A Three-Molecule Model of Structural Plasticity: the Role of the Rho family GTPases in
Local Biochemical Computation in Dendrites

by

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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

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ABSTRACT

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Abstract

It has long been appreciated that the process of learning might invoke a physical change in the brain, establishing a lasting trace of experience. Recent evidence has revealed that this change manifests, at least in part, by the formation of new connections between neurons, as well as the modification of preexisting ones. This so-called structural plasticity of neural circuits – their ability to physically change in response to experience – has remained fixed as a primary point of focus in the field of neuroscience.

A large portion of this effort has been directed towards the study of dendritic spines, small protrusions emanating from neuronal dendrites that constitute the majority of recipient sites of excitatory neuronal connections. The unique, mushroom-like morphology of these tiny structures has earned them considerable attention, with even the earliest observers suggesting that their unique shape affords important functional advantages that would not be possible if synapses were to directly contact dendrites. Importantly, dendritic spines can be formed, eliminated, or structurally modified in response to both neural activity as well as learning, suggesting that their organization reflects the experience of the neural network. As such, elucidating how these structures undergo such rearrangements is of critical importance to understanding both learning and memory.
As dendritic spines are principally composed of the cytoskeletal protein actin, their formation, elimination, and modification requires biochemical signaling networks that can remodel the actin cytoskeleton. As a result, significant effort has been placed into identifying and characterizing such signaling networks and how they are controlled during synaptic activity and learning. Such efforts have highlighted Rho family GTPases – binary signaling proteins central in controlling the dynamics of the actin cytoskeleton – as attractive targets for understanding how the structural modification of spines might be controlled by synaptic activity. While much has been revealed regarding the importance of the Rho GTPases for these processes, the specific spatial and temporal features of their signals that impart such structural changes remains unclear.

The central hypotheses of the following research dissertation are as follows: first, that synaptic activity rapidly initiates Rho GTPase signaling within single dendritic spines, serving as the core mechanism of dendritic spine structural plasticity. Next, that each of the Rho GTPases subsequently expresses a spatially distinct pattern of activation, with some signals remaining highly localized, and some becoming diffuse across a region of the nearby dendrite. The diffusive signals modify the plasticity induction threshold of nearby dendritic spines, and the spatially restricted signals serve to keep the expression of plasticity specific to those spines that receive synaptic input. This combination of differentially spatially regulated signals thus equips the neuronal dendrite with the ability to perform local biochemical computations, potentially
establishing an organizational preference for the arrangement of dendritic spines along a dendrite. Finally, the consequences of the differential signal patterns also help to explain several seemingly disparate properties of one of the primary upstream activators of these proteins: brain-derived neurotrophic factor (BDNF).

The first section of this dissertation describes the characterization of the activity patterns of one of the Rho family GTPases, Rac1. Using a novel Förster Resonance Energy Transfer (FRET)-based biosensor in combination with two-photon fluorescence lifetime imaging (2pFLIM) and single-spine stimulation by two-photon glutamate uncaging, the activation profile and kinetics of Rac1 during synaptic stimulation were characterized. These experiments revealed that Rac1 conveys signals to both activated spines as well as nearby, unstimulated spines that are in close proximity to the target spine. Despite the diffusion of this structural signal, however, the structural modification associated with synaptic stimulation remained restricted to the stimulated spine. Thus, Rac1 activation is not sufficient to enlarge spines, but nonetheless likely confers some heretofore-unknown function to nearby synapses.

The next set of experiments set out to detail the upstream molecular mechanisms controlling Rac1 activation. First, it was found that Rac1 activation during sLTP depends on calcium through NMDA receptors and subsequent activation of CaMKII, suggesting that Rac1 activation in this context agrees with substantial evidence linking NMDAR-CaMKII signaling to LTP in the hippocampus. Next, in light of recent evidence linking
structural plasticity to another potential upstream signaling complex, BDNF-TrkB, we explored the possibility that BDNF-TrkB signaling functioned in structural plasticity via Rac1 activation. To this end, we first explored the release kinetics of BDNF and the activation kinetics of TrkB using novel biosensors in conjunction with 2p glutamate uncaging. It was found that release of BDNF from single dendritic spines during sLTP induction activates TrkB on that same spine in an autocrine manner, and that this autocrine system was necessary for both sLTP and Rac1 activation. It was also found that BDNF-TrkB signaling controls the activity of another Rho GTPase, Cdc42, suggesting that this autocrine loop conveys both synapse-specific signals (through Cdc42) and heterosynaptic signals (through Rac1).

The next set of experiments detail one the potential consequences of heterosynaptic Rac1 signaling. The spread of Rac1 activity out of the stimulated spine was found to be necessary for lowering the plasticity threshold at nearby spines, a process known as synaptic crosstalk. This was also true for the Rho family GTPase, RhoA, which shows a similar diffusive activity pattern. Conversely, the activity of Cdc42, a Rho GTPase protein whose activity is highly restricted to stimulated spines, was required only for input-specific plasticity induction. Thus, the spreading of a subset of Rho GTPase signaling into nearby spines modifies the plasticity induction threshold of these spines, increasing the likelihood that synaptic activity at these sites will induce structural plasticity. Importantly, these data suggest that the autocrine BDNF-TrkB loop
described above simultaneously exerts control over both homo- and heterosynaptic structural plasticity.

The final set of experiments reveals that the spreading of GTPase activity from stimulated spines helps to overcome the high activation thresholds of these proteins to facilitate nearby plasticity. Both Rac1 and RhoA, the activity of which spread into nearby spines, showed high activation thresholds, making weak stimuli incapable of activating them. Thus, signal spreading from a strongly stimulated spine can lower the plasticity threshold at nearby spines in part by supplementing the activation of high-threshold Rho GTPases at these sites. In contrast, the highly compartmentalized Rho GTPase Cdc42 showed a very low activation threshold, and thus did not require signal spreading to achieve high levels of activity to even a weak stimulus. As a result, synaptic crosstalk elicits cooperativity of nearby synaptic events by first priming a local region of the dendrite with several (but not all) of the factors required for structural plasticity, which then allows even weak inputs to achieve plasticity by means of localized Cdc42 activation.

Taken together, these data reveal a molecular pattern whereby BDNF-dependent structural plasticity can simultaneously maintain input-specificity while also relaying heterosynaptic signals along a local stretch of dendrite via coordination of differential spatial signaling profiles of the Rho GTPase proteins. The combination of this division of spatial signaling patterns and different activation thresholds reveals a unique...
heterosynaptic coincidence detection mechanism that allows for cooperative expression of structural plasticity when spines are close together, which in turn provides a putative mechanism for how neurons arrange structural modifications during learning.
Dedication

This work is first and foremost dedicated to my mother and father, who set their dreams – and often and their wellbeing – aside to give me the life I wanted. You were understanding in times that bred little more than confusion, kind in times that seemed to warrant only anger, and supportive in times that seemed to offer only defeat. This dissertation and this degree are documented proof that you overcame the vicious cycles of home and the damnation of poverty that leave so many with so little. And what’s more, you did it for someone other than yourselves. You, my parents, far more than I, should be very, very proud.

This work is also dedicated to my late brother. In death, you revealed the me the unwavering responsibility that every individual shoulders for the sake of those around them: to live.

To my brilliant niece, Brooke-Lynn: you were the first light of my life. Never have I found so much joy in living for someone else. You have reminded me that the truest happiness that you can bring to yourself is always the happiness of someone else. Know that sadness has the power to teach you great things. Know that I believe that you can do anything in the world that you truly want to do. And finally, know that you are loved... tirelessly, deeply, and fully.

To my friends and brothers, Chase and Curtis, who had the patience of putting up with me for years. In truth, I will never understand why you did, but I will always

x
remember it. With all sincerity, your friendship has meant more than you will ever know. I am honored and privileged to have been a part of your lives for so many years, and I hope that never really ends.

And finally, to Jessica Bolton, my Zelda. You are one of the few people who shares my insatiable curiosity for almost everything, and time with you has been a brilliant tangle of philosophy, science, happiness, and maybe an occasional normal human thing. I love and respect you more than you could possibly know. Where I am irritable, cynical, and frustrated, you are invariably calm, optimistic, and meditative. You are what I would want to be if I weren’t so stubborn...and yet, you still somehow make me feel good to be me. I am eternally grateful for the life that you have given me; I am a happier and a better person because of you. I hope that I can save you the way you saved me.
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1. Introduction

1.1 Learning, memory, and neuronal plasticity

1.1.1 Background

The ability of an animal to learn requires that the brain be able to encode and store—as well as retrieve—information about the animal’s experience. These traces of past events in the brain must be at once long lasting and malleable, allowing preservation of learned information while simultaneously allowing the trace to be updated in light of new salient information. In a similar dichotomy, these traces must maintain specificity so as to correctly reflect the encoding of a particular stimulus, while at the same time preserving the ability to associate any trace with new information. These seemingly paradoxical, yet astonishingly routine features of learning and memory have kept this topic fixed as a central point of fascination in neuroscience since the emergence of the field.

The capacity of the brain to change so as to encode experience—referred to as neural plasticity—is now thought to underlie not only normal physiological processes such as learning and memory, but also pathological states, such as addiction and post-traumatic stress disorder (PTSD). By co-opting the brain’s ability to selectively strengthen certain pathways, these diseases effectively manifest as deleterious ‘learning’, maintaining harmful behavioral patterns or emotional states long after the initial insult. Thus, understanding the processes by which the brain encodes information could
provide some clues as to how to prevent it from encoding ‘unwanted’ information (or even reverse the encoding of such information).

Conversely, some of the most burdensome diseases to society at large are those associated with the inability to correctly form new memories, such as Alzheimer’s disease (AD) and other neurodegenerative diseases. As such, acquiring a clear understanding of the various tools and strategies that the brain uses to encode experience is critical for being able to treat these diseases. In addition, being able to artificially enhance or induce neuronal plasticity is a promising possibility for rehabilitation for individuals recovering from brain trauma, and even for promoting adult learning of novel tasks such as language acquisition.

1.1.2 Long-term potentiation (LTP)

Despite the central importance of learning and memory to animal and human behavior alike, specific biological mechanisms explaining these processes remained incredibly elusive until only very recently. Even working off the assumption that physical changes should occur in the brain with learning, identifying the locus of such change has been extremely difficult. After all, the gross structural anatomy of the brain is largely similar from individual to individual, and so whatever structural traces might appear are certainly on a different scale than the ultrastructure of the brain. Similarly, even by changing scale and visualizing individual cells gives few clues as to the past histories of the circuits they occupy, and reveals little more than the staggering
complexity of even the basic building blocks of the brain. As a result, identifying the locus of experience-associated physical changes in the brain is effectively impossible without visualizing the brain directly during the event. As such, any mechanistic explanations of these phenomena were largely speculative.

It was not until the discovery of long-term potentiation (LTP), a process whereby strong synaptic activity increases the sensitivity of active synapses to future input, that there was an attractive model of cellular learning in the brain (Bliss 1973; Bliss 1973). Along with its counterpart, long-term depression (LTD), which reduces the sensitivity to future input, LTP allows the tuning of synaptic strength according to the history of the activity of any particular synapse. Certain patterns (typically strong and fast bouts of activity) lead to LTP, while other distinct patterns (typically slower, prolonged activity) lead to LTD. This form of cellular pattern recognition allows the associated synapses to reflect the occurrence of a particular pattern of synaptic activity long after the stimulus has ended, which, in a very real sense, shows that these synapses are capable of storing this information.

Importantly, a number of features of LTP and LTD further suggested that these phenomena might be associated with behavioral learning. First, these phenomena were first described in the hippocampus, an area of the brain critical for the formation of new memories. Second, these changes are both long lasting and input-specific, and thus parallel many of the features associated with behavioral learning described above. While
these features by no means establish a causal connection, they do fit the known constraints of the system, and thus provide an attractive target. Third, a large body of work has since made it clear that several human mutations that cause impairments in learning and memory (such as those seen in mental retardation) also impair the induction and/or expression of LTP. Thus, the molecular requirements for both learning/memory and LTP (or LTD) are similar, consistent with the possibility that LTP and/or LTD underlie learning and memory.

