BDNF-TrkB Signaling in Single Spine Structural Plasticity

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Neurobiology in the Graduate School of Duke University

2015
ABSTRACT

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Abstract

Multiple lines of evidence reveal that activation of the tropomyosin related kinase B (TrkB) receptor is a critical molecular mechanism underlying status epilepticus (SE) induced epilepsy development. However, the cellular consequences of such signaling remain unknown. To this point, localization of SE-induced TrkB activation to CA1 apical dendritic spines provides an anatomic clue pointing to Schaffer collateral-CA1 synaptic plasticity as one potential cellular consequence of TrkB activation. Here, we combine two-photon glutamate uncaging with two photon fluorescence lifetime imaging microscopy (2pFLIM) of fluorescence resonance energy transfer (FRET)-based sensors to specifically investigate the roles of TrkB and its canonical ligand brain derived neurotrophic factor (BDNF) in dendritic spine structural plasticity (sLTP) of CA1 pyramidal neurons in cultured hippocampal slices of rodents. To begin, we demonstrate a critical role for post-synaptic TrkB and post-synaptic BDNF in sLTP. Building on these findings, we develop a novel FRET-based sensor for TrkB activation that can report both BDNF and non-BDNF activation in a specific and reversible manner. Using this sensor, we monitor the spatiotemporal dynamics of TrkB activity during single-spine sLTP. In response to glutamate uncaging, we report a rapid (onset less than 1 minute) and sustained (lasting at least 20 minutes) activation of TrkB in the stimulated spine that depends on N-methyl-d-aspartate receptor (NMDAR)-
Ca²⁺/Calmodulin dependent kinase II (CaMKII) signaling as well as post-synaptically synthesized BDNF. Consistent with these findings, we also demonstrate rapid, glutamate uncaging–evoked, time-locked release of BDNF from single dendritic spines using BDNF fused to superecliptic pHluorin (SEP). Finally, to elucidate the molecular mechanisms by which TrkB activation leads to sLTP, we examined the dependence of Rho GTPase activity – known mediators of sLTP – on BDNF-TrkB signaling. Through the use of previously described FRET-based sensors, we find that the activities of ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 (Cdc42) require BDNF-TrkB signaling. Taken together, these findings reveal a spine-autonomous, autocrine signaling mechanism involving NMDAR-CaMKII dependent BDNF release from stimulated dendritic spines leading to TrkB activation and subsequent activation of the downstream molecules Rac1 and Cdc42 in these same spines that proves critical for sLTP. In conclusion, these results highlight structural plasticity as one cellular consequence of CA1 dendritic spine TrkB activation that may potentially contribute to larger, circuit-level changes underlying SE-induced epilepsy.
Dedication

I dedicate this dissertation to my wife, Mary, and my daughter, Ausley. Their love, support, and most importantly, patience for me have been truly essential towards the successful completion of this dissertation. I thank you both and am eager for the three of us to start the next chapter of our lives together.
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Acknowledgements

To begin, I would like to acknowledge all of my friends and family who have supported me along this incredible journey. This has truly been a team effort and with your love and support, I am not sure I would have completed this projection.

I especially want to thank my wife Mary and my daughter Ausley. These past few years have been incredibly exciting but also quite challenging. Your patience, understanding, and continuous encouragement have helped me preserve as I toiled away in the lab. I am truly blessed to have both of you in my life.

I want to thank my Mom and Dad. You all have provided me such wonderful opportunities throughout my life. Most importantly, you all ensured that I had the best education possible growing up. I may not have realized it at the time but I recognize now how much my education has paid off. It has fostered a passion and love for learning that fills me with excitement each and every day. The gift of education was truly the best gift you all could have ever given me, thank you!!!

I want to recognize my wife’s family as well. Through these past eight years, you all have been incredibly supportive. Your kindness and generosity has been astounding. I don’t say it enough but I will say it here . . . thank you, I truly do love you all!
In addition to family and friends, there are countless others I would like to acknowledge – the entire McNamara and Yasuda Labs, Rich Mooney, Steve Lisberger, Pate Skeen, Allan Friedman, and Anne West. I have been very lucky to have tremendous support throughout this entire process. However, there are four individuals I want to single out.

First, Shenyu Zhang. Shenyu is a former graduate student of the Yasuda Lab who helped me perform several of the experiments that are presented in Figure 9.

Second, Enhui Pan. Enhui provided incredible help in completing the functional plasticity experiments outlined in this dissertation (Section 3.2.5). His insight and technical expertise made these experiments not only successful but very easy to accomplish.

Third, Nathan Hedrick. Nathan is the reason why this project worked. His patience, guidance, and insight truly helped me figure out to do the experiments I describe in this dissertation. In fact, several of the key findings I describe here are largely in thanks to ideas and efforts from Nathan (Section 3.3.3 as well as many of the experiments in chapters 6 and 7). With all this said, I am happy to say that we may have started as fellow graduate students but we are going to finish as great friends. Thank you Nathan for everything!

And finally, Jim . . . I do not think words can begin to do justice to the gratitude I owe you. You have not only been a tremendous mentor, you have been a true friend.
When I graduate, I realize that I will always have a home here at Duke. You have set a wonderful example of how to be a successful physician scientist and even better human being. It is bittersweet that I must start the next chapter of my career but luckily you have given me tremendous guidance and insight over these past six years . . . thank you!

I promise to use it well.
1. Introduction

1.1 Epilepsy

Epilepsy is a devastating neurologic disease affecting nearly 1% of the world’s population (Bialer and White, 2009; Forsgren et al., 2005; Löscher et al., 2013). Its clinical hallmark is recurrent episodes of unprovoked, uncontrolled, and synchronous neuronal activity manifesting as behavioral seizures (Berg et al., 2010; Goldberg and Coulter, 2013). Despite its classic constellation of symptoms, epilepsy is not a single disease. In fact, the International League against Epilepsy (ILAE) Commission currently recognizes over 30 distinct epilepsy syndromes (Berg et al., 2010). Identified causes of these epilepsies include genetic mutations in voltage-gated ion channels, traumatic brain injury, and cortical developmental abnormalities (Berg et al., 2010). Consistent with this heterogeneity in origin, behavioral manifestations of seizures are quite diverse and can range from short, commonly undetected events to prolonged periods of bilateral muscle convulsions and loss of consciousness lasting minutes to hours (Berg et al., 2010; Goldberg and Coulter, 2013).

There are many treatment options for epilepsy including both pharmacologic and surgical interventions. In terms of medical therapy, over the past few decades, numerous anti-epileptic drugs have been developed (Löscher et al., 2013; Löscher and Schmidt, 2011). These agents generally act to either reduce neuronal excitability through inhibition of the various ion channels (voltage-gated sodium channels for example) or
enhance neuronal inhibition by increasing the activity of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Bialer and White, 2009). Regardless of their mechanisms of action, none of these agents are disease modifying – they all treat the symptoms of epilepsy, namely seizures, without addressing the underlying pathology (Löscher et al., 2013; Löscher and Schmidt, 2011). For many patients, these therapeutic interventions are effective in that they limit or completely prevent seizure occurrence. However, for approximately 30% of patients, adequate symptom control with medical therapy remains elusive (Brodie et al., 2012; Löscher et al., 2013; Sillanpää and Schmidt, 2006). For these patients with pharmacoresistant epilepsy, one of the last remaining options is surgical resection of affected brain tissue. In the setting of a clearly defined anatomic focus for seizure initiation and propagation, surgery can be highly effective (Téllez-Zenteno et al., 2010). For most patients though, pinpointing the exact anatomic location for seizure origin can be quite challenging thus precluding surgery as a viable option.

Because of the clear limitations in both pharmacologic and surgical therapies for epilepsy, there is a tremendous need to develop novel, more effective, and truly disease modifying interventions. To this end, it is critical that we gain improved understanding of the molecular and cellular mechanisms contributing to the pathology underlying epilepsy.
1.1.1 Temporal lobe epilepsy

Of the epilepsies, temporal lobe epilepsy is one of the most common accounting for nearly 40% of all adult epilepsy cases (McNamara et al., 2006). TLE is an example of focal epilepsy with seizures commonly arising from within the limbic system, particularly the hippocampus (Bartolomei et al., 2005; Engel, 1996; Engel, 2001). Often developing during childhood or early adulthood, TLE can progress over time in severity, become resistant to pharmacologic treatment, and frequently be associated with other behavioral and psychiatric disorders such as depression and anxiety (Arroyo et al., 2002; Berg et al., 2006; Cascino, 2009; Pitkänen and Sutula, 2002; Shukla and Prasad, 2011). For these reasons, TLE is often considered to be one of the most devastating forms of epilepsy.

Development of TLE has been associated with a preceding episode of severe and prolonged seizure activity – termed status epilepticus (SE) – in childhood. Specifically, retrospective analysis of patients diagnosed with TLE has revealed a common history of childhood SE arising in the setting of fever (French et al., 1993). In parallel with this, longitudinal studies have revealed that of patients experiencing SE, nearly 50% develop epilepsy later in life (Annegers et al., 1987; Tsai et al., 2008). For those patients that do develop epilepsy following SE, the occurrence of spontaneous, recurrent seizures is not immediate. Rather, there seems to be a latent period after initial SE that can last for months to years and in rare cases decades before epilepsy onset (French et al., 1993).
This gap between initial SE and epilepsy onset suggests a period of epileptogenesis following SE where a normal brain gradually becomes epileptic. Because this latent period has been recognized clinically, many have suggested that this time period may afford a therapeutic window in which one can intervene after SE and potentially inhibit epilepsy development. To this end, much work has been put forth to identify the molecular and cellular mechanisms by which a single episode of SE can evolve into TLE.

1.1.2 Animal models of SE-induced TLE

Ideally, mechanisms by which SE induces TLE would be examined in human patients where this process is occurring. However, due to obvious ethical concerns and technical limitations, such work is not possible. As such, researchers have turned towards animal models as a means to study human diseases with the aim of developing improved therapies. With respect to TLE, multiple animal models have been developed. Two of these models are discussed below:

1.1.2.1 The pilocarpine model

First developed in rats, this model utilizes systemic administration of pilocarpine – a non-selective muscarinic cholinergic receptor agonist – to induce SE (Turski et al., 1983a; Turski et al., 1983b). Depending on the protocol, SE continues until its natural termination several hours after induction or until its pharmacologically stopped, usually with a benzodiazepine, after a set time period. Following SE termination, animals do not initially display either behavioral or electrographic seizure activity. However, after
a period of several days to several weeks, seizures develop that persist for the remainder of the animal’s life (Cavalheiro et al., 1991; Curia et al., 2008). Because these seizures are spontaneous and recurrent, the animal is considered epileptic.

The phenotype of these animals models human TLE in several respects. First, like human TLE, these animals develop epilepsy in response to SE. Consistent with humans, epilepsy onset is not immediate but rather is preceded by a latent period. Once seizures do develop, there is evidence that some seizures may originate from limbic structures consistent with temporal lobe pathology (Cavalheiro et al., 1991; Turski et al., 1983b). Additionally, with time, these animals develop several hippocampal pathologic changes – hippocampal sclerosis and mossy fiber sprouting – that recapitulate findings observed in human TLE patients (Curia et al., 2008).

1.1.2.2 The kainic acid model

Kainic acid (KA) is an agonist of the kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtypes of glutamate receptors (Hampson and Manalo, 1997). Its application systemically leads to increased neuronal excitation that like pilocarpine can result in SE (Williams et al., 2009). Following SE termination either naturally or pharmacologically, these animals do not initially develop SRS; rather, there is a delay of a few days between SE and epilepsy onset analogous to the latent period observed in humans (Williams et al., 2009). However, once SRS do develop, they
generally are consistent with a limbic origin and they persist for the life of the animal (Raol and Brooks-Kayal, 2011).

In addition to systemic administration, several variations of this model have been developed that utilize targeted KA injection. One that has been extensively used by the McNamara Lab is KA infusion into the basolateral amygdala – KA microinfusion model (Ben-Ari et al., 1979; Helgager et al., 2013; Mouri et al., 2008). Like systemic administration, amygdala infusion leads to SE that will in turn yield SRS approximately one week later (Liu, 2013). One of the main advantages of this targeted KA injection is that it specifically engages limbic circuitry to induce SE and thus may be more closely aligned with human TLE than systemic administration models.

1.1.3 Molecular mechanisms of SE-induced TLE – the roles of BDNF and TrkB

Over the past few decades, work using the animal models described above has led to multiple molecular mechanisms being proposed for SE-induced epileptogenesis. Of these mechanisms, the most substantial and compelling evidence exists for the neurotrophin brain-derived neurotrophic factor (BDNF) and its high-affinity receptor tropomyosin related kinase B (TrkB). Below we review both the molecular biology of BDNF-TrkB signaling as well as evidence supporting its role within SE-induced TLE.

1.1.3.1 Molecular biology of BDNF-TrkB signaling

TrkB is a member of the tropomyosin-related kinase (Trk) family of plasma membrane receptors with other members including TrkA and TrkC (Barbacid, 1995).
Each receptor has a unique expression pattern in the nervous system. Of the three, TrkB is the mostly highly expressed in the central nervous system, especially the hippocampus (Klein et al., 1990). Within the hippocampus, TrkB has been localized to both excitatory and inhibitory cell populations as well as numerous subcellular compartments including the pre- and post-synaptic terminals of multiple hippocampal synapses (Drake et al., 1999; Helgager et al., 2013; Ji et al., 2005; Lai et al., 2012). Because of such ubiquitous expression, it is no surprise that TrkB has been implicated in a variety of physiologic and pathologic processes occurring in the CNS.

Activation of TrkB is primarily mediated by its canonical ligand, the neurotrophin brain-derived neurotrophic factor (BDNF). Initially, BDNF is generated as a precursor or pro-neurotrophin that requires cleavage to generate its mature form – the form that can activate TrkB (Mowla et al., 2001). Cleavage of proBDNF to mature BDNF can occur through multiple intracellular and extracellular mechanisms involving various protease enzymes including furin, pro-convertases, plasmin, and MMPs (Keifer et al., 2009; Matsumoto et al., 2008; Mizoguchi et al., 2011; Mowla et al., 2001; Pang et al., 2004).

Regardless of the mechanism, cleavage results in a 12 kDa mature BDNF that will complex with another mature BDNF molecule to non-covalently form a stable dimer (Haniu et al., 1997). It is this homodimer that can then bind to TrkB leading to its activation.
Similar to TrkB, BDNF is expressed throughout the central nervous system (CNS) and is implicated in a wide variety of functions – neuronal survival, neuron development, and synaptic function and plasticity (Chao, 2003; Huang and Reichardt, 2000; McAllister et al., 1998). To be effective, BDNF must be released into the extracellular space so that it can then bind to the extracellular domain of TrkB (Haniu et al., 1997). BDNF release has been extensively studied and has been shown in certain contexts to be activity-dependent (Balkowiec and Katz, 2002; Kohara et al., 2001). However, the site of BDNF release – axonal versus dendritic – has proven highly controversial (for a more detailed discussion, see chapter 6) (Dieni et al., 2012). Despite discrepancy about release site, it is clear that BDNF has functional significance at both pre- and post-synaptic locations (Chao, 2003; Minichiello, 2009).

For BDNF to have functional impact, it must first bind to and activate the TrkB receptor. Being released as a homodimer, BDNF binding to TrkB initiates receptor dimerization, similar to what occurs for other receptor tyrosine kinases (Barbacid, 1995; Cunningham and Greene, 1998; Haniu et al., 1997). Following dimerization, the intrinsic kinase domain within TrkB is activated leading to phosphorylation of various tyrosine residues within the autoregulatory loop of the kinase domain (Chao, 2003; Huang and Reichardt, 2002; McNamara et al., 2006). Such phosphorylation modulates the kinase’s conformation that in turn allows it to phosphorylate various tyrosine residues within TrkB’s intracellular domain (Huang and Reichardt, 2002; Lai et al., 2012; McNamara et
al., 2006). These phosphorylated residues serve as docking sites for adaptor proteins: 1) phosphorylated tyrosine 515 and the Src homology 2 domain containing protein (Shc) and 2) phosphorylated tyrosine 816 and phospholipase-C gamma-1 (PLCγ1) (Huang and Reichardt, 2002; Lai et al., 2012; Middlemas et al., 1994; Patapoutian and Reichardt, 2001). Additionally, it has been shown that BDNF binding to TrkB can induce phosphorylation of serine 478 in a Cdk5-dependent manner. Once phosphorylated, serine 478 serves as an adaptor protein for the T-lymphoma invasion and metastasis-inducing protein 1 (Tiam1) (Lai et al., 2012).

Following binding of these proteins, TrkB’s kinase will phosphorylate and activate them thereby triggering various signaling cascades downstream of TrkB – Tiam1 leading to Rac1 signaling, Shc leading to Ras-ERK and PI3K-Akt signaling, and PLCγ1 leading to IP3 and diacylglycerol (DAG) production plus release of intracellular Ca^{2+} (Figure 1A, B, and C) (Chao, 2003; Huang and Reichardt, 2002 1994).

In addition to BDNF, other mechanisms contributing to TrkB activation have been identified. Examples include activation via G-protein-coupled receptors (GPCRs), epidermal growth factor receptor (EGFR), and the divalent cation Zn^{2+} (Huang et al., 2008; Lee and Chao, 2001; Puehringer et al., 2013). Since these mechanisms are independent of neurotrophin binding and indirectly lead to receptor activation, they are defined as transactivation (Nagappan et al., 2008). Of these, the cation Zn^{2+} has been
suggested to be of potential importance for TrkB signaling in epileptogenesis (Huang et al., 2008; Pan et al., 2011).

In the nervous system, Zn$^{2+}$ is found as a both free and bound ion. In its free form, Zn$^{2+}$ is commonly localized to clear synaptic vesicles from which it can be released into the extracellular space in an activity-dependent manner (Choi and Koh, 1997; Frederickson and Danscher, 1990; Frederickson et al., 2005). Such free Zn$^{2+}$ is particularly prominent in the axonal boutons of mossy fibers in the hippocampus. The bound form of Zn$^{2+}$ is typically found in association with the intracellular protein metallothionein 3 (MT-3) (Huang and McNamara, 2012; Maret, 2000). This zinc-binding protein is found throughout the CNS including multiple neuronal populations in the hippocampus. To activate TrkB, this ion must be within the cytosol (Huang et al., 2008). Here, intracellular Zn$^{2+}$ binds to the C-terminal Src kinase (Csk) found within the Src-family kinase proteins (SFKs). Normally, Csk activity acts to prevent SFK activation. However, when bound to Zn$^{2+}$, Csk activity is impaired and thus its inhibition of SFK is relieved. In its active form, SFK can directly phosphorylate various tyrosine and serine residues within TrkB’s intracellular domain (Huang and McNamara, 2010; Huang et al., 2008). Like BDNF-mediated TrkB activation, phosphorylation of these various residues serves as docking sites for distinct adaptor proteins that trigger initiation of specific signaling cascades downstream of TrkB (Figure 1D, E, and F).
Figure 1: BDNF and Zn\(^{2+}\) mediated TrkB activation and signaling mechanisms

A) Non-active TrkB is found within the plasma membrane as a monomer. B) In response to BDNF binding, two TrkB monomers dimerize leading to receptor activation and subsequent autophosphorylation of various tyrosine residues within TrkB’s kinase domain (Y705 and Y706) and intracellular domain (Y515 and Y816). Additionally, receptor activation leads to activation of the kinase Cdk5 that in turn phosphorylates S478. C) Once phosphorylated, these serine and tyrosine residues serve as docking sites for various adaptor proteins that in turn initiate multiple signaling pathways. D) In the absence of BDNF, Zn\(^{2+}\) can activate TrkB through a transactivation mechanism. To do this, Zn\(^{2+}\) activates SFKs. Of note, we show TrkB as a dimer here but it is currently unknown if Zn\(^{2+}\) transactivation requires receptor dimerization. E) Once active, SFK will directly phosphorylate various tyrosine residues within TrkB’s kinase (Y705 and Y706) and intracellular domains (Y515 and Y816). We do not show S478 in this figure as it is currently unknown whether Zn\(^{2+}\)-mediated transactivation leads to S478
Following phosphorylation, these tyrosine residues serve as docking sites for various adaptor proteins from which multiple signaling pathways arise.

1.1.3.2 BDNF-TrkB signaling in SE-induced epilepsy

Multiple lines of evidence support the hypothesis that BDNF-TrkB signaling is required for SE-induced epilepsy. To begin, in situ hybridization targeted at BDNF messenger RNA (mRNA) has revealed that following SE, BDNF mRNA levels increase in the hippocampus and other brain regions (Ernfors et al., 1991; Isackson et al., 1991). Building on these findings, utilization of a BDNF-specific antibody in conjunction with immunohistochemistry has shown that SE leads to increased BDNF protein content throughout the hippocampus, especially the stratum lucidum region – the site of the mossy fiber pathway (Conner et al.).

Since TrkB is the primary receptor for BDNF, it was hypothesized that this observed increase in hippocampal BDNF might lead to a concurrent increase in TrkB activity. This question was probed utilizing antibodies binding to the phosphorylated form of TrkB. Consistent with the hypothesis, enhanced TrkB phosphorylation – a surrogate measure for TrkB activation (Segal et al., 1996) – was found in the hippocampus following SE (Binder et al., 1999a; Helgager et al., 2013). These results provide correlational evidence that in response to SE, both BDNF protein content and TrkB activation increase. This evidence is further supported by findings of similar
enhancements for BDNF content and TrkB activity in other, non-SE epilepsy models
(Danzer et al., 2003; He et al., 2004; He et al., 2002; He et al., 2010).

Regarding the requirement for both BDNF and TrkB in epilepsy, initial work did not utilize SE models for epilepsy but rather the kindling model of epileptogenesis. In this model, subconvulsive, intracerebral, focal electrical stimuli are repeatedly administered and with time, the seizures progress in duration, severity, and extent of brain structures recruited – a process called kindling (Goddard, 1967; Goddard et al., 1969). With enough stimuli, animals begin to display SRS thus defining them as epileptic.

Despite multiple differences between the kindling and SE-induced epilepsy models, insight into mechanisms underlying SE-induced epilepsy can still be gained through kindling. Specifically, kindling is impaired in the setting of reduced BDNF content achieved by either genetic (Bdnf heterozygous mice or Bdnf condition knockout mice) or pharmacologic means (intraventricular infusion of BDNF scavenging antibodies – TrkB-Fc) (Binder et al., 1999b; He et al., 2004). Similarly, reduced TrkB content in a Trkb conditional knockout animal also leads to impaired kindling (He et al., 2004). Together, these results provide additional evidence highlighting a critical role for BDNF and TrkB in epilepsy.

Building on TrkB’s established role in epilepsy, mutant mice containing various point mutations within TrkB were utilized to examine the downstream signaling
pathways of TrkB required in epilepsy (Minichiello et al., 2002; Minichiello et al., 1998). By comparing mutant mice containing either the Y515F or Y816F mutations, it was found that development of kindling was profoundly inhibited in the presence of the Y816F and not the Y515F mutation (He et al., 2010). This finding not only establishes a critical role for TrkB in SE-induced epilepsy but it also identifies the signaling pathway arising from TrkB that may be contributing to epilepsy, namely PLCγ1.

This critical role for TrkB signaling within SE-induced epilepsy makes it a very promising therapeutic target. However, an important question with clinical implications is whether TrkB inhibition is anti-convulsant or anti-epileptogenic. Unfortunately though, the genetic TrkB perturbations described above do permit distinguishing these possibilities. Rather, what is needed is a method to acutely and transiently inhibit TrkB activity following SE. Such an experiment would allow one to dissect the anti-epileptogenic potential of TrkB inhibition. The McNamara Lab recently accomplished this using a previously described chemical-genetic approach for specific and reversible TrkB inhibition (Chen et al., 2005). With this approach, the McNamara Lab demonstrated that two weeks of TrkB inhibition commencing immediately after SE termination is able to inhibit epilepsy onset during the treatment period as well as in the weeks that follow (Helgager et al., 2013). Since this chemical-genetic approach is rapidly reversible, the duration of the effects on seizures observed strongly argue that acute, post-SE TrkB inhibition is impairing the epileptogenic process and as such, it can have
long-lasting impact on seizure occurrence, well after the effects on TrkB activity wear off.

Of all the results described above, this final result – transient TrkB inhibition following SE prevents epilepsy development – provides the most compelling argument that TrkB activity is required for SE-induced epileptogenesis. As such, it defines TrkB signaling as one potential molecular mechanism through which SE can convert a normal brain into an epileptic one. Furthermore, it identifies TrkB as a novel therapeutic target with disease-modifying potential.

1.1.4 Cellular mechanisms of SE-induced TLE – a role for structural plasticity?

With TrkB activation identified as one molecular mechanism contributing to SE-induced epilepsy, the next question is what are the cellular consequences of such activation. To this end, the McNamara Lab has recently shown that one locale for SE-induced TrkB activation is within apical dendritic spines of hippocampal CA1 pyramidal cells (Helgager et al., 2013). Such spine localization provides clues to both the anatomic locale as well as the manifestation of the cellular consequences downstream of TrkB activation. Specifically, CA1 apical dendritic spines are the post-synaptic component of the Schaffer collateral-CA1 synapse within the hippocampus (Harris et al., 1992). During SE, the Schaffer collateral axons experience repetitive and synchronous firing that leads to robust glutamate release into the synaptic cleft (McNamara et al., 2006). Similar patterns of glutamate release can be induced artificially with either high
frequency or tetanic stimulation of Schaffer collateral axons via a stimulating electrode (Kumar, 2010; Minichiello, 2009). Such electrical stimulation has been associated with both functional and structural potentiation of the Schaffer collateral-CA1 synapse (Kumar, 2010; Matsuzaki et al., 2004). These observations led us to propose that one cellular consequence of SE-induced dendritic spine TrkB activation could be synaptic potentiation.

To explore this hypothesis, we sought a simplified and reduced experimental preparation. We ultimately chose two-photon glutamate uncaging evoked structural plasticity of CA1 dendritic spines (Lee et al., 2009; Matsuzaki et al., 2004; Murakoshi et al., 2011; Yasuda et al., 2006) because of its ease of implementation and its parallels to seizure activity. With this approach, we began our work by explore TrkB’s role, if any, within structural plasticity.

1.2 Functional and structural plasticity of the Schaffer Collateral – CA1 synapse

How does the brain learn and remember? This has been a question that has fascinated neuroscientists for centuries. Over time, numerous and sometimes wild speculations have been put forth. Of these though, it was the hypothesis of Ramon y Cajal that has emerged dominant. He was the first to note the existence of small protrusion from the dendrites of neurons. These protrusions, later known as dendritic spines, led Ramon y Cajal to describe the neuron doctrine in which he argues that neurons are connected and communicate via synapses. Based on this idea, Ramon y
Cajal went on to suggest that the mechanism underlying how the brain learns and remembers is modulation of the strength of these synapses.

For years after Ramon y Cajal’s initial hypothesis, the cellular and molecular mechanisms underlying synaptic modulation in learning and memory remained elusive. However, all this changed thanks to the work of Bliss and Lomo in the 1960s. By studying the hippocampi of rabbits, these two neuroscientists uncovered the cellular phenomenon of long-term potentiation (LTP) (Bliss and Lomo, 1973). With this finding, the budding sub-field of synaptic plasticity originated within Neurobiology.

Since the pioneering working of Bliss and Lomo, our understanding of synaptic plasticity has blossomed. We know recognize multiple, distinct forms of long-lasting plasticity including LTP as well as LTD (long-term depression). We realize that such plasticity occurs in multiple mammalian species (Douglas and Goddard, 1975; Nosten-Bertrand et al., 1996; Urban et al., 1995) including humans (Beck et al., 2000; Chen et al., 1996) as well as at numerous synapses throughout the CNS. And recently, we have begun to definitively associate synaptic plasticity with learning and memory thanks to recent work demonstrate that synaptic plasticity may be the cellular mechanism underlying these processes (Abel et al., 1997; Bliss and Collingridge, 1993; Malenka and Bear, 2004; Martin et al., 1999; Nabavi et al., 2014).

Despite the heterogeneity of synaptic plasticity in terms of its direction and synaptic location, LTP of the hippocampal Schaffer collateral-CA1 synapse has been one
of the most extensively studied (Figure 2). Because this synapse is also implicated in SE-induced epilepsy, our discussion below focuses on LTP mechanisms observed at this synapse.

