ROLE OF DENDRITIC BDNF SYNTHESIS IN ADULT NEUROGENESIS
AND SPINE MORPHOGENESIS

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ROLE OF DENDRITIC BDNF SYNTHESIS IN ADULT NEUROGENESIS AND SPINE MORPHOGENESIS

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ABSTRACT

Substantial evidence has demonstrated that dendritic protein synthesis is an important source of new protein during the expression of synaptic plasticity. Dendritically synthesized brain-derived neurotrophic factor (BDNF) has been shown to be key in the expression of lasting synaptic plasticity. However little is known about the function of dendritic protein synthesis in other cellular forms of plasticity. Here we investigated the role of dendritic BDNF synthesis in adult neurogenesis and spine morphogenesis.

Adult neurogenesis in the subgranular zone (SGZ) of the dentate gyrus is regulated by hippocampal network activity. However the interplay between glutamatergic and GABAergic transmission during adult neurogenesis remains unclear. Here we report that dendritic synthesis of BDNF mediates this interplay. Activation of the N-methyl-D-aspartic-acid receptor (NMDAR) stimulated dendritic BDNF synthesis in hippocampal neurons and the effect of NMDAR antagonism on adult neurogenesis was abolished in mice lacking dendritic BDNF synthesis. These animals also exhibited deficits in GABAergic innervation to the SGZ and differentiation of precursor cells, which was rescued by administration of a GABA_A agonist. Furthermore, we observed similar neurogenesis deficits in mice where BDNF signaling was selectively abolished.
in parvalbumin-expressing GABAergic (PV) interneurons. We propose that these data establish a model in which NMDAR activation stimulates dendritic synthesis of BDNF, which facilitates GABA release from PV interneurons, leading to differentiation of precursor cells.

A sequence in the Bdnf mRNA long 3’untranslated region (3’UTR) has been shown to be necessary and sufficient to target Bdnf mRNA into the dendrites. Furthermore, an animal model deficient in dendritic Bdnf mRNA exhibits alterations in spine morphogenesis. We investigated what possible role single nucleotide polymorphisms (SNPs) in the human Bdnf 3’UTR might have on Bdnf mRNA trafficking and spine morphogenesis. We found that one of the eight reported SNPs in the human Bdnf 3’UTR impeded dendritic trafficking of Bdnf mRNA in cultured hippocampal neurons. In addition, neurons transfected with these constructs showed impairments in spine morphogenesis. Mature neurons had longer, thinner, more densely clustered spines that resembled immature neuronal spines. These data suggest that humans carrying this SNP may have impairments in dendritic BDNF synthesis and spine morphogenesis.
The research and writing of this thesis
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Chapter One: Dendritic BDNF synthesis and synaptic plasticity

General introduction to dendritic protein synthesis and BDNF

Synaptic plasticity is the activity-dependent selective strengthening or weakening of individual synapses so that information can be represented, processed, and stored in complex neural networks. This process most often occurs at excitatory synapses with dendritic spines as the postsynaptic site (Sheng and Hoogenraad, 2007). Intense investigation has therefore focused on how neurons with thousands of dendritic spines can differentially modify signals at individual synapses. Activity-dependent local protein synthesis at or near individual spines has emerged as a mechanism for how this type of fine-tuned synaptic plasticity might occur. Although studies conducted as early as 1965 demonstrated that RNA did localize outside of the neuronal soma (Bodian, 1965; Koenig, 1965), it was not until the early 1980s that both mRNAs and polyribosomes were localized to dendritic compartments and to regions beneath individual dendritic spines (Palacios-Pru et al., 1981; Steward and Levy, 1982; Steward and Falk, 1985, 1986). Since then, extensive research has focused on processes by which mRNA might be transported into the dendritic compartment and locally translated there in response to stimulation (Bramham and Wells, 2007).

Brain derived neurotrophic factor (BDNF) is a crucial regulator of activity-dependent synaptic plasticity (Arancio and Chao, 2007; Lu et al., 2008). It belongs to a family of small, closely related, secreted proteins called neurotrophins that also includes nerve growth factor (NGF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5).
Neurotrophins exert their influence on neurons mainly through Trk receptor tyrosine kinases: NGF activates TrkA; BDNF and NT4/5 activate TrkB; and NT3 activates TrkC. In addition, they achieve some biological functions through a common receptor p75\textsuperscript{NTR} (Reichardt, 2006). One recent surprising discovery is that the unprocessed pro-forms of neurotrophins, termed proneurotrophins, are secreted and have a unique biological function through the p75\textsuperscript{NTR}/sortilin receptor complex (Lee et al., 2001; Nykjaer et al., 2004). BDNF is widely expressed in both the developing and the mature brain (Hofer et al., 1990; Maisonpierre et al., 1990; Phillips et al., 1990). In addition to its role in promoting the proliferation, differentiation, and survival of neurons (Davies, 1994; Bothwell, 1995), BDNF has been shown to be crucial in regulating synaptic activity and plasticity through both functional and structural changes in neurons (Arancio and Chao, 2007; Lu et al., 2008).

This chapter outlines the role of BDNF in synaptic plasticity, concentrating on postsynaptic mechanisms in the adult hippocampus. First, we review findings that implicate BDNF in hippocampal synaptic plasticity. Then, we describe specific mechanisms that restrict BDNF to active synapses. Finally, we discuss two processes by which localized BDNF may affect synaptic plasticity: phosphorylation of local proteins and induction of local protein synthesis.

**BDNF and synaptic plasticity in the mature hippocampus**

No paradigm of synaptic plasticity has been better studied than that of long-term potentiation (LTP) in the hippocampus. LTP refers to the relative strengthening of
synapses such that subsequent stimuli will more readily produce a postsynaptic response. The majority of fibers in the hippocampus follow a circuit that starts and ends in the entorhinal cortex. Layers II/III of entorhinal cortex project via the perforant pathway to granule cells of the hippocampal dentate gyrus. The granule cells project via mossy fibers to pyramidal cells of Ammon's horn, subfield 3 (CA3), which then project, via Schaffer collaterals, to the pyramidal cells of Ammon’s horn subfield 1 (CA1). CA1 pyramidal cells project to deep layers of the entorhinal cortex completing this circuit: entorhinal cortex to granule cells to CA3 pyramidal cells to CA1 pyramidal cells back to entorhinal cortex (Witter M.P., 1989). The preponderance of LTP research has focused on the synapses between presynaptic CA3 region Shaffer collateral axon terminals and postsynaptic CA1 dendrites. By inducing tetanic stimulation in presynaptic CA3 neurons, and recording the amplitude of excitatory postsynaptic potentials (EPSPs) in CA1 neurons researchers have been able to tease apart important aspects of LTP, and much of this work has focused on the role of BDNF in these processes.

It is generally accepted that LTP can be divided into an early phase (E-LTP), and a late phase (L-LTP). E-LTP lasts between one and two hours and is dependent on the modification of existing proteins (Malenka and Bear, 2004). L-LTP has been documented to last up to days (Abraham, 2003), and is dependent on de novo protein synthesis (Frey et al., 1988; Kandel et al., 2001). There is substantial evidence to support a critical role for BDNF in the expression of hippocampal long-term potentiation (LTP), in both the early and late phases (Minichiello et al., 1999; Pozzo-
Miller et al., 1999; Xu et al., 2000; Kovalchuk et al., 2002; Zakharenko et al., 2003; Gartner et al., 2006; Rex et al., 2006; Lu et al., 2008). Importantly, BDNF mediates different processes for early and late phase LTP. Evidence suggests that BDNF mediates E-LTP via release of existing presynaptic BDNF pools and subsequent modification of existing synaptic proteins (Ponzzo-Miller et al., 1999; Jovanovic et al., 2000; Zakharenko et al., 2003). By contrast, evidence suggests that BDNF mediates L-LTP via induction of postsynaptic BDNF synthesis and release (Pang et al., 2004; Kang et al., 1997), and subsequent stimulation of de novo protein synthesis of proteins crucial for the induction and maintenance of L-LTP (Kang and Schuman, 1996; Yin et al., 2002; Schratt et al., 2004). This rise in postsynaptic BDNF during L-LTP is likely due to both the increased translation of existing synaptic BDNF mRNA (Pang et al., 2004; An et al., 2008) and the transcription and translation of new BDNF mRNA (Patterson et al., 1992; Castren et al., 1993). However, pools of newly transcribed BDNF mRNA probably function to refresh depleted dendritic BDNF mRNA for the maintenance and not the induction of L-LTP, as the timeframe for transcription and trafficking would be prohibitive for the induction of L-LTP. Furthermore, BDNF has been shown to be secreted in an activity-dependent manner from both postsynaptic spines and presynaptic terminals (Hartmann et al., 2001; Kohara et al., 2001; Kojima et al., 2001), and the TrkB receptor has been localized to both pre- and postsynaptic sites (Drake et al., 1999). It is therefore likely that BDNF is a dynamic regulator of synaptic plasticity by mediating changes on both sides of the synapse to facilitate the expression of E-LTP and L-LTP.
LTP refers to a physiological change in the strength of synaptic transmission; however, this physiological change is also accompanied by structural alterations in synapses (Tanaka et al, 2008). BDNF is able to locally mediate molecular changes that alter both the physiology and structure of synapses. In the subsequent sections we outline mechanisms by which BDNF is restricted to activated synapses, and explain how synapse-restricted BDNF effects molecular changes that alter both synapse physiology and structure.

**Restriction of BDNF action to active synapses**

One signature feature of synaptic plasticity is that synaptic strengthening or weakening only occurs at synapses with altered activity. If BDNF is a key regulator of synaptic plasticity, then it is important to understand how BDNF, as a secreted and diffusible molecule, can achieve such spatial specificity. From recent work four mechanisms have emerged that may restrict the action of BDNF to active synapses: (1) local synthesis of BDNF and TrkB in dendrites; (2) activity-dependent release of BDNF from pre- and postsynaptic sites; (3) activity-dependent insertion of TrkB into the plasma membrane; and, (4) cleavage of proBDNF to mature BDNF. These four mechanisms likely work in tandem to ensure the specificity of BDNF action.

It has been shown that BDNF transcripts are transported to the dendritic compartment in cultured hippocampal neurons in response to KCl-induced depolarization (Tongiorgi et al., 1997), in the rat hippocampus in response to epileptogenic stimulation (Simonato et al., 2002; Tongiorgi et al., 2004; Chiaruttini et al.,
TrkB mRNA is also transported to dendrites of cultured hippocampal neurons in response to depolarization (Tongiorgi et al., 1997). Some of the effect of activity on dendritic trafficking of BDNF and TrkB mRNAs appears to result from activity-induced BDNF release, because pulse application of BDNF to cultured neurons increased accumulation of BDNF and TrkB mRNAs in dendrites via a phosphatidylinositol-3 kinase (PI3K) dependent pathway (Righi et al., 2000). It is generally believed that dendritically localized mRNAs remain translationally inactive until stimulation (Bramham and Wells, 2007). Thus translation of mRNAs for BDNF and TrkB in response to activity would restrict newly synthesized BDNF and TrkB to activated postsynaptic sites.

A wide range of studies have provided evidence that BDNF is released both pre- and postsynaptically in an activity-dependent manner (Lessmann et al., 2003; Lu, 2003). Recent advancements in our understanding of activity-regulated BDNF release have mainly come from imaging of green fluorescent protein (GFP)-tagged BDNF (BDNF-GFP) in cultured neurons. In cultured cortical neurons BDNF-GFP was targeted into secretogranin II positive dendritic secretory granules of the regulated pathway in the vicinity of synaptic junctions (Haubensak et al., 1998), suggesting that BDNF may be secreted through the postsynaptic membrane in an activity-dependent fashion. Indeed, BDNF was released postsynaptically in response to network activity, as revealed by time-lapsed imaging of BDNF-GFP in hippocampal neurons (Kuczewski et al., 2008). LTP-inducing high-frequency stimulation was found to elicit release of
BDNF from secretory granules localized at glutamatergic synapses, and activity-induced BDNF release at the postsynaptic site was dependent on calcium influx and activation of ionotropic glutamate receptors (Hartmann et al., 2001). In cultured hippocampal neurons, network activity also caused BDNF-GFP to be transported to and released from the presynaptic site, and be transferred to the postsynaptic site (Kohara et al., 2001; Kojima et al., 2001). Furthermore, BDNF is a “sticky” molecule likely due to its abundance of positively charged moieties (pI=9.6), so that locally released BDNF should remain in the vicinity of where it is released from, and not spread to distant non-activated synapses.

Activity-dependent surface expression of TrkB further restricts the action of BDNF to stimulated synapses. The concept that surface expression of TrkB is regulated emerged from work examining the survival of retinal ganglion cells (RGCs) in culture. Meyer-Franke found that the survival of cultured postnatal RGCs was low when treated with BDNF unless their intracellular cAMP was increased pharmacologically or their activity was enhanced by KCl-induced depolarization or activation of glutamate receptors (Meyer-Franke et al., 1995). It turned out that few TrkB receptors were localized to the plasma membrane in cultured RGCs, although high levels of TrkB were present intracellularly. Depolarization or cAMP elevation greatly increased surface TrkB levels within minutes by stimulating translocation of intracellularly stored TrkB to the plasma membrane (Meyer-Franke et al., 1998). If activity-dependent TrkB surface expression occurs at postsynaptic sites, the insertion of TrkB into the plasma membrane can strengthen the response to BDNF at a single dendritic spine while leaving nearby
synapses unchanged. Du et al demonstrated that there was a rapid (<30 min), and therefore not protein synthesis dependent, membrane insertion of the TrkB receptor in response to high frequency stimulation in cultured hippocampal neurons (Du et al., 2000). This membrane insertion of the TrkB receptor was shown to depend on calcium influx and Ca$^{2+}$/calmodulin-dependent kinase II (CaMKII). Fluorescent immunocytochemistry revealed that TrkB receptors were preferentially moved from intracellular pools to the plasma membrane of dendrites (Du et al., 2000). Interestingly, it was reported that within 15 seconds of BDNF application, TrkB receptors were up-regulated at the plasma membrane of hippocampal neurons (Haapasalo et al., 2002). This observation suggests that increased TrkB surface expression in response to cellular stimulation is at least in part mediated by activity-dependent BDNF release.

The cleavage of proBDNF to mature BDNF also appears to play a role in the restriction of BDNF to activated synapses. It has only recently been discovered that proBDNF has a unique biological function through the p75NTR/sortilin receptor complex (Nykjaer et al., 2004). Evidence suggests that proBDNF may play a role in the regulation of hippocampal synaptic plasticity by facilitating long term depression (LTD) (Woo et al., 2005), and in peripheral neurons by facilitating apoptosis (Teng et al., 2005). It has been postulated that proBDNF is converted to mature BDNF intracellularly by pro-protein convertase 1/3 (PC1/3) in secretory granules and by furin in trans-Golgi networks (Seidah et al, 1996; Mowla et al., 2001). However, extracellularly, plasmin and matrixmetalloprotease-7 appear to mediate the conversion of proBDNF to mature BDNF (Lee et al., 2001). In tissue, plasmin is mainly present as
an inactive precursor form, plasminogen, which is converted into plasmin by tissue plasminogen activator (tPA) (Castellino and Ploplis, 2005). The conversion of proBDNF to mature BDNF by tPA/plasmin is necessary for hippocampal late-LTP (Pang et al., 2004), suggesting that proBDNF is also secreted in the brain, at least at synapses. Because of the difficulty in detecting proBDNF, ascertaining the amount of BDNF that is secreted as proBDNF in nervous tissues has remained controversial (Matsumoto et al., 2008). However recently, research conducted by the research teams of Bai Lu and Barbara Hempstead have generated novel antibodies to detect proBDNF and have reported that both proBDNF and mature BDNF are secreted from neurons, with proBDNF being the primary form released from dendrites (Nagappan et al., 2009; Yang et al., 2009). Specifically, Nagappan and colleagues (2009) reported that both proBDNF and mature BDNF were predominantly secreted by the regulated pathway, and that high frequency (HFS) versus low frequency stimulation (LFS) favored the extracellular accumulation of mature BDNF due to HFS dependent release of tPA. Importantly, the authors showed that in tPA -/- mice, HFS resulted in increases in extracellular proBDNF and not mature BDNF, indicating that proBDNF is the main form secreted from dendrites and that HFS-induced release of tPA converts these pools to mature BDNF. Yang and colleagues also found that HFS increased extracellular mature BDNF while LFS increased extracellular proBDNF, and further reported that by inhibiting plasmin generation extracellularly in cultured hippocampal neurons mature BDNF levels were undetectable in the media, unless TrkB-Fc receptor antibodies were added to the media to bind to extracellular mature BDNF, suggesting that in the absence
of extracellular plasmin, any mature BDNF released is in low enough quantities that it readily binds to surface TrkB or is degraded. Taken together, these studies depict a model in which dendritic LFS and HFS primarily promote the release of proBDNF, with HFS increasing the release of tPA into the extracellular space, thus shifting the extracellular balance from proBDNF to mature BDNF.