Finally, recent work has demonstrated that particular aspects of behavioral memory can be inactivated and reactivated by inducing LTD and LTP, respectively, at synapses associated with the behavior (Nabavi 2014). Thus, LTP and LTD are phenomena causally associated with learning and memory that relate synaptic history to behavior.

1.1.3 Structural plasticity and structural LTP (sLTP)

While the functional consequences of LTP are numerous, the central hallmark of its expression classically reduces to the postsynaptic cell’s ability to increase its response to presynaptic glutamatergic input for a prolonged period of time. This so-called ‘functional’ LTP is often read out as an increased inward current in the postsynaptic cell in response to activation of a pathway that has undergone LTP. At the hippocampal Schaffer Collateral synapse, this sensitization process is largely driven by an increase in the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-
mediated current (Shi 1999), suggesting that either entirely new AMPAR-containing synapses are formed (Engert 1999; Maletic-Savatic 1999), or that new AMPARs are inserted into the postsynaptic membrane of existing synapses (Patterson, M 2010), partially accounting for the increased sensitivity to presynaptic neuronal activity. Both of these mechanisms have also been described in a variety of other synapses in the brain, suggesting a relatively generalizable mechanism of LTP across excitatory neurons. As a result of this, electrophysiological readouts focusing on activity-induced enhancements of excitatory postsynaptic currents have remained the predominant indicator of the occurrence of LTP.

However, LTP is additionally associated with a number of structural changes to the cell, including the elaboration of the dendritic arbor, as well as both the formation of new synapses and the modification of existing ones. This so-called ‘structural plasticity’ likely subserves the functional changes that occur in parallel, as the two show a high degree of correlation, and typically share common mechanisms. The addition of new synapses, for example, represents an increase in the number of recipient sites of excitatory neurotransmission, and thus can contribute to the increase in postsynaptic response to presynaptic activity. This is by definition a structural change to the neuron that requires modification of the existing cellular infrastructure that permits the stable formation of synapses.
1.1.3.1 sLTP of dendritic spines

Interestingly, long before the discovery of LTP, there were surprisingly accurate speculations as to the locus of a learning-induced physical change in the brain. The most surprising of such speculations was made by Santiago Ramón y Cajal, the pioneer of modern neuroscience, who surmised that dendritic spines – small, mushroom-like protrusions emanating from the dendrites of neurons – served as connection points between axons and dendrites, and as such, were attractive targets for providing fast functional modifications of synaptic transmission. He went so far as to speculate that the movement, growth, or retraction of spines could underlie different behavioral states of animals.

It is now appreciated that dendritic spines are, in fact, the primary sites of excitatory neuronal transmission in the brain, and as such are primary candidates for the site of activity-dependent modifications in synaptic strength, as with those seen in LTP and LTD. With the advent of molecular tools (such as green fluorescent protein, GFP) that enabled the longitudinal visualization of living cells, it has also been demonstrated that dendritic spines are not rigid structures, but rather highly plastic ones that change markedly over time. These changes occur both spontaneously (in the absence of neuronal activity) (Tashiro, A., 2000) and in rapid response to LTP- and LTD-inducing stimuli (Engert 1999; Maletic-Savatic 1999; Matsuzaki 2004; Murakoshi 2011; Oh 2013), as
well as experience (Trachtenberg 2002; Xu, T 2009; Roberts 2010; Fu, M 2012; Peters 2014).

Given this, structural plasticity of dendritic spines has received considerable attention as a reduced preparation to understand the underlying mechanisms of LTP at their most basic unit. In the extreme case, inducing plasticity in single dendritic spines would allow an effectively minimized preparation of LTP. In order to isolate a single dendritic spine, however, it was first necessary to reduce the input stimulus to one that could target a single spine. To this end, photolysis of chemically caged glutamate, which has a very low affinity for glutamate receptors until optically uncaged, has become a critical tool (Matsuzaki 2004). By combining caged glutamate with two-photon excitation, which has a cross section much smaller than single-photon excitation, it became possible to selectively stimulate a single dendritic spine with glutamate, mimicking presynaptic release events (Matsuzaki 2004; Harvey, C 2008; Lee, S-JRJ 2009; Murakoshi 2011). When spines are stimulated with a train of such stimuli, they undergo both functional LTP (as measured by an increase in the AMPAR-mediated current) as well as sLTP. These changes are remarkably input-specific, as nearby spines show no changes in either AMPAR-mediated current or spine volume.

Single-spine sLTP is characterized by two distinct phases: a large transient phase, in which the spine shows an increase in volume several times its original size over a few minutes, and a slower, more modest sustained phase, in which the spine volume relaxes
to ~150-200% of its original size (Fig. 1) (Matsuzaki 2004; Lee, S-JRJ 2009; Murakoshi 2011). The sustained phase lasts for at least several hours, and correlates with the degree of AMPAR-mediated glutamate sensitization. Importantly, this process has been largely mechanistically inseparable from the ‘functional’ expression hallmarks of LTP at any particular synapses, suggesting that these processes are largely intermingled, and perhaps interdependent. Further, the two phenomena share a significant number of molecular requirements for their expression. As such, the same events that elicit functional LTP are necessarily recruiting machinery that is required for structural LTP, suggesting that they likely occur at the same time in response to the same signals. At the hippocampal CA3-CA1 synapse, for instance, both functional and structural LTP require calcium current through N-methyl-D-aspartate receptors (NMDARs) and subsequent activation of Ca²⁺-calmodulin-dependent kinase II (CaMKII), small GTPases, and actin polymerization for normal expression (Matsuzaki 2004; Harvey, C 2008; Lee, S-JR 2009; Murakoshi 2011). Thus, the structural plasticity of single dendritic spines can be effectively used as a readout of the occurrence of LTP, and can be used as a minimal functional model.
Figure 1: Spine volume change upon induction of LTP at a single dendritic spine.

Schematic of volume changes in dendritic spine induced by 2p-glutamate uncaging-based LTP protocols. Stimulation of a single spine leads to a rapid and robust transient change in volume that lasts <5 min, which is then followed by a sustained elevation of spine volume that lasts for at least 30 minutes.

It should be noted, however, that there are instances of the separation of functional and structural LTP. First, structural plasticity actually seems to precede externalization of AMPARs (Kopec, Charles D., 2006), suggesting that structural changes can exist without the accompanying functional changes, however briefly. An
important caveat of this study is that LTP was induced chemically, bringing into question whether these findings represent a physiologically relevant occurrence. Indeed, more recent evidence exploring single-spine LTP with the methods described above suggests that the two phenomena occur simultaneously, at least under glutamatergic stimulation paradigms (Patterson, M 2010).

Like with LTP, some studies have revealed a separation between functional LTD from structural changes to spines (in the case of LTD, spine shrinkage), suggesting that these phenomena might be interactive, but not entirely interdependent (Sdrulla 2007; Wang 2007). One of these studies, however, also showed that the functional LTD that they observed in the absence of spine shrinkage was blocked in the presence of an actin polymerization inhibitor, suggesting that ‘unseen’ or internal structural changes were still responsible for the functional effects they observed (Wang 2007). Another study showed that structural plasticity could be forced to occur in the absence of structural plasticity by overexpression of the C-terminal tail of the GluA1 subunit of AMPARs (Kopec, C. D., 2007). While this data does show a separation between functional and structural plasticity, the manipulation was somewhat artificial, and indeed, the authors conclude that these two forms of plasticity are linked by the AMPA receptor itself, with increased receptors simultaneously causing an increased synaptic response and serving as a critical molecular scaffold that maintains structural plasticity. Consistent with the idea that these two phenomena are largely linked, more recent evidence has revealed
that single-spine stimulation paradigms similar to that described above for sLTP cause simultaneous spine shrinkage and reduction of EPSP amplitude. In one study, spines on basolateral dendrites of hippocampal pyramidal CA1 neurons stimulated with low-frequency glutamate uncaging bouts showed spine-specific spine shrinkage and depression of EPSP amplitude (Oh 2013). In a very different paradigm, another study described a form of LTD was not spine specific, and required simultaneous GABAergic stimulation (Hayama 2013). Despite the very different protocol, structural and functional LTD were nonetheless effectively synonymous, with both stimulated and adjacent spines undergoing decreases in volume and experiencing a reduction in EPSP amplitude. Thus, while certain contexts reveal a divorce between structural and functional plasticity, the two phenomena are largely linked, and perhaps inseparable at their core forms of expression.

The exact functional purpose of dendritic spine sLTP remains unclear, but several mechanisms – including passive changes in input properties or even long-term changes in the molecular scaffolding within spines (Bosch 2014) – have been proposed. Long-term changes in molecular scaffolding could potentially subserve functions such as synaptic maintenance, as well as offer a form of synaptic tag (Bosch 2014). As structural plasticity is associated with an increase in post-synaptic density (PSD) size (as well as the actual influx of PSD protein), which is in turn associated with increased synaptic efficacy (Harris 1989) and calcium influx (Matsuzaki 2004), the structural
enlargement of a spine likely reflects a collaborative process between many molecular players that serve to modify a variety of features of dendritic spines, including – but not limited to – the functional enhancement of synaptic efficacy.

Thus, in addition to the long-lived changes in synaptic transmission efficacy, LTP is also characterized by structural changes to synaptic connections on neurons, thereby providing a long sought-after targetable and defined locus of the physical trace of a memory.

1.2 Spatiotemporal regulation and gating of structural plasticity

Several lines of evidence suggest that the formation, elimination, and modification of dendritic spines that occur surrounding synaptic activity or learning are not random, but rather are highly specific processes that occur with exquisite temporal and spatial precision. This precision suggests that neurons have several mechanisms to select when and where structural plasticity can occur, which in turn suggests that such precision might be a critical feature of how structural plasticity might subserve learning and memory.

Consistent with this, some structural phenomena only occur during a particular phase of learning, or within a certain window of synaptic activity, suggesting that the timing of these events is a critical element of their occurrence. Similarly, some LTP and learning paradigms show a spatial preference for structural plasticity, resulting in
modifications only at certain cellular loci (Peters 2014) or in certain spatial patterns (Harvey, C 2007; Fu, M 2012). As such, there are likely a variety of cues that create a permissive environment for structural plasticity, or otherwise overcome barriers to its occurrence, so as to signal the appropriate spatiotemporal setting for its induction. These cues are likely utilized so as to optimize where and when structural plasticity occurs so as to give rise to learning only in the appropriate context. What these cues are, and how they are recruited to define the appropriate spatiotemporal windows of plasticity, however, remains largely unknown.

To assist in identifying these cues, it has been immensely helpful to first understand the constraints and requirements imposed by learning itself.

1.2.1 Spine addition and elimination with different phases of learning

Does the addition of a dendritic spine represent the formation of a memory? Is the removal of spines tantamount to forgetting? While the actual physical representation of a single memory is still debated, one clue lies in the fact that both spine addition and elimination occur during learning (Roberts 2010). Recent work, however, has illustrated that the addition and elimination of dendritic spines occur at specific time windows during certain forms of learning. By monitoring dendrites of the motor cortex while an animal learned a novel motor task, it was found that spine formation is a hallmark of the early phases of learning, in a phase when the animal’s performance is only beginning to improve (Peters 2014). Subsequently, spine addition begins to decline, and spine
elimination starts to occur, a transition that correlates with the improved performance of the animal at the motor task. These data suggest that different forms of structural plasticity occur over different phases of the learning process, and likely subserve the different requirements of learning as it progresses.

Thus, different forms of structural plasticity serve temporally distinct functions during learning. As such, it is critical that the system be able to tightly regulate the time windows over which each type of plasticity occurs. The consequence of this is that the cellular and molecular processes that govern these two distinct forms of plasticity must be carefully maintained over the temporal window of learning. How such control is conferred is unclear.

1.2.2 Precise timing of structural plasticity via molecular gating

In some contexts, structural plasticity may require the coordination of a number of other events before it can be effectively induced. This kind of ‘coincidence detection’ would ensure that structural plasticity would only occur in certain situations, and likely imbues the neuron with increased computational strategies.

In one study, it was found that excitatory cortico-stratial synapses in the mammalian striatum can exhibit glutamatergic, Ca\(^{2+}\)-CaMKII-dependent structural plasticity, much like that seen in the hippocampus, but that this only occurs if there is concomitant dopaminergic input (Sho 2014) activating a PKA-dependent pathway to ‘ungate’ CaMKII-dependent plasticity. Importantly, the overlap of these signals shows a
very specific temporal requirement: structural plasticity was only induced if
dopaminergic inputs were activated approximately 1 second after glutamatergic
stimulation. This temporal window overlaps well with reward timing in behavioral
settings, and thus provides an attractive cellular and molecular model for how
reinforcement learning is coordinated.

