNF probe set (Affymetrix Rat Expression Array 230 2.0 probe set 1368677_at, which will be referred to as "BDNF77") and two reliable TrkB probe sets (Affymetrix Rat Expression Array 230 2.0 probe sets 1368972_at and 1370687_a_at, which will be referred to as "TrkB72" and "TrkB87", respectively) are reported.

Real-time PCR

Unused RNA from the samples in Experiment I used for gene chip hybridization was re-assessed to ensure maintenance of quality and to re-measure concentration. Seventeen of the twenty-two samples used for the array, and thirty-eight of forty samples from Experiment II, were found to remain of sufficient quantity and quality for quantitative real-time PCR. Of the thirty-eight animals in Experiment II whose motor cortex samples were used, thirty-seven parieto-occipital cortical samples were found to be of sufficient quantity and quality for use as control tissue. First-strand cDNA synthesis was carried out according to the manufacturer's instructions using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) primed with oligo(dT). Each reaction was performed using 500 ng RNA, both with and without reverse transcriptase. The cDNA was then treated with RNaseH (2 U/reaction).

For quantitative real-time PCR, BDNF and TrkB primers were designed using Primer Express™ 1.5 (Applied Biosystems, Foster City, CA). β-actin was used as an endogenous control to normalize expression levels of the genes of interest across samples. The β-actin primers were used previously (79), and the reverse primer spanned an intron. All primers were synthesized and purified by Invitrogen Life Technologies (Carlsbad, CA) or by Allele Biotechnology and Pharmaceuticals (San Diego, CA). The sequences of primers were as follows: BDNF forward 5’

CCATAAGGACGCGGACTTGT 3', BDNF reverse 5'

GAGGCTCCAAAGGCACTTGA 3', TrkB forward 5'

GGTGATTGCCTCTGTGGTAGGA 3', TrkB reverse 5'

GGAATGTCTCGCCAACTTGAG 3', β-actin forward 5'

CAATGAGCGGTTCCGATG 3', β-actin reverse 5'

TGGGTATGGAATCCTGTGGC 3'.

Quantitative real-time PCR was performed using a BioRad iQ iCycler Detection System (BioRad Laboratories, Hercules, CA). Thermal cycling conditions were as follows: an initial incubation at 95 °C to calculate the well factors for 8.5 minutes followed by 45 cycles of 95 °C for 15 seconds and 58 °C for 30 seconds. After the amplification cycles were completed, the plate was heated to 95˚C for 1 minute, then to 55˚C for 1 minute. Finally, to check the quality of amplification, a melting profile was generated for the products over a temperature range of 55 °C to 95 °C. Melt curves were then converted into melting peaks by plotting the negative derivative of fluorescence versus temperature. The presence of a single, double-stranded product was confirmed by the presence of a single peak. Reactions were performed in a total volume of 25 µl – 12.5µl RT^{2}Real-Time SYBR Green/Fluorescein PCR Master Mix containing HotStart DNA Taq polymerase, dNTPs, SYBR Green dye, Fluorescein reference dye, and buffer (SuperArray Bioscience Corporation, Frederick, MD), 0.25 µl of each primer at 100µM concentration for BDNF and TrkB and 75µM for β-actin, and 12 µl of the diluted cDNA template. Reactions were carried out using three to

five replicates for each sample. The genes of interest and endogenous control β-actin were amplified in separate tubes. Negative controls (minus reverse transcriptase) and negative controls using water in the reverse transcription reaction instead of RNA were also run to assess specificity and indicate potential contamination.

Real-time PCR Data Analysis

PCR quantification curves were generated showing the characteristic exponential relationship between relative fluorescence units (RFU) and cycle number. Figures 2-4A, 2-4B, and 2-4C show representative standard curves for BDNF, TrkB, and β-Actin, respectively. For each curve, a threshold cycle (C_t) value was obtained by setting the relative fluorescent units threshold to the maximum automatically calculated value among all of the plates for the reference gene, β-actin. This value corresponded to 10x the standard deviation of the background signal. Quantitative PCR yields information about the amount of a transcript in a given sample *relative* to the amount in a reference sample. The reference used for the analysis was the 0 days condition.

For each sample, the ΔC_t value, representing the difference in threshold cycle between the target (BDNF or TrkB) and control (β-actin) genes, was determined by subtracting the C_t value of the β-actin curve from the C_t values for curves derived from each of the target genes. The $\Delta\Delta C_t$ value was derived from the subtraction of the

resultant ΔC_t values from the mean ΔC_t value of the 0 days conditions. The data are expressed as $2^{(-\Delta\Delta Ct)}$, or the n-fold difference relative to the reference. The fold-change values for each target gene at each of the training time points were plotted and analyzed.

Statistics

PCR results were analyzed using a one-way analysis of variants (ANOVA) for changes in gene expression as a function of training day and using a two-way ANOVA for effect of experimental group, training day, and the interaction between them. For post-hoc pair-wise mean comparisons, a Fisher's protected least significant difference (Fisher's PLSD) test was used.

Results

Behavioral Results

The performance of animals trained in the skilled reach task whose RNA met quality control standards is shown in Figure 2-5 for Experiment I (microarray analysis followed by real-time PCR). Figure 2-6 shows the combined behavioral results for animals trained for three or 14 days from Experiment I and Experiment II (real-time PCR).

Array Results from Experiment I

The RMA-generated expression values for BDNF and TrkB as a function of training are graphed in Figure 2-7 for BDNF, Figure 2-8 for TrkB Probe Set 72, and Figure 2-9 for TrkB Probe Set 87. For the trained animals at the one day time point and the 10 day time point, there was one array per group, whereas for every other condition, there were two arrays per group. Because of the small numbers, statistical analyses were not done on these data. There was a suggestion, however, of initial BDNF downregulation, followed by gradual upregulation. TrkB expression did not appear to be modulated by training.

Real-time PCR Results

Based on the results obtained from the array experiment, a larger expression study using real-time PCR was conducted. BDNF and TrkB expression were examined as a function of training for three days (a time point at which there was a trend toward BDNF downregulation) and 14 days (at which point BDNF expression appeared to be upregulated relative to earlier time points). The results from all animals trained in both Experiment I and II for 0 days (n=9 for BDNF, n=8 for TrkB), three days (trained, n= 9 for BDNF, 8 for TrkB; active, n=9 for BDNF and TrkB), and 14 days (trained, n=10 for BDNF and TrkB; active, n=9 for BDNF and TrkB) for

BDNF and TrkB are shown in Figures 2-10 and 2-11, respectively. The TrkB fold change values included two outliers, one in the 0 days condition (fold change 18.7) and one in the three days trained condition (fold change 8.5), which were eliminated from analysis because they were greater than two standard deviations from the means of other subjects in the group; the mean and standard deviation for the 0 day condition was 2.7 +/- 6.0, and the mean and standard deviation for the three days trained condition were 1.7 +/- 2.6. Both the trained and active control groups show an increase in BDNF expression as a function of training (ANOVA, $p=0.02$ for trained and $p=0.002$ for active controls). For the trained group, a post-hoc analysis revealed a trend toward increased expression of 56 % from 0 to three days (1.06 +/- 0.12 fold change at 0 days, compared to 1.65 +/- 0.25 fold change at 3 days, mean +/- S.E.M., $p=0.07$), and a statistically significant difference of 82% between 0 and 14 days training (1.06 +/- 0.12 fold change at 0 days, compared to 1.93 +/- 0.24 fold change at 14 days, $p=0.008$). For the active control group, both three days unskilled reach training (1.06 +/- 0.12 fold change at 0 days, 1.70 +/- 0.09 fold change at 3 days, 60% increase, $p=0.006$) and 14 days unskilled reach training (1.06 +/- 0.12 fold change at 0 days, 1.87 +/- 0.20 fold change at 14 days, 77% increase, $p=0.007$) were significantly different than no training. A similar pattern of modulation of TrkB expression was suggested, with increasing expression from 0 to 3 to 14 days of training, but this was not significant (trained ANOVA, $p=0.43$; active control ANOVA $p=0.20$). A two-factor ANOVA comparing trained and active control groups and the interaction between them reveals that for both BDNF and TrkB expression, there is no effect of

training group (BDNF p=0.98, TrkB p=0.86), nor is there an interaction between training group and training day (BDNF p=0.96, TrkB p=0.95).

The modulation of BDNF expression observed in the forepaw motor cortex was not observed in the parieto-occipital cortex. One outlier in the 14 days trained condition (fold change 3.0; mean and standard deviation 1.5 +/- 0.7) was excluded from analysis because its value were greater than two standard deviations from the group mean. Neither the trained nor the active control groups showed a modulation in BDNF expression as a function of training (ANOVA, p=0.11 for trained and p=0.11 for active controls).

Discussion

The current study examined changes in cortical gene expression over the course of motor learning, in particular, the transcriptional modulation of the neurotrophic factor BDNF and its high affinity receptor, TrkB. Real-time PCR results show that BDNF expression, specifically in the forepaw motor cortex, increased as a function of learning as well as motor activity. TrkB expression was not modulated as a function of training.

BDNF expression was upregulated three days following the initiation of motor training, the first time point at which others have found task performance to be

significantly improved over baseline (58) . BDNF expression continued to rise at 14 days, when behavioral performance has plateaued (56), and cortical map reorganization is apparent (58). It is possible, given the role of BDNF in mediating synaptic changes, that the initial upregulation of BDNF leads to structural and physiological synaptic plasticity, which may underlie the subsequent cortical map plasticity. Indeed, there is much evidence that a causal relationship may exist between synaptic plasticity and map reorganization (80-82, reviewed in 84). It is also possible that the early upregulation of BDNF is related to structural and electrophysiological synaptic plasticity, and that, independently, the continued upregulation may permit cortical map reorganization. These possibilities have yet to be investigated.

The trends observed in the array experiment, indicating an initial downregulation of BDNF, followed by an upregulation, were not confirmed in the dataset generated by real-time PCR. However, array samples were few in number and did not permit statistical comparison, whereas PCR data were generated from a larger and therefore statistically reliable sample set. The correlation of gene array data with "confirmatory" methods of expression, including *in situ* hybridization, PCR, or immunolabeling, is generally good. However, discrepancies can occur. For example, Affymetrix gene chips were used to assess differential expression of genes in the hippocampi of transgenic mice constitutively expressing CREB versus in wild type mice (63). Though the array results were in general agreement with the results of *in situ* hybridization performed for confirmation, one of 10 genes tested revealed a

discrepancy between the techniques. In a study of an *in vitro* model of phenylketonuria (85), embryonic rat neurons were cultured, and RNA from experimental versus control cultures were hybridized to Affymetrix gene chips. Of the 174 genes differentially expressed, the expression of three was further investigated using real-time PCR. The up- and down-regulation was confirmed for the three genes, but the fold change was inconsistent between the two methods. One gene was upregulated 2.1 fold on as measured on the array, but 55.5 fold as measured using PCR. These studies indicate that, while different methods of gene expression quantification are generally accepted and considered reliable, comparisons between techniques may not yield consistent results.

In the present study, experimental rats learned a novel fine motor skill – reaching for and grasping a small sugar pellet, whereas control rats underwent forelimb activity in the absence of skilled grasping behavior. BDNF was upregulated in both groups, which does not suggest a specific role for BDNF in learning, as opposed to activity. Indeed, it is known that neuronal activity robustly increases BDNF expression (reviewed in 8, 9). The lack of BDNF modulation in the parieto-occipital cortex argues against the notion that the BDNF upregulation observed was a result of increased global activity not specific to the motor cortex. It remains possible, however, that increased levels of BDNF that result from motor activity could support plastic changes in the motor cortex that underlie learning, a possibility that was explored in subsequent experiments.

Conclusions and Future Directions

This study has shown that BDNF expression is modulated specifically in the motor cortex during learning of a novel motor skill and during learning of a stereotyped motor procedure. This finding raises a number of additional questions: Is the modulation of BDNF expression necessary for this motor learning? Though the literature suggests that motor activity alone does not lead to other forms of cortical plasticity, such as structural and electrophysiological changes (86-88), is the modulation of BDNF observed in this study, in fact, specific to procedural (both skilled and unskilled) learning, or is the upregulation of BDNF a result of neuronal activity, and perhaps a prerequisite for learning? What, if any, is the relationship between BDNF modulation and the other forms of plasticity observed in the motor cortex in association with this task, such as motor map reorganization and dendritic hypertrophy? It is hoped that these results will prompt additional studies in order to better characterize the nature and function of BDNF in motor learning.

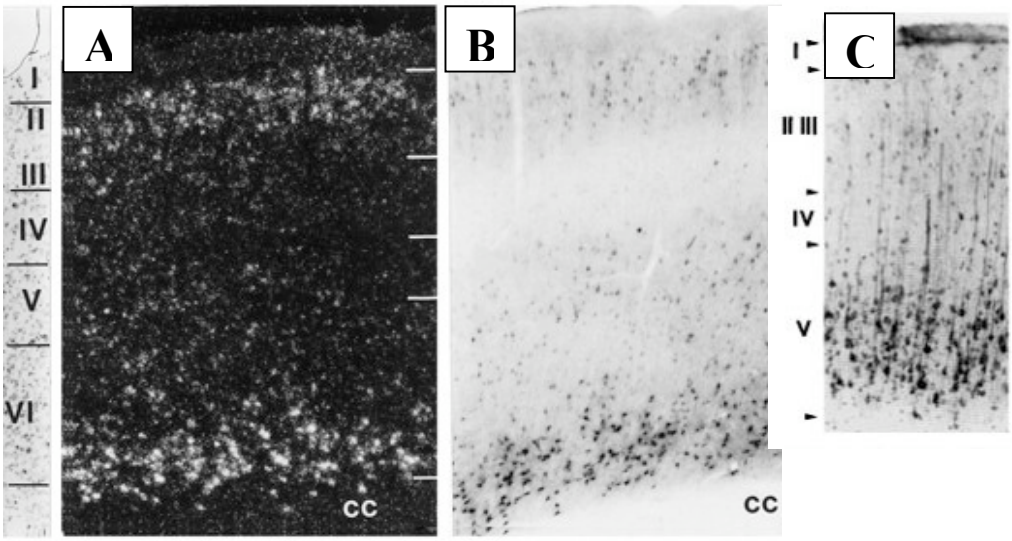


Figure 2-1. Endogenous Expression of BDNF and TrkB. (**A)**, (**B)**, from (2) and (**C)** from (3) (**A)**. BDNF message in the parietal cortex. (**B)**. BDNF IR in the parietal cortex. (**C)**. TrkB IR in the frontal cortex. For this figure, image (**C)** was scaled such that the level of cortical layer IV, as delineated by Yan, et al, roughly corresponds to the level of layer IV, as delineated by Conner, et al.

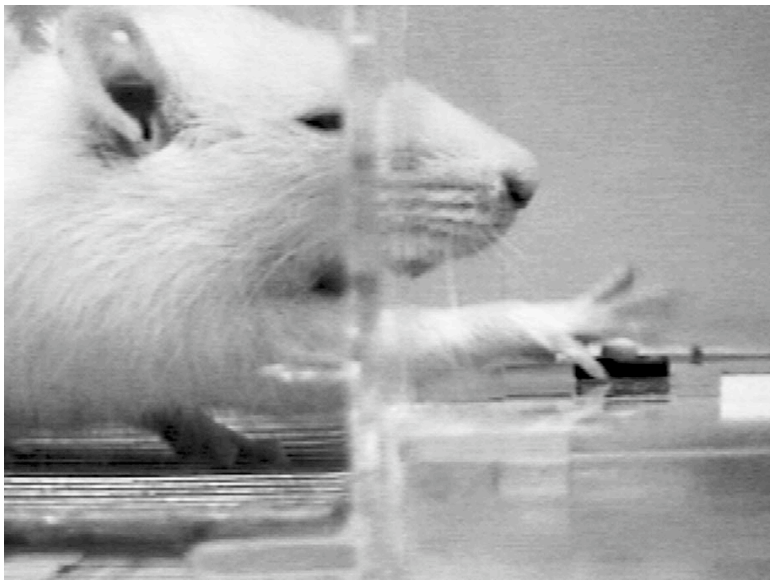


Figure 2-2. Whishaw Forepaw Reach Task. Adult male Fischer 344 reaching through slot to retrieve reward pellet. From Weidner et al, 2001 [1].

Table 2-1. Summary of the training regimen, spectrophotometer, and bioanalyzer readings for RNA samples used for the Affymetrix gene expression analysis. The ratio of 28S/18S ribosomal RNA is greater than 1.4 in all samples which passed RNA quality control criteria. Note that two samples (#6 and #17) failed due to low cDNA yields and unacceptable RNA quality, respectively.

Sample	Treatment	Spectro-photometer Concentration Reading (ng/µl)	Spectro-photometer Absorbence Ratio 260nm/280nm	Bio-Analyzer Ratio 28S/18S	
1	Handle 1 week	267.5	2.07	1.5	
2	Handle 1 week	191.0	2.07	2.6	
3	Handle 4 weeks	322.5	2.11	1.6	
4	Handle 4 weeks	272.5	2.08	2.8	
5	Reach Training 1 day	327.5	2.06	2.2	
6	Reach Training 1 day	216.3	2.07	2.5	failed due to low cRNA yields
7	Control Training 1 day	295.0	2.08	2.3	
8	Control Training 1 day	155.0	2.11	2.3	
9	Reach Training 3 days	275.0	2.05	2.3	
10	Reach Training 3 days	214.3	2.09	2.2	
11	Control Training 3 days	252.5	2.07	1.6	
12	Control Training 3 days	171.5	2.1	2.3	
13	Reach Training 7 days	247.3	2.1	1.8	
14	Reach Training 7 days	231.0	2.06	2.4	
15	Control Training 7 days	226.5	2.08	2.0	
16	Control Training 7 days	186.5	2.11	2.1	
17	Reach Training 10 days	255.0	2.1	0.0	failed due to RNA QC
18	Reach Training 10 days	257.5	2.08	2.3	
19	Control Training 10 days	260.0	2.09	2.6	
20	Control Training 10 days	262.5	2.1	2.2	
21	Reach Training 14 days	290.0	2.08	2.5	
22	Reach Training 14 days	305.0	2.04	2.5	
23	Control Training 14 days	300.0	2.08	2.2	
24	Control Training 14 days	285.0	2.05	2.5	

Table 2-2. Summary of the training regimen, spectrophotometer, and bioanalyzer readings for the RNA samples used for real-time PCR. The ratio of 28S/18S ribosomal RNA is greater than 1.4 in all samples which passed RNA quality control criteria. Note that two samples (#15 and #38) failed due to unacceptable RNA quality and over-dilution resulting in too low a concentration, respectively.

Sample	Treatment	Spectrophotometer Concentration Reading (ng/µl)	Spectrophotometer Absorbence Ratio 260nm/280nm	BioAnalyzer Ratio 28S/18S	
1	Handle 1 week	53.2	2.1	2.6	
3	Handle 4 weeks	63.6	2.08	2.8	
4	Handle 4 weeks	50.0	2.15	2.3	
5	Reach Training 1 day	67.6	2.05	2.3	
9	Reach Training 3 days	54.8	2.11	2.1	
10	Reach Training 3 days	48.1	1.98	2.4	
11	Control Training 3 days	52.5	2.01	2.9	
13	Reach Training 7 days	50.4	1.99	2.4	
14	Reach Training 7 days	51.6	2.04	2.4	
15	Control Training 7 days	36.7	2.12	2.2	
18	Reach Training 10 days	54.2	2.07	2.6	
19	Control Training 10 days	51.1	1.9	3.2	
20	Control Training 10 days	59.7	2.09	2.7	
21	Reach Training 14 days	46.2	2	2.7	
22	Reach Training 14 days	67.3	2.06	2.4	
23	Control Training 14 days	51.9	2.08	2.6	
24	Control Training 14 days	43.7	2.07	2.4	
MB 1	Handle 1 week	36.4	2.07	2.3	
MB 2	Handle 1 week	26	1.99	2.2	
MB 3	Handle 1 week	39.2	2.03	2.2	
MB 4	Handle 1 week	21.3	2.01	2.2	
MB 5	Handle 1 week	28.3	2.11	2.4	
MB 6	Handle 1 week	24.4	2.03	2.4	
MB 7	Handle 1 week	34.9	2.01	2.3	
MB 8	Handle 1 week	27.4	1.99	2.4	
MB 9	Reach Training 3 days	34.2	1.97	1.9	
MB 10	Control Training 3 days	23.4	2	2.4	
MB 11	Reach Training 3 days	36.4	2.08	2.4	
MB 12	Control Training 3 days	29.2	2.11	2.3	
MB 13	Reach Training 3 days	29.7	2.03	2.4	
MB 14	Control Training 3 days	29	2.12	2.5	
MB 15	Reach Training 3 days	6.96	2.22	0	failed due to RNA QC
MB 16	Control Training 3 days	36.3	2.08	2.6	
MB 17	Reach Training 3 days	35.7	2.13	2.5	
MB 18	Control Training 3 days	31.4	2	1.9	
MB 19	Reach Training 3 days	40.8	2.02	2.9	
MB 20	Control Training 3 days	18.1	2.05	2.5	
MB 21	Reach Training 3 days	32.6	2.08	2.7	
MB 22	Control Training 3 days	31.6	2.11	2	
MB 23	Reach Training 3 days	34.1	2.05	2.1	
MB 24	Control Training 3 days	23.8	2.15	2.1	
MB 25	Reach Training 14 days	20.8	1.88	1.7	
MB 26	Control Training 14 days	32.6	2.03	2.4	
MB 27	Reach Training 14 days	28.4	1.9	1.4	
MB 28	Control Training 14 days	35.7	1.92	2.2	
MB 29	Reach Training 14 days	38.6	1.95	2.3	
MB 30	Control Training 14 days	21.6	2.05	2.4	
MB 31	Reach Training 14 days	50.4	1.86	2.5	
MB 32	Control Training 14 days	32.3	1.97	2.3	
MB 33	Reach Training 14 days	30.3	1.91	2.1	
MB 34	Control Training 14 days	23.8	2.07	2.3	
MB 35	Reach Training 14 days	28.4	1.9	2.1	
MB 36	Control Training 14 days	26.5	2.02	2	
MB 37	Reach Training 14 days	26.6	1.84	2.3	
MB 38	Control Training 14 days	19.8	1.51	2.1	failed due to over-dilution
MB 39	Reach Training 14 days	15.1	1.83	2.3	
MB 40	Control Training 14 days	46.1	1.95	1.7	

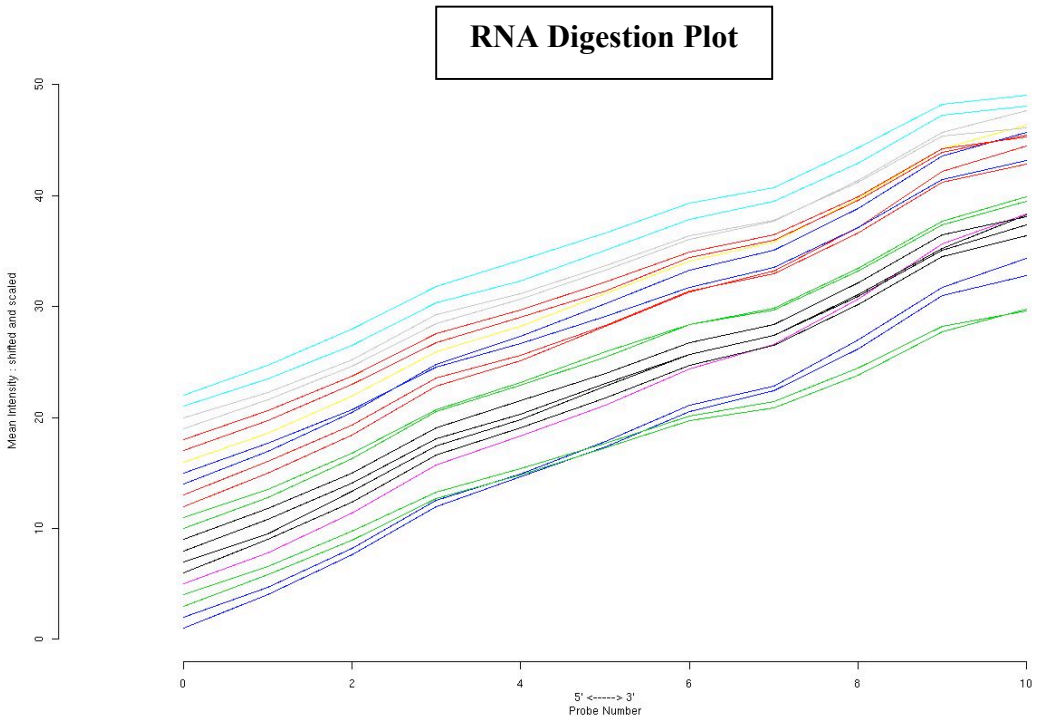


Figure 2-3. RNA digestion plot of all 22 chips. Signal intensities are greater for probes closer to the 3' end of the transcript. The relationship of intensities from 5' to 3' is upward sloping and linear, as expected for high quality RNA. Parallelism indicates consistency of RNA quality among chips.