Still unknown, however, is the mechanism by which BDNF mRNA in distal dendrites--far beyond dendritic branch points where Golgi outposts have been documented--is translated into proBDNF and packaged into dense core granules to be secreted via the regulated secretory pathway. Golgi outposts, defined by their characteristic mini-Golgi stack ultra-structure and immunoreactivity to the Golgi matrix protein GM130, have only been found as far as dendritic branch points along the dendritic shaft (Gardiol et al., 1999; Horton et al., 2005). Nonetheless, the Golgi markers giantin and α-mannosidase II have been found in dendritic spines (Pierce et al., 2001). It is therefore possible that as yet unknown dendritic organelles, whether adapted from and similar to prototypical Golgi networks, or completely different in structure and function from existing organelles, might mediate the activity-regulated secretion of pro- and/or mature BDNF from dendritic spines. The possibility of additional dendritic organelles is supported by findings indicating that without distal dendritic BDNF mRNA, distal dendritic BDNF protein is greatly reduced (An et al., 2008). Consequently it is not likely that BDNF is primarily synthesized at proximal Golgi outposts and then trafficked to distal dendrites. The possibility of additional dendritic organelles would explain current discordant findings and demonstrate the complex
regulation of both proBDNF and mature BDNF action at activated synapses. In this model, BDNF synthesized in the cell body and proximal dendrites would undergo processing and packaging for the regulated secretory pathway in traditional Golgi-structures, destined for axonal or dendritic release. Interestingly, Lochner and colleagues recently showed that BDNF, tPA and plasmin were co-packaged in dense core granules and transported into dendrites. Using fluorescent chimeras of BDNF, tPA, and plasmin the authors demonstrated that all three constructs co-localized in dense core granules along the dendritic shaft and into dendritic spines. Although the authors state that it is proBDNF they are measuring, no measures were taken to distinguish mature-from proBDNF. Furthermore, due to the construct 3’UTR, these chimeric mRNAs were likely translated and packaged in the soma before being trafficked to distal dendrites. It is therefore likely that these chimeric constructs were translated, packaged, and processed to maturity in the soma, and then trafficked to distal dendrites. This is supported by findings that proBDNF is processed intracellularly by Golgi structures and endoplasmic reticule to generate mature BDNF (Mowle et al., 2001; Kolarow et al., 2007). Alternatively, BDNF mRNA trafficked to distal dendrites might undergo packaging in Golgi-like structures that predominantly yield release of proBDNF, thus allowing for spatially discrete HFS-induced release of tPA, and local conversion to mature BDNF (Nagappan et al., 2009; Yang et al., 2009). This model would also account for a recent report that proBDNF release, when combined with LFS, yields long term depression (Woo et al., 2005), a electrophysiological state that has been associated with dramatic shrinkage in spine size (Zhou et al., 2004). Thus, dendritic synaptic HFS
would: (1) cause the release of locally synthesized pools of proBDNF; (2) cause the release of tPA, in turn converting extracellular proBDNF to mature BDNF; and, (3) cause the trafficking of dense core granules containing mature BDNF to activated synapses for local release. Alternatively, LFS would also cause the release of proBDNF, but not tPA/plasmin, thus leaving proBDNF in the synapse to initiate cellular cascades though the p75/sortillin receptor complex causing LTD and spine shrinkage. This model of complex regulation would allow for fine-tuned and varied responses to disparate input stimuli. Future work investigating how disparate stimuli might differentially activate these processes is needed to provide a clearer picture of the processing and release of BDNF.

**BDNF-mediated phosphorylation of synaptic proteins**

In the previous section we discussed several mechanisms that may restrict the action of BDNF to activated synapses. Evidence accumulated during the past 15 years suggests that BDNF can exert its fast effect on synaptic transmission through posttranslational modifications of synaptic proteins. Application of BDNF to cultured neurons has been shown to potentiate synaptic transmission through either a presynaptic (Lohof et al., 1993) or postsynaptic mechanism (Levine et al., 1995).

Presynaptically, BDNF has been shown to modify Synapsin I and RIM1α (Jovanovic et al., 1996; Jovanovic et al., 2000; Simsek-Duran and Lonart, 2008). Synapsin I, a protein found on small synaptic vesicles, aids in the attachment of synaptic vesicles to actin filaments near the presynaptic membrane, thereby restricting
their release. Phosphorylation of synapsin I results in vesicle detachment and an increased probability of exocytosis (Greengard et al., 1993). BDNF was shown to induce phosphorylation of synapsin I via the mitogen-activated protein kinase (MAPK) cascade in cortical neurons and PC12 cells (Jovanovic et al., 1996). The inhibition of MAPK with PD98059 caused a significant decrease in BDNF-induced synapsin I phosphorylation, and in neurotransmitter release. Furthermore, the stimulation of neurotransmitter release by BDNF was markedly attenuated in synaptosomes prepared from mice lacking synapsins (Jovanovic et al., 2000). Taken together, these studies suggest that BDNF induces phosphorylation of synapsin I, thus increasing docking of synaptic vesicles and neurotransmitter release. Presynaptic BDNF also acts on the Rab3a signaling pathway to increase efflux of glutamate from CA1 nerve terminals. This was first demonstrated when neurons cultured from Rab3a knock out mice did not display BDNF-enhanced neurotransmitter release (Thakker-Varia et al., 2001; Alder et al., 2005). Rab3a signaling involves the phosphoprotein RIM1α as an effector molecule (Lonart et al., 2003). RIM1α has been shown to be necessary for L-LTP (Huang et al., 2005) and necessary for BDNF-enhanced glutamate release from CA1 synaptoneurosomes (Simsek-Duran and Lonart, 2008). BDNF appears to augment this pathway via ERK2 dependent phosphorylation of RIM1α (Simsek-Duran and Lonart, 2008).

At the postsynaptic site, phosphorylation of N-methyl-d-aspartate (NMDA) receptor subunits has been shown to potentiate NMDA currents in hippocampal neurons (Wang and Salter, 1994), and BDNF appears to at least partially mediate this
phosphorylation (Suen et al., 1997). In synaptoneurosome preparations, BDNF application has been demonstrated to rapidly cause phosphorylation of the NMDA receptor subunit 1 (NR1) (Suen et al., 1997). Moreover, BDNF application has also been demonstrated to increase phosphorylation of the NMDA receptor subunit 2B (NR2B) in postsynaptic densities to 165% of control levels within 5 minutes of application (Lin et al., 1999), and BDNF application to hippocampal slices was shown to increase the amplitude of spontaneous excitatory postsynaptic currents (Levine et al., 1995). These observations have thus demonstrated that BDNF signaling has the ability to stimulate protein modification that functionally alters synapses, independent of protein synthesis.

BDNF also appears to have a role in modifying local proteins that modulate structural plasticity. When paired with theta-burst stimulation, BDNF applied to hippocampal slice culture significantly enhanced the number of phalloidin labeled (F-actin) dendritic spines (Rex et al., 2007). This finding suggests that BDNF may regulate dendritic actin dynamics. In the same set of experiments BDNF application was shown to increase the phosphorylation of p21-activated kinase and coflin (two dendritically localized actin-regulatory proteins implicated in spine plasticity) in an acute and dose-dependent manner, thus suggesting a mechanism by which BDNF regulates spine dynamics (Rex et al., 2007).
Regulation of local protein synthesis by BDNF

Application of BDNF has been shown to induce long-lasting LTP in hippocampal slices (Kang and Schuman, 1995), which is dependent on local protein synthesis in dendrites (Kang and Schuman, 1996). Local protein synthesis in dendrites is also required for other paradigms of enduring synaptic plasticity (Huber et al., 2000; Miller et al., 2002). These observations suggest that BDNF may modulate synaptic plasticity by regulating dendritic local protein synthesis. A growing number of mRNAs have been localized in neuronal dendrites (Steward and Schuman, 2003). Recent studies indeed found that BDNF stimulates the local synthesis of many different proteins in dendrites via both increasing dendritic mRNA trafficking and facilitating translation of existing dendritic mRNAs.

Moving mRNAs from somata into dendrites is thought to be accomplished by holding mRNAs translationally silent in RNA granules and transporting them into the dendritic compartment via molecular motors (Kindler et al., 2005; Hirokawa, 2006; Martin and Zukin, 2006). The immediate early gene activity-regulated cytoskeleton-associated protein (Arc) has been shown to be crucial in LTP consolidation at the synapse, and both in vitro and in vivo studies have demonstrated that Arc synthesis is necessary for BDNF to have an effect on LTP at the synapse (Yin et al., 2002; Ying et al., 2002; Messaoudi et al., 2007). Discrete in vivo application of BDNF to the dentate gyrus increased dendritic Arc mRNA, suggesting that BDNF stimulation increases Arc mRNA dendritic trafficking (Ying et al., 2002). However, in this same study overall levels of Arc mRNA were increased, leaving the possibility that BDNF could solely be
inducing transcription. Zipcode binding protein 1 (ZBP1), an mRNA binding protein, was shown to be associated with β-actin mRNA and targeted into the dendritic compartment in response to cellular depolarization (Tiruchinapalli et al., 2003). Interestingly, BDNF-induced spine head growth was reduced in cells where ZBP1 was knocked down (Eom et al., 2003). These findings suggest two possibilities. The first possibility is that BDNF stimulates dendritic trafficking of β-actin mRNA via a ZBP1-dependent mechanism. The second possibility is that BDNF induces local translation of β-actin mRNA, but could not do so when local mRNA pools were depleted due to knocked-down ZBP1 and therefore decreased ZBP1-dependent trafficking of β-actin mRNA to dendrites. The strongest support for the possibility that BDNF does stimulate dendritic trafficking of mRNA can be found in a study reporting that BDNF application increased dendritic levels of mRNAs for both TrkB and BDNF (Righi et al., 2000). This study showed that BDNF application to cultured hippocampal neurons in the absence of KCl stimulation was sufficient to increase dendritic levels of both BDNF and TrkB mRNAs in the presence of the transcription inhibitor, actinomycin (Righi et al., 2000). Furthermore, inhibition of BDNF action with scavenger TrkB-IgG, decreased levels of BDNF and TrkB mRNAs in the dendritic compartment by 53% after KCl stimulation (Righi et al., 2000). Collectively, these data suggest that BDNF induces an increase in trafficking of mRNA from somata to dendrites.

A great deal of research has been conducted to address the question of which dendritic mRNAs are translationally induced by BDNF. In cultured neurons, application of BDNF induces the local synthesis of several synaptic proteins in dendrites (Aakalu et
Mechanisms mediating the effect of BDNF on local protein synthesis

It is currently believed that mRNAs are held translationally silent in at least three types of protein-RNA granules found in dendrites, processing bodies (P-bodies), ribonucleoprotein particles (RNPs), and stress granules (SGs), until a signal de-represses the mRNA and allows for translational activation (Kindler et al., 2005; Schuman et al., 2006; Bramham and Wells, 2007; Zeitelhofer et al., 2008). One way in which BDNF induces local translation is by de-repressing RNA granules. It has been shown that BDNF application to cultured hippocampal neurons leads to a 67%
reduction in the number of P-bodies in dendrites, suggesting that BDNF is de-repressing dendritic mRNA, thereby enabling the mRNA to be translationally active (Zitelhofer et al., 2008). RNA granule protein 105 (RNG 105) is a RNA binding protein that associates with RNA granules in hippocampal dendrites and suppresses translation in vivo and in vitro (Shiina et al., 2005). Application of BDNF has been shown to induce the dissociation of RNG105 from RNA granules (Shiina et al., 2005). Importantly, BDNF application to dissociated dendritic cultures was shown to shift the key plasticity mRNAs CaMKIIα, TrkB, BDNF, and CREB from association with RNA granules to association with translationally-active polysomes, ultimately increasing dendritic protein levels (Shiina et al., 2005). Taken together, these findings suggest that BDNF increases dendritic protein synthesis, in part, by dissociating mRNA from repressing RNA granules.

BDNF has also been shown to facilitate local protein synthesis by activating translation initiation factors and modulating elongation factors. The rate-limiting step in cap-dependent translation initiation is the phosphorylation of translation initiation factor 4E (eIF4E), which results in an increased rate of translation (Gingras et al., 2004). Alternatively, phosphorylation of eIF4E binding protein (eIF4E-BP) will also lead to translation initiation via release of eIF4E. In cultured cortical neurons using an 35S-met radiolabeled protein synthesis assay, BDNF was found to activate protein translation via two pathways, the PI3K-mTOR pathway and the MAPK pathway (Takei et al., 2001). BDNF increased translation initiation via MAPK-induced phosphorylation of eIF4E and via mTOR-induced phosphorylation of eIF4E-BP1 (Takei et al., 2001). These results
were extended to demonstrate the local effect of BDNF on translation initiation by utilizing synaptoneuroosomes, in which vesicularized pre- and postsynaptic membranes are isolated. In synaptoneuroosomes BDNF-mTOR signaling led to phosphorylation of e1F4E-BP and increased translation initiation (Takei et al., 2004). Furthermore, BDNF treatment was shown to result in a rapid and transient activation of eIF4E and the increased expression of CaMKIIα in synaptoneurosome preparations (Kanhema et al., 2006). It also appears that BDNF signaling may increase translation by inducing the local synthesis of ribosomal proteins and translation factors. Transcripts encoding these proteins usually contain a terminal oligopyrimidine tract (TOP) in their 5’UTR, and mTOR activation of ribosomal protein S6 kinase (p70S6K) induces translation of 5’TOP containing mRNAs. BDNF application to synaptoneurosome preparations activated (phosphorylated) p70S6K in an mTOR dependent manner (Takei et al., 2004), and similarly, BDNF induced phosphorylation of p70S6K in cultured neurons at or near synapses (Schratt et al., 2004).

Fractionation studies have revealed that BDNF can induce translocation of eIF4E to mRNA granule fractions, suggesting that de-repressed mRNA will be released from translational repression for proximal translation (Smart et al., 2003). In the same set of experiments, using immunocytochemistry assays, it was reported that BDNF induced translocation of eIF4E to dendritic spines, thus ensuring that eIF4E is in the right place for local translation initiation (Smart et al., 2003). Using FRET analysis to show translation initiation via a direct interaction of two translation initiation factors, eIF5 and eIF2, Miyata et al. found that the simultaneous application of BDNF and
ephrins potentiated local protein synthesis in the dendrites of hippocampal neurons (Miyata et al., 2005). It also appears that BDNF can induce translation initiation by stimulating the dissociation of eIF4E from the repressive mRNA-binding protein CYF1P in the dendritic compartment (Napoli et al., 2008). Taken together, these studies support a model of BDNF as a dynamic facilitator of dendritic translation initiation.