Importantly, these experiments also illustrate that there is precedent for the
temporal gating of structural plasticity to achieve a potentially behaviorally relevant
phenomenon.

1.2.3 Spine clustering

While it is difficult to appreciate any organizational patterns of dendritic spines
when each pyramidal cell has on the order of 10,000 such structures, several lines of
evidence have suggested that spines show very specific organizational patterns across
the dendritic arbor.

In particular, significant effort has been placed into identifying whether synaptic
inputs are dispersed randomly across dendrites, or are spatially ‘clustered’ in specific
regions. Some evidence suggests that the induction of LTP itself is sufficient to induce
the clustered formation of spines along a dendrite (De Roo 2008), suggesting that this
process somehow creates local environments along neuronal dendrites that favor the
formation of new synapses. Other studies suggest a ‘functional’ clustering of synapses,
wherein the activities of closely opposed spines are linked.
One study revealed that repeated motor learning preferentially induces the formation of spines in close proximity to one another, clustering the spines together as the animal refined the task (Fu, M 2012). Importantly, learning a different motor task would still cause new dendritic spines to form, but these spines were not added to the cluster associated with the original task. Thus, clusters of dendritic spines seem to correlate with a particular learned task, suggesting that spine clustering might subserve some aspect of repeated learning.

Another study showed that sensory experience leads to a patterned distribution of AMPA receptors in small clusters along a dendrite, potentially serving as the ‘functional’ correlate of the physical clustering of spines (Makino 2011). These AMPA receptor clusters were of a similar size as the spatial scale of structural spine clustering mentioned above (~8 µm). Interestingly, this clustering effect could be eliminated by only two mutations on the AMPA receptors. These residues are sensitive to phosphorylation by intracellular kinases, suggesting that the clustering phenomenon observed in this study is regulated by postsynaptic intracellular signaling. Furthermore, phosphorylation at these residues is known to modify the threshold for the insertion of AMPA receptors to the synaptic surface (Hu 2007), suggesting that such threshold modification is critical for the formation of these clusters. These data highlight not only the fact that clustered synapses have both structural (the spatial proximity of groups of spines) and functional (differential AMPA receptor recruitment at these clusters)
characteristics, but also the fact that these clusters are arranged by postsynaptic intracellular signaling.

Importantly, the consequences of a clustered arrangement of synapses are far greater than their physical proximity alone. It has been suggested that the close juxtaposition of dendritic spines could give rise to sufficient local depolarization to recruit active conductance mechanisms (Poirazi, P., 2001), such as those governed by voltage-gated ion channels (Magee 2005), leading to local nonlinearities in depolarization. Such nonlinearities would cause even a small number of synaptic inputs to have large depolarizing effects on the postsynaptic cell (Losonczy, A., 2008), which would then impart a preference for clustered groups of synapses in generating action potential output.

1.2.4 Synaptic crosstalk

One structural phenomenon that invokes both precise timing and defined spatial limitations is synaptic crosstalk, a process whereby the induction of LTP at a single dendritic spine lowers the threshold for LTP induction at spines within about 10µm of the site of stimulation (Harvey, C 2007). This allows even weak inputs that would otherwise not generate LTP to reach the necessary threshold at these nearby spines. If no subsequent stimulus is received within the critical time window, however, no long-term changes occur at these nearby spines, and the LTP event remains specific to the original spine. This phenomenon is presumed to be conveyed by an intracellular signal, as very
nearby spines (even within the 10µm limit) on different dendrites do not experience the threshold modification effect. Consistent with this, synaptic crosstalk seems to require the heterosynaptic signals conveyed by the small GTPase Ras (Harvey, C 2008). Taken together, these data suggest that the induction of LTP activates a signal that invades a local region of the dendrite (Ras, e.g.), thus allowing the preferential strengthening of synapses that are in close proximity to each other along a dendritic branch. Interestingly, this is one putative mechanism by which synaptic clusters (described above) might arise.

1.3 Molecular mechanisms of structural plasticity

1.3.1 Upstream Activators

1.3.1.1 NMDAR-CaMKII

Central to a surprisingly large proportion of the mechanisms of plasticity that have been studied in the brain is calcium influx through N-methyl-D-aspartate-class glutamate receptors (NMDARs) and subsequent activation of Ca\(^{2+}\)-calmodulin-dependent kinase II (CaMKII). The reason for this commonality is likely the unique properties of these molecules that equip the system with features that are exceptionally useful in the encoding of specific information. NMDARs, for instance, allow the filtering of most synaptic inputs as inappropriate for LTP induction by virtue of a voltage-sensitive Mg\(^{2+}\) blockade. This features makes presynaptic release of glutamate alone insufficient to cause calcium influx through NMDARs, thus preventing the induction of
plasticity. However, when sufficient depolarization of the postsynaptic cell is achieved (either through sufficient temporal or spatial summation of nearby non-NMDAR-dependent current or by virtue of back-propagating action potentials), the Mg\(^{2+}\) blockade is relieved, and calcium can enter the synapse, activating the signaling cascades required for LTP induction. As such, NMDARs function as a coincidence detector, allowing plasticity only when the pre- and postsynaptic neurons are sufficiently co-active. This feature makes NMDARs indispensible for LTP and the structural plasticity of dendritic spines (Matsuzaki 2004; Lee, S-JR 2009; Murakoshi 2011).

The Ca\(^{2+}\) influx through NMDARs subsequently binds to calmodulin, which in turn binds to CaMKII, activating the kinase. CaMKII then goes on to activate many downstream proteins that are necessary for LTP (see below). Importantly, CaMKII possesses the ability to maintain its own activity even after calmodulin unbinds via autophosphorylation. Since CaMKII forms a large dodecamer, potentially creating structural hub conducive to a cycle of auto-activation, it has been proposed that this autophosphorylation could lead to prolonged activation of CaMKII, which might account for the longevity of LTP (Lisman, John, 1994; Lisman, J., 2012). While more recent evidence has shown that CaMKII activity itself is not necessarily prolonged for the duration of plasticity, its presence and normal autophosphorylation are nevertheless required for normal structural LTP at single dendritic spines (Lee, S-JR 2009), functional LTP (Giese 1998; Ohno 2002), as well as learning (Giese 1998).
1.3.1.2 BDNF-TrkB

Another one of the more widely studied molecules associated with LTP is brain-derived neurotrophic factor (BDNF) and its receptor, tropomyosin receptor kinase B (TrkB). BDNF is a secreted factor that can be released from neurons in a variety of contexts. Upon its release, it binds to TrkB receptors on the surface of the same or nearby cells, and this bound complex subsequently activates a variety of signaling cascades that subserve a multitude of functions.

Early studies revealed that the induction of LTP increases levels of BDNF mRNA, suggesting a connection of this molecule to potentiated cells (Patterson, SL 1992; Pozzo-Miller 1999). It was then shown that BDNF is actually required for the normal expression of LTP, as mice with a genetic deletion of the BDNF locus show impairments in LTP that can be rescued with exogenous BDNF (Korte 1995; Korte 1996). Importantly, the requirement of BDNF seems to be acute, as these deficits are also observed when an extracellular scavenger of BDNF (TrkB-Ig) is bath applied (Figurov 1996; Chen, G 1999).

BDNF signaling has also been shown to give rise to a number of structural changes to neurons, including the formation of new spines and the outgrowth of dendrites.

It has been suggested that neuronal depolarization could give rise to BDNF release, an event that might link BDNF signaling to neuronal activity. Indeed, one study
suggested that depolarization-induced BDNF release and subsequent TrkB activation is required for certain paradigms of single-spine sLTP (Tanaka 2008).

BDNF has also been shown to stabilize individual spines by causing the accumulation of the postsynaptic scaffolding protein Homer in the postsynaptic density during the late phase of sLTP (Bosch 2014). This result has several implications, including not only a molecular connection of BDNF to structural changes in spines, but also the possibility that BDNF could promote the longevity of LTP by virtue of increasing the molecular scaffolding within a spine that might allow the recruitment of several plasticity-related proteins.

Consistent with the role of BDNF in LTP, it has also been demonstrated that the primary receptor for BDNF, TrkB, is required for LTP, as mice lacking a functional TrkB receptor show deficits in functional LTP (Minichiello, L. , 1999; Pozzo-Miller 1999; Xu, B 2000).

Given all of this, it is likely that BDNF has a central role in relaying neuronal activity to structural modifications of the activated cells, and as such could serve as a link between certain neural activity patterns and structural plasticity. Indeed, several studies have suggested a connection between BDNF-TrkB signaling and the Rho GTPases Rac1 and Cdc42, further supporting the possibility that BDNF serves as a critical intermediate between neuronal activity and structural plasticity.
1.3.2 Downstream effectors

1.3.2.1 Actin and Rho GTPases

In order for structural plasticity to take shape, the mechanisms that control the exquisite spatial and temporal regulation of structural plasticity described above must ultimately converge on a biochemical signaling pathways that control the internal architecture of the cell, and in particular, the spines themselves. Recent work using scanning electron microscopy has revealed that dendritic spines are composed principally of the cytoskeletal protein actin (Korobova 2010), a globular monomer that can be polymerized into long helical chains that provide internal structure to the surface of the cell. Therefore, structural plasticity of dendritic spines must involve biochemical signaling mechanisms that can alter the actin network so as to modify (or create) the structure of spines.

The most prominent of such biochemical mechanisms almost universally recruit the Rho GTPases, a subfamily of Ras-related small GTPases that act as binary switches relating extracellular cues to intracellular signals. The most widely studied Rho GTPases, RhoA, Cdc42, and Rac1, have been extensively characterized in regards to their control over the actin cytoskeleton. Through a variety of signaling pathways, the Rho GTPases can cause the stabilization, elongation, branching, and collapse of actin filaments, which can rapidly and drastically alter the local morphology of the cell. As a result, the Rho GTPases are indispensible in a vast array of cellular functions that
involve physical mobilization of the cell in response to external cues, including cell migration, differentiation, and phagocytosis, just to name a few.

Given this, it is not surprising that the Rho GTPases are also involved in structural plasticity. Indeed, a variety of studies have shown these proteins to be essential for spine formation, spine motility (Tashiro, A., 2000; Tashiro, Ayumu 2004), and many LTP-like phenomena (Xie 2007; Hayashi-Takagi 2010; Murakoshi 2011; Lai 2012). One study revealed that the Rho GTPases connect experience-dependent neuronal activity to dendritic arborization during development (Sin 2002). In this context, heterologous expression of constitutively active variants of Rac1 and Cdc42 in the tadpole optic tectum led to an enhancement of light-induced dendritic arborization, while dominant negative variants had the opposite effect. The inverse was observed for RhoA, suggesting that RhoA negatively regulates dendritic growth, a fact that has been supported by other studies (Nakayama 2000). Taken together, these data suggest that Rac1 and Cdc42 are responsible for activity- and experience-induced elaboration of the dendritic arbor by stabilizing and elaborating the actin cytoskeleton, while RhoA has a negative effect on dendritic outgrowth and spine morphogenesis, perhaps through a depolymerizing effect on actin. Notably, these activity-dependent changes required NMDAR function, as pharmacological blockade of these receptors precluded activity-dependent changes, as well as the enhancements seen by GTPase signal modification.
Collectively, these data suggest that NMDAR activity signals through Rho GTPases to afford activity-dependent structural plasticity by modulating the actin cytoskeleton.