![Hippocampal Anatomy](image)

**Figure 2: Hippocampal Anatomy**

The classic circuit for the hippocampal formation involves excitatory projection from layer II cells in the entorhinal cortex (EC) projecting to granule cells in the dentate gyrus (DG). These granule cells then send axons, called mossy fibers, into area CA3 where they synapse on thorny excrescences on the apical dendrites of CA3 pyramidal cells. These CA3 cells then project to area CA1 as Schaffer collateral axons that synapse on spines of the apical dendrite of CA1 pyramidal cells. CA1 will then complete the circuit by projecting back to the EC. It should be noted, that this is a greatly simplified description of hippocampal anatomy, but it does define the main synapses implicated in both epilepsy and plasticity, namely the mossy-fiber-CA3 synapse and the Schaffer collateral-CA1 synapse.

### 1.2.1 Functional plasticity at the Schaffer Collateral-CA1 synapse

Functional plasticity is defined as long-lasting changes in strength and duration of synaptic transmission. As we mention above, such plasticity is bidirectional in that it can be an enhancement (LTP) or a reduction (LTD) in a synapse’s functional properties.
Exploration of synaptic plasticity at the hippocampal Schaffer collateral-CA1 synapse has helped define some of general properties of synaptic plasticity found at this synapse as well as throughout the CNS.

To begin, study of this synapse has revealed that plasticity induction and expression are dependent on the activity pattern preceding their development. For example, multiple stimulation paradigms have been used to induce LTP at the Schaffer collateral-CA1 synapse including high frequency stimulation, theta-burst stimulation, and paired pre-synaptic stimulation with post-synaptic depolarization. Each of these paradigms yields a functional enhancement of the synapse’s properties (LTP) but, the kinetics, duration, and properties of the produced LTP vary greatly depending on the paradigm (Collingridge et al.; Grover; Grover and Teyler, 1990; Harris et al.; Morris et al., 1985). A second rule for plasticity elucidated from this synapse is that it is input-specific meaning that only stimulated synapses gets potentiated (Andersen et al.; Lynch et al., 1977). With this said however, a third rule of plasticity is that it is associative in that even though non-stimulated synapses are not initially potentiated, their threshold for future potentiation is lowered by the fact that nearby synapses underwent potentiation. As such, a weak, normally non-LTP inducing stimulus can now be used to potentiate these neighboring synapses (Lu et al., 2011).

In addition to these general rules for plasticity, study of the Schaffer collateral-CA1 synapse has identified many of the mechanisms underlying LTP. Specifically,
multiple lines of evidence show that LTP is comprised of three distinct functional events – induction, maintenance, and expression (Sweatt, 1998). Induction is defined as the event that actually triggers the initial onset of LTP. For example, in response to synaptic stimulation, induction is commonly defined as Ca\textsuperscript{2+} influx resulting from glutamate binding to various glutamate receptors. Maintenance builds from induction and consists of the biochemical changes (signaling cascades activated) that arise in response to synaptic stimulation. For example, Ca\textsuperscript{2+} influx during induction can activate a wide array of proteins, such as CaMKII and protein kinase C (PKC), which will in turn trigger a series of signaling pathways with long-lasting effects. The long-lasting effects of these biochemical changes are defined as expression. Examples include changes in gene transcription and protein translation that can lead to increased post-synaptic glutamate sensitivity or enhanced neurotransmitter release at the pre-synapse.

At the Schaffer collateral-CA1 synapse, induction is largely considered to be post-synaptic in origin (Kumar, 2010). Specifically, Schaffer collateral stimulation leads to glutamate release into the synaptic cleft. Here, glutamate will bind to various post-synaptic glutamate receptors including both AMPARs and NMDARs. When glutamate binds to AMPARs, the receptor’s ion channel opens leading to Na\textsuperscript{+} influx (Kumar, 2010; Sweatt, 1998). Conversely, when glutamate binds to NMDARs, the receptor’s ion channel does not initially open because of its voltage-dependent Mg\textsuperscript{2+} block. However, if glutamate binding occurs in parallel with membrane depolarization – as may result
from AMPAR-mediated Na$^+$ influx – then the Mg$^{2+}$ block is relieved and the NMDAR’s ion channel opens becoming permeable to various cations (Kumar, 2010; Minichiello, 2009). Of these ions, the most prevalent and most important for plasticity is Ca$^{2+}$. Its influx into the post-synaptic compartment leads to a rapid rise in intracellular Ca$^{2+}$ concentrations that proves to be a key event underlying LTP induction – without it, LTP will not ensue (Kumar, 2010; Sweatt, 1998).

Following this rapid rise in intracellular Ca$^{2+}$ levels, several Ca$^{2+}$ sensitive proteins become activated including CaMKII and PKC (Lisman, 1994; Nishizuka, 1992). Once active, both of these kinases phosphorylate and activate various second messenger molecules thereby triggering multiple signaling pathways that underlie LTP maintenance (Klann et al., 1993; Lisman, 1994; Sacktor et al., 1993)

The signaling pathways triggered by CaMKII and PKC activation primarily involve transient biochemical changes – for example protein phosphorylation and dephosphorylation. How these transient changes are converted into long-lasting changes underlying LTP expression largely depends on the sub-cellular locale of expression – pre- versus post-synaptic.

Pre-synaptic mechanisms of expression manifest as enhanced neurotransmitter release (Minichiello, 2009). Multiple mechanisms have been proposed for such enhancement. For one such mechanism, it has been hypothesized that following induction of post-synaptic signaling, a retrograde signal is transmitted from the post-
synapse to the pre-synapse where it then initiates a second round of signaling. This pre-
synaptic signaling culminates in modifications of the pre-synaptic terminal that yield
increased neurotransmitter release. A second proposed mechanism ignores the post-
synapse entirely and instead suggests that pre-synaptic activity leads to release of a
neuromodulator that then acts in an autocrine fashion to enhance neurotransmitter. One
such neuromodulator implicated in this process is BDNF. Regardless of the mechanism,
with more neurotransmitter, the synapse is activated more strongly and has thus been
potentiated (Kumar, 2010; Minichiello, 2009).

Post-synaptic mechanisms of expression can manifest in several ways. First,
CaMKII activation during the maintenance phase can trigger a signaling cascade that
leads to insertion of AMPARs into the post-synaptic density (Murakoshi and Yasuda,
2012; Patterson and Yasuda, 2011). With more receptors, this terminal is now more
sensitive to glutamate and can thus respond more strongly. A second mechanism is
through modulation of gene transcription and subsequent protein expression. For
example, one molecule downstream of CaMKII is the GTPase Ras (Yasuda et al., 2006).
It has been shown that during LTP, CaMKII activation leads to transient Ras activation
that lasts for only 15 to 20 minutes (Yasuda et al., 2006). However, downstream of Ras
is ERK, a kinase that when active, gets transported to the nucleus where it
phosphorylates and regulates the activity of various transcription factors. Through this
action, transient Ras-ERK signaling can mediate long-lasting changes in gene
transcription and subsequent protein translation (Zhai et al., 2013). This altered protein expression is critical for mediating the long-lasting changes that support and maintain long-lasting functional enhancement of stimulated synapses.

1.2.2 Structural plasticity of CA1 dendritic spines

In the central nervous system, most post-synaptic terminals of excitatory synapses are found within dendritic spines. These spines have a characteristic appearance: they typically have a spherical head separated from the dendrite by a long, thin neck. Based on this structure, it has been suggested that spines act as self-contained, sub-cellular compartments where biochemical signaling can occur independently of the dendrite and neighboring spines.

During synaptic potentiation of the Schaffer Collateral-CA1 synapse, the size of these dendritic spines has been shown to increase. The pattern of this increase is quite stereotyped in that immediately after stimulation, spine size rapidly increases and after a few minutes, it peaks. Following this rapid growth, the spine volume slowly decays and around 10 to 20 minutes after initial stimulation, the spine volume stabilizes to a level that is higher than its pre-stimulation state (Figure 3) (Lee et al., 2009; Matsuzaki et al., 2004; Murakoshi et al., 2011; Yasuda et al., 2006). The functional significance of the initial, transient rise in spine volume remains unclear. However, the sustained elevation in spine size seen after 10 minutes correlates with the extent of functional enhancement of the synapse’s properties (Lee et al., 2009; Matsuzaki et al., 2004; Murakoshi et al.,
This connection between functional and structural plasticity is attributed to the accumulation of AMPARs in the spine during LTP – the more AMPARs that are inserted, the larger the spine’s volume and also the greater its synaptic potentiation (Patterson et al., 2010). Like synaptic LTP, the enhanced spine volume can persist for hours and as such, it is commonly called structural LTP (sLTP).

Figure 3: Time course of dendritic spine volume change in response to glutamate uncaging

Following two-photon glutamate uncaging (30 stimulations, 0.5 Hz, 4-6 ms pulse duration) targeted just beyond the head of a single dendritic spine, spine volume rapidly increases by 400-500% and peaks shortly after termination of glutamate uncaging.
(transient phase, defined as average spine volume 1-2 minutes after onset of stimulation). Following this peak, spine volume decays to a stable volume that is approximately 80 to 100% larger than baseline (sustained phase, defined as spine volume >10 minute after onset of stimulation).

sLTP is associated with multiple stimulation paradigms – high frequency electrical stimulation of Schaffer Collateral axons, theta-burst electrical stimulation of Schaffer Collateral axons, and two-photon glutamate uncaging. Interestingly, despite the distinct stimulation paradigms, all produce similar patterns of spine volume change – transient rise, decay, and then sustained elevation. However, of these paradigms, two-photon glutamate uncaging has recently risen in popularity due to its presumed ability to selectively activate the post-synaptic terminal. By isolating induced activity to the post-synapse, experimental design and interpretation are greatly simplified.

Central to two-photon glutamate uncaging is the molecule MNI-L-glutamate (caged glutamate) (Matsuzaki et al., 2004). This synthetic molecule consists of the neurotransmitter glutamate surrounded by a chemical cage (Matsuzaki et al., 2001). One can apply this molecule to a hippocampal slice and then using two-photon laser excitation, the chemical cage can be photo-activated leading to release of glutamate (Matsuzaki et al., 2001; Matsuzaki et al., 2004). Because of the point-spread-function for two-photon lasers, the diameter of glutamate uncaging in the x-y plane is approximately 1 µm or the width of an average dendritic spine. As such, the area of glutamate release is quite small and thus can be targeted to single dendritic spines (Figure 4) (Lee et al., 2009; Matsuzaki et al., 2001; Matsuzaki et al., 2004). If performed in the absence of
extracellular Mg\(^2+\), glutamate uncaging is sufficient to activate post-synaptic NMDARs in a manner mimicking pre-synaptic glutamate release (Lee et al., 2009).

![Diagram of axon and dendrite with caged and free glutamate](image)

**Figure 4: Single spine two-photon glutamate uncaging**

In our experiments, we utilize a two-photon laser tuned to 720 nm to focally activate caged glutamate at the head of single dendritic spines. Due to the point-spread function of the two-photon laser, the diameter of uncaging is approximately 1 µm – slightly smaller than the average size of a dendritic spine. As such, our stimulation only activates caged glutamate near the head of the spine thus allowing for spine specific stimulation.

Utilizing glutamate uncaging, the mechanisms underlying sLTP have begun to be delineated. To begin, glutamate binds post-synaptic NMDARs and since these are commonly performed in the absence of Mg\(^2+\), the ion channel of the receptor opens permitting Ca\(^{2+}\) influx (Matsuzaki et al., 2004; Sobczyk et al., 2005). Once in the spine head, this Ca\(^{2+}\) leads to CaMKII activation via the Ca\(^{2+}\) binding protein calmodulin (Lee et al., 2009; Lisman, 1994). CaMKII activation then initiates multiple signaling pathways involving several of the Rho GTPases – Cdc42, RhoA, and Rac1 – as well as Ras (Murakoshi et al., 2011; Murakoshi and Yasuda, 2012; Patterson and Yasuda, 2011;
Yasuda et al., 2006). The activity of these GTPases induces actin cytoskeleton reorganization, membrane trafficking, and the insertion of AMPARs into the post-synaptic membrane – all events that contribute to the structural and functional enhancement of the spine (Figure 5) (Murakoshi and Yasuda, 2012; Patterson et al., 2010). As highlighted here, the ease of two-photon glutamate uncaging plus the parallels between functional and structural plasticity have established sLTP as an excellent system for dissecting molecular mechanisms underlying synaptic plasticity.

![Diagram of molecular mechanisms underlying sLTP](image)

**Figure 5: Current molecular mechanisms underlying sLTP**

In response to glutamate uncaging, NMDARs activate and become permeable to Ca$^{2+}$. Ca$^{2+}$ into the spine activates CaMKII leading to the activation of various downstream
GTPases – Ras, RhoA, Cdc42, and Rac1. These GTPases, through their ability to modulate actin cytoskeleton organization, contribute to sLTP induction and expression.

**1.2.3 BDNF-TrkB signaling in functional and structural plasticity at the Schaffer collateral-CA1 synapse**

Like SE-induced epilepsy, multiple lines of evidence implicate BDNF and TrkB as critical mediators of plasticity at the Schaffer collateral-CA1 synapse. Regarding functional plasticity, BDNF and TrkB have been implicated in both the maintenance and expression phases of LTP. First, using *in situ* hybridization, it was shown that BDNF mRNA levels increase within CA1 pyramidal cells in response to LTP-inducing stimulations (Patterson et al., 1992; Pozzo-Miller et al., 1999). Building on this correlation, later work demonstrated that in mice lacking BDNF, LTP is impaired but can be rescued with either exogenous BDNF application or virally induced BDNF expression in CA1 pyramidal cells exclusively. Additionally, application of an extracellular BDNF scavenging antibody (TrkB-Ig) has been shown to block LTP induced through a variety of paradigms (Chen et al., 1999; Figurov et al., 1996; Kang et al., 1997; Korte et al., 1997). Consistent with these findings for BDNF, mice lacking TrkB also show impairments in LTP (Minichiello et al., 1999; Pozzo-Miller et al., 1999; Xu et al., 2000). Finally, there is also evidence that BDNF can facilitate LTP (Kovalchuk et al., 2002). Collectively, these results delineate a critical role TrkB and its canonical ligand BDNF in functional plasticity of the Schaffer collateral-CA1 synapse.
Despite all this evidence for BDNF-TrkB signaling in functional plasticity, its role in structural plasticity is not as clear. To date, there are only a few lines of evidence supporting a role for BDNF and TrkB signaling in structural plasticity (Lai et al., 2012; Rex et al., 2007; Tanaka et al., 2008). However, multiple technical issues confound interpretation of these results (see chapter 3). As such, defining a role for both BDNF and TrkB in sLTP is currently an open question needing further investigation.
2. Materials and Methods

2.1 Reagents

Human recombinant BDNF and human recombinant beta-nerve growth factor (NGF) were purchased from Millipore. K252a, D-2-amino-5-phosphonovalerate (AP5), and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) were purchased from Tocris. 1-(1,1-dimethylethyl)-3-(1-naphthalenylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (1NMPP1) was purchased from Santa Cruz. Human-IgG was purchased from Sigma. TrkB-Ig was a gift from Regeneron. The tat-CN21 peptide (YGRKKRRQRRRKRRPPKLGQIGRSKRVIEDDR) was synthesized by GenScript.

2.2 Plasmids

2.2.1 TrkB-mEGFP

TrkB-mEGFP was prepared by inserting the coding sequence of mouse TrkB – obtained from a previously described plasmid (Huang and McNamara, 2010) – into pEGFP-N1 (Clontech) containing the A206K monomeric mutation in EGFP (Zacharias et al., 2002) and the CAG promoter. The linker between TrkB and mEGFP is TGRH.

TrkB\textsuperscript{Y816F}-mEGFP was prepared by introducing a point mutation using the Site-Directed Mutagenesis Kit (Stratagene).
2.2.2 mRFP-PLC-mRFP

mRFP-PLC-mRFP was prepared by inserting the coding sequence for the carboxy-terminal SH2 domain of human PLCγ1 (659-769; obtained from full-length, human PLCγ1 purchased from Origene) into a tandem-mRFP plasmid containing the CAG promoter. The linkers between the mRFPs and PLCγ1 (659-769) are RSRAQASNS for the amino-terminus and GSG for the carboxy-terminus.

2.2.3 BDNF-SEP

HA-BDNF-Flag was a kind gift of Dr. Anne West. The coding sequence for SEP – obtained from SEP-GluA (Patterson et al., 2010) – was incorporated onto the 3’ end of HA-BDNF-Flag to generate HA-BDNF-Flag-SEP (BDNF-SEP). HA-BDNF-Flag-mRFP was generated in a similar fashion (BDNF-mRFP).

2.2.4 POMC-mCherry

POMC-mCherry was generated by amplifying the POMC peptide – MMWCLESSQCQDLTTESNLLACIRACRLDL (Lou et al., 2005) – using overhang PCR with a C-terminal linker –

GGGGGGGGGCGGGGGGGGGGGGGGGGGMADQLTEEWHRGPGS. This amplicon was then inserted into the tandem mCherry plasmid by replacing the coding sequence of the first mCherry.
2.2.5 mEGFP-Rac1

mEGFP-Rac1 was prepared by inserting the coding sequence of human Rac1 – a kind gift from Dr. M. Matsuda – into pEGFP-C1 (Clontech) containing the A206K monomeric mutation in EGFP (Zacharias et al., 2002) and the CAG promoter.

2.2.6 mCherry-Pak2-mCherry

mCherry-Pak2-mCherry was prepared by inserting a fragment of human Pak1 coding sequence (65 – 118) – a kind gift from Dr. Scott Soderling – into a tandem mCherry plasmid that has previously been described (Murakoshi et al., 2011). The CRIB domain of Pak2 was generated by introduction mutations R27C and S79A into the Pak1 using a Site-Directed Mutagenesis kit (Strategene).

2.2.7 Additional plasmids

A plasmid containing mEGFP-CaMKIIα-REACh (green-Camuia; the CaMKII FRET sensor) was prepared as previously described (Lee et al., 2009). Plasmids containing mEGFP-RhoA and mCherry-Rhotekin-mCherry (RhoA FRET sensor) was prepared as previously described (Murakoshi et al., 2011). Plasmids containing mEGFP-Cdc42 and mCherry-Pak3-mCherry (Cdc42 FRET sensor) was prepared as previously described (Murakoshi et al., 2011). A plasmid containing mCherry-IRES-TeTX (Tetanus toxin light chain) was a kind gift of Dr. Michael Ehlers. pCAG-mCherry-mCherry was generated as previously described (Murakoshi et al., 2011).
2.3 Mammalian cell and slice preparations

2.3.1 HeLa cells

HeLa cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin/streptomycin (100 units/100 µg per mL; Sigma). Cells were maintained in a humidified incubator at 37°C and 5% CO₂. Cells were transfected with Lipofectamine 2000 using the manufacturer’s protocol (Invitrogen). Concentrations used were 0.5 µl/mL Lipofectamine and 1 µg/mL total cDNA (1:1 ratio of TrkB-mEGFP to mRFP-PLC-mRFP plasmid DNA).

For imaging experiments, culture media was replaced after 24 to 48 hours of transfection with imaging HEPES-buffered artificial cerebral spinal fluid (iHACSF: 25 mM HEPES pH 7.4, 127 mM NaCl, 25 mM NaHCO₃, 25 mM D-glucose, 2.5 mM KCl, 1.25 mM Na₂HPO₄, and 310 mOsm). After a 30 min equilibration period, transfected cells were imaged using 2pFLIM as described below. Cell stimulation was performed by directly adding BDNF (100 ng/ml) or vehicle to the cells.

For biochemistry experiments, cultured media was replaced after 24 to 48 hours of transfection with biochemistry HEPES-buffered artificial cerebral spinal fluid (bHACSF: 10 mM HEPES pH 7.35, 150 mM NaCl, 20 mM glucose, 3 mM KCl, and 310 mOsM). After a 30 min equilibration period, cells were stimulated by directly adding BDNF (100 ng/ml) to the cells.
2.3.2 Mixed cortical cultures

Mixed cortical cultures were prepared as described previously (Xiong and McNamara, 2002). Briefly, cerebral cortices from embryonic day 18 rat embryos were dissected and then dissociated with Hank’s balanced salt solution (HBSS; Gibco) free of Ca\(^{2+}\) and Mg\(^{2+}\) but supplemented with 10 mM HEPES (pH 7.4), 33.3 mM glucose, 5 \(\mu\)g/ml gentamicin, 100 \(\mu\)g/ml DNase, and 0.25% trypsin for 12 minutes at 37°C. The cortices were then washed in plating media [Neurobasal (Gibco) supplemented with 10% heat-inactivated FBS (Sigma), 2% B27 supplement without vitamin A (Gibco), 1% glutamax I (Gibco), and 100 units/100 \(\mu\)g per mL of penicillin/streptomycin (Sigma)] followed by trituration through a fire-polished Pasteur pipette. Neurons were pelleted through microcentrifugation at 16,000 × g for 10 min at 4°C and then plated at a density of 1,250,000 cells per 3.5 cm dish. Dishes used had previously been coated with poly-D-lysine (Sigma). Neurons were maintained in a humidified incubator at 37°C with 5% CO\(_2\). Plating media was completely replaced by culture media (Neurobasal supplemented with 2% B27 supplement and 1% glutamax I) at DIV 1. From this point forward, half-media changes were done every 2 to 3 days until neurons were used (typically around DIV 10 to 12).

Neurons were transfected with Lipofectamine 2000 using a modified protocol. 1 \(\mu\)l Lipofectamine was mixed with plasmid DNA (1 \(\mu\)g per construct transfected) in 100 \(\mu\)l of culture media for 20 minutes. Culture media was removed from the 3.5 cm dish
until only 1 ml remained. The Lipofectamine/DNA solution was added to the neurons for 45 minutes. At this point, all the media was removed and replaced with 2 mL conditioned culture media. 24 to 48 hours later, culture media was replaced with iHACSF (described above). For zinc experiments, a modified HACSF lacking NaHCO₃ and NaH₂PO₄ was used, because in the presence of these molecules, zinc precipitated out of solution. After a 30 min equilibration period, transfected neurons were imaged using 2pFLIM as described below. To stimulate cells, we added BDNF, NGF, or zinc directly to the neurons. 30 min after stimulation, we then added K252a to the neurons.

2.3.3 Cultured hippocampal slices

Cultured hippocampal slices were prepared from postnatal day 5 to 7 rats or mice, as previously described (Stoppini et al., 1991), in accordance with the animal care and use guidelines of Duke University Medical Center. Briefly, we deeply anaesthetized the animal with isoflurane, quickly decapitated it, and then excised the brain. The hippocampi were dissected and cut into 350 μm coronal sections using a McIlwain tissue chopper. Hippocampal slices were plated on tissue culture inserts (Millicell) and fed with tissue media (for 2.85 L: 20.95 g MEM, 17.9 g HEPES, 1.1 g NaHCO₃, 5.8g D-Glucose, 120 μL 25% ascorbic acid, 12.5 mL L-Glutamine, 2.5 mL Insulin, 500 mL Horse Serum, 5 mL 1M MgSO₄ and 2.5 mL 1 M CaCl₂ with pH and osmolality adjusted to 7.5 and 310 mOsm respectively). Slices were maintained in a humidified incubator with 3% CO₂ and 35°C (the final pH of the media in the incubator was ~ 7.2).
After 5 to 12 days in culture, CA1 pyramidal neurons were transfected with biolistic gene transfer using gold beads (12 mg; Biorad) coated with plasmids containing 20 to 40 µg of total cDNA (Table 1). Of note, in order to utilize the TrkB sensor in the setting of post-synaptic BDNF knockout (Bdnf<sup>fl/fl</sup> plus Cre), we had to transfect for at least 5 days to ensure adequate inhibition of BDNF expression. Unfortunately, we discovered that if TrkB sensor expression was too high, neurons commonly did not survive the required 5 days. To limit expression and promote neuron survival, we found that co-transfection of the TrkB sensor with mCherry sufficiently limited TrkB sensor expression so as to minimally impact neuron health.

**Table 1: Plasmid DNA amounts for Biolistic Transfection**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Transfection Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>mEGFP (Rat and TrkB&lt;sup&gt;F616A&lt;/sup&gt; slices)</td>
<td>20 µg mEGFP</td>
</tr>
<tr>
<td>mEGFP (TrkB&lt;sup&gt;+/+&lt;/sup&gt; and Bdnf&lt;sup&gt;+/+&lt;/sup&gt; slices – Cre negative cells)</td>
<td>20 µg mEGFP</td>
</tr>
<tr>
<td>mEGFP (TrkB&lt;sup&gt;+/+&lt;/sup&gt; and Bdnf&lt;sup&gt;+/+&lt;/sup&gt; slices – Cre positive cells)</td>
<td>20 µg mEGFP, 10 µg tdTomato-&lt;wbr/&gt;Cre</td>
</tr>
<tr>
<td>TrkB Sensor (Hela cells, cultured neurons, and rat Slices)</td>
<td>15 µg TrkB-&lt;wbr/&gt;mEGFP, 15 µg mRFP-&lt;wbr/&gt;PLC-&lt;wbr/&gt;mRFP</td>
</tr>
<tr>
<td>Plasmids</td>
<td>Transfection Time</td>
</tr>
<tr>
<td>----------</td>
<td>------------------</td>
</tr>
<tr>
<td>5 µg TrkB-mEGFP</td>
<td>5 µg mRFP-PLC-mRFP</td>
</tr>
<tr>
<td>5 µg TrkB-mEGFP</td>
<td>5 µg mRFP-PLC-mRFP</td>
</tr>
<tr>
<td>5 µg TrkB-mEGFP</td>
<td>5 µg mRFP-PLC-mRFP</td>
</tr>
<tr>
<td>20 µg BDNF-SEP</td>
<td>10 µg mCherry-mCherry</td>
</tr>
<tr>
<td>20 µg BDNF-SEP</td>
<td>10 µg mCherry-IRES-TeTX</td>
</tr>
<tr>
<td>20 µg BDNF-SEP</td>
<td>10 µg POMC-mCherry</td>
</tr>
<tr>
<td>10 µg mEGFP</td>
<td>10 µg mCherry-mCherry</td>
</tr>
<tr>
<td>10 µg mEGFP</td>
<td>10 µg mCherry-mCherry</td>
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<tr>
<td>10 µg mEGFP</td>
<td>10 µg mCherry-mCherry</td>
</tr>
<tr>
<td>10 µg mEGFP</td>
<td>10 µg mCherry-mCherry</td>
</tr>
<tr>
<td>20 µg green-Camuia</td>
<td>10 µg mCherry-mCherry</td>
</tr>
</tbody>
</table>
2.3.4 Acute hippocampal slices

Acute hippocampal slices were prepared from mice ages P28 to P42 as described previously (Pan et al., 2011). Briefly, mice were anesthetized with pentobarbital,
decapitated, and the hippocampi dissected out. Using this isolated hippocampus, 400 μm slices were cut and then used on the day of cutting.

2.4 Biochemical analysis of Hela cells

2.4.1 Immunoprecipitation

HeLa cells were transfected with the TrkB sensor (TrkB-EGFP and RFP-PLC-RFP) using Lipofectamine 2000 and stimulated with BDNF as described above. Following stimulation, cells were washed in ice-cold phosphate buffered saline (PBS, Gibco), and then lysed in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, and protease inhibitors) for 10 minutes on ice. The supernatant was collected after a 10 min centrifugation at 16,000 x g at 4°C. At this point, a small volume of the supernatant was added to 5x-SDS-Sample Buffer (250 mM Tris pH 6.8, 10% sodium dodecyl sulphate, 30% glycerol, 2 mg bromophenol blue, and 5% beta-mercaptoethanol) and saved as the “Cell Lysate” sample. The remaining supernatant was pre-cleared using protein G-Sepharose beads (25 μl, Roche) for 30 min at 4°C. After pre-clearing, the supernatant was incubated with 20 μg mouse monoclonal anti-phosphotyrosine (BD Transduction Labs) at 4°C overnight. The immunocomplexes were precipitated with protein G-Sepharose beads (50 μl) for 3 hours at 4°C and then analysed with Western blotting as described below.
2.4.2 Western Blotting

Western blotting was conducted as previously described (He et al., 2010). Antibodies used include TrkB (1:1000; Millipore), GFP (1:1000; Abcam), β-actin (1:10000; Sigma), and pTrkB(Y515) (Sigma). Secondary antibodies used were horseradish peroxidase conjugated goat-anti-rabbit. Visualization of secondary antibody binding was accomplished using enhanced chemiluminescent (ECL) reagents from GE Healthcare.