The effect of BDNF on translation elongation appears to be modulatory rather than strictly facilitory. Elongation factor 2 (eEF2), a GTP binding protein, regulates transfer of peptidyl tRNAs from the A-site to the P-site in ribosomes during elongation. Phosphorylation of eEF2 typically results in release of eEF2 from the ribosome and elongation arrest (Browne and Proud, 2002), however translation of CaMKIIα mRNA has been shown to be increased with phosphorylation of eEF2 (Scheetz et al., 2000). In vivo infusion of BDNF into the rat dentate gyrus increased levels of phosphorylated eEF2, while the infusion of a MAPK inhibitor into the dentate gyrus blocked induction of LTP by BDNF and the increase in phosphorylated eEF2 (Kanhema et al., 2006). Interestingly, in the same set of experiments, BDNF treatment was shown to have no effect on eEF2 in synaptoneurosomes (Kanhema et al., 2006). In cultured cortical neurons, however, BDNF application increased the rate of elongation (as shown by ribosomal transit time), while decreasing the phosphorylation of eEF2 via an mTOR-dependent pathway (Inamura et al., 2005). These findings might indicate that the role of BDNF and eEF2 in elongation are both site and substrate specific. Further investigation is needed to better elucidate how BDNF, and the phosphorylation status of eEF2, control elongation.
Local synthesis of BDNF and its role in spine morphology and synaptic plasticity

Tongiorgi and colleagues found that BDNF mRNA was transported to dendrites of cultured hippocampal neurons when the neurons were depolarized (Tongiorgi et al., 1997). Their subsequent studies showed that BDNF mRNA was also targeted to dendrites in vivo after epileptogenic stimulation (Simonato et al., 2002; Tongiorgi et al., 2004; Chiaruttini et al., 2008). These observations suggested that translation of dendritically localized BDNF mRNA may play a crucial role in some activity-dependent processes. It has been known for a while that there are two pools of BDNF mRNAs with either a short 3' UTR or a long 3' UTR (Timmusk et al., 1993; Ghosh et al., 1994), but it had been a puzzle why cells need two species of transcripts encoding exactly the same protein (Fig. 1A). An et al (2008) found that the short 3' UTR BDNF mRNA was restricted to cell bodies whereas the long 3' UTR BDNF mRNA was also localized to dendrites. They further demonstrated that the long 3' UTR was sufficient to target transcripts to dendrites of cultured hippocampal neurons and that truncation of the long 3' UTR abolished dendritic targeting of BDNF mRNA in vivo. These data indicate that the long 3' UTR controls dendritic targeting of BDNF mRNA and provide an example where mRNAs containing the same coding sequence but distinct 3'UTRs can have distinct subcellular localization and function.

The trkB gene encodes two proteins, one full-length TrkB receptor and one truncated TrkB receptor which lacks the tyrosine kinase domain (Klein et al., 1990). TrkB mRNA has been shown to be localized in dendrites in response to activity (Tongiorgi et al., 1997; Simonato et al., 2002), suggesting that TrkB, like BDNF, is also
locally synthesized in dendrites. Interestingly, transcripts for both TrkB receptors have either a short 3′ UTR or a long 3′ UTR, and the two sets of 3′ UTRs are completely different (Fig. 1B). Further investigation is necessary to determine whether the long 3′ UTRs target TrkB mRNAs to dendrites and why the trkB gene needs two sets of 3′ UTRs. It was estimated that more than half of all human genes have multiple polyadenylation sites (Zhang et al., 2005). Because these multiple polyadenylation sites could generate transcripts with different lengths of 3′ UTRs that encode the same protein and are expressed in the same cell, an important question for future research is to determine if alternative polyadenylation is a common way that neurons spatially restrict protein expression. Future research is also needed to explore the possible mechanisms that regulate alternative polyadenylation.

In addition to two different lengths of 3UTR, each BDNF transcript can have one of several distinct alternatively spliced 5′ UTRs (Liu et al., 2006; Aid et al., 2007). BDNF mRNAs containing different 5′ UTRs were found to be targeted to dendrites of cortical and hippocampal neurons with different efficiencies after epileptogenic stimulation (Pattabiraman et al., 2005; Chiaruttini et al., 2008). Given that the long BDNF 3′ UTR is necessary and sufficient for dendritic targeting of BDNF mRNA (An et al., 2008), it is possible that some of the BDNF 5UTRs can modulate the efficiency of BDNF mRNA dendritic targeting mediated by the long 3′ UTR. Alternatively, neuronal activity may increase the usage of the second polyadenylation site for BDNF transcripts containing the 5′ UTRs that are preferentially targeted to dendrites.
It remains to be determined which sequence elements in the long 3′ UTR and which proteins control trafficking of BDNF mRNA to dendrites. The long BDNF 3′ UTR contains multiple cytoplasmic polyadenylation elements (CPEs) (Du and Richter, 2005) and an AU-rich element (ARE), which is a putative binding site for Hu proteins (HuR, HuB, HuC, and HuD) (Okano and Darnell, 1997). CPEs and their binding protein CPEB1 have been shown to facilitate mRNA transport to dendrites and local protein synthesis in dendrites (Wu et al., 1998; Huang et al., 2003). Interaction of HuR with the ARE sequence is thought to facilitate the relief of microRNA-mediated translational repression of certain mRNAs (Bhattacharyya et al., 2006). While HuR is widely expressed, the other three members of the Hu family are specifically expressed in neuronal tissues (Okano and Darnell, 1997). In the middle of the long BDNF 3′ UTR, there is a G-rich sequence with a potential to form a G quartet structure, a binding motif for fragile X mental retardation protein (FMRP). Recent studies show that FMRP is important for dendritic trafficking of mRNAs (Dictenberg et al., 2008; Estes et al., 2008) and for stimulation-dependent protein translation at synapses (Weiler et al., 2004; Muddashetty et al., 2007). It would be interesting to determine whether these proteins play a role in regulating dendritic trafficking and translation of the long 3′ UTR BDNF mRNA.

By taking advantage of an existing mouse strain in which the long BDNF 3′ UTR is truncated, An et al. demonstrated that the long 3′ UTR was necessary for dendritic targeting of BDNF mRNA in vivo (An et al., 2008). Despite the truncation, the Bdnf mouse mutant still produced a normal amount of BDNF mRNA and BDNF
protein. Thus, the mouse mutant provides an ideal system to examine the physiological function of local protein synthesis. Although local protein synthesis is important for long-lasting synaptic plasticity in hippocampal slices (Kang and Schuman, 1996; Huber et al., 2000; Miller et al., 2002), the in vivo physiological function of dendritically localized mRNAs largely remains unknown because it is technically challenging to selectively abolish dendritic local protein synthesis without affecting the total protein level in a neuron. One surprising observation from studying the \textit{Bdnf} mutant is that levels of BDNF protein are lower in dendrites and higher in somata in hippocampal neurons isolated from the mutant mice (An et al., 2008). This result suggests that vesicles containing BDNF synthesized in the soma are not transported to dendrites efficiently and that dendritic BDNF is mainly derived from local synthesis. The \textit{Bdnf} mouse mutant displays an elevated spine density and reduced spine head diameter in distal apical dendrites of CA1 hippocampal neurons at two months of age (An et al., 2008). This observation provides the first direct evidence tying local protein synthesis to morphology of dendritic spines. The spine phenotype in the \textit{Bdnf} mouse mutant likely results from deficits in spine pruning, because the spine density measured at the end of spinogenesis is not altered in the mutant hippocampus (An et al., 2008). Future studies using in vivo imaging are necessary to confirm that local BDNF synthesis plays a key role in spine pruning, which is dependent on sensory experience and has been implicated in activity-dependent refinement of synaptic connections (Zuo et al., 2005).

The work done by An et al clearly indicates that dendritically synthesized BDNF regulates spine morphology. We propose a model of activity-dependent spine changes
to integrate their findings and findings from other groups (Fig. 2). Although BDNF is secreted as both mature BDNF and proBDNF, dendritically released BDNF is primarily secreted in the pro-form (Nagappan et al., 2009; Yang et al., 2009). This is likely due to the fact that Golgi-like organelles are not widely present in distal dendrites (Gardiol et al., 1999; Horton et al., 2005), such that dendritic proBDNF packaging and release probably utilizes a novel mechanism. Extracellular proBDNF can be converted to mature BDNF by the tPA/plasmin system (Pang et al., 2004), which is secreted in an activity-dependent manner (Lochner et al., 2006), thus the action of mature BDNF is limited to stimulated spines and can activate TrkB in an autocrine manner. As discussed in the previous sections, TrkB signaling should induce the local synthesis of proteins important for spine growth and plasticity within and/or underneath the stimulated spines. We speculate that these locally synthesized proteins then promote actin dynamics and AMPA receptor trafficking in spines, which leads to growth of the spine head and formation of stable LTP. This model is based on the following observations. First, the activation of TrkB-mediated signaling cascades has been shown to induce the local synthesis of several proteins, including Arc, CaMKIIα, Homer2, and Limk1 (Kang and Schuman, 1996; Yin et al., 2002; Schratt et al., 2004). While CaMKIIα is a key regulator of AMPA receptor trafficking at synapses (Derkach et al., 2007), the other three proteins promote actin polymerization required for LTP consolidation (Bramham and Wells, 2007) and the maturation and enlargement of spine heads (Sala et al., 2001; Meng et al., 2002). Second, the gradual phase of spine enlargement induced by synaptic stimulation is dependent on protein synthesis and BDNF action (Tanaka et al., 2008).
This model predicts that selective inhibition of dendritic BDNF synthesis would decrease levels of released proBDNF and therefore extracellular levels of mature- and/or proBDNF. This decreased pool of extracellular proBDNF would impair expression of both LTP and LTD depending upon the type of incident stimulation; HFS or LFS respectively. Furthermore, dendritic spines would display a reduction in size and an increase in number; the phenotypes that An et al. observed in mice lacking local BDNF synthesis (An et al., 2008)
Transcripts encoding BDNF and TrkB full length (TrkB-FL) and truncated (TrkB-T) undergo alternative polyadenylation generating two mRNA species with identical coding sequences but different length 3’UTRs. The short form of BDNF mRNA is restricted to the soma, however the long form is targeted to the dendrites (An et al., 2008). Although not demonstrated, it is possible that this same sub-cellular localization mechanism is utilized for TrkB mRNA as well.
Figure 2. Mechanisms of BDNF Induced Synaptic Plasticity.

In the non-stimulated state, dendritic spines remain “silent”: mRNAs are repressed in RNA granules, pro-BDNF, tpa, and AMPA receptors are held intracellularly, and actin dynamics leave the spine head in an immature form. Following synaptic activation, repressed mRNAs are disinhibited, TrkB is inserted into the plasma membrane, pro-BDNF and tpa are packaged and released into the synaptic cleft, pro-BDNF is converted into BDNF by Plasmin, and BDNF binds to TrkB on the local dendritic membrane. Activation of TrkB by BDNF increases translation of CaMKII, GluR1, Arc, and LimK1, leading to increased AMPA receptor formation (GluR1) and membrane insertion (CaMKII) and increased actin polymerization (Arc and LimK1). TrkB signaling also induces phosphorylation of NMDA receptors, synapsin-1, and p21 activated kinase and Cofilin, thus increasing receptor activity, vesicle-plasma membrane fusion and neurotransmitter release, and polymerization of actin respectively.
Chapter Two: Research aims and hypotheses

Role of dendritic BDNF synthesis in adult neurogenesis

Adult neurogenesis in the dentate gyrus of the hippocampus has been found to generate new neurons throughout life. This process has been shown to be regulated by BDNF, but a clear picture of the regulatory mechanisms has remained elusive due to conflicting reports of BDNF's effects. In particular, previous research had not considered a role for dendritic BDNF protein synthesis in adult neurogenesis. We postulated that dendritic synthesis of BDNF might be regulating adult neurogenesis and might help to explain the disparate findings in the field.

Aim 1: To investigate what stages of hippocampal adult neurogenesis are regulated by dendritic BDNF synthesis.

Aim 2: To establish a possible mechanism by which dendritic BDNF synthesis controls hippocampal adult neurogenesis.

Effect of human SNPs in the Bdnf 3’UTR on dendritic mRNA trafficking and spine morphogenesis

A region in the Bdnf long 3’UTR has been shown to be necessary and sufficient to target Bdnf mRNA into the dendritic compartment for local protein synthesis. Without this isoform of Bdnf mRNA animals have spine dysmorphology resembling an immature neuronal spine phenotype. This is caused by deficits in spine pruning. The
human long *Bdnf* 3’UTR has seven SNPs. We theorized that one or more of these seven SNPs might influence *Bdnf* mRNA dendritic trafficking or spine morphogenesis.

*Aim 1:* To determine possible effects of SNPs in the human long 3’UTR *Bdnf* mRNA on dendritic mRNA trafficking.

*Aim 2:* To determine possible effects of SNPs in the human long 3’UTR *Bdnf* mRNA on spine morphogenesis.
Chapter Three: Activity-dependent dendritic synthesis of BDNF controls adult neurogenesis through parvalbumin interneurons

INTRODUCTION

Adult hippocampal neurogenesis generates new neurons in the subgranular zone (SGZ) of the mature dentate gyrus (DG) throughout life (Altman and Das, 1965; Cameron et al., 1993; Eriksson et al., 1998; Gould et al., 1999; Kempermann et al., 1997; Kuhn et al., 1996; Seki and Arai, 1991). This process produces functionally integrated granule cells that participate in the existing hippocampal circuitry (Dupret et al., 2008; Ramirez-Amaya et al., 2006; van Praag et al., 2002; Zhang et al., 2008; Zhao et al., 2006). Approximately 6% of the granule cell population is generated each month (Cameron and McKay, 2001), without which specific deficits in spatial memory occur (Dupret et al., 2008; Zhang et al., 2008). While it is clear that hippocampal network activity regulates adult neurogenesis (Bengzon et al., 1997; Bernabeu and Sharp, 2000; Cameron et al., 1995; Ge et al., 2006; Malberg et al., 2000; Tozuka et al., 2005), the specific mechanism by which this regulation occurs has remained elusive. Mounting evidence supports two key players: the receptor for N-methyl-D-aspartic acid (NMDAR), and the neurotransmitter gamma-aminobutyric acid (GABA). Interestingly, application of an NMDAR agonist reduced precursor proliferation, while application of an NMDAR antagonist increased precursor proliferation (Cameron et al., 1995; Nacher et al., 2001). Similarly, ablation of the main excitatory input to the DG or blockade of
either the $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) or kainate receptor also resulted in increased proliferation of precursor cells (Bernabeu and Sharp, 2000; Cameron et al., 1995). These studies suggest that increased excitatory activity in the DG decreases the proliferative population, possibly due to increased differentiation and maturation of existing precursor cells. In support of this idea, some hippocampal-dependent learning tasks have been shown to decrease the number of proliferating cells (Dobrossy et al., 2003). Although precursor cells express NMDAR subunits (Kitayama et al., 2004; Nacher et al., 2007), newly generated neurons do not receive glutamatergic input until two weeks after birth (Tozuka et al., 2005), and do not show excitation following application of an AMPA or NMDA receptor agonist (Tozuka et al., 2005; Wang et al., 2005). It is therefore highly possible that the effect of glutamate on precursor cells is indirect, most likely directly acting on mature granule neurons. However, it is unknown how NMDAR activation of granule neurons affects precursor cells.

Like NMDAR activation, GABAergic stimulation also decreases the rate of proliferation (Chan et al., 2008; Tozuka et al., 2005). This effect, however, is directly mediated through GABA$_A$ receptors on precursor cells, and is due to GABA’s excitatory action on neuronal differentiation (Ge et al., 2006; Tozuka et al., 2005). Expression of the Na$^+$-K$^+$-2Cl$^-$ ion co-transporter on adult precursor cells results in GABA-mediated depolarization, increased expression of the transcription factor NeuroD, and neuronal differentiation (Liu et al., 2000; Miyata et al., 1999; Tozuka et al., 2005). GABA signaling therefore facilitates neuronal differentiation of precursor cells.
cells, thereby decreasing the size of the proliferative population. It is unclear, however, what the source of GABA is.

Brain-derived neurotrophic factor (BDNF) is a key regulator of NMDAR-mediated synaptic plasticity in the brain (Figurov et al., 1996; Kang and Schuman, 1995; Korte et al., 1995; Patterson et al., 1996). Furthermore, BDNF promotes maturation of GABAergic inhibitory networks (Hong et al., 2008; Huang et al., 1999). These observations suggest that BDNF may play a key role in the glutamate- and GABA-mediated effects on adult neurogenesis. Considerable evidence links BDNF and adult neurogenesis (Bergami et al., 2008; Chan et al., 2008; Lee et al., 2002a; Li et al., 2008; Sairanen et al., 2005; Scharfman et al., 2005); however, these studies have reported conflicting results. While exogenous BDNF injected into the murine hippocampus was reported to increase proliferation (Scharfman et al., 2005), Bdnf conditional knock-out mice lacking BDNF in mature neurons displayed increased proliferation as well (Chan et al., 2008). Reports of neurogenesis in Bdnf heterozygous mice have also been inconclusive, with some groups reporting increased proliferation (Sairanen et al., 2005) and others reporting decreased proliferation (Lee et al., 2002b). One explanation for the discrepant findings concerning the role of BDNF in adult neurogenesis might lie in the complex regulation of BDNF expression. For instance, the Bdnf gene is transcribed from multiple promoters (Aid et al., 2007), and each transcript is polyadenylated at one of two alternative sites, leading to distinct populations of mRNA species: those with a short 3′ untranslated region (3′ UTR) and those with a long 3′ UTR (Ghosh et al., 1994; Timmusk et al., 1993). It has been shown that short 3′ UTR
$Bdnf$ mRNA is restricted to the soma, whereas long 3′UTR $Bdnf$ mRNA is also targeted to dendrites for local translation (An et al., 2008).