Similarly, several lines of indicate that the Rho GTPases are involved in the structural plasticity of dendritic spines. Similar manipulations using constitutively active and dominant negative variants of these proteins revealed that negative regulation of Rac1 causes a progressive collapse of spines (Nakayama 2000), while activation of Rac1 causes supernumerary (but smaller) spines (Luo 1996). Conversely, upregulation of RhoA activity through deletion of a RhoA-inactivating protein (Govek 2004) or by stimulation of certain RhoA-activating receptors (Fu, W-Y 2007) leads to reduction in spine number. Similarly, the opposing roles of Rac1 and RhoA have been demonstrated in spine morphology of existing spines, with Rac1 positively regulating motility, and RhoA showing the inverse effect (Tashiro, A., 2000; Tashiro, Ayumu 2004). Despite the general consensus that RhoA negatively regulates spine structure, recent lines of evidence have suggested that this GTPase is also required for certain forms of structural plasticity. For instance, both RhoA and Cdc42 were been shown to be essential for single-spine structural plasticity (Murakoshi 2011). By combining two-photon glutamate uncaging with two-photon fluorescence lifetime imaging (2pFLIM) to visualize the activity of the Rho GTPases, it was shown that RhoA and Cdc42 are rapidly activated in the stimulated spine during sLTP induction, and that this activity endures for at least 40 minutes. Both RhoA and Cdc42 activity was shown to be initiated during –
and required for structural plasticity of a single spine, as either pharmacological blockade or their signal, or shRNA-based knock-down of the proteins caused deficits in the expression of structural plasticity. It was further shown that the activation of these two GTPases was NMDAR-CaMKII-dependent, not only forming a strong connection with LTP, but also illustrating that Rho GTPases transform transient signals of neuronal activity from NMDARs to long-lasting structural changes at the sites of synaptic connection. Interestingly, this study also showed that while both Rho GTPases were activated in the stimulated spine, Cdc42 remained restricted to the stimulated spine while RhoA activity then rapidly spread into the dendrite and nearby spines. This combination of different spatial signaling profiles might allow the cell to maintain input specificity of synaptic signals while at the same time conferring messages to nearby synapses. Nonetheless, this heterosynaptic signal is clearly not sufficient for structural plasticity, as no overall changes in spine volume are observed during glutamate uncaging-induced structural plasticity, suggesting that RhoA activity is not sufficient for spine enlargement.

In summary, substantial literature suggests that the Rho GTPases are essential for the structural plasticity of both dendrites and spines, and are thus attractive candidates as a surrogate for monitoring the molecular underpinnings of structural plasticity. By visualizing the activity of these proteins, it would be possible to identify
the spatial patterns of molecular signals that underlie eventual structural modifications of dendritic spines.

The primary focus of the following dissertation will be describing the spatial and temporal characteristics of the molecular signaling components that are critical for single-spine structural plasticity, with a particular focus on the Rho GTPases. By monitoring the spatial patterns of activity of the Rho GTPases and the upstream molecular signals that control them, inferences can be made regarding the characteristics of input-specific spine structural plasticity as well as other potentially masked or latent forms of structural plasticity. Finally, an understanding of the different molecular signals that confer such ‘structurally relevant’ signals will provide information as to the potential contexts in which such phenomena might occur.

As such, the basic properties of signaling proteins will be used to inform the pursuit of higher-order functions of both spines and dendrites, which ultimately subserve even higher-order functions of neurons, circuits, and ultimately, the brain as a whole.
2. Materials and Methods

2.1 Reagents

Human recombinant BDNF was purchased from Millipore, N-2 amino-5-phosphonovalerate (N-AP5), NSC-23766, and EHT-1864 were purchased from Tocris, and TrkB-Ig was a gift from Regeneron. The tat-CN21 peptide (YGRKKRRQRRRKRPPKLGQIGRSKRVIEDDR) was synthesized by GenScript.

2.2 Plasmids

2.2.1 Rac1 biosensor plasmids

2.2.1.1 mEGFP-Rac1

The plasmid containing human Rac1 was a kind gift of M. Matsuda. The Rac1 FRET donor construct was created by inserting the Rac1 coding sequence into the pEGFP-C1 (Clontech) vector containing the monomeric mutation (A206) in the EGFP coding sequence.

2.2.1.2 mCherry-PBD2(R71C,S78A)-mCherry

The plasmid containing human Pak1(65-118) was a kind gift of S. Soderling. The CRIB domain of Pak2 (PBD2) was prepared by introducing mutations R72C and S79A into Pak1 (60-118) using a Site-Directed Mutagenesis kit (Stratagene). These mutations correspond to residues R71 and S78 in human Pak2, thus this construct is referred to as PBD2(R71C, S78A). The Rac1 FRET biosensor acceptor construct was created by inserted
the PBD2(R71C,S78A) coding sequence into a tandem mCherry, described previously (Murakoshi 2011).

### 2.2.2 Dendritic inhibitor plasmids

#### 2.2.2.1 W56-mCherry-MTBD

W56-mCherry-MTBD was prepared by amplifying the Rac1 inhibitory peptide W56 (Gao, Y., 2001) using overhang PCR with a C-terminal linker (GGGGGGGGGGGGGGGGGGGGGGMADQLTEWHRGTAGPGS) and inserting it into pCAG-mCherry-mCherry (Murakoshi 2011) by removing the first mCherry with EcoRI+KpnI restriction digest and replacing it with the W56-Linker amplicon, creating pCAG-W56-(Linker)-mCherry. In parallel, the microtubule-binding domain (MTBD) of human MAP2c (272-end) (Zhong 2009) was isolated from a human cDNA library using PCR amplification. This amplicon was then further amplified with overhang PCR to contain a linker (same as above), and then inserted into pCAG-mCherry-mCherry using BamHI-NotI restriction digest to produce pCAG-mCherry-(Linker)-MTBD. The two constructs were then combined using BamHI+NotI restriction digest to create pCAG-W56 - (Linker) - mCherry - (Linker) - MTBD.

#### 2.2.2.2 DNRhoA-mCherry-MTBD & DNCdc42-mCherry-MTBD

Dendritic inhibitor constructs for RhoA and Cdc42 were generated by PCR amplification of dominant negative (T17N) variants of RhoA and Cdc42 into the Rac1 dendritic inhibitor construct, inserting an additional MfeI site into the C-terminus of
the GTPase signal sequences and removing the stop codon to allow for continuous translation of DN(GTPase) - (Linker) - mCherry - (Linker) - MTBD.

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, M 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 TrkB biosensor plasmids

2.2.4.1 mEGFP-TrkB

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 2002) and the CAG promoter. The linker between TrkB and mEGFP is TGRH.

TrkBY816F-mEGFP was prepared by introducing a point mutation using the Site-DirectedMutagenesis Kit (Stratagene).

2.2.4.2 mRFP-PLCγ-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.

### 2.2.5 POMC-mCherry

POMC-mCherry was generated by amplifying the POMC peptide – MMWCLESSQCQDLTEESNLACIRACRLDL (Lou et al., 2005) – using overhang PCR with a C-terminal linker - GGGGGGGGGGGGGGGGGGGGGGGGGMADQLTEEWHRGTAGPGS. This amplicon was then inserted into the tandem mCherry plasmid by replacing the coding sequence of the first mCherry.

### 2.2.6 CA-Cdc42

The constitutively active (CA) Cdc42 plasmid was a kind gift of Scott Soderling.

### 2.3 Preparation

#### 2.3.1 Organotypic hippocampal slice cultures

Hippocampal slices were prepared from postnatal day 5-7 rats or mice in accordance with the animal care and use guidelines of Duke University Medical Centre. Briefly, we deeply anaesthetized the animal with isoflurane, after which the animal was quickly decapitated, and the brain removed. The hippocampi were isolated and cut into 350µm sections using a McIlwain tissue chopper. Hippocampal slices were plated on tissue culture inserts (Millicell) fed by tissue medium (for 2.5 L: 20.95 g MEM, 17.9 g HEPES, 1.1 g NaHCO₃, 5.8 g D-Glucose, 120 µL 25% ascorbic acid, 12.5 mL L-Glutamine,
2.5 mL Insulin, 500 mL Horse Serum, 5 mL 1 M MgSO$_4$, 2.5 mL 1 M CaCl$_2$). Slices were incubated at 35°C in 3% CO$_2$ and fed every 2-3 days with a 50% media replacement.

### 2.3.2 ‘Biolistic’ gene transfer

After 1-2 weeks in culture, CA1 pyramidal neurons were transfected with 1µm ballistic gene transfer using gold beads (8-12 mg) coated with plasmids containing 30-40µg of total cDNA. Cells expressing only mEGFP were imaged 1-5 days post transfection, cells expressing TrkB were imaged 1-2 days post transfection, and all other plasmid combinations were imaged 2-5 days post transfection.

For structural plasticity experiments, conditional knockout slices ($BDNF^{+/−}$, $TrkB^{+/−}$, and $Rac1^{+/−}$) were transfected with either GFP alone or GFP and tdTomato-Cre (1:1) for 3-7 days prior to imaging. For sensor experiments in these slices, the sensors were used in the ratios listed above with an amount of Cre recombinase equal to the amount of donor DNA. The presence of Cre was confirmed by nuclear-localized tdTomato signal.

### 2.3.3 Mammalian cell lines

HeLa and HEK293T cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum at 37°C in 5% CO$_2$. Transfection was performed at ~50-90% cell confluency using Lipofectamine (Invitrogen) and 2µg/mL of total cDNA/35mm dish, following the ratios listed below.
### Table 1: Ratios of plasmid DNA for various experiment types

<table>
<thead>
<tr>
<th>Construct</th>
<th>Donor</th>
<th>Acceptor</th>
<th>Cell fill</th>
<th>Other</th>
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<td>mEGFP-C1</td>
<td></td>
<td>mEGFP</td>
<td>20µg</td>
<td></td>
</tr>
<tr>
<td>mEGFP + Cre</td>
<td></td>
<td>mEGFP</td>
<td>20µg</td>
<td>tdTomato-Cre</td>
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<tr>
<td>mEGFP + Dendritic Inhibitor</td>
<td></td>
<td>mEGFP</td>
<td>25µg</td>
<td>W56-mCherry-MTBD or DNX-mCherry-MTBD</td>
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2.4 Imaging

2.4.1 Two-photon fluorescence intensity imaging

Two-photon imaging was performed using a Ti-sapphire laser (MaiTai, Spectraphysics) tuned to a wavelength of 920nm, allowing simultaneous excitation of mEGFP and mCherry. All samples were imaged using <2mW laser power as measured at the objective. Fluorescence emission was collected using an immersion objective (60x, numerical aperture 0.9-1.0, 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).

2.4.2 Fluorescence lifetime imaging

FRET imaging using a custom-built two-photon fluorescence lifetime imaging microscope was performed as previously described (Yasuda, Ryohei, 2006; Murakoshi 2008; Murakoshi 2011) (Fig. 2). 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, Ryohei, 2006), while the red channel signal was acquired using a separate data acquisition board (PCI-6110) controlled with Scanimage software (Pologruto 2003).
Figure 2: Schematic of two-photon excitation microscopy and two-photon fluorescence lifetime imaging (2pFLIM).

(a) Setup of two-photon excitation microscopy. A femtosecond pulsing laser is directed towards x-y scanning mirrors, which scan the designated imaging field. The scanning and tube lenses direct the beam through a dichroic mirror into the back aperture of the objective, which then focuses the scanning beam onto the imaging plane. Fluorescence signals from the sample are then collected by the objective, and reflected off the dichroic mirror and focused onto a photomultiplier tube (PMT). The photocurrent received is paired with the position information from the x-y scanning mirrors to assign intensity information to a particular pixel corresponding to that location on the imaging field. The time of arrival of the photocurrent can also be paired with reference pulses from the laser to calculate the fluorescence lifetime of the sample.

(b) Schematic of fluorescence lifetime calculation. The offset between a reference laser pulse and the photon arrival time from the sample corresponds to the fluorescence lifetime of the sample. A histogram is built corresponding to the arrival time of all the photons collected for a given image. This histogram can be curve-fitted to describe the decay of the fluorescence emission.

(c) Schematic of the effect of FRET
on fluorescence lifetime. A free mEGFP molecule has a long fluorescence lifetime (green curve), while mEGFP linked to a FRET acceptor molecule (sREACH) transfers energy non-radiatively to the acceptor, and therefore emits photons for a shorter time.

d) Sample image of fluorescence lifetime imaging in HeLa cells. In addition to intensity information, the image also shows the fluorescence lifetime value (pseudocolored) of each pixel.

2.4.3 BDNF-SEP imaging

In order to capture fast release events, BDNF-SEP imaging experiments were performed by imaging at an 8Hz frame-scanning frequency. Glutamate uncaging pulses (described below) were interleaved into the imaging time course every 16 frames (2 seconds) after a 32-frame baseline. Multiple spines (≤ 30) were imaged on each neuron.