2.5 Two-photon fluorescence lifetime imaging (2pFLIM)

FRET imaging using a custom-built two-photon fluorescence lifetime imaging microscope was performed as previously described (Lee et al., 2009; Murakoshi et al., 2008; Murakoshi et al., 2011; Yasuda, 2006; Yasuda et al., 2006). Briefly, two-photon imaging was performed using a Ti-sapphire laser (MaiTai, Spectraphysics) tuned to a wavelength of 920nm, allowing simultaneous excitation of mEGFP, mRFP, mCherry, and SEP. All samples were imaged using <2mW laser power measured at the objective. Fluorescence emission was collected using an immersion objective (60x, numerical aperture 0.9, Olympus), divided with a dichroic mirror (565nm), and detected with two separate photoelectron multiplier tubes (PMTs) placed downstream of two wavelength filters (Chroma, HQ510-2p to select for green and HQ620/90-2p to select for red). The green channel was fitted with a PMT having a low transfer time spread (H7422-40p; Hamamatsu) to allow for fluorescence lifetime imaging, while the red channel was fitted
with a wide-aperture PMT (R3896; Hamamatsu). Photon counting for fluorescence lifetime imaging was performed using a time-correlated single photon counting board (SPC-140; Becker and Hickl) controlled with custom software (Yasuda, 2006), while the red channel signal was acquired using a separate data acquisition board (PCI-6110) controlled with Scanimage software (Pologruto et al.).

## 2.6 Two-photon glutamate uncaging

A second Ti:Sapphire laser tuned at a wavelength of 720 nm was used to uncage 4-Methoxy-7-nitroindolinyl-caged-L-glutamate (MNI-caged L-glutamate) in extracellular solution with a train of 4 to 6 ms, 4 to 5 mW pulses (30 times at 0.5 Hz) near a spine of interest. Experiments were performed in Mg$^{2+}$ free artificial cerebral spinal fluid (ACSF; 127 mM NaCl, 2.5 mM KCl, 4 mM CaCl$_2$, 25 mM NaHCO$_3$, 1.25 mM NaH$_2$PO$_4$ and 25 mM glucose) containing 1 µM tetrodotoxin (TTX) and 4 mM MNI-caged L-glutamate aerated with 95% O$_2$ and 5% CO$_2$. Experiments were performed at 24 to 26°C (room temperature) or 30 to 32°C using a heating block holding the ACSF container. Temperature measurements were made from ACSF within the perfusion chamber holding the slice.

## 2.7 2pFLIM data analyses

To measure the fraction of donor bound to acceptor, we fit a fluorescence lifetime curve summing all pixels over a whole image with a double exponential function convolved with the Gaussian pulse response function:
\[ F(t) = F_0 [P_D H(t, t_0, \tau_D, \tau_G) + P_D H(t, t_0, \tau_{AD}, \tau_G)] \]  \hspace{1cm} \text{Eq. S1}

where \( \tau_{AD} \) is the fluorescence lifetime of donor bound with acceptor, \( P_D \) and \( P_{AD} \) are the fraction of free donor and donor bound with acceptor, respectively, and \( H(t) \) is a fluorescence lifetime curve with a single exponential function convolved with the Gaussian pulse response function:

\[
H(t, t_0, \tau_D, \tau_G) = \frac{1}{2} \exp \left( \frac{\tau_G^2}{2\tau_D^2} \left( \frac{t - t_0}{\tau_D} \right) \right) \text{erfc} \left( \frac{\tau_G^2 - \tau_D(t - t_0)}{\sqrt{2\tau_D\tau_G}} \right) \]  \hspace{1cm} \text{Eq. S2}

in which \( \tau_D \) is the fluorescence lifetime of the free donor, \( \tau_G \) is the width of the Gaussian pulse response function, \( F_0 \) is the peak fluorescence before convolution and \( t_0 \) is the time offset, and erfc is the error function.

We fixed \( \tau_D \) to the fluorescence lifetime obtained from free mEGFP (2.59 ns). To generate the fluorescence lifetime image, we calculated the mean photon arrival time, \( \langle t \rangle \), in each pixel as:

\[
\langle t \rangle = \frac{\int tF(t) \, dt}{\int F(t) \, dt}, \]  \hspace{1cm} \text{Eq. S3}

then, the mean photon arrival time is related to the mean fluorescence lifetime, \( \langle \tau \rangle \), by an offset arrival time, \( t_0 \), which is obtained by fitting the whole image:

\[
\langle \tau \rangle = \langle t \rangle - t_0. \]  \hspace{1cm} \text{Eq. S4}

For small regions-of-interest (ROIs) in an image (spines or dendrites), we calculated the binding fraction (PAD) as:

\[
P_{AD} = \tau_D (\tau_D - \langle \tau \rangle)(\tau_D - \tau_{AD}) \tau_D^2 (\tau_D + \tau_{AD} - \langle \tau \rangle)^{-1}. \]  \hspace{1cm} \text{Eq. S5}
2.8 BDNF-SEP imaging

BDNF-SEP imaging was performed by interleaving 8 Hz two-photon imaging with two-photon glutamate uncaging (30 pulses at 0.5Hz). Multiple (1 to 30) spines were imaged on each neuron. Change in BDNF-SEP fluorescence was measured as $\Delta F/F_0$ after subtracting background fluorescence. Uncaging-triggered averages were calculated as the average increase in SEP fluorescence after each individual uncaging pulse. Red fluorescence increase was smoothed using a 16-frame window.

2.9 BDNF-mRFP imaging

For visualizing BDNF-mRFP localization in CA1 pyramidal neurons, transfected slices were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde. Following fixation, slices were washed briefly in PBS and then mounted on a glass slide using a coverslip and Vectashield Mounting Media with Dapi (Vector Labs). Images were obtained using a Leica SP5 laser scanning confocal microscope (Leica).

2.10 Spine volume analysis

For two-photon imaging without 2pFLIM, spine volume was measured using the green fluorescent intensity from mEGFP. The one exception here is when we utilized the BDNF-SEP construct. Because of SEP’s fluorescence, we instead measured spine volume using fluorescent intensity from mCherry. For 2pFLIM imaging, spine volume was reported using the red fluorescent intensity from mRFP or mCherry. In all experiments, spine volume was measured as the integrated fluorescent intensity after
subtracting background ($F$). Spine volume change was calculated by $F/F_0$ where $F_0$ is the average spine volume prior to stimulation.

2.11 Electrophysiological stimulation and recording

Using acute hippocampal mouse slices, a whole cell patch was established on a CA1 pyramidal cell. A stimulating electrode was then positioned within the Schaffer Collateral pathway. Prior to making baseline recordings, maximal stimulation intensity was established. Using 33% of this maximum intensity, the Schaffer Collateral axons were stimulated every 30 seconds for ten minutes (0.5 Hz) and post-synaptic responses were collected. Following baseline recording, a train of 150 stimuli (33% of maximal stimulation intensity) was administered at 2 Hz (75 second total train duration) and paired with post-synaptic depolarization to 0 mV. After stimulation, post-synaptic responses were once again collected at 30-second intervals for the next 20 to 30 minutes.

2.12 Treatments

2.12.1 1NMPP1

1NMPP1 was dissolved in dimethyl sulfoxide (DMSO) at 100 mM and stored at -20°C. In preparation for use, the frozen stock was thawed and then diluted to a working concentration of 1 mM using solubilization buffer containing 0.9% NaCl and 2.5% Tween-20. To control for non-specific effects of the solubilization buffer, DMSO was dissolved in the solubilization buffer in a manner analogous to 1NMPP1. This DMSO/solubilization buffer solution was then used as a “vehicle” control.
2.12.2 TrkB-Ig and human-IgG in cultured hippocampal slices

Prior to treatment with either TrkB-Ig or human-IgG, slices were pre-treated with 1 µg/ml of bovine serum albumin (BSA) for at least 10 minutes in an effort to minimize non-specific binding of these antibodies to the perfusion tubing.

2.12.3 BDNF in cultured hippocampal slices

Prior to treatment with BDNF, slices were pre-treated with 1 µg/ml BSA for at least 10 minutes in an effort to minimize non-specific binding of BDNF to the perfusion tubing.

2.13 Mice

2.13.1 Trkb\(^{F616A}\)

TrkB\(^{F616A}\) mutant mice were provided by Dr. David Ginty (Chen et al., 2005).

2.13.2 Bdnf\(^{fl/fl}\) and Trkb\(^{fl/fl}\)

Bdnf\(^{fl/fl}\) and Trkb\(^{fl/fl}\) mice were provided by Dr. Luis Parada. Please see figure 6 for a diagram of the mouse Trkb and Bdnf genes with the position of the inserted lox-p sites represented.

2.13.3 Trkb\(^{Y515F}\)

Trkb\(^{Y515F}\) mice were generated as previously described (He et al., 2010; Minichiello et al., 1998).
Figure 6: Schematic of Trkb<sup>fl/fl</sup> and Bdnf<sup>fl/fl</sup> genes

A) Trkb<sup>fl/fl</sup> was generated by inserting lox-p sites on either side of exon 2 of Trkb within chromosome 13. This exon contains DNA encoding the signal peptide and first 40 amino acids of the N-terminus of TrkB. B) Bdnf<sup>fl/fl</sup> was generated by inserting lox-p sites on either side of exon 9 of Bdnf within chromosome 2. BDNF has multiple splice variants that may or may not contain exons 1 – 8. However, all these BDNF variants contain exon 9. The untranslated regions of exons are represented in red while the translated portions are depicted in black.

2.13.4 Trkb<sup>Y816F</sup> and Trkb<sup>Y816Y</sup>

Trkb<sup>Y816F</sup> and Trkb<sup>Y816Y</sup> mice were generated as previously described (He et al., 2010; Minichiello et al., 2002). Of note, unlike the Trkb<sup>Y515F</sup> mice where TrkB expression levels are unaffected by generation of the mutation, the Trkb<sup>Y816F</sup> mice display a 50% reduction in TrkB expression levels compared to wild-type littermate controls (Minichiello et al., 2002; Minichiello et al., 1998). The source of this reduced protein
expression is the cDNA “knockin” approach utilized to introduce the mutation into the endogenous TrkB locus. Because of the reduced TrkB content in the mutant animals, their wild-type littermate controls are not the appropriate control for evaluating the effects of this TrkB mutation. To resolve this issue, an additional mouse line was generated using the same cDNA “knockin” approach but this time, the wild-type nucleotide sequence was introduced thus generating a TrkbY816Y mouse. Similar to the TrkbY816F, the TrkbY816Y mouse has a 50% reduction in TrkB protein expression (Minichiello et al., 2002). As such, for all experiments utilizing the TrkbY816F mice, results were compared to both wild-type littermates as well as age-matched TrkbY816Y animals.
3. Contribution of BDNF-TrkB signaling to structural and functional plasticity at the CA3-CA1 synapse

The neurotrophin brain derived neurotrophic factor (BDNF) and its canonical receptor tropomyosin receptor kinase B (TrkB) play important roles in many forms of neuronal plasticity (Bekinschtein et al., 2007; Figurov et al., 1996; Kang et al., 1997; Korte et al., 1996; Korte et al., 1997; Kovalchuk et al., 2002; Lohof et al., 1993; Minichiello, 2009; Minichiello et al., 1999; Patterson et al., 1996; Xu et al., 2000). One such plasticity is activity induced, long-term dendritic spine enlargement (also called structural plasticity, structural long-term potentiation, or sLTP) (Lai et al., 2012; Rex et al., 2007; Tanaka et al., 2008) – a structural correlate of long-term potentiation (LTP) as well as an animal’s learning and memory (Kim et al., 2013a; Kim et al., 2013b; Matsuzaki et al., 2004; Okamoto et al., 2004; Tanaka et al., 2008). Despite multiple lines of evidence detailing a clear role for BDNF and TrkB in sLTP, questions remain: 1) what is the cellular source and subcellular locale of BDNF/TrkB signaling during sLTP and 2) what are the signaling pathways downstream of TrkB that contribute to sLTP? Here, we demonstrate that sLTP of CA1 apical dendritic spines requires expression of TrkB and BDNF in the post-synaptic cell. Additionally, we show that sLTP requires tyrosine 816 but not 515 within TrkB’s intracellular domain. Further, we show that BDNF alone is insufficient to induce sLTP but when paired with a weak stimulus (a stimulus that uncages but at a level insufficient to induce structural plasticity), sLTP results. Finally, we demonstrate
that extracellular BDNF is required for functional plasticity at the CA3-CA1 synapse induced by low-frequency Schaffer Collateral stimulation paired with depolarization of the post-synaptic CA1 cell – an LTP inducing paradigm considered to require post-synaptic mechanisms for its induction and expression (Kang et al., 1997). Together, these findings build on previously published results and clearly establish a critical role for post-synaptic BDNF-TrkB signaling in structural plasticity of single spines of the apical dendrite of CA1 pyramidal cells as well as functional plasticity of the CA3-CA1 synapse.

3.1 Introduction

3.1.1 BDNF-TrkB signaling in sLTP

BDNF and TrkB have long been implicated as critical players in various types of synaptic plasticity (Bekinschtein et al., 2007; Figurov et al., 1996; Kang et al., 1997; Korte et al., 1996; Korte et al., 1997; Kovalchuk et al., 2002; Lohof et al., 1993; Minichiello, 2009; Minichiello et al., 1999; Patterson et al., 1996; Xu et al., 2000). Consistent with these findings, several lines of evidence have recently suggested that BDNF-TrkB signaling may be critical for sLTP (Lai et al., 2012; Rex et al., 2007; Tanaka et al., 2008).

Specifically, in 2008, Tanaka and colleagues demonstrated that blocking BDNF action through application of the extracellular BDNF scavenger TrkB-Ig or inhibiting TrkB activity with the kinase inhibitor K252a impairs structural plasticity of CA1 apical dendritic spines. Because the authors utilized a pairing protocol involving laser evoked
glutamate uncaging combined with post-synaptic back-propagating action potentials, they argued that their stimulation activates the dendritic spine specifically without involving the pre-synaptic terminal. Based on this point, the authors then concluded that the BDNF-TrkB signaling required for sLTP is post-synaptic in origin. This conclusion may be correct but it is controversial in that the stimulation paradigm utilized may not be as specific as the authors argued. Namely, the glutamate uncaging, even though targeted towards a single dendritic spine, may also act on the pre-synaptic terminal. As such, it is possible that sLTP may be mediated by pre- and/or post-synaptic mechanisms. Returning to the question of the cellular locale of BDNF-TrkB signaling, the perturbations of BDNF-TrkB signaling utilized by Tanaka et al. are exogenously applied and thus will globally affect BDNF-TrkB signaling throughout the cell, both pre- and post-synaptically. Consequently, these results highlight an important role for BDNF and TrkB in sLTP but they do not clarify the specific cellular locale for either protein.

3.1.2 BDNF-TrkB signaling pathways and synaptic plasticity

BDNF induces TrkB activation by first inducing receptor dimerization that leads to phosphorylation of various tyrosine and serine residues within TrkB’s intracellular domain (Chao, 2003; Dechant et al., 1993; Lai et al., 2012; Middlemas et al., 1994; Rodriguez-Tébar and Barde, 1988). Each of these residues has been linked to distinct signaling pathways, some of which have been implicated in neuronal plasticity (Chao,
51

Serine 478 (S478) is an amino acid near the juxtamembrane domain of TrkB that is phosphorylated in response to BDNF. However, unlike its tyrosine counterparts, S478 is not auto-phosphorylated by the intrinsic kinase domain within TrkB. Rather, BDNF binding to TrkB leads to activation of the serine/threonine kinase cyclin-dependent kinase 5 (Cdk5) (Cheung et al., 2007). Once active, Cdk5 phosphorylates S478. In its phosphorylated form, S478 serves as a docking site for the guanine nucleotide exchange factor Tiam1. Once Tiam1 binds to S478, TrkB then phosphorylates it leading to its activation and ultimately to activation of the Rho GTPase Rac1. This BDNF-TrkB-Tiam1-Rac1 pathway has been shown to be critical for functional plasticity in acute hippocampal slices and dendritic spine structural plasticity in dissociated hippocampal neuron cultures (Lai et al., 2012).

Tyrosine 515 (Y515) is another amino acid within TrkB that is phosphorylated in response to BDNF. Following BDNF binding and receptor dimerization, Y515 is autophosphorylated by TrkB’s intrinsic kinase domain allowing it to serve as a docking site for the Src homologous collagen-like (Shc) adaptor protein (Chao, 2003). After Shc binding, TrkB phosphorylates and activates this protein leading to signaling through two pathways – PI3K/Akt and Ras/ERK (Chao, 2003; Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). Even though Ras and ERK have both been implicated
in LTP, previous work has demonstrated that Y515 phosphorylation is not required for LTP at the CA3-CA1 hippocampal synapse (Korte et al., 2000; Minichiello et al., 2002).

Finally, tyrosine 816 (Y816) is also phosphorylated in response to the binding of BDNF to TrkB. Like Y515, Y816 phosphorylation is an autophosphorylation event due to TrkB’s intrinsic kinase activity (Chao, 2003). In its phosphorylated form, Y816 serves as a docking site for phospholipase C gamma-1 (PLCγ1) (Middlemas et al., 1994). Once bound, PLCγ1 is phosphorylated by the TrkB kinase thus activating the protein. In its active form, PLCγ1 initiates a signaling pathway that ultimately results in IP3 and DAG production as well as the release of intracellular Ca\(^{2+}\) (Rhee, 2000). Previous work has demonstrated a critical role for phosphorylation of this amino acid in LTP at the CA3-CA1 synapse but to date, no one has examined its role in sLTP (Gruart et al., 2006; Minichiello et al., 2002)

### 3.2 Results

#### 3.2.1 Post-synaptically expressed TrkB is necessary for single spine structural plasticity

To determine if TrkB activation is necessary for glutamate uncaging evoked structural plasticity of CA1 dendritic spines, we biolistically transfected rat cultured hippocampal slices with monomeric enhanced green fluorescent protein (mEGFP). After at least 24 hours of transfection, we bathed the slices in MNI-glutamate (4 mM). Using two-photon microscopy, we imaged transfected CA1 pyramidal cells while simultaneously inducing sLTP with a low-frequency train of two-photon pulses (30
pulses at 0.5 Hz) in order to uncage glutamate at a single dendritic spine (Figure 7). In response, spine volume transiently increased by ~450% (transient phase defined as 1 to 2 minutes following the start of uncaging) before relaxing to a sustained state of ~100% (sustained phase defined as 10 to 40 minutes following the start of uncaging) (Ctrl; Figure 8A, B, and C). Following successful induction of sLTP, we applied the kinase inhibitor K252a (200 nM) to the slice for at least 30 minutes and then attempted to induce sLTP using glutamate uncaging on another dendritic spine from the same cell. In the presence of K252a, we found an impairment of both the transient and sustained phases of sLTP (K252a; Figure 8A, B, and C).
Rodent Brain (P5 - P7) ➔ 350 μm Hippocampal Slices

Biolistic Transfection
- Plasmid DNA
- 1μm Gold Particles

EGFP Transfection
- 2p-Uncaging at 720 nm
- 2p-Imaging at 920 nm

CA1 Pyramidal Cell
- Apical Dendritic Spine
- Axon

Caged Glutamate
Free Glutamate
Uncaging beam

CA2
CA3
DG
Figure 7: Hippocampal slice preparation, biolistic transfection, and two-photon imaging and uncaging

In our experimental preparation, we prepare 350 µm hippocampal slices from P5 to P7 mice and rats. These slices are cultured at 35°C and 3% CO₂ for 1 to 2 weeks and then biolistically transfected with 1 µm gold particles coated with plasmid DNA (EGFP for example). Because of the low efficiency of biolistic transfection, only a few neurons across the hippocampal slice are typically transfected. For our experiments, we specifically use apical dendritic spines of transfected CA1 pyramidal cells using two-photon imaging (920 nm) in combination with two-photon glutamate uncaging (720 nm) to induce and visualize single spine structural plasticity.

Since K252a is a non-specific kinase inhibitor and thus can act on a variety of non-TrkB molecules, many of which have been implicated in plasticity, we sought to verify sLTP’s requirement for TrkB using a chemical-genetic approach that would enable specific inhibition of TrkB signaling. In this approach, we prepared cultured hippocampal slices from mice containing a single amino acid, knockin mutation within the TrkB locus (F616A). This single amino acid change is within the intracellular kinase domain and renders the kinase susceptible to inhibition by the synthetic small molecule 1’-Naphthylmethyl-4-amino-1-tert-butyl-3-(p-methylphenyl)pyrazolo[3,4-d] pyrimidine (1NMPP1). In the absence of this molecule, mutant and wild-type TrkB are equivalent in function. However, in the presence of 1NMPP1, kinase function for TrkB containing the mutation is impaired thus leading to a blockade of kinase-dependent TrkB signaling.

Utilizing Trkb^{F616A} cultured hippocampal slices, we once again imaged single dendritic spines of CA1 pyramidal cells transfected with mEGFP using two-photon microscopy. In response to glutamate uncaging, we found a transient increase in spine volume to around 400% that then relaxed to a sustained state of 60% (Ctrl; Figure 8D, E
and F). Following successful induction of sLTP at one spine on a given dendrite, we then added either vehicle (DMSO) or 1NMPP1 (1 µM) to the slice for at least 30 minutes prior to uncaging glutamate over a second spine on the same cell. In the presence of vehicle, we did not observe any impairment in sLTP (Veh, 8E and F); however, in the presence of 1NMPP1, we did find a reduction in both the transient and sustained phases of structural plasticity (1NMPP1; Figure 8D, E, and F). These results are consistent with those obtained using K252a and together confirm previous work that TrkB is required for normal sLTP.

Since K252a and 1NMPP1 will inhibit TrkB activity throughout the slice – pre- and post-synaptically – these results do not reveal the cellular locale of TrkB required for sLTP. To determine if post-synaptically expressed TrkB is required for single spine structural plasticity, we prepared cultured hippocampal slices from Trkb<sup>fl/fl</sup> mice. These mice contain lox-<i>p</i> sites flanking the endogenous TrkB locus. In the presence of the protein Cre-recombinase (Cre), the DNA in between these two sites is excised (Abremski and Hoess, 1984). For the Trkb<sup>fl/fl</sup> mouse, such excision results in removal of DNA critical for TrkB expression and thus leads to a knockout of TrkB protein levels (He et al., 2004). Using slices from these animals, we transfected them with either mEGFP alone (referred to as Cre negative) or mEGFP plus Cre-tdTomato (referred to as Cre positive). Due to the sparse nature of biolistic transfection, this method led to TrkB knockout in only a few CA1 pyramidal cells (2 to 3 cells on average) per Cre positive hippocampal slice.
thus yielding what is essentially a “single cell knockout” (Lu et al., 2009). After at least 5 days of transfection, we imaged transfected CA1 pyramidal cells two-photon microscopy and induced sLTP on single dendritic spines using glutamate uncaging. In the Cre Negative cells, spine volume transiently increased to ~400% before relaxing to a sustained level of ~80% (Cre Neg; Figure 8G, H, and I). In comparison, spine volume for the Cre Positive cells was impaired in both the transient and sustained phases of sLTP (Cre Pos; Figure 8G, H, and I). These results indicate that post-synaptic TrkB is necessary for sLTP.
Figure 8: Effect of TrkB perturbation on single spine structural plasticity

Representative two-photon images (A, D, and G), time courses (B, E, and H), and quantification (C, F, and I) of glutamate uncaging-induced spine volume change during the transient (averaged over 1-2 minutes) and sustained (averaged over 10-40 minutes) phases. A, B, and C) Before (Ctrl, black trace, n = 6 spines) and after K252a application (K252a, red trace, n = 7 spines) in rat hippocampal slices. D, E, and F) Before (Ctrl, black trace, n = 15 spines) and after Vehicle (Veh, blue trace, n = 13 spines) or 1NMPP1 application (1NMPP1, red trace, n = 19 spines) in Trkb<sup>fl/fl</sup> hippocampal slices. G, H, and I) Cells transfected with mEGFP alone (Cre Neg, black trace, n = 10 spines) or mEGFP plus Cre-tdTomato (Cre Pos, red trace, n = 12 spines) in Trkb<sup>fl/fl</sup> hippocampal slices. Error bars are s.e.m. Asterisks denote statistical significance (p < 0.05) as determined by ANOVA followed by post-hoc tests (when appropriate) with the least significant difference.

3.2.2 Phosphorylation of tyrosine 816 but not 515 is required for single spine structural plasticity

Within the TrkB intracellular domain, there are multiple tyrosine and serine residues (S478, Y515, and Y816) that become phosphorylated in response to TrkB
activation (Chao, 2003; Dechant et al., 1993; Lai et al., 2012; Middlemas et al., 1994). Once phosphorylated, these residues serve as starting points for various molecular signaling pathways (S478 and Tiam1-Rac1, Y515 and SHC/Ras/ERK, and Y816 and PLCγ1) (Chao, 2003; Cheung et al., 2007; Kaplan and Miller, 2000; Lai et al., 2012; Middlemas et al., 1994). Several of these residues have proven critical for synaptic plasticity at the CA3-CA1 synapse – S478 and Y816 (Gruart et al., 2006; Lai et al., 2012; Minichiello et al., 2002). With respect to sLTP, only S478 has been examined (Lai et al., 2012).

To determine the involvement of Y515 and Y816 in sLTP, we utilized two distinct mouse lines – TrkB<sup>Y515F</sup> and TrkB<sup>Y816F</sup>. For each line, the tyrosine of interest has been mutated to a phenylalanine – an amino acid that cannot be phosphorylated and as such, prevents binding of the appropriate adaptor protein and initiation of the associated signaling pathway (Minichiello et al., 2002; Minichiello et al., 1999). Utilizing slices from mutant animals and their appropriate controls (see Materials and Methods for description of controls), we imaged transfected CA1 pyramidal cells using two-photon microscopy while simultaneously inducing sLTP of dendritic spines using glutamate uncaging. For spines from TrkB<sup>Y515F</sup>, we found no significant difference in the transient or sustained phases of sLTP compared to littermate controls (Figure 9A, B, and C). For spines from TrkB<sup>Y816F</sup> however, we found significant reduction in both the transient and sustained phases of sLTP compared to littermate and TrkB<sup>Y816Y</sup> controls (Figure 9D, E,
and F). Additionally, when we compared the magnitude of sLTP from wild-type littermates for TrkbY816F and TrkbY816Y to sLTP from TrkbY816Y, we were unable to detect any significant differences (Figure 9E and F). Taken together, these results identify tyrosine 816 but not 515 within TrkB as a critical amino acid residue for mediating sLTP.
Figure 9: Effect of mutation of specific tyrosine residues within TrkB on single spine structural plasticity
Representative two-photon images (A and D), time courses (B and E), and quantification (C and F) of glutamate uncaging-induced spine volume change during the transient (averaged over 1 to 2 minutes) and sustained (averaged over 10 to 40 minutes) phases. A, B, and C) Spines from wild-type (TrkbWT, black trace, n = 11 spines) and Y515F mutant (TrkbY515F, red trace, n = 11 spines) mouse hippocampal slices. D, E, and F) Spines from wild-type (TrkbWT, black trace, n = 10 spines), Y816Y mutant mice (TrkbY816Y, blue trace, n = 7 spines), and Y816F mutant (TrkbY816F, red trace, n = 6 spines) mouse hippocampal slices. Error bars are s.e.m. Asterisks denote statistical significance (p < 0.05) as determined by ANOVA followed by post-hoc tests (when appropriate) with the least significant difference.

3.2.3 Post-synaptically expressed BDNF is necessary for single spine structural plasticity

BDNF is the canonical ligand for initiation of TrkB kinase activity and the resulting signaling that develops (Chao, 2003; Rodriguez-Tébar and Barde, 1988). Since TrkB kinase activity is necessary for sLTP (Figure 8), BDNF is the most likely source for TrkB activation during this event. To determine if BDNF is in fact necessary for sLTP, we transfected cultured rat hippocampal slices with mEGFP and then imaged transfected CA1 pyramidal cells while inducing sLTP with two-photon glutamate uncaging. Similar to previous results, we found that spine volume transiently increased to ~400% before decaying to a sustained state of ~85%. In cells that successfully demonstrated sLTP, we then applied either TrkB-Ig (an extracellular scavenger of BDNF; 2 ug/ml) or human-IgG (a control for the Ig portion of TrkB-Ig) for at least 30 minutes. In response to glutamate uncaging, we found that TrkB-Ig but not human-IgG significantly impaired both the transient and sustained phases of sLTP (Trk-Ig; H-IgG; Figure 10A, B, and C).
Similar to the pharmacologic perturbations of TrkB, TrkB-Ig application does not distinguish between pre- and post-synaptic sources of BDNF. As such, to determine if post-synaptic BDNF is necessary for sLTP, we prepared cultured hippocampal slices from Bdnffl/fl mice. Like the Trkbfl/fl mice described above, in the Bdnffl/fl mice, two lox-p sites flank the Bdnf locus. In the presence of Cre-recombinase (Cre), the DNA in between these two sites is excised. For Bdnffl/fl mice, this excision event results in removal of Bdnf DNA and thus leads to a reduction of BDNF levels. Utilizing slices from these animals, we transfected them with mEGFP (Cre Negative) or mEGFP plus Cre-tdTomato (Cre Positive). In response to glutamate uncaging, spines from Cre Negative cells underwent normal sLTP – transient phase of ~450% and a sustained phase of ~80% (Figure 10D, E, and F). However, for the Cre positive cells, these spines showed significant impairments in both phases (Figure 10D, E, and F).