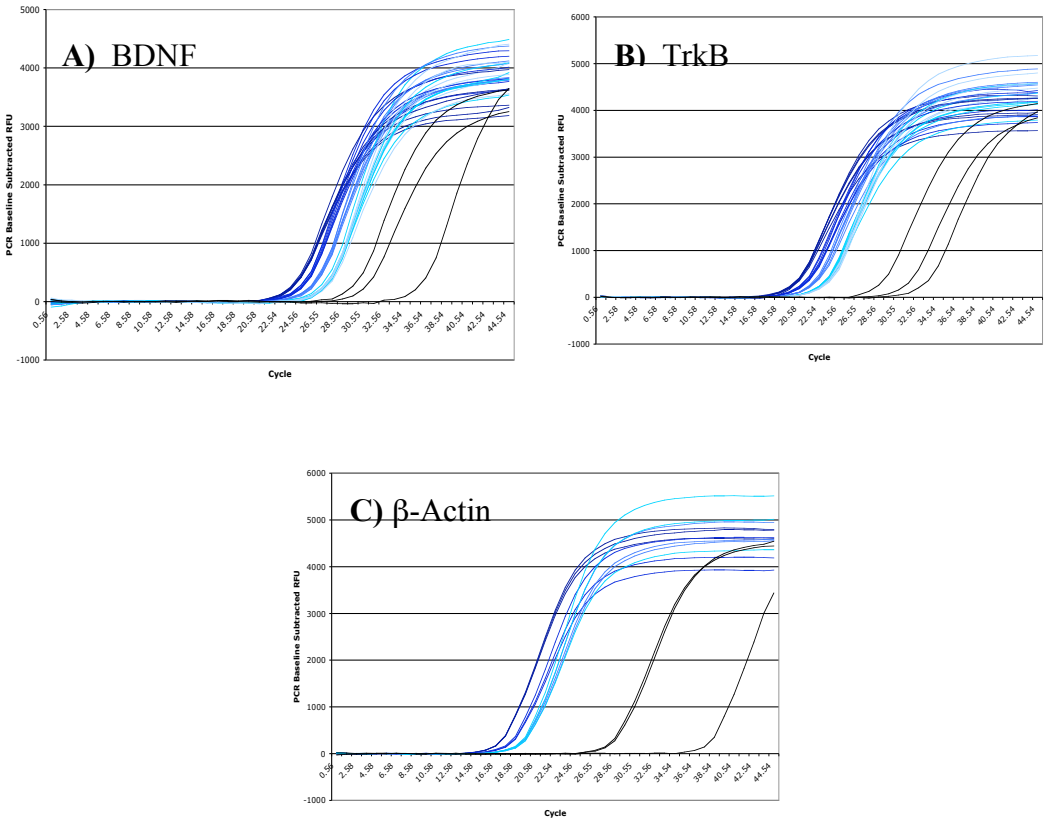


Figure 2-4. Real-time PCR standard curves for (**A**) BDNF, (**B**) TrkB, and (**C**) β-Actin. Values shown are the PCR baseline subtracted relative fluorescence units, expressed as a function of cycle number. The curves represent a 2-fold dilution series of samples, with higher to lower concentration proceeding from darker to lighter color designation. Black curves represent negative control samples in which reverse transcriptase was omitted from the cDNA synthesis reaction. Samples whose curves depart from baseline at a lower cycle number contain more starting material. A 2-fold dilution should result in a separation of 1 cycle between dilutions.

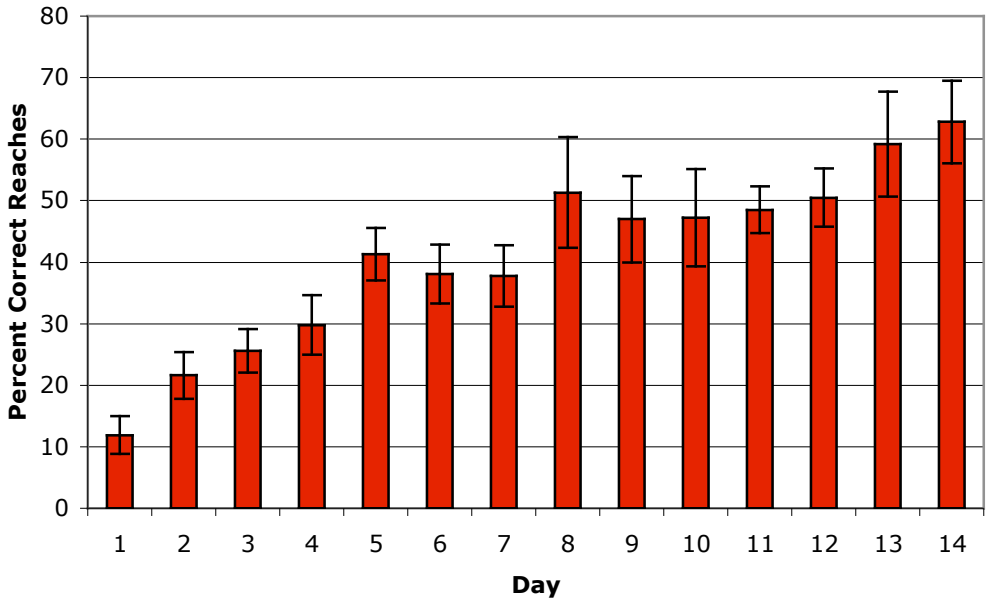


Figure 2-5. Behavioral results for animals trained in the skilled reaching task whose RNA was used for array analysis (Experiment I). On Day 1, n=16; Day 2-3, n=14; Day 4-7, n=10; Day 8-10, n=6; Day 11-14, n=4. (Animals which learned to reach without grasping are not shown, as they, by definition, performed no correct reaches.) Performance is expressed as percent correct reaches per group per day. Error bars are +/- S.E.M.

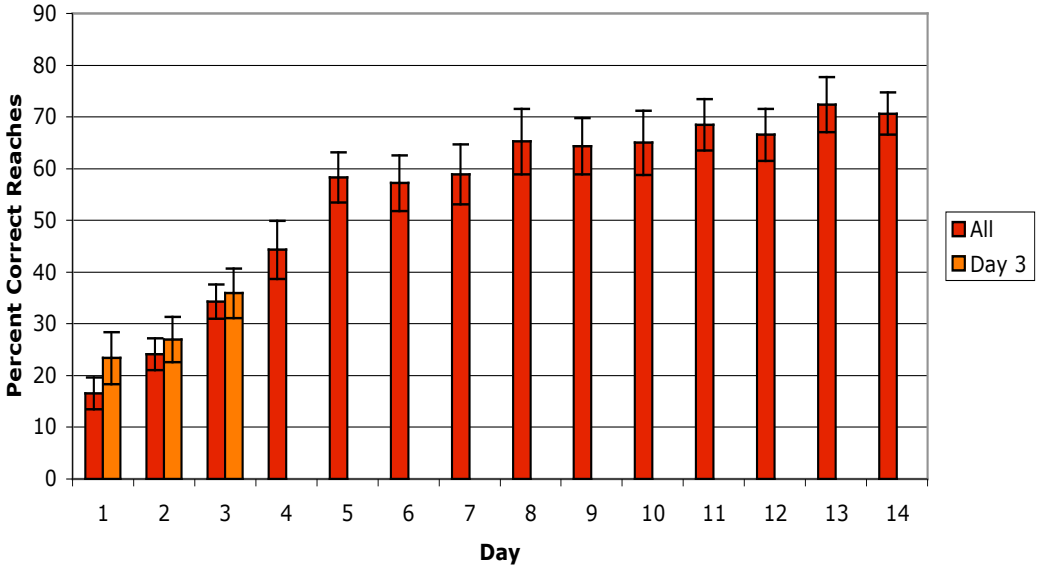


Figure 2-6. Behavioral results for animals trained in the skilled reaching task whose RNA was used for the real-time PCR study (Experiment I + Experiment II). On Days 1-3, n=24 and on Days 4-14, n=12. (Animals which learned to reach without grasping are not shown, as they, by definition, performed no correct reaches.) Performance is expressed as percent correct reaches per group per day. Error bars are +/- S.E.M.

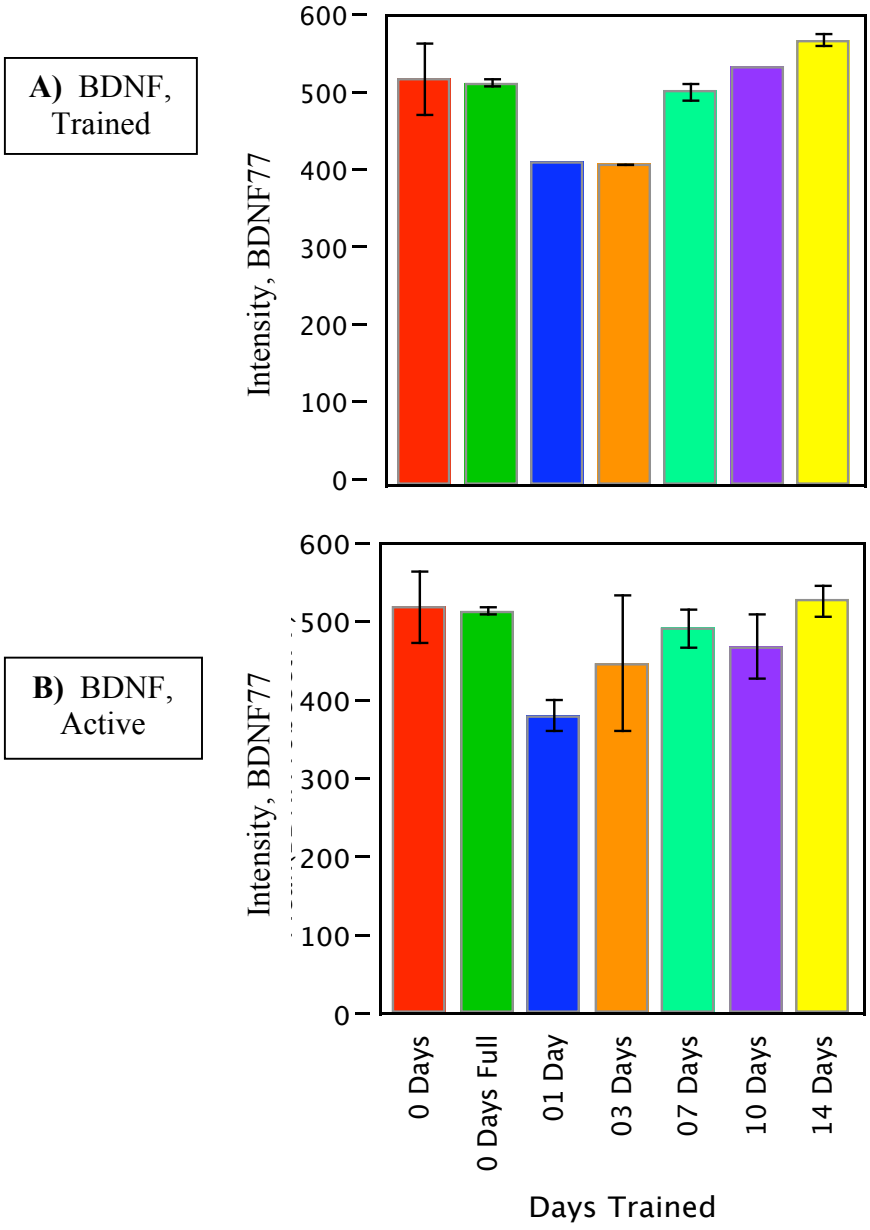


Figure 2-7. RMA-generated Affymetrix signal intensities from BDNF Probe Set 77 for arrays containing data from animals trained for 0 days, for 0 days but handled for the full training period, and trained for 1-14 days. (**A**) Data from animals trained in the skilled reaching task and from (**B**) active motor control animals.

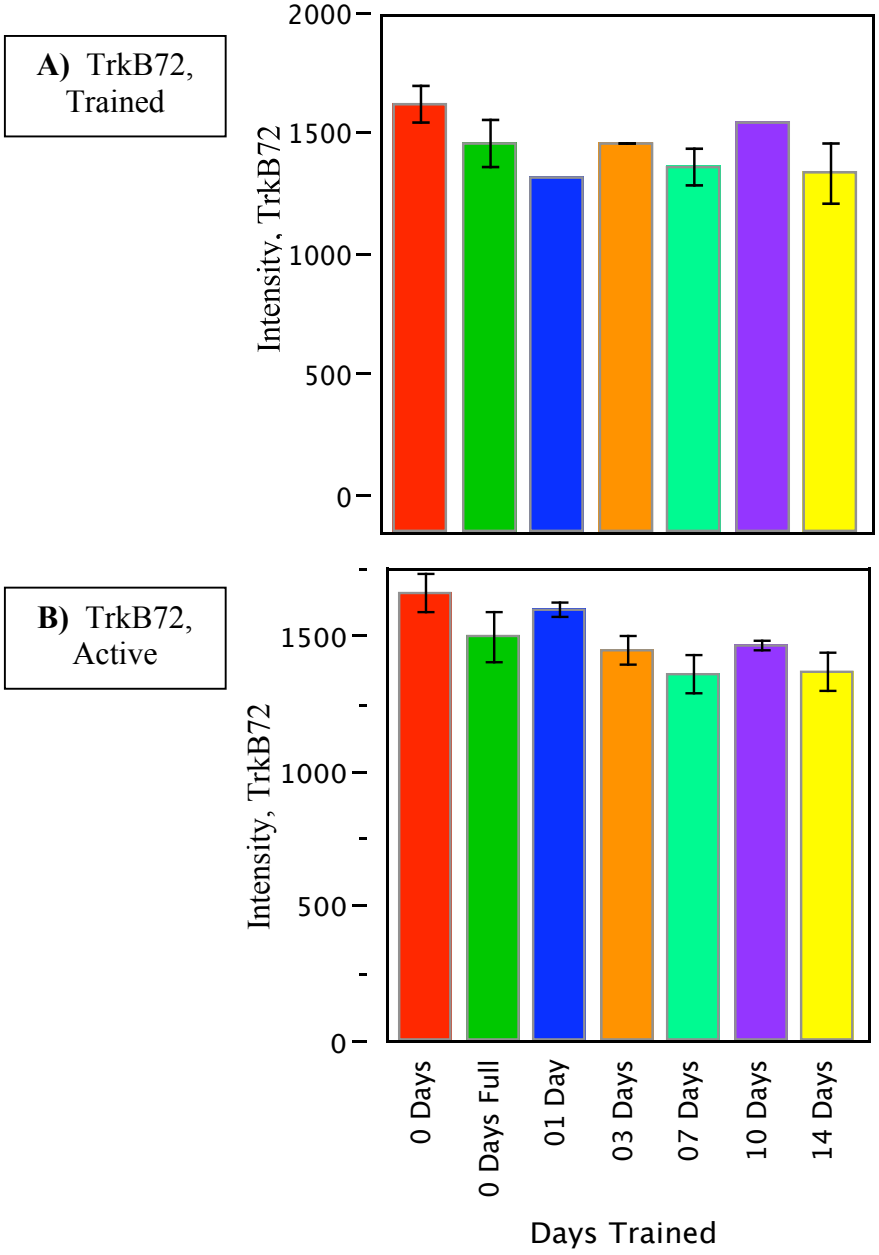


Figure 2-8. RMA-generated Affymetrix signal intensities from TrkB Probe Set 72 for arrays containing data from animals trained for 0 days, for 0 days but handled for the full training period, and trained for 1-14 days. (**A**) Data from animals trained in the skilled reaching task and from (**B**) active motor control animals.

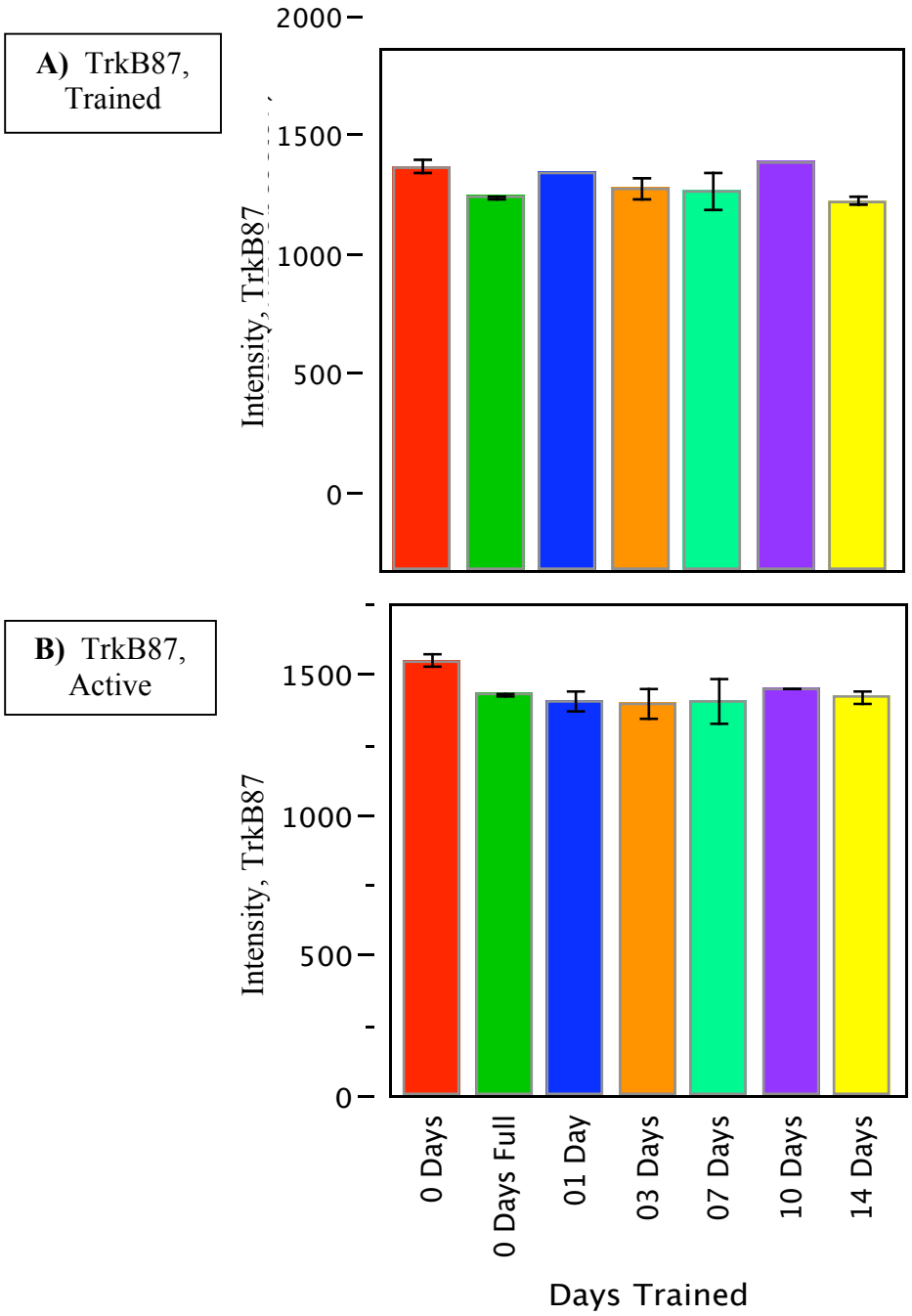


Figure 2-9. RMA-generated Affymetrix signal intensities from TrkB Probe Set 87 for arrays containing data from animals trained for 0 days, for 0 days but handled for the full training period, and trained for 1-14 days. (**A**) Data from animals trained in the skilled reaching task and from (**B**) active motor control animals.

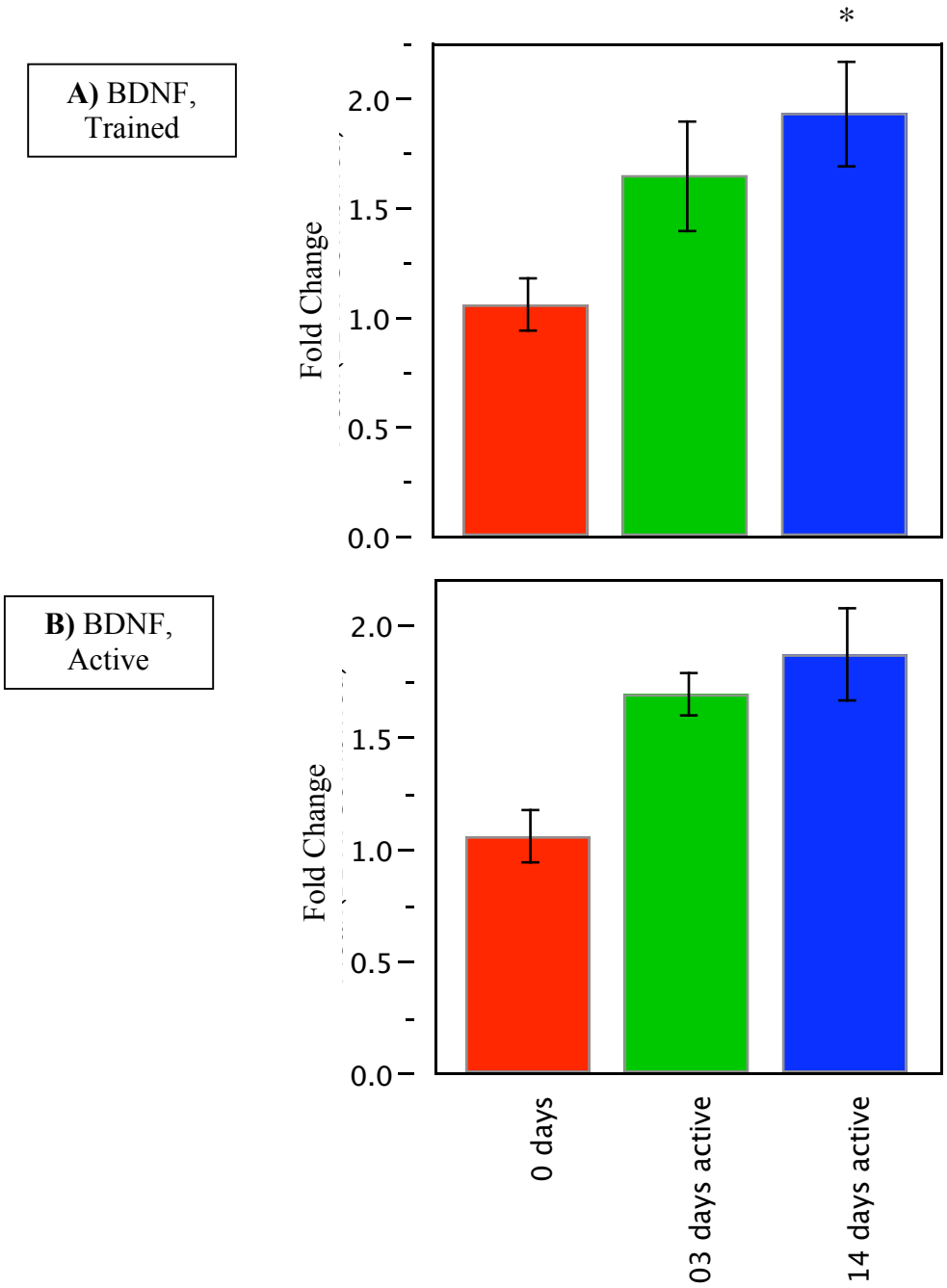


Figure 2-10. Real-time PCR expression profiles of BDNF in trained animals (**A**, p=0.02) and active controls (**B**, p=0.002) over the course of learning. Error bars are +/- SEM. For the trained group at 0. 3, and 14 days, n=9, 9, and 10, respectively. For the active control group, n=9, 9, and 9. * (p<0.01), ** (p<0.01), *** (p<0.001) in comparison to 0 days.