The present studies investigated the relationship between dendritically synthesized BDNF, excitatory synaptic transmission, and GABA signaling with regard to their roles in adult neurogenesis. We found that an NMDAR agonist stimulated dendritic BDNF synthesis in cultured hippocampal neurons and that the effect of NMDAR antagonism on adult neurogenesis was abolished in mice lacking dendritic BDNF synthesis. In addition, in mice lacking dendritic BDNF synthesis, GABAergic innervation to the SGZ was reduced, and differentiation and maturation of SGZ precursor cells was impaired, which was rescued by administration of a GABA$_A$ agonist. Furthermore, we found similar neurogenesis deficits in mutant mice lacking the BDNF receptor, TrkB, on GABAergic PV interneurons. We have therefore established both a possible source of GABAergic input to the neurogenic niche, as well as a mechanism through which excitatory synaptic transmission and BDNF might regulate adult neurogenesis.

RESULTS

Long 3′UTR $Bdnf$ mRNA Regulates Adult Neurogenesis

Local translation of dendritic mRNAs is essential for lasting synaptic plasticity in hippocampal slices (Huber et al., 2000; Kang and Schuman, 1996) and in *Aplysia* neural circuits in culture (Martin et al., 1997; Wang et al., 2009); however, it is unknown what effect local protein synthesis has on adult neurogenesis. The $Bdnf$ long 3′UTR is
necessary to localize \( Bdnf \) mRNA to the dendrites of hippocampal neurons (An et al., 2008). \( Bdnf^{klox/klox} \) mice lack dentritic \( Bdnf \) mRNA due to a truncation of the long 3'UTR and thus are deficient in local dendritic BDNF synthesis (An et al., 2008). To determine if dentritic \( Bdnf \) mRNA is necessary for adult neurogenesis regulation, we first assessed proliferation in \( Bdnf^{klox/klox} \) mice by measuring the number of cells that incorporate the DNA synthesis marker 5-bromo-2'-deoxyuridine (BrdU). Mice were injected with BrdU intraperitoneally (IP) at 6 weeks of age and euthanized at varying time intervals post injection; including 1 hour, 24 hours, 3 days, 1 week, 3 weeks, 6 weeks, or 12 weeks. We were surprised to find that 24 hours following BrdU administration \( Bdnf^{klox/klox} \) mice had a 1.7 fold increase in the density of BrdU-labeled cells in the DG compared to WT controls (Figures 3A and 3B). In addition, \( Bdnf^{klox/klox} \) mice had a modest but significant increase in BrdU-labeled cells at the 1 hour time point compared to WT controls (Figure 3B). However, \( Bdnf^{klox/klox} \) mice showed no significant differences in the number of BrdU-labeled cells at all other time points analyzed, from 3 days to 12 weeks (Figure 3B). These results indicate that over the course of 24 hours \( Bdnf^{klox/klox} \) mice were accumulating more BrdU-labeled cells compared to WT mice, and that the extra newly labeled BrdU-positive cells present at 24 hours were dying off by 1 week post labeling. To test if the DG proliferative population was expanded in \( Bdnf^{klox/klox} \) mice, we counted the number of cells positive for Ki-67, an endogenous marker for actively cycling cells, and found that \( Bdnf^{klox/klox} \) mice did not have a significantly expanded proliferative population compared to WT controls (Figures 4A and 4B). In addition, we found no significant difference in granule
cell layer thickness between WT and $Bdnf^{kloxfloxclo}$ mice (Figures 4C and 4D), indicating that the increase of newborn cells at the early time points was temporary and ultimately did not result in a thicker DG.

Previous groups investigating adult neurogenesis in $Bdnf$ heterozygous mice have reported conflicting findings, with one study demonstrating a small but significant increase in proliferation (Sairanen et al., 2005) and another study demonstrating a small but significant decrease in proliferation (Lee et al., 2002b). Although these reports are conflicting, they do demonstrate that alterations in total levels of BDNF can modulate proliferation in the DG. We therefore investigated whether our finding of a transient increase in precursor cells might result from reduced $Bdnf$ expression in the DG of $Bdnf^{kloxfloxclo}$ mice. To explore this possibility we conducted radioactive in situ hybridization on brain sections from WT and $Bdnf^{kloxfloxclo}$ mice using a probe against the $Bdnf$ coding region. This experiment revealed only a modest decrease in levels of DG $Bdnf$ mRNA in $Bdnf^{kloxfloxclo}$ mice (Figures 3D and 3E). This result is largely in agreement with the previous observation that $Bdnf$ mRNA levels are normal in the $Bdnf^{kloxfloxclo}$ hippocampus, as revealed by Northern hybridization (An et al., 2008). Because it is difficult to dissect the DG, we measured the concentration of BDNF in whole hippocampal lysates using ELISA, which revealed that $Bdnf^{kloxfloxclo}$ mice had a 48% reduction of total hippocampal BDNF compared to WT mice (Figure 3C). These observations indicate that $Bdnf^{kloxfloxclo}$ mice exhibit reduced BDNF levels in addition to the lack of dendritic $Bdnf$ mRNA in the hippocampus.
In order to determine whether the neurogenesis deficit observed in $Bdnf^{lox/lox}$ mice is due to a lack of dendritic $Bdnf$ mRNA or a global reduction in BDNF protein levels, we conducted a BrdU labeling study of $Bdnf$ heterozygous mice ($Bdnf^{+/-}$), which carry only one copy of the functional $Bdnf$ allele and should express half the amount of total BDNF. Unlike $Bdnf^{lox/lox}$ mice, $Bdnf^{+/-}$ mice still have long 3'UTR $Bdnf$ mRNA, and therefore retain pools of dendritic $Bdnf$ mRNA. We found that 24 hours after BrdU administration, $Bdnf^{+/-}$ mice did not show significant increases in BrdU-labeled cells compared to WT mice, but $Bdnf^{lox/lox}$ mice did (Figure 3F). Collectively, these observations reveal that the lack of dendritic $Bdnf$ mRNA, and not the reduction of total BDNF, resulted in the drastic increase in numbers of BrdU-labeled cells in the first 24 hours after BrdU administration in $Bdnf^{lox/lox}$ mice.
Figure 3. Long 3′UTR Bdnf mRNA Regulates Adult Neurogenesis

(A) Representative images of BrdU immunostaining in the dentate gyrus 24 hours post BrdU injection of 6-week-old WT (+/+) and Bdnf<sup>lox/lox</sup> (k/k) mice. Scale bar, 50 µm.

(B) Quantification of the density of BrdU-immunoreactive cells in the dentate gyrus of +/+ and k/k mice. Mice were treated with BrdU at 6 weeks of age and euthanized at varying time intervals post BrdU administration, from 1 hour to 12 weeks (n=4 mice per time-point, per genotype). Error bars indicate standard errors.

(C) ELISA analysis of hippocampal BDNF levels in 6-week-old +/+ and k/k mice (n=5 mice per genotype). Error bars indicate standard errors.

(D) Representative images of radioactive in situ hybridization of +/+ and k/k coronal brain sections.

(E) Quantification of radioactive in situ hybridization signal in the granule cell layer of the dentate gyrus of +/+ and k/k mice (n=4 mice per genotype). Error bars indicate standard errors.

(F) Quantification of the density of BrdU-immunoreactive cells in the dentate gyrus of +/+, Bdnf<sup>+/−</sup> (+/−), and k/k mice. Mice were treated with BrdU at 6 weeks of age and euthanized 24 hours post injection. Error bars indicate standard errors.
**Figure 4. Bdnf^lox/lox^ Mice Have Normal Precursor Pool Size and Granule Cell Layer Thickness**

(A) Representative images of Ki-67 immunostaining in the DG of 6-week-old WT (+/+) and Bdnf^lox/lox^ (k/k) mice. Scale bar, 50 µm.
(B) Quantification of Ki-67^+^ cells in the DG of 6-week-old +/+ and k/k mice. Error bars indicate standard errors.
(C) Representative images of Nissl-stained dentate gyri in +/+ and k/k mice. Black bars represent the thickness of the granule cell layer. Scale bars, 50 µm.
(D) Quantification of DG granule cell layer thickness in +/+ and k/k mice. Error bars indicate standard errors.
Long 3'UTR Bdnf mRNA Promotes Neuronal Differentiation

Our finding that Bdnf<sup>klox/klox</sup> mice had a temporarily expanded pool of BrdU-labeled cells, which increased in magnitude from 1 hour to 24 hours post BrdU administration, indicates that dendritic Bdnf mRNA is necessary for neuronal differentiation. We reasoned that if proliferative cells were not receiving proper neuronal differentiation signals, they would stay in the cell cycle longer and the BrdU-labeled population would expand. To determine if Bdnf<sup>klox/klox</sup> mice have differentiation deficits, we first evaluated cell-cycle exit of BrdU-labeled cells 3 days post BrdU labeling in the DG. Cells that remained in the cell cycle following that period would still express Ki-67 (Ki-67<sup>+</sup>BrdU<sup>+</sup>); however, cells that had exited the cell cycle would retain their BrdU labeling, but lose Ki-67 expression (BrdU<sup>+</sup>). We found that Bdnf<sup>klox/klox</sup> mice did have impaired cell-cycle exit, such that a significantly higher percentage of cells that were BrdU positive, also stained for Ki-67 in Bdnf<sup>klox/klox</sup> mice compared with WT controls (Figures 5A and 5B).

To further investigate neuronal differentiation deficits in Bdnf<sup>klox/klox</sup> mice, we used a neuronal differentiation index. Three days after BrdU administration we colocalized BrdU and the neuronal marker, NeuN in the DG. If cells differentiated out of the cell cycle and into the neuronal phenotype, they would co-stain for both NeuN and BrdU (NeuN<sup>+</sup>BrdU<sup>+</sup>); however, if cells had not attained the neuronal phenotype, they would only contain BrdU (BrdU<sup>+</sup>). We found that Bdnf<sup>klox/klox</sup> mice had a significantly lower percentage of BrdU positive cells that also stained for NeuN.
(Figures 5C and 5D), indicating that $\text{Bdnf}^{\text{klox/klox}}$ mice display deficits in neuronal differentiation.

Finally, we assessed differentiation deficits using the neuronal differentiation marker NeuroD. Upon excitation of precursor cells, calcium signaling stimulates expression of NeuroD (Deisseroth et al., 2004), which is required for the differentiation of precursor cells into granule neurons (Liu et al., 2000). Twenty-four hours after BrdU administration, we co-localized BrdU and NeuroD in the DG of WT and $\text{Bdnf}^{\text{klox/klox}}$ mice. Precursor cells that had received NeuroD differentiation signaling would contain both NeuroD and BrdU (NeuroD$^+$BrdU$^+$), whereas cells that had not would only be labeled by BrdU (BrdU$^+$). We found a significantly lower percentage of BrdU positive cells that also stained for NeuroD in $\text{Bdnf}^{\text{klox/klox}}$ mice compared with WT controls (Figures 5E and 5F), indicating impaired differentiation signaling in $\text{Bdnf}^{\text{klox/klox}}$ mice. Collectively, these data argue that dendritic $\text{Bdnf}$ mRNA is necessary for precursor cell differentiation out of the cell cycle and into the neuronal phenotype.

Because $\text{Bdnf}^{\text{klox/klox}}$ mice did not show expanded BrdU labeling 1-12 weeks post BrdU administration (Figure 3B), and did not show increased granule cell layer thickness (Figures 4C and 4D), we sought to determine if apoptosis was occurring at higher rates in $\text{Bdnf}^{\text{klox/klox}}$ mice. We stained $\text{Bdnf}^{\text{klox/klox}}$ and WT sections from 7 week old mice with an antibody against the apoptotic marker cleaved caspase-3 (CC3) and with cresyl violet to reveal the density of CC3 positive cells in the SGZ (Figure 5G). $\text{Bdnf}^{\text{klox/klox}}$ mice had an increased density of CC3 positive cells in the SGZ (Figure 5H), indicating increased apoptosis.
Figure 5. Long 3’UTR Bdnf mRNA Promotes Neuronal Differentiation

(A) Representative confocal images of BrdU colocalized with Ki-67 in WT (+/+) and Bdnf<sup>klox</sup>klox (k/k) mice. Mice were treated with BrdU at 6 weeks of age and euthanized 3 days post injection for immunohistochemistry.
(B) Quantification of BrdU and Ki-67 colocalization. The graph denotes the percentage of BrdU<sup>+</sup> cells that also express Ki-67 (n=4 mice per genotype). Error bars indicate standard errors.
(C) Representative confocal images of BrdU colocalized with NeuN in +/+ and k/k mice. Mice were treated with BrdU at 6 weeks of age and euthanized 3 days post injection for immunostaining.

(D) Quantification of BrdU and NeuN colocalization. The graph denotes the percentage of BrdU⁺ cells that also express NeuN (n=4 mice per genotype). Error bars indicate standard errors.

(E) Representative confocal images of BrdU colocalized with NeuroD in +/+ and k/k mice. Mice were treated with BrdU at 6 weeks of age and euthanized 24 hours post injection for immunostaining. Scale bar, 25 µm.

(F) Quantification of BrdU and NeuroD colocalization. The graph denotes the percentage of BrdU⁺ cells that also express NeuroD (n=4 mice per genotype). Error bars indicate standard errors.

(G) Representative images of sections that were immunostained for cleaved caspase-3 (CC3) and counterstained with Nissl in +/+ and k/k mice. Scale bars, 25 µm.

(H) Quantification of the density of CC3-positive cells in the SGZ of the dentate gyrus of +/+ and k/k mice. SGZ was defined as cells in the first two cell layers of the granule cell layer adjacent to the hilus. Error bars indicate standard errors.
Long 3'UTR Bdnf mRNA Facilitates Neuronal Maturation and Integration

To determine if dendritic Bdnf mRNA is required for normal maturation and integration of granule cells into the DG, we administered BrdU to WT and Bdnf<sup>klox/klox</sup> mice and waited 6 weeks to allow the newly labeled cells to mature. Sections were then stained with antibodies to BrdU and to one of four markers identifying different stages of granule cell maturation: (1) the microtubule-associated protein doublecortin (DCX), which is expressed early in maturation and marks immature neurons (Francis et al., 1999; Gleeson et al., 1999); (2) NeuN, which is a soluble nuclear protein and expressed in post-mitotic neuroblasts and mature neurons (Ming and Song, 2005); (3) calretinin, which is a calcium binding protein and expressed transiently by immature neurons after DCX expression (Brandt et al., 2003); and, (4) calbindin, which is also a calcium binding protein, is expressed after calretinin, and marks mature granule neurons (Brandt et al., 2003). We found that Bdnf<sup>klox/klox</sup> mice showed increased co-localization of BrdU with the immature neuronal marker DCX and decreased colocalization with the intermediate neuronal marker calretinin and the mature neuronal marker calbindin (Figures 6A and 6B). We saw no difference in colocalization of BrdU with NeuN (Figures 6A and 6B), most likely because this population of neurons encompasses some cells expressing DCX and all cells expressing calretinin and calbindin. In a separate experiment, we wanted to determine if dendritic BDNF promotes maturation of glial cells in the DG. We found that 6 weeks after BrdU labeling, significantly fewer BrdU positive cells in Bdnf<sup>klox/klox</sup> mice expressed the glial cell marker glial fibrillary acidic
protein (GFAP) (Figures 7A and 7B). Taken together, these experiments indicate that dendritic \textit{Bdnf} mRNA facilitates both neuronal and glial maturation in the DG.