2.5 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 glutamate) in extracellular solution with a train of 4-6 ms, 4-5 mW pulses (30 times at 0.5 Hz) near a spine of interest (‘sLTP stimulus’). Experiments were performed in Mg^{2+} free artificial cerebral spinal fluid (ACSF; 127 mM NaCl, 2.5 mM KCl, 4 mM CaCl\textsubscript{2}, 25 mM NaHCO\textsubscript{3}, 1.25 mM NaH\textsubscript{2}PO\textsubscript{4} and 25 mM glucose) containing 1 µM tetrodotoxin (TTX) and 4 mM MNI-caged L-glutamate aerated with 95% O\textsubscript{2} and 5% CO\textsubscript{2} at 30°C, as described previously. ‘Subthreshold’ stimuli were delivered using a train of 1ms, 4-5mW pulses (30 times at 0.5 Hz). Crosstalk experiments were performed by first delivering an sLTP stimulus (4-
6ms, based on the measured depth of the targeted spine, where 4, 5, and 6ms pulses were used for depths of 10-20, 20-30, and 30-40µm depth, respectively), then delivering a subthreshold stimulus to a nearby (within ~2-5µm) spine ~90 seconds later, as previously described (Harvey, C 2007). Anywhere from 1-5 spines were stimulated per cell, and a maximum of 3 crosstalk experiments were performed on a single cell.

2.6 Data Analysis

2.6.1 Spine volume analysis

Spine volume was calculated as the background-subtracted integrated fluorescence intensity over a region of interest around the dendritic spine head (Fluorescence, F). Change in spine volume was measured as $\Delta F/F_0$, where $\Delta F = F_t - F_0$, and $F_0$ is the average fluorescence intensity of baseline acquisitions prior to stimulation. Analysis of two-photon images outside of the context of 2pFLIM was performed in ImageJ.

2.6.2 BDNF-SEP intensity analysis

The change in BDNF-SEP fluorescence intensity was calculated as $\Delta F/F_0$ after subtracting background fluorescence. Red fluorescence intensity was simultaneously measured and calculated in the same manner.
2.6.3 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:

\[
F(t) = F_0 [P_D H(t, t_0, \tau_D, \tau_G) + P_{AD} H(t, t_0, \tau_{AD}, \tau_G)] \tag{Eq. S1}
\]

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:

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

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 \(\text{erfc}\) is the error function.

We fixed \(\tau_D\) to the fluorescence lifetime obtained from free mEGFP (2.6 ns). To generate the fluorescence lifetime image, we calculated the mean photon arrival time, \(<t>\), in each pixel as:

\[
<t> = \frac{\int tF(t) \, dt}{\int F(t) \, dt},
\]
then, the mean photon arrival time is related to the mean fluorescence lifetime, $<\tau>$, by an offset arrival time, $t_0$, which is obtained by fitting the whole image:

$$<\tau> = <t> - t_0.$$  

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

$$P_{AD} = T_D (T_D - <\tau>) (T_D - T_{AD})^{-1}(T_D + T_{AD} - <\tau>)^{-1}.$$  \hspace{1cm} \text{Eq. S3}

2.7 Measurements of the affinity between Rho GTPases and RBDs

Polyhistidine-tagged super-folder GFP (sfGFP)-Rac1, mCherry-Cdc42 / Rac1 binding domain (CRIB) of Pak2 and their mutants were cloned into the pRSET bacterial expression vector (Invitrogen). Proteins were overexpressed in Escherichia coli (DH5α), purified with a Ni$^2+$-nitrilotriacetate (NTA) column (HiTrap, GE Healthcare), and desalted with a desalting column (PD10, GE Healthcare) equilibrated with phosphate buffer saline (PBS). The concentration of the purified protein was measured by the absorbance of the fluorophore (sfGFP, $A_{489} = 83,000$ cm$^{-1}$M$^{-1}$ (Shaner 2004); mCherry, $A_{587} = 72,000$ cm$^{-1}$M$^{-1}$ (Pédelacq 2005)).

Purified sfGFP-Rac1 was loaded with GppNHp (2',3'-O-N-methyl anthraniloyl-GppNHp) and GDP by incubating in the presence of 10-fold molar excess of GppNHp...
and GDP in MgCl$_2$-free PBS containing 1 mM EDTA for 10 min, respectively. The reaction was terminated by adding 10 mM MgCl$_2$. sfGFP-Rac1 and mCherry-CRIB were mixed and incubated at room temperature for 20 min. FRET between sfGFP and mCherry was measured under 2pFLIM, and the fraction of sfGFP-Rac1 bound to mCherry-CRIB was calculated by fitting the fluorescence lifetime curve with a double exponential function (Eq. S1). The dissociation constant was obtained by fitting the relationship between the binding fraction and the concentration of mCherry-CRIB ([mCherry-CRIB]) with a Michaelis-Menten function (Fig. 3).

![Graphs showing binding fractions for Rac1 and Cdc42](image)

**Figure 3: Measurement of affinity between Rac1 vs. Cdc42 and substrate, PBD2 (R71C, S78A)**

Comparison of affinities of the Rac1 biosensor acceptor (PBD2(R71C, S78A)) to the active (GTP-bound; red curves) and inactive (GDP-bound; black curves) forms of
both Rac1 (left graph) and Cdc42 (right graph). Binding fraction was calculated based on the fluorescence lifetime of super-folding GFP (sfG), which shortens when undergoing FRET with monomeric Cherry (mC). The binding of sfG-tagged proteins to mC-tagged substrates thus results a co-localization of the two fluorophores, shortening the lifetime of sfG, which then serves as a surrogate measurement of substrate-binding. Varying the concentration of the substrate thus yields a representation of the affinity of a protein for its substrate. The shown $K_d$ values were determined by fitting the data (red) to a Michalis-Menten function.

3. Imaging the spatiotemporal dynamics of Rac1 signaling during sLTP

3.1 Introduction

In order to begin to understand how structural plasticity might be spatially and temporally coordinated in response to synaptic activity, it is critical to first understand how the molecular signals that underlie plasticity – in particular, the Rho GTPases – are activated in response to such stimuli. Previous studies using two-photon fluorescence lifetime imaging (2pFLIM) in combination with FRET-based sensors revealed that the Rho GTPases Cdc42 and RhoA are rapidly activated in dendritic spines in response to glutamate uncaging, and that such activation is necessary for spine structural plasticity (Murakoshi 2011). As their activity was NMDAR-CaMKII-dependent, RhoA and Cdc42 form a critical link in a chain reaction that transduces transient calcium entry during synaptic activity to short-lived (on the order of minutes) kinase activity, which then results in long lasting (>30 min) activation of the Rho GTPases. As such, these proteins act a persistent trace of the occurrence of strong synaptic input.
Interestingly, despite their comparable temporal activation patterns, these two GTPases displayed distinct spatial profiles, with Cdc42 showing synapse-restricted activation and RhoA showing a more diffuse, heterosynaptic pattern of activity (Fig. 4). Importantly, since this paradigm elicits sLTP only at the stimulated spine, the diffusive activity of RhoA is not sufficient to induce structural plasticity at nearby spines. The potential functions of this differential spatial signalling within a dendrite have thus remained elusive, but it is likely that such divergence conveys structural signals relating synaptic activity (through some unknown mechanism) to nearby synapses while at the same time maintaining input specificity.
Figure 4: Spatial profiles of RhoA and Cdc42

(a) Representative 2pFLIM images of Cdc42 (left) and RhoA (right) biosensors during 2p-glutamate uncaging-induced sLTP. (b) The change in binding fraction of Cdc42 (left) and RhoA (right) in response to glutamate uncaging, plotted as a function of distance from the stimulated spine. The spine data is plotted on the y-axis. A spreading index (S.I.) was calculated as the ratio of the spine activation with the activation in the dendrite 1-5μm away from the spine from 1-2 minutes after stimulation.
3.2 Results

3.2.1 Design of a FRET-based Rac1 biosensor

The spatiotemporal behaviour of other structural proteins – such as the Rho GTPase Rac1 – in response to synaptic activity is unclear. To obtain the full spatiotemporal profile of Rho GTPase proteins, a new FRET-based sensor for Rac1 was developed following the basic design of the Cdc42 and RhoA sensors: monomeric enhanced green fluorescent protein (mEGFP) is fused to the N-terminus of Rac1, serving as the fluorescence donor construct, and a Rac1 binding partner, the Cdc42-Rac binding domain of Pak2 with affinity-adjusting mutations (R71C, S78A; Fig 3), is flanked by mCherry fluorophores, serving as the fluorescence acceptor (Fig. 5a). In its active state, Rac1 switches bound guanine nucleotide diphosphate (GDP) for guanine nucleotide triphosphate (GTP). As GTP-bound Rac1 has a much higher affinity for mCh-Pak2(R71C, S78A)-mCh (and Pak2 in general), the acceptor construct binds preferentially to active Rac1, allowing the co-localization of the mEGFP and mCherry fluorophores, thus inducing FRET when mEGFP is optically excited. By deconvolving the FRET and non-FRET populations of fluorescence lifetime from mEGFP-Rac1, a ‘binding fraction’ of the Rac1 population can be extracted from the mean photon arrival time (Fig. 5b) (Yasuda, Ryohei, 2006).
Figure 5: Rac1 FRET-based sensor design

a) Schematic of the Rac1 biosensor design. Upon activation/ GTP binding, Rac1 binds to its substrate, which forces the mEGFP and mCh fluorophores into proximity with one another, inducing a FRET reaction when mEGFP is excited. b) The amount of FRET occurring at any given time can be measured as a change in the fluorescence lifetime of mEGFP. While free (unbound) mEGFP shows a characteristic long lifetime (~2.59ns; green curve), the occurrence of FRET shortens this lifetime (red curve), and a mixed population of bound and unbound mEGFP-Rac1 is measured as an intermediate between these curves. The binding fraction of mEGFP-Rac1 can then be calculated by deconvolving the FRET and non-FRET components.
3.2.2 Characterization of the Rac1 biosensor

In order to ensure that the Rac1 sensor FRET signal was indicative of actual Rac1 activation, the biosensor was expressed in HEK293T cells with multiple known variations (with characterized effects on Rac1 signalling), and subjected to established stimuli. First, dominant negative (Rac1 (T17N)), substrate-binding null (Rac1(Y40C)), and constitutively active (Rac1(Q61L)) variants of the Rac1 sensor were introduced into HEK293T cells, and the effect of these mutations on the basal binding fraction of the Rac1 sensor was tested. Consistent with the expected direction of effect, Rac1(Y40C) significantly lowered the basal binding of the Rac1 sensor, and Rac1(Q61L) significantly raised it (Fig. 6a,b). Notably, Rac1(T17N) did not significantly affect the basal binding fraction of the sensor (Fig. 6a,b), suggesting that either this sensor design is incapable of resolving any difference with this mutation, or that this mutation does not affect basal binding of Rac1 to its substrate.

To further test the specificity of the FRET signal from the Rac1 sensor, a known activator (the Rac1 guanine nucleotide exchange factor (GEF), Tiam1) and inactivator (the Rac1 GTPase-activating protein (GAP), ARHGAP15) of Rac1 were co-transfected along with the wild-type Rac1 sensor (Fig. 6a,b). Once again, consistent with the expected direction of effect, Tiam1 significantly elevated the basal binding of the Rac1 sensor, while ARHGAP15 significantly lowered it, suggesting that the Rac1 sensor can
be bi-directionally regulated by enzymes with known activity towards endogenous Rac1.