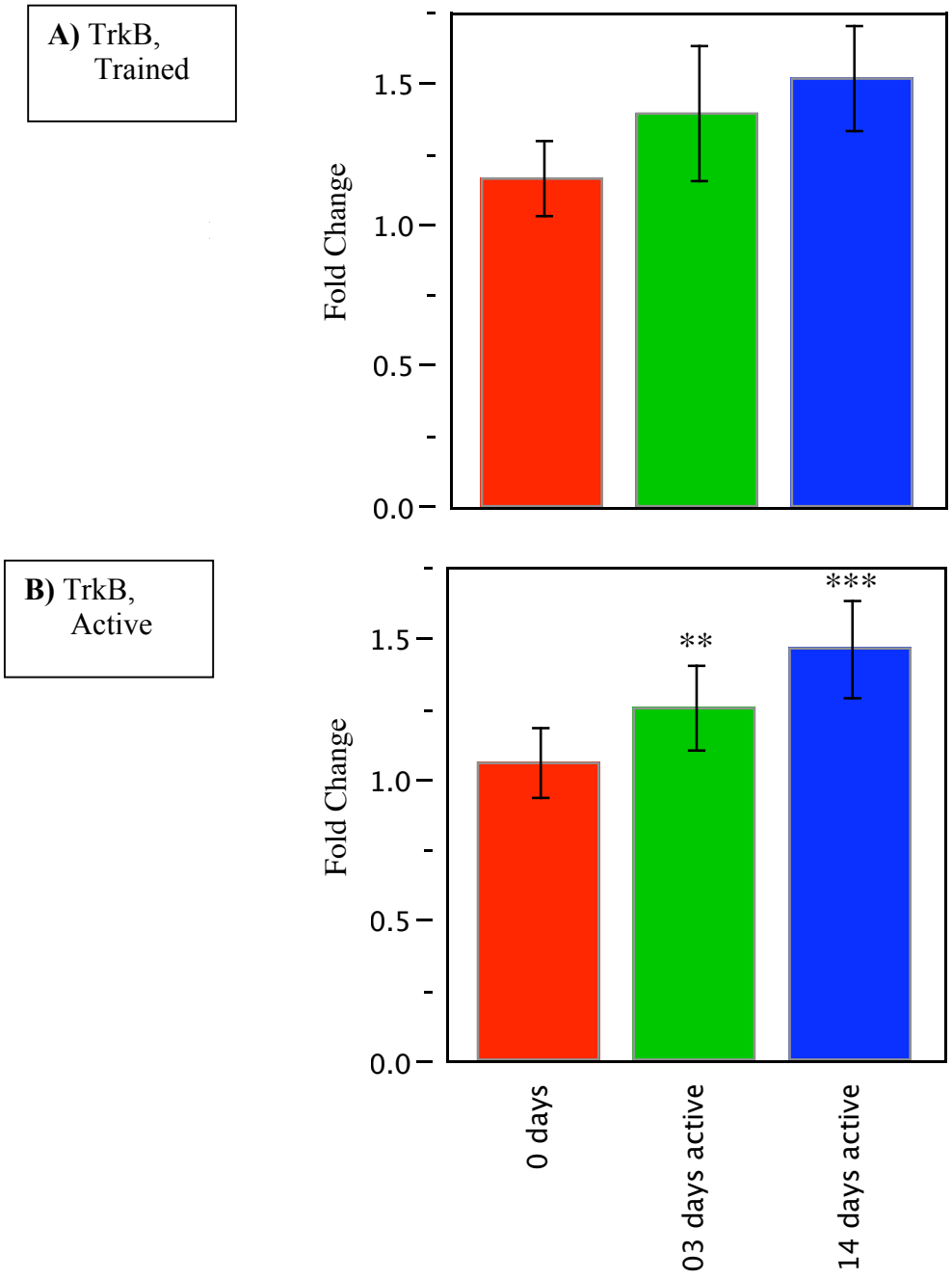


Figure 2-11. Real-time PCR expression profiles of TrkB in trained animals (**A**, p=0.43) and active controls (**B**, p=0.20) over the course of learning. Error bars are +/- SEM. For the trained group at 0. 3, and 14 days, n=8, 8, and 10, respectively. For the active control group, n=8, 9, and 9.

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Chapter 3: Effect of RNAi-mediated BDNF Reduction on Cortical Plasticity

Abstract

Brain-derived neurotrophic factor (BDNF) is upregulated with, and essential for, a variety of learning tasks. To address the second and third specific aims of this dissertation, I sought to investigate the role of BDNF in mediating learning and functional organization in the motor cortex of adult rats. An siRNA construct directed against BDNF was created which targets BDNF mRNA for degradation, thereby "knocking-down" BDNF levels. *In vitro*, the lentiviral siRNA construct reduced BDNF protein levels by 81% compared to uninfected cells and 85% compared to control virus-infected cells. *In vivo*, BDNF ELISAs showed that siRNA in the motor cortex significantly reduced BDNF protein levels by 22% compared to uninjected cortices and 34% compared to control virus-injected cortices. This reduction of cortical BDNF, however, did not lead to impairments in motor learning or changes in motor map organization. It is concluded that a reduction of BDNF protein levels in the motor cortex by 22% does not affect motor learning or motor map organization.

Introduction

The neurotrophin brain-derived neurotrophic factor (BDNF) has been widely investigated as an activity-dependent modulator of neuronal structure and function in the adult brain. In particular, its role in learning and memory-related plasticity has been the subject of intensive study.

Activity-dependent changes in synaptic strength appear to underlie cellular and synaptic plasticity. BDNF is a molecule whose expression (reviewed in 3-6) and secretion (7-9) are tightly regulated by neuronal activity. It is able to modulate synaptic transmission (reviewed in 10-12), and cause or facilitate changes in synaptic efficacy (13-26). BDNF has also been shown in many paradigms to be regulated by (27-38) and have a role in many types of memory (27, 29, 34, 36, 38-52). Among the most informative methods by which to examine its role in the adult brain is to block or attenuate its availability and examine subsequent effects on structural, physiological, and behavioral plasticity.

To assess the role of endogenous BDNF in learning and memory, a variety of techniques have been employed to block BDNF signaling in the brain, such as function-blocking antibodies, antisense oligonucleotides directed against BDNF, and dominant negative forms of the high-affinity receptor for BDNF, TrkB.

The studies examining the function of BDNF blocking-antibodies and non-functioning TrkB receptors will be reviewed further in the next chapter. The

mechanism of blocking BDNF using antisense oligonucleotides is similar to the mechanism of RNA interference-based blocking in that both attenuate BDNF at the mRNA level. A number of studies have used BDNF antisense to investigate the role of BDNF in normal adult learning.

In one study, antisense BDNF oligonucleotides were shown to impair reference and working memory in a hippocampal-dependent eight-arm radial arm maze (38). Deficits were seen in memory formation, as indicated by experiments in which infusions of BDNF antisense began three days before training, as well as in memory retention, as indicated by a greater number of reference and working memory errors secondary to infusions of BDNF antisense beginning 29 days after training.

Antisense BDNF injected directly into the dentate gyrus of the hippocampus was shown to lead to an impairment in an inhibitory avoidance learning task (29). In this study, BDNF antisense treatment impaired learning when administered before training and during hippocampal memory consolidation (from two days before training, to 12 hours after training), but not when administration began only after consolidation had presumably taken place, at six and 12 hours after training.

In another study using BDNF antisense to investigate its role in hippocampal memory, contextual fear conditioning was found to be impaired after infusion of the blocking oligonucleotides into the dorsal hippocampus (50). In this study the infusion

occurred 90 minutes before training, and specifically impaired intermediate term (retested at 24 hours) memory and not short term memory (retested at three hours).

The effects of antisense BDNF on memory have been examined outside of the hippocampus as well. The phenomenon of ocular dominance plasticity has also been shown to be modulated by BDNF (53). Young rats (aged 22-23 days) were subjected to monocular deprivation, which caused a characteristic shift of preference of visual cortical neurons to the non-deprived eye. The authors showed that they were able to compensate for the effects of the visual deprivation by injecting exogenous BDNF into the deprived eye, or by injecting BDNF antisense oligonucleotides into the open eye; both treatments counteracted the ocular dominance plasticity caused by the monocular deprivation. Further, in animals reared with normal visual stimulation to both eyes, a combination of exogenous BDNF in one eye and antisense BDNF in the other, to create maximal BDNF imbalance between the two eyes, caused a shift in ocular dominance toward the eye injected with exogenous BDNF.

In another study using sensory deprivation-mediated plasticity (54), guinea pigs were subjected to unilateral labyrinthectomy (UL), a manipulation which results in an initial disruption of vestibular reflexes, followed by a gradual and partial recovery called “vestibular compensation”. The authors found that infusions of antisense BDNF into the vestibular nucleus ipsilateral to the UL led to an

intensification of one of the symptoms of UL, roll head tilt, significantly delaying compensation of this postural symptom.

These blocking experiments indicate that BDNF is involved in the establishment and/or storage of several types of memories in a variety of brain structures. To date, no study has examined the effect of blocking BDNF in the context of cortical motor learning, and in the characteristic cortical reorganization concomitant with this learning. Also, no study to date has used RNA interference in the adult brain to examine learning-related plasticity.

The identification and characterization of RNA interference, or RNAi, has provided a valuable tool for investigating gene function in mammalian systems. First discovered in the nematode worm Caenorhabditis elegans (55), RNAi has been used in a variety of applications with the goal of significantly attenuating the production of a target gene product (56, 57-60). The mechanism underlying RNAi is that short, double-stranded RNAs bind to an mRNA transcript containing a complementary sequence to one of the strands of the short, double-stranded RNAs. Cellular machinery consisting of proteins with endonucleolytic cleavage activity recognize the bound RNA complex and cleave the mRNA transcript, rendering it un-translatable (61-63; see schematic in Figure 3-1).

There are many ways to introduce short, double-stranded RNAs into a cell (2; see schematic in Figure 3-2). Cells may be directly transfected with 19-22-mer small interfering RNAs (siRNAs), the sense strand of which binds an mRNA transcript targeted for knock-down. Alternatively, longer double-stranded RNAs (dsRNAs) which are complementary to a longer span of the target gene may be cleaved into several siRNAs, each of which is a complement to a different site of the target mRNA transcript, and the mixture of random siRNAs may be introduced into a cell. DNA can also be introduced into a cell (either as a plasmid or as a viral vector), which encodes for the sense and antisense strands of a short interfering RNA. These strands then anneal to form an siRNA. In cases where interfering RNAs are to be transcribed from DNA, an RNA Polymerase III promoter is placed upstream of each short sequence in order for the short RNA transcripts to remain un-polyadenylated and unmodified. Finally, DNA can be introduced into a cell which encodes a moiety known as a short-hairpin RNA (shRNA). The RNA Polymerase III promoter sits upstream of a DNA sequence encoding the sense strand, then a short hairpin loop, and then the antisense strand of an shRNA. The resulting transcript folds in on itself with the short hairpin loop connecting the sense and antisense strands. The complementary strands anneal, and endogenous proteins within the cell cleave the loop, leaving a functioning siRNA.

In order to reduce its availability in the forepaw motor cortex and thereby examine its role in normal learning and in cortical map plasticity in the adult animal, a

lentivirus encoding a short hairpin RNA directed against the transcript for BDNF was created.

The model of cortical motor learning used to investigate the involvement of BDNF in learning and learning-related plasticity was the Whishaw Forepaw Reach Task (64), in which animals reach through a small slot in a plexiglass chamber and grasp a food reward. At the beginning of their training, their successful grasp rate is quite low, but by the end of the training period, they become skilled at reaching for the reward, grasping it, and bringing it to their mouths. Using this paradigm, the effects of BDNF on cortical motor learning, and on cortical map reorganization that occurs when rats learn this skilled reaching task (65-67), were examined.

To test the hypothesis that BDNF plays a role in motor learning and in motor map organization, siBDNF or control virus was injected into the forepaw area of the motor cortex of adult male rats, who were then trained on the forepaw reach task. When they reached a performance plateau, maps were made of their motor cortices using intracortical microstimulation (ICMS). ICMS, developed by Stoney et al in 1968 (68), provides a means to investigate movements elicited via electrical stimulation of relatively focal motor cortical stimulation sites.

Based on the results of the experiments described above, it is concluded that decreasing the availability of BDNF protein in the motor cortex by 22% (compared to

uninjected cortices) or 34% (compared to control virus-injected cortices) does not have an effect on motor learning or on motor map organization.

Materials and Methods

Cloning the H1 promoter into a lentiviral plasmid

The general cloning strategy, the plasmid pSuper-like containing the H1 RNA Polymerase III promoter, as well as a lentiviral backbone plasmid containing the GFP sequence under the direct control of a CMV promoter and an Nhe1 restriction site in the U3 untranslated region (LV-CMV-GFP-U3Nhe1) were generously provided by Dr. Inder Verma (69; Salk Institute for Biological Sciences, La Jolla, CA). The H1 promoter was amplified out of the pSuper-like plasmid by PCR using a forward primer containing a 5' Xba1 site and a reverse primer containing a 3' Xho1 site and an Nhe1 site. Their sequences are shown in Figure 3-3.

The PCR product was digested with Xba1 and Nhe1 and cloned into p LV-CMV-GFP-U3Nhe1 after linearization with Nhe1. The resulting plasmid will be referred to as LV-CMV-GFP-U3-H1.

Cloning an shRNA behind the H1 promoter

Several web-based siRNA design programs (InvivoGen, http://www.sirnawizard.com/design.htm, Promega, http://www.promega.com/siRNADesigner/program/default.asp, and Ambion, http://www.ambion.com/techlib/misc/siRNA_finder.html) were used to evaluate 19-mer interfering sequences from the BDNF gene. A list of candidate sequences was generated by each software program, using both proprietary and user-defined criteria, such as GC content, limitations on consecutive base repeats, and selection of initiating bases. The sequence lists were compared, and sequences which appeared on multiple lists were given preference. BLAST searches were conducted on selected sequences to reduce the likelihood of off-target effects, and, of these, three 19-mer sequences were chosen to be tested as potential shRNAs (also referred to as siRNAs). The sequences were: 1) AGACTGCAGTGGACATGTC, 2) AAGGCCAACTGAAGCAATA, and 3) TCATAAGGATAGACACTTC. Leading strand and complementary strand oligonucleotides for each of the three sequences were made, which, when annealed, contained partial 5' Xho1 and 3' Nhe1 overhangs, as shown below:

5' TCGACCC-19-TTCAAGAGA-19-TTTTTGGAAA 3'
3' GGG-19-AAGTTCTCT-19-AAAAACCTTTGATC 5'

LV-CMV-GFP-U3-H1 was then cut with Xho1 and Nhe1 and each of the three putative siRNAs was cloned in downstream of the promoter, creating three different plasmids for testing, each containing one siRNA sequence downstream of the H1 promoter and the GFP gene downstream of the cytomegalovirus (CMV) promoter. A schematic of the resulting plasmid is shown in Figure 3-4.

In vitro testing of three putative siRNA-containing plasmids

Testing of the effectiveness of each of the three candidate siRNAs was carried out using a stable line of transduced fibroblasts overexpressing and releasing BDNF. The generation and characterization of the cell line were described previously (70); briefly, primary rat fibroblasts from skin biopsies were genetically modified to produce human BDNF. The BDNF transgene was constitutively expressed by the long terminal repeat (LTR) and was secreted by the cells into the medium, as verified by a two-site enzyme-linked immunosorbent assay (ELISA).

BDNF-transduced fibroblasts were grown to confluence and infected with replication-incompetent, HIV-1-based lentivirus (see below for description of lentiviral production) produced from either a plasmid containing only the H1 promoter (LV-CMV-GFP-U3-H1, which from this point forward will be referred to as siControl), or one of the three siRNA-containing plasmids. Successful incorporation of the virus was confirmed by the presence of green, GFP-expressing cells. Supernatants were collected from cells twenty-four hours after transduction with one of the four viruses (either the siControl virus, or one of the three putative BDNF siRNA viruses). BDNF protein levels in the supernatants were then measured by an ELISA (71). Costar 3590, 96-well EIA/RIA plates were coated with a 1:4,000 dilution of either capture antibody (Chemicon, AB1779) or normal rabbit serum and incubated

overnight at 4˚C. Plates were washed in wash buffer consisting of PBS with 0.4M NaCl and 0.5% Triton X-100, blocked in PBS containing 3% bovine serum albumin for 60 minutes, and washed again. Known quantities of purified BDNF protein or fibroblast supernatants were added and allowed to incubate overnight at 4˚C. Plates were washed, and a 1:2500 dilution of chicken anti-BDNF (Promega G1641) was added and allowed to incubate overnight at 4˚C. Plates were washed and peroxidase-conjugated anti-chicken IgY (Promega G1351, 1:1000 dilution) was added and allowed to incubate overnight at 4˚C. Following extensive washes, a soluble calorimetric product was generated by adding a solution containing 0.033 M sodium phosphate, 0.016 M citric acid, 0.05% o-phenylenediamine and 0.02% H_2O_2. Plates were then incubated in the dark for approximately 5 min before stopping the reaction with the addition of a 10% H_2SO_4 solution. Optical density measurements were read on a microplate reader at an absorbance of 490 nm.

Of the three siRNA sequences tested, sequence #2 (which from this point forward will be referred to as siBDNF) was particularly successful at reducing BDNF levels, and was then further tested on fibroblasts overexpressing BDNF, but under the control of a regulatable tetracycline-responsive promoter. Tetracycline-regulatable systems make use of promoters which are either inactive at baseline and activated in the presence of tetracycline (or its analog, doxycycline [DOX]; “tet-on”; [72]), or which are constitutively active until the addition of tetracycline or doxycycline represses them (“tet-off”; [73]). Fibroblasts expressing tet-on regulated BDNF were

created in our laboratory (74). Adding DOX to the medium of these cells activates the promoter and increases levels of BDNF expression by approximately 15-fold compared to the minus DOX condition, in which very low amounts of BDNF are detectable in the supernatants. The tet-on BDNF fibroblasts were plated at a density of 300,000 cells per 60 mm plate, grown overnight, and infected with either siBDNF lentivirus (1 µl, titer 283 µg/ml p24 and 9.5 x 10^9 IU/ml) or siControl lentivirus (3 µl, titer 105 µg/ml p24 and 9 x 10^8 IU/ml). Cells with no virus infection were also grown in parallel. Virus-infected cells were then sorted using Fluorescence Activated Cell Sorting (FACS) into fractions weakly expressing GFP (19x background fluorescence), or strongly expressing GFP (127x background fluorescence for siBDNF cells and 151x background fluorescence for siControl cells), as a rough indication of the amount of shRNA they were expressing. Five groups of cells (those strongly expressing siBDNF, weakly expressing siBDNF, strongly expressing siControl, weekly expressing siControl, and those not infected with any virus) were grown separately. Cells were plated at a density of 50,000 cells per well in six-well plates. DOX was either added (1 µg/ml medium; "+DOX") or not added ("-DOX") to wells of each cell type, such that 10 total conditions existed, with three replicates per condition. Twenty-four hours after plating and +/- DOX treatment, supernatants for all of the wells of all of the conditions were then collected and BDNF protein measured via ELISA.

Making lentiviral vectors siControl and siBDNF

The siBDNF plasmid and the siControl plasmid were packaged into concentrated lentiviral vectors for use in *in vivo* studies. The procedure for producing lentiviral vectors has been previously described (75, 76); briefly, 293T cells were grown to subconfluence and simultaneously transfected, using CaCl, with a plasmid containing the sequence of interest (either siControl or siBDNF, each of which also expresses GFP) and three packaging plasmids containing viral coat and packaging genes. The medium was changed 24 hours after transfection, and then supernatants were collected at two 24-hour intervals. The virus-containing supernatants were then centrifuged at 24,000 rpm, and pellets were resuspended in 100-200µl of Hank's Balanced Salt Solution (Invitrogen, Carlsbad, CA). The concentrated virus was then split into 10µl aliquots and frozen. To determine viral titer, test aliquots were taken and the amount of the viral capsid protein p24 was measured using a p24-specific ELISA kit (DuPont, Billerica, MA) as described previously (75). Viral titer was expressed as µg p24 per ml (µg/ml p24). Viral infectivity was also measured using a cell based infectious assay, as has been previously described (77). Briefly, 293T cells were seeded at a density of 50,000 cells per well in a 24 well plate, with 1 ml of medium per well. They were then infected with 4 10-fold serial dilutions of virus (from 10^{-2} µl to 10^{-5} µl), with each dilution repeated in triplicate. After a two day incubation period, GFP-positive colonies were counted in each well. Each colony was assumed to have been infected by one infectious unit, and viral infectivity was expressed as infectious units per ml (IU/ml).

Surgical technique

All animal procedures were performed in compliance with AAALAC and institutional guidelines and regulations for the humane treatment of animals. Adult male Fischer 344 rats were anesthetized using a cocktail (2ml/kg) of ketamine (25 mg/ml), xylazine (1.3 gm/ml), and acepromazine (0.25 mg/ml) and placed in a stereotaxic head frame. Injections of either siControl lentivirus or siBDNF lentivirus were made into the forepaw region of the motor cortex.

For cortical injections, the bone over the cortex was either removed or was carefully drilled until a very thin layer was left, through which small burr holes could be made. Virus (average titer siBDNF 197 μg/ml p24 and 3.2×10^9, average titer siControl 223 μg/ml p24 and 1.7×10^{10}) was injected at 8 sites per side (Bregma + 0.3 mm AP and +/- 2.8 mm and +/- 3.5 mm ML, Bregma + 0.8 mm AP and +/- 3.1 mm and +/- 3.9 mm ML, Bregma + 1.3 mm AP and +/- 2.8 mm and +/- 3.5 mm ML, and Bregma + 1.3 mm AP and +/- 3.1 mm and +/- 3.9 mm ML) at a depth of 1.5mm. At each of the 8 sites, approximately 250nl of virus was injected at a rate of approximately 1μl per minute. The animals were allowed to recover two weeks after surgery before motor skill training began.

Behavior

Adult male Fischer-344 rats (n=43; aged 3-5 months) were trained in the Whishaw Forepaw Reach Task, in which rats are trained to reach for and grasp a food pellet through a small slot in a plexiglass cage (64). The behavioral paradigm was described in detail in the previous chapter. In this study, all of the animals were trained in the skilled motor task, wherein they reached for and grasped pellets, and all of the animals were trained for the entire two-week period.

Intracortical microstimulation

Maps of the motor cortices bilaterally were constructed using intracortical microstimulation techniques previously described (66). Animals were anesthetized initially with 70 mg/kg ketamine i.p, then, after 5-10 minutes, with 5 mg/kg xylazine i.p. Throughout the mapping procedure, they received supplementary doses of the ketamine/xylazine mixture and/or isofluorane gas anesthesia as needed. The bone over the motor cortices was removed, but, in most cases, the dura was left intact over most of the exposed cortex. This significantly reduced cortical damage due to drying, swelling, and vascular insult. Pulled glass electrodes were filled with 3 M NaCl, and a chlorided silver wire was placed into the electrode such that its tip was well submerged in the NaCl solution. Penetrations with the microelectrode were made systematically at 500 μm intervals across the motor cortex at a depth of 1.7mm-1.8mm, corresponding to cortical layers V-VI. At each site, two 30 ms pulse trains were delivered 1.2 s apart. Each pulse train consisted of 200 μs monophasic cathodal

pulses delivered at 333 Hz from an electrically isolated, constant current stimulator (Axon Instruments, Union City, CA) under the control of a programmable pulse generator (AMPI, Jerusalem, Israel). Body movements evoked by the stimulation were observed with the animal in a prone position. Pairs of pulse trains were delivered and the current delivered by each stimulus (10 μA to 250μA) was gradually increased until a movement could be discerned. The current at which this occurred was considered the "threshold" current for the movement at that site. The area of cortex devoted to a particular movement (wrist, elbow, shoulder, etc…) was calculated by multiplying the number of sites evoking that movement by $0.25mm^2$. If two movements occurred at the threshold current in a given site, they were both recorded, and each was considered to occupy one half of the cortical area represented by that site.

Histology

Animals with motor cortex injections were either decapitated for quantification of siBDNF-mediated reduction two weeks after virus injections or, if they were part of the skilled motor learning experiments, they were mapped, and their tissue was processed to confirm adequate spread of virus into the cortex (via GFP immunofluorescence) and to assess whether any cortical damage resulted from the virus injections (via Nissl staining) which could have interfered with learning and maps.