Late-phase long-term potentiation (L-LTP) and memory consolidation require expression of immediate early genes (Bozon et al., 2002; Guzowski et al., 2000). Two such genes, \textit{Arc} and \textit{Zif268}, are induced by activity in newly generated adult-born DG neurons (Bruel-Jungerman et al., 2006; Kee et al., 2007). In order to investigate if adult-born DG neurons in \textit{Bdnf}^{\text{klox/klox}} mice show integrational deficits, we used seizure induction to activate the expression of \textit{Arc} and \textit{Zif268} in BrdU-labeled DG neurons from WT and \textit{Bdnf}^{\text{klox/klox}} mice (Cole et al., 1990; Snyder et al., 2009). Mice were injected with BrdU at 4 weeks of age, and left for 4 additional weeks to allow the newly labeled precursor cells to mature and integrate. At 8 weeks of age, grade 4-5 seizures were induced using kainate, and animals were euthanized two hours after seizure induction. BrdU-labeled cells that had integrated into the hippocampal circuitry would be more readily activated by this stimulation. Cells that showed strong network integration would contain BrdU and either Arc or Zif268 protein expression (BrdU$^+$Arc$^+$ or BrdU$^+$Zif268$^+$), while cells that had not strongly integrated would only immunostain for BrdU (BrdU$^+$). We found that \textit{Bdnf}^{\text{klox/klox}} mice had significantly lower levels of colocalization between Arc and BrdU compared WT controls (Figures 6C and 6D), demonstrating that fewer adult-born cells had integrated into the hippocampal network in \textit{Bdnf}^{\text{klox/klox}} mice. In addition, although not significant, we saw a trend toward decreased colocalization between Zif268 and BrdU (Figures 8A and 8B). This integrational deficit appears to be exclusive to adult-born neurons, because the overall
density of Arc and Zif268 positive cells in the DG was not significantly different in WT and $Bdnf^{K\text{lox}/K\text{lox}}$ mice (Figures 8C-8F). Collectively, these findings demonstrate that dendritic $Bdnf$ mRNA facilitates maturation and integration of adult-born neurons.
Figure 6. Long 3'UTR Bdnf mRNA Facilitates Neuronal Maturation and Integration

(A) Representative merged confocal images depicting colocalization of BrdU with doublecortin (DCX), NeuN, calretinin, or calbindin in WT (+/+) and Bdnf<sup>flox/flox</sup> (k/k) mice. Mice were treated with BrdU at 6 weeks of age and euthanized 6 weeks post BrdU injection for immunostaining. Scale bar, 50 µm.

(B) Percentage of BrdU<sup>+</sup> cells that were immunoreactive to DCX, NeuN, calretinin, or calbindin 6 weeks post BrdU injection (n=4 mice per genotype). Error bars indicate standard errors.

(C) Representative confocal images of activity-dependent Arc expression in adult-born cells. Mice were treated with BrdU at 4 weeks of age. Four weeks post BrdU injection, kainate (35 mg/kg IP) was used to induce grade 4-5 seizures. Two hours post seizure induction, animals were euthanized for immunohistochemistry.

(D) Percentage of BrdU<sup>+</sup> cells that also expressed Arc 4 weeks post BrdU injection and 2 hours post seizure induction (n=3 mice for +/+ and n=4 mice for k/k). Scale bar, 50 µm.
Figure 7. BDNF Derived from Long 3’UTR Bdnf mRNA Promotes Maturation of Glial Cells in the DG

(A) Representative confocal images of BrdU (red) colocalized with GFAP (green) in WT (+/+) and Bdnf<sub>klox/klox</sub> (k/k) mice. Mice were treated with BrdU at 6 weeks of age and euthanized 6 weeks post BrdU injection. Scale bars, 50 µm.

(B) Percentage of BrdU<sup>+</sup> cells that expressed GFAP 6 weeks post BrdU injection (n=4 mice per genotype). Error bars indicate standard errors.
Figure 8. Induction of Zif268 and ArC Is Normal in the DG of Bdnf\textsuperscript{klox/klox} Mice

(A) Representative confocal images of colocalization of BrdU with Zif268 in the dentate gyrus of WT (+/+) and Bdnf\textsuperscript{klox/klox} (k/k) mice. Mice were treated with BrdU at 4 weeks of age. Four weeks post BrdU injection, kainate was used to induce grade 4-5 seizures. Two hours post seizure induction animals were euthanized for immunostaining. Scale bar, 50 µm.

(B) Percentage of BrdU\textsuperscript{+} cells that expressed Zif268 four weeks post BrdU injection and 2 hours post seizure induction (n=3 mice for +/+ and n=4 mice for k/k). Error bars indicate standard errors.

(C) Representative images of Zif268 immunohistochemistry in the DG of WT (+/+) and Bdnf\textsuperscript{klox/klox} (k/k) mice. Mice were treated as described in (A). Scale bar, 50 µm.

(D) Density of Zif268\textsuperscript{+} cells in the DG of +/+ and k/k mice. Error bars indicate standard errors.

(E) Representative images of Arc immunohistochemistry in the DG of +/+ and k/k. Mice were treated as described in (A). Scale bar, 50 µm.

(F) Density of Arc\textsuperscript{+} cells in the DG of +/+ and k/k mice. Error bars indicate standard errors.
Activation of the NMDA Receptor Modulates Adult Neurogenesis by Stimulating BDNF Synthesis

It is known that activation of the NMDA receptor regulates adult neurogenesis (Cameron et al., 1995; Nacher et al., 2001); however, the specific mechanism has remained unclear. Although precursors express NMDA receptor subunits (Kitayama et al., 2004; Nacher et al., 2007), newly generated neurons do not receive glutamatergic input until 2 weeks after birth (Tozuka et al., 2005), and do not show excitation following application of AMPA or NMDA receptor agonists (Tozuka et al., 2005; Wang et al., 2005). These findings suggest that the effect of NMDAR activation on this precursor population is indirect. We hypothesized that NMDAR activation might be stimulating dendritic synthesis of BDNF to regulate adult neurogenesis. To test this, we utilized an in vitro local protein synthesis reporter assay based on green fluorescent protein (GFP) expression. Myr-d1GFP-nls reporter constructs used in this study contain four key elements: (1) a sequence encoding a myristoylation peptide (myr) that brings the GFP fusion protein to the plasma membrane, (2) a nuclear localization signal (nls) at the C-terminus that further restricts protein diffusion, (3) a sequence encoding a destabilized green fluorescent protein (GFP) with a half life of 1 hour, and (4) a genomic sequence encoding either the Bdnf long 3′UTR (myr-d1GFP-A*B) or short 3′UTR (myr-d1GFP-A). Once this construct is expressed, GFP will attach to the membrane or move into the nucleus to restrict movement, while destabilized GFP will be degraded quickly, thus reporting the site at which mRNA from the construct is translated. This strategy has been successfully employed to examine local synthesis of
CaMKIIα and LIMK1 in dendrites (Aakalu et al., 2001; Schratt et al., 2006). Cultured hippocampal neurons were transfected with either myr-d1GFP-A*B or myr-d1GFP-A on DIV14. One day later, neurons were treated with either vehicle or 50 µM NMDA for 1 hour before fixation, and then analyzed for GFP signal intensity along the main dendrite. We had previously established that long 3′UTR Bdnf mRNA is trafficked to the dendrites and short 3′UTR Bdnf mRNA is restricted to the soma (An et al., 2008), thus we expected to see dendritic changes in local synthesis only in neurons transfected with the long 3′UTR construct. We found that NMDA significantly increased GFP signal intensity along the dendrites of neurons transfected with myr-d1GFP-A*B but not myr-d1GFP-A (Figures 9A-9B). Thus, NMDA stimulates dendritic local synthesis of BDNF in hippocampal neurons.

To further test our hypothesis that NMDAR activation stimulates dendritic synthesis of BDNF to regulate adult neurogenesis we next examined an in vivo model. Previous studies have shown that NMDAR antagonism resulted in increased numbers of BrdU-labeled cells in the DG (Cameron et al., 1995; Nacher et al., 2001). We reasoned that if dendritic synthesis of BDNF is necessary for NMDAR modulation of adult neurogenesis, than mice in which dendritic synthesis of BDNF is abolished (Bdnf<sup>lox/lox</sup> mice) should not respond, or have a diminished response to, NMDAR antagonism. To test this, we injected 6 week old WT and Bdnf<sup>lox/lox</sup> mice with the competitive NMDAR antagonist CGP37849. Two days after CGP37849 administration, we administered BrdU and euthanized the animals 1 hour after the last BrdU injection. WT mice showed a robust and significant increase in BrdU labeled cells in the DG after
NMDAR antagonism; however, this effect was abolished in $Bdnf^{\text{lox/lox}}$ mice (Figures 9C and 9D). Taken together, we conclude that NMDAR activation stimulates dendritic synthesis of BDNF and that dendritically synthesized BDNF is necessary for NMDAR’s modulation of adult neurogenesis.
Figure 9. NMDA Receptor Activation Stimulates Dendritic BDNF Synthesis and Adult Neurogenesis

(A) Whole cell images of cultured rat hippocampal neurons expressing either myr-d1GFP-A or myr-d1GFP-A*B. Neurons were transfected at DIV14 and treated with vehicle (control) or 50 μm NMDA (NMDA) at DIV15 for one hour and fixed for analysis. Scale bar, 50 μm.

(B) Quantification of myr-d1GFP fluorescence in dendrites. Fluorescence intensities on distal dendrites (100-150 μm and 150-200 μm away from somata) were measured and normalized to control levels (n=24 for A control, n=22 for A NMDA, n=22 for A*B control, and n=25 for A*B NMDA). Error bars indicate standard errors.

(C) Representative images of BrdU immunostaining in the dentate gyrus of WT (+/+) and Bdnf<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> (k/k) mice treated with either saline (control) or the NMDA antagonist CGP37849 (CGP, 5 mg/kg IP). Scale bar, 100 μm.

(D) Quantification of the density of BrdU positive cells in the dentate gyrus of +/- or k/k mice treated with saline (control) or CGP37849 (CGP) (n=7 for +/- control, n=7 for +/- CGP, n=4 for k/k control, n=4 for k/k CGP). Error bars indicate standard errors.
Long 3′UTR Bdnf mRNA Is Required for GABAergic Innervation

GABAergic depolarization of precursor cells regulates neuronal differentiation (Tozuka et al., 2005), maturation, and integration (Ge et al., 2006). Importantly, BDNF has been shown to promote maturation of GABAergic circuitry (Huang et al., 1999). Furthermore, BDNF has been found to facilitate the effects of GABA on adult neurogenesis (Chan et al., 2008). Based on this previous work, we reasoned that the deficits in adult neurogenesis in Bdnf<sup>klox/klox</sup> mice might be due to altered GABAergic input to the precursor cell population. In order to investigate this possibility, we immunostained brain sections from 7 week old WT and Bdnf<sup>klox/klox</sup> mice with antibodies to the presynaptic GABA synthesizing enzyme, glutamic acid decarboxylase 65 (GAD65), or the presynaptic vesicular glutamate transporter 1, VGLUT1. We found that Bdnf<sup>klox/klox</sup> mice had decreased GAD65 staining intensity (Figures 10A and 10B), but normal VGLUT1 staining intensity in the SGZ compared to WT controls (Figures 10C and 10D), indicating that without dendritic Bdnf mRNA there is deficient GABAergic innervation, but normal glutamatergic innervation to the SGZ. In order to determine if the decrease in GAD65 staining intensity in Bdnf<sup>klox/klox</sup> mice is due to a decrease in the number of GABAergic interneurons, we immunostained brain sections from WT and Bdnf<sup>klox/klox</sup> mice with antibodies to the interneuron markers parvalbumin (PV), somatostatin (SOM), or neuropeptide Y (NPY). We found no significant difference in the density of PV (Figures 10E and 10F), SOM (Figures 10G and 10H), or NPY (Figures 10I and 10J) positive cells in the DG. These data suggest that dendritic
*Bdnf* mRNA regulates GABAergic innervation of the SGZ, but not interneuron cell number.
Figure 10. GABAergic Innervation Is Impaired in Bdnf<sup>klox/klox</sup> Mice

(A) Representative confocal images of GAD65 immunostaining in the DG in WT (+/+) and Bdnf<sup>klox/klox</sup> (k/k) mice.
(B) Quantification of GAD65 immunostaining signal intensity in the SGZ of +/+ and k/k mice (n=5 mice per genotype). Error bars indicate standard errors.
(C) Representative confocal images of VGLUT1 immunostaining in the DG of +/+ and k/k mice. Scale bars, 50 µm.
(D) Quantification of SGZ VGLUT1 immunostaining signal intensity in +/+ and k/k mice (n=5 per genotype). Error bars indicate standard errors.
(E-J) Long 3′UTR Bdnf mRNA does not regulate the number of GABAergic interneurons. Representative images from immunohistochemistry against parvalbumin (PV; panel E), somatostatin (SOM; panel G), and neuropeptide Y (NPY; panel I) in 6-week-old +/+ and k/k mice. (F, H, J) Normal cell density was found for interneurons expressing PV, SOM, or NPY in the dentate gyrus of 6-week-old k/k mice (n=4 mice per genotype). Error bars indicate standard errors. Scale bars, 50 µm.
**GABA\(\text{A}\) Receptor Agonist Rescues Neurogenesis Deficits in \(Bdnf^{\text{klox/klox}}\) Mice**

GABA\(\text{A}\) receptor signaling stimulates DG precursor differentiation into neurons via NeuroD induction (Tozuka et al., 2005). We found that dendritic \(Bdnf\) mRNA is required for precursor differentiation and for GABAergic innervation of the SGZ (Figures 5 and 10). We therefore reasoned that decreased GABAergic signaling to the precursor population might be the cause of the deficits in neuronal differentiation in \(Bdnf^{\text{klox/klox}}\) mice. To test this, we attempted to rescue the neurogenesis deficits in \(Bdnf^{\text{klox/klox}}\) mice using the GABA\(\text{A}\) receptor agonist, phenobarbital. Based on the protocol established by Tozuka et al. (2005), we gave 6-week-old WT and \(Bdnf^{\text{klox/klox}}\) mice phenobarbital systemically once a day for three days, and on the fourth day administered BrdU. Twenty-four hours post BrdU administration the animals were euthanized, and their brains were prepared for immunohistochemistry with antibodies to BrdU and NeuroD. Without phenobarbital, \(Bdnf^{\text{klox/klox}}\) mice had a decreased percentage of BrdU-labeled cells that also expressed NeuroD (BrdU\(^+\)NeuroD\(^+\)), indicating impaired neuronal differentiation. However, phenobarbital administration restored WT levels of NeuroD and BrdU colocalization in \(Bdnf^{\text{klox/klox}}\) mice (Figures 11A and 11B), demonstrating that activation of the GABA\(\text{A}\) receptor is sufficient to rescue the impaired differentiation phenotype observed in \(Bdnf^{\text{klox/klox}}\) mice.

We originally hypothesized that the reason we saw an expansion of the BrdU-labeled pool of cells 24 hours post BrdU administration in \(Bdnf^{\text{klox/klox}}\) mice was due to deficits in neuronal differentiation and therefore a temporary increase in the number of dividing cells. If this is true, then rescuing the impaired differentiation phenotype via
GABA_A agonism should also reduce 24-hour BrdU-labeled cell levels to those found in WT mice. Indeed, we found that phenobarbital administration reduced the density of BrdU-labeled cells 24 hours post BrdU administration to WT levels (Figures 11C and 11D). We conclude that dendritic Bdnf mRNA stimulates differentiation of precursor cells via GABA_A receptor signaling.
Figure 11. GABA<sub>A</sub> Receptor Agonist Rescues Neurogenesis Deficits in Bdnf<sup>klox/klox</sup> mice

(A) Representative confocal images of immunostaining against BrdU and NeuroD in 6-week-old WT (+/+) and Bdnf<sup>klox/klox</sup> (k/k) mice, either untreated or treated with phenobarbital. Treated mice were given phenobarbital once a day for three days. On the fourth day, treated and untreated mice were given BrdU injections. Mice were euthanized 24 hours post BrdU injections. Scale bar, 50 µm.

(B) Quantification of colocalization of BrdU with NeuroD in +/+ and k/k mice, either untreated or treated with phenobarbital (n=4 mice for untreated groups per genotype; n=3 mice for treated groups per genotype). Error bars indicate standard errors.

(C) Representative images of BrdU immunostaining in either treated or untreated 6-week-old +/+ or k/k mice. Mice were treated as described in (A). Scale bar, 50 µm.