Given the powerful trophic effects of BDNF, the possibility arose that the impairment of sLTP is a nonspecific consequence of an unhealthy cell or loss synaptic integrity. To address these possibilities, we first asked whether acute application of BDNF to the cultured slice could rapidly rescue the impairment of sLTP in the setting of post-synaptic BDNF knockout. We therefore applied 20 ng/ml recombinant human BDNF to Cre Positive cells for 10 minutes and then induced sLTP with glutamate uncaging. We found a partial, non-significant rescue of the transient phase but a
complete rescue of the sustained phase (Figure 10D, E, and F). Such rescue argues sLTP does require cell-autonomous, post-synaptically expressed BDNF.
Figure 10: Effect of BDNF perturbation on single spine structural plasticity
Representative two-photon images (A and D), time courses (B and E), and quantification (C and F) of glutamate uncaging-induced spine volume change during the transient (averaged over 1-2 minutes) and sustained (averaged over 10-40 minutes) phases. A, B, and C) Before (Ctrl, black trace, n = 6 spines) and after Human-IgG (H-IgG, blue trace, n = 6 spines) or TrkB-Ig application (TrkB-Ig, red trace, n = 8 spines) in rat hippocampal slices. D, E, and F) Cells transfected with mEGFP (Cre Neg, black trace, n = 14 spines) or mEGFP plus Cre-tdTomato in the absence (Cre Pos, red trace, n = 31 spines) or presence of exogenous BDNF (Cre Pos + BDNF, green trace, n = 7 spines). Error bars are s.e.m. Asterisks denote statistical significance (p < 0.05) as determined by ANOVA followed by post-hoc tests with the least significant difference.

3.2.4 BDNF enables a subthreshold stimulus to induce single spine structural plasticity

The above-mentioned experiments clearly position BDNF-TrkB signaling as necessary for sLTP but they do not address the issue of sufficiency. To determine if BDNF alone is sufficient to induce sLTP, we first devised a stimulation paradigm that would lead to glutamate uncaging (read out by an initial change in spine volume) but would not result in sLTP (sustained spine volume changes following uncaging). Using this sub-threshold paradigm, we found that in cultured rat hippocampal slices transfected with mEGFP, spine volume increased in the transient phase by ~100% but then decayed almost back to baseline values (~20%) during the sustained phase (11A, B). We then applied recombinant human BDNF (20 ng/ml) to these same slices for 10 minutes. During this incubation period, we monitored spine volume of unstimulated spines and did not observe any noticeable change in volume. After the 10-minute incubation period, we then stimulated a single dendritic spine using the sub-threshold paradigm and found a trend towards an increase in the transient phase with a clear,
significant increase in the sustained phase (Figure 11A, B). These data indicate that BDNF alone is insufficient for sLTP but in the presence of low-levels of activity (sub-threshold stimulus), it is able to induce sLTP.

![Figure 11](image_url)

**Figure 11: Effect of exogenous BDNF on subthreshold induction of single spine structural plasticity**

Time courses (A) and quantification (B) of the transient (averaged over 1-2 minutes) and sustained (averaged over 10-30 minutes) phases of spine volume changes in response to a sub-threshold glutamate uncaging stimulus in the absence (Sub, black trace, n = 6 spines) or presence of exogenous BDNF (Sub + BDNF, green trace, n = 7 spines). Error bars are s.e.m. Asterisks denote statistical significance (p < 0.05) as determined by an ANOVA.

### 3.2.5 BDNF is required for post-synaptic LTP at the CA3-CA1 synapse

Single CA1 dendritic spine structural plasticity has consistently been associated with a functional correlate, namely potentiation of the electrical properties of the synapse. However, to assume that single spine structural plasticity is always equivalent to functional plasticity may be incorrect. As such, to determine if BDNF-TrkB signaling is necessary for functional enhancement of CA1 dendritic spines, we analyzed LTP of CA1 pyramidal cells in acute mouse hippocampal slices using a stimulation paradigm
that favors post-synaptic mechanisms of induction and expression – low-frequency pre-
synaptic stimulation paired with post-synaptic depolarization. Using this paradigm, we
found that EPSC amplitudes increased by ~155% representing LTP 10 to 20 minutes
following stimulation (Figure 12A, B). In the presence of TrkB-Ig but not human-IgG,
we found a significant reduction in EPSC amplitude potentiation compared to controls
(Figure 12A, B). These results suggest that extracellular BDNF is critical for a post-
synaptic mechanism of LTP at the CA3-CA1 synapse and thus provide additional
evidence that the BDNF-TrkB signaling requirement for sLTP is likely indicative of its
requirement in functional plasticity as well.

Figure 12: Effect of BDNF perturbation on a post-synaptic mechanism of Schaffer
Collateral-CA1 LTP
A and B) Representative current traces (A) and average EPSC amplitude changes (B) recorded in CA1 pyramidal cells evoked by Schaffer collateral stimulation before and after LTP induction in the absence (Ctrl, n = 22 animals) or presence of Human-IgG (H-IgG, n = 9 animals) or TrkB-Ig (n = 12 animals). C) Quantification of EPSC amplitude changes averaged over 10-20 minutes following LTP induction.

3.3 Discussion

Here we utilized two-photon glutamate uncaging and electrophysiology in conjunction with pharmacologic perturbations as well as genetically modified mice to test the hypothesis that BDNF-TrkB signaling is involved in structural and functional plasticity of CA1 dendritic spines. We find that single spine structural plasticity requires post-synaptically expressed BDNF and TrkB as well as tyrosine 816 but not 515 within TrkB. We further find that BDNF alone is insufficient to induce sLTP but in combination with sub-threshold activity, it is able to yield sLTP. Finally, we provide electrophysiological evidence that extracellular BDNF is required for a post-synaptic mechanism of LTP induction and expression at CA1 pyramidal neurons. All together, these experiments clearly detail a critical role for post-synaptic BDNF-TrkB signaling within CA1 dendritic spines for establishing and maintaining plasticity.

3.3.1 Post-synaptic TrkB and sLTP

Prior work utilizing electron microscopy as well as immunohistochemistry has identified the dendritic spine as one locale for TrkB (Drake et al., 1999; Ji et al., 2005). Additionally, recent work from the McNamara lab has shown that in response to high levels of activity (status epilepticus), this spine population of TrkB can be activated.
(Helgager et al., 2013). Because of its locale in spines as well as the activity-dependent nature of its activation, it has long been suggested to be a critical player in hippocampal plasticity. Prior to our work however, most investigations have explored TrkB’s role in the later phases of plasticity, namely those occurring minutes to hours after initial stimulation (Bekinschtein et al., 2007; Kang et al., 1997; Korte et al., 1997; Minichiello, 2009). Here though, we clearly demonstrate that post-synaptic TrkB is required not only for the later aspects of sLTP (the sustained phase) but also the initial development of sLTP during the transient phase. These observations suggest two possible roles for TrkB in sLTP – permissive and/or instructive. It should be noted that these two roles are not exclusive in that TrkB could be assuming both during sLTP induction and expression. Unfortunately, the experiments outlined in this chapter do not permit investigating these potential roles of TrkB for either phase of plasticity. However, we do address this question in chapters 4 and 5 by developing and utilizing a dynamic, FRET-based sensor for monitoring TrkB activation before, during, and after sLTP induction.

Regarding the role of pre-synaptic TrkB in plasticity, there is also much experimental evidence. Here though, we do not directly examine the role of pre-synaptic TrkB in sLTP. The uncaging stimulation paradigm is assumed to be quite specific for the post-synaptic dendritic spine (Matsuzaki et al., 2004; Murakoshi and Yasuda, 2012; Tanaka et al., 2008) but as discussed above, this may not entirely be true.
As such, we cannot rule out the possibility that pre-synaptic TrkB is playing some role in sLTP. Examination of this possibility is an important direction for future investigation.

In addition to TrkB’s involvement in plasticity, there has been much work done to dissect the specific signaling pathways arising from TrkB necessary for such plasticity (Gruart et al., 2006; Minichiello et al., 2002). As discussed above, much of this work has identified tyrosine 816 and the associated activation of the PLCγ1 pathway as critical for LTP at the CA3-CA1 synapse. In line with these findings, we show that tyrosine 816 but not 515 is critical for sLTP of single CA1 dendritic spines. These results only further bolster the overlaps between structural and functional plasticity on a molecular level.

### 3.3.2 Post-synaptic BDNF and sLTP

Like TrkB, there is an abundance of evidence linking BDNF to plasticity. However unlike TrkB, the cellular locale of BDNF is not as clear. Over the past few decades, there has been evidence suggesting both a pre- and post-synaptic population of BDNF that contributes to plasticity at the CA3-CA1 synapse (Carvalho et al., 2008; Gärtner et al., 2006; Hartmann et al., 2001; Jovanovic et al., 2000; Kolarow et al., 2007; Korte et al., 1995; Korte et al., 1996; Matsuda et al., 2009; Tanaka et al., 2008; Zakharenko et al., 2003). However, identifying endogenous post-synaptic BDNF within CA1 dendrites and spines has proven quite challenging (Dieni et al., 2012). For this reason, some have begun to question the existence of post-synaptic BDNF within CA1 pyramidal cells.
Our results directly address this controversy by providing new evidence that post-synaptic, cell-autonomous BDNF is required for sLTP. Similar to TrkB, our results here do not rule out the possible involvement of pre-synaptic BDNF in this process. Rather, they position post-synaptic BDNF as one BDNF population required for sLTP.

As with any knockout approach, there is a concern for the health of the targeted cell. Specifically, does removal of BDNF adversely impact the health of the cell thus leading to impaired plasticity? We first address this concern by demonstrating a rescue of sLTP utilizing acute application of human recombinant BDNF. The fact that rescue can occur with such an acute application argues that the targeted cell is healthy and capable of undergoing plasticity but it does not since it lacks BDNF. We further address this concern in chapter 5 where we examine the activation of CaMKII – a known mediator of sLTP believed to be upstream of BDNF-TrkB signaling – in the setting of reduced post-synaptic BDNF.

In addition to the post-synaptic locale of the BDNF required for sLTP, the fact that it is cell autonomous is quite intriguing. This finding suggests that even though CA1 pyramidal cells sit in a population of thousands of cells in close proximity, it is BDNF from itself that is necessary for sLTP. Such autocrine actions of BDNF are intriguing considering that this is a protein that is released into the extracellular space. How BDNF is able to stay local to the cell it arose from is currently an unanswered but important question for future examination.
Lastly, the fact that BDNF, like TrkB, is required for the transient and sustained phases of plasticity implies multiple possibilities as to when it may have been released. Unfortunately, these current experiments do not distinguish between BDNF released prior to, during, or after the stimulus. We address this specific question in chapter 6.

### 3.3.3 BDNF alone is insufficient for inducing plasticity

Whether BDNF is sufficient to induce plasticity has been a question of interest for quite some time (Kang and Schuman, 1995). Like the cellular locale of BDNF required for plasticity, this question has proven quite controversial. The consensus seems to be that BDNF alone is insufficient to induce plasticity. Rather, BDNF plus activity provides the appropriate combination of factors that proves sufficient to induce LTP. Similar to these previous findings, we found that BDNF plus sub-threshold activity is sufficient to induce sLTP. Further, our results support the notion that BDNF acts only on active synapses. Specifically, we found sLTP only on those spines we stimulated in the presence of BDNF. The unstimulated spines did not show any spine volume change.

### 3.3.4 Post-synaptic BDNF-TrkB signaling and functional plasticity

Historically, dendritic spine enlargement has been directly linked to functional enhancement of the electrical properties of the spine. Despite these links, it may not be necessarily valid to assume that structural plasticity always equates to functional plasticity. For this reason, we investigated whether the post-synaptic BDNF-TrkB signaling required we observed in sLTP also applied in a functional plasticity
preparation. The paradigm we utilized is one that is considered to be largely post-synaptic nature. The pre-synaptic stimulation is low frequency and thus places minimal burden on the pre-synaptic machinery while the post-synaptic compartment is depolarized engaging many distinct signaling pathways. As such, the observed impairment of LTP in the presence of TrkB-Ig suggests that extracellular BDNF arising from the post-synaptic terminal is required. However, these results are not definitive for two reasons. One, the stimulation paradigm utilized may be largely post-synaptic in nature but it does not exclude potential involvement of pre-synaptic mechanisms or signaling pathways. Two, TrkB-Ig will scavenge BDNF from all cellular sources (pre- and post-synaptic) and thus it too does not exclude potential pre-synaptic BDNF release.

Moving forward, the experiment needed is to utilize the single-cell knockout approach detailed above in conjunction with the functional plasticity paradigm we used here. If the results parallel those seen with sLTP, namely knocking out BDNF in the post-synaptic cell impairs LTP, then we can confidently conclude that post-synaptic BDNF is necessary for both sLTP and functional LTP.

**3.3.5 Conclusion**

In conclusion, we have demonstrated that post-synaptic BDNF-TrkB signaling is critical for both the transient and sustained phase of structural plasticity. However, these results do not inform about the time course and sub-cellular profile (dendrite versus dendritic spine) for such signaling. Specifically, it is unclear if BDNF release and
subsequent TrkB activation is required before, during, or after sLTP induction. To address this question, what we need is a dynamic readout of BDNF release and TrkB activity that can be used in a living neuron undergoing plasticity. In the chapters that follow, we develop such tools and utilize them to clarify the spatiotemporal dynamics of BDNF-TrkB signaling during sLTP.
4. Design, development, and characterization of a FRET-FLIM sensor for TrkB activation

4.1 Introduction

We previously demonstrated that post-synaptic BDNF and TrkB are required for sLTP. However, because of the nature of these experiments, we do not know when – before, during, or after glutamate uncaging – or where within the post-synaptic cell – dendritic spine, dendritic shaft, or adjacent spines – that BDNF-TrkB signaling occurs. To address these questions, we designed, developed, and characterized a novel FRET-based sensor for TrkB activation that allows for real-time imaging of TrkB activity with high temporal and sub-cellular spatial resolution in a living cell. We show in both HeLa cells and dissociated cultured neurons that this sensor is sensitive to known TrkB activating stimuli – BDNF and zinc – and it is specific for phosphorylation of tyrosine 816. Additionally, we show that the sensor is reversible thus indicating that it can report both activation and inactivation. All together, these results highlight the development of a novel biosensor for TrkB that can be utilized in living cells to monitor changes in TrkB activity in real-time.

4.1.1 Imaging TrkB activation in real time

As described above, TrkB activation can be initiated through two primary sources: 1) the neurotrophin brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) which bind to the extracellular domain of TrkB leading to
receptor dimerization and activation (Chao, 2003; Haniu et al., 1997; Huang and Reichardt, 2002) or 2) non-neurotrophins such as zinc (Zn²⁺), EGF, adenosine and pituitary adenylate cyclase-activating polypeptide (PACAP) that induce activation through (Huang et al., 2008; Lee and Chao, 2001; Rajagopal et al., 2002 2013) ligand-independent mechanism. Regardless of the mechanism, activation of TrkB leads to autophosphorylation of specific tyrosine residues within its intracellular domain that in turn serve as initiation sites for various signaling pathways. For example, phosphorylation of tyrosine 515 is associated with Ras/MAPK/ERK signaling while tyrosine 816 is associated with PLCγ-1 signaling (Chao, 2003; Huang and Reichardt, 2002; Middlemas et al., 1994). It has been shown that inhibition of tyrosine 816 phosphorylation specifically impairs PLCγ-1 signaling but not Ras/MAPK/ERK signaling while inhibition of tyrosine 515 phosphorylation impairs only Ras/MAPK/ERK signaling (Minichiello et al., 2002; Minichiello et al., 1999). As such, phosphorylation of TrkB at its various residues reports receptor activation and more specifically activation of individual signaling pathways arising from TrkB (Segal et al., 1996).

Due to this link between tyrosine phosphorylation and TrkB activation, much work investigating this receptor has utilized phospho-specific antibodies in either analyzing brain extracts with Western Blot or fixed tissue samples in immunohistochemistry. These techniques have provided a wealth of information about mechanisms underlying TrkB activation, the signaling pathways associated with TrkB,
and the functional consequences of TrkB activation – both physiologic and pathologic. However, these techniques are limited in their temporal and spatial resolution. Using phospho-specific antibodies does not provide a dynamic readout of TrkB activation patterns but rather a static snapshot in time and space. Elucidating the spatiotemporal properties of TrkB activation is critical because as has been shown many times before, these properties often have tremendous influence on the signaling pathways and functions mediated by specific receptors. This is especially true in polarized cells like neurons where the locale and timing of receptor activation may directly dictate its local signaling (Dehmelt and Bastiaens, 2010; Grecco et al., 2011; Yasuda, 2006). Thus, what is needed for TrkB is a dynamic readout that in the same experiment can provide spatial and temporal information about its activity.

4.1.2 FRET based sensors and FLIM imaging

The issue of dynamically measuring a protein’s activity in a living cell is not new. In fact, over the past decade or so, much work has been done to develop biosensors to accomplish this goal. Some of the most successful attempts have utilized sensors that rely on fluorescence resonance energy transfer (FRET). In fact, many of these sensors have been developed for molecules known to be critical in both LTP and sLTP and several of these sensors been utilized for imaging enzyme activity during sLTP induction (Murakoshi and Yasuda, 2012; Yasuda, 2006; Yasuda et al., 2006). For example, sensors for CaMKII, Ras, Cdc42, and RhoA have all been developed and
successfully used to image the activity of these proteins during sLTP. Not surprisingly, each protein has yielded a unique spatiotemporal profile that informs our understanding of how each protein contributes to mechanisms of plasticity (Lee et al., 2009; Murakoshi et al., 2011; Yasuda et al., 2006). Based on this success, we decided to utilize a similar FRET-based approach to develop a sensor for TrkB activity.

Fluorescence resonance energy transfer (FRET) is a technique dating back more than six decades. However, in the past decade, utilization of this technique in studying living cells has risen significantly. FRET is a physical process where an excited donor fluorophore non-radiatively transfers energy to an acceptor fluorophore and thereby causes donor emission to decrease and acceptor emission to increase (Figure 13A, C). The efficiency of FRET is strongly dependent on the distance separating the donor and acceptor molecules; more specifically, for effective FRET to occur, the two molecules must be within a few nanometers of each other (Selvin, 1995; Stryer; Yasuda, 2006). Because of this strong distance-dependence, FRET can be used to dynamically report interactions between fluorescently labeled proteins and as such, it has been utilized to make sensors for protein-protein interactions, enzyme activity, and even intracellular ion dynamics within living cells (Berglund et al., 2006; Lee et al., 2009; Murakoshi et al., 2011; Sorkin et al., 2000; Takao et al., 2005; Yasuda et al., 2006; Zhai et al., 2013).

Multiple approaches have been used to image FRET – fluorescence intensity, fluorescence lifetime, and fluorescence anisotropy (Yasuda, 2006). Of these, we have
chosen fluorescence lifetime imaging (FLIM). In this method, the fluorescence lifetime of the donor molecule is recorded. As the efficiency of FRET increases (the proximity of donor and acceptor increases), the donor fluorescence lifetime decreases (Figure 13A, B, C, and D). In a typical biological context, there is a mixed population of donor molecules, namely those that are free plus those that are bound to their acceptor. As such, the fluorescence lifetime curve generated for the entire population of donor molecules consists of both normal and shortened lifetimes due to the absence or presence of FRET respectively. If the donor molecule on its own displays single exponential decay, then the mixed population will yield a curve with a double exponential decay. From this curve, one can then calculate the fraction of donor molecules bound to an acceptor (binding fraction; see chapter 2 for more detail) and in turn measure the extent of interaction between two, labeled proteins of interest (Lee et al., 2009; Murakoshi et al., 2011; Yasuda, 2006; Yasuda et al., 2006).
Figure 13: FRET and its effects on fluorescence lifetime

A) In the absence of a protein-protein interaction, GFP and RFP are not in close proximity. As such, when GFP is excited, it produces robust emission without transferring any energy to RFP via FRET. B) In the absence of FRET, GFP fluorescence decays with an average lifetime of $\tau_D$. C) Following protein-protein interaction, GFP and RFP are brought in close proximity. As a result, when GFP is excited, some of its energy is transferred to RFP via FRET. The result is a decrease in GFP emission and an increase in RFP emission. D) Due to FRET, GFP fluorescence lifetime shortens ($\tau_{AD}$).

Because FLIM imaging is compatible with two-photon microscopy, the two have been combined (2pFLIM) to dynamically image a variety of FRET sensors (Lee et al., 2009; Murakoshi et al., 2011; Yasuda, 2006; Yasuda et al., 2006). Building from this
previous work, we will also utilize 2pFLIM to dynamically image our TrkB sensor so as to record TrkB activity before, during, and after glutamate uncaging. Of note, unlike these previous FRET-FLIM sensors, our sensor is the first to image the dynamic activity of a receptor tyrosine kinase.

4.2 Results

4.2.1 Sensor design

Similar to previously designed FRET-FLIM sensors (Murakoshi et al., 2011; Yasuda, 2006; Yasuda et al., 2006), the TrkB sensor consists of two components: 1) full-length TrkB fused to mEGFP (TrkB-mEGFP; Figure 14A) and 2) a fragment of the TrkB binding partner phospholipase-C-gamma-1 (PLCγ1; amino acids 659 – 769) containing the C-terminal SH2 domain with copies of monomeric red fluorescent protein fused to both its N- and C-termini (mRFP-PLC-mRFP; Figure 14A, B).

For TrkB-mEGFP, we utilized full-length TrkB as opposed to a TrkB fragment (the intracellular domain for example) so as to ensure proper trafficking, activation, and signaling from this recombinant protein. Additionally, the mEGFP fluorophore was selected because it has previously been demonstrated to be an excellent donor fluorophore for 2pFLIM (Murakoshi et al., 2011; Yasuda, 2006; Yasuda et al., 2006). Specifically, this fluorophore is bright facilitating two-photon imaging and it has a single exponential fluorescence lifetime decay curve simplifying fluorescence lifetime imaging (Yasuda, 2006; Yasuda et al., 2006)
For mRFP-PLC-mRFP, the C-terminal SH2 domain of PLCγ1 was selected for several reasons. As described above, phosphorylation of various tyrosine and serine residues within TrkB can serve as a surrogate measure of the receptor’s activation (Segal et al., 1996). As such, a potential sensor for TrkB activation would be one that reports the phosphorylation status of TrkB. Of the various residues within TrkB that are phosphorylated during activation, we chose to focus on tyrosine 816 because as shown in chapter 3, this residue and its phosphorylation are critical for sLTP. Thus, we hypothesized that during sLTP induction, tyrosine 816 is likely phosphorylated and that monitoring the phosphorylation state of this residue would be one way to report TrkB activation. It has been shown previously that when TrkB is phosphorylated at tyrosine 816, the protein PLCγ1 will bind to this residue via its SH2 domains (Bae et al., 2009; Felder et al., 1993; Middlemas et al., 1994; Schlessinger and Lemmon, 2003). The specific SH2 domain mediating this interaction is unfortunately not known since the crystal structure of TrkB bound to PLCγ1 is not currently available. However, a crystal structure for PLCγ1 interacting with the fibroblast growth factor receptor (FGFR; a receptor tyrosine kinase similar to TrkB) was recently produced (Bae et al., 2009). From this structure, it became clear that both the N- and C-terminal SH2 domains of PLCγ1 (amino acids 545 – 649 and 663 – 765 respectively; Figure 14B) are capable of interacting with an activated FGFR that is phosphorylated at tyrosine 766 – a residue analogous to tyrosine 816 in TrkB. However, it turns out that the N-terminal SH2 domain has a
higher affinity (nanomolar) for the receptor because once bound to phosphorylated tyrosine 766, a free portion of this domain binds to a secondary site within FGFR. This two-part binding establishes a very stable, high affinity interaction. In comparison, the C-terminal SH2 domain only binds to the phosphorylated tyrosine and thus has a much lower affinity for the receptor (micromolar). We extrapolated these findings to the TrkB/PLCγ1 interaction and thus predicted that the C-terminal SH2 domain would likely bind to active TrkB with lower affinity than the N-terminal domain. It is the Yasuda lab’s experience that acceptor constructs with micromolar affinity are optimal because such affinity seems to allow for reversible binding of the acceptor to the donor enabling bidirectional changes in the sensor’s signal, namely reporting both activation and deactivation. As such, we chose the C-terminal domain alone for use in our sensor.

We chose mRFP as the acceptor fluorophore because it has proven to be an excellent partner with mEGFP in FLIM imaging. Also, we included two mRFP molecules (one C-terminal and one N-terminal) per PLCγ1 fragment because previous work has demonstrated that mRFP has approximately a 50% probability of folding correctly and thus being a functional fluorophore. Since mRFP must be functional to contribute to FRET, this 50% functionality will reduce both the FRET efficiency and the corresponding FRET signal. It has been previously shown that including two mRFP fluorophores within a sensor will enhance both the FRET efficiency and signal that is
produced (Yasuda, 2006; Yasuda et al., 2006). Based on this, we also included two mRFP molecules per PLCγ1 fragment.

Figure 14: TrkB sensor design

A) Schematic of the TrkB FRET sensor consisting of TrkB-mEGFP and mRFP-PLC-mRFP. When TrkB-mEGFP is activated, mRFP-PLC-mRFP will bind. As a result, the mEGFP and mRFP molecules are brought in close proximity enabling FRET to occur. B) Protein fragment from full-length PLCγ1 used to generate mRFP-PLC-mRFP.

4.2.2 Sensor characterization in HeLa cells

To test the functionality of the TrkB sensor, we first expressed it in HeLa cells – a mammalian cell line that does not normally express TrkB – using Lipofectamine transfection. The first question we examined was whether TrkB-mEGFP would traffic to the plasma membrane. Demonstrating such trafficking is critical since TrkB must be at the plasma membrane to be activated by the extracellular protein BDNF. To determine if TrkB-mEGFP trafficked appropriately, we imaged transfected cells using two-photon
microscopy. Consistent with plasma membrane localization, we found that TrkB-mEGFP fluorescence was most prevalent at the cell periphery. In contrast, mRFP-PLC-mRFP fluorescence was found in equivalent amounts throughout the cell suggesting a cytoplasmic distribution (Figure 15).

![Figure 15: TrkB sensor expression in HeLa cells](image)

HeLa cells were transfected with the TrkB sensor (TrkB-mEGFP and mRFP-PLC-mRFP) and then imaged using two-photon microscopy.

To demonstrate that the TrkB sensor can be activated appropriately, we stimulated HeLa cells transfected with the TrkB sensor with 100ng/ml BDNF for 10 minutes and then collected the cellular lysates. We performed two separate experiments with these lysates to determine whether TrkB-mEGFP was in fact being activated in response to BDNF.

For the first analysis, we performed an immunoprecipitation (IP) using an antibody against the phosphorylated form of tyrosine (pTyr). We then probed the IP sample using antibodies against TrkB and green fluorescent protein (GFP) in a Western
Blot. As predicted, for the BDNF-treated cells, we found an increased immunoreactive signal with both the TrkB and GFP antibodies as compared to the signals for the vehicle-treated cells (Figure 16A). The observed signal was at approximately 160kDa, a molecular weight consistent with the predicted size of TrkB-mEGFP. Additionally, to confirm that equivalent amounts of protein were loaded into each IP sample, we probed the original lysate samples with antibodies against TrkB, GFP, and actin in a Western Blot and found similar immunoreactive signals for the BDNF- and vehicle treated cells. These results are consistent with the phosphorylated tyrosine antibody precipitating with a greater amount of TrkB-mEGFP in the BDNF-treated cells thus suggesting an increased tyrosine phosphorylation of TrkB-mEGFP following BDNF treatment.