Freshly extracted brains from decapitated animals (n=12) were placed in a brain blocker, and the 2mm x 2.5mm section corresponding to the approximate area of the viral injections bilaterally was dissected. The mediolateral borders of this area are from 2 mm lateral to midline to 4.5 mm lateral to midline, and the rostrocaudal borders are from bregma to 2 mm rostral to bregma. The entire volume of grey matter (all VI cortical layers) corresponding to this area was dissected and frozen at –70˚C. Fresh-frozen dissections of forepaw motor cortex were sonicated in homogenization buffer (PBS with 0.4 M NaCl, 0.5% BSA, 0.1 M PMSF, 20 RI/ml aprotinin, 5 mM EDTA and 0.1% Triton X-100) while being kept cool in a crushed dry ice/ethanol bath. The solution was spun down (14,500 rpm for 30 minutes at 4˚C) and the supernatant was collected for BDNF ELISA (described above).

Tissue from animals which were part of the motor learning experiment (n=32) was used for histological confirmation of virus spread and extent of injection-mediated damage. Experimental subjects were perfused with 75 ml phosphate buffered saline followed by 250 ml 4% paraformaldehyde (PFA). Their brains were removed and post-fixed in PFA overnight, then transferred to 30% sucrose for several days. Brains were then blocked and 40µm coronal slices through the motor cortex were made on a sliding microtome. Adjacent series of sections (a series is one in six slices, such that the interval between sections in a series is 240µm) were processed for Nissl staining and for GFP fluorescent immunolabeling. Sections were washed in 0.1 M Tris-

buffered saline (TBS) and blocked in 5% donkey serum plus 0.25% Triton X-100 in TBS for one hour. They were then incubated in goat anti-GFP (Rockland, Inc; 1:1500 dilution in blocking solution) overnight at 4˚ C. They were rinsed in TBS plus 0.25% Triton X-100 and incubated in the same solution with a 1:150 dilution of secondary antibody (Alexa 488 flourophore donkey anti-goat, Molecular Probes, Eugene, OR) for 2.5 hours at room temperature. Sections were finally rinsed in TBS, mounted on gel-subbed slides, dried for 30 minutes, and coverslipped using Fluoromount G (Southern Biotech, Birmingham, AL).

For the motor learning experiment, a total of 43 animals were injected with either the siBDNF virus (n = 19) or siControl virus (n = 13), or received sham operations (n = 11), from which a total of 32 animals met the analysis criteria (n = 12 siBDNF, 8 siControl, and 11 sham-operated). Criteria for exclusion are discussed below, and included lesions of the cortex contralateral to the preferred paw, as assessed by Nissl staining, and/or lack of appreciable virus spread in the forelimb motor cortex contralateral to the preferred paw, as assessed by GFP immunoreactivity.

To assess lesion severity, Nissl stained sections through the motor cortex were classified on a four-point scale as exhibiting no lesion, mild, moderate, or severe lesions to the brain parenchyma. Mild lesions included those in which there was some swelling of the cortex through the bone opening, and/or hypercellularity around the virus injection site, and/or mild tissue damage of limited spatial extent. Moderate

damage included mild tissue tearing or other forms of damage extending beyond a small focal area. Severe lesions were observed where large areas of tissue were torn and/or where there was evidence of large areas of red blood cell infiltration, and/or tissue necropsy. Animals which exhibited moderate or worse lesions to the cortex contralateral to the preferred paw were excluded from analysis.

The amount of virus spread was assessed by measuring the mediolateral and dorsoventral extent of GFP immunoreactivity in each slice of a series (one in six 40 μm slices) through the motor cortex, from which an area of virus spread per slice was derived. An approximate volume of spread was obtained by a summing the area of spread per slice, multiplied by 240 μm (the rostrocaudal distance between slices in a series). The average spread of virus in siBDNF-injected animals was 2.25 mm^3, and in siControl animals, 3.11 mm^3. Animals which had less than 1.5 mm^3 of virus spread were excluded from analysis.

Statistics

All analyses were done using a Student's t-test or an analysis of variants (ANOVA). For the behavioral data, learning across days was analyzed using a two-way repeated measures ANOVA to assess the effect of treatment group, training day, and any interaction between the two. Where the null hypothesis was rejected, post-

hoc pairwise comparisons were done using a Fisher's protected least significant difference (Fisher's PLSD).

Results

ShRNAs directed against BDNF effective in vitro

The ability of three candidate siRNAs to reduce BDNF protein levels in BDNF-over-expressing fibroblasts was examined. Supernatants from cells infected with all three siRNAs exhibited reduced BDNF protein levels compared to supernatants from siControl infected cells. SiRNA sequence #1 reduced BDNF protein levels by 51%, #2 by 80%, and #3 by 35% of those of the control condition. Levels of BDNF were most effectively reduced by siRNA sequence #2 (from 3,622 pg BDNF/10^6 cells/24 hours to 711 pg BDNF/10^6 cells/24 hours). This construct was therefore utilized for additional *in vitro* and *in vivo* studies.

To assess whether siRNA sequence #2 (also known as siBDNF) could successfully reduce both high and low levels of BDNF, a cell line was used which expresses BDNF under the control of a regulatable promoter. Cells both with and without DOX in the medium were infected with either siBDNF virus, siControl virus, or no virus at all. The results of this experiment are shown in Figure 3-5.

In the no virus condition, in the absence of DOX, these cells produced 93% less BDNF than when DOX was present (+Dox 1,380 +/-180 pg/mg BDNF, -Dox 94 +/-19 pg/mg BDNF, mean +/- S.E.M.). In conditions of high levels of BDNF expression (+ DOX), a high number of siBDNF copies per cell as well as a low copy number per cell were able to significantly reduce BDNF production (by 82% and 89%, respectively, compared to siControl condition, and by 74% and 89%, respectively, compared to the "No Virus" condition; Fisher's PLSD $p<0.0001$ in all cases). In conditions where very low levels of BDNF are produced (-DOX), low copy numbers of siBDNF were not sufficient to have any effect, whereas a high copy number of siBDNF per cell were able to reduce low levels of BDNF production to undetectable levels, though this effect was not significant ($p=0.12$ compared to siControl condition). This and the previous experiment indicate that the siBDNF virus significantly reduces BDNF expression in transduced fibroblasts *in vitro*.

Quantification of siBDNF-mediated reduction in adult rat motor cortex

Figure 3-6 shows examples of cortical injections of siBDNF and siControl viruses in the motor cortices of animals which were trained on the forepaw reach task and mapped, with the spread of virus indicated by GFP-fluorescence. To quantify the degree of siBDNF-mediated BDNF reduction in the cortex, six rats received motor cortical injections of siBDNF on one side and siControl on the contralateral side. BDNF ELISA was used to measure BDNF protein levels in siBDNF versus siControl

motor cortices. Three naive animals were also sacrificed and their 6 forepaw motor cortices were analyzed in parallel. The ELISA results are shown in Figure 3-7. The overall ANOVA comparing the three groups shows a statistically significant difference in BDNF levels ($p<0.05$). A Fisher's post-hoc analysis reveals that the siBDNF-injected cortices contained a significant 34% less BDNF than the siControl-injected cortices (siBDNF 0.57 +/- 0.11 pg/mg BDNF, siControl 0.87 +/- .05 pg/mg BDNF, $p<0.05$). A trend toward a reduction in siBDNF versus naïve cortices was observed (naïve, 0.79 +/- 0.06 pg/mg BDNF, 28% reduction, $p<0.07$, Fisher's PLSD), but this was not significant. This trend was confirmed in a second experiment comparing siBDNF-injected to naïve cortices, revealing a significant ($p<0.05$) si-BDNF-mediated reduction of BDNF protein of approximately 22% (siBDNF 2.56 +/- 0.10 pg/mg BDNF, naïve 3.28 +/-0.29 pg/mg BDNF; Figure 3-8).

Lack of effects of siBDNF on learning and map plasticity

To determine the effects of BDNF attenuation on cortical motor learning and on cortical map plasticity, animals receiving sham operations, or bilateral injections of siBDNF virus or siControl virus into their forepaw motor cortices were trained in the skilled reach task. The assessments on the behavioral and electrophysiological effects of siBDNF assume a 22% reduction of BDNF availability in the forepaw motor cortex when comparing siBDNF-injected to sham-operated animals, and a 34% reduction

when comparing siBDNF-injected to siControl-injected animals, based on the ELISA measurements in other animals.

Behavioral data from the siBDNF (n=12), siControl (n=8), and sham-operated (n=11) animals are shown in Figure 3-9. A repeated measures ANOVA reveals that there was a significant learning effect ($p<0.0001$), but there was no effect of treatment group. When rats reached a performance plateau, maps were made of their motor cortices bilaterally for six siBDNF animals, six siControl animals, and three sham-operated animals. Representative maps from an animal in each group are shown in Figure 3-10. One siControl and one sham-operated animal reached with both paws, so data reported are the average from both sides of the cortex. Otherwise, only map data from the cortex contralateral to the preferred paw are reported. In previous studies describing map reorganization on motor learning, different aspects of the motor map were found to reorganize. One group found that the size of the entire caudal forelimb area (CFA) increased with learning (65, 66), and the other found that, while the size of the CFA remains constant, the percentage of the CFA devoted to distal (wrist) versus proximal (elbow/shoulder) movements increased (67). Both measures are therefore reported in Figure 3-11, as are sample maps from one animal in each of the three experimental groups. There was no significant effect of treatment on either size of the CFA (Figure 3-11A) or percentage of the CFA devoted to wrist movements (Figure 3-11B).

Discussion

A short hairpin interfering RNA directed against BDNF was created that can be delivered to cells via lentivirual vectors, where it is processed into a functioning siRNA. This construct reduced BDNF protein levels *in vitro* by 81% (compared to uninfected cells) and 85% (compared to siControl-infected cells), and *in vivo* in the motor cortex by 22% (compared to naïve cortices) and 34% (compared to siControl-injected cortices). The siBDNF was injected into the forepaw area of the rat motor cortex and rats were trained on the Whishaw Forepaw Reach Task to test the hypothesis that BDNF is involved in motor learning and motor map organization, and it was observed that skilled motor learning appeared to be unaffected by the siBDNF-mediated reduction. Rats receiving motor cortex injections of the siBDNF virus performed the same as control rats injected with siControl virus and as sham-operated rats. Further, the caudal forelimb area (CFA), as assessed by ICMS-generated maps, was also unchanged among the three groups. The size of the CFA in siBDNF-injected rats was reduced by approximately 21% compared to sham-operated animals, corresponding to the 22% siBDNF-mediated reduction in siBDNF versus naïve cortices, but siControl-injected rats also exhibited a 21% reduction in the size of their CFA, suggesting a non-specific or damage-mediated effect of the viruses.

There is previous precedent for a moderate *in vivo* reduction of BDNF having significant biological effects. In a study using anti-sense oligonucleotides directed against BDNF and infused into the dentate gyrus (29), the authors reported a significant (83%) impairment in inhibitory avoidance learning. This behavioral impairment was observed despite the fact that the antisense-mediated reduction of BDNF mRNA was a moderate, but statistically significant, 26%. In another study using BDNF antisense, learning in an eight-arm radial maze task was almost completely abolished, despite a reduction of BDNF mRNA of less than 20% and a concomitant protein reduction of less than 35% (38). The contention that a relatively small siRNA-mediated BDNF reduction is potentially physiologically relevant is also supported by a study by Baker-Hermann, et al in which siRNAs directed against BDNF were injected into rat spinal cord (78). In that study, naked siRNAs (four different siRNA constructs, pooled) were injected intrathecally and BDNF was subsequently measured in the ventral gray of C4-C5. The siRNAs did not reduce baseline BDNF levels, but they prevented a ~20% increase in BDNF after intermittent hypoxia in anesthetized rats. This small "knock-down" *in vivo* was enough to completely eliminate a ~70% phrenic nerve long-term facilitation that normally occurs after intermittent hypoxia. In this same study, an *in vitro* experiment showed that the pooled naked siRNAs were able to reduce BDNF mRNA by 75% in a hippocampal cell line. These results parallel the ones reported here; a substantial *in vitro* effect with a moderate *in vivo* effect. In that study, however, this moderate reduction was sufficient to cause a physiological response.

The siBDNF construct used in the studies reported here, the most effective of three tested *in vitro*, was able to reduce BDNF levels *in vitro* by 81% compared to uninfected cells and 85% compared to siControl-infected cells. The knock-down was enough to significantly reduce protein production *in vivo*, by 22% compared to naïve cortices or 34% compared to siControl-injected cortices. When compared to an 81%-85% knock-down *in vitro*, however, this reduction is moderate.

There are several potential explanations for this observation. In general, quantitatively comparing *in vitro* with *in vivo* results is difficult, given the different environments surrounding cells *in vitro* versus in an adult brain. Additionally, in these experiments specifically, cell culture work was done on fibroblasts because of their robustness in culture, and because of the large amounts of BDNF they had been transduced to express. *In vivo*, infected cells were almost exclusively neuronal. Thus, in addition to the cells' differing surroundings and differing extracellular matrices, there could be differences in lentiviral infectivity or lentiviral gene production between these cell types. Another factor may be that, *in vitro*, relatively homogenously infected populations of cells were assayed, whereas *in vivo*, a much smaller percentage of cells were infected per unit area assayed, diluting the effect of the lentiviral constructs.

Finally, BDNF protein is actively transported away from the motor cortical cells which produce it, as well as into the motor cortex from other areas (79,80, 81). This robust transport of BDNF protein offers another potential explanation for the moderate reduction of BDNF protein measured in the cortex secondary to siBDNF infection. The local cells producing BDNF in the cortex (and subject to the knock-down) are not necessarily the only or even the major source of local BDNF protein. This confounding factor could be eliminated by an injection of the microtubule transport inhibitor colchicine, which arrests axonal transport of many proteins, including BDNF (81, 82). However, because colchicine disrupts the trafficking of numerous molecules essential to normal neuronal function, behavioral data obtained under these conditions would be unreliable.

Conclusions and Future Directions

The results of the present study show that a BDNF-blocking lentiviral vector has been created that is effective for *in vitro* applications, and that could potentially be effective *in vivo* where a moderate BDNF reduction is desired. In the context of skilled motor learning, a 22% (siBDNF compared to naive) or 34% (siBDNF compared to siControl) reduction of BDNF protein was not sufficient to lead to a change in performance, nor was it sufficient to affect cortical map organization. Future attempts to examine the effects of BDNF in this paradigm should therefore focus on other means of attenuating BDNF signaling, perhaps at the level of the

protein rather than the message, such as ligand capture with receptor bodies or blocking antibodies. Alternatively, if BDNF signaling is more completely blocked using another technique, it may be found that BDNF has no role in modulating motor cortical learning or motor cortex reorganization in the context of motor learning.

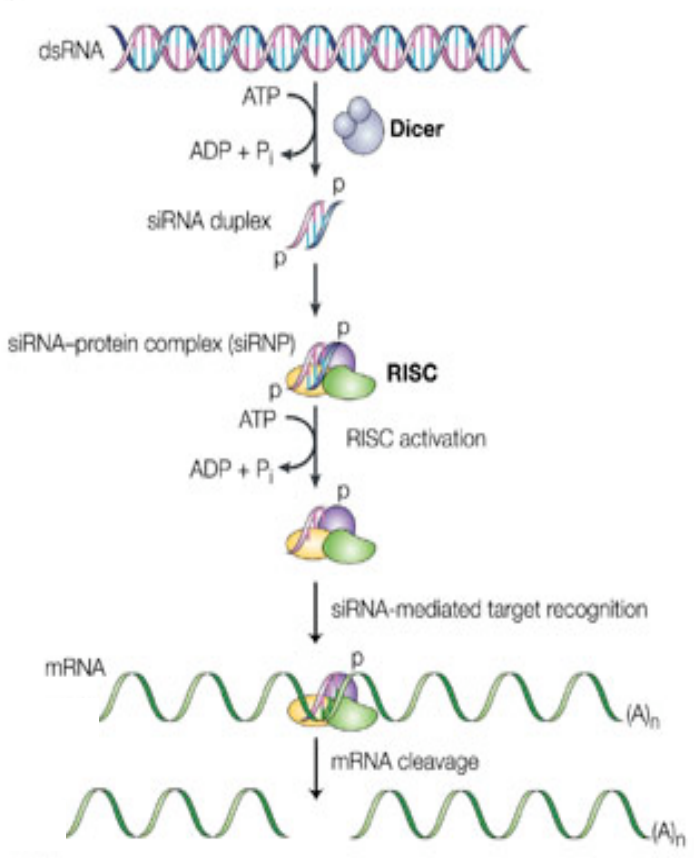


Figure 3-1. Schematic of RNA interference mechanism, reprinted by permission from Macmillan Publishers Ltd: Nat Rev Mol Cell Biol 4:457, 2003 (1).

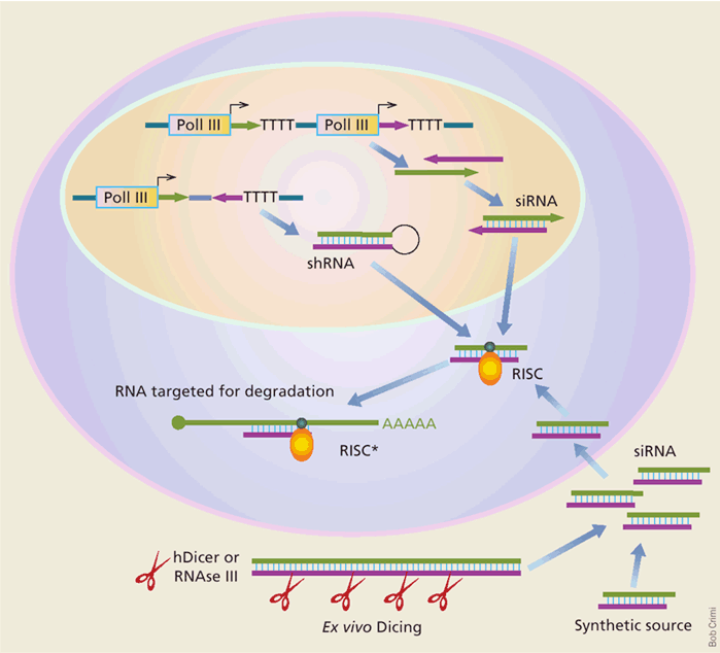


Figure 3-2. Schematic summary of methods of introducing siRNAs into a cell, reprinted by permission from Macmillan Publishers Ltd: Nat Biotechnol 21:1457, 2003 (2).

H1F1 (30mer):

H1R1 (39mer):

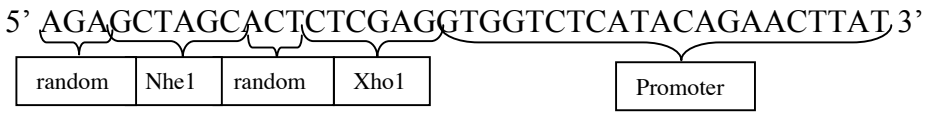


Figure 3-3. Forward and reverse primers used to amplify the H1 promoter out of the pSuper-like plasmid.

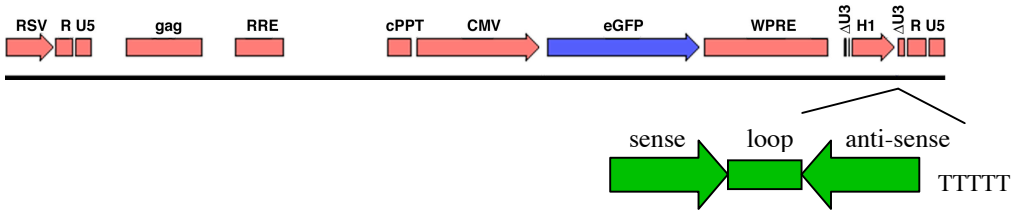
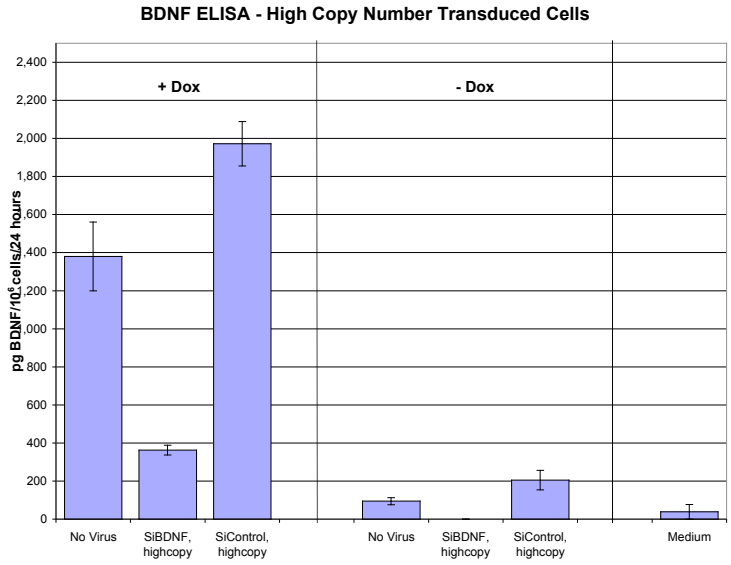


Figure 3-4. Schematic of the shRNA plasmid.

A)



B)

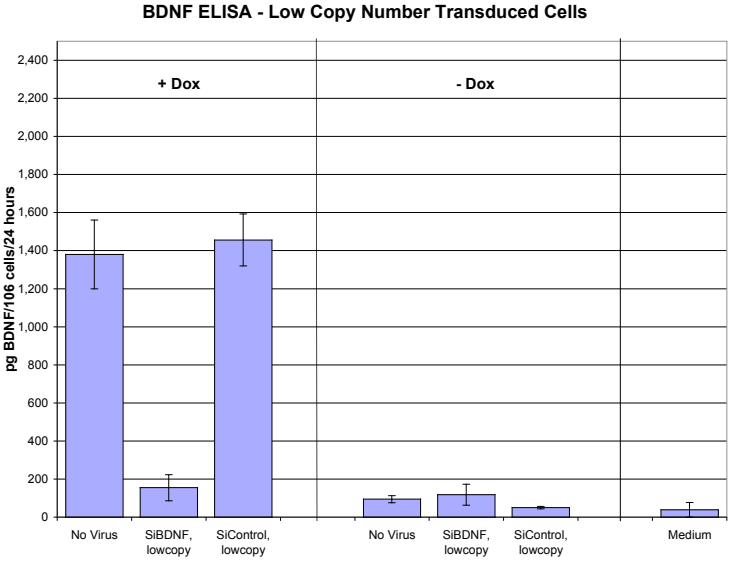


Figure 3-5. BDNF ELISA of supernatants of FACS sorted cells infected with the siControl virus or siBDNF virus with or without doxycycline in the cell medium illustrating the effect of siBDNF-expressing virus on cells expressing high amounts of BDNF under doxycycline-mediated activation or lower constitutive amounts. BDNF protein measured is pg BDNF per million cells per 24 hours. Values shown are mean +/- S.E.M. **A**) BDNF ELISA results from cells in the FACS-sorted partition containing high levels of green fluorescence, indicating high copy-number infection (overall ANOVA $p<0.0001$), and **B**) low levels of green fluorescence, indicating low copy-number infection (overall ANOVA $p<0.0001$).

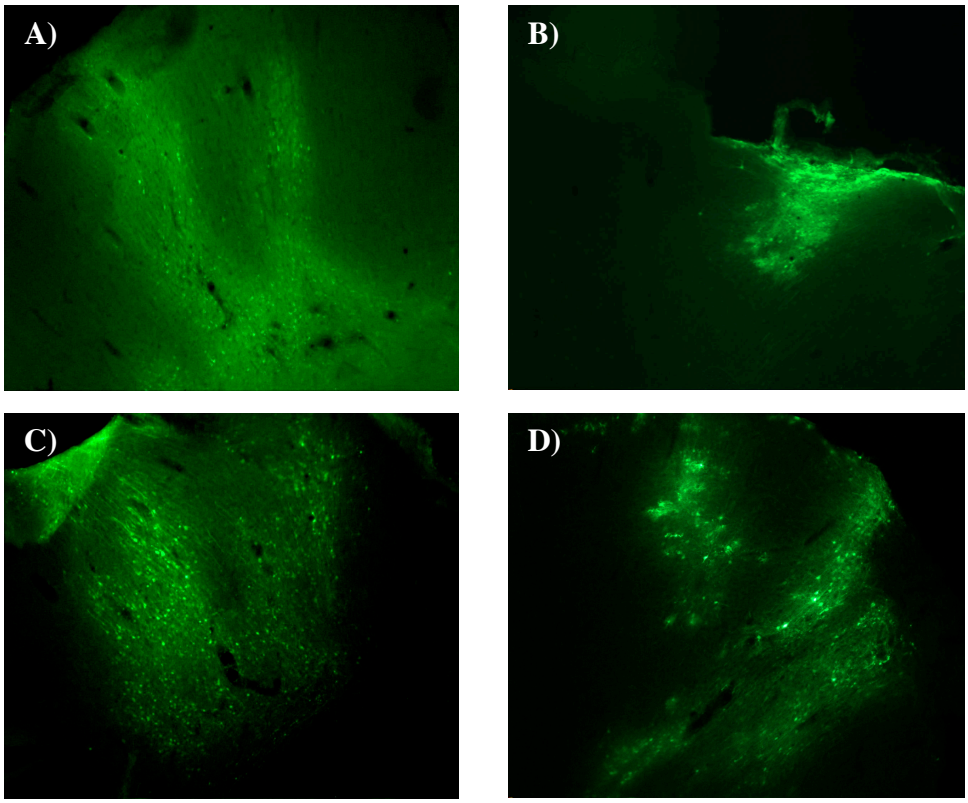


Figure 3-6. GFP immunoreactivity illustrating virus injections into forepaw motor cortex. **A**), **B**) siBDNF virus injections. **C**), **D**) siControl virus injections. **A**), **C**) Animals with adequate virus spread (>1.5mm^3) **B**), **D**) Animals with inadequate virus spread (<1.5mm^3).