(D) The density of BrdU<sup>+</sup> cells in the DG of untreated and phenobarbital-treated mice, 24 hours following the last BrdU injection (n=4 mice for untreated groups per genotype; n=3 mice for treated groups per genotype). Error bars indicate standard errors.
**Deletion of the TrkB Gene in PV Interneurons Causes Neurogenesis Deficits**

The DG has multiple subtypes of GABAergic interneurons, which have been classified based on morphology, electrophysiology, and expression of calcium-binding proteins and neuropeptides (Houser, 2007). PV interneurons of the DG comprise between 20-25% of the total DG interneuron population (Jinno and Kosaka, 2002). Since PV neurons form perisomatic GABAergic synapses (Freund, 2003) and their maturation and action potential firing are stimulated by BDNF (Berghuis et al., 2004; Huang et al., 1999), we hypothesized that PV neurons might provide the pathway through which BDNF synthesized in the dendrites of granule neurons promotes differentiation and maturation of neural precursor cells.

First we investigated whether TrkB is expressed in PV interneurons of the DG, and therefore could be stimulated by dendritic BDNF synthesis. We performed immunohistochemistry against parvalbumin and β-galactosidase on brain sections from TrkB<sup>LacZ</sup>/+ mice where expression of β-galactosidase is under the control of the TrkB promoter and thus recapitulates the expression pattern of the TrkB gene (Xu et al., 2000a), and found that 100% of parvalbumin positive neurons expressed TrkB (Figure 12A).

We employed BAC Parvalbumin-Cre (PV-Cre) mice (Tanahira et al., 2009) and floxed TrkB (fB) mice to selectively delete the TrkB gene in parvalbumin-expressing cells (Zheng et al., manuscript in preparation). We hypothesized that fB/fB;PV-Cre mutant mice would display an impaired neurogenesis phenotype similar to that found in Bdnf<sup>klox/klox</sup> mice. To investigate this possibility, we injected fB/fB (control) mice and
fB/fB;PV-Cre mice with BrdU and euthanized the animals 24 hours later for immunohistochemistry with antibodies to BrdU. Indeed, we found that similar to Bdnf<sup>lox/lox</sup> mice, fB/fB;PV-Cre mice had a 1.97 fold increase in BrdU-labeled cells compared to control mice (Figures 12B and 12C). In order to examine if TrkB deletion in PV neurons affects interneuron survival, we immunostained sections from fB/fB and fB/fB;PV-Cre mice with an antibody to PV to determine the density of PV neurons in the DG. We found no significant difference in the density of PV neurons between genotypes (Figures 12D and 12E), indicating that the demonstrated increase in BrdU-labeled cells is due to deficits in TrkB signaling in this population, and not due to decreased interneuron cell number. Taken together, these data suggest that dendritically synthesized BDNF acts on the TrkB receptor on PV interneurons to regulate adult neurogenesis in the DG.
Figure 12. Deletion of the TrkB Gene in Parvalbumin Neurons Causes Neurogenesis Deficits

(A) Confocal images showing colocalization of parvalbumin (PV) and TrkB in the dentate gyrus. Sections were taken from TrkB$^{LacZ/+}$ mice. Scale bar, 50 µm.

(B) Representative images of BrdU immunostaining in 6-week-old $fB/fB$ and $fB/fB;PV-Cre$ mice. Mice were treated with BrdU and euthanized 24 hours post BrdU injection. Scale bar, 50 µm.

(C) Quantification of BrdU$^+$ cells in the dentate gyrus of $fB/fB$ and $fB/fB;PV-Cre$ mice 24 hours post BrdU administration. Error bars indicate standard errors.

(D) Representative images of parvalbumin neurons in the dentate gyrus of $fB/fB$ and $fB/fB;PV-Cre$ mice. Scale bar, 50 µm.

(E) The density of parvalbumin interneurons in the dentate gyrus of $fB/fB$ and $fB/fB;PV-Cre$ mice. Error bars indicate standard errors.
DISCUSSION

It is known that excitatory cortical input to the hippocampus via the perforant pathway and GABA release from interneurons are both crucial for the proliferation, differentiation, and maturation of precursor cells in the dentate gyrus. However, the means by which glutamate regulates adult neurogenesis and the source of GABA have not been determined. In the set of experiments presented here we provide evidence that supports a model in which glutamate released from the perforant pathway binds to NMDARs to stimulate local translation of long 3′UTR Bdnf mRNA in the dendrites of granule neurons, which in turn promotes GABA release from PV interneurons to influence adult neurogenesis. We delineated this pathway by employing the unique Bdnf^klox/klox mouse mutant, in which local dendritic BDNF synthesis is abolished, in combination with an in vitro local protein synthesis assay and a conditional mouse mutant in which the TrkB gene is selectively deleted in PV interneurons.

Several recent studies have shown that BDNF regulates adult neurogenesis, but a clear understanding of how BDNF regulates adult neurogenesis has been difficult to determine. We believe that this difficulty stems from the dynamic and widespread expression of BDNF in many cell types found in the DG. We found that BDNF and its receptor TrkB are expressed in proliferating precursor cells (Ki-67^+), in immature neurons (DCX^+), and in mature and immature neurons (NeuN^+) (Figure 13). Furthermore, the Bdnf gene is transcribed from multiple promoters in response to developmental and environmental cues, and each of its transcripts can generate either short 3′UTR Bdnf mRNA or long 3′UTR Bdnf mRNA due to two alternative
polyadenylation sites (Timmusk et al., 1993), which enables the post-transcriptional regulation of BDNF expression in different subcellular compartments (An et al., 2008; Lau et al., 2010). Thus far, studies investigating the role of BDNF in adult neurogenesis have not addressed this important aspect of BDNF regulation. It is highly likely that BDNF acts in many ways to regulate adult neurogenesis, dependent upon cell type and BDNF regulatory mechanisms. Dividing cells, immature neurons, and mature neurons have distinct morphologies and are in distinct niches, and therefore likely regulate BDNF expression differently. Because proliferating precursor cells and undifferentiated post-mitotic cells in the SGZ do not have a dendritic compartment, a lack of local BDNF synthesis should not impact BDNF expression in these cells. In fact, we theorize that this is the reason why we did not observe decreased proliferation of precursor cells and reduced DG thickness in Bdnf<sup>flox/flox</sup> mice, which was reported in mutant mice where the Bdnf or TrkB gene was deleted in precursor cells (Li et al., 2008). This discrepancy illustrates that depending on cell type and microenvironment, BDNF appears to regulate adult neurogenesis in different ways. Furthermore, this suggests that total BDNF expression in precursor cells is necessary for proliferation of precursor cells and maintenance of DG volume (Li et al., 2008), whereas BDNF synthesized in the dendrites of mature neurons regulates differentiation and maturation of adult-born neurons. In agreement with this view, deletion of the Bdnf gene in mature or nearly mature neurons, using a Cre transgene under the control of the promoter for the α subunit of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II, also leads to an increase in
BrdU-labeled cells 1 day post BrdU injection, as well as impaired maturation of adult-born neurons (Chan et al., 2008).

*Bdnf*\textsuperscript{\textit{klox/klox}} mice have multiple SV40 polyadenylation sites inserted into the long *Bdnf* 3’UTR, and therefore have a truncated long 3’UTR and abolished dendritic synthesis of BDNF (An et al., 2008). In addition to this deficit in dendritic BDNF, *Bdnf*\textsuperscript{\textit{klox/klox}} mice also have half of total BDNF levels in the hippocampus compared to WT mice. We previously showed that these animals exhibit abnormal dendritic spines and deficits in long-term potentiation in the hippocampus. These defects may lead to alterations in neuronal activity, which would cause changes in the expression of BDNF in the hippocampus, as neuronal activity regulates *Bdnf* gene transcription (Hong et al., 2008) and *Bdnf* mRNA translation (Lau et al., 2010). Furthermore, loss of the long *Bdnf* 3’UTR should diminish activity-dependent translation of *Bdnf* mRNA in *Bdnf*\textsuperscript{\textit{klox/klox}} mice because activity stimulates translation of long 3’UTR *Bdnf* mRNA and suppresses translation of short 3’UTR *Bdnf* mRNA (Lau et al., 2010). We hypothesize that the combination of alterations in network activity and the loss of activity-dependent translation result in the large reduction of BDNF levels in the hippocampus of *Bdnf*\textsuperscript{\textit{klox/klox}} mice. The normal BDNF levels in *Bdnf*\textsuperscript{\textit{klox/klox}} mice, as demonstrated by Western blots in our previous study (An et al., 2008), may be due to the inclusion of nonspecific proteins at the proBDNF band. Even though *Bdnf*\textsuperscript{\textit{klox/klox}} mice have decreased total BDNF levels, the results reported here indicate that the neurogenesis deficits in *Bdnf*\textsuperscript{\textit{klox/klox}} mice result from a lack of local dendritic BDNF synthesis instead
of a global reduction in BDNF levels, because $Bdnf^{+/-}$ mice did not display the neurogenesis deficits that $Bdnf^{klo{x}/klo{x}}$ mice did.

Local protein synthesis is crucial for lasting synaptic plasticity in the hippocampus (Bramham and Wells, 2007; Waterhouse and Xu, 2009). Here we establish for the first time the connections between activity, local dendritic BDNF synthesis, and neurogenesis regulation. We found that mice lacking dendritic BDNF synthesis ($Bdnf^{klo{x}/klo{x}}$ mice) displayed increased proliferation, decreased differentiation, and decreased maturation of neural precursors. It has also been reported that blocking NMDARs in vivo leads to increased proliferation of precursors (Cameron et al., 1995; Nacher et al., 2001), indicating that NMDARs and dendritically synthesized BDNF might be working through the same mechanism to control neurogenesis. Using a local protein synthesis reporter assay, we were able to link these two phenomena by showing that NMDA stimulates local translation of $Bdnf$ mRNA in cultured hippocampal neurons. In strong support of this hypothesis, we were able to further show in vivo that without dendritic synthesis of BDNF, NMDAR antagonism cannot modulate adult neurogenesis. The connection between activity and local BDNF synthesis has already been established. Stimulation in vitro and in vivo has been shown to increase trafficking of $Bdnf$ mRNA from the somata to dendrites of hippocampal neurons (Tongiorgi et al., 1997), and our previous work demonstrated that dendritic pools of $Bdnf$ mRNA can be locally translated (An et al., 2008). Furthermore, synaptic activity can stimulate dendritic BDNF synthesis by modulating eukaryotic elongation factor 2 (Verpelli et al., 2010). Based on these previous studies and our results in the present study, it is likely
that one way in which activity regulates adult neurogenesis is by activating NMDAR on the dendrites of granule cells and stimulating local BDNF synthesis.

The neurogenic niche is a complex and poorly understood microenvironment, with regulatory factors coming from local and non-local origins (Suh et al., 2009). GABA is a modulator of precursor cells in the SGZ neurogenic niche (Ge et al., 2006; Tozuka et al., 2005); however, the origin of GABAergic innervation has not been established. It has been shown that GABA binding to GABA_A receptors located on precursor cells facilitates precursor differentiation and maturation into the neuronal phenotype (Ge et al., 2006; Tozuka et al., 2005). It has also been well established that BDNF regulates inhibitory synaptic transmission both \textit{in vitro} and \textit{in vivo}. \textit{In vitro}, BDNF has been shown to facilitate several aspects of inhibitory transmission, including GABA synthesis, neurite outgrowth, and synaptic inhibitory strength (Marty et al., 1996; Rutherford et al., 1997; Widmer and Hefti, 1994). \textit{In vivo}, BDNF has been shown to promote maturation of GABAergic inhibitory networks in the visual cortex (Huang et al., 1999), and without activity-dependent BDNF synthesis, cortical inhibitory networks show decreased spontaneous inhibitory events, decreased inhibitory synapses, and decreased expression of presynaptic inhibitory markers (Hong et al., 2008; Sakata et al., 2009). In the present study, we found that mice lacking dendritic BDNF synthesis displayed a similar phenotype to those treated with a GABA_A antagonist (Tozuka et al., 2005). This led us to hypothesize that local dendritic BDNF synthesis and release might be acting on GABAergic interneurons of the dentate gyrus, facilitating inhibitory synaptic transmission to the neurogenic niche, thus stimulating neuronal differentiation.
and maturation of precursor cells. In clear support of this hypothesis, we were able to rescue the differentiation deficits observed in \textit{Bdnf}^{loox/klox} mice with systemic application of a GABA\textsubscript{A} agonist. We further probed this hypothesis by investigating a possible GABAergic interneuron population that might be mediating this effect. By knocking down \textit{TrkB} selectively in PV-expressing interneurons, we were able to recapitulate the large increase in proliferation demonstrated in \textit{Bdnf}^{loox/klox} mice. This experiment provides evidence that TrkB stimulation of this specific interneuron population is at least partially responsible for the control of adult neurogenesis by dendritically synthesized BDNF, and furthermore establishes a likely source for GABAergic innervation to the neurogenic niche.

The cell bodies of PV interneurons predominantly reside at the base of the granule cell layer (GCL), with their dendrites ascending into the GCL and molecular layer (ML), and their axons terminating in the GCL and SGZ (Houser, 2007; Kneisler and Dingledine, 1995; Ribak et al., 1990). It is therefore possible that BDNF released from the dendrites of granule cells could bind to TrkB receptors on PV interneurons to promote dendritic growth and enhance the efficacy of glutamatergic synapses, and thus increase excitatory inputs into these interneurons. Additionally, TrkB activation on interneuron dendrites could start transcriptional programs in cell bodies and increase GABAergic innervation of neuronal precursors, as GABAergic innervation in the SGZ is reduced in \textit{Bdnf}^{loox/klox} mice. Since dendrites contain components necessary for endocytosis and retrograde transport (Kapitein et al., 2010; Kennedy and Ehlers, 2006), one way for dendritically synthesized BDNF to activate signaling cascades in cell
bodies might be through the transport of the BDNF-TrkB complex from dendrites to cell bodies, a mechanism similar to retrograde neurotrophic signaling in axons (Cosker et al., 2008). Both increased excitatory input and expanded GABAergic innervation would amplify GABA release from PV interneurons to the SGZ.

In conclusion, we have presented evidence that supports a model in which glutamate coming from the perforant path stimulates NMDA receptors on granule cells to facilitate local dendritic BDNF synthesis. Release of locally synthesized BDNF from granule cells may then stimulate TrkB receptors on the dendrites of PV-positive GABAergic interneurons to increase GABA input to neural precursors, thus stimulating their differentiation into neurons (Figure 14). To our knowledge, the data presented here provide the first evidence of local protein synthesis regulating adult neurogenesis. In addition, these data provide evidence for a mechanism by which the NMDAR may be indirectly modulating neurogenesis, and provide evidence for a possible population of interneurons that are the source of GABAergic stimulation of the neurogenic niche.
Figure 13. Expression of BDNF and TrkB in the Dentate Gyrus

(A-F) Representative images of BDNF and TrkB colocalized with the maturation markers Ki-67 (dividing cells), DCX (immature neurons), and NeuN (immature and mature neurons) in the dentate gyrus. *Bdnf* ^lacZ/+^ and *TrkB* ^lacZ/+^ sections were stained with antibodies against β-galactosidase and Ki-67 (A, D), DCX (B, E), or NeuN (C, F). Scale bar, 50 µm.

(G and H) Percentage of BDNF⁺ (G) or TrkB⁺ (H) cells that express Ki-67, DCX, or NeuN.
Figure 14. Schematic Representation of a Possible Mechanism by Which Dendritic BDNF Synthesis Controls Adult Neurogenesis

(A) Previously it was shown that NMDAR agonism and perforant path stimulation result in decreased size of the proliferative population; however, the mechanism through which this occurs was unknown. Previous studies also determined that GABA is a key regulator of adult neurogenesis, stimulating differentiation and maturation of precursors; however, the source of GABA to the neurogenic niche was not determined. Abbreviation: Glu, glutamate; PV, parvalbumin; ML, molecular layer; GCL, granule cell layer; SGZ, subgranular zone.