Finally, to test whether the Rac1 sensor could dynamically report changes in the activation state of Rac1, HEK293T cells overexpressing the Rac1 sensor were exposed to epidermal growth factor, a stimulus known to activate endogenous Rac1 (Fig. 6c). Consistent with reports describing the effect of EGF application on endogenous Rac1, the Rac1 sensor reported a rapid increase in substrate binding within five minutes. This activity then gradually decayed, returning to baseline within approximately 10-15 minutes. Importantly, neither the Rac1(T17N) nor Rac1(Y40C) sensor variants showed any change in binding fraction upon application of EGF, suggesting that the sensor is capable of dynamically reporting signals that are representative of Rac1 activation. Additionally, inclusion of a Rac1 inhibitory peptide, W56 (Gao, Y., 2001), reduced the EGF-induced increase in Rac1 binding fraction, further supporting the specificity of the sensor’s signal. Taken together, these data suggest that the Rac1 sensor presented here is a specific biosensor capable of dynamically and reversibly reporting changes in the activity of Rac1.
Figure 6: Characterization of the Rac1 biosensor

a) Representative 2pFLIM images of Rac1 sensor expression in HEK293T cells. Sensor variants include: dominant negative (T17N), substrate-binding null (Y40C), and constitutively active (Q61L).

b) Quantification and summary of data in (a).

c) Activation of the Rac1 sensor in response to bath application of 100 ng/mL epidermal growth factor (EGF) onto HEK293T cells.
3.3 Imaging Rac1 in single dendritic spines

To image the spatiotemporal dynamics of Rac1 activation in neurons, we transfected organotypic hippocampal slices of rodents with the Rac1 sensor using biolistics (Stoppini 1991; McAllister, 2000) (Fig. 7) and imaged CA1 pyramidal neurons with 2pFLIM (Yasuda, R., 2006). This approach allows the sparse labelling of neurons, which is favourable for the identification of individual dendritic spines.

In order to monitor Rac1 activation in a reduced preparation of sLTP, 2pFLIM was combined with two-photon glutamate uncaging, allowing the visualization of Rac1 activation during the induction of sLTP at a single dendritic spine (Fig. 7). For 2pFLIM, imaging was interleaved with glutamate uncaging pulses during the stimulation window so as to achieve near-simultaneous imaging and stimulation. In this way, the fine-scale activation kinetics of Rac1 during glutamatergic stimulation could be resolved.
Figure 7: Approach for single-spine sLTP induction and imaging

Coronal sections of postnatal day 5-7 rodent hippocampi are isolated and cultured for at least 7 days prior to transfection. Using ballistic transfection of a desired gene labeled with a fluorescent protein, individual cells can be visualized within the hippocampal structure. Pyramidal cells transfected in area CA1 were identified based on hippocampal anatomy and cellular morphology as seen under epifluorescence. Once identified, the dendrites of the CA1 cell were targeted using two-photon microscopy. Individual spines can then be stimulated using two-photon glutamate uncaging. If presented with a specific pattern of uncaging pulses (30 pulses at 0.5 Hz), the volume of the stimulated spine rapidly increases, and remains elevated for at least 30 minutes.
3.3.1 Rac1 activation during sLTP

When sLTP was induced in single dendritic spines with two-photon glutamate uncaging (Matsuzaki 2004; Honkura 2008), the Rac1 sensor was rapidly (within ~1 min) activated in the stimulated spine, and then remained active for at least 30 min (Figure 1b, c). The prolonged nature of this activation is likely not an artefact of the sensor, as its activation is reversible in other contexts (Fig. 6c). Additionally, the sustained activation showed a mild decay from the peak activation during uncaging, suggesting that the sensor’s activation is at least partially reversible in this context. The time course of Rac1 activation in spines was similar to the reported pattern of RhoA and Cdc42 activation (Murakoshi 2011), but with a more pronounced sustained phase, perhaps suggesting a differential contribution of Rac1 during this phase of structural plasticity. Thus, like RhoA and Cdc42, Rac1 serves as a lasting trace of synaptic activity at a single spine.

The activation of Rac1 showed limited diffusion for the first minute, after which it slowly spread over ~10 μm of the parent dendrite until the dendritic fraction nearly equalized with the activity in the spine (Fig. 8a, c, Fig. 9a)
Figure 8: Rac1 activation during single-spine sLTP

(a) Representative images of Rac1 activation during the induction of sLTP. Images represent the average of multiple frames over the time bins indicated above each image. Warm colors correspond to a shorter lifetime (more Rac1 activation), while cooler colors correspond to a longer lifetime (less Rac1 activation). (b) Spine volume change during sLTP induction in Rac1 sensor-overexpressing cells illustrating the successful induction of sLTP. (c) Quantification of Rac1 activation over various regions of interest during sLTP induction. Inset cartoon illustrates the regions of interest, with color corresponding to the different binding fraction curves.
3.3.2 The spatial profile of Rac1 activation

As previously mentioned, the Rho GTPases RhoA and Cdc42 showed distinct spatial profiles of activation after the induction of sLTP: Cdc42 activity was highly compartmentalized within the stimulated spine, while RhoA activity spread into nearby regions of dendrite and surrounding spines. The spatial profile of Rac1 showed a unique pattern, with a mixture of compartmentalized activation in the early phase and a spreading pattern in the later phase (Fig. 8a, 9a). As such, Rac1 displays spatial signalling characteristic of both Cdc42 (compartmentalization) and RhoA (signal diffusion) across different time scales. The functional significance of this phenomenon is unclear, though it is possible that the enrichment of Rac1 activity in the spine in the early phase of sLTP is critical for the large transient increase in spine volume during this same epoch.

To compare the spatial profile of the GTPases, we defined a spreading index (S.I.), corresponding to the ratio of the activity in the dendrite (1-5 µm from the stimulated spine) to the maximal spine activation 1-2 minutes after stimulation. In comparison to Rac1 (S.I. = 0.42), Cdc42 activation was restricted to the stimulated spine (S.I. = 0.12), while RhoA showed more immediate but more limited (S.I. = 0.30) spreading over ~5 µm (Fig 9b), as previously reported (Murakoshi 2011). Thus, despite showing early compartmentalization, Rac1 expresses the highest degree of dendritic activity of all of the Rho GTPases (with respect to its peak activation in the spine).
### 3.3.3 Pharmacological characterization of Rac1 activity during sLTP

Previous reports have indicated that activity-dependent structural modifications to neurons requires calcium influx through N-methyl-D-aspartate (NMDA) receptors (Sin 2002; Tolias 2005; Xie 2007; Saneyoshi 2008). To test whether the activation of the Rac1 sensor that we observed during single-spine sLTP also requires NMDARs, we applied

**Figure 9: Time-dependent spatial spreading of Rac1 activation**

a) Spatial profile of Rac1 activation during sLTP induction. Colored lines represent different time bins, plotted as activation of Rac1 along a dendritic segment as a function of distance from the stimulated spine. The stimulated spine activation during that time bin is plotted on the y-axis. b) Comparison of the spatial profile of Rac1 vs. the other Rho GTPases, RhoA and Cdc42, normalized to peak activation. All data plotted as mean ± sem.
100µM of the competitive NMDAR antagonist (2R)-amino-5-phosphonovaleric acid (AP5) to prevent caged glutamate from activating this receptor. In this context, Rac1 activation was eliminated (Fig. 10), suggesting that Rac1 signaling in response to glutamate uncaging requires calcium entry through NMDARs.

Given the tight connection of NMDAR calcium and the activation of calcium/calmodulin-dependent kinase II (CaMKII) (Lee, S-JR 2009), we next explored whether Rac1 activation also required CaMKII activity. To do this, we applied 10µM of the cell-permeable selective CaMKII inhibitory peptide TAT-CN21 (Vest, R 2007). Consistent with previous reports suggesting the association of CaMKII and Rac1 signaling pathways (Tolias 2005; Xie 2007), Rac1 activity was heavily attenuated under the blockade by CN21 (Fig. 10). Notably, however, activity was only significantly affected in the early phase, but not after ~10 minutes. This could suggest that a different signaling pathway regulates the more sustained aspects of Rac1 signaling.

Taken together, the above data illustrate that glutamatergic stimulation of dendritic spines rapidly induces Rac1 activation at those synapses in an NMDAR-CaMKII-dependent manner. These data connect Rac1 activation to a large body of literature implicating NMDAR-CaMKII in both structural and functional plasticity of dendritic spines.
Figure 10: Rac1 activation during uncaging-evoked sLTP requires NMDAR-CaMKII signaling

a) Effect of inhibition of NMDAR-CaMKII signaling on Rac1 activation during uncaging-evoked sLTP. Pharmacological inhibitors of NMDRs (AP5; 100µM) or CaMKII (CN21; 10µM) were bath applied and incubated for >20-30 minutes prior to beginning the experiment. Summary of data presented in (a). The transient phase was calculated as the average change in binding fraction from 1-2 minutes for each spine. The sustained phase was calculated as the average from 10-20 minutes for each spine. All data plotted as mean ± sem.
3.4 Controlling for the overexpression of Rac1

It is possible that the overexpression of Rac1 could affect the activation profile observed. In order to address this, we first estimated the endogenous expression levels of Rac1 in the hippocampus so as to determine the level of overexpression of the Rac1 sensor. By comparing the level of endogenous Rac1 visualized in a western blot to a standard curve established by several known purified concentrations of His-Rac1, it was estimated that the endogenous concentration of Rac1 in these cells is 872nM (Fig. 11).

We next correlated the various parameters of Rac1 signalling observed with the Rac1 sensor with the overexpression level of mEGFP-Rac1. The overexpression level of mEGFP-Rac1 was estimated by comparing the fluorescence intensity of a large portion of the proximal primary apical dendrite to a standard curve of purified mEGFP fluorescence intensity vs. imaging laser power. Using this approach, we found that most experiments utilized the Rac1 sensor showed expression levels similar to that of the endogenous protein (Fig. 11), suggesting that most experiments were performed in the context of ~2x overexpression of Rac1. Despite the abundance of the protein, however, no correlation was seen between concentration and the degree of Rac1 activation, the basal binding fraction, the change in spine volume, or the spreading of Rac1 signalling.
into the dendrite.

Figure 11: Measurement of effects of overexpression of the Rac1 sensor

a) Estimation of endogenous Rac1 in area CA1 of the hippocampus. Known concentrations of His-tagged Rac1 (purified from *E. coli*) were run on SDS-PAGE along with dissected CA1, and blotted using an anti-Rac1 antibody. These data were used to create a standard curve of anti-Rac1 antibody staining intensity vs. the concentration of Rac1 protein (right). The estimated concentration of Rac1 was found to be 872 nM. b-e) Effect of Rac1 sensor overexpression on various readouts of the sensor activity and structural plasticity. The overexpression level of the Rac1 sensor was estimated by comparing the fluorescence intensity to a standardized curve of known concentrations of GFP (data not shown) at known imaging power. The majority of experiments showed overexpression levels similar to the endogenous
expression level of Rac1, suggesting an approximate 2-fold increase in the levels of Rac1 protein. Overexpression did not have a significant effect on the change in binding fraction/activation of the Rac1 sensor (b), the change in spine volume during sLTP (c), the basal binding of the Rac1 sensor (c), or the amount of activation in the parent dendritic (d).

3.5 **Determining the requirement of Rac1 signalling for sLTP**

While the above experiments clearly illustrate that Rac1 can be activated by an sLTP-inducing stimulus, the role of this signalling in sLTP per se was not yet clear. In order to test whether the observed activation of Rac1 is actually necessary for the induction and/or expression of sLTP, we next utilized both pharmacological and genetic approaches to reduce or eliminate functional Rac1 from cells.

First, we bath applied 120µM of the Rac1-specific inhibitor NSC23766 (Gao, Yuan 2004) to hippocampal slices with mEGFP-expressing CA1 pyramidal cells for at least 30 minutes, then tested the integrity of structural plasticity evoked by 2p glutamate uncaging. In this condition, both the transient (1-2 minutes) and sustained (10+ minutes) of sLTP were significantly impaired (Fig. 12a,c), suggesting that Rac1 signaling is required for the expression of normal sLTP.