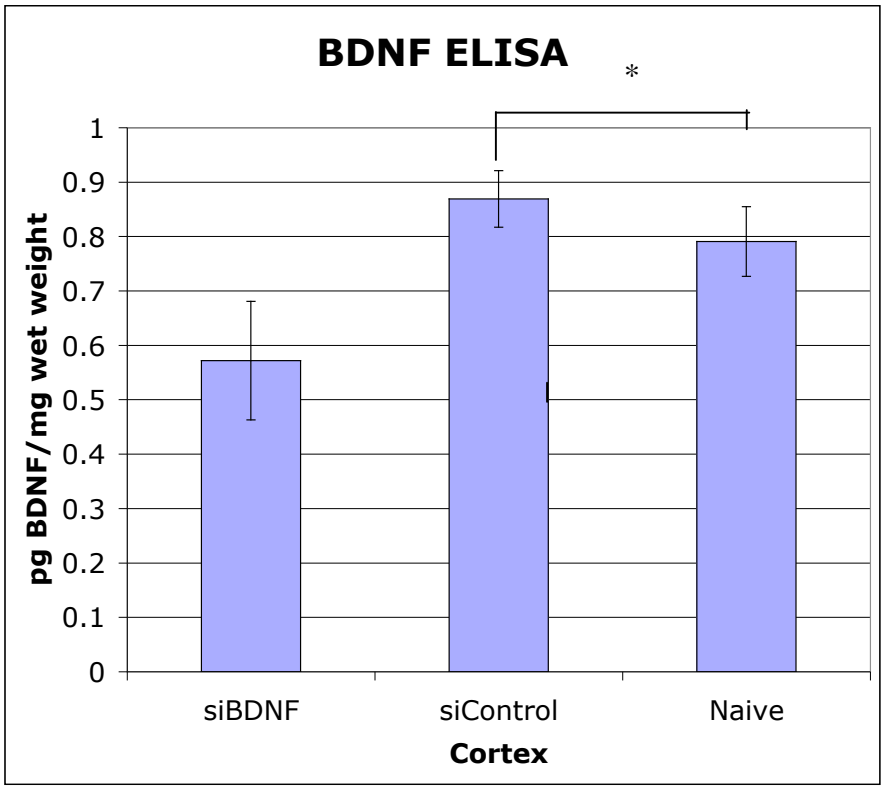


Figure 3-7. BDNF ELISA of siBDNF-injected, siControl-injected, and naive motor cortices. BDNF protein measured is pg BDNF per mg wet weight tissue. Values are expressed as means +/- S.E.M. Overall ANOVA $p<0.05$. * $p<0.05$

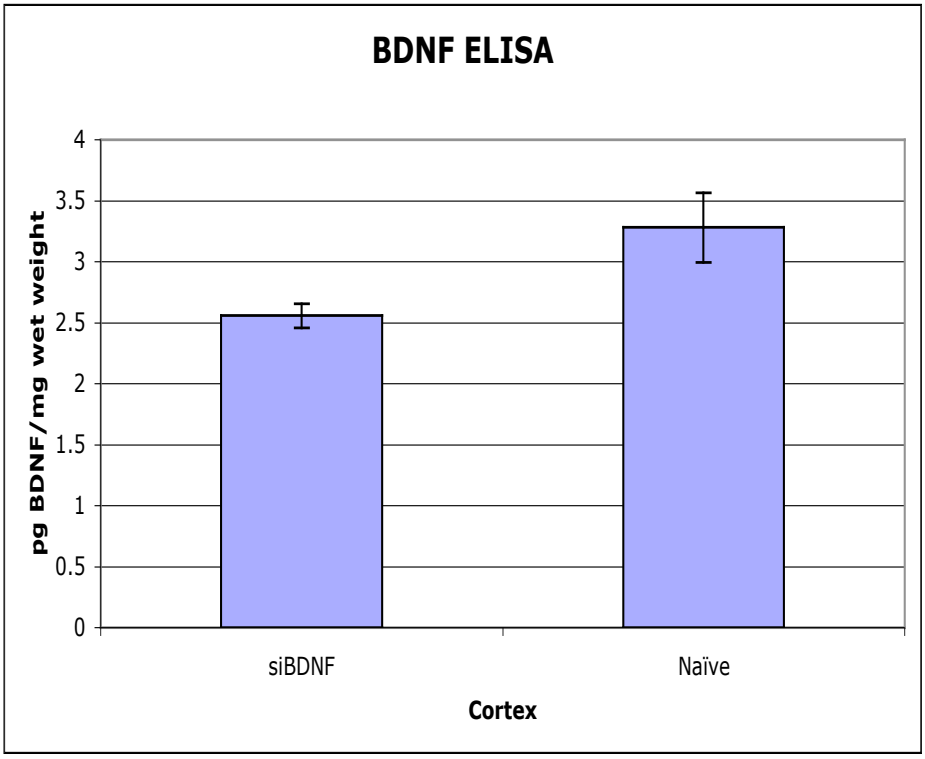


Figure 3-8. BDNF ELISA of siBDNF-injected and naive motor cortices. BDNF protein measured is pg BDNF per mg wet weight tissue. Values are expressed as means +/- S.E.M. Student's t-test $p<0.05$.

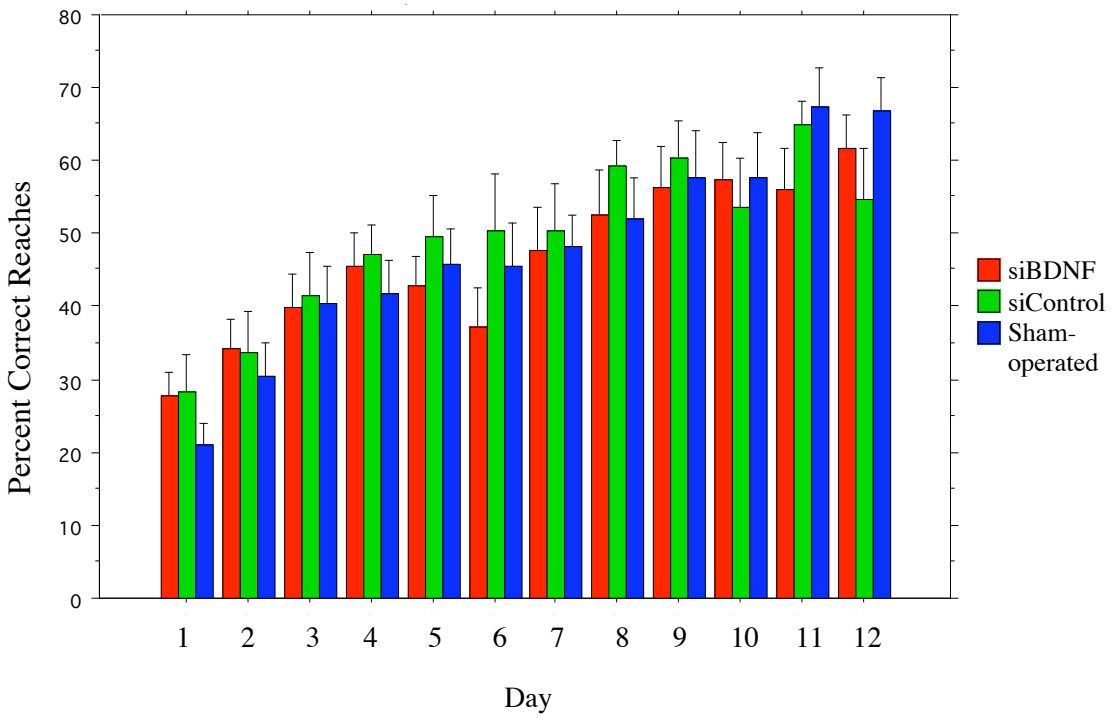


Figure 3-9. Task performance expressed as percent correct reaches on each training day for siBDNF-injected (n = 12), siControl-injected (n = 8), and sham-operated (n = 11) animals. Error bars shown are +/- S.E.M. Repeated measures two-way ANOVA $p<0.0001$ for task acquisition, $p=0.88$ for treatment group, $p=0.42$ for acquisition by treatment group.

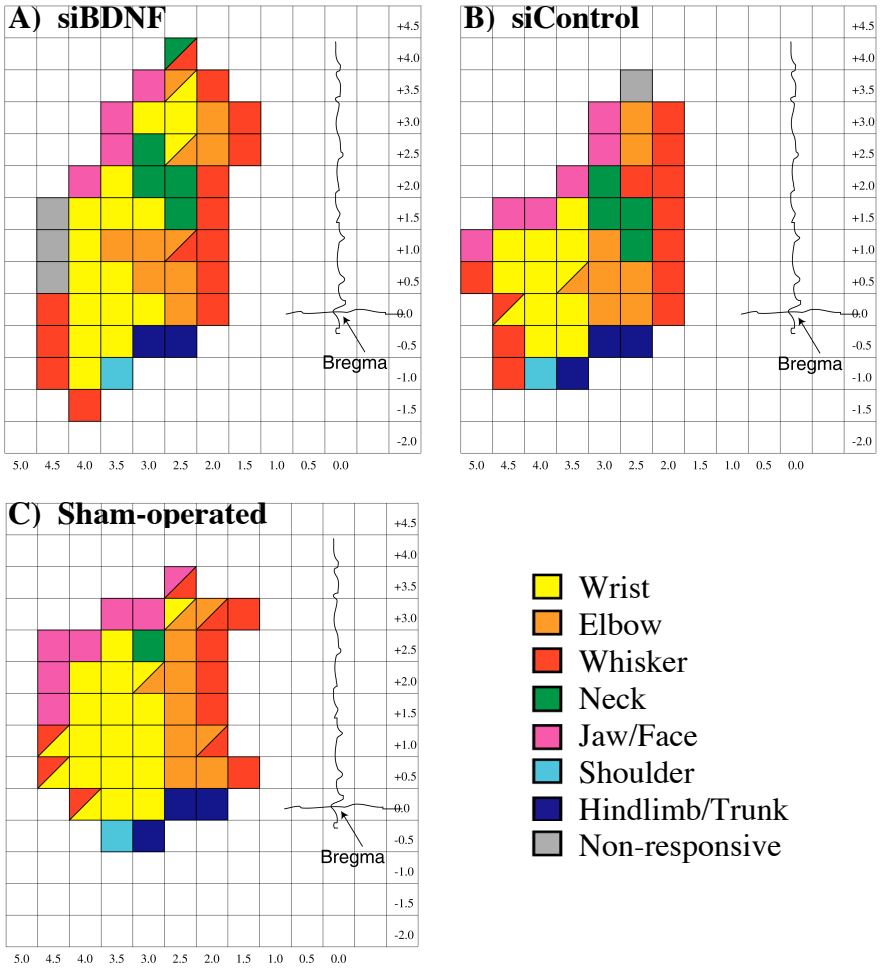


Figure 3-10. Representative motor maps from **A**) siBDNF-injected, **B**) siControl-injected, and **C**) sham-operated animals. Horizontal and vertical numerical designations on the grid represent mediolateral and rostrocaudal coordinates of the motor cortex (expressed in mm from Bregma), respectively. Each square on the grid represents one ICMS penetration site, with color corresponding to body part in which movement was elicited.

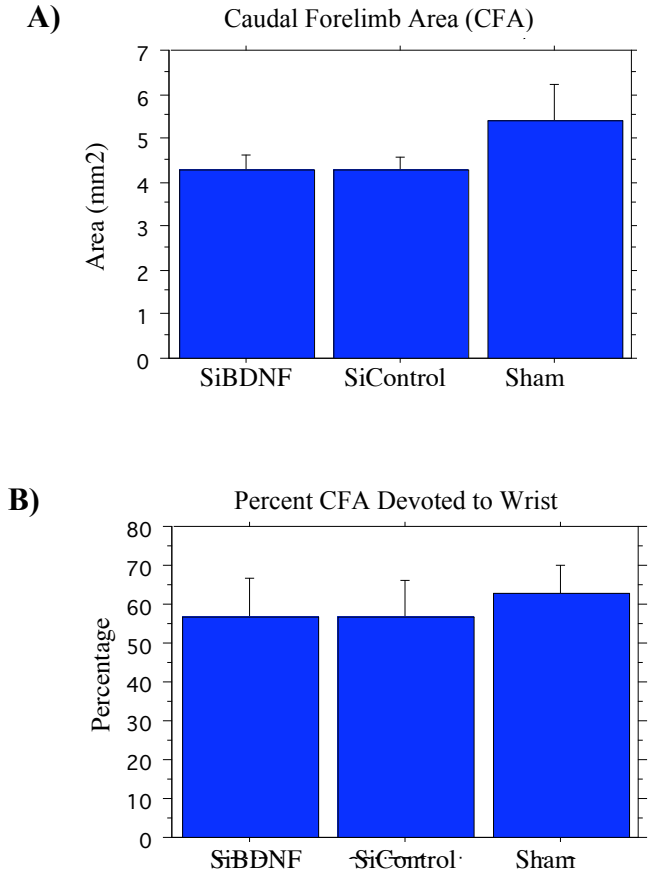


Figure 3-11. Motor map data for siBDNF-injected (n=6), siControl-injected (n=6), and sham-operated (n=3) animals. **A**) Size of the caudal forelimb area in the cortex contralateral to the preferred paw, expressed in mm^2. ANOVA p=0.21. **B**) Percentage of the preferred caudal forelimb area devoted to wrist movements. ANOVA p=0.92. Error bars shown are +/- S.E.M.

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Chapter 4: Effect of Sequestering BDNF on Cortical Plasticity

Abstract

Brain-derived neurotrophic factor (BDNF) has been shown to be involved in learning and synaptic plasticity in the adult brain. To further investigate the second and third specific aims of this dissertation, I sought to test the hypothesis that BDNF blockade in the motor cortex of normal adult rats would block cortical motor map plasticity and functional acquisition of skilled forelimb reaching by infusing a BDNF sequestering molecule (TrkB-Fc receptor body) that would reduce BDNF accessibility to its high affinity receptor, TrkB. The TrkB receptor body blocked BDNF activity *in vitro* but did not alter the acquisition of a motor skill or motor map organization *in vivo*. It is concluded that attenuating BDNF signaling in the motor cortex using a BDNF sequestering construct does not have an effect on skilled motor learning or on motor map organization.

Introduction

The neurotrophin brain-derived neurotrophic factor has been widely investigated as an activity-dependent modulator of neuronal structure and function in the adult brain. In particular, its role in learning and memory-related plasticity has been the subject of intensive study.

Activity-dependent changes in synaptic strength appear to underlie cellular and synaptic plasticity. BDNF is a molecule whose expression (reviewed in 1-4) and secretion (5-7) are tightly regulated by neuronal activity. It is able to modulate synaptic transmission (reviewed in 8-10), and cause or facilitate changes in synaptic efficacy (11-24). BDNF has also been shown in many paradigms to be regulated by (25-36) and have a role in learning-related plasticity (25, 29, 33-35, 37-50). Among the most informative methods by which to examine its role in the adult brain is to block or attenuate its availability and examine subsequent effects on structural, physiological, and behavioral plasticity.

To assess the role of endogenous BDNF in learning and memory, a variety of techniques have been employed to block BDNF signaling in the brain: function-blocking antibodies, antisense oligonucleotides directed against BDNF, and dominant negative forms of the high-affinity receptor for BDNF, TrkB. The studies examining the function of antisense oligonucleotides directed against BDNF mRNA were reviewed in the preceding chapter. Two laboratories have also used BDNF blocking-antibodies, and one has used non-functioning TrkB receptors consisting of the extracellular domain of TrkB, conjugated to the Fc region of human IgG (referred to as TrkB-Fc or TrkB-IgG) to investigate the role of BDNF in normal adult learning.

One study showed that infusion of BDNF blocking antibodies into the parietal cortex (0.5 μg/side) immediately after training led to impaired performance 24 hours after training in a one-trial inhibitory avoidance task in rats (45). In another study by the same laboratory using infusion of BDNF blocking antibodies, this time into the CA fields of the hippocampus (29), BDNF was found to be important for short- and intermediate-term inhibitory avoidance memory.

In another study (49) of BDNF function in hippocampal-dependent learning, antibodies to BDNF were infused intraventricularly and led to learning impairments in the Morris Water Maze. It should be mentioned, however, that this laboratory is the same source from which we received the BDNF antibodies, which, in our hands, neither blocked, nor even bound, BDNF.

A study of fear conditioning in the amygdala found that intra-amygdala infusion of TrkB-IgG (35) resulted in an impairment of fear conditioning, as assessed by the fear-potentiated startle response. The TrkB-IgG construct did not, in this study, impair basal amygdala function, performance, or expression of fear.

BDNF-blocking receptor bodies and antibodies have also been used to explore the role of endogenous BDNF in cellular and synaptic plasticity, events which may be correlates of learning.

Many studies have described the impairment of hippocampal LTP secondary to application of BDNF antibodies and/or TrkB receptor bodies (21, 51-54, reviewed in 55). In one of these studies, TrkB-Fc was shown to block consolidation of LTP as well as a BDNF-mediated increase in spine filamentous actin secondary to high-frequency stimulation (54). TrkB receptor bodies were shown to reduce a BDNF-mediated increase in AMPA receptor subunits (56), as well as a BDNF-mediated modulation of vesicular release during hippocampal long-term potentiation (57). Sequestration of BDNF increased the frequency of spontaneous inhibitory post-synaptic potentials (IPSPs), increased paired pulse depression of evoked IPSPs (58), and impaired paired pulse depression at excitatory synapses (59), all of which have the effect of decreasing excitation in the hippocampus.

The involvement of endogenous BDNF in LTP in perirhinal slices (60), as well as in the visual system, has also been demonstrated using TrkB receptor bodies (12, 20). Further, Trkb-IgG was found to mimic the effects of activity blockade on intrinsic excitability in visual cortical slices (61), leading to disruptions of LGN axonal segregation into eye-specific layers in the visual cortex with an associated disruption of ocular dominance column formation (62).

TrkB-IgG was found to block a BDNF-mediated enhancement of cognitive function (63) and increased expression of synaptic vesicle proteins (64) in response to

voluntary exercise. Finally, TrkB-Fc prevented a BDNF-mediated increase in spine number in cerebellar cultures (65).

In the present study, to test the hypothesis that BDNF exerts a role in cortical motor learning and cortical map plasticity, BDNF signaling was blocked using two different approaches: a TrkB receptor body fusion protein (TrkB-Fc), and BDNF function-blocking antibodies.

The TrkB-Fc fusion protein binds extracellular BDNF, effectively decreasing the amount of BDNF available for downstream signaling via the wild type TrkB receptor. This receptor body has been shown to specifically quench endogenous BDNF activity (66, 62). We also obtained a BDNF blocking antibody (X-F Zhou, Flinders University, Adelaide, Australia), which had been used *in vivo* and elicited a behavioral effect (49). As discussed above, BDNF blocking antibodies have been used successfully *in vivo* (29, 44, 45) to block endogenous BDNF function.

Using a cell proliferation/survival assay, both the TrkB-Fc and the BDNF blocking antibody were tested *in vitro*. The receptor body effectively reduced BDNF bioactivity, whereas the BDNF antibody did not block, or even bind, BDNF. Lentiviruses expressing the TrkB-Fc contruct were then injected into the forepaw area of the motor cortex, and no effect on motor skill acquisition or on motor map organization was observed. These findings lead to the conclusion that blocking BDNF

signaling using this construct does not have an effect on cortical motor learning or motor map topography.

Materials and Methods

Making TrkB-Fc virus

A plasmid containing the coding sequence for TrkB-Fc was provided by Genentech, South San Francisco, CA. The construct was excised from this plasmid and cloned into a plasmid containing an internal ribosomal entry site (IRES) upstream of the GFP reporter gene. The TrkB-Fc construct was cloned in upstream of the IRES such that the resulting construct was TrkB-Fc-IRES-GFP. This was then excised from the plasmid and cloned into a lentiviral expression plasmid. The final pLV-TrkB-Fc-IRES-GFP plasmid was amplified, purified, and packaged into a functional lentivirus. The lentiviral production procedure has been described elsewhere (67, 68) and is summarized in the preceding chapter.

In vitro bioassay for TrkB-Fc

Experiment #1

To test the effectiveness of the TrkB-Fc receptor body *in vitro*, NIH 3T3 cells expressing wild-type TrkB (a generous gift from J. McCarty, UC Santa Barbara, CA) were used. These cells are responsive to BDNF for their proliferation and/or survival (69). The cells were plated on a 24-well plate at a density of 160,000 cells per well. In one well, cells were infected with the TrkB-Fc virus (153 µg/ml p24 and 4.5×10^8 IU/ml) at a concentration of 4 µl virus in 200 µl medium. The cell medium was replaced 24 hours after infection, and forty-eight hours after infection, cells were trypsinized and re-plated on a T75 flask. In order to achieve a pure population of infected cells, they were then sorted using Fluorescence Activated Cell Sorting (FACS), and the partition containing green, GFP-expressing cells was replated and grown to confluence, as were uninfected cells that were FACS sorted as a control. A poly-L-lysine/fibronectin-coated 24-well plate was plated at a density of 20,000 cells per well. Twelve wells were seeded with FACS sorted TrkB-Fc-expressing cells, and twelve with uninfected, FACS sorted (to control for any possible deleterious effects of the sorting process) cells. Serum-free defined medium was used in order to control the composition and concentration of trophic support. Twenty four hours after seeding, one of five mixtures was added to the cells: medium containing fetal bovine serum (positive control), or defined medium containing BDNF at 0 ng/ml, 10 ng/ml, 50 ng/ml, or 100 ng/ml. Each condition was done in duplicate wells for each cell type (TrkB-Fc or control), except the 0 ng/ml BDNF, which was done in quadruplicate. Every 24 hours, fresh medium containing the specified components was added to the plates. On the fifth day from seeding, cells were trypsinized and counted.

Experiment # 2

In order to eliminate any potential confounds from effects that virus infection may itself have had on the growth rate of the NIH 3T3 WT TrkB cells, the experiment above was repeated, without direct infection of the cells with virus. Instead, they were exposed to supernatants retrieved from 293T cells transiently transfected with plasmids containing either the TrkB-Fc construct or the gene for GFP. The day after the transient transfection of the 293T cells, medium was removed and replaced with defined medium. Thirty-six hours later, supernatants were collected and frozen for use on NIH 3T3 WT TrkB cells.

In order to increase the number of cells starting the experiment, the initial plating of NIH 3T3 WT TrkB cells was done in normal, serum-containing medium at a density of 100,000 cells/well in each of three, poly-L-lysine/fibronectin-coated 6-well plates. Twenty four hours after seeding, supernatants from GFP or TrkB-Fc expressing cells (1 ml defined medium) were added to the cells together with BDNF at 0 ng/ml, 50 ng/ml, or 100 ng/ml. Each condition was performed in triplicate. Every 24 hours, fresh supernatant containing the specified BDNF concentration was added to the plates. On the fifth day from seeding, cells were trypsinized and counted.