For the second analysis, we probed cell lysates from the BDNF- and vehicle-treated cells with antibodies against phosphorylated tyrosine 515 within TrkB, TrkB, GFP, and actin. Consistent with the IP results, we found a BDNF-dependent increase in TrkB phosphorylation at tyrosine 515 (Figure 16B). Unfortunately, we did not have an effective antibody for phosphorylation of tyrosine 816 and thus could not evaluate this site. However, previous work in the McNamara Lab and others has consistently shown that BDNF application phosphorylates both tyrosine 515 and 816 (Huang and McNamara, 2010; Huang and McNamara, 2012; Huang et al., 2008). Thus, our ability to demonstrate phosphorylation of tyrosine 515 is likely predictive of tyrosine 816 phosphorylation as well. Taken together, the results from these two analyses indicate
that TrkB-mEGFP is able to respond to BDNF in a manner analogous to that observed with endogenous sources of TrkB.

**Figure 16:** BDNF-mediated tyrosine phosphorylation of the TrkB sensor in HeLa cells

**A** (Upper) Western blot analysis (IB) of cell extracts from HeLa cells stimulated with either BDNF or vehicle. Extracts were immunoprecipitated with an antibody for phosphorylated tyrosine residues (pTyr) and then probed with antibodies for TrkB and GFP. (Lower) IB of BDNF and vehicle stimulated cell extracts prior to IP using antibodies for TrkB, GFP, and actin. **B** IB of cell extracts from HeLa cells stimulated with BDNF or vehicle using antibodies for TrkB phosphorylated at Y515 (pTrkB(Y515)), TrkB, GFP, and actin.

With evidence that the TrkB sensor is functional, we next sought to image the sensor in HeLa cells using 2pFLIM. Based on the sensor design, we predicted that the observed BDNF-induced phosphorylation would increase the affinity of mRFP-PLC-mRFP for TrkB-mEGFP. As a result, mEGFP and mRFP would be brought in close
proximity allowing FRET to occur (Figure 13C, 14A). This FRET reaction would in turn lead to a decrease in the fluorescence lifetime of mEGFP (Figure 13C, D). Using the fluorescence lifetime decay for mEGFP, we could then calculate the fraction of TrkB-mEGFP bound to mRFP-PLC-mRFP (binding fraction) and in turn utilize changes in binding fraction as a surrogate measure of TrkB activation – an increase in binding fraction would represent increased TrkB activation and vice versa. As expected, when we expressed the sensor in HeLa cells and then applied 100ng/ml BDNF, we observed an increase in the sensor’s binding fraction via 2pFLIM consistent with TrkB activation (Figure 17A, B, and C). When we applied vehicle, we did not see a similar increase (Figure 17B, C). These results thus indicate that the sensor is sensitive to a known TrkB activating stimulus, namely BDNF.

To address the specificity of the sensor’s signal, we mutated tyrosine 816 within the sensor to phenylalanine – an amino acid that cannot be phosphorylated. Following application of BDNF, we did not observe an increase in the sensor’s binding fraction (Figure 17A, B, and C). Since previous work has demonstrated preserved phosphorylation of other tyrosine residues within TrkB receptors containing the Y816F mutation (Minichiello et al., 2002), the lack of an increased binding fraction in response to BDNF suggests that our sensor is specifically reporting phosphorylation of tyrosine 816 and not other phosphorylated residues within TrkB.
Collectively, these results demonstrate that our sensor design is effective in that it can be expressed in a living cell, it is responsive to BDNF application, and its activation can be dynamically imaged using 2pFLIM.

Figure 17: Characterization of the TrkB sensor in HeLa cells

A) FLIM images of TrkB and TrkB<sub>Y816F</sub> sensor activation acquired before and 2-6 minutes after BDNF stimulation (single image was acquired by averaging multiple images over 5 minutes). Warmer colors represent shorter mEGFP fluorescent lifetimes and thus higher TrkB activity. B) Time course of TrkB and TrkB<sub>Y816F</sub> sensor activation measured as the change in binding fraction of TrkB-mEGFP or TrkB<sub>Y816F</sub>-mEGFP bound to mRFP-PLC-mRFP before and after BDNF or vehicle stimulation. Sample number (cells/experiment) is 22/8 for TrkB plus BDNF (black trace), 9/4 for TrkB plus vehicle (blue trace), and 11/4...
for TrkB\textsuperscript{Y816F} plus BDNF (red trace). C) Quantification of TrkB and TrkB\textsuperscript{Y816F} sensor activation for experiments in (B) (averaged over 6-10 minutes). Error bars are s.e.m. An asterisk denotes statistical significance (p < 0.05) as determined by an analysis of variance (ANOVA) followed by post-hoc tests using the least significant difference.

4.2.3 Sensor characterization in cultured cortical neurons

To further characterize the sensor’s efficacy and utility, we expressed it in dissociated mixed neuron cultures using Lipofectamine transfection. One to two days following transfection, we applied BDNF (30 ng/ml) and found a rapid increase in sensor binding fraction that peaked at approximately 5 minutes, remained elevated for at least 30 minutes, and then was reversed by the TrkB kinase inhibitor K252a (200 nM; Figure 18A, B, and C). In contrast, we found that application of nerve growth factor (NGF; 30 ng/ml), a ligand for TrkA but not TrkB, did not yield any significant increase in binding fraction (Figure 18B, C).

Additionally, to examine non-BDNF mechanisms of TrkB activation, we applied the divalent cation zinc (500 µM) – an ion known to transactivate TrkB in a BDNF-independent manner (Huang and McNamara, 2010; Huang et al., 2008). Once again, we observed a rapid increase in sensor binding fraction that peaked at around 5 minutes and then remained elevated for at least 30 minutes following initial stimulation (Figure 18D, E). Additionally, this increase in binding fraction was reversed by application of K252a (Figure 18D, E).
Altogether, these results collected in HeLa cells and cultured neurons demonstrate that the TrkB sensor is sensitive to various TrkB stimuli, it is specific for tyrosine 816, and it is bidirectional in that it reports both activation and inactivation.

Figure 18: Characterization of the TrkB sensor in cultured cortical neurons

A) FLIM images of TrkB activation in a cultured neuron transfected with the TrkB sensor before and after BDNF stimulation followed by K252a application at 30 minutes. Warmer colors represent shorter mEGFP fluorescent lifetimes and higher TrkB activity.
B) Time course of TrkB sensor activation in transfected cultured neurons measured as the change in binding fraction of TrkB-mEgFP bound to mRFP-PLC-mRFP before and after BDNF or NGF stimulation followed by K252a application at 30 minutes. The number of samples (neurons) is 8 for BDNF (black trace) and 4 for NGF (red trace). C) Quantification of TrkB sensor activation for experiments in (B) averaged over 10-30 minutes following BDNF/NGF stimulation and 3-5 minutes following K252a application. D) Time course of TrkB sensor activation in transfected cultured neurons measured as described in (B). E) Quantification of TrkB sensor activation for experiments in (D) averaged over 10-30 minutes following zinc stimulation and 3-5 minutes following K252a application. Sample number (neurons) is 6. Error bars are s.e.m. A single asterisk denotes statistical significance (p < 0.05) as determined by an analysis of variance (ANOVA) followed by post-hoc tests (when appropriate) using the least significant difference. A double asterisk indicates statistical significance (p < 0.05) as determined by a two-tailed paired samples t-test.

4.3 Discussion

In this chapter, we designed, developed, and characterized a novel FRET-FLIM sensor for TrkB activation. Based on data collected, the sensor is able to traffic to the plasma membrane and then be phosphorylated in response to BDNF. Additionally, we provide evidence that through 2pFLIM, we are able to monitor changes in the phosphorylation state of tyrosine 816 specifically in response to both BDNF and zinc (known TrkB activators). We further demonstrate that the change in binding fraction reported by the sensor is reversible through TrkB inhibition thus suggesting that our sensor can report both TrkB activation and inactivation.

4.3.1 Functionality of the TrkB sensor

For the TrkB sensor to be an effective measure of endogenous TrkB activity, it must function in a manner analogous to the endogenous TrkB population. Specifically, the sensor should traffic to the appropriate sub-cellular locations and in turn be
responsive to known TrkB stimuli. To start, TrkB is known to localize to the plasma membrane where it in turn is able to bind to BDNF, a cell membrane impermeable, extracellular ligand. Similarly, our sensor shows an expression pattern in HeLa cells consistent with plasma membrane localization, namely it is prevalent at the periphery of the cell. Furthermore, in response to BDNF application, the sensor displays robust activation in both HeLa cells and cultured neurons. Such sensitivity to BDNF is promising for the sensor’s ability to detect and report TrkB activation during sLTP. As shown in chapter 3, sLTP depends critically on post-synaptic BDNF expression. As we discussed above, sLTP induction may lead to BDNF induced activation of TrkB. Because of our sensor’s ability to respond to BDNF, it is likely that we will be able to use the sensor to detect this event.

In addition to being activated by BDNF, endogenous TrkB is also activated by a variety of non-BDNF stimuli through transactivation mechanisms. A recently discovered source of TrkB activation is the divalent action zinc. Consistent with these findings, we also demonstrate that our sensor can respond to zinc application. Even though we do not currently have evidence demonstrating a role for zinc in sLTP of CA1 dendritic spines, it is conceivable that zinc could be a critical player. Specifically, there is evidence for zinc in clear synaptic vesicles colocalized with glutamate at the CA3-CA1 synapse and there is precedent for zinc being an important regulator of plasticity at another hippocampal synapse – the mossy fiber-CA3 synapse (Frederickson et al., 2005;
Pan et al., 2011). If zinc is indeed mediating some TrkB activation during sLTP, our sensor is capable of detecting such activation.

Lastly, the kinetic activation profiles of a variety of proteins involved in sLTP show a high degree of dynamism with both activation and inactivation. For example, CaMKII activity quickly increases, peaks, and then decays back to baseline in response to sLTP induction. Correctly identifying and monitoring this pattern of activity has proven informative with respect to CaMKII’s functional role with sLTP. As such, it is likely that TrkB activity may show a similar dynamic pattern and thus an effective sensor must be able to report both activation and inactivation. For our sensor, we found inhibition of the TrkB kinase with K252a following BDNF- and zinc-stimulation could reverse the sensor’s signal and return it to baseline. These results indicate that our sensor is bidirectional and suggest that our sensor may be able to detect both increases and decreases of TrkB activity during sLTP induction.

4.3.2 Specificity of the TrkB sensor

Within TrkB, there are multiple residues that can be phosphorylated in response to both BDNF and non-BDNF stimuli. Each residue is associated with a unique signalling pathway. As discussed in chapter 3, not all of these residues and their corresponding signalling pathways are critical for either functional or structural plasticity of CA1 dendritic spines. Based on these results, it is possible that during sLTP some but not all residues within TrkB may get phosphorylated and further, for those
that are phosphorylated, the kinetics of these events may be distinct. As such, an ideal sensor would detect only one such event, preferably the one that is most important for sLTP. Here, our sensor was designed to detect phosphorylation of tyrosine 816 – residue we previously demonstrated to be critical for sLTP. Consistent with this, we find that mutation of this residue within the sensor impairs development of a BDNF-induced signal. Since previous work has shown that mutation of tyrosine 816 does not impair phosphorylation of other residues within TrkB, it is likely that other residues within TrkB(Y816F)-mEGFP, most notably tyrosine 515, are still being phosphorylated. As such, the fact that we did not find any change in binding fraction for the sensor indicates that the mRFP-PLC-mRFP selectively detects phosphorylation of tyrosine 816 and not other residues within TrkB.

4.3.3 Development of other TrkB and receptor tyrosine kinase sensors

The current sensor we developed was specific for phosphorylation of tyrosine 816 within TrkB. However, because of the success of this first sensor, we have begun to develop a sensor specific for TrkB activation at tyrosine 515. Like the current sensor, we will use full-length TrkB-mEGFP as the donor construct since we have previously shown that in response to BDNF, this recombinant protein is phosphorylated at tyrosine 515. For the acceptor construct, we substituted a fragment of the Shc adaptor protein for the C-terminal SH2 domain for PLCγ1. Previous work has shown that the Shc protein will bind to phosphorylated tyrosine 515 similar to how PLCγ1 binds to phosphorylated
tyrosine 816. The utility of a sensor specific for tyrosine 515 is that it may reveal a spatiotemporal profile distinct from our current sensor. If true, this would suggest that tyrosine 515 and 816 might get phosphorylated by independent mechanisms that could explain their distinct roles in various neuronal functions.

In addition to sensors for TrkB activation, the design utilized here could also be extended to other receptor tyrosine kinases important for neuron physiology and pathology. Specifically, we have shown that a two-component design can yield an effective FRET-FLIM sensor capable of dynamically reporting changes in the activity of a receptor tyrosine kinase. Application of this approach to novel receptors would further enhance our understanding of the mechanisms underlying the activation of these receptors as well as their ultimate contributions to a variety of neuronal processes.

4.3.4 Conclusion

In conclusion, we have developed a novel biosensor for TrkB that can be used to dynamically image the activity of this receptor in living cells, specifically neurons. In the next chapter, we will utilize this sensor to study TrkB activity in single CA1 dendritic spines undergoing sLTP. The successful design and implementation of this sensor will hopefully foster development of additional sensors for both TrkB as well as other receptor tyrosine kinases. As has been shown previously, the ability to dynamically image the activity of a protein with high temporal and spatial resolution yields tremendous mechanistic and functional insight. Consequently, development of novel
biosensors is critical for continued gains in understanding how plasticity as well as a wide array of other neuronal processes work.
5. Spatiotemporal properties of TrkB activation during single spine structural plasticity

5.1 Introduction

In chapter 3, we identified a critical role for post-synaptic BDNF-TrkB signaling in single spine structural plasticity. To further examine this role, we utilized the FRET-based TrkB sensor developed in chapter 4 to image the spatiotemporal dynamics of TrkB activation before, during, and after sLTP induction. Here, we report a fast (onset within 1 minute) and sustained (lasting longer than 20 minutes) activation of TrkB in response to glutamate uncaging. Initially, this activation is largely restricted to the stimulated spine; however, with time, activation develops in the adjacent dendrite and surrounding spines as well. Mechanistically, this activation depends on NMDAR-CaMKII signaling as well as post-synaptically synthesized BDNF. In sum, these results identify an autocrine BDNF-TrkB signaling loop in which activity induces a rapid, initially spine specific activation of TrkB that depends on BDNF from the stimulated cell.

5.1.1 Known mechanisms contributing to sLTP

To induce sLTP of CA1 dendritic spines, we utilize a two-photon laser to focally uncage MNI-L-glutamate (caged glutamate) near the head of a single dendritic spine. Following uncaging, glutamate dissociates from its chemical cage thereby enabling it to bind to nearby glutamate receptors (Matsuzaki et al., 2001; Matsuzaki et al., 2004). One such receptor is the post-synaptic NMDAR. Normally, glutamate binding alone is
insufficient to open NMDARs because of their voltage-dependent block by Mg\(^{2+}\) (Paoletti et al., 2013). However, since we perform our experiments in ACSF with minimal levels of Mg\(^{2+}\), there is no Mg\(^{2+}\) block and thus glutamate binding alone is sufficient to activate these receptors on the dendritic spine leading to Ca\(^{2+}\) influx (Lee et al., 2009; Matsuzaki et al., 2004; Zhai et al., 2013). Once in the spine, this Ca\(^{2+}\) binds to calmodulin leading to activation of CaMKII (Lisman, 1994). Active CaMKII then initiates signaling pathways leading to the activation of a variety of downstream molecules including the small GTPases – Ras, RhoA, Rac1, and Cdc42 – which ultimately culminate in increased spine volume, AMPAR trafficking to the post-synaptic density, and a functional potentiation of the electrical properties of the spine (Lee et al., 2009; Murakoshi et al., 2011; Patterson and Yasuda, 2011; Yasuda et al., 2006). As we have shown in chapter 3, post-synaptic BDNF-TrkB signaling is a critical component of these mechanisms underlying sLTP. However, how BDNF release and the resulting TrkB activation relate to the above described signaling mechanisms for sLTP is not known. To address this question, we sought to visualize TrkB activity before, during, and after sLTP induction using our FRET-based sensor for TrkB. With such spatial and temporal information regarding TrkB activation, we hoped to gain functional insight into how and where BDNF-TrkB signaling contributes to sLTP thus allowing us to build on these current mechanistic models for sLTP.
5.1.2 Utilizing 2pFLIM to image TrkB during sLTP

One of the primary advantages of combining two-photon glutamate uncaging with 2pFLIM is the ability to image FRET-based sensors during the uncaging stimulation. Specifically, by interleaving 2pFLIM with two-photon uncaging, one can collect data from the FRET sensor while simultaneously activating a spine with glutamate uncaging (Lee et al., 2009; Murakoshi et al., 2011; Yasuda, 2006; Yasuda et al., 2006). As depicted in Figure 19, during fast FLIM imaging, two-photon uncaging and two-photon imaging alternate. Individual FLIM images produced are actually the average of 8 imaging epochs with one epoch occurring in between two uncaging pulses. As such, our sample rate for sensor activation is approximately 16 seconds. Put another way, a single FLIM image during fasting imaging is a snapshot of TrkB activation in 16-second blocks – a temporal resolution much greater than traditional biochemical and immunohistochemical methods for reporting TrkB activation (Figure 19). Thanks to fast FLIM imaging, we can monitor a protein’s activity throughout the entire course of structural plasticity – before, during, and after induction. Such ability is critical as it may be necessary to fully capture a protein’s entire time course of activation. For example, with CaMKII, its activity develops, peaks, and then decays back to baseline all during the stimulation epoch (Lee et al., 2009). In light of these previous findings, we utilize our TrkB sensor to visualize TrkB activity before, during, and after sLTP induction.
In order to collect sensor activation data in the midst of two-photon uncaging, we interleave uncaging with imaging. So as to acquire enough photos for a usable image, we average 8 individual imaging epochs. As such, our sensor can report changes in TrkB activity with a temporal resolution down to 16 seconds.

### 5.2 Results

#### 5.2.1 Temporal and spatial properties of TrkB activation during single spine structural plasticity

Our findings in chapter 3 highlight a critical role for post-synaptic TrkB during sLTP. Two questions arising from these findings are when does TrkB activation develop during sLTP and where does such activation occur within the post-synaptic compartment. To address these questions, we biolistically transfected rat cultured hippocampal slices with the TrkB sensor. One to two days following transfection, we utilized 2pFLIM to image single dendritic spines of transfected CA1 pyramidal cells bathed in ACSF containing 4mM Ca\(^{2+}\), 0mM Mg\(^{2+}\), 1µM tetrodotoxin (TTX), and 4mM
caged glutamate. While imaging, we induced sLTP using a low-frequency train of two-photon glutamate uncaging pulses (30 pulses at 0.5Hz with each pulse lasting 4-6ms) positioned at the head of a single dendritic spine. To estimate the spine volume throughout sLTP induction, we utilized the fluorescence intensity of mRFP-PLC-mRFP.

In response to glutamate uncaging, spine volume rapidly increased to 192±20% (transient phase) before relaxing to a persistent, elevated state of 87±14% lasting at least 20 minutes (sustained phase) (Stim Spine; Figure 20A, B, C).

In parallel to these changes in spine volume, we also found a rapid increase in TrkB activity in the stimulated spine as reported by the TrkB sensor. This increase in TrkB activity developed within seconds following the onset of the uncaging stimulus, it peaked at around 1 to 2 minutes, and then remained persistently elevated for at least 20 minutes (Stim Spine; Figure 20A, D, and E). To determine if TrkB activity is restricted to the stimulated spine, we also examined TrkB activation at the spine base (Spine base), in neighboring spines (Adj spine), and in the adjacent dendrite (Dendrite). We did find increases in activity in these adjacent areas but they developed with slower kinetics and lower magnitude compared to the stimulated spine (Figure 20A, D, E).
Figure 20: Spatiotemporal dynamics of TrkB activation during single spine structural plasticity

A) Averaged FLIM images of TrkB sensor activation during single spine structural plasticity. Arrowhead indicates the stimulated spine. Uncaging indicates images collected during the glutamate uncaging stimulus. Warmer colours indicate shorter lifetimes and higher TrkB activity. B) Time course of spine volume change in response to glutamate uncaging. C) Transient (averaged over 1-2 minutes) and (averaged over 10-20 minutes) spine volume change for experiments in (B). D) (Left) Time course of TrkB sensor activation for experiments in (B) measured as change of TrkB-mEGFP bound to mRFP-PLC-mRFP in the stimulated spine (Stim spine, n = 45 spines), the base of the spine neck (Spine base, n = 45 spines), adjacent spines (Adj spine, n = 47 spines), and the dendritic shaft adjacent to the stimulated spine (Dendrite, n = 45 dendrites). (Right) A closer view of TrkB sensor activation during the uncaging epoch. E) Peak (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) TrkB sensor activation for experiments in (D). Error bars are s.e.m. Asterisks denote statistical significance (p <0.05) as determined by ANOVA followed by post-hoc tests using the least significant difference.
To further compare TrkB activity in the stimulated spine and adjacent dendrite, we examined the spatial profile of TrkB activity within the dendrite as a function of distance from the stimulated spine (Figure 21A). During the first two minutes following glutamate uncaging, we found that TrkB activity was largely restricted to the stimulated spine with only a small fraction spreading into the dendrite. To quantify the degree of this initial compartmentalization, we calculated the spreading index (S.I.) for TrkB activity 1 to 2 minutes after stimulation and found it to be 0.14 – we define the spreading index as the ratio of TrkB activity in the dendrite 1 to 5 µm from the stimulated spine compared to maximal spine activation. In comparison to other molecules studied in sLTP, TrkB’s initial spreading index is most consistent with Cdc42 – a molecule whose activity is largely restricted to the stimulated spines as evident by an S.I. of 0.12 – as compared to Rac1 and RhoA – two molecules whose activity spreads from the stimulated spine out into the dendrite as evident by S.I. of 0.42 and 0.31, respectively (Figure 21B and Table 2). Further comparisons between TrkB and the activity of these three Rho GTPases are analysed and discussed in chapter 7. Despite this initial restriction of activity to the spine, dendrite activity does rise with time suggesting a loss of compartmentalization for TrkB activity during later phases of sLTP (Figure 20D, E, and 21A).
Figure 21: Spatial profile of TrkB activity single spine structural plasticity

A) Spatial profile of TrkB sensor activation within the dendrite measured as the change in TrkB-mEGFP bound to mRFP-PLC-mRFP and plotted as a function of distance away from the stimulated spine. Time epochs examined were 1-2 minutes (black trace) and 11-20 minutes after glutamate uncaging (green trace). B) Comparison of TrkB’s spatial profile 1-2 minutes after glutamate uncaging to that of three other molecules involved in sLTP – Rac1, RhoA, and Cdc42. Each spatial profile is normalized to its maximal spine activation 1-2 minutes after glutamate uncaging. Error bars are s.e.m.

Table 2: Spreading indices for TrkB, Cdc42, RhoA, and Rac1

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<th>Spreading Index</th>
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<tr>
<td>TrkB</td>
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<tr>
<td>Cdc42</td>
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<tr>
<td>RhoA</td>
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<td>Cdc42</td>
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To confirm the validity of these changes in TrkB activity reported by the TrkB sensor, we examined the dependence – or lack thereof – of the sensor’s signal on TrkB kinase activity, temperature, and sensor concentration. To evaluate its dependence on kinase activity, we applied K252a prior to inducing sLTP. Compared to untreated
spines, we found nearly complete inhibition of TrkB activity with a corresponding reduction in spine volume change (Figure 22). To determine if TrkB activity depends on ACSF temperature, we compared TrkB activity during sLTP induced at either room or near physiological temperatures (30-32°C). In both conditions, we found comparable spine volume change and TrkB activity (Figure 23). Lastly, to determine if the observed patterns of activity and sLTP depended on TrkB sensor concentration, we compared the sensor’s concentration (measured at the apical dendrite immediately adjacent to the soma) to the corresponding spine volume change during the transient phase of sLTP, basal binding fraction reported by the sensor, and peak change in binding fraction observed in both the stimulated spine and adjacent dendrite. For all four measures, we did not find any correlation (Figure 24). Cumulatively, these results provide strong evidence that our sensor is in fact reporting TrkB activity in a specific manner. As such, the results reported above with the TrkB sensor suggest that glutamate uncaging induces a rapid and persistent increase in TrkB activity that is initially restricted to the stimulated spine but with time spreads into the adjacent dendrite and surrounding spines.
Figure 22: Effect of TrkB kinase inhibition glutamate uncaging induced TrkB activation

A) Time course of TrkB sensor activation measured as the change in TrkB-mEGFP bound to mRFP-PLC-mRFP before (Ctrl, n = 45 spines) and at least 30 min after K252a application to the perfusion bath (K252a, n = 9 spines).  B) Peak (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) TrkB sensor activation for experiments in (A).  C) Time course of spine volume change for experiments in (A).  D) Transient (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) spine volume change for experiments in (A).  Error bars are s.e.m. Asterisks denote statistical significance (p<0.05) as determined by an ANOVA.
Figure 23: Effect of temperature on glutamate uncaging induced TrkB activation

Time course of TrkB sensor activation measured as the change in TrkB-mEGFP bound to mRFP-PLC-mRFP in either room temperature or near-physiologic temperature ACSF (30°C-32°C).

Figure 24: Effect of TrkB sensor concentration on glutamate caging induced TrkB activation and spine volume change
Effect of TrkB-mEGFP concentration as measured in individual neurons on the corresponding change in binding fraction of the stimulated spine (A), change in spine volume (B), binding fraction prior to uncaging (basal binding fraction) (C), and change in binding fraction of the dendrite (D) during single spine structural plasticity.

5.2.2 Mechanisms underlying TrkB activation during single spine structural plasticity

Since single spine sLTP is known to be critically dependent on Ca\textsuperscript{2+} influx through NMDARs and subsequent downstream CaMKII activation, we asked whether glutamate-uncaging induced TrkB activation also requires the NMDAR-CaMKII pathway. To examine the dependence of TrkB activity on both NMDARs and CaMKII, we first induced sLTP at single, CA1 dendritic spines in cultured rat hippocampal slices while simultaneously imaging TrkB activity using 2pFLIM (Ctrl; Figure 25). Following successful induction of both sLTP and TrkB activation, we applied either the NMDAR inhibitor APV (100 µM) or CaMKII inhibitor peptide CN21 (10 µM) (Vest et al., 2007) to the slice for at least 30 minutes and then attempted to induce sLTP while imaging the TrkB sensor on another dendritic spine from the same cell. In the presence of APV, we found a near complete reduction in TrkB activity during both its peak and sustained phases with a corresponding blockade on spine volume during the transient and sustained phases of sLTP (AP5; Figure 25). Similarly, in the presence of CN21, we found a robust impairment of TrkB activity during its peak and sustained phases with a parallel reduction in spine volume (CN21; Figure 25). These results indicate that TrkB activation during sLTP lies downstream of both NMDARs and CaMKII activation.
Figure 25: Effect of NMDAR and CaMKII inhibition on glutamate uncaging induced TrkB activation

A) Averaged FLIM images of TrkB sensor activation during single spine structural plasticity in the absence (Ctrl) or presence of AP5 and CN21. Arrowhead indicates the stimulated spine. Uncaging indicates images collected during the glutamate uncaging
stimulus. Warmer colours indicate shorter lifetimes and higher TrkB activity. **B)** Time course of TrkB sensor activation measured as the change in TrkB-mEGFP bound to mRFP-PLC-mRFP before (Ctrl, black trace, n = 19 spines) and at least 30 min after AP5 (blue trace, n = 10 spines) or CN21 (red trace, n = 17 spines) application. **C)** Peak (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) TrkB sensor activation for experiments in **B**. **D)** Time course of spine volume change for experiments in **B**. **E)** Transient (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) spine volume change for experiments in **B**. Error bars are s.e.m. Asterisks denote statistical significance (p<0.05) as determined by an ANOVA followed by post-hoc tests using the least significant difference.