(B) Based on previous work and the data presented here we propose a possible mechanism by which local dendritic BDNF synthesis ties the actions of NMDA receptor activation and GABA release in the regulation of adult neurogenesis. Perforant path stimulation releases glutamate onto the NMDA receptor on granule cell dendrites. NMDA receptor stimulation induces dendritic synthesis of BDNF in granule neurons. Locally synthesized BDNF can then undergo activity-dependent release and bind to TrkB receptors on parvalbumin positive interneurons. TrkB signaling then enhances release of GABA into the neurogenic niche to promote neuronal differentiation and maturation.
Chapter Four: A single nucleotide polymorphism in the human BDNF 3’UTR impedes dendritic trafficking of BDNF mRNA and alters spine morphogenesis

INTRODUCTION

The majority of variation in the human genome arises from single nucleotide polymorphisms (SNPs) which occur about once in every 1000 nucleotides (Sachidanandam et al., 2001; Wang et al., 1998). While many SNPs may have no functional correlates, certain SNPs are associated with complex disease phenotypes. SNPs in coding DNA sequences can have dramatic effects by altering the function of key regulatory proteins (Egan et al., 2003; Meyre et al., 2005; Polymeropoulos et al., 1997; Thorleifsson et al., 2009). However it has become increasingly clear that SNPs in non-coding DNA sequences can also have significant effects on neural function (Harold et al., 2009; Meyre et al., 2005; Thorleifsson et al., 2009). According to the National Center for Biotechnology Information SNP database there are 8 identified SNPs in the human \textit{Bdnf} 3’UTR (Fig 15A). Although the prevalence of SNP1-5 and SNP7-8 are unknown, 12% of the population is homozygous for SNP6. Interestingly, sub-population variation for homozygosity of SNP6 (<1% of Asian descent, 13% of European decent, and 27% of Sub-Saharan African decent) exists.

Due to alternative polyadenylation, the human and rodent \textit{Bdnf} gene produces two mRNA isoforms, one with a short 3’UTR (~0.4 kb) and one with a long 3’UTR (~3.0 kb) (Ghosh et al., 1994; Timmusk et al., 2003). Our lab has demonstrated that a signal in the long 3’UTR \textit{Bdnf} mRNA is necessary and sufficient to target that mRNA species into the
dendritic compartment of neurons (An et al., 2008). Furthermore, we showed that mutant mice with truncated long 3’UTR Bdnf mRNA exhibit spine dymorphogenesis, with deficits in spine maturation and pruning (An et al., 2008). Because the human long Bdnf 3’UTR has 7 SNPs (SNP1 is in the short 3’UTR), thus far without known function, we set out to examine if any of the 7 SNPs has an effect on dendritic Bdnf mRNA trafficking or spine morphogenesis. In the work presented here we report that SNP6 (Fig. 15A) impedes dendritic trafficking of Bdnf mRNA (Fig. 15D) and leads to deficits in spine maturation and pruning (Fig. 16B-16E).

RESULTS

SNP6 abolishes dendritic localization of long 3’UTR Bdnf mRNA

Because a signal in the long 3’UTR is responsible for targeting Bdnf mRNA into dendrites, we hypothesized that one or more of the human long 3’UTR Bdnf mRNA SNPs might affect dendritic targeting of Bdnf mRNA. To test this, in situ hybridization on cultured hippocampal neurons was used to measure subcellular mRNA localization. The genomic sequence encoding the human Bdnf long 3’UTR was PCR amplified and cloned into a GFP-pcDNA vector using the Not1 and Kpn1 enzymatic restriction sites. Site specific mutations for each of the seven SNPs were made to generate eight different DNA constructs (containing either the non-mutated or SNP2-SNP8 mutated Bdnf 3’UTRs). SNP1 was not generated because it occurs in the short 3’UTR which has been shown to not localize to dendrites. The constructs were transfected into cultured hippocampal neurons and fluorescent in situ hybridization with a GFP anti-sense
riboprobe was performed to determine subcellular mRNA location. GFP signal intensity was quantified in 10 µm segments along the dendrite extending out from the soma. We found that SNP6 impeded dendritic trafficking of construct mRNA (Fig. 15B and 15D). No other SNP had a significant effect on dendritic mRNA trafficking (Fig15E-15G). Furthermore, using northern blot we demonstrated that both non-mutated and SNP6 mutated DNA constructs generated similar amounts of long and short mRNA isoforms (Fig 15C).
Figure 15. SNP6 Abolishes Dendritic Localization of Long 3’UTR Bdnf mRNA

(A) SNPs in the human Bdnf 3’UTR. The location and nucleotide change for each SNP is labeled. The 1st polyadenylation site is at 316 nucleotides downstream of the Bdnf coding sequence (CDS).

(B) Subcellular localization of GFP mRNA in hippocampal neurons expressing GFP constructs linked to BGH 3’UTR, h3’UTR, or h3’UTRsnp6.

(C) Northern blot of RNA collected from HEK-293 cells transfected with GFP-h3’UTR constructs with a riboprobe against GFP.

(D-G) Levels of GFP mRNAs along the main dendrites of transfected neurons. Data were obtained from 17-22 transfected neurons grown on 3 coverslips for each construct and presented in arbitrary unit. pGFP-h3’UTR constructs are compared to pGFP-BGH. T-test: *, p < 0.05; **, p < 0.01, ***, p < 0.0001.
SNP6 impairs spine maturation and pruning

Immature dendritic spines are produced in abundance during early postnatal development, followed by a period of spine pruning and maturation. This process of dendritic spine overproduction, pruning, and maturation occurs in both humans and rodents. Selective pruning of spines leads to about a 40% reduction in spine number, while selective maturation of spines alters spine morphology from thin and long (immature), to stubby and short (mature) (Bhatt et al., 2009; Galvez et al., 2005; Grutzendler et al., 2002; Holtmaat et al., 2005; Zuo et al., 2005).

Previous work in our lab has shown that mutant mice with deficient dendritic targeting of \textit{Bdnf} mRNA show deficits in spine pruning and maturation (An et al., 2008). Because SNP6 led to decreased dendritic \textit{Bdnf} mRNA in cultured hippocampal neurons we hypothesized that hippocampal neurons transfected with DNA constructs containing SNP6 might have altered spine morphogenesis. In order to explore this we set up an \textit{in vitro} assay in which neurons were transfected with DNA constructs at DIV7, and then fixed and analyzed for spine morphology at DIV14, DIV21, or DIV28. The genomic sequence encoding the human \textit{Bdnf} 3’UTR (either non-mutated or SNP6 mutated) was cloned into a pBluescript (pBK) vector containing the BDNF coding region with a c-terminal MYC tag, generating two plasmids that would produce long 3’UTR \textit{Bdnf} mRNA containing either non-mutated or SNP6 mutated 3’UTR (pBDNFmyc-h’UTR and pBDNFmyc-h3’UTR\textsuperscript{snp6} respectively). Empty pBK was used as a negative control. One of these three constructs was co-transfected into primary
hippocampal neurons with a construct expressing actin-GFP (pActin-GFP) to label spines.

Over-expression of \( Bdnf \) mRNA containing SNP6 lead to a significant increase in spine density along the main dendrite of hippocampal neurons fixed at DIV28 (Fig. 16A and 16B). To more closely investigate the pruning and maturation of spines we looked at spine density, spine head diameter, and spine length, at DIV14, DIV21, and DIV28 for each construct. We found that neurons transfected with control pBK and pBDNFmyc-h3’UTR showed a significant decrease in spine density from DIV21-DIV28, indicating spine pruning in these cultures, however this effect was abolished in cultures transfected with pBDNFmyc-h3’UTR\(^{\text{SNP6}}\) (Fig. 16C). We also found that neurons transfected with pBK and pBDNFmyc-h3’UTR showed significant increases in spine head diameter and decreases in spine length from DIV21-DIV28, indicating spine maturation in these cultures, however this effect was also abolished in cultures transfected with pBDNFmyc-h3’UTR\(^{\text{SNP6}}\) (Fig. 16D and 16E). Taken together, these results indicated that the presence of SNP6 in the \( Bdnf \) 3’UTR resulted in deficits in the pruning and maturation of dendritic spines. In addition, we found that over-expression of BDNF using the pBDNFmyc-h3’UTR construct led to an increase in spine head diameter at DIV21 and DIV28 compared to the control plasmid pBK. These findings indicated that exogenous increases in dendritically synthesized BDNF can lead to the hyper-maturation of spines.

Spine formation, however does not appear to be impaired in cultures transfected with pBDNFmyc-h3’UTR\(^{\text{SNP6}}\). Instead, we found that over-expression of BDNF,
attached to either the mutated or non-mutated 3’UTR showed increased spine density compared to pBK control at DIV14 and DIV21 (Fig. 16C), indicating that BDNF synthesized in the soma is necessary for spine formation. Therefore, in addition to identifying that SNP6 leads to impairments in spine pruning and maturation, our data suggest that somatically synthesized BDNF is necessary for spine formation, while dendritically synthesized BDNF is necessary for spine pruning and maturation.
Figure 16. SNP6 Impairs Spine Maturation and Pruning

(A) Representative dendrites of rat hippocampal neurons containing pBK, pBDNFmyc-h3'UTR, or pBDNFmyc-h3'UTR\textsuperscript{sn6} at DIV28. Scale bar, 25 µm.

(B) Hippocampal neurons harboring pBDNFmyc-h3'UTR\textsuperscript{sn6} at DIV28 have significantly higher spine density along main dendrites when compared with those expressing either pBDNFmyc-h3'UTR or pBK ($F_{2, 27}=159.6$, $p<0.0001$ by two-way ANOVA). N=10 dendrites and 10 neurons.

(C-E) Average spine density, spine head diameter, and spine length were measured from 10 neurons per condition at DIV14, DIV21, and DIV28. T-test: (*) compares within construct; *, $p < 0.05$; **, $p < 0.01$, ***, $p<0.0001$; (#) compares between constructs within a time point. #, $p < 0.05$; ##, $p < 0.01$, ###, $p<0.0001$. 


DISCUSSION

Recent work has demonstrated that SNPs in non-coding DNA can have significant effects on neural function (Harold et al., 2009; Meyre et al., 2005; Thorleifsson et al., 2009). The human Bdnf long 3’UTR contains seven SNPs (Fig 15A) and it has been shown that a signal in the Bdnf long 3’UTR targets Bdnf mRNA into the dendritic compartment (An et al., 2008). Sequences in the 3’UTR of microtubule associated protein 2 (MAP2), calcium calmodulin-dependent protein kinase II alpha (CaMKIIα), and β-actin mRNA are also necessary for dendritic targeting of these mRNA species (Blichenberg et al., 2001; Eom et al., 2003; Tiruchinapalli et al., 2003). Mounting evidence suggests that mRNAs are transported into the dendritic compartment in ribonucleoprotein particles, where local signals can control protein synthesis and the expression of synaptic plasticity (Hirokawa et al., 2006; Kindler et al., 2005). The evidence predicts that mutations in cis-elements of mRNA or trans-elements of binding proteins could cause alterations in dendritic targeting and local protein synthesis. RNA β-hairpin, α-helix, and helix–bend–helix secondary structures have been shown to be critical in binding of proteins to RNA that aid in RNA trafficking (Martin et al., 2006; Weiss et al., 1998). We hypothesize that SNP6 may alter the formation of mRNA secondary structures or may alter the binding sites for trafficking proteins to the mRNA secondary structures, thus disrupting the trafficking process.

We found that a SNP in the Bdnf 3’UTR (SNP6) impeded dendritic trafficking of BDNF mRNA and resulted in decreased spine pruning and maturation in vitro (Fig 15 and Fig 16). The resultant hippocampal cultures transfected with SNP6-containing DNA
displayed dendritic spines that appeared immature because they were longer, thinner and more densely clustered compared to neurons transfected with both control plasmid and non-mutated plasmid (Fig 16). Converging evidence suggests that many disease states are associated with spine pathology (Spronsen and Hoogenraad, 2010). There is also evidence that SNP6 has a high prevalence in the human population. Consequently the findings reported here have the potential to contribute to our understanding of both spine dynamics and human disease.
Chapter Five: Materials and Methods

Animals. $Bdnf^{klax/+}$, $Bdnf^{LacZ/+}$, and $TrkB^{LacZ/+}$ mice were previously described (An et al., 2008; Xu et al., 2000b) and maintained on the C57BL/6 background. $Bdnf^{klax/klax}$ mice and wild-type littermates were obtained from intercrosses of $Bdnf^{klax/+}$ mice. $TrkB$ mutant $fB/fB;PV-Cre$ and control $fB/fB$ mice were obtained from crosses between $fB/fB$ and $fB/+;PV-Cre$. The generation of the $fB$ allele and the $PV-Cre$ allele were previously described (Liu et al., 2007; Tanahira et al., 2009). All procedures described were approved by the Institutional Animal Care and Use Committee at Georgetown University and were in compliance with the NIH guide for the care and use of laboratory animals. All animals were given free access to food and water and housed in a 12-hour light/dark cycle.

Radioactive In Situ Hybridization. In situ hybridization of tissue sections was performed as described previously (Xu et al., 2003). In brief, mouse brains were dissected and frozen immediately in an isopentane-dry ice bath. Brains were sectioned at 10 μm using a cryostat, and in situ hybridization was performed on sections using a $^{35}$S-labeled antisense riboprobe derived from cDNA encoding BDNF. After hybridization and washes, sections were exposed to Kodak BioMax MR Hyperfilm. For each mouse, images from eight sections were scanned at 1,200 dpi, and the optical
density of *in situ* signal in the granule cell layer of the dentate gyrus was determined using NIH Image J software.

**BDNF ELISA.** Hippocampi were dissected from 5 pairs of *Bdnf*<sup>klox/klox</sup> mice and WT littermates at approximately 6 weeks of age, weighed, and homogenized in an ice-cold lysis buffer (100 mM Tris-HCl, 2% bovine serum albumin, 1 M NaCl, 4 mM EDTA, 2% Triton X-100, and protease inhibitors, pH 7). The lysates were kept on ice for 30 min and centrifuged at 12,500 rpm at 4°C for 20 min. Supernatants were recovered as hippocampal protein extract. The amount of BDNF in hippocampal protein extract was measured using the BDNF ELISA kit from Millipore (Temecula, CA).

**Pharmacological Treatments.** BrdU was administered intraperitoneally (50 mg/kg) three times, once every two hours, into mice either at 4 weeks of age (the activation study; Figure 3), or at 6 weeks of age (the proliferation, differentiation, and maturation studies; Figures 1-4, 5, and 6). Phenobarbital was administered intraperitoneally (80 mg/kg) once a day for three days prior to BrdU administration (the proliferation and differentiation rescue studies; Figure 6). Kainate was administered intraperitoneally (35 mg/kg) to 8-week-old animals to induce seizures, 4 weeks after BrdU administration, and 2 hours prior to euthanasia. All animals achieved at least grade 4 seizures in which rearing was observed (Racine et al., 1972). The NMDA antagonist CGP37849 (5 mg/kg) was administered intraperitoneally at 6 weeks of age, 2 days prior to BrdU administration.
Local Protein Synthesis Assay. Rat hippocampal neuron culture and the local protein synthesis constructs were previously described (An et al., 2008). A sequence encoding the Src myristoylation peptide was added to the 5′ end of a PCR-amplified d1EGFP insert from plasmid pd1EGFP-N1, which was inserted into a plasmid downstream of the human synapsin promoter, generating phSYN-myr-d1GFP. The mouse sequences for the BDNF 3′UTRs short (A) and long (A*B) (where the first polyadenylation signal AATAAA was mutated to TTTTTT) were cloned into phSYN-myr-d1GFP, generating phSYN-myr-d1GFP-A (myr-d1GFP-A) and phSYN-myr-d1GFP-A*B (myr-d1GFP-A*B). These two constructs were transfected into cultured rat hippocampal neurons on DIV14. One day after transfection, the cultured neurons were treated with 50 μM NMDA or vehicle for 1 hr and then fixed. The longest dendrites of transfected neurons were analyzed by quantification of the fluorescent intensity of a line drawn through the center of the dendrite using NIH Image J software, and mean intensity values for each condition were calculated in 50 μm bins.