In vitro bioassay for BDNF blocking antibody

Experiment #1

To test the effectiveness of the BDNF blocking antibody *in vitro*, the NIH 3T3 WT TrkB cells used to test the TrkB-Fc protein were utilized. Six dilutions of the antibody (Ab) were used, along with a specified concentration of BDNF (50 ng/ml, a dose that in the TrkB-Fc assays was determined to be effective at promoting cell proliferation and/or survival). As before, a poly-L-lysine/fibronectin-coated 24-well plate was plated at a density of 20,000 cells per well. Twenty four hours after seeding, one of 8 solutions was added to the cells: defined medium containing 0 ng/ml BDNF and 0 µg/ml Ab, 50 ng/ml BDNF and 0 µg/ml Ab, 0 ng/ml BDNF and 100 µg/ml Ab (highest concentration of Ab used), 50 ng/ml BDNF and 100 µg/ml Ab, 50 ng/ml BDNF and 10 µg/ml Ab, 50 ng/ml BDNF and 1 µg/ml Ab, 50 ng/ml BDNF and 0.1 µg/ml Ab, or 50 ng/ml BDNF and 0.01 µg/ml Ab. Each condition was repeated in triplicate. Every 24 hours, fresh medium containing the specified components was added to the plates. On the fifth day from seeding, cells were trypsinized and counted.

Experiment #2

Because of the unexpected result obtained after the first experiment, the *in vitro* bioassay to test the BDNF blocking antibody was repeated. In addition to the

procedure described above, the poly-L-lysine was filtered to remove any potential contaminants and fibronectin was centrifuged at 1,000 r.p.m. for five minutes to remove potential precipitate. Furthernore, the final cell count was done on the sixth day from seeding.

BDNF antibody binding

To assess the binding affinity of BDNF antibodies to BDNF, a two-site enzyme-linked immunosorbent assay (ELISA) was employed (70). Costar 3590, 96-well EIA/RIA plates were coated with a 1:4,000 dilution of the capture antibody (Chemicon, AB1779) used in our laboratory as a positive control, with a 1:4,000 dilution of the BDNF blocking antibody, or with normal rabbit serum as a negative control and incubated overnight at 4˚C. Plates were washed in wash buffer consisting of PBS with 0.4M NaCl and 0.5% Triton X-100, blocked in PBS containing 3% bovine serum albumin for 60 minutes, and washed again. Known quantities of purified BDNF protein were added and allowed to incubate overnight at 4˚C. Plates were washed, and a 1:2500 dilution of chicken anti-BDNF (Promega G1641) was added and allowed to incubate overnight at 4˚C. Plates were washed and peroxidase-conjugated anti-chicken IgY (Promega G1351, 1:1000 dilution) was added and allowed to incubate overnight at 4˚C. Following extensive washes, a soluble calorimetric product was generated by adding a solution containing 0.033 M sodium phosphate, 0.016 M citric acid, 0.05% o-phenylenediamine and 0.02% H_2O_2. Plates

were then incubated in the dark for approximately five minutes before stopping the reaction with the addition of a 10% H_2SO_4 solution. Optical density measurements were read on a microplate reader at an absorbance of 490 nm.

Surgical technique

All animal procedures were performed in compliance with AAALAC and institutional guidelines and regulations for the humane treatment of animals. Adult male Fischer 344 rats were given anesthetic cocktail (2 ml/kg) of ketamine (25 mg/ml), xylazine (1.3 gm/ml), and acepromazine (0.25 mg/ml) and placed in a stereotaxic head frame. Injections of either siControl lentivirus (average titer 223 µg/ml p24 and 1.7 x 10^{10} IU/ml) or TrkB-Fc lentivirus (average titer 114 µg/ml p24 and 4.3 X 10^8 IU/ml) were made bilaterally into the forepaw region of the motor cortex. The calvarium over the cortical area of interest was carefully drilled until it was thin enough for small burr holes to be made. Virus was injected at eight sites per side (Bregma + 0.3 mm AP and +/- 2.8 mm and +/- 3.5 mm ML, Bregma + 0.8 mm AP and +/- 3.1 mm and +/- 3.9 mm ML, Bregma + 1.3 mm AP and +/- 2.8 mm and +/- 3.5 mm ML, and Bregma + 1.3 mm AP and +/- 3.1 mm and +/- 3.9 mm ML) at a depth of 1.5mm. At each of the eight sites, approximately 250 nl of virus was injected at a rate of approximately 1µl per minute. The animals were allowed to recover two weeks after surgery before beginning motor skill training.

Behavior

Adult male Fischer-344 rats (n=39, aged 3-5 months) were trained in the Whishaw Forepaw Reach Task, in which rats are trained to reach for and grasp a food pellet through a small slot in a plexiglass cage (71). The behavioral paradigm was described in detail in Chapter 2. Briefly, A 31 cm (length) x 21 cm (width) x 19 cm (height) clear Plexiglass chamber was constructed with a 1.5 cm-wide slot on the front wall. Outside the slot was an adjustable tray whose distance from the slot could range from 1-2 cm. The tray was fitted with an indented well where the reward, a 45 mg sucrose pellet (Research Diets, New Brunswick, NJ), was placed. The training paradigm included one week of handling and habituation before training, followed by 12 days of skilled reach training. The rats were maintained at 85%-95% of their free-feeding weight in order to encourage them to reach for sugar pellets. A reach was counted if the rat extended his forepaw through the slot and retracted it. The percentage of reaches that were "hits" were reported as "percent correct reaches". The order in which the rats were trained was randomized each day, and the paw preference of each rat was recorded.

Intracortical microstimulation

Maps of the motor cortices bilaterally were constructed using the intracortical microstimulation technique described in the preceding chapter.

Histology

After mapping, animals were perfused with 75 ml phosphate buffered saline followed by 250 ml 4% paraformaldehyde (PFA). The brains were removed and post-fixed in PFA overnight, after which they were immersed in 30% sucrose for several days until the sucrose penetrated the tissue. Brains were then blocked and 40 μm coronal slices through the forepaw motor cortex were made on a sliding microtome. A one in six series of sections was processed for GFP immunofluorescence (control virus-injected animals) or double GFP fluorescent and Fc light-level immunolableing (TrkB-Fc-injected animals) to confirm adequate spread of virus injected into the cortex. A Nissl stain was done on an adjacent series of sections to assess whether there was cortical damage that resulted from the virus injections which could have interfered with learning and maps. The technique for GFP fluorescent immunolabeling was described in the previous chapter. For Fc light-level/GFP fluorescent double-immunolableing, sections were washed in 0.1 M Tris-buffered saline (TBS) and blocked in 0.6% hydrogen peroxide in TBS for 30 minutes. They were then blocked in 5% rabbit serum plus 0.25% Triton X-100 in TBS and incubated with biotinylated rabbit anti-human IgG (FC specific) (Jackson ImmunoReasearch, West Grove, PA) at 1:500 in blocking solution for 1 hour at room temperature. Sections were then rinsed in TBS and incubated in a 1:50 dilution of avidin-biotin peroxidase reagent in TBS (ABC Elite, Vector Labs) at room temperature for one

hour. They were rinsed in TBS and incubated in TBS containing 0.25 mg/ml diaminobenzidine tetrahydrochloride, 0.04% nickel chloride, and 0.015% hydrogen peroxide until adequate staining was observed (usually three to ten minutes). Sections were then rinsed in TBS and incubated in goat anti-GFP (Rockland, Inc; 1:1500 dilution in blocking solution) overnight at 4˚ C. They were rinsed in TBS plus 0.25% Triton X-100 and incubated in the same solution with a 1:150 dilution of secondary antibody (Alexa 488 flourophore donkey anti-goat, Molecular Probes, Eugene, OR) for 2.5 hours at room temperature. Sections were finally rinsed in TBS, mounted on gel-subbed slides, dried for 30 minutes, and coverslipped using Cytoseal 60 mounting medium (Richard-Allan Scientific, Kalamazoo, MI).

A total of 39 animals were injected with either the TrkB-Fc virus (n = 15) or control virus (n = 13), or received sham operations (n = 11), from which a total of 24 animals met the analysis criteria (n = 5 TrkB-Fc, 8 control virus, and 11 sham-operated). Criteria for exclusion are discussed below, and included lesions of the cortex contralateral to the preferred paw, as assessed by Nissl staining, and/or lack of appreciable virus spread in the forelimb motor cortex contralateral to the preferred paw, as assessed by Fc immunoreactivity.

To assess lesion severity, Nissl stained sections through the motor cortex were classified on a four-point scale as exhibiting no, mild, moderate, or severe lesions to the brain parenchyma. Mild lesions included those in which there was minimal

swelling of the cortex through the calvarial opening, and/or hypercellularity around the virus injection site, and/or mild tissue damage of limited spatial extent. Moderate damage included mild tissue tearing or other forms of damage extending beyond a small focal area. Severe lesions were observed where large areas of tissue were torn and/or where there was evidence of large areas of red blood cell infiltration and/or tissue necropsy. Animals which exhibited moderate or severe lesions to the cortex contralateral to the preferred paw were excluded from analysis.

The amount of virus spread was assessed by measuring the mediolateral and dorsoventral extent of Fc immunoreactivity in each slice of a series (one in six 40 μm slices) through the motor cortex, from which an area of virus spread per slice was derived. An approximate volume of spread was obtained by a summing the area of spread per slice, multiplied by 240 μm (the rostrocaudal distance between slices in a series). The average spread of virus in TrkB-injected animals was 1.55 mm^3, and in control virus-injected animals, 3.11 mm^3. Animals which had less than 1.5 mm^3 of virus spread were excluded from analysis.

Statistics

All analyses were done using analysis of variance (ANOVA). For the behavioral data, learning across days was analyzed using a two-way repeated measures ANOVA, to assess the effect of treatment group, training day, and any

interaction between the two. Where the null hypothesis was rejected, post-hoc pairwise comparisons were done using a Fisher's protected least significant difference (Fisher's PLSD).

Results

TrkB-Fc blocks BDNF bioactivity *in vitro*

The results of two experiments in which the TrkB-Fc fusion protein was tested on NIH 3T3 cells expressing full-length TrkB are shown in Figure 4-1 and 4-2. In the first experiment (Figure 4-1), experimental cells were directly infected with a TrkB-Fc virus, and control cells were uninfected. In the second experiment, full-length TrkB-expressing cells were exposed to supernatants from 293T cells which had been transiently transfected with either TrkB-Fc or GFP as a control (Figure 4-2). In both experiments, BDNF was shown to promote cell survival and/or proliferation in a dose-dependent manner, while TrkB-Fc significantly attenuated or even abolished this BDNF-mediated effect on cell survival and/or proliferation. Statistical analysis revealed a significant BDNF dose effect (ANOVA $p<0.0001$ for Experiment #1; $p<0.002$ for Experiment #2) and TrkB-Fc treatment effect ($p<0.005$ for Experiment #1; $p<0.0001$ for Experiment #2). There was also a dose by treatment effect ($p<0.05$ for both experiments), suggesting that in the presence of the TrkB-Fc receptor body, increasing doses of BDNF were not utilized as effectively by the cells.

BDNF "blocking" antibodies are inactive *in vitro*

The NIH 3T3 full-length TrkB cells described above were also used to test the effectiveness of the BDNF blocking antibodies. The results of two essentially identical experiments are shown in Figure 4-3. In both experiments, there is an overall effect of treatment ($p<0.05$ and $p<0.0001$, respectively). Also, in both experiments, the 100 µg Ab/50 ng BDNF condition (and, notably, not the 100 µg AB/0 ng BDNF condition) promoted the survival and/or proliferation of significantly more cells than any other combination of antibody and BDNF dose (Fisher's PLSD $p<0.05$ compared to all other conditions in first experiment, and $p<0.0001$ compared to all other conditions in second experiment), indicating an agonistic, rather than antagonistic effect. None of the other conditions, in the post hoc analyses, were different from each other.

Given the unexpected agonist effect of the BDNF antibody on the NIH 3T3 WT TrkB cells, its properties were further investigated. An ELISA binding assay was conducted, wherein the BDNF antibody was used as the capture antibody. Recombinant human BDNF protein was added in amounts as high as 100 pg/well. The BDNF antibody, in the concentration used (1:4,000 in 50 µl coating buffer), did not bind BDNF at any concentration. The absorbance readings from all BDNF antibody wells were below detection and were comparable to the absorbance readings

for the wells coated with normal rabbit serum. The absorbance readings for the positive control wells coated with the capture antibody used in our laboratory generated a linear standard curve.

Having determined that the BDNF antibodies neither blocked nor, apparently, bound BDNF, they were not used for *in vivo* experiments.

TrkB-Fc does not affect cortical motor learning or map organization

Because lentivirus expressing TrkB-Fc fusion proteins efficiently blocked BDNF activity *in vitro*, effects of TrkB-Fc virus on skill learning and motor map organization were examined *in vivo* after injection into the forepaw motor cortex. TrkB-Fc virus-injected animals (n=15) were compared to animals whose forepaw motor cortices were injected with GFP-expressing siControl virus described previously (n=8, used in this experiment as a control for non specific virus- and/or injection-mediated effects) and with animals that received sham operations (n=11). Of 15 animals receiving TrkB-Fc virus injections, 10 were excluded from analyses due to poor virus spread within the forepaw motor cortex or insufficient expression of TrkB-Fc ($<1.5mm^3$), as described above (Figure 4-4). While there was a significant improvement in performance as a function of training day (Figure 4-5, $p<0.0001$), there was no difference in the behavioral performance among the three groups

($p=0.85$). The ability of the TrkB-Fc construct to affect cortical map organization was then examined.

When rats reached a performance plateau in the skilled reach task, bilateral motor cortex maps were made for four TrkB-Fc animals, six control virus animals, and three sham-operated animals. Representative maps from an animal in each group are shown in Figure 4-6. One control virus and one sham-operated animal reached with both paws, so data reported are the average from both sides of the cortex. Otherwise, only map data from the cortex contralateral to the preferred paw are reported. In previous studies describing map reorganization on motor learning, different aspects of the motor map were found to reorganize. One group found that the size of the entire caudal forelimb area (CFA) increased with learning (72, 73), and the other found that, while the size of the CFA remains constant, the percentage of the CFA devoted to distal (wrist) versus proximal (elbow/shoulder) movements increased (74). Both measures are therefore reported in Figure 4-7. There was no significant effect of treatment on either size of the CFA (Figure 4-7A) or percentage of the CFA devoted to wrist movements (Figure 4-7B).

Discussion

In this study, the efficacy of blocking BDNF function *in vitro* and *in vivo* using a TrkB receptor body fusion protein was tested. Though the TrkB-Fc construct was

effective *in vitro*, reducing full-length TrkB-mediated effects on cell survival and/or proliferation, there was no effect of blocking BDNF using TrkB-Fc on cortical motor learning, reminiscent of the results described in Chapter 3 using siRNA directed against BDNF. Several factors may account for the lack of TrkB-Fc-mediated effects: 1) TrkB-Fc expressed by lentivirus-transduced cells might not have adequate access to BDNF *in vivo*, particularly at synaptic sites where BDNF is likely to exert its effect, 2) TrkB-Fc might not act as a function-blocking molecule *in vivo*, 3) the distribution and/or amount of TrkB-Fc were insufficient to sequester adequate amounts of BDNF. Finally, it is possible that BDNF does not have an effect on motor learning or cortical map plasticity in the skilled reach task.

The possibility that TrkB-Fc has different biological activities *in vivo* than *in vitro* is suggested by a study in which TrkB-Fc was shown to facilitate diffusion of BDNF through brain parenchyma (75). The study examined the ability of exogenous BDNF to increase expression of neuropeptide Y (NPY) in the hippocampus and cortex of adult rats, which was expected to be attenuated by coinfusion of TrkB-Fc. The results indicated that the receptor body had the opposite effect; a greater volume of tissue showed upregulation of NPY, a consequence of TrkB-Fc-mediated increased diffusion of BDNF protein throughout the hippocampus and cortex. Further, co-infusion of BDNF and TrkB-Fc increased phosphorylation of full-length endogenous TrkB, presumably due to the increased distribution of BDNF. Finally, diffusion of radiolabeled BDNF into the brain from intraventricular infusion, which is normally

limited, possibly due to BDNF sequestration by periventricular TrkB and truncated TrkB receptors (76, 77), was greatly increased when TrkB-Fc was co-infused intraventricularly. The data from this study indicate that TrkB-Fc may act to facilitate, rather than block the function of BDNF. This is in contrast to numerous other findings (12, 20, 21, 51-54, 56-65) that TrkB-Fc blocks the function of BDNF, but most these studies were carried out *in vitro*, where the dynamics of BDNF availability and diffusion are considerably different than they are *in vivo*, and none of these studies examined the role of BDNF in the cortex. There should also be a distinction made between facilitating the diffusion of exogenous BDNF, as in the study described above, and the effects of TrkB-Fc on the distribution of endogenous BDNF, as in the present study. It is not known whether TrkB-Fc might bind and "carry" endogenous BDNF, effectively increasing its availability and possibly counteracting the local blocking effect, but this possibility could explain the lack of effect of TrkB-Fc on learning and motor map organization observed in the present study.

The data discussed in the previous chapter describing siBDNF-mediated BDNF blocking and the data discussed in this chapter describing TrkB-Fc-mediated BDNF blocking are consistent, in that no effect was observed by either manipulation on motor skill learning or motor map topography. However, in both experiments, the attenuation of BDNF availability achieved was incomplete, suggesting that the negative results might be due to technical limitation, rather than the biological function of BDNF in cortical motor learning and plasticity. This contention is

supported by the results described in Chapter 2, where an upregulation of BDNF was seen with skilled and unskilled motor learning. Techniques which more fully block BDNF availability in the cortex are therefore necessary to conclusively assess the role of BDNF in cortical motor learning and motor map plasticity.

The antibody tested in this study for BDNF blocking *in vivo* has previously been reported to exhibit BDNF antagonist properties (49), with behavioral consequences. However, the data from the present study did not confirm this report. On the contrary, the BDNF antibody exhibited agonist properties, significantly increasing cell survival and/or proliferation in a BDNF-dependent cell line. It may bind TrkB directly, and, at high concentrations, act in concert with BDNF as an agonist. This interpretation is supported by the fact that the antibody's positive effect on cell proliferation and/or survival occurred only in the presence of BDNF, making a non-specific mechanism of action unlikely. Indeed, antibody agonism has been shown to occur (78), and would be an interesting possibility to explore.

Conclusions and Future Directions

Because the result obtained from this study appears to support the hypothesis that blocking BDNF using TrkB-Fc does not affect motor learning or cortical map plasticity, future studies using this construct must first address the potential technical issues of virus titer and spread discussed above. Improved delivery of TrkB-Fc

lentivirus may be explored, such as an increased number of injection sites and/or a higher volume of concentration virus per site. Alternatively, a non-lentiviral delivery approach delivering concentrated, purified TrkB-Fc protein to the motor cortex via osmotic pump could be employed. Only after all technical issues have been addressed and significant TrkB-Fc-mediated BDNF blocking has been demonstrated *in vivo* can a firm conclusion about the role of BDNF in motor cortex plasticity and learning be drawn.

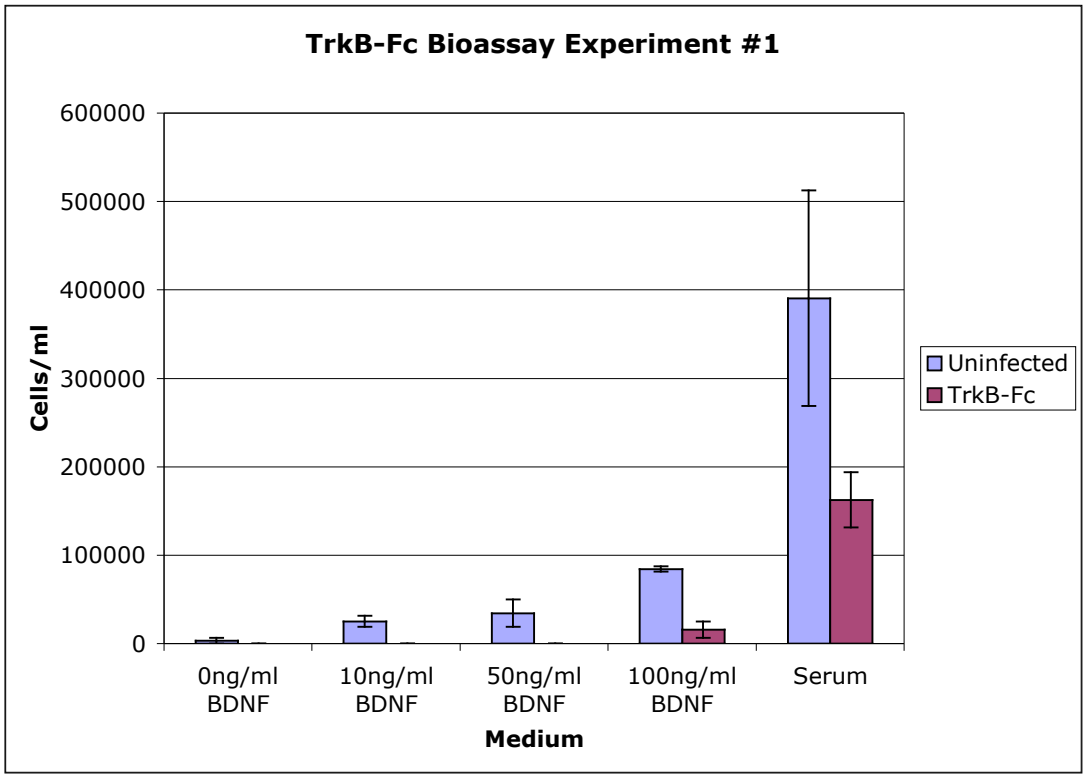

Figure 4-1. Effect of TrkB-Fc on NIH 3T3 WT TrkB cells *in vitro*. Cells counted in this experiment (Experiment #1) were directly infected with TrkB-Fc virus, and survival/proliferation was compared to uninfected cells. Relative to no virus, TrkB-Fc virus reduced cell number by 100% in the 0ng/ml BDNF, 10 ng/ml BDNF, and 50 ng/ml BDNF conditions, by 81% in the 100 ng/ml BDNF condition, and by 58% in the serum positive condition. BDNF dose effect, $p<0.0001$, TrkB-Fc effect, $p<0.005$. Interaction between dose and treatment, $p<0.05$. Error bars are +/- S.E.M.

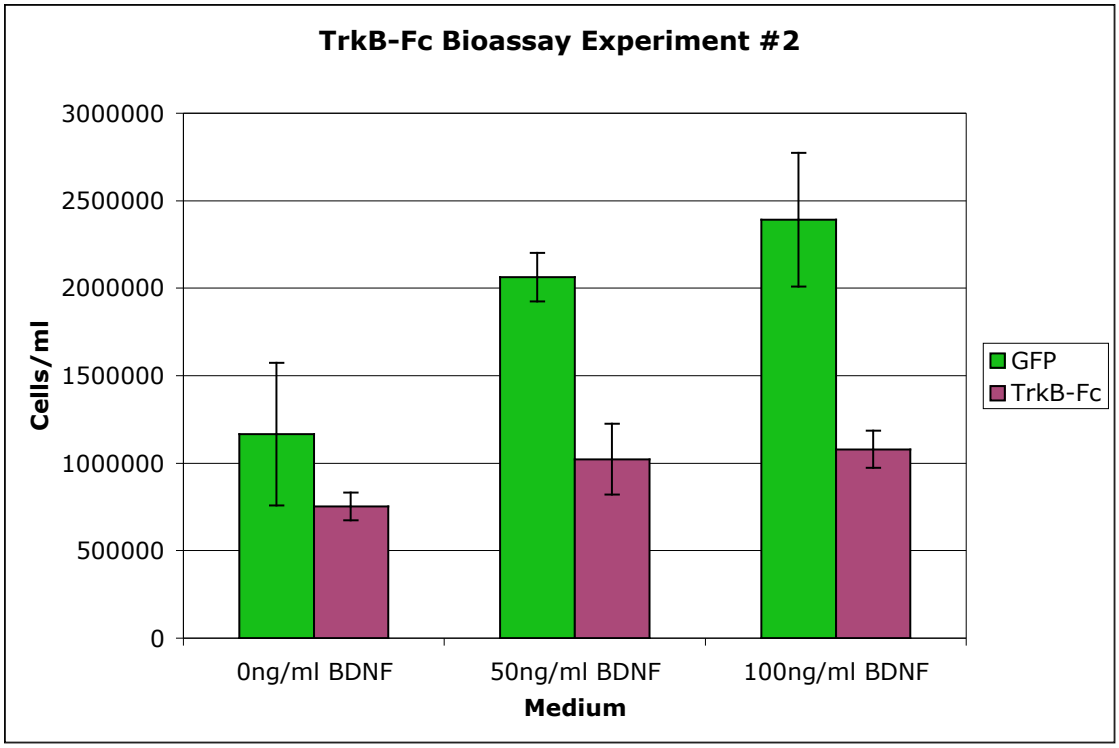

Figure 4-2. Effect of TrkB-Fc on NIH 3T3 WT TrkB cells *in vitro*. In this experiment (Experiment #2), cells were exposed to supernatants from 293T cells transfected with GFP or TrkB-Fc plasmids. Relative to GFP, TrkB-Fc reduced cell number by 35% in the 0 ng/ml BDNF condition, by 50% in the 50 ng/ml BDNF condition, and by 55% in the 100 ng/ml BDNF condition. BDNF dose effect, $p<0.002$, TrkB-Fc effect, $p<0.0001$. Interaction between dose and treatment, $p<0.05$. Error bars are +/- S.E.M.