Next, in order to ensure both specificity of the requirement of Rac1 as well as specifically target the postsynaptic cell, we utilized Rac1^{f/f} mice and biolistically introduced Cre-recominase alongside mEGFP. In this way, only the transfected cells lack Rac1, and due to the sparseness of transfection of biolistic gene transfer, it can be safely
assumed that only the postsynaptic cell lacks the gene of interest. By comparing the expression of sLTP to Rac1<sup>fl/fl</sup> littermate controls into which no Cre-recombinase was introduced, one can accurately assess the requirement of Rac1 for sLTP in a single cell. In this context, the transient phase of sLTP was drastically reduced, and the sustained phase of sLTP was eliminated (Fig. 12<sup>b,c</sup>), suggesting that the presence of Rac1 protein is necessary for both the induction and expression of sLTP.
Figure 12: Rac1 is required for single-spine sLTP

a) In the presence of 120μM NSC23766 (orange curve), both the transient and sustained phases of structural plasticity are reduced compared to control (black curve). b) When Rac1 is removed from a single cell via the introduction of Cre recombinase into Rac1fl/fl mouse slices, both the transient and sustained phases of sLTP are nearly eliminated. c) Summary of data presented in (a) and (b). All data plotted as mean ± sem.
3.6 Discussion

The experiments described in this section reveal a rapid and sustained activation of the small Rho-family GTPase Rac1 during glutamatergic stimulation of single dendritic spines in area CA1 of the rodent hippocampus. The pharmacological characterization of this activation revealed that it shares many of the hallmark features of the expression of LTP and sLTP: namely, that it depends on calcium through NMDA receptors and subsequent activation of CaMKII. Consistent with this, functional Rac1 signaling was found to be required for the induction and expression of sLTP, suggesting that, like Cdc42 and RhoA, Rac1 transduces transient signals from the NMDAR-CaMKII pathway into persistent changes in the stimulated spine that are relevant for the structural modification and stability of the actin cytoskeleton in that subcellular domain.

Interestingly, the activation of Rac1 was also observed in the neighboring dendrites and spines after the stimulation of only a single dendritic spine. It is unlikely that this signal spreading is an artifact of the sensor, given that the Rac1 sensor is reversible (meaning that activation in nearby regions is not due to ‘overflow’ of permanently activated Rac1 from the stimulated spine) and that its overexpression level did not correlate well with the degree of dendritic activation. Like the spreading of RhoA activity after sLTP induction, the diffusive activation of Rac1 is likely not sufficient for the expression of sLTP, as nearby spines showed no average increase in spine volume. It should be noted, however, that this does completely rule out the
possibility of Rac1 activation being sufficient for sLTP in certain contexts. Since RhoA has been associated with negative regulation of the actin cytoskeleton and structural plasticity, it is possible that the spreading activity of RhoA and Rac1 balance each other out at nearby spines, preventing any change. Their balance in the stimulated spine might be overturned by spine-specific Cdc42 activity, which is also a positive regulator of actin polymerization.

Taken together, this suggests that even the combination of Rac1 and RhoA activity in nearby spines is not sufficient for sLTP expression. Given that RhoA has been suggested to have a role in activity-dependent actin depolymerization in neurons (Sin 2002), it could be that Rac1 and RhoA signaling balance each other out in this context. Tipping this balance in the favor of actin polymerization and spine volume change might involve Cdc42 activation, another actin polymerization signal that is required for sLTP and whose activity is highly compartmentalized in the stimulated spine. The compartmentalization of Cdc42 would thus be sufficient to maintain input specificity of sLTP. Whether Rac1 and RhoA are conveying meaningful signals to nearby spines – or are simply cancelling each other out – is not clear from this data.
4. Upstream control of structural signals: Demonstration of postsynaptic autocrine BDNF release during sLTP

4.1 Introduction

In order to more fully appreciate the implications of the spatiotemporal features of Rac1 activity during sLTP, it is critical to have a full understanding of the upstream mechanisms controlling this activation. In addition to upstream NMDAR-CaMKII signaling, recent evidence has suggesting that Rac1 activation during structural plasticity might depend on the tropomysin-receptor kinase B (TrkB) and its ligand, brain-derived neurotrophic factor (BDNF) (Lai 2012). Consistent with this, many lines of evidence support a role of BDNF-TrkB in the expression of LTP. First, early work demonstrated that the induction of LTP caused increased in the levels of BDNF mRNA in CA1 pyramidal cells (Patterson, SL 1992; Pozzo-Miller 1999). It was then shown that BDNF is actually required for the normal expression of LTP, as mice with a genetic deletion of the BDNF locus show impairments in LTP that can be rescued with exogenous BDNF (Korte 1995; Korte 1996). Importantly, the requirement of BDNF seems to be acute, as these deficits are also observed when an extracellular scavenger of BDNF (TrkB-Ig) is bath applied (Figurov 1996; Chen, G 1999). More recent evidence has shown that BDNF is required for the increase in F-actin-rich spines resulting from theta-burst induction of LTP (Rex 2007), and for sLTP and LTP induced by pairing of postsynaptic depolarization and glutamate uncaging (Tanaka 2008). Taken together, these data suggest that BDNF-TrkB signaling is required for LTP, and that this activity is likely due
to the acute release of BDNF and subsequent binding to TrkB in response to LTP-inducing stimuli.

Despite this, the role of BDNF-TrkB in plasticity has been largely inferential, and no direct measurement of BDNF release nor TrkB activation during LTP has been achieved. Further, the nature of TrkB activation by BDNF, and in particular the cellular source of this BDNF, has not been fully described. Namely, it is not clear whether BDNF is released from the presynaptic terminal, as other neurotransmitters like glutamate, or whether BDNF is released from some other cellular source. Adding to the likelihood of the latter possibility, several studies have identified LTP- or learning-functions for other cellular sources of BDNF, such as those from glial cells (Parkhurst 2013), or the dendrites of postsynaptic cells (Tanaka 2008). The differential localization of BDNF sources suggests that different sources of BDNF might confer distinct functions, and as such, it is critical to investigate the contribution of each source of BDNF for different forms of plasticity.

4.2 Requirement of postsynaptically synthesized BDNF for sLTP

While the presence and potential functions of axonal BDNF have been described, the contribution – and even existence – of other sources of BDNF, most notably postsynaptic BDNF, has remained controversial.
In order to explore the potential contribution of postsynaptic BDNF to LTP, we first targeted single dendritic spines of CA1 hippocampal pyramidal neurons from organotypic slices from mice with a conditional deletion of the BDNF locus (BDNF<sup>fl/fl</sup>) with 2-photon glutamate uncaging. By introducing Cre recombinase and mEGFP into these cells with biolistics, which results in incredibly sparse transfection, we could remove BDNF from only a few cells. In this condition, targeting a labeled CA1 cell that shows no labeled presynaptic counterpart ensures that only the postsynaptic cell is lacking BDNF. When sLTP was induced in the dendritic spines of CA1 pyramidal cells lacking BDNF, we observed a pronounced reduction in both the early and late phases of volume change, suggesting that postsynaptically synthesized BDNF is required for sLTP (Fig. 13).

Importantly, given that several days of incubation were required for a phenotype to emerge, it is possible that the prolonged absence of BDNF from even a single cells is sufficient to compromise the health of the cell. To address this, we applied exogenous BDNF for a short period before the induction of sLTP in a Cre(+) cell. In this condition, sLTP was fully rescued (Fig. 13), suggesting that the effects of BDNF in sLTP are acute, and can be fully restored when BDNF is supplemented. Thus, the deficit of sLTP observed by removal of postsynaptic BDNF are likely not due to long-term changes of the cell, but rather only the absence of functional BDNF at the postsynaptic cell.
Figure 13: Requirement of postsynaptically synthesized BDNF for sLTP

(top) sLTP curves for spines from BDNF<sup>fl/fl</sup> mouse slices in the absence and presence of Cre recombinase in the postsynaptic cell. 20ng/mL exogenous BDNF was bath-applied to rescue Cre-dependent removal of BDNF. (bottom) Summary of data presented above. Transient phase comprises 1-2 minutes post stimulation; sustained phase comprises 10+ minutes post stimulation. All data plotted as mean ± sem.
In order to further characterize the general viability of cells lacking postsynaptic BDNF, we next explored the functional integrity of the synaptic signaling holoenzyme, CaMKII. As CaMKII activity depends primarily on calcium influx through NMDARs and subsequent binding of calcium-bound calmodulin, we hypothesized that the prolonged absence of BDNF would not affect CaMKII signaling in response to glutamate uncaging. We found that CaMKII activation during sLTP is completely preserved in this context (Fig. 14), suggesting that the canonical plasticity machinery – namely, NMDAR-Ca++-CaMKII - is not affected by the prolonged absence of BDNF from a single cell.
Figure 14: CaMKII signaling is unaffected by the prolonged absence of BDNF

(a) Change in fluorescence lifetime of the CaMKII biosensor (Camuiα) during the induction of sLTP in BDNF\textsuperscript{fl/fl} mouse slices in absence (‘Cre Neg’) and presence (‘Cre Pos’) of Cre recombinase. (b) Summary of data shown in (a). (c) Corresponding sLTP curves for CaMKII-expressing BDNF\textsuperscript{fl/fl} cells. (d) Summary of data presented in (c). Transient phase comprises 1-2 minutes post stimulation; sustained phase comprises 10+ minutes post stimulation. Asterisks indicate a statistically significant difference as determined by an independent-samplse t-test. All data plotted as mean ± sem.
Taken together, these data suggest that postsynaptically synthesized BDNF is a critical component of sLTP at single dendritic spines. This effect seems to be acute, suggesting the possibility that BDNF release during glutamate uncaging is the central action of this source of BDNF. How postsynaptic BDNF relays this signal to affect the spine was still unclear.

Recent evidence has indicated that the receptor for BDNF, TrkB, is phosphorylated in response to strong synaptic activity (Helgager 2013) at both the spines and dendrites of CA1 pyramidal cells. Thus, there is precedent for TrkB activation occurring at these sites. If postsynaptic BDNF is released from dendrites and/or spines, then the TrkB expressed at these locales should presumably be activated during sLTP. Thus, we next sought to visualize TrkB activation during sLTP induction.

4.3 Direct visualization of TrkB activation during sLTP

4.3.1 Design and characterization of TrkB FRET-based biosensor

In order to visualize TrkB receptor activation during sLTP (which should presumably occur if postsynaptic BDNF is released) we developed a FRET-based sensor for TrkB to image the spatiotemporal dynamics of its activation during this process. The sensor consists of two components: 1) a donor – full-length TrkB fused to monomeric enhanced green fluorescent protein (TrkB-mEGFP) on its C-terminus and 2) an acceptor – an SH2 domain of the TrkB binding partner phospholipase-C-gamma-1 (PLCγ1)
(Middlemas 1994) with a copy of monomeric red fluorescent protein fused to both its N- and C-termini (mRFP-PLC-mRFP; Fig. 15a). Based on our sensor design, we predicted that BDNF-mediated phosphorylation of Y816 would increase the affinity of mRFP-PLC-mRFP for TrkB-mEGFP (Middlemas 1994), which would in turn would allow FRET to occur between the fluorophores leading to a shortening of mEGFP’s fluorescence lifetime (Fig. 15a). By measuring this change in lifetime with 2pFLIM, we could then determine the change in binding fraction of TrkB-EGFP bound to mRFP-PLC-mRFP (Yasuda, Ryohei, 2006; Harvey, CD 2008; Lee, S-JRJ 2009; Murakoshi 2011) with a higher binding fraction indicating increased TrkB activity. As expected, BDNF application (100 ng/ml) increased the sensor’s binding fraction indicating sensor sensitivity to known TrkB activating stimuli (Fig. 15). Conversely, mutation of a critical phosphorylation site of TrkB (Y816F) (Middlemas 1994) abolished this change, thus demonstrating sensor specificity (Fig. 15b-d). In additional tests in mixed cortical cultures, BDNF application (30 ng/ml) induced a rapid increase of sensor binding fraction that peaked at ~5 minutes and remained elevated for at least 30 minutes (Fig. 16). By contrast, 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 (Fig. 16). These BDNF-induced changes were reversed by application of the TrkB kinase inhibitor K252a (200 nM) (Fig. 16). Taken together, these results validate that our FRET-based TrkB sensor is sensitive to BDNF-
mediated activation, is specific to phosphorylation of tyrosine 816, and is bidirectional in that it can report both increases and decreases of activity.

**Figure 15: Design and characterization the TrkB biosensor**

(a) Schematic of the design of the TrkB FRET-based biosensor. Full-length TrkB was C-terminally tagged with monomeric enhanced GFP to form the donor construct. The acceptor construct was created by the C-terminal SH2 domain of PLCγ1 flanked by monomeric RFP fluorophores. Activated/phosphorylated TrkB binds to the C-SH2 domain of PLC, causing the juxtaposition of mEGFP and mRFP, causing FRET when mEGFP is excited. (b) Representative 2pFLIM images of HeLa cells expressing the TrkB sensor when exposed to exogenous BDNF. The TrkB-816 Tyrosine was mutated to phenylalanine to illustrate phospho-site specificity of the
sensor. Warmer colors represent a shorter lifetime/ more activation. (c) Quantification of BDNF application on TrkB sensor (and mutant) -expressing HeLa cells as compared to vehicle. (d) Summary of data presented in (c). All data plotted as mean ± sem.

**Figure 16: Reversibility and ligand-specificity of TrkB biosensor in neurons**

(a) Representative 2pFLIM images of TrkB sensor activation upon bath application of BDNF (30ng/mL) in cortical neurons. (b) Quantification of experiments presented in (a). The neurotrophin NGF (a ligand for TrkA, but not TrkB) was also used to show ligand-specificity of the TrkB sensor for BDNF. The general kinase inhibitor K252a was applied at t = 30min to reverse TrkB activation. (c) Summary of data presented in (b). All data plotted as mean ± sem.
4.3.2 Imaging TrkB activation during single spine sLTP

Using the newly developed TrkB sensor, we transfected cultured rat hippocampal slices with biolistics and imaged CA1 pyramidal neurons under 2pFLIM. In response to a low-frequency train of two-photon glutamate uncaging pulses (30 pulses at 0.5 Hz) targeted to a single dendritic spine, TrkB was rapidly activated in the spine, peaking at ~1-2 min and then remaining elevated for at least 20 min (Fig. 17,18). In the adjacent dendrite and surrounding spines, TrkB activation developed more slowly and to a lesser extent, but also remained sustained for at least 20 min (Fig. 17, 18).
Figure 17: TrkB activation during single-spine sLTP

Representative 2pFLIM images of activation of the TrkB FRET-based biosensor during induction of sLTP by 2p glutamate uncaging. Arrowhead indicates the target of glutamate uncaging. Warmer colors indicated a shorter fluorescence lifetime/ higher activation. Note that activation is not restricted to, but is highly enriched in, the stimulated spine.
Figure 18: Quantification of TrkB activation during sLTP

a) Representative images of TrkB activation during single-spine sLTP. Arrow indicates targeted point of glutamate uncaging. Note that the activity is not specific to, but is highly enriched in the stimulated spine. b) Quantification of TrkB activation during sLTP, measured across different regions of interest (colored curves). c) Summary of data presented in (b). All data plotted as mean ± sem.
Since single spine sLTP is known to be critically dependent on Ca\(^{2+}\) influx through NMDARs (Matsuzaki 2004; Tanaka 2008; Lee, S-JRJ 2009; Murakoshi 2011) and subsequent downstream CaMKII activation (Matsuzaki 2004; Lee, S-JRJ 2009), we asked whether glutamate-uncaging induced TrkB activation also requires the NMDAR-CaMKII pathway. Application of the NMDAR inhibitor AP5 (100 µM) or the CaMKII inhibitor peptide CN21 (Vest, RS 2007) (10 µM) impaired TrkB activation during both the transient and sustained phases of sLTP while also inhibiting associated spine volume change (Fig. 19). These results indicate that TrkB activation during sLTP lies downstream of both NMDAR and CaMKII activation.
Figure 19: TrkB sensor activation requires NMDAR-CaMKII signaling

(a) Quantification of TrkB sensor activation in control conditions compared to NMDAR (AP5) or CaMKII (CN21) blockade. (b) Summary of data presented in (a). (c) sLTP curves for the experiments presented in (a). (d) Summary of data presented in (c). Transient phase comprises 1-2 minutes post stimulation. Sustained phase comprises 10+ minutes post stimulation. All data plotted as mean ± sem.
4.3.3 The contribution of postsynaptic BDNF to TrkB activation at spines during sLTP

4.3.3.1 Global reduction of extracellular BDNF

To determine whether BDNF mediates the observed activation of the TrkB sensor, we first applied the extracellular BDNF scavenger TrkB-Ig (6-8 µg/ml) and found impaired TrkB activation throughout the time course of sLTP, with a parallel impairment of the associated spine volume change (Fig. 20). These data suggest that extracellular (and therefore, presumably secreted) BDNF contributes to at least a large fraction of the observed TrkB activation during sLTP induction. The source of the remaining TrkB activation is unclear, but could be due to other known BDNF-independent mechanisms, such as transactivation (Huang 2008).
Figure 20: TrkB sensor activation requires extracellular BDNF

(a) Quantification of TrkB sensor activation in control conditions as compared to the presence of the extracellular BDNF scavenger, TrkB1g. Human IgG (HIgG) was used as a control for the BDNF specificity of TrkB1g. (b) Summary of data presented in (a). (c) Corresponding sLTP curves for the experiments presented in (a). (d) Summary of data presented in (c). Transient phase comprises 1-2 minutes post stimulation. Sustained phase comprises 10+ minutes post stimulation.
4.3.3.2 Specific elimination of postsynaptic BDNF

While the above experiments implicate BDNF-mediated signalling as a significant fraction of the total TrkB activation, TrkB-Ig globally reduces BDNF from all sources, and thus does not yield any information regarding the cellular source(s) of BDNF. To better understand the specific cellular source(s) of BDNF that result in TrkB activation during sLTP, we sparsely transfected the TrkB sensor along with Cre-recombinase (Cre) in slices from \textit{BDNF}^{fl/fl} mice. Using this single-cell knockdown approach, we reduced BDNF synthesized in the post-synaptic cell (Lu 2009), and once again found glutamate-uncaging–evoked TrkB activation to be largely inhibited, with a parallel impairment in sLTP (\textbf{Fig. 21}). These results implicate post-synaptically synthesized BDNF as a primary means of activating spine TrkB during single spine structural plasticity.
Figure 21: TrkB sensor activation requires postsynaptic BDNF

(a) Quantification of TrkB sensor activation in BDNF fl/fl cells in the absence (Cre-) and presence (Cre+) of Cre recombinase to remove postsynaptic BDNF. (b) Summary of data presented in (a). (c) Corresponding sLTP curves for the experiments presented in (a). (d) Summary of data presented in (c). Transient phase comprises 1-2 minutes post stimulation. Sustained phase comprises 10+ minutes post stimulation. All data plotted as mean ± sem.
4.4 Direct visualization of BDNF release during sLTP

4.4.1 Design and characterization of BDNF-SEP

The rapid kinetics of glutamate-uncaging induced TrkB activation and its dependence on post-synaptic BDNF suggests equally rapid, post-synaptic BDNF release during the transient phase of sLTP. To directly visualize this event, we used biolistics to transfect CA1 pyramidal cells with full-length BDNF containing SEP on its C-terminus (Dean 2009; Matsuda 2009) (Fig. 22). Previously, BDNF fused to fluorescent proteins in this manner has been demonstrated to be processed and trafficked in a manner similar to endogenous BDNF (Dean 2009; Matsuda 2009). In our experiments, while BDNF-SEP was not visible in spines under basal conditions, BDNF-mRFP puncta were found in spines and dendritic shafts similar to these previous reports (Fig. 22) suggesting that fluorophore-tagged BDNF (like BDNF-SEP) may be a good proxy for endogenous BDNF. Additionally, because of SEP’s pH-sensitivity, its fluorescence is quenched in acidic conditions (such as those present in endosomes), but is de-quenched in neutral-to-basic conditions (such as those present in the extracellular space)(Miesenböck 1998), thus enabling its signal to represent endosome release events.
Figure 22: Design of BDNF-SEP

a) Schematic of BDNF-SEP sensor design. A BDNF construct flanked on the N-terminus by an HA-tag and on the C-terminus by a FLAG-tag was further modified to include super ecliptic pHluorin (SEP). An RFP-containing variant was produced to visualize total fluorescence. b) Model demonstrating the hypothesized increase in SEP fluorescence upon Ca\(^{2+}\)-induced BDNF release. The acidic pH of intracellular vesicles quenches the SEP molecules, preventing them from fluorescing. Externalization of the vesicular cargo then exposes the SEP molecules to the higher pH of the extracellular fluid, increasing the dequenching SEP and causing green fluorescence. c) Localization of total BDNF by RFP-tagged BDNF. d) Quantification of the pH-dependence of the BDNF-SEP signal. When the extracellular pH is lowered, the signal drops, and when it is raised, it is slightly enhanced, consistent with the known pH-dependence of this signal. All data plotted as mean ± sem.
4.4.2 Visualization of BDNF-SEP during sLTP

In response to glutamate uncaging, we observed an increase in SEP fluorescence largely restricted to the stimulated spine (Fig. 23), perhaps correlating with activity-induced release of endogenous BDNF. This fluorescence increase had two distinct kinetic profiles: the first was a transient, spike-like increase time-locked to each uncaging pulse (Fig. 23). The second was a slow increase of fluorescence commencing with the start of the uncaging train and peaking at its end, perhaps due to build-up of released BDNF-SEP and associated spine volume change (Fig. 23).

Several lines of evidence indicated that these fast increases in SEP fluorescence are in fact due to BDNF 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 ACSF of pH 6.5 (Fig. 22). Second, this fluorescence signal also depended on exocytosis, since it was abolished by co-expression with tetanus toxin (TeTX; an inhibitor of VAMP-dependent exocytosis) (Fig. 23). Finally, the signal was dependent on BDNF sorting machinery, since it was inhibited by co-expression of POMC, a peptide that prevents trafficking of BDNF to its activity-dependent release pathway (Lou 2005) (Fig. 23). Together, these results demonstrate that the observed increase in SEP signal reports glutamate-dependent exocytosis and likely corresponds to 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 (AMPARs) with AP5 and NBQX (Fig. 23c,d). Further, we found the increase in SEP fluorescence to be partially inhibited by CaMKII inhibition with CN21 (Fig. 23c, 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 23: BDNF release from single dendritic spines during sLTP

a) Representative images illustrating BDNF-SEP externalization during glutamate uncaging at a single dendritic spine. Image time courses for each row correspond to the uncaging-triggered average of 16 images/glutamate uncaging pulse (imaged at 8Hz) for the indicated epochs. b) Quantification of the change in BDNF-SEP fluorescence intensity during glutamate uncaging. Black bars at top correspond to the timing of each glutamate uncaging pulse. Inset figure corresponds to the change in red fluorescence intensity of an mCherry cell fill, and thus represents the
change in spine volume due to glutamate uncaging. The red intensity values were smoothed with a 16-frame multidimensional image filter. c) Uncaging-triggered average of the response of BDNF-SEP to a glutamate uncaging pulse. TeTx: tetanus toxin, a VAMP inhibitor that eliminates vesicular release; POMC: inhibitor of BDNF trafficking; AP5: Blocker of NDMARs; NBQX: blocker of AMPARs; CN21: inhibitor of CaMKII d) Summary of the peak response of the uncaging-triggered average data shown in (c). All data plotted as mean ± sem.

The data collected with both the TrkB sensor and the BDNF-SEP construct provide converging evidence that autocrine BDNF-TrkB signalling is rapidly activated during single spine structural plasticity.

4.5 Visualization of endogenous BDNF in dendritic spines

Despite the functional evidence presented so far, the only method of visualizing BDNF in dendritic spines relies on the overexpression of the protein, which could affect how the protein is targeted and the degree to which it exists in any subcellular compartment. As such, it is also critical to visualize endogenous BDNF in dendritic spines to show that the native protein actually exists in these structures. This is critically important for the interpretation of the functional data presented above, which might result from cell-wide deficits induced by the absence of BDNF.

In order to address this, transmission electron micrograph sections of HA-BDNF mice, which have an HA tag added to the endogenous locus of BDNF gene to enhance antibody-mediated detection of endogenous BDNF, were prepared using immunogold
labeling of the HA-tag. In this preparation, HA-BDNF immunogold labeling was present in both spines and dendrites of HA-BDNF animals (and absent in wildtype animals) (Fig. 24), suggesting that endogenous BDNF is present in these subcellular compartments.

Figure 24: Verification of the presence of endogenous BDNF in dendrites and spines

Representative transmission electron micrographs of HA-BDNF mouse slices using immunogold labeling of the HA tag. HA-BDNF was identified in axons terminals (a), dendrites (b), and dendritic spines (c). (d) The observed HA-BDNF was primarily identified in neurons. (e) Summary of observations of HA-BDNF in HA-BDNF mice vs. WT mice.
4.6 Discussion

In this section, we have demonstrated that sLTP