Immunohistochemistry and Nissl stains. Mice were anaesthetized with avertin and transcardially perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde sequentially. Brains were removed from the skull, post-fixed in 4% paraformaldehyde overnight, and soaked in 30% sucrose. Coronal brain sections (50 μm) were obtained from the whole rostro-caudal extent of the hippocampus using a sliding microtome. For non-fluorescent staining, sections were incubated with 10% methanol-3% hydrogen peroxide in Tris-buffered saline (TBS) to quench endogenous peroxidases. HCl antigen
retrieval was used for BrdU and Ki-67 immunostaining (2N HCl at 37 °C for 1 hour before blocking). After incubation with blocking buffer (0.4% Triton X-100, 2.5% bovine serum albumin, and 10% horse serum in TBS) for 1 hour, the sections were incubated with primary antibody diluted in blocking buffer overnight at room temperature. The following primary antibodies were used: rat anti-BrdU (1:500; Accurate Chemical, Westbury, NY), rabbit anti-Ki-67 (1:100, Thermo-Scientific Fisher, Waltham, MA), mouse anti-NeuN (1:500, Chemicon, Billerica, MA), goat anti-NeuroD (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-cleaved caspase-3 (1:200, Cell Signaling, Danvers, MA), goat anti-DCX (1:250, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-calretinin (1:1,000, Chemicon, Billerica, MA), mouse anti-calbindin (1:400, Sigma, St. Louis, MO), rabbit anti-GFAP (1:400; Sigma, St. Louis, MO), mouse anti-Arc (1:50, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Zif268 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-GAD65 (1:400, Boehringer Mannheim, Ingelheim, Germany), guinea pig anti-VGLUT1 (1:1,000, Millipore, Billerica, MA), mouse anti-parvalbumin (1:2,000, Sigma, St. Louis, MO), rabbit anti-somatostatin (1:1,000; Immunostar, Hudson, WI), rabbit anti-NPY (1:2,000, Sigma, St. Louis, MO), and rabbit anti-β-galactosidase (1:2,000, Cappel, Durham, NC). After three washes in TBS, the sections were incubated with the appropriate secondary antibody in blocking buffer. For co-localization studies, the appropriate fluorescent-conjugated secondary antibodies were used followed by three washes, after which sections were mounted using gelvatol fluorescent mounting media. For non-fluorescent staining, the appropriate biotinylated secondary antibody, followed
by the avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA) was used according to the manufacturer’s protocol. Sections were developed in 0.05% 3-3′-diaminobenzidine tetrahydrochloride (DAB) and 0.003% hydrogen peroxide in 0.1 M Tris–HCl (pH 7.5), mounted onto slides, dehydrated, and coverslipped with DPX. Nissl-staining was performed by submerging mounted sections in cresyl violet for 20 min prior to dehydration.

**Quantitative Analysis for Neurogenesis Studies.** Stereological counts of DAB labeled cells were performed using Stereo Investigator software (MicroBrightField Inc, Williston, VT, USA). Twelve series of brain sections were taken, and all sections from one well containing the whole rostro-caudal extent of the DG were analyzed. The granule cell layer was outlined using a 20x objective and all stained cells within the outlined area were counted using a 60x objective to calculate cell density. Pictures for co-localization quantification were acquired using a confocal microscope with a 40x oil immersion lens. GAD65 and VGLUT1 staining intensities in the SGZ were measured using NIH Image J software. A box was drawn around the first two cell layers of the granule cell layer adjacent to the hilus and staining intensity was quantified.

**Culture of Primary Neurons.** Rat hippocampal neuron culture was performed as previously described (An et al., 2008). Briefly, hippocampal neurons were isolated from E18.5 Sprague-Dawley rat embryos and cultured onto 10mm glass coverslips at a concentration of 1.8 X 10^5 cells per well in 12-well plates. Coverslips were soaked in
nitric acid, washed with double distilled water, and baked overnight. Coverslips were then coated with poly-D-lysine and laminin prior to culture. Cultures were maintained in Neurobasal medium (Invitrogen Corporation, Carlsbad, CA) supplemented with B27, 0.5 mM L-glutamine, 25 µM glutamate, and 1% penicillin-streptomycin. Every 7 days, one-half of the medium was removed and replaced with an equal volume of fresh Neurobasal medium.

**DNA Plasmids and Transfection For In situ Hybridization Studies.** The genomic sequence encoding the human BDNF long 3’UTR was cloned into a pcDNA-GFP vector using the Not1 and Kpn1 enzymatic restriction sites. Site-directed mutagenesis was used to create seven constructs, each containing one of the seven SNPs found in the human BDNF long 3’UTR. A pcDNA-GFP vector containing the bovine growth hormone 3’UTR was used as a negative control because it is not targeted into dendrites, while the non-mutated BDNF long 3’UTR was used as a positive control because it is targeted into the dendrites. Plasmids were transfected into DIV6 hippocampal cultures using Lipofectamine 2000 and 0.2µg of DNA per kB of plasmid to correct for number of moles transfected. Twenty-four hours after transfection the neurons were fixed and fluorescent *in situ* hybridization was performed.

**DNA Plasmids and Transfection for Spine Studies.** The mouse genomic sequences encoding the *Bdnf* coding region (containing a Myc tag at the C-terminus) were obtained by PCR and inserted downstream of the cytomegalovirus (CMV) promoter of
the pBluescript (pBK) plasmid (Addgene Inc., Cambridge, MA). The genomic sequence for the human BDNF 3’UTR containing either the non-mutated or SNP6 mutated site were then cloned downstream of the BDNF coding region, generating two plasmids containing the BDNF coding region and the long 3’UTR, either WT or SNP6. Empty pBK was used as negative control. The pActin-GFP construct used was previously described (Fischer et al., 1998). Plasmids were transfected into DIV7 hippocampal cultures using Lipofectamine 2000 and 0.2µg of DNA per kB of plamid to correct for number of moles transfected. Seven days, 14 days, or 21 days after transfection, neurons were fixed for immunocytochemistry.

**Fluorescent In situ hybridization.** In situ hybridization of cultured hippocampal neurons was performed using n anti-GFP DIG-labeled riboprobe and the TSA Plus Fluorescein System (PerkinElmer, Waltham, MA) as previously described (An et al., 2008). Cells were fixed with 4% paraformaldehyde for 1 hour. Cells were rinsed in PBS containing 0.1% Tween-20, and then permeabilized for 10 minutes in PBS containing 0.3% Triton X-100. Cells were rinsed in PBS containing 0.1% Tween-20, and then acetylated for 10 minutes in TEA containing 0.25% acetic anhydride. Cells were rinsed, and incubated in a pre-hybridization mix for one hour at 55°C. Cells were then incubated overnight at 55°C in hybridization mix containing 50 ng/mL of antisense riboprobe for the GFP sequence. After hybridization, cells were rinsed with SSC buffer and treated with RNase A at 55°C for 1 hour to remove unannealed probes. Following a series of washes in SSC buffer, endogenous peroxidases were removed by incubating cells in PBS.
containing 10% methanol and 1% \( \text{H}_2\text{O}_2 \) for 30 minutes. Cells were then incubated for one hour at room temperature with blocking buffer containing 1X Roche blocking solution (Roche Applied Science, Indianapolis, IN), 0.15 M NaCl and 0.1 M Tris-HCl pH 7.5. Cells were then incubated overnight at 4°C in blocking buffer containing primary antibody. The primary antibody, mouse anti-MAP2 (Clontech Laboratories Inc., Mountain View, CA), was used at a dilution of 1:200. The following day, cells were rinsed in double distilled water containing 0.15 M NaCl, 0.1 M Tris-HCl pH 7.5 and 0.05% Tween 20. Cells were then incubated for one hour at room temperature in blocking buffer containing secondary antibody. The secondary antibody, texas-red goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used at a 1:500 dilution. Cells were then washed in double distilled water containing 0.15 M NaCl, 0.1 M Tris-HCl pH 7.5 and 0.05% Tween 20, and incubated at room temperature with the TSA Plus Fluorescein System (PerkinElmer, Waltham, MA) at a 1:50 dilution for 10 minutes. Cells were washed in double distilled water containing 0.15 M NaCl, 0.1 M Tris-HCl pH 7.5 and 0.05% Tween 20, and then mounted onto glass coverslips using gelvetol fluorescent mounting medium.

**Northern blot.** Plasmid DNA, pcDNA-GFP-hBDNF 3’UTR (non-mutated or SNP6 mutated) was transfected into HEK-293 cells at 50% confluence. Three days post transfection, cells were harvested and RNA was extracted using Trizol reagent (Life Technologies, GibocoBRL). The northern hybridization probe used was a DIG labeled GFP riboprobe against the GFP coding sequence.
**Immunocytochemistry.** Cells were fixed with 4% paraformaldehyde for 20 minutes. Cells were then rinsed once in PBS, and endogenous peroxidases were removed using a solution of 10% Methanol, 3% H$_2$O$_2$ in 1X PBS for 20 minutes at room temperature. Cells were rinsed once in PBS, and then permeabilized with 0.25% Triton X-100 in PBS at room temperature for 10 minutes. Following one rinse with PBS, cells were incubated in blocking solution containing 10% BSA and 0.1% Triton X-100 in PBS for one hour. Cells were then incubated overnight at 4°C with primary antibody in solution containing 1% BSA and 0.1% Triton-X 100. The rabbit anti-GFP primary antibody (Clontech Laboratories Inc., Mountain View, CA) was used at a 1:5000 dilution. The following day, cells were rinsed with PBS and then incubated with secondary antibody solution containing 1% BSA and 0.1% Triton-X 100 in PBS for one hour at room temperature. The goat anti-rabbit biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used at a 1:200 dilution. Cells were rinsed with PBS and then incubated in an avidin-biotin complex solution (Vector Labs, Burlingame, CA) used at a dilution of 1:300. Cells were rinsed in 100mmM Tris pH 7.4, and then exposed to solution containing 0.05% DAB and 0.003% H$_2$O$_2$ in 100 mM Tris. After 5 minutes, colorimetric reaction was complete, and cells were rinsed with PBS and mounted onto glass slides using DPX mounting medium.

**mRNA Trafficking Analysis.** Transfected cultures were fixed at DIV 7 and *in situ* hybridization was performed using an antisense GFP riboprobe. Images of transfected neurons were taken using a confocal microscope. Dendritic signal intensity was measured
using image J software (NIH). Signal intensity in 10µm sections along the dendrite, extending out from the soma, was compared between DNA constructs.

**Spine Analysis.** Transfected cultures were fixed at DIV14, DIV21, and DIV28 and immunostained with an antibody to GFP. Dendritic arbors of transfected neurons were traced, and spines were counted on one main dendrite per neuron using Neurolucida software (MicroBrightField, Inc) at 60X magnification using an oil-immersion lens. Spine density was calculated using NeuroExplorer software. At least 10 neurons from multiple coverslips were analyzed for each construct, at each time point and condition. To analyze the morphology of spines, high magnification images (60X) using an oil-immersion lens were taken of stained dendrites. Spine head diameter and length were measured using Image J software. Spine head diameter and spine length are defined as the maximum width of the spine head and the distance from the tip of the spine head to the interface with the dendritic stalk, respectively. Spines were measured along the 50-100 µm segment of one main dendrite per neuron. At least 10 neurons from multiple coverslips and approximately 50 spines per neuron were analyzed for each construct, at each time point and condition.

**Statistical Analysis.** All data are expressed as mean ± SEM. Data were analyzed using an unpaired Student’s t test (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).
Chapter Six: Final conclusions and future directions

Dendritic targeting of mRNA and local protein synthesis are crucial regulators of synaptic plasticity (Bramham et al., 2007; Hirokawa et al., 2006; Pfeiffer et al., 2006). Decreases in dendritic pools of key plasticity-inducing mRNAs have been shown to result in altered LTP and structural dysmorphology of hippocampal neurons (An et al., 2008; Tiruchinapalli et al., 2007; Miller et al., 2002). Unpublished work from our lab has shown that Bdnf$^{klox/klox}$ mice, with a truncated Bdnf long 3’UTR, display significant impairments in the Morris water maze, indicating decreased spatial memory function (data not shown).

In the work reported here we demonstrated that deficits in dendritic targeting of Bdnf mRNA resulted in impairments in adult neurogenesis in vivo, with animals displaying decreased differentiation, maturation, and integration of newly born neurons from the SGZ of the hippocampus. We also identified a possible mechanism by which dendritically synthesized BDNF controls adult neurogenesis. In our proposed model, perforant path glutamate binds to NMDA receptors to facilitate local BDNF synthesis and subsequent release onto TrkB receptors of PV interneurons. To our knowledge, this is the first evidence of local protein synthesis controlling adult neurogenesis.

Despite these initial exciting findings there are key questions that remain to be answered. It is still unclear how the binding of BDNF to TrkB receptors on PV interneurons leads to an increase in GABA release into the neurogenic niche. It is possible that BDNF-TrkB signaling leads to alterations in the dendritic arbor of
interneurons, thus allowing for increases in cell firing following additional excitatory input. In future research we could explore this hypothesis by comparing the dendritic arbors of PV interneurons in PV-GFP mice (crossed with either WT or $Bdnf^{\text{lox/lox}}$ mice). However, it is also possible that instead of gradual sculpting of the dendritic arbor, BDNF-TrkB signaling may elicit more acute effects either directly at the synapse or via signaling endosomes. As described extensively in chapter one, BDNF can induce local plasticity at synapses, however, in addition, BDNF-TrkB signaling endosomes may be transported from the dendrite to the cell body to initiate cellular cascades that increase the synthesis and/or release of GABA. Testing this possibility would require the generation of an inducible TrkB KO mouse in PV interneurons. If we crossed a GFP-labeled estrogen receptor-Cre transgene under the PV promoter to a floxed TrkB mouse line, we would be able to acutely and selectively induce knockout of TrkB receptors in PV+ neurons using tamoxifen. Using these mice we could record from labeled PV+ neurons after perforant path stimulation to test the strength of the molecular layer synapses. In addition, by stimulating labeled PV+ interneurons and recording from their postsynaptic contacts, or by co-staining interneurons for GAD65 we could investigate if GABA synthesis or release is altered. Taken together, these studies have the possibility of demonstrating how binding of BDNF to TrkB receptors on PV interneurons alters GABA transmission in the SGZ. We would also like to further characterize the neurogenic phenotype of the $PV-Cre;fB/fB$ mouse. Thus far, we have shown that these mice have a similar increase in BrdU labeled cells compared to $Bdnf^{\text{lox/lox}}$ mice. Research is still needed to look at the differentiation, maturation, and
integration phenotypes of these mice. Finally, we are also interested in whether other populations of interneurons release GABA into the neurogeneic niche and are regulated by local BDNF synthesis. Somatostatin (SOM) and Neuropeptide Y (NPY) are two other potential interneuron populations that may mediate BDNFs effects on neurogenesis. After we demonstrate co-localization of TrkB receptors and SOM or NPY in the DG, we could cross SOM-Cre or NPY-Cre mice to floxed TrkB mice to selectively knock out TrkB in these populations and assess these mice for similar alterations in neurogenesis.

In chapter four we provided data that demonstrated identification of a frequent human SNP (SNP6) which results in decreased dendritic trafficking of Bdnf mRNA, and decreased spine pruning and maturation in vitro. Because 12% of the human population is homozygous for SNP6, findings from this work could have a significant impact on human health. Important future work is needed to understand the role of the Bdnf 3’UTR in neural function and the role of SNP6 in neural pathology. Identification of specific cis-acting elements in long 3’UTR Bdnf mRNA that orchestrate dendritic trafficking is necessary to isolate cellular functions of dendritic trafficking. Furthermore, little is known about the trans-acting factors that bind to Bdnf mRNA to aid in trafficking mRNA into the dendritic compartment. Finally, it will be important to create a knockin mouse harboring the human Bdnf 3’UTR either with or without the SNP6 mutation. These mouse models will allow us to test for possible in vivo effects of SNP6, including important molecular, morphological, electrophysiological, and behavioral abnormalities.
Dendritic spines are the sites for most excitatory synapses in the brain, and although the majority of spine plasticity occurs during development, dendritic spines continue to form, mature, and retract throughout adult life (Bhatt et al., 2009; Holtmaat et al., 2009; Yoshihara et al., 2009). Spine alterations have been documented in a number of diseases and disease models, including Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD), schizophrenia, addiction, autism, and mental retardation (Bhatt et al., 2009). Although causation has not been established between these cellular processes and diseases, the links between spine abnormalities and disorders suggest that the work presented here may be of value as initial groundwork for future research examining altered dendritic mRNA trafficking in the etiology and pathophysiology of disease.
Chapter Seven: References


Dobrossy, M.D., Drapeau, E., Aurousseau, C., Le Moal, M., Piazza, P.V., and Abrous, D.N. (2003). Differential effects of learning on neurogenesis: learning increases or decreases the number of newly born cells depending on their birth date. Mol Psychiatry 8, 974-982.


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


Woo, N.H., Teng, H.K., Siao, C.J., Chiaruttini, C., Pang, P.T., Milner, T.A., Hempstead, B.L., Lu, B., 2005. Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. Nat Neurosci 8, 1069-1077.