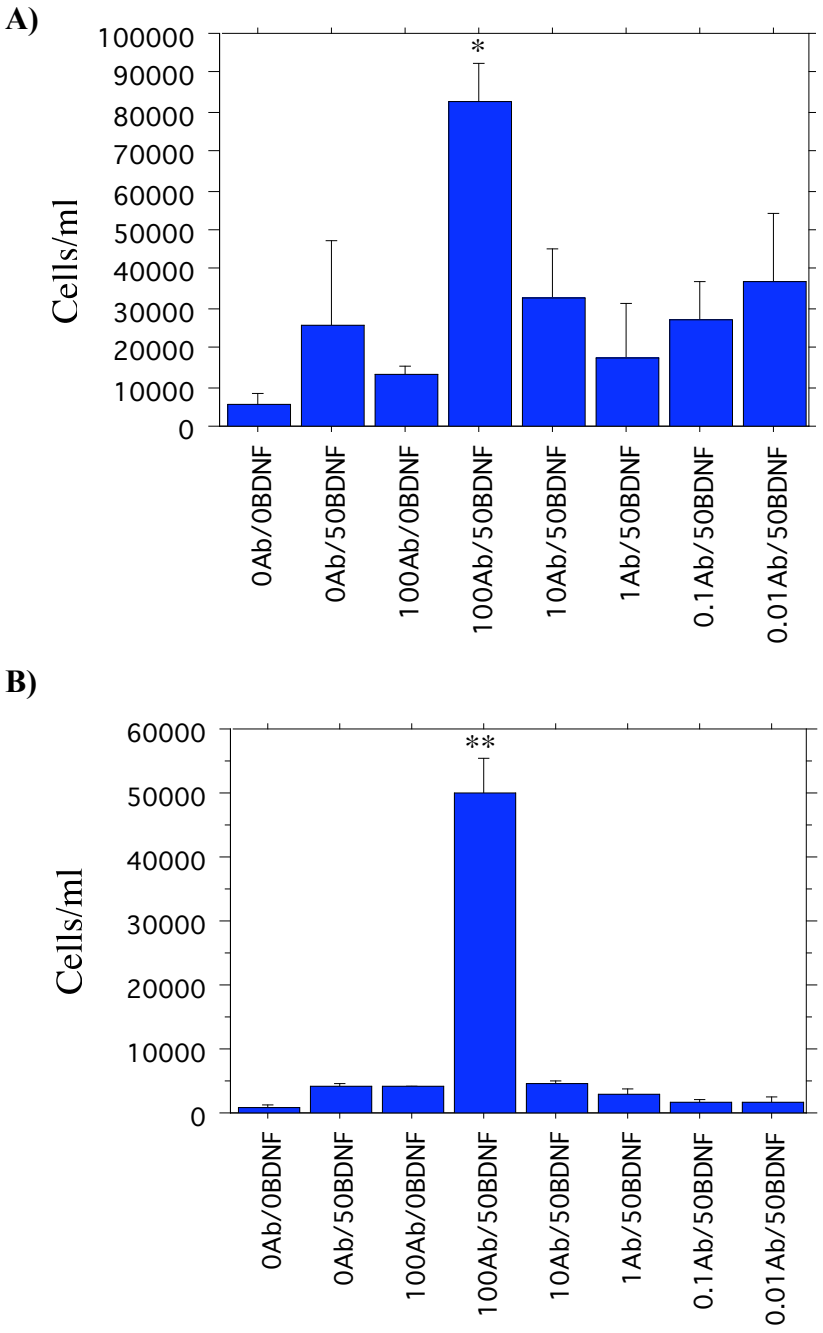

Figure 4-3. BDNF antibody bioassays. Cell survival/proliferation as a function of differing concentrations of BDNF and BDNF antibody. **A)** Experiment #1, overall ANOVA $p<0.05$. **B)** Experiment #2, overall ANOVA $p<0.0001$. * $p<0.05$, ** $p<0.0001$.

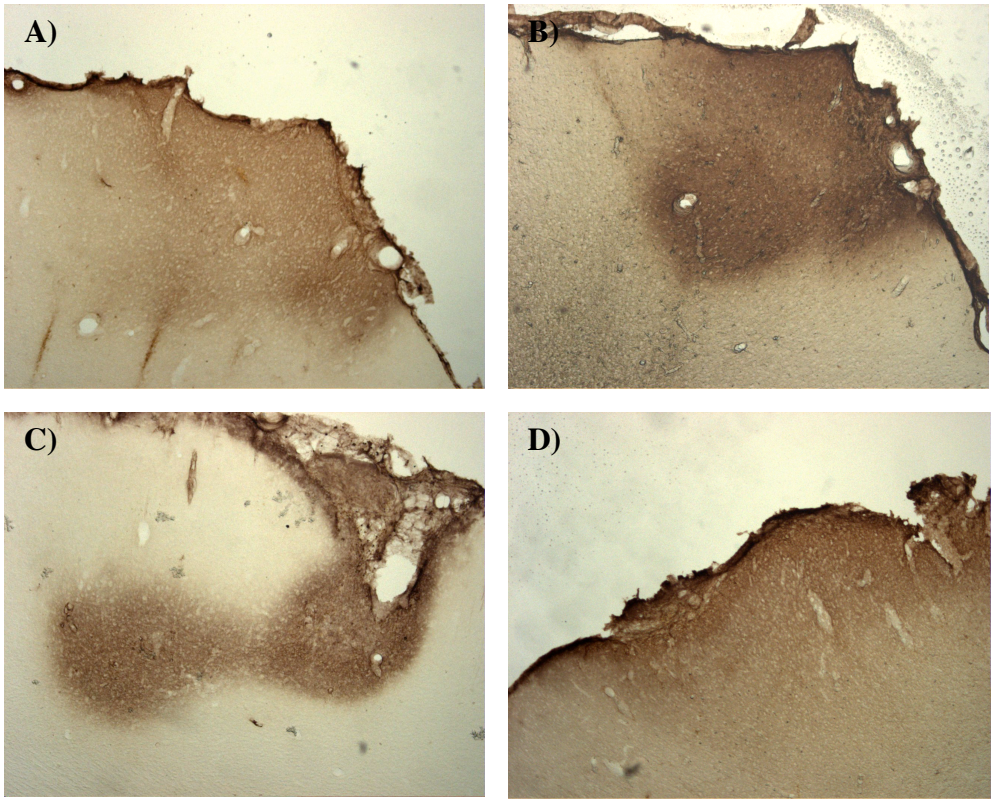

Figure 4-4. Fc immunolabeling in the forepaw motor cortices of TrkB-Fc virus-injected animals illustrating adequate virus spread (>1.5mm^3; **A, B, C**), and inadequate virus spread (<1.5mm^3; **D**).

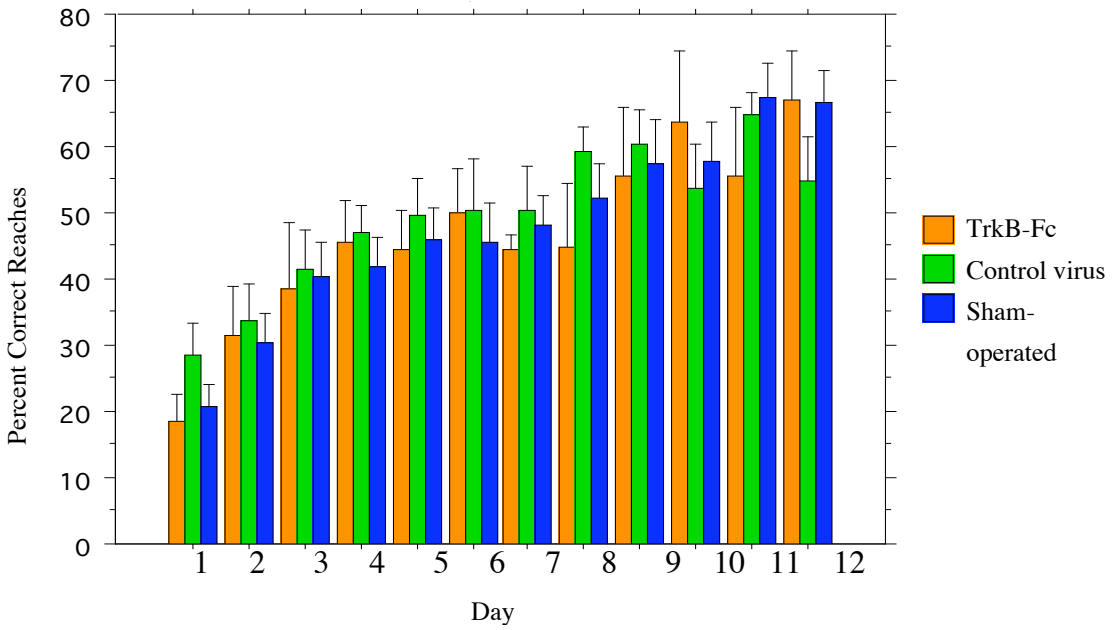

Figure 4-5. Task performance expressed as percent correct reaches on each training day for TrkB-Fc-injected (n = 5), control virus-injected (n = 8), and sham-operated (n = 11) animals. Error bars shown are +/- S.E.M. Repeated measures two-way ANOVA $p<0.0001$ for task acquisition, $p=0.91$ for treatment group, $p=0.61$ for acquisition by treatment group.

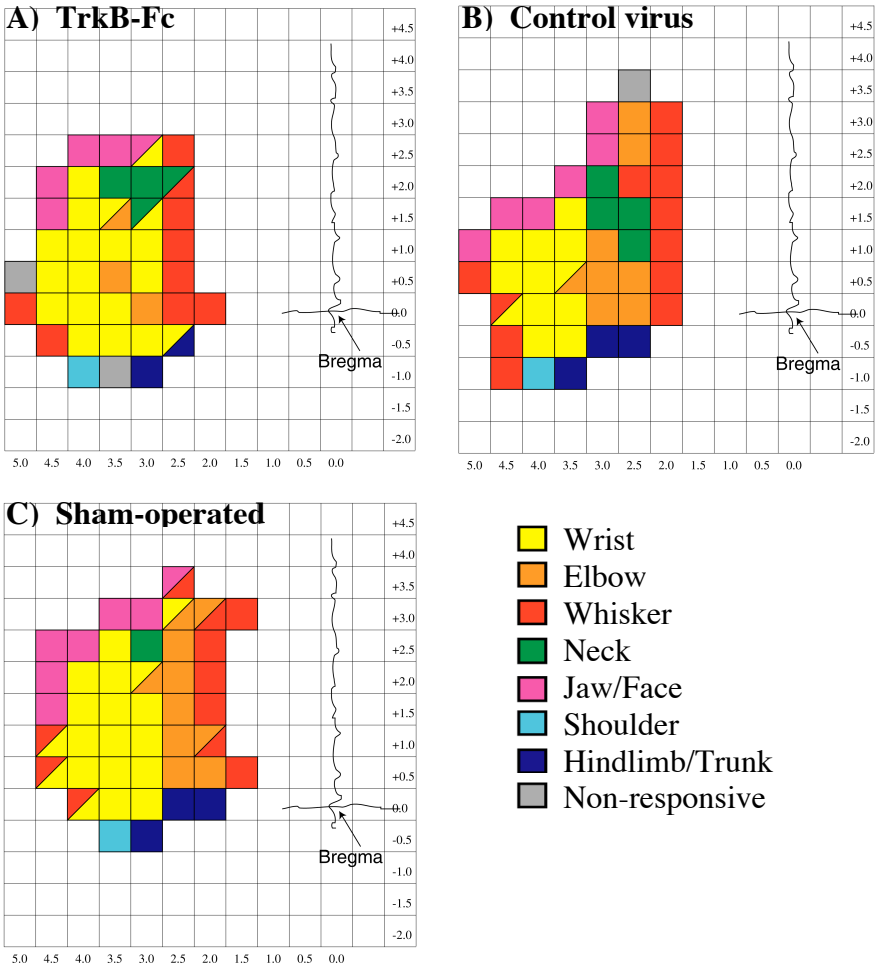

Figure 4-6. Representative motor maps from **A)** TrkB-Fc-injected, **B)** control virus-injected, and **C)** sham-operated animals. Horizontal and vertical numerical designations on the grid represent mediolateral and rostrocaudal coordinates of the motor cortex (expressed in mm from Bregma), respectively. Each square on the grid represents one ICMS penetration site, with color corresponding to body part in which movement was elicited.

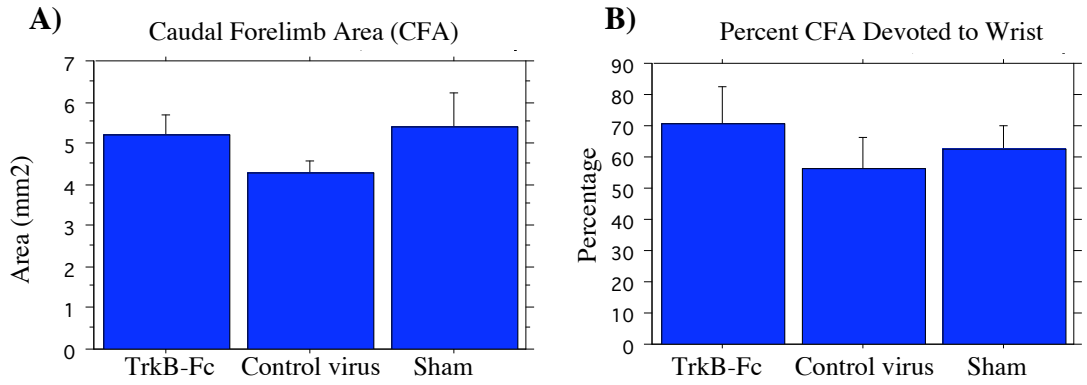

Figure 4-7. Motor map data for TrkB-Fc-injected (n=4), control virus-injected (n=6), and sham operated (n=3) animals. **A)** Size of the caudal forelimb area in the cortex contralateral to the preferred paw, expressed in mm^2. ANOVA p=0.19. **B)** Percentage of the preferred caudal forelimb area devoted to wrist movements. ANOVA p=0.61. Error bars shown are +/- S.E.M.

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Chapter 5: General Discussion and Conclusions

Summary of Results:

1) BDNF expression increases as a function of both skilled and unskilled motor learning.
2) An siRNA lentiviral construct (siBDNF) was created which reduces BDNF protein levels *in vitro*, by 81% compared to uninfected cells and 85% compared to control virus-infected cells.
3) A TrkB receptor-body fusion protein (Trk-Fc) lentivirus was created which impairs BDNF bioactivity *in vitro*.
4) SiBDNF virus injections into the adult rat motor cortex reduced BDNF protein levels, two weeks after injection, by 22% compared to uninjected cortices, and 34% compared to control virus-injected cortices.
5) An siBDNF-mediated 22% (compared to uninjected cortices) or 34% (compared to control-virus-injected cortices) reduction of BDNF protein levels, or TrkB-Fc-mediated sequestration of BDNF protein did not affect motor skill learning or cortical map organization.

The overall conclusion that can be made based on these results is that, while BDNF appears to be modulated by motor experience, reducing BDNF protein levels in the forepaw motor cortex by 22% (compared to uninjected cortices) or 34%

(compared to control-virus-injected cortices) using siBDNF, or to an unknown degree using TrkB-Fc, has no effect on learning acquisition or on cortical map organization.

I have analyzed the implications of these results in the preceding chapters. I will therefore devote this section to a synthesis of the relevant literature as it relates to plasticity in the motor cortex and the potential role of BDNF therein. First I will discuss map plasticity, including the mechanisms by which it might occur and its limited duration in the motor cortex. Next I will elaborate on aspects of synaptic plasticity in the motor cortex, including its relationship to structural and map plasticity. Finally, based on these ideas, I will propose a model of motor learning, and discuss how BDNF might contribute to motor plasticity with the idea that, in the future and with improved experimental techniques, its involvement might be further investigated.

Mechanisms of Motor Map Plasticity

To investigate the role of BDNF in cortical learning, the present studies utilize a motor skill learning task which had been shown previously to require the motor cortex (1, 2), and to lead to structural and circuit plasticity in the motor cortex (3-10). Though many have investigated skilled motor learning, and, in particular, associated cortical map reorganization, the mechanism for such plasticity has yet to be determined.

The reorganization of the map seen in motor learning paradigms is specific to skill-learning, and is not due solely to increased use of the muscles controlled. Rats trained to press a lever (7), reach for an unattainable pellet (8), engage in increased motor activity (11), or increase forepaw strength (12), do not exhibit an expansion of movement representations. Similarly, squirrel monkeys trained to retrieve food pellets from a large well, a task that does not require the development of novel motor skill, do not show any reorganization of digit movement representations (13).

How might motor maps reorganize? There is evidence that the functional reorganization that occurs in the motor cortex with experience is anatomically constrained. This was demonstrated in two studies using a combination of electrical stimulation and axon labeling (14, 15). In the first study (14), maps of forelimb and vibrissal movements were constructed via ICMS. Injections were made into the vibrissae area, and labeled axons were restricted to the whisker representation and areas at the whisker/forelimb border. Similarly, injections into the wrist representation labeled projections restricted to the forelimb area (interestingly, not just to the wrist) and to the border with the vibrissae area. The second study (15) used a peripheral lesion to reorganize the motor map. Acute functional reorganization of the motor cortex occurs when the facial nerve is cut such that the forelimb representation expands medially into part of the territory previously devoted to vibrissae movements. The remainder of the vibrissae area becomes silent. Injections placed into vibrissae

cortex corresponding with the newly expanded forelimb representation gave rise to labeled axons which crossed the forelimb/vibrissae border and extended well into forelimb area. Injections placed into silent vibrissae cortex gave rise to axons which remained restricted to the original vibrissae representation. These results suggest a mechanism of restricted map reorganization within the motor cortex, wherein the reorganization is constrained by the existing horizontal connections within motor cortical subregions and in specific inter-subregion areas.

What delineates the borders between body representations if there is anatomical overlap between them? And, if major architectural rearrangement does not underlie the map plasticity observed during learning and other manipulations, what might? One can imagine a system of inhibitory circuitry which refines the between-region excitatory connections to create discreet body representations. Such an organizational principle is evident in sensory systems, in the form center-surround inhibition, which is observed in the auditory system (16), the somatosensory system (17, 18), the olfactory system (19), and at multiple levels of the visual system (20-23). The mechanism of center-surround antagonism is lateral inhibition, wherein a cell which is stimulated sends inhibitory, lateral projections to cells in the surround of its receptive field, with the effect of refining receptive fields and increasing contrast and edge discrimination. In the motor cortex, there are extensive and long-reaching intracortical excitatory connections between regions that would suggest that activation in one region would lead to activation in neighboring regions. However, the presence

of local inhibitory connections between regions limits the group of cells activated by intracortical stimulation or by natural activity, and could therefore serve to define the topography seen in the motor cortex.

Such a system of inhibitory control could be modulated in the context of learning to mediate map reorganization. Indeed, it is known that removing GABAergic control reveals occult excitatory circuitry and "expanded" motor maps (24). In this study, peripheral nerve transection was used to create new patterns of motor representation. It had previously been shown that within hours after transection of the facial motor nerve, which normally controls movement of the contralateral vibrissa, movements of non-vibrissa body parts normally represented in adjacent motor cortical areas could be evoked by stimulation within the former vibrissa area (25). The authors of this study sought to recapitulate this injury-induced map reorganization by adjusting intracortical inhibition. A GABA antagonist, bicuculine, was injected into a site within the forelimb representation. Within five to 65 minutes of this injection, a test site within the vibrissa representation which formerly elicited only vibrissa movements was able, when stimulated, to elicit both vibrissa and forelimb movements. This shift in representation lasted only as long as bicuculine was present. The authors tested the possibility that this shift occurred simply as a result of increased excitation, instead of a release of inhibition. However, substitution of bicuculine with glutamate or acetylcholine did not lead to this short term shift in representation, indicating that the mechanism of the reorganization was indeed a

release of inhibition. Evidence corroborating this hypothesis can be found in the somatosensory cortex. As in the motor cortex, peripheral denervation of sensory input to an area of cortex causes body areas represented in surrounding cortical areas to activate the denervated cortex (reviewed in 26). The authors of a study using peripheral denervation to somatosensory cortex in monkeys found that immunoreactivity for GABA was markedly decreased in the deprived area of cortex on a time course paralleling the sensory map reorganization (27). Together, these findings suggest that moment to moment motor cortical organization likely depends on a balance between excitation and inhibition.

The observation that map reorganization occurs during motor skill learning does not speak to the relationship between map plasticity and learning. However, the notion that map plasticity could underlie behavioral performance is not hard to imagine. It has been observed for decades that, in the motor cortex, the greater the area devoted to a body part, the greater the dexterity of those muscles. Maps can be thought of not simply as the reflection of motor representations on the surface of the cortex, but as a substrate, themselves, for enabling complex movements and for learning new movements. Indeed, ICMS studies in cats show that motor maps develop as does dexterity; they are absent at postnatal day 45 and become more complex as the cats' movements do (28, 29). Further, it has been shown that removing cholinergic inputs to the motor cortex prior to, but not after, skill training prevents both map reorganization in the rat motor cortex and impairs motor learning

(4).

Limits to Map Plasticity

It is possible that transient motor map plasticity occurs early during training (30, 31), but that permanent map reorganization reflects learning consolidation, and requires more robust, repeated exposure to the task.

One study (30) illustrating this found that there were performance gains after just one session of motor sequence learning in humans, but that reorganization of motor representations occurred over several weeks, and were in accordance with the time during which performance gains reached an asymptote. The map plasticity then persisted for months, though it has been shown that with the introduction of a task competing for cortical resources, cortical map plasticity from an already learned task can be reversed (32). In that study, squirrel monkeys learned a skilled digit manipulation task, leading to the characteristic expansion of digit representations. Interestingly, subsequent training in these same animals on a skilled forearm manipulation task resulted in an expansion of the forearm representation at the expense of the digit representation. This not only further demonstrates the lability of motor maps, but also indicates that cortical resources are limited and expanded cortical area is allocated to tasks only until competing tasks require them. In fact, it would be difficult to conceive of a system in which motor maps expanded indefinitely each time a new motor task was learned. It was not discussed in the study whether the

behavioral performance on the already learned task was affected by the introduction of the competing task and the resultant shift in cortical map plasticity. The answer to this question would be of interest, as it would help elucidate the nature of the relationship between map plasticity and task performance.

A study in humans (33) was done in which maps were made of cortical motor potentials evoked by focal transcranial magnetic stimulation (TMS) in normal subjects during learning of a repetitive motor sequence. The authors found that primary motor cortex topography changed such that muscles involved in the task were over-represented. A serial reaction time test was used wherein some subjects were prompted to execute button press sequences, including a particular repeated sequence, whereas others executed non-repeating sequences. Those who executed the repeating sequence eventually developed an explicit knowledge that a sequence was being repeated, and, as this knowledge developed, their motor maps rapidly returned to their baseline topography, despite continued improved motor performance. The return of the maps suggests that as the motor sequence is explicitly learned, motor cortical involvement is attenuated and other structures assume more active roles in the execution of the task. Indeed, learning of a complicated sequence of voluntary finger movements is associated with increases in cortical and cerebellar regional cerebral blood flow (rCBF), whereas having learned the motor skill results in rCBF increases in the striatum (34).