### 5.2.3 Contribution and cellular locale of BDNF underlying TrkB activation during single spine structural plasticity

As demonstrated in chapter 3, sLTP depends on both BDNF and TrkB and since BDNF is the canonical ligand for TrkB, we hypothesized that the observed TrkB activation during sLTP is BDNF-mediated. To evaluate this hypothesis, we once again induced sLTP at single, CA1 dendritic spines while simultaneously imaging TrkB activity using 2pFLIM (Ctrl; Figure 26). Following the successful induction of both sLTP and TrkB activation, we applied the extracellular BDNF scavenger TrkB-Ig (6-8 µg/ml) or a control molecule (Human-IgG, 8 µg/ml) to the slice for at least 30 minutes before inducing sLTP on another dendritic spine from the same cell. In the presence of TrkB-Ig, we found impaired TrkB activation during both the peak and sustained phases with similar reductions in the associated spine volume change (TrkB-Ig; Figure 26). In the presence of human-IgG however, TrkB activity and spine volume change were comparable to those observed in untreated control spines (H-IgG; Figure 26) thus confirming the specificity of TrkB-Ig’s effect on sLTP and TrkB activation.
Figure 26: Effect of scavenging extracellular BDNF on glutamate uncaging induced TrkB activation

A) Averaged FLIM images of TrkB sensor activation during single spine structural plasticity in the absence (Ctrl) or presence of TrkB-Ig. Arrowhead indicates the stimulated spine. Uncaging indicates images collected during the glutamate uncaging stimulus. Warmer colours indicate shorter lifetimes and higher TrkB activity. B) Time course of TrkB sensor activation measured as the change in TrkB-mEGFP bound to mRFP-PLC-mRFP before (Ctrl, black trace, n = 18 spines) and at least 30 min after
Human-IgG (HIgG, blue trace, n = 11 spines) or TrkB-Ig (red trace, n = 15 spines) application. C) Peak (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) TrkB sensor activation for experiments in (B). D) Time course of spine volume change for experiments in (B). E) Transient (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) spine volume change for experiments in (B). Error bars are s.e.m. Asterisks denote statistical significance (p<0.05) as determined by an ANOVA followed by post-hoc tests using the least significant difference.

These results indicate that extracellular BDNF is a primary means for TrkB activation during sLTP but they do not inform us about the cellular source of this BDNF. To determine if post-synaptically expressed BDNF underlies this activation, we biolistically transfected cultured hippocampal slices from Bdnf\textsuperscript{fl/fl} mice with either the sensor plus mCherry (Cre negative) or the sensor, mCherry, and Cre-tdTomato (Cre positive). As described above, the sparse transfection associated with this technique led to sensor and Cre expression in only a few cells in the slice (Lu et al., 2009). Five to seven days following transfection, we once again induced sLTP on single, CA1 dendritic spines while simultaneously imaging TrkB activity using 2pFLIM. For spines from Cre negative cells, we found robust TrkB activation in the stimulated spine with corresponding changes in spine volume (Cre Neg, Figure 27). For spines from Cre positive cells, we found reduced but not completely impaired TrkB activation with parallel reductions in sLTP (Cre Pos, Figure 27). These results implicate post-synaptically synthesized BDNF as a primary means of activating spine TrkB during single spine structural plasticity.
Figure 27: Effect of post-synaptic BDNF knockout on TrkB activation during single spine structural plasticity

A) Time course of TrkB sensor activation measured as the change in TrkB-mEGFP bound to mRFP-PLC-mRFP in cells transfected with the TrkB sensor (Cre Neg, black trace, n = 15 spines) or the TrkB sensor plus Cre-tdTomato (Cre Pos, red trace, n = 17 spines). B) Peak (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) TrkB sensor activation for experiments in (A). C) Time course of spine volume change for experiments in (A). D) Transient (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) spine volume change for experiments in (A). Error bars
are s.e.m. Asterisks denote statistical significance (p<0.05) as determined by an ANOVA followed by post-hoc tests using the least significant difference.

5.2.4 CaMKII activity during inhibition of BDNF signaling

In chapter 3, we addressed concerns regarding neuronal health in the setting of post-synaptic BDNF knockout by rescuing the sustained phase of sLTP with acute application of BDNF. To further address the concerns regarding cell health and synaptic integrity following post-synaptic BDNF knockout, we sought to visualize CaMKII activation. Since we previously showed that BDNF-TrkB signaling depends on CaMKII, we hypothesized that its activation should be independent of BDNF and could thus be used as a measure of dendritic spine signaling integrity upstream of both BDNF and TrkB. To evaluate this hypothesis, we biolistically transfected cultured hippocampal slices from Bdnf\textsuperscript{fl/fl} with either previously developed CaMKII sensor (Lee et al., 2009) plus mCherry (Cre Negative) or the CaMKII sensor mCherry, and Cre (Cre Positive). After 5 to 7 days of transfection, we induced sLTP on single, CA1 dendritic spines using two-photon glutamate uncaging while simultaneously imaging CaMKII activity using 2pFLIM. For spines from Cre negative cells, we found robust CaMKII activation with corresponding spine volume change consistent with previously published findings (Cre Neg, Figure 28). For spines from Cre positive cells, we found reduced sLTP consistent with post-synaptic BDNF knockout but unimpaired CaMKII activity (Cre Pos, Figure 28). These results confirm that CaMKII activation is upstream of BDNF-TrkB signaling. Moreover, these results also demonstrate preserved upstream signaling mechanisms in
the setting of post-synaptic BDNF knockout. Such integrity of NMDR-mediated CaMKII activation and signalling argues against global ill health of the neuron induced by reduction of BDNF expression.

**Figure 28: Effect of post-synaptic BDNF knockout on glutamate uncaging induced CaMKII activation**

**A)** Time course of CaMKII sensor activation measured as the change in fluorescent lifetime of mEGFP-CaMKII in cells transfected with the CaMKII sensor (Cre Neg, black trace, n = 13 spines) or the CaMKII sensor plus Cre-tdTomato (Cre Pos, red trace, n = 15 spines).  
**B)** Peak (averaged over 0-48 seconds) CaMKII sensor activation for experiments in **(A)**.  
**C)** Time course of spine volume change for experiments in **(A)**.  
**D)** Transient (averaged over 1-2 minutes) and sustained (averaged over 8-10 minutes) spine volume...
change for experiments in (A). Error bars are s.e.m. Asterisks denote statistical significance (p<0.05) as determined by an ANOVA.

5.3 Discussion

Here we combined two-photon glutamate uncaging with 2pFLIM to examine the spatiotemporal properties of TrkB activation during single spine structural plasticity. We find that in response to glutamate uncaging, TrkB activates rapidly (onset less than 1 minute) and persistently (lasting for at least 20 minutes). Initially, this activity is restricted to the stimulated spine but with time, it develops in the adjacent dendrite and neighboring spines. We further report that this activity depends on NMDAR and CaMKII activation as well as post-synaptic BDNF. All together, these results highlight an activity-induced autocrine BDNF-TrkB signalling loop within dendritic spines that contributes to sLTP induction and expression.

5.3.1 Kinetics of TrkB activation

Prior to our FRET-based sensor for TrkB activation, the kinetics of TrkB activity was examined primarily by Western Blotting of neuron culture lysates. With these techniques, the fastest observed TrkB activation was within 1 to 2 minutes of either activity onset or BDNF stimulation (Chao, 2003). Such a temporal profile was puzzling because BDNF itself had known effects on neurons with much more rapid kinetics. Specifically, it had been shown that BDNF induces a rapid, inward current with CA1 pyramidal cells within a few milliseconds of its application (Blum et al., 2002). In addition, this effect of BDNF required TrkB kinase activity thus implying that the
observed current was due to BDNF-TrkB signaling (Kafitz et al., 1999). The rapid, activity-evoked, BDNF-dependent TrkB activation we observe with our sensor helps address this discrepancy. Based on our data, it is clear that BDNF-TrkB signaling can be initiated in response to activity on the order of seconds, not minutes as had been previously suggested. Why we find more rapid TrkB activation kinetics is likely a reflection of the differences in temporal resolution between previous techniques, namely Western Blotting, and the FLIM imaging with FRET-based sensors we perform here.

The rapid onset of TrkB activity observed here provides insight into why TrkB is required for both the transient and sustained phases of sLTP as shown in chapter 3 as well as for early and late LTP as shown previously. To start, glutamate uncaging evoked TrkB activation precedes the onset of spine enlargement (Figure 29) thus suggesting that activity-induced TrkB activation may serve an instructive role during the induction of sLTP. One way to directly address this question would be to simultaneously induce sLTP while acutely blocking TrkB activation. Unfortunately, application of traditional TrkB and BDNF inhibitors (TrkB-Ig, K252a, etc.) is sub-optimal as their time of onset depends on penetration into the slice. As such, correctly timing the onset of sLTP induction with the onset of BDNF/TrkB inhibition in a cell of interest would be impossible. An alternative solution is to use a previously developed “caged” BDNF antibody (Kossel et al., 2001). Similar to caged glutamate, this antibody is contained within a chemical cage and it only becomes functional once released following photo-
activation. By combining this antibody with MNI-L-glutamate, one could simultaneously uncage both glutamate and the BDNF antibody. Such temporal correlation would provide a means to acutely inhibit BDNF function thereby allowing one to dissect whether BDNF and TrkB have permissive versus instructive roles in sLTP.

Building on this finding of rapid TrkB activation, numerous questions arise. First, how is such rapid TrkB activation accomplished? Is it due to an equally rapid release of BDNF from the stimulated spine? Our findings that the peak phase of TrkB activation depends on post-synaptic BDNF seem to suggest yes. We explore this question further in chapter 7. In addition to rapid, BDNF release, TrkB’s rapid kinetic profile could also be due to activity-induced TrkB exocytosis. Multiple lines of evidence suggest that neuron activity and BDNF can both induce the externalization of TrkB receptors leading to their activation by nearby, extracellular BDNF (Guo et al., 2014;
Meyer-Franke et al., 1998). To determine if such an exocytosis event underlies the activation we observe here, two experiments could be done. First, one could measure TrkB activation while simultaneously blocking exocytosis with tetanus toxin. Finding inhibition of TrkB activation in the presence of tetanus toxin would suggest that TrkB activity depends on exocytosis. However, because BDNF release is also tetanus toxin sensitive, interpreting these results will likely be challenging. A second approach to explore the question of TrkB exocytosis would be to design a TrkB construct containing a pH-sensitive N-terminal tag such as super-ecliptic pHluorin (SEP). Because of its pH-sensitivity, SEP is a great tool for visualizing exocytosis events (Miesenböck et al., 1998) (see Chapter 7 for more information regarding the properties of SEP). Using SEP-TrkB, one could visualize activity-induced TrkB exocytosis events. Such information would be quite informative regarding the movement of TrkB molecules during sLTP. However, what this experiment cannot answer though is the exact contribution such exocytosis events have on TrkB activation during sLTP. To address this question, what is needed is a method for blocking TrkB exocytosis specifically. Unfortunately, such a method does not yet exist.

Moving beyond TrkB’s rapid onset of activation, our sensor reports a sustained phase that persists for at least 20 minutes. Concerns have been raised about the fidelity of such sustained activation suggesting that it is an artifact of the sensor. However, several key pieces of evidence argue against this idea. First, the observed persistence of
TrkB activation following sLTP induction is consistent with previous work demonstrating TrkB activation outlasting stimulation for minutes to hours. As such, prolonged TrkB activation is not unfounded. Second, in chapter 3, we demonstrate that the sensor is bidirectional and does report both activation and inactivation. Based on these results, we would expect our sensor to be bidirectional during sLTP induction. Third, in sLTP experiments, we show that TrkB activity increases, peaks, and then decays to a sustained but elevated level. The decay of activity from its peak highlights the sensor’s bidirectional nature. Taken together, these results demonstrate that our sensor can in fact report both activation and inactivation and the fact that inactivation is not observed implies that it is not actually occurring.

With this said, multiple question arise. First, how long does the activity remain elevated? We demonstrate here TrkB activity lasting for at least 20 minutes. In other experiments not shown here, we have imaged the TrkB sensor for as long as 45 minutes without finding a reduction in TrkB activation. Future work probing the full temporal profile of TrkB activation would help address its role throughout the entire time course of sLTP. Second, what are the mechanisms underlying the maintenance of this activity. Does continued TrkB activity imply continuous BDNF release throughout sLTP? Does BDNF binding to TrkB induce internalization of the BDNF/TrkB complex into a “signalling endosome” (Howe and Mobley, 2004; Howe and Mobley, 2005) that can serve to protect and maintain TrkB activation? Does this activity require protein
synthesis as has been shown for later phases of sLTP (beyond 60 minutes) (Bosch et al., 2014; Govindarajan et al., 2011; Tanaka et al., 2008)? Exploring such questions promises to inform mechanisms of TrkB activation as well as its role in sLTP.

Regardless of the mechanism, TrkB’s sustained activity suggests a critical role for TrkB activation in the maintenance-expression of sLTP. In support of this, none of our perturbations explored here yielded preserved TrkB activation with impaired spine volume or vice versa. However, this observation is only correlational. To directly test the relationship between TrkB activity and the sustained phase of sLTP, one can impair TrkB activity at various time points following sLTP induction. These results could then define a critical window for when TrkB activity is necessary for sLTP expression similar to what has been done for TrkB during late LTP.

As highlighted here, the kinetic profile of TrkB activity during sLTP induction has proven informative for explaining its functional role during this process. Consequently, it would be interesting to examine if the same kinetic profile exists for pre-synaptic TrkB, TrkB within other synapses in the hippocampus and beyond, and TrkB activation during other, non-sLTP neuronal events. Furthermore, it would be useful to develop FRET-based sensors for other receptor tyrosine kinases within the central nervous system. It is conceivable that other receptors may be able to match the speed of TrkB activation and if so, this information would provide significant functional insight analogous to the knowledge gained here regarding TrkB’s role in sLTP.
5.3.2 Spatial profile of TrkB activation

Previous work utilizing antibodies against phosphorylated TrkB in immunohistochemistry has revealed evidence for activity-induced TrkB activation in both the dendrite and dendritic spines (Helgager et al., 2013). However, utilization of our FRET-based sensor provides a more sophisticated analysis and reveals a dynamic spatial profile for TrkB that evolves with time following initial stimulation. Initially, TrkB activity is largely restricted to the stimulated spine in a manner analogous to spatial profiles observed for CaMKII and Cdc42 (Lee et al., 2009; Murakoshi et al., 2011). With time however, TrkB activity increases in the dendrite suggesting a spreading of TrkB activity out of the spine and into adjacent regions analogous to the spatial profiles observed with RhoA, Rac1, and Ras (Murakoshi et al., 2011; Yasuda et al., 2006). Thus, the spatial profile identified here is consistent with previous of TrkB activation in both dendrites and dendritic spines (Drake et al., 1999; Helgager et al., 2013; Ji et al., 2005).

This initial restriction of TrkB activation to the spine may provide mechanistic insight into the spine specific nature of sLTP. Namely, by compartmentalizing TrkB activation to the stimulated spine to start, the pro-sLTP effects of TrkB signalling are similarly restricted to the stimulated spine. However, how TrkB activity is initially restricted in the spine is currently not known. Possible explanations include reduced diffusion of TrkB out of the spine following activity and/or the presence of inhibitory molecules at the spine neck that inactivate any TrkB molecule exiting the spine.
Even though it is initially restricted, TrkB activity does spread out into the dendrite. How this spreading occurs is currently unclear. One possibility is that activity spreading is due to diffusion of active TrkB molecules out of the stimulated spine and into adjacent regions. This possibility can be examined in the future through the use of TrkB fused to a photoactivatable GFP (Patterson and Lippincott-Schwartz, 2002). Such a construct would enable visualization of the trafficking of labelled TrkB receptors before, during, and after glutamate uncaging (Murakoshi et al., 2011). Another possibility is that activity spreading is a result of diffusion of TrkB stimuli that can activate TrkB positioned in the spine as well as in adjacent regions. BDNF is a TrkB stimulus that may be capable of such diffusion. Our findings suggest that post-synaptic BDNF release occurs in response to glutamate-uncaging. Once released into the extracellular space, BDNF could diffuse from the site of initial activity, namely the dendritic spine, and with time activate TrkB in the dendrite and neighboring spines. A third possibility is that TrkB stimuli, such as BDNF, are initially released in a spine-specific manner thereby activating local TrkB. With time, the area of TrkB stimuli release may expand beyond the stimulated spine and out into adjacent regions thus leading to TrkB activation in these areas as well. We begin to explore these questions surrounding BDNF release in chapter 7.

In addition to mechanisms underlying the spreading of TrkB activity, the functional consequence of this spreading is not clear. One idea is that TrkB activity
spreading may serve as a synaptic tag that would lower the threshold for inducing plasticity in nearby regions. Previous work has demonstrated a central role for TrkB in mediating heterosynaptic plasticity (Barco et al., 2005; Lu et al., 2011). Combining these findings, one possible functional consequence for TrkB’s spatial profile could be to promote spine specific plasticity to start (within the first 1-2 minutes following stimulation) but then to promote enhanced plasticity at nearby spines at later time points. A fellow graduate student in the Yasuda Lab, Nathan Hedrick, is currently exploring this specific question in his thesis work.

5.3.3 Role of TrkB activation within known sLTP signalling pathways

Activation of both NMDARs and CaMKII has been previously linked to TrkB activation (Brigadski et al., 2005; Hartmann et al., 2001; Marini et al., 1998). Consistent with this, we find that in sLTP, TrkB activation is also downstream of the NMDAR-CaMKII pathway. Comparing the temporal profiles of CaMKII and TrkB reveals that CaMKII activation precedes TrkB activation and is unaffected by post-synaptic BDNF knockout (Figure 30 right panel). However, while CaMKII activity sharply decays to baseline around 1 to 2 minutes, TrkB activity persists for the duration of sLTP (Figure 30). This divergence in kinetics suggests that the persistence of TrkB activation may be a functional means for extending the influence of CaMKII activity beyond its robust but transient increase. Additionally, CaMKII activity is associated with a wide variety of downstream signalling pathways. TrkB with its multiple associated signalling pathways
could provide a mechanism for the divergence of CaMKII activity. Despite being positioned downstream of CaMKII, one question that remains is how TrkB mechanistically mediates sLTP. We explore this question specifically in chapter 6 where we examine the dependence of Rho GTPase activity – molecules that are regulators of the actin cytoskeleton and known to be critical for sLTP (Hall, 1998; Murakoshi et al., 2011; Murakoshi and Yasuda, 2012; Takai et al., 2000)– on BDNF-TrkB signaling.

![Graph showing temporal dynamics of glutamate uncaging induced TrkB activation, CaMKII activation, and spine volume change](image)

**Figure 30: Comparison of temporal dynamics of glutamate uncaging induced TrkB activation, CaMKII activation, and spine volume change**

Time course of TrkB sensor activation (green trace), CaMKII activation (purple trace), and spine volume change (black trace) normalized to percent of maximal activity or volume change.

### 5.3.4 Dependence of TrkB activation on post-synaptic BDNF

Multiple lines of evidence have linked BDNF to both the induction and expression of LTP (Bekinschtein et al., 2007; Figurov et al., 1996; Kang et al., 1997; Korte...
et al., 1996; Korte et al., 1997; Kovalchuk et al., 2002; Lohof et al., 1993; Minichiello, 2009; Minichiello et al., 1999; Patterson et al., 1996; Xu et al., 2000). Here we demonstrate that BDNF is critical for both sLTP and post-synaptic TrkB activation. Specifically, using TrkB-Ig, we find reduction in both sLTP and TrkB activation; however, these impairments are not complete. One explanation could be that TrkB-Ig is incomplete in its scavenging of extracellular BDNF. For example, with activity, BDNF may be rapidly released from the post-synaptic compartment and due to the speed of its release and its proximity to post-synaptic TrkB receptors, BDNF may bind to the receptor before it can be scavenged by TrkB-Ig. Another explanation however is that the residual TrkB activation in the presence of TrkB-Ig is due to non-BDNF TrkB stimuli, namely transactivation stimuli. A potential non-BDNF source could be the divalent cation zinc. As discussed above, synaptically released as well as intracellular sources of zinc are capable of transactivating TrkB (Huang and McNamara, 2010; Huang et al., 2008; Pan et al., 2011). Further, at the CA3-CA1 synapse, there is evidence for zinc being present, albeit at low levels (Frederickson et al., 2005). Future work should explore if zinc (or other non-BDNF sources of TrkB activation) contribute to both sLTP and TrkB activation through both pharmacologic and genetic perturbation.

Despite consensus that BDNF is critical for plasticity, the cellular locale of this BDNF (pre- versus post-synaptic) has been intensely debated (Dieni et al., 2012; Korte et al., 1996; Kovalchuk et al., 2002; Minichiello, 2009; Park et al., 2014; Tanaka et al., 2008;
Xu et al., 2000; Zakharenko et al., 2003). Here, we provide evidence supporting the idea that post-synaptically synthesized BDNF is critical for both sLTP and post-synaptic TrkB activation. However, in the setting of post-synaptic BDNF knockout, we find reduced but not eliminated TrkB activation. In comparison to the results obtained with TrkB-Ig, there appears to be more residual TrkB activation following post-synaptic BDNF knockout. Consequently, these results suggest that post-synaptic BDNF may be one of several sources of BDNF contributing to both sLTP and TrkB activation, namely BDNF from the pre-synaptic terminal, astrocytes, and/or glia. Future work should investigate these additional sources of BDNF and detail their involvement – or lack thereof – in sLTP and TrkB activation. On this point, there are luckily numerous genetic strategies for selectively targeting and knockout BDNF in a variety of cell populations. Utilization of such strategies could directly address the question of BDNF cellular locale during sLTP.

Regardless of these other sources, the impaired sLTP and TrkB activation clearly highlight post-synaptic BDNF as critical. Since post-synaptic BDNF knockout impairs both the peak and sustained phases of TrkB activation, this implies that BDNF from the post-synaptic terminal may be released with kinetics matching that of TrkB activity, namely rapid and persistent. We address this question further in chapter 7.
5.3.5 Conclusion

In conclusion, we have provided further evidence that post-synaptic BDNF-TrkB signalling occurs during and is required for the transient and sustained phases of single spine structural plasticity. Stemming from these results are questions regarding the kinetics and subcellular locale of post-synaptic BDNF-release and mechanisms through which BDNF-TrkB signalling contributes to sLTP. In the chapters that follow, we utilize previously developed FRET-based sensor for various Rho GTPases known to be critical for sLTP to probe their dependence on BDNF-TrkB signalling. Additionally, we explore the existence of post-synaptic BDNF release using a previously developed sensor for BDNF exocytosis.
6. Visualization of activity-induced post-synaptic BDNF release

The rapid, robust, initially spine specific profile of glutamate-uncaging induced TrkB activation and its dependence on post-synaptic BDNF suggests that glutamate uncaging may produce an equally rapid, release of BDNF from the stimulated spine. To directly visualize this event, we utilized a previously designed BDNF release sensor and demonstrate that BDNF is in fact released from the spine and that such release depends on BDNF sorting machinery, exocytosis, NMDARs, AMPARs, and CaMKII activation. These results confirm that glutamate uncaging initiates a rapid, spine-specific, autocrine BDNF-TrkB loop that is critical for sLTP induction and expression.

6.1 Introduction

6.1.1 The BDNF controversy – pre-synaptic versus post-synaptic release

The subcellular locale of activity-induced BDNF release is quite controversial. Most everyone agrees that endogenous BDNF can be released from pre-synaptic terminals of a variety of neurons throughout the central nervous system (Dieni et al., 2012; Heldt et al., 2014; Zakharenko et al., 2003). However, there is much disagreement about whether similar release events occur from post-synaptic terminals, namely dendrites and dendritic spines (Dieni, 2012; Xu, 2000; Dean, 2009; Matsuda, 2009; Lou, 2005). The difficulty in addressing this question stems from the fact that endogenous BDNF is present at very low levels in the CNS (Barde et al., 1981). We have recently
produced evidence for endogenous BDNF within dendritic spines of rat cultured embryonic hippocampal neurons using a BDNF antibody validated for specificity (Dieni et al., 2012; Matsumoto et al., 2008) (Figure 31). However, using this same antibody with immunohistochemistry and electron microscopy, Yves Barde and colleagues found clear BDNF localization in axons and boutons but not dendrites and spines in slices prepared from adult mouse hippocampi (Dieni et al. 2012).

**Figure 31: Endogenous BDNF localization in cultured hippocampal neurons**

Confocal images of a cultured hippocampal neuron virally infected with mCherry and then stained with an antibody against BDNF.
In light of this discrepancy in BDNF localization, concern has been raised about whether cultured neurons accurately represent in vivo BDNF biology (Dieni et al. 2012). Specifically, cultured neurons develop in an environment quite distinct from their in vivo counterparts. Cultured neurons form fewer synaptic connections, are surrounded by an incomplete extracellular matrix, and commonly are grown in the absence of glia and astrocytes (Dieni et al., 2012; Mellman and Nelson, 2008). All these factors could affect BDNF expression, trafficking, and secretion and thus contribute to cultured neurons displaying a BDNF expression and localization profile distinct from neurons found in vivo.

Despite the controversy surrounding techniques used to localize neuronal BDNF, most would agree that the current absence of clear, structural data localizing BDNF to post-synaptic structures does not definitively exclude a functional role for post-synaptic BDNF. On this point, in chapters 3 and 5, we have presented multiple lines of evidence supporting the existence and functional importance of post-synaptic BDNF in cultured slices. Specifically, we have shown that selectively knocking out post-synaptic BDNF impairs post-synaptic TrkB activation and sLTP. We have also shown that the sustained phase of sLTP can be rescued in the setting of post-synaptic BDNF knockout by acutely applying exogenous BDNF. Furthermore, we report that CaMKII activity – a molecule we demonstrate to be upstream of TrkB activation – is intact despite post-synaptic BDNF knockout thus suggesting that synaptic responsiveness and signaling are intact.
up to the point of BDNF-TrkB signaling. Collectively, these results provide functional evidence for post-synaptic BDNF existing and serving a critical role in sLTP. Building on these results, we sought to develop a method for visualizing post-synaptic BDNF release during sLTP induction.

Visualizing BDNF release has been an actively pursued question for quite some time (Brigadski et al., 2005; Dean et al., 2009; Hartmann et al., 2001; Haubensak et al., 1998; Matsuda et al., 2009). Unfortunately though, visualizing endogenous BDNF release has proven technically challenging. Our TrkB sensor, thanks to its ability to report BDNF-mediated TrkB activation, may be the best method to date for visualizing and reporting endogenous BDNF release. With this method, we have demonstrated rapid, post-synaptic TrkB activation that depends on post-synaptic BDNF. The rapid kinetics of TrkB activation in the spine suggests an equally rapid release of BDNF from the post-synaptic compartment. However, since our sensor reports TrkB activation and not specifically BDNF release, we are unable to discern the specific kinetics and subcellular locale of BDNF release during sLTP induction. To address this issue, we sought to develop a biosensor for visualizing and monitoring BDNF release.

In the past, attempts to visualize BDNF release have used a variety of tagged BDNF constructs (BDNF-GFP, BDNF-RFP, etc) (Hartmann et al., 2001; Haubensak et al., 1998). The most successful and the most useful for our purposes has been BDNF tagged to a pH sensitive variant of GFP called pHluorin (Dean et al., 2009; Matsuda et al., 2009).
The fluorescence of GFP is pH sensitive in that its fluorescent intensity varies as a function of acidity – fluorescence is decreased in acidic conditions but increases in basic conditions (Miesenböck et al., 1998). By introducing multiple mutations at key amino acids, GFP’s pH sensitivity was enhanced such that the mutant protein (super-ecliptic pHluorin – SEP) displays a more robust loss of fluorescence with decreasing pH (Miesenböck et al., 1998; Sankaranarayanan et al., 2000). In fact, at pH values less than 6.0, the fluorescence of SEP is essentially zero.

Such pH-sensitivity of SEP is ideal for monitoring exocytosis events, such as BDNF release, thanks to the differing environments found inside an endosome compared to the extracellular space. Endosomes commonly have a very acidic interior (pH less than 6.0). Conversely, the extracellular space is more basic with a pH around 7.4. Following endosome fusion with the plasma membrane during exocytosis, the intra-endosome space becomes contiguous with the extracellular environment causing the pH inside the endosome to increase. Because SEP is pH sensitive, changes in its fluorescence intensity can be used as proxy for exocytosis (Dean et al., 2009; Granseth et al., 2006; Matsuda et al., 2009; Miesenböck et al., 1998; Patterson et al., 2010; Sankaranarayanan et al., 2000). Namely, prior to exocytosis, SEP within the endosome will be quenched but following exocytosis and the resulting increase in pH, SEP fluorescence will rise thereby indicating a release event.
SEP has been successfully utilized to visualize exocytosis of a variety of proteins at both pre- and post-synaptic terminals (Dean et al., 2009; Granseth et al., 2006; Matsuda et al., 2009; Miesenböck et al., 1998; Patterson et al., 2010; Sankaranarayanan et al., 2000). Regarding BDNF, several BDNF-pHfluorin constructs have been used to visualize BDNF release (Dean et al., 2009; Matsuda et al., 2009). To date, all these experiments have been done in cultured neurons and in response to whole cell stimuli such as electrical or chemical stimulation.