The Site of a Motor Memory May Shift from the Motor Cortex

Further evidence of a time-limited consolidation process, wherein motor memory may shift from one structure to another, comes from studies in which after a motor task is learned, interference tasks are introduced at various time points. In one study (35), human subjects learned to adapt to a force-field through which they made a series of arm movements, demonstrated by improved performance 24-hours after the training session. If a second force-field was introduced immediately after the first, there was no improvement in performance for the first sequence at the 24-hour time point. If, however, the second sequence was introduced four to six hours after the first, it did not interfere, at the 24-hour time point, with the memory for the first. Conversely, the longer the interval between the first and second fields, the better the subjects learned the second sequence as well, indicating both anterograde as well as retrograde interference. Similar results were seen in a study in which human subjects performed a digit tapping sequence (36). Subjects showed a memory (as demonstrated by increased speed and accuracy) for the sequence 24-hours after the training session. If a second sequence was introduced immediately after the first, there was no improvement in accuracy for the first sequence, though there was for the second sequence, at the 24-hour time point. If, however, the second sequence was introduced six hours after the first, it did not interfere, at the 24-hour time point, with the memory for the first.

These findings notwithstanding, other experiments show that secondary tasks interfere with visuomotor learning, no matter how long the delay between tasks (37-39). Given the fact, however, that the interference is in effect over every interval of time examined, it is most likely that the interfering task in these studies impairs the retrieval or execution, and not the consolidation, of the first task. Nevertheless, while the studies examined in the last paragraph indicate the existence of a consolidation process wherein motor memories become less susceptible to disruption over time, they are not, alone, an indication that the consolidation process involves a shift of memories from one motor structure to another, and certainly not an indication that motor memories shift specifically from the motor cortex to other structures.

It is reasonable to hypothesize, however, that there might be situations wherein tasks become independent of the motor cortex, and that, as they do, their execution is subserved by subcortical motor structures, such as the basal ganglia, and/or by the cerebellum. Indeed, much evidence has accumulated regarding the role of the striatum and cerebellum in motor learning (reviewed in (40-48)). These reviews survey many studies in which striatal or cerebellar activation is observed in the context of implicit learning, or where performance on various procedural tasks, such as rotary pursuit, sequence learning, and serial reaction time tasks, are impaired in the context of striatal damage (such as in patients with Parkinson's Disease) or in the context of cerebellar lesions. Also, a series of studies in rats showed that acrobatic motor learning, and not motor activity, lead to structural changes in the cerebellum, in the form of increased

dendritic hypertrophy (49), and increased synaptogenesis (50, 51) which persisted for at least one month (52), even in the absence of continued training.

Specifically, the involvement of the cerebellum and striatum in learning may relate to these structures' role in motor memory consolidation and storage. Indeed, many studies show that the basal ganglia and/or the cerebellum (in particular, the deep cerebellar nuclei) are involved in the later stages of learning, or that they are likely involved in the ultimate storage of motor skill representations (34, 53-63).

In one study elucidating the role of subcortical structures (58), 3 groups of patients were studied: those with striatal damage secondary to Parkinson's Disease (PD), those with cerebellar lesions, and those with well-circumscribed frontal lobe lesions. The three groups were subjected to a visuomotor learning task, and only the PD patients and those with cerebellar lesions had difficulty improving performance, with the difficulty emerging only in the later stages of training. None of the patients had any deficits in explicit memory for the task. The implication is that the striatum and cerebellum have a role in implicit motor memory improvement beyond, in this case, the initial three days of training. The same laboratory studied these patients again 10-18 months later to assess their ability to retain the memory for the task (59). The patients whose PD had advanced, as well as the cerebellar patients, showed impaired memory retention, while again having no trouble with the declarative aspects of the testing sessions. Together, these two studies lend support to the assertion that

the cerebellum and basal ganglia may be sites of transfer of motor cortical memories, and may aid in long term storage of such memories.

One elegant study (60) in which single neurons in monkeys were recorded extracellularly during performance of already learned or newly learned motor sequences revealed that the caudate and anterior putamen were more involved in new sequences, whereas the posterior putamen was more activated by already learned sequences. This suggests, not surprisingly, that different parts of the basal ganglia may be involved in acquisition, consolidation, and storage of motor memories. The same group had previously done a study in which partial, reversible blockage (using muscimol, a GABA receptor agonist) of the caudate and anterior putamen of monkeys preferentially impaired performance of novel motor sequences, whereas muscimol injections into the middle and posterior putamen impaired performance of well-learned sequences (61). In yet another study conducted by the same group (62), once again, muscimol-induced blockade was employed, but this time to the deep cerebellar dentate nucleus. The authors found that the number of errors increased significantly for already learned sequences but not for new sequences, further suggesting a role for the cerebellum in long-term storage of motor memories.

Finally, studies in humans using positron emission tomography (PET) showed that subjects learning a finger movement sequence initially exhibited decreases in

rCBF in the basal ganglia, followed by increases during the advanced learning phase (34, 63).

Taken together, the preceding studies suggest that motor map reorganization is a mechanism of storage of motor experience, at least for an initial period after learning, or possibly for longer, as in the case of animals which have no further experience requiring increased cortical allocation. The mechanism of this motor map reorganization appears to be related to existing anatomical interconnectivity which is unmasked by a release of inhibition. When motor cortical resources are further called upon, or simply in the normal course of motor memory consolidation, motor map topography may revert to its original state, with other structures, such as the basal ganglia or cerebellum, assuming control of the new motor skill.

LTP/LTD

Regardless of the amount of time a motor memory trace is stored in the motor cortex, the map plasticity discussed above is one manifestation of changes which occur in the cortex following procedural motor learning. Another form of plasticity observed in the motor cortex is synaptic plasticity. If skill learning occurs through a shift in the balance between excitation and inhibition, it can be said that the motor cortex must be able to undergo processes analogous to LTP and LTD, such that some synapses are strengthened while others are weakened.

Would it be reasonable to expect that motor learning might cause global changes in synaptic efficacy? It has been reported in slice studies that motor learning is sufficient to elicit enhancement of horizontal field potentials via an LTP-like mechanism (64-66), but this result has been difficult to confirm (personal communication, J.M. Conner) and has been called into question (67). It would actually be surprising that a learning-mediated net change would be observed in nonspecific field potential recordings, which reflect average ensemble activity over large numbers of synapses. Complex activity patterns evoked during motor learning, including both increases and decreases in synaptic efficacy, might more accurately be predicted to occur on a synapse-by-synapse basis, with no lasting net change in overall activity. Indeed, in the context of skilled reach training in rats (9), it was found that evoked field potentials were significantly different than baseline in the cortex contralateral to the trained paw eight days after training commenced, following the phase of steep improvement in behavioral performance, but not at 15 days after the beginning of training, when behavioral improvement has plateaued. A study in humans using TMS during training on a pegboard task (68) showed that muscle-evoked potentials were facilitated, and that the facilitation was selective to the muscles engaged in gripping the pegs, but that the enhancement lasted only a short time (<15 minutes) after the training session. These findings suggest that overall changes in synaptic efficacy occur during the course of motor learning, but that a homeostasis is

eventually reached, representing a balance between facilitated and depressed circuits encoding the memory trace.

Another very elegant experiment was done in humans (69) using paired associative stimulation of the right median nerve and TMS of the left hand area in M1. The authors were able to induce LTP-like increases in synaptic efficacy in the motor cortex using this protocol, and they showed that motor learning occluded further increases in synaptic efficacy and enhanced further decreases in synaptic efficacy. This finding presents evidence that motor learning in humans may occur via LTP- and LTD-like mechanisms, but the effect was measured only up to 30 minutes after training. It is not known, therefore, whether this learning paradigm would induce long-lasting changes in synaptic efficacy. However, based on the fact that, in the study, experimental subjects were tested on different paradigms at one week intervals, it can be assumed that, by one week after training, the motor learning-associated enhancement of synaptic efficacy had reverted to baseline levels.

In a study of elite raquetball players (70), it was shown that their primary motor cortices exhibit higher amplitude motor-evoked potentials and decreased activation thresholds than casual players. It is possible that in humans, wherein the motor repertoire is broader and the capacity for skill learning is greater than that of rodents, learning a complex motor skill over the course of years does, in fact, induce long-lasting changes in synaptic efficacy. The players in this study were studied once,

after a two-week suspension of play, such that the temporal extent of the enhanced synaptic activity is not known. However, the fact that these changes developed only after years of skilled play suggests that they might be maintained for years.

If LTP of motor cortical circuits is important, which circuits might be potentiated? As discussed in Chapter 1, vertical pathways leading from the thalamus are a candidate, as are local circuits within the motor cortex itself. An especially intriguing possibility for synaptic modification are intracortical horizontal pathways, particularly from the sensory cortex. Indeed, stimulating sensory pathways has been shown to elicit motor cortical LTP (71-73). Intuitively, it follows that as motor tasks such as fine grasp skills are learned, information from sensory areas provides essential feedback used to progressively refine the learned movement. In support of this, a study in monkeys suggests that the sensory cortex might have an instructive role in motor learning (74). The authors showed that removing the hand area of the sensory cortex unilaterally, followed by training in two skilled motor tasks, led to an impairment in skill acquisition with the limb contralateral, and not ipsilateral, to the removed sensory cortex. After skill acquisition, the other sensory cortex was removed, and no change was observed in performance by either limb. Further, performance of an unskilled control motor task was unaffected by sensory cortical lesions. These data suggest that the sensory cortex had a specific role in establishing the skill, and not in aiding in task performance.

The Relationship Among Structural, Synaptic, and Map Plasticity

I have thus far described structural, synaptic, and map plasticity which occur in the motor cortex secondary to experimental manipulations or motor learning. What is the relationship between these phenomena, and is it causal? It has been shown that synaptic/structural modifications precede map expansion (8, 9). In these studies, the number of synapses per neuron became significantly increased at seven or eight days into training, whereas a significant expansion of the distal movement representation did not occur until 10 days into training. Further, neither structural nor electrophysiological plasticity were evident until well after significant improvement in reach accuracy had occurred (at three days).

The modulation of synaptic strength which occurs in the motor cortex may result from synapse-specific LTP-like and LTD-like modifications, and may be related to learning, but is there a connection between synaptic plasticity and map plasticity (reviewed in 75)? Indeed, LTP-inducing repetitive stimulation has been shown to lead to map expansion. In one study, microelectrodes were introduced into ICMS-defined "border" regions, and repetitive current pulses were delivered. Border shifts occurred, wherein the movement represented at the repetitively stimulated site was expanded. These effects were reversible, indicating map lability (76). LTP induced via corpus callosal stimulation in awake, behaving rats, was also shown to lead to map reorganization (77), as have local application of GABA antagonists (24) and repeated

seizure activity, which is also associated with enhanced cortical field potentials (78). Manipulations which lead to decreases in synaptic efficacy have also been shown to cause motor map reorganization. LTD of callosal projections to the motor cortex for 20-25 days resulted in ~30% smaller caudal forelimb area (79) in freely behaving rats.

The data described above suggest that there is an association between synaptic and map plasticity, but is there a structural basis for LTP/LTD? It is known that induction of LTP in the motor cortex *in vivo* leads to increased spine density and altered dendritic morphology (80). In one study, it was shown that LTP in the motor cortex lead to increased synaptogenesis, increased length of post-synaptic densities, and an increased number of perforated synapses and synapse-associated polyribosomes, both hallmarks of synaptogenesis (81). It has also been shown that LTD induction is related to decreases in dendritic length (but not complexity) and spine density both in layers III and V as well as a decrease in dendritic branch complexity in layer V of the sensorimotor cortex, as assessed by analysis of Golgi-Cox stained sections (82).

The connection between electrophysiological and structural synaptic plasticity has been explored at the single-synapse level. In an elegant study using two-photon local uncaging of glutamate, it was shown that spine enlargement occurs with repeated stimulation, specifically at the stimulated spine (83).

Finally, there are studies which illustrate an association among structural, electrophysiological, and map plasticity from the same manipulation. LTP induced via corpus callosal stimulation in awake, behaving rats, was shown in one study to lead to both map reorganization and dendritic hypertrophy (77). In another study, LTD induction resulted in smaller (by ~30%) motor maps of the caudal forelimb area, with a reduction of distal movement representations accounting for most of this reduction. There were also accompanying structural changes - a reduction in the density of excitatory perforated synapses and an increase in the density of inhibitory synapses in layer V of the sensorimotor neocortex. (79).

A Model of Motor Learning

Based on a synthesis of the studies described above examining cortical reorganization and associated structural and electrophysiological synaptic plasticity, I propose the following model:

As a result of training specific motor programs, certain synapses are strengthened while others are weakened. As shorter term synaptic modifications become more permanent, new synapses (and potentially new synaptic spines) are created and neuronal structure is modified. In this way, trained movements are allocated more circuitry. That is, a circuit (i.e., a set of specific neurons in a given pathway) controlling a certain movement is active at a lower threshold than it had

been previously because specific synaptic components of that circuit are strengthened at the expense of specific synaptic components of competing circuits. So, after skilled reach training, more cortical area will elicit, say, a concerted digit flexion resulting in a grasp, than it did before training. On a gross level, this is manifested as increased synaptogenesis and a reorganization of the motor map.

In the setting of cortical map plasticity and motor learning in the adult, where changes in the balance between inhibition and excitation may occur, BDNF seems particularly well-suited to mediating these effects. At the synapse level, BDNF has the ability to mediate changes in number and/or strength of both GABAergic and glutamatergic synapses. At the circuit level, BDNF may act via release of inhibition in cortical areas bordering body region representations and by simultaneously potentiating excitatory circuits representing newly active movements in areas of expanded representations. Indeed, in a recent study in humans expressing a valine to methoionine (Val/Met) polymorphism in the BDNF gene (84), map plasticity and motor-evoked potentials were shown to be affected by BDNF genotype. In this study, 9 control (Val/Val), and 17 polymorphic (Val/Met or Met/Met) subjects were trained on three fine motor tasks. Although there were no baseline differences in corticospinal output or motor map area as a function of genotype, TMS revealed that the brief period of motor training enhanced corticospinal output and increased motor map area in Val/Val but not in Val/Met or Met/Met subjects. This is the first direct

evidence of a causal relationship between BDNF and cortical map plasticity in the motor cortex.

At the molecular level, in the context of skilled reach learning, it is known that protein synthesis is necessary for improvements in task performance (85, 86), for map expression, number and size of synapses, and cortical field potentials (85). In fact, protein synthesis inhibitors abolished motor maps, and motor skill learning reinstated them (85). Based on its ability to modify neuronal structure, neuronal excitability and synaptic efficacy, excitatory and inhibitory circuitry acutely and in the long term, and cortical organization (87), BDNF may be one of the proteins whose synthesis is required to mediate these plastic changes. The next section will focus on the molecular mechanisms by which it might do so.

BDNF and Intracellular Signaling

How might BDNF signaling lead, ultimately, to changes in neuronal structure and excitability? As was alluded to in the introduction, BDNF signaling via TrkB is upstream of a number of intracellular cascades which have been associated with a variety of cellular functions. One of these, the MAPK/ERK cascade, is of particular interest, as it is most often linked to cellular plasticity and learning (88-91, but see also 92), including the formation of stable dendritic filopodia (93). Briefly, the cascade is as follows: BDNF binds TrkB, which dimerizes and becomes autophosphorylated.

Phosphorylation on the receptor of tyrosine residue Y490 recruits and phosphorylates Shc, an adaptor protein. This leads to recruitment to the membrane of a complex of the adaptor Grb-2 and the Ras exchange factor son of Sevenless (SOS), thereby stimulating transient activation of Ras. Ras activates cRaf, a MAPKKK. cRAF activates MEK1/2, a MAPKK. MEK1/2 activates ERK1/2, a MAPK. ERK/MAPK then phosphorylates other cytoplasmic proteins or translocates to nucleus, where it phosphorylates CREB (94-96), Elk-1, Fos (97), and other transcription factors, leading directly or indirectly to gene transcription. Within the motor cortex, increases in transcription factor expression have been shown to occur during the early stages of motor acquisition (98), suggesting that such intracellular mechanisms may be involved in motor learning.

There have been many studies which link BDNF signaling to plasticity via ERK/MAPK activation. In the invertebrate model, Aplysia, BDNF, along with a serotonin pulse, can produce long-term synaptic facilitation. This BDNF-mediated effect requires ERK activation (99). BDNF-LTP has also been shown to occur *in vivo* in the hippocampus, is associated with phosphorylation of ERK and phosphorylation of CREB in an ERK-dependent manner, and is abolished by inhibitors of ERK phosphorylation (100). In another pair of studies investigating the role of BDNF and ERK signaling in the hippocampus, anti-BDNF antibody was shown to reduce ERK2 and CREB activation, and administration of recombinant human BDNF subsequently increased ERK1/2 and CREB activation. This increase in activation was specific to

this pathway – activated p38, activated PKC-isoforms, and activated-AKT were unaltered. There was also no change on alpha and beta PKA catalytic subunits in the nucleus. In these studies, endogenous BDNF was found to be required for both short-term and long-term memory formation in the hippocampus, and this requirement appeared to involve ERK1/2-dependent and -independent mechanisms (101, 102). The same group examined the role of BDNF and ERK signaling on spine density in hippocampal cultures. They had previously found that BDNF increases apical dendrite spine density on CA1 pyramidal neurons (103, 104). They then investigated whether BDNF-mediated ERK signaling mediated this effect, and found that MEK (ERK kinase) inhibitors, in the presence of BDNF, prevented the increase in spine density in a dose-dependent manner (105). In the absence of BDNF, the MEK inhibitors had no effect on spine density. They also showed that BDNF increased levels of phosphorylated ERK1 and ERK2, and that this effect was eliminated when slices were pre-incubated with MEK inhibitors.

In a study of intracellular mechanisms of BDNF-mediated plasticity in the amygdala (106), the signaling cascade from BDNF to MAPK was examined as a function of time after training on a fear conditioning task. The authors first showed that exogenous BDNF enhanced, and blocking BDNF attenuated, fear-potentiated startle. They then showed that, following training, there was an increase in TrkB co-immunoprecipitation with Shc, and Shc co-immunoprecipitation with Ras. Levels of activated (versus inactivated) Ras were also increased as a function of training, as

were levels of activated, or phosphorylated (versus unphosphorylated) MAPK. Further, inhibitors of Ras and MAPK activation impaired fear-conditioning and the BDNF-mediated enhancement thereof. Finally, bath-applied BDNF to amygdala slices lead to increases in activated Ras and MAPK, effects which were abolished when slices were pretreated with K252a.

Taken together, these data indicate that the effects of BDNF on both structural and electrophysiological synaptic plasticity, as well as on learning, appear to be mediated by the MAPK/ERK intracellular cascade.

What effectors might be downstream of the BDNF-MAPK cascade that could lead to these various forms of plasticity? BDNF, in an ERK-dependent manner, has been shown to increase the expression of the immediate early genes c-fos, early growth response gene 1 (EGR1), and activity-regulated cytoskeletal-associated protein (Arc) (100, 107), an immediate early gene that has been shown to be required for generation of late, mRNA synthesis-dependent LTP, and learning (108). BDNF not only mediates changes in the transcription of plasticity related genes, but it also regulates translation of a number of gene products, a process which has been shown to involve ERK (109). For example, BDNF enhances the synthesis of Arc and of calcium/calmodulin-dependent protein kinase II alpha (CamKIIα) (110, 111). CaMKII is crucial for many aspects of synaptic plasticity, and is a leading candidate in the search for the molecular basis for learning and memory (reviewed recently in 112-

115). For example, it has been shown to enhance the efficacy of synaptic transmission, be activated by LTP, and be necessary for LTP induction. Mutations that prevent activation of CaMKII block plasticity and memory.

The regulation of synaptic proteins has also been shown to be mediated by BDNF. The translation of Homer 2, a cytoskeletal scaffolding protein that has been implicated in the growth of dendritic spines (116), is increased by BDNF (111). BDNF also enhances translation of the glutamate receptor subunits GluR1 and NR1/3 (111). BDNF (and not NGF or NT-3) was shown to trigger an increase in AMPA receptor subunit protein (not mRNA) in cultured neocortical neurons (117). BDNF is also able to regulate the surface expression/translocation of AMPA receptor subunits to the cell membrane (118, 119).

The intracellular machinery involved in cortical motor learning has not yet been fully explored, nor has the intracellular signaling that might occur downstream of BDNF as cortical motor tasks are learned. However, given the possible outcomes of BDNF intracellular signaling, namely, initiation of transcription cascades which can result in increases in synaptic efficacy and even increases in synaptic/spine numbers, BDNF remains a good candidate molecule for mediating such forms of plasticity in the motor cortex.

Conclusions and Future Directions

The results described in this dissertation, taken as a whole, suggest the following conclusions:

1) BDNF mRNA is upregulated during motor skill learning and during acquisition of a non-skilled, procedural motor task.
2) TrkB mRNA does not appear to be modulated by motor learning.
3) An siRNA construct directed against BDNF mRNA reduces BDNF protein production *in vitro*, by 81% compared to uninfected cells and 85% compared to control-virus infected cells.
4) The lentiviral siBDNF reduces BDNF protein by 22% compared to uninjected cortices and 34% compared to control-virus-injected cortices, as measured by ELISA in the forepaw motor cortex.
5) The lentiviral siBDNF construct does not have an effect on cortical motor skill learning or on the associated cortical map reorganization.
6) A TrkB-Fc lentiviral construct blocks BDNF bioactivity *in vitro*.
7) The TrkB-Fc fusion protein, delivered to the forepaw motor cortex in unquantified amounts and with unknown effectiveness, does not have an effect on cortical motor skill learning or on the associated cortical map reorganization.

8) A BDNF-blocking antibody which has been reported to elicit behavioral effects does not bind BDNF *in vitro*, and does not block BDNF bioactivity *in vitro*.
9) The antibody tested for BDNF blocking effects *in vitro* may, at high concentrations and in concert with BDNF, act as a TrkB agonist.

The results of these studies suggest several further lines of investigation. Though unrelated to the specific questions within the scope of the hypothesis tested by the studies described, other genes of interest, such as those encoding for molecules in the intracellular cascades downstream of BDNF signaling, and synaptic proteins, including receptor subunits, might also be modulated, and differentially modulated, during learning. The array data from this experiment may offer a wealth of preliminary information on the molecular events associated with the laying down of motor cortical memory.

Another unrelated but potentially interesting question which arises from these results is related to the observation that the BDNF "blocking" antibody seemed, at a high concentration, to play an agonistic, rather than antagonistic role. It might therefore be of interest to use biochemical techniques to determine whether the antibody does, indeed, bind TrkB, and to explore additional bioassays to determine whether it acts as an agonist in other settings. For these experiments, an examination of concentrations between 10 μg/ml and 100 μg/ml might be warranted. If blocking

activity is detected in this or in other preparations at a lower concentration within this range, perhaps a critical concentration will be observed, at which antibody activity switches from antagonism to agonism.

Finally, the present results suggest further exploration of the questions related to the role of BDNF in cortical motor learning. BDNF was found to increased at three days into training, though this increase represented a trend and was not significantly different than baseline. This is concomitant with the significant performance gain reported previously in this task (8). Also, BDNF continued to rise, to a level significantly different than baseline at 14 days, by which time cortical map reorganization is apparent (8). It is possible that activity-driven experience initiates early transcription factor cascades which activate promoters on the BDNF gene, leading to the initial upregulation of BDNF. This upregulation might lead to further, BDNF-mediated intracellular signaling, leading to structural and physiological synaptic plasticity, which may in turn underlie the subsequent cortical map plasticity. It is also possible that early upregulation of BDNF is related to structural and electrophysiological synaptic plasticity, and that, independently, continued upregulation may reflect the processes involved in cortical map reorganization. Finally, it is possible that the upregulation of BDNF is unrelated to the associated motor cortical plasticity.

To answer these questions, investigations into the mechanism(s) and role(s) of the modulation of BDNF during learning could be carried out. Biochemical experiments could be used to assess levels, binding, and phosphorylation states of intracellular molecules downstream of BDNF as a function of learning of this task. Pharmacological manipulations using inhibitors or agonists of signaling molecules and synaptic proteins, such as ion channels, would elucidate the roles of various components of the signaling cascade leading from BDNF to the various forms of plasticity observed in this paradigm. Finally, electrophysiological studies would be valuable in assessing the time course of cellular and map activity secondary to these manipulations.

The experiments described in this dissertation describe attempts to block BDNF signaling using 3 different tools, which act in mechanistically distinct ways. Because of the limited BDNF protein reduction achieved using the siBDNF and TrkB-Fc constructs in the forepaw motor cortex *in vivo*, further attempts to block BDNF signaling might best be focused on an effective BDNF blocking antibody. The present data indicate that a partial attenuation of BDNF signaling is insufficient to affect motor learning or motor map organization. However, only at the point when the signaling block is deemed sufficient in magnitude and physical extent, could a negative result be interpreted as a lack of BDNF effect in this system.

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