Here, we utilize a previously developed BDNF construct (HA-Pro-BDNF-Flag) whose expression, processing, and trafficking has been validated (Chen et al., 2004). We fused SEP to the C-terminus of this construct to generate BDNF-SEP (Figure 32A). By visualizing the change in SEP fluorescence, we can record its release from an acidic endosome to the more basic extracellular space (Figure 32B).
**Figure 32: Design and validation of BDNF-SEP**

A) Schematic of BDNF-SEP and BDNF-mRFP. HA – hemagglutinin tag; Pro – amino acids 19-128 of human BDNF; BDNF – amino acids 129 – 247 of human BDNF corresponding to the mature chain; FLAG – FLAG tag; SEP – supercliptic pHluorin; mRFP – monomeric red fluorescent protein.  
B) Mechanistic model linking changes in SEP fluorescence with BDNF release.  
C) Confocal images of a CA1 pyramidal neuron transfected with mEGFP and BDNF-mRFP. Arrowheads indicate dendritic spines.  
D) Change in BDNF-SEP fluorescence following glutamate uncaging under control (Ctrl), acidic (pH 6.5), and basic (pH 8.0) conditions.
6.2 Results

6.2.1 BDNF-SEP expression and cellular localization

Even though the BDNF construct we used to generate BDNF-SEP has been previously validated (Chen et al., 2004; Matsuda et al., 2009), we sought to confirm that BDNF-SEP retains an expression and localization profile similar to endogenous BDNF despite addition of a C-terminal fluorophore tag, namely SEP. However, since SEP fluorescence is pH dependent and BDNF is predominantly localized to acidic endosomes, we struggled to visualize the BDNF-SEP signal in the absence of activity, thus preventing us from visualizing its basal localization in the neuron. To address this, we replaced SEP with mRFP (Figure 32A), a fluorophore of similar size as SEP but with much lower pH sensitivity. Using confocal imaging, we found that BDNF-mRFP was distributed in a punctate pattern in the soma, surrounding dendrites (including the apical dendrite), and dendritic spines (Figure 32C). This pattern is consistent with previous studies visualizing both fluorophore tagged and endogenous BDNF including the work by the Yasuda Lab described above (Figure 31) (Matsuda et al., 2009).

To further validate the appropriate processing and trafficking of BDNF-SEP, we asked if BDNF-SEP expression could rescue sLTP in the setting of post-synaptic knockout of endogenous BDNF. We transfected cultured hippocampal slices from Bdnffl/fl mice with one of three plasmid DNA combinations: 1) mEGFP and mCherry (Cre Negative), 2) mEGFP, mCherry, and tdTomato-Cre (Cre Positive), and 3) BDNF-SEP,
mCherry, and tdTomato-Cre (Cre Positive + BDNF-SEP). After at least 5 days of transfection, we induced sLTP with glutamate uncaging. Similar to the results found with acute application of exogenous BDNF (Figure 12C, D), expression of BDNF-SEP in the Cre positive cells restored the sustained but not the transient phase of sLTP (Figure 33). These results suggest that BDNF-SEP can effectively replace endogenous BDNF and restore some neuronal functions that would otherwise have been lost due to post-synaptic BDNF knockout.

![Graph A](image1)

**Figure 33: Effect of BDNF-SEP expression on single spine structural plasticity in the setting of post-synaptic BDNF knockout**

**A)** Time course of spine volume change for cells without Cre-recombinase (Cre Neg, black trace, n = 13 spines), with Cre-recombinase (Cre Pos, red trace, n = 11 spines), or with Cre-recombinase plus BDNF-SEP (Cre Pos + BDNF-SEP, green trace, n = 13 spines). **B)** Transient (averaged over 1-2 minutes) and sustained (averaged over 10-40 minutes) spine volume change for experiments in (A). Error bars are s.e.m. Asterisks denote statistical significance (p<0.05) determined by ANOVA followed by post-hoc tests using the least significant difference.
6.2.2 Uncaging evoked BDNF-SEP release

The rapid kinetics of glutamate-uncaging evoked TrkB activation and its dependence on post-synaptic BDNF suggest an equally rapid, post-synaptic BDNF release during the transient phase of sLTP. To determine if such release does in fact occur, we biolistically transfected CA1 pyramidal cells in cultured rat hippocampal slices with BDNF-SEP as well as mCherry for visualization of the transfected cell. After 3 to 7 days of transfection, we imaged the SEP signal using two-photon microscopy. At baseline, the SEP fluorescence signal was quite low and in many instances was indistinguishable from background fluorescence. However, in response to glutamate uncaging, we observed a robust and consistent increase in SEP fluorescence mostly restricted to the stimulated spine (Figure 34A, B). This increase in SEP fluorescence had two kinetic profiles: 1) a transient, spike-like increase time-locked to each individual uncaging pulse and 2) a gradual increase in fluorescence commencing with the first uncaging pulse and peaking sixty seconds later at the termination of the uncaging train (Figure 34A, B, and C).

Focusing primarily on the first kinetic profile, several pieces of evidence indicated that this rapid, increase in SEP fluorescence was likely due to BDNF-SEP release from the spine. First, the observed fluorescence signal in our experiments depended on pH, as expected for SEP, since it was greatly attenuated by replacing the normal ACSF (pH 7.4) with acidic ACSF (pH 6.5) (Figure 32D). Second, this
fluorescence signal depended on exocytosis since it was inhibited by co-expression with tetanus toxin (TeTX, an inhibitor of VAMP-dependent exocytosis) (Figure 34C, D). Finally, the signal was dependent on known BDNF sorting machinery, since it was abolished by co-expression of POMC, a peptide that prevents trafficking of BDNF to its activity-dependent release pathway (Lou et al., 2005) (Figure 34C, D). All together, these results demonstrate that the observed increase in SEP signal reports glutamate-dependent exocytosis and release of BDNF from stimulated spines.

Next, we explored the mechanisms underlying this glutamate-dependent BDNF release. We found the SEP signal to be largely blocked by NMDAR inhibition with AP5, and completely blocked by simultaneous inhibition of NMDARs and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) with AP5 and NBQX (Figure 34C, D). Further, we found the increase in SEP fluorescence to be partially inhibited by CaMKII inhibition with CN21 (Vest et al., 2007) (Figure 34C, D). Together, these data suggest that BDNF release from the stimulated spine is NMDAR-CaMKII dependent, consistent with the results obtained with the TrkB sensor.
Figure 34: Spatiotemporal dynamics of post-synaptic BDNF release during single spine structural plasticity

A) Two-photon images of glutamate uncaging-evoked changes in BDNF-SEP fluorescence in dendritic spines of CA1 hippocampal neurons. Each row of images represents the uncaging-triggered average of the BDNF-SEP signal in response to individual uncaging pulses for the designated time window and associated uncaging stimuli. B) Averaged time course of BDNF-SEP fluorescence change in spines and adjacent dendritic shafts in response to glutamate uncaging (30 pulses at 0.5Hz; timing of glutamate pulses indicated by black bars (top)). Inset shows the change in mCherry fluorescence (red) in response to glutamate uncaging, indicative of spine volume
change. C) Uncaging-triggered average of the increase in BDNF-SEP fluorescence with glutamate uncaging. Data using genetic and pharmacologic inhibitors is also shown (Ctrl – control, n = 182 spines; TeTx – neurons transfected with tetanus toxin, an inhibitor of exocytosis, n = 80 spines; POMC – neurons transfected with the POMC peptide, an inhibitor of activity-dependent BDNF release, n = 29 spines; AP5 – NMDAR inhibitor, n = 50 spines; AP5+NBQX – NMDAR and AMPAR inhibitors, n = 46 spines; and CN21 – CaMKII inhibitor, n = 41 spines). Time course represents the average response over the duration of a single uncaging pulse. D) Peak (64ms) of the uncaging-triggered averaged increase of BDNF-SEP fluorescence in (C). Asterisks indicate a statistically significant difference (p<0.05) between the indicated condition and control condition as determined by ANOVA followed by post-hoc tests using the least significant difference.

6.3 Discussion

Utilizing two-photon imaging with a biosensor for BDNF release (BDNF-SEP), we examine the spatiotemporal properties of activity-induced post-synaptic BDNF release. We find that during glutamate uncaging evoked structural plasticity of single CA1 dendritic spines, BDNF is rapidly released from the stimulated spine in a manner time-locked to the stimulation. We further report that this release depends on NMDARs, AMPARs, CaMKII activation, and BDNF-sorting machinery. Collectively, these experiments reveal an activity-dependent BDNF release event from a single dendritic spine with a temporal and spatial profile matching that of TrkB activation.

6.3.1 Spatiotemporal profile of BDNF release

In chapter 5, we demonstrate largely spine-specific TrkB activation during the first few minutes of sLTP induction. Consistent with this localization, we show here that glutamate-uncaging evoked BDNF release arises predominantly from the stimulated spine. As such, these findings suggest that one mechanism underlying TrkB’s initial spatial profile is the spine specific nature of BDNF release. Additionally, the tight
correlation between the locales of TrkB activation and BDNF release during uncaging suggests that once externalized, BDNF’s sphere of influence on TrkB is spatially restricted. Analogous to Ca$^{2+}$ influx into a dendritic spine, it seems that BDNF release may yield a nanodomain of extracellular BDNF that only activates TrkB receptors in the immediate vicinity, namely those on the stimulated spine (Lee et al., 2009). Such limited spatial influence for BDNF helps explain why this extracellular molecule is able to have such spine-specific effects.

However, because our experiments only imaged BDNF release during uncaging, it is unclear if BDNF release continues after uncaging and if it spreads into adjacent regions analogous to our findings for TrkB activity. Extending the time course of our experiments to examine BDNF release after the termination of stimulation will prove informative in explaining mechanisms of TrkB activity spreading. Additionally, such experiments would also address questions surrounding TrkB’s sustained activity.

### 6.3.2 Mechanisms underlying post-synaptic BDNF release

Previous reports have suggested that post-synaptic BDNF release is mediated by Ca$^{2+}$ dependent exocytosis (Kuczewski et al., 2008; Matsuda et al., 2009). Consistent with these findings, we report that BDNF release during sLTP requires both NMDARs and AMPARs and that it is sensitive to tetanus toxin. Additionally, we find that full release requires CaMKII activation. Putting these results together, we propose that in response to activity, Ca$^{2+}$ enters the dendritic spine through either NMDARs and/or AMPARs.
Once in the spine, Ca\textsuperscript{2+} on its own can initiate BDNF release by engaging known post-synaptic exocytosis machinery. However, for maximal release to occur, Ca\textsuperscript{2+} mediated CaMKII activation is also required. Our data suggests that CaMKII activity may serve to potentiate BDNF release. On this point, we have preliminary evidence suggesting that with each subsequent pulse during the uncaging train, the magnitude of BDNF release increases. Interestingly though, in the presence of a CaMKII inhibitor, BDNF release still occurs but the increase over time does not. This potential ability of CaMKII to potentiate BDNF release is consistent with current theories of CaMKII being an integrator of Ca\textsuperscript{2+}-influx into the spine (Lee et al., 2009). As such, it is believed that CaMKII activation may serve as a proxy for a spine’s level of stimulation. Combining these points, it seems that the amount of BDNF release may serve as a threshold detector. In low activity, CaMKII activation is minimal and as such, BDNF release is low. However, in high activity, CaMKII activation is strong leading to potentiated BDNF release and ultimately sLTP.

Despite the mechanism detailed here, numerous questions still remain regarding post-synaptic BDNF release. First, what is the organelle within the spine in which BDNF resides? Is it in dense-core vesicles? Is it endosomes? Unfortunately the difficulties with localizing BDNF to the post-synaptic compartment have precluded addressing these questions. Second, where within the spine does BDNF release occur? Is it at the post-synaptic density or is extra-synaptic? Third, how does CaMKII
activation potentiate BDNF release? Exocytosis is known to be a Ca\textsuperscript{2+} dependent event. However, because of its rapid time course, exocytosis has not traditionally been associated with phosphorylation events. Thus, it is puzzling how activation of kinase like CaMKII can translate into enhanced exocytosis.

**6.3.4 Conclusion**

In conclusion, we have demonstrated that in response to glutamate uncaging, post-synaptic BDNF rapidly releases from a single dendritic spine in a CA\textsuperscript{2+} dependent manner. The kinetics and spatial profile of this release provides a mechanism explaining the similarly rapid and spine specific TrkB activation during sLTP. Moving forward, it would be interesting to determine if other neuropeptides are released on the same time course as BDNF. If true, this would imply kinetics for neuromodulation that rivals that of neurotransmission.
7. BDNF-dependence of Rac1 and Cdc42 but not and RhoA activity during single spine structural plasticity

BDNF and its receptor TrkB are critical for the induction and expression of CA1 dendritic spine structural plasticity, yet the molecular mechanisms by which BDNF-TrkB signaling contributes to spine enlargement are not clear. Candidate molecules downstream of BDNF and TrkB include the Rho GTPases – a family of molecules known to regulate the actin cytoskeleton and dendritic spine morphology (Hall, 1998; Heasman and Ridley, 2008). In fact, recent work has demonstrated that during single spine structural plasticity, the Rho GTPases Race, Cdc42, and RhoA are all activated and that such activation is necessary for full expression of sLTP (Murakoshi et al., 2011). Here, we report that during sLTP, Cdc42 and Rac1 activity but not RhoA activity are dependent on post-synaptic BDNF. These findings position BDNF-TrkB signaling upstream of these two GTPases and thus provide a signaling mechanism by which TrkB activation can lead to actin cytoskeleton reorganization in the setting of the structural plasticity.

7.1 Introduction

7.1.1 Contributions of the Rho GTPases to single spine structural plasticity

Ras-related C3 botulinum toxin substrate 1 (Rac1), cell division cycle 42 (Cdc42) and Ras homolog (Rho) – all members of the Rho family of GTPases – are key regulators of the actin cytoskeleton in both neuronal and non-neuronal cells (Hall, 1998; Murakoshi
and Yasuda, 2012; Saneyoshi et al., 2010; Takai et al., 2000). In general, Rac1 and Cdc42 activities encourage actin polymerization while RhoA activity leads to actin depolymerization (Takai et al., 2000). Within neurons, the pro-polymerization functions of Rac1 and Cdc42 have been associated with spine formation and enlargement, axon extension and guidance, and dendrite growth while the pro-depolymerization function of RhoA has been linked to spine loss, spine shrinkage, and neurite collapse (Hall, 1998; Saneyoshi et al., 2010; Takai et al., 2000).

Through their abilities to regulate the actin cytoskeleton, these three GTPases serve critical functions in mediating synaptic plasticity (Murakoshi et al.; Murakoshi and Yasuda). Focusing on sLTP, perturbing each GTPase individually produces unique effects on sLTP induction and expression. Starting with Rac1, post-synaptic knockout or pharmacologic inhibition of its activation leads to reduction of both the transient and sustained phases of sLTP (unpublished data). For Cdc42, post-synaptic knockout selectively impairs the sustained but not the transient phase of sLTP (Murakoshi et al., 2011). Interestingly, for RhoA, the opposite effect is noted in that post-synaptic knockout selectively reduces the transient but not the sustained phase of sLTP (Murakoshi et al., 2011).

Consistent with their distinct involvements in sLTP, each GTPase presents with a unique temporal and spatial activity profile during sLTP. Starting with Rac1, in response to glutamate uncaging, it rapidly activates in the stimulated spine (within 1
minute of stimulus onset), it peaks around 1 to 2 minutes, decays slightly
(approximately 25%), and then stabilizes at a sustained level that persists for at least 30
minutes (unpublished data). This activity begins in the stimulated spine but quickly
spreads out into the parent dendrite and neighboring spines. For Cdc42, it also rapidly
activates in response to glutamate uncaging. This activity begins within 1 minute of
stimulus onset, it peaks around 1 to 2 minutes, and then it decays by more than 50%
before stabilizing a sustained level that persists for at least 40 minutes (Murakoshi et al.,
2011). In terms of spatial profile, unlike Rac1, Cdc42’s activity remains restricted to the
stimulated spine with limited diffusion into adjacent regions (Murakoshi et al., 2011).
For RhoA, like Cdc42 and Rac1, it is rapidly activated in response to glutamate uncaging
(within 1 minute of stimulus onset), it peaks at around 1 to 2 minutes, and then it decays
by more than 50% before stabilizing at a sustained level that persists for at least 40
minutes (Murakoshi et al., 2011). Unlike Cdc42 but similar to Rac1, RhoA’s activity
begins in the spine but quickly spreads out into the dendrite and adjacent spines
(Murakoshi et al., 2011).

<table>
<thead>
<tr>
<th>Cdc42</th>
<th>1-2 minutes</th>
<th>&gt;50%</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>RhoA</td>
<td>1-2 minutes</td>
<td>&gt;50%</td>
<td>Yes</td>
<td>Yes (dendrite and neighboring spines)</td>
</tr>
<tr>
<td>Rac1</td>
<td>1-2 minutes</td>
<td>&lt;25%</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Despite such distinct temporal and spatial activity profiles, each GTPase depends on activation of both NMDARs and CaMKII (Murakoshi et al.) (unpublished data). As such, these GTPases all share a common origin of activation, namely Ca\(^{2+}\) influx through activated NMDARs that in turn triggers activation of CaMKII (Murakoshi et al., 2011; Okamoto et al., 2009). However, in sLTP, CaMKII activation is transient, lasting only a few minutes while GTPase is sustained and long lasting (Lee et al. 2009; Murakoshi et al. 2011). This dichotomy raises the critical question of how fleeting CaMKII activity can induce sustained GTPase activity. Here we explore whether BDNF-TrkB signaling downstream of CaMKII could be the missing link.

### 7.1.2 Molecular and functional links between BDNF/TrkB signaling and the Rho GTPases

As demonstrated in chapters 3 and 5, BDNF-TrkB signaling is necessary for single spine structural plasticity. One possible mechanism by which BDNF-TrkB signaling may mediate sLTP is through regulation of the actin cytoskeleton. Previously, BDNF signaling has been associated with synaptogenesis, axon growth cone extension and guidance, as well as dendrite growth and morphogenesis – events that all require actin cytoskeleton rearrangement (Cheung et al., 2007; Lai et al., 2012; Saito et al., 2013; Sheng and Hoogenraad, 2006). In fact, many of these BDNF-mediated processes have proven dependent on the Rho GTPases Cdc42 and Rac1 (Chen et al., 2006; Cheung et al., 2007; Myers et al., 2012; Shen et al., 2006; Yuan et al., 2002). Based on these findings, it
has been suggested that BDNF-TrkB signaling can lead to activation of various Rho GTPases.

In support of this idea, BDNF has been associated with activation of several guanine exchange factors (GEFs) – proteins that mediate Rho GTPase activation by inducing the exchange of GDP for GTP. Specifically, BDNF has been shown to activate Vav2 and Tiam1 (Lai et al., 2012; Shen and Cowan, 2010; Zhou et al., 2007). Through activation of these GEFs, BDNF application has also been linked to Cdc42 and Rac1 activation in both neuronal and non-neuronal cells (Chen et al., 2006; Cheung et al., 2007; Guo et al., 2012; Miyamoto et al., 2006; Myers et al., 2012; Shen et al., 2006; Yuan et al., 2002). One proposed mechanism underlying these activation events is BDNF-mediated activation of Cdk5, which in turn phosphorylates serine 478 within TrkB’s intracellular domain. Once phosphorylated, this residue serves as a docking site for the GEF Tiam1. Following Tiam1 binding, TrkB phosphorylates and thus activates this GEF leading to activation of downstream GTPases. This mechanism has been experimentally demonstrated for Rac1 activation but only proposed for Cdc42 activation (Cheung et al.; Lai et al., 2012)

Regarding RhoA, a link to BDNF-TrkB signaling has not been clearly established. There is evidence in Schwann cells that BDNF can induce RhoA activation; however, this activation is TrkB-independent and instead involves p75NTR – the low-affinity neurotrophin receptor (Yamauchi et al., 2004). Additionally, others have demonstrated
that BDNF can induce increased RhoA synthesis that in turn yields greater RhoA activation (Briz et al.). However, a direct link from BDNF-TrkB signaling to RhoA activity has yet to be identified.

Considering that there is experimental evidence connecting BDNF-TrkB signaling with both Cdc42 and Rac1, activation of these GTPases by BDNF-TrkB signaling may be one mechanism underlying the roles of both BDNF and TrkB in sLTP. We address this question below by examining the dependence – or lack thereof – of Rac1, Cdc42, and RhoA activity on BDNF.

7.2 Results

7.2.1 Rac1 activity during inhibition of BDNF signaling

Unpublished work from the Yasuda lab reveals a critical role for the Rho GTPase Rac1 in both the transient and sustained phases of sLTP (unpublished data). Additionally, it has been previously shown that Rac1 activation can be induced through a BDNF-TrkB dependent mechanism (Lai et al., 2012). Given the requirement of BDNF, TrkB, and Rac1 for single spine structural plasticity and the known link between TrkB and Rac1, we hypothesized that Rac1 activation during sLTP may be downstream of BDNF-TrkB signaling. To examine this hypothesis, we transfected cultured rat hippocampal slices with a previously developed Rac1 sensor (unpublished data). Like the TrkB sensor, the Rac1 sensor consists of two components: 1) full-length Rac1 fused to mEGFP on its C-terminus (Rac1-mEGFP) and 2) a fragment of the Rac1 binding partner
Pak2 containing its Cdc42-Rac binding domain with copies of the mCherry fluorophore fused to both its C- and N-termini (mC-Pak2-mC). Of note, two mutations were introduced into the Pak2 fragment (R71C and S478A) that biases this domain’s affinity for Rac1 over Cdc42. Following at least 24 hours of sensor transfection, we utilized 2pFLIM to image Rac1 activity in single dendritic spines of transfected CA1 pyramidal cells while simultaneously inducing sLTP with two-photon glutamate uncaging as described in chapter 5. To estimate the spine volume throughout sLTP induction, we utilized the fluorescence intensity of mC-Pak2-mC. In response to glutamate uncaging, we found that Rac1 rapidly activates in the stimulated spine, peaks around 1 to 2 minutes following stimulus onset, and then decays slightly before establishing a sustained state lasting the duration of the experiment (at least 20 minutes) (Ctrl; Figure 35A, B). In parallel with Rac1 activation, we also saw robust spine enlargement consistent with sLTP (Figure 35C, D). Following successful induction of both Rac1 activation and sLTP, we applied TrkB-Ig (2 µg/ml) to the slice for at least 30 minutes and then attempted to induce sLTP with glutamate uncaging while simultaneously imaging Rac1 activation on another dendritic spine from the same cell. In the presence of TrkB-Ig, we found impaired Rac1 activity with a corresponding reduction in sLTP (TrkB-Ig; Figure 35). These findings suggest that glutamate-uncaging evoked Rac1 activity is dependent on extracellular BDNF.
Figure 35: Effect of scavenging extracellular BDNF on glutamate uncaging induced Rac1 activation

A) Time course of Rac1 sensor activation measured as a change in mEGFP-Rac1 bound to mC-Pak2-mC before (Ctrl, black trace, \(n = 11\) spines) and at least 30 minutes after TrkB-Ig application (Trk-Ig, red trace, \(n = 14\) spines). B) Peak (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) Rac1 sensor activation for experiments in (A). C) Time course of spine volume change for experiments in (A). D) Transient (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) spine volume change for experiments in (A). Error bars are s.e.m. Asterisks denote statistical significance (p<0.05) as determined by an ANOVA.

To determine if Rac1 activity depends specifically on post-synaptic BDNF, we biolistically transfected cultured hippocampal slices from BDNF<sup>fl/fl</sup> mice with either the Rac1 sensor alone (Cre negative) or the Rac1 sensor plus Cre-tdTomato (Cre positive).
As described earlier, the sparse transfection associated with this technique led to essentially single-cell BDNF knockout in the Cre positive cells. After 5 to 7 days of transfection, we once again induced sLTP on single, CA1 dendritic spines while simultaneously imaging Rac1 activity using 2pFLIM. For spines from Cre negative cells, we found robust Rac1 activation in the stimulated spine with corresponding changes in spine volume (Cre Neg; Figure 36). For spines from Cre positive cells, we found a nearly complete impairment of Rac1 activation with a corresponding reduction in sLTP (Cre Pos; Figure 36). Taken together, these results position Rac1 signalling downstream of post-synaptic BDNF-TrkB signalling thus demonstrating that one mechanism recruited by TrkB to mediate spine volume change is through Rac1.
Figure 36: Effect of post-synaptic BDNF knockout on glutamate uncaging induced Rac1 activation

A) Time course of Rac1 sensor activation measured as a change in mEGFP-Rac1 bound to mC-Pak2-mC in spines from Bdnf<sup>fl/fl</sup> mouse slices transfected with either the Rac1 sensor alone (Cre Neg, black trace, n = 13 spines) or the Rac1 sensor plus Cre-recombinase (Cre Pos, red trace, n = 16 spines). B) Peak (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) Rac1 sensor activation for experiments in (A). Time course of spine volume change for experiments in (A). D) Transient (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) spine volume change for experiments in (A). Error bars are s.e.m. Asterisks denote statistical significance (p<0.05) as determined by an ANOVA.
7.2.2 Cdc42 activity during inhibition of BDNF signaling

Like Rac1, previous reports have highlighted a link between BDNF-TrkB signaling and activation of the Rho GTPase Cdc42 (Chen et al., 2006; Cheung et al., 2007; Myers et al., 2012; Shen et al., 2006). Additionally, the Yasuda Lab has shown that Cdc42 is activated during sLTP and that its activation is critical for full expression of single spine structural plasticity (Murakoshi et al., 2011). To determine if Cdc42 activity is BDNF-dependent, we transfected cultured rat hippocampal slices with a previously developed Cdc42 sensor. Similar to TrkB and Rac1, this sensor consists of two components: 1) full-length Cdc42 fused with mEGFP (mEGFP-Cdc42) and 2) the Rho GTPase binding domain of Pak3 fused to mCherry fluorophores at both its N- and C-termini (mC-Pak3-mC) (Murakoshi et al., 2011). As described above, we utilized 2pFLIM in conjunction with two-photon glutamate uncaging to simultaneously image Cdc42 activity while inducing sLTP. To estimate the spine volume throughout sLTP induction, we utilized the fluorescence intensity of mC-Pak3-mC. In response to glutamate uncaging, we found that Cdc42 rapidly activates in the stimulated spine, peaking at around 1 to 2 minutes, and then decaying to a sustained state lasting the duration of the experiment (at least 20 minutes) (Ctrl; Figure 37A, B). In parallel with this Cdc42 activation, we also found robust spine enlargement consistent with sLTP induction (Figure 37C, D). Following successful induction of both Cdc42 activation and sLTP, we applied TrkB-Ig (2 µg/ml) to the slice for at least 30 minutes and then
attempted to induce sLTP with glutamate uncaging while simultaneously imaging Cdc42 activity on another dendritic spine from the same cell. In the presence of TrkB-Ig, we found impaired Cdc42 activity with a corresponding reduction in sLTP (TrkB-Ig; Figure 37). These findings suggest that glutamate-uncaging evoked Cdc42 activity is dependent on extracellular BDNF.

Figure 37: Effect of scavenging extracellular BDNF on glutamate uncaging induced Cdc42 activation

A) Time course of Cdc42 sensor activation measured as a change in mEGFP-Cdc42 bound to mC-Pak3-mC before (Ctrl, black trace, n = 12 spines) and at least 30 minutes after TrkB-Ig application (TrkB-Ig, red trace, n = 12 spines). B) Peak (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) Cdc42 sensor activation for experiments in (A). Time course of spine volume change for experiments in (A). D) Transient (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes)
spine volume change for experiments in (A). Error bars are s.e.m. Asterisks denote statistical significance (p<0.05) as determined by an ANOVA.

To determine if Cdc42 activity depends specifically on post-synaptic BDNF, we biolistically transfected cultured hippocampal slices from \textit{BDNF}^{+/+} mice with either the Cdc42 sensor alone (Cre negative) or the Cdc42 sensor plus Cre-tdTomato (Cre positive). After 5 to 7 days of transfection, we once again induced sLTP on single, CA1 dendritic spines while simultaneously imaging Cdc42 activity using 2pFLIM. For spines from Cre negative cells, we found robust Cdc42 activation in the stimulated spine with corresponding changes in spine volume (Cre Neg; Figure 38). For spines from Cre positive cells, we found a nearly complete impairment of Cdc42 activation with a corresponding reduction in sLTP (Cre Pos; Figure 38). Taken together, these results are similar to those obtained with Rac1 and thus position Cdc42 signalling downstream of post-synaptic BDNF-TrkB signalling.
**Figure 38: Effect of post-synaptic BDNF knockout on glutamate uncaging induced Cdc42 activation**

**A)** Time course of Cdc42 sensor activation measured as a change in mEGFP-Rac1 bound to mC-Pak2-mC in spines from Bdnf<sup>fl/fl</sup> mouse slices transfected with either the Cdc42 sensor alone (Cre Neg, black trace, n = 13 spines) or the Cdc42 sensor plus Cre-recombinase (Cre Pos, red trace, n = 16 spines).  

**B)** Peak (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) Cdc42 sensor activation for experiments in (A).  

**C)** Time course of spine volume change for experiments in (A).  

**D)** Transient (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) spine volume change for experiments in (A).  

Error bars are s.e.m. Asterisks denote statistical significance (p<0.05) as determined by an ANOVA.

### 7.2.3 RhoA activity during inhibition of BDNF signaling

Unlike Rac1 and Cdc42, RhoA has not been directly linked to BDNF-TrkB signaling. However, since RhoA is critical for sLTP like Rac1 and Cdc42 (Murakoshi et
al., 2011), it is possible that RhoA activity may represent a BDNF-independent, parallel signaling pathway during sLTP. To address if RhoA activity is BDNF dependent, we transfected cultured rat hippocampal slices with a previously developed RhoA sensor consisting of two components: 1) full-length RhoA fused with mEGFP (mEGFP-RhoA) and 2) the Rho GTPase binding domain of Rhotekin fused to mCherry fluorophores at both its N- and C-termini (mC-RBD-mC) (Murakoshi et al. 2011). As described above, we utilized 2pFLIM in conjunction with two-photon glutamate uncaging to simultaneously image Cdc42 activity while inducing sLTP. To estimate the spine volume throughout sLTP induction, we utilized the fluorescence intensity of mC-RBD-mC. In response to glutamate uncaging, we found that RhoA rapidly activates in the stimulated spine, peaks around 1 to 2 minutes, and then decays to a sustained state lasting the duration of the experiment (at least 20 minutes) (Ctrl; Figure 39A, B). In parallel with this RhoA activation, we also found robust spine enlargement consistent with sLTP induction (Figure 39C, D). Following successful induction of both RhoA activation and sLTP, we applied TrkB-Ig (2 µg/ml) to the slice for at least 30 minutes and then attempted to induce sLTP with glutamate uncaging while simultaneously imaging RhoA activity on another dendritic spine from the same cell. In the presence of TrkB-Ig, we found no impairment of RhoA activity. However though, we also found seemingly normal sLTP despite the presence of TrkB-Ig.
Figure 39: Effect of scavenging extracellular BDNF on glutamate uncaging induced RhoA activation

A) Time course of RhoA sensor activation measured as a change in mEGFP-RhoA bound to mC-RBD-mC before (Ctrl, black trace, n = 8 spines) and at least 30 minutes after TrkB-Ig application (TrkB-Ig, red trace, n = 20 spines)  
B) Peak (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) RhoA sensor activation for experiments in (A).  
C) Time course of spine volume change for experiments in (A).  
D) Transient (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) spine volume change for experiments in (A). Error bars are s.e.m. Asterisks denote statistical significance (p<0.05) as determined by an ANOVA.

The absence of impaired sLTP in the setting of TrkB-Ig – a finding inconsistent with our results in chapter 3 as well as the results presented above for the Rac1 and Cdc42 sensors – complicates interpreting the RhoA activity data. As such, we decided to examine this question in the setting of post-synaptic BDNF knockout. Being a genetic
perturbation, we thought it might be a more robust method of impairing post-synaptic BDNF. Similar to single cell knockout experiments described above, we biolistically transfected cultured hippocampal slices from \textit{BDNF}^{fl/fl} mice with either the RhoA sensor alone (Cre negative) or the RhoA sensor plus Cre-tdTomato (Cre positive). After 5 to 7 days of transfection, we induced sLTP on single, CA1 dendritic spines using glutamate uncaging while simultaneously imaging RhoA activity with 2pFLIM. In the Cre positive cells, we once again found no evidence for impaired RhoA activity (Figure 40A, B). However, unlike the experiments utilizing TrkB-Ig, in the setting of post-synaptic BDNF knockout, we did observe impaired sLTP (Figure 40C, D). These findings suggest that RhoA is likely BDNF-independent and thus exists in a signaling cascade that acts in parallel with BDNF-TrkB-Rac1/Cdc42 to promote sLTP.
A) Time course of RhoA sensor activation measured as a change in mEGFP-RhoA bound to mC-RBD-mC in spines from Bdnf<sup>fl/fl</sup> mouse slices transfected with either the RhoA sensor alone (Cre Neg, black trace, n = 11 spines) or the RhoA sensor plus Cre-recombinase (Cre Pos, red trace, n = 14 spines). B) Peak (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) RhoA sensor activation for experiments in (A). Time course of spine volume change for experiments in (A). D) Transient (averaged over 1-2 minutes) and sustained (averaged over 10-20 minutes) spine volume change for experiments in (A). Error bars are s.e.m. Asterisks denote statistical significance (p<0.05) as determined by an ANOVA.

7.3 Discussion

Here, we utilized 2pFLIM to visualize glutamate uncaging induced Rac1, Cdc42, and RhoA activation in single dendritic spines so as to determine the dependence of their activity on BDNF. Our results reveal that Rac1 and Cdc42 but not RhoA activity...
depends on post-synaptic BDNF. As such, these experiments provide a mechanistic link from BDNF-TrkB signaling to spine volume regulation via the Rho GTPases Cdc42 and Rac1 but not RhoA.

7.3.1 BDNF-TrkB-Rac1 signaling

Previous reports have demonstrated that BDNF-TrkB signaling can lead to Rac1 activation and that such activation is one mechanism enabling BDNF-TrkB signaling to modulate actin cytoskeleton organization (Lai et al., 2012). Consistent with these findings, we demonstrate post-synaptic BDNF knockout leads to a nearly complete impairment of glutamate uncaging induced Rac1 activation. Such inhibition suggests a tight correlation between the activities of both TrkB and Rac1. Building on this observation, the temporal profiles of these two proteins appear more similar to each other than to the profiles of other molecules studied in sLTP – Cdc42, RhoA, Ras, and CaMKII. Specifically, for TrkB and Rac1, both display a rapid rise in activity that first develops within 1 minute of the onset of glutamate uncaging. After peaking between 1 to 2 minutes, activities for both proteins begin to decay before reaching a lower, but still elevated level of sustained activity. The sustained phase of both TrkB and Rac1 activity is at least 50% of the peak activation. This is in contrast to CaMKII, RhoA, and Cdc42 where there is either no sustained phase (CaMKII) or this phase is less than 50% of the maximal activation (RhoA and Cdc42).
Despite such similarities in the temporal profile of their activities, the spatial profiles of Rac1 and TrkB are quite distinct. As described in chapter 5, the spatial pattern of TrkB activation reveals it to be confined to the spine undergoing sLTP following glutamate uncaging. This is in contrast to the spatial profile of Rac1 that shows significant spreading out of the spine undergoing sLTP and into the adjacent dendrite and neighboring spines (unpublished data). These findings in combination with Rac1’s strong dependence on BDNF suggest that Rac1 may be a means for TrkB’s spine restricted activity to have functional influence beyond the confines of the stimulated spine. However, how Rac1 activity spreads – whether it is spreading of active Rac1 molecules or of Rac1 stimuli – is not known. This and similar questions regarding the functional link between BDNF, TrkB, and Rac1 are currently being investigated by Nathan Hedrick, a fellow graduate student in the Yasuda Lab.

7.3.2 BDNF-TrkB-Cdc42 signaling

Like Rac1, there is abundant evidence linking Cdc42 activity to BDNF-TrkB signaling in the setting of actin cytoskeleton reorganization (Chen et al., 2006; Cheung et al., 2007; Myers et al., 2012; Shen et al., 2006). Consistent with these findings, we demonstrate that Cdc42 activity during sLTP is also dependent on post-synaptic BDNF. Unlike Rac1 however, the impairment is incomplete. In the peak phase, Cdc42 activity is largely inhibited by post-synaptic BDNF knockout but in the sustained phase, activity is not impaired. These findings thus suggest that Cdc42 depends largely but not wholly
on BDNF. This residual Cdc42 activity could be due to a parallel, BDNF-independent pathway mediating Cdc42 activation. If true, this could imply that to achieve full Cdc42 activation, both BDNF and non-BDNF mediated mechanisms must be employed. Supporting this idea is the fact that TrkB and Cdc42 present with distinct temporal profiles. Both proteins reach peak activity between 1 to 2 minutes following the onset of glutamate uncaging. However, after reaching their peaks, the activity profiles of these two proteins diverge. For Cdc42, its activity rapidly decays by more than 50% before stabilizing at a sustained state. For TrkB, the observed decay in activity is much smaller, less than 50%. Further support for the idea that Cdc42 activation arises from multiple mechanisms comes from Cdc42’s spatial profile. Comparing their individual spatial profiles, there are clear similarities as well as some critical differences. Initially, the two are quite similar showing prominent restriction of activity to the stimulated spine. However, with time, TrkB activity begins to spread into the adjacent dendrite and neighboring spines while Cdc42 remains restricted. These observations that Cdc42 and TrkB activities do not overlap in both time and space suggests that BDNF-TrkB signaling may be one of multiple mechanisms culminating in Cdc42 activation.

7.3.3 BDNF-independence of RhoA activity

Unlike Rac1 and Cdc42, there is little prior evidence establishing a mechanistic link between BDNF-TrkB signaling and RhoA activity. Consistent with this, we report no impairments in RhoA activity in the setting of BDNF inhibition – either through
extracellular scavenging with TrkB-Ig or post-synaptic knockout. These results suggest that RhoA activation may arise from an activity-dependent but BDNF-independent pathway contributing in parallel with BDNF-TrkB signaling to sLTP.

The idea of a pathway running parallel to BDNF-TrkB signaling during sLTP is appealing in that it may explain the incomplete impairments on sLTP we observe while simultaneously imaging RhoA activation. Specifically, in the context of TrkB-Ig application, we did not observe a reduction in either the transient or sustained phases of sLTP. Furthermore, in the single-cell BDNF knockout experiments, while expressing the RhoA sensor, sLTP is only affected in the sustained phase. The lack of an effect for TrkB-Ig and a partial effect for post-synaptic BDNF knockout on sLTP are in stark contrast to results obtained in multiple, other experiments presented here – see Figures 3A an B, 19C and D, 28B and C, and 30B and C. Thus, our findings with the RhoA sensor could suggest that the RhoA sensor expression may rescue sLTP in the setting of BDNF impairment.

Despite the concerns outlined above, we did not find evidence for BDNF being necessary for RhoA activation. As such, this suggests the existence of two signaling pathways in sLTP – one depending on BDNF, one independent of BDNF. The functional significance of this could be that the two pathways together may serve as a coincidence detector for sLTP. Namely, in order for sLTP to arise, one must have activation of both pathways. This dual pathway idea may explain why BDNF
application on its own is insufficient to induce sLTP in that one gets Rac1 and Cdc42 activity but not RhoA. To address this idea, one could express constitutively active RhoA (CA-RhoA) in CA1 pyramidal cells, apply exogenous BDNF, and then record changes in spine volume. The prediction would be that BDNF would activate Rac1 and Cdc42 and that such activation on the background of CA-RhoA would provide the BDNF-dependent and independent components necessary for sLTP leading to spine volume enlargement.

7.3.4 Mechanisms of BDNF-TrkB-Rho GTPase signaling

Building on links established between BDNF-TrkB signaling and Rac1 and Cdc42 activation, it is critical to dissect the mechanisms underlying this signaling pathway. For both Rac1 and Cdc42, previous work has identified Cdk5 phosphorylation of serine 478 within TrkB as a critical event for their activation by BDNF-TrkB signaling (Lai et al., 2012). However, how BDNF-TrkB signaling activates Cdk5 is unclear. For example, the amino acid residue within TrkB mediating this signaling is not known. Additionally, whether TrkB directly activates Cdk5 or if it initiates a signaling cascade culminating in Cdk5 activation are not known. One potential method to address these questions could be to utilize the Rac1 and Cdc42 FRET sensors in cultured hippocampal slices from mutant mice containing point mutations for the various amino acids within TrkB known to be critical for downstream signaling – S478, Y515, Y705/Y706, and Y816. By examining both Rac1 and Cdc42 activation in the background of these various TrkB
mutations, we can begin to dissect the signaling mechanisms connecting BDNF, TrkB, and these two Rho-GTPases.

An additional question to explore pertains to non-BDNF mediated mechanisms of TrkB activation. The results here only speak to the dependence of these three GTPases on BDNF. For both Cdc42 and RhoA, where we find either partial or no impairment in the setting of BDNF inhibition, it is possible that non-BDNF mediated TrkB activation, namely transactivation, could be contributing. Two simple ways to explore this question would be to use the chemical-genetic approach to TrkB inhibition or the single-cell TrkB knockout method – both described in chapter 3 – to impair TrkB activity while simultaneously imaging Cdc42 and RhoA activation during sLTP. If these impairments proved more efficacious than those used for BDNF, this could suggest that during sLTP, multiple sources of TrkB activation exist that culminate in activation of distinct signaling pathways.

**7.3.5 Conclusion**

In conclusion, we have demonstrated that Cdc42 and Rac1 but not RhoA activity are dependent on post-synaptic BDNF during sLTP. These two GTPases provide a mechanism by which BDNF-TrkB signaling can directly regulate the actin cytoskeleton and thus influence dendritic spine size and morphology. RhoA’s independence suggests that BDNF-TrkB signaling alone is insufficient for sLTP and that a parallel, BDNF-independent pathway must also exist.
8. Discussion

Utilizing two-photon microscopy in combination with 2pFLIM and glutamate uncaging, we define here a role for post-synaptic BDNF-TrkB signaling within CA1 apical dendritic spines undergoing sLTP. By developing and utilizing a FRET-based sensor for TrkB, we further demonstrate that during sLTP, post-synaptic TrkB rapidly activates within stimulated spines in an NMDAR, CaMKII, and post-synaptic BDNF dependent manner. Building on these findings, we go on to show that the activity of the Rho GTPases Rac1 and Cdc42 depends on post-synaptic BDNF-TrkB signaling. We then conclude by optimizing and utilizing a BDNF release sensor (BDNF-SEP) to demonstrate that in response to glutamate uncaging, post-synaptic BDNF rapidly releases from the stimulated spine via exocytosis in an AMPAR, NMDAR, and CaMKII dependent manner.

Combining all these results, we propose the existence of a cell-autonomous, spine-specific, autocrine BDNF-TrkB signaling loop that contributes to activation of GTPases that in turn mediate actin cytoskeleton changes underlying sLTP. Thus returning to our original question – what are the cellular consequences of TrkB activation within CA1 dendritic spines – we propose that one answer is that TrkB activation mediates structural plasticity at this synapse and through this mechanism, it
may enhance the functional properties of the synapse contributing to epilepsy
development.

8.1 Signaling pathways in sLTP

We demonstrate here that post-synaptic BDNF/TrkB signaling is critical for sLTP.
Combining these results with previously published findings, we now propose the
following signaling pathway model for sLTP (Figure 41). First, glutamate binds to
NMDARs and in the absence of Mg$^{2+}$, such binding induces ion channel opening. Once
open, the NMDAR is activated and is permeable to Ca$^{2+}$ ions. Because of the
electrochemical gradient for Ca$^{2+}$, it flows into the cell leading to a rise of Ca$^{2+}$ levels in
the spine. Once inside the cell, Ca$^{2+}$ complexes with the Ca$^{2+}$ binding protein calmodulin
(CaM). This Ca$^{2+}$/CaM complex will then bind to one of the subunits within CaMKII.
Following CaM binding, CaMKII changes its conformation and becomes active. In its
activated state, CaMKII can initiate a multitude of signaling events. One such event is to
induce the release of BDNF from stimulated dendritic spines. Of note, we also propose
that BDNF release can result directly from Ca$^{2+}$ influx in a CaMKII-independent manner.
Regardless of the mechanism, once released, BDNF binds to TrkB found within the
dendritic spine in an autocrine and spine-specific fashion. Once active, TrkB becomes
phosphorylated at various residues within its intracellular domain – of these, we
propose that Y816 is the most critical for sLTP. TrkB activation in turn leads to
activation of the Rho GTPases Cdc42 and Rac1. TrkB activation of these GTPases is likely an indirect process by which TrkB first activates one of the GEFs – Tiam1 for example – which in turn induces Cdc42/Rac1 activation. Once active, these GTPases will then initiate a series of events culminating to actin cytoskeleton reorganization – a process critical to structural plasticity.

In addition to these BDNF-TrkB dependent mechanisms, we also want to highlight that CaMKII activation initiates a BDNF-independent pathway that ultimately results in RhoA activation – another molecule capable of regulating actin cytoskeleton reorganization. As such, based on our results, we propose that CaMKII is a divergence point with the initiation of both BDNF-dependent and independent signaling pathways, both of which are critical for sLTP.
8.2 Mechanisms of TrkB activation – BDNF and Zn$^{2+}$ transactivation

In our experiments, we define a role for post-synaptic BDNF in sLTP. Because BDNF is the canonical ligand for TrkB, we initially assumed that observed TrkB activation during sLTP would be entirely BDNF-dependent. However, in the presence of multiple BDNF perturbations (application of TrkB-Ig and post-synaptic BDNF-knockout), we saw impaired but not fully eliminated TrkB activation during sLTP. This
residual TrkB activity suggests that in addition to BDNF, non-BDNF stimuli may be contributing to TrkB activation during sLTP.

As we describe in chapter 1, several non-BDNF stimuli for TrkB activation have been identified. These include TrkB transactivation by PACAP, adenosine, EGF, and Zn\(^{2+}\) (Huang and McNamara, 2010 2005; Huang et al., 2008; Lee and Chao, 2001; Rajagopal et al., 2002); 2) the kinetics of Zn\(^{2+}\)-mediated TrkB activation are consistent with temporal profile of sLTP induced TrkB activation (Huang and McNamara, 2010; Huang et al., 2008); and 3) Zn\(^{2+}\) has recently been shown to have a modulatory role in plasticity of another hippocampal synapse – the mossy fiber-CA3 synapse (Pan et al., 2011).

With all this said, it is unclear exactly how Zn\(^{2+}\) could contribute to TrkB activation during sLTP? There are several possibilities. First, active NMDARs are zinc-permeable (Frederickson et al., 2005). As such, when NMDARs open in response to glutamate uncaging, they could provide a means through which extracellular Zn\(^{2+}\) in the synaptic cleft could gain access to the intracellular space. Once inside the cell, Zn\(^{2+}\) could transactivate TrkB in a Src Family Kinase dependent manner (SFK). Second, Zn\(^{2+}\) is present in clear synaptic vesicles within pre-synaptic terminals (Frederickson et al., 2005). Within the hippocampus, vesicular Zn\(^{2+}\) is most prominent at mossy fiber giant boutons but there is also evidence for it within Schaffer collateral axons (Frederickson et
al., 2005). With this said, glutamate uncaging could induce pre-synaptic vesicular release of Zn$^{2+}$ that could cross the synaptic cleft and access the intracellular space of dendritic spines via active NMDARs or other routes. Third, the zinc-binding protein MT-3 tightly binds intracellular Zn$^{2+}$. MT-3 is found in CA1 pyramidal cells including their dendrites. It has been shown that generation of reactive oxygen species can lead to Zn$^{2+}$ release from MT-3 that can in turn transactivate TrkB (Huang and McNamara, 2012). On this point, it has been demonstrated that robust NMDAR activation can trigger the generation of reactive oxygen species (Gunasekar et al., 1995). If true, this could potentially lead to an increase intracellular Zn$^{2+}$ levels that could contribute to both TrkB activity and sLTP. These are both questions that we plan to actively explore in future experiments.

Of note, we would like to also point out that this discussion of TrkB transactivation would not have arisen if not for the TrkB FRET-based sensor. Specifically, perturbation of BDNF inhibited sLTP in a manner similar to TrkB inhibition and post-synaptic TrkB knockout. As such, these results did not suggest involvement of additional TrkB stimuli. However, upon visualization of TrkB activation with the FRET-based sensor, we did uncover residual TrkB activity in the setting of both pharmacologic and genetic BDNF impairment. This point only further validates the utility of studying the spatiotemporal dynamics of protein activation in living cells – sometimes the
mechanisms underlying activation are more complicated than previously considered and it is only with visualization that such complexity can be fully appreciated.

8.3 NMDARs and TrkB

Both NMDARs and TrkB have been shown to be critical for various physiologic and pathologic processes in the CNS including synaptic plasticity, epilepsy, and learning and memory (Kumar, 2010; McNamara et al., 2006; Minichiello). This commonality in function has always suggested a potential mechanistic link between these two receptors but this was hard to reconcile with the seemingly different activation kinetics of these two receptors, namely NMDARs activating rapidly on the order of milliseconds and TrkB activating slowly on the order of minutes. In retrospect, this discrepancy was likely a result of differences in temporal resolution for the various techniques used to measure receptor activity – a patch pipette for NMDARs versus Western Blotting with TrkB. Here though, using a FRET-based sensor with temporal resolution vastly improved over Western Blotting, we demonstrate that TrkB activation is much faster than previously demonstrated and that it parallels the time course of NMDAR-mediated Ca²⁺. As such, our findings demonstrate a mechanistic link between NMDARs and TrkB that could potentially explain the various functional parallels between these two receptors.
In terms of dissecting the molecular players linking NMDAR activity with TrkB activation, our BDNF release sensor proved quite helpful. Using this sensor, we identify the Ca\textsuperscript{2+} dependent, release of BDNF from dendritic spines as one molecular pathway that is triggered by NMDAR activation and culminates in TrkB activation. Interestingly, this NMDAR-BDNF release-TrkB molecular pathway is not unique to CA1 dendritic spines. In fact, recent event highlights the existence of a similar mechanism within pre-synaptic terminals within the striatum (Park et al., 2014). As such, it seems that NMDAR triggered BDNF release with subsequent TrkB activation may be a pervasive molecular mechanism underlying many aspects of both neuronal physiology and pathology.

8.4 Mechanisms of BDNF release – the V66M mutation

Using the BDNF release sensor, we provide compelling evidence for post-synaptic BDNF release. However, as we discuss in chapter 6, the mechanisms underlying this release largely remain unknown. To this end, an interesting and clinically relevant question to address pertains to the effects of the human BDNF polymorphism V66M on BDNF release properties.

V66M is a mutation that was recently discovered in humans. Most cases of this mutation are heterozygous and have been associated with impairments in cognitive and memory function (Egan et al., 2003; Hariri et al., 2003; Neves-Pereira et al., 2002;
Ventriglia et al., 2001). Because of the clinical impact of this mutation, its functional significance has been extensively studied in various animal models. Based on this work, it appears that this mutation leads to marked deficits in synaptic plasticity, learning, and memory (Hariri et al., 2003). The proposed mechanism for how this mutation impairs these functions is through reduced activity-dependent BDNF release (Chen et al., 2004).

Our BDNF release sensor in combination with two-photon glutamate uncaging provides a potential method to more fully investigate these links between the V66M mutation and BDNF release properties. Information gained from these experiments could prove clinically relevant in terms of designing targeted therapies for ameliorating the cognitive deficits observed in humans containing this mutation.

### 8.5 Microcompartment autocrine signaling at other synapses?

Through the combined use of the TrkB sensor, BDNF-SEP, and two-photon glutamate uncaging, we have identified a novel BDNF-TrkB autocrine signaling loop that arises and acts on single dendritic spines – a microcompartment with an approximate volume of 0.1 femtoliter. To our knowledge, identification of an autocrine signaling loop fully contained within such a small subcellular compartment is unprecedented and truly unique to biology. As such, an important question moving forward is to determine whether this signaling loop can be found at other synapses in the hippocampus and beyond. One synapse of particular interest is the Mossy Fiber –
CA3 synapse within the hippocampus. It has previously been shown that at this synapse, LTP engages primarily pre-synaptic mechanisms that involve BDNF and TrkB. In fact, it has been suggested but not yet directly shown that pre-synaptic BDNF release in response to synaptic activity may act on pre-synaptic TrkB receptors to induce LTP at this synapse. This hypothesis could be directly probed through the use of the tools we developed here, namely the TrkB sensor and BDNF-SEP. If an autocrine signaling loop was in fact identified, this would be quite exciting, as it would extend our results to another synapse as well as another cellular microcompartment – the pre-synaptic terminal.

**8.6 Physiology informing pathology – sLTP and epilepsy**

Our primary goal with this project was to utilize sLTP to explore potential cellular consequences of TrkB activation in CA1 dendritic spines. Based on our results, it seems clear that one cellular consequence of post-synaptic TrkB activation is the induction of structural plasticity. Building on this observation, the next step is to explore how these findings might inform various molecular and cellular mechanisms occurring as a consequence of seizures. To this end, we want to propose two potential mechanisms by which sLTP may contribute to epilepsy.

To begin, sLTP is the start of a series of changes that can have long-lasting and profound impact on the functional state of a cell (Murakoshi and Yasuda, 2012). Here
we only examine sLTP at a single spine. However, in the setting of SE, it is easy to imagine sLTP occurring concomitantly at many spines across the cell. The Yasuda Lab recently examined the functional impact of such multi-spine activity. Specifically, they found that stimulation of as few as 5 spines was sufficient to induce nuclear activation of the transcriptional regulator ERK. (Zhai et al., 2013) If only 5 spines can initiate activity, the activation with hundreds of spines in the setting of SE must be profound.

Another interesting observation is that enhanced TrkB activation is sustained following a brief (1 minute) epoch of glutamate uncaging. How such sustained activity occurs and the functional significance of this persistent activity is currently unclear. However, we do know that following a seizure, enhanced activation of TrkB occurs and remains elevated for some time. What might persistent TrkB activation being doing in both sLTP and seizure? One possibility is that it may be enhancing a spine’s sensitivity for future input. As we show in chapter 3, BDNF application and presumably TrkB activation are sufficient to lower the threshold for sLTP induction. A similar process may be occurring in the setting of prolonged TrkB activation following sLTP induction or seizure activity. If true, this enhancement of a spine’s sensitivity could explain the classic notion of “seizures beget seizures.” Namely, once a seizure happens, TrkB activation in a spine promotes additional spine activation with subsequent, potentially weaker stimuli. The end result is a domino effect where over time, synapses become
more and more sensitive that in terms of seizures, translates into increased susceptibility for synchronous, uncontrolled neuronal firing that can culminate in behavioral seizures.

**8.7 Future Directions**

Stemming from this work, multiple future directions exist. First, we have identified sLTP as one cellular consequence of TrkB activation within CA1 dendritic spines. The next step is to elucidate the molecular mechanisms by which TrkB mediates this cellular consequence. To this end, we have already identified signaling PLCγ1, Rac1 activity, and Cdc42 activity as critical downstream molecules. However, how these distinct signaling mechanisms relate to each other and how they specifically contribute to sLTP requires further exploration.

Second, we have defined a novel, autocrine BDNF-TrkB signaling loop that underlies sLTP. Because of its importance for mediating plasticity at this synapse, this mechanism is likely utilized at other synapses as well. Identification of these synapses plus their respective functional consequences could prove quite informative.

Third, TrkB-mediated sLTP appears to be a cellular consequence of SE but whether this structural plasticity and/or its underlying TrkB activation are necessary events for epilepsy development are not known. Now that we have identified these molecular and cellular mechanisms in a reduced preparation, it will be critical to explore these in an *in vivo* setting to confirm their applicability to epilepsy.
Finally, as we have mentioned before, many of the findings and observations presented here are in large part thanks to the development of a novel, FRET-based sensor for TrkB. Moving forward continued utilization of this sensor in a variety of physiologic and pathologic contexts will undoubtedly inform the mechanisms by which TrkB performs its many ascribed functions. More importantly though, our work will hopefully foster continued development of new FRET-based sensors for a wide array of neuronal proteins. As we show here, understanding a protein’s functional role within a neurologic process is not as simple as determining whether it gets activated or not. Rather, having a detailed description of its temporal and spatial profile affords insight into how this protein functions and how such function fits into the larger signaling mechanisms found within neurons. It is with this knowledge that one can truly begin to understand both physiology as well as pathology and then use such understanding to develop new and more efficacious therapies for diseases like epilepsy.
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Biography

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Publications


**Posters**


“Dynamic FRET-FLIM imaging of TrkB activation in living cells.”


“Identifying small molecule inhibitors of the TrkB/PLC-gamma interaction using TR-FRET.”

Presentations


Awards

2007 Inducted to the Alpha Omega Alpha Medical Honor Society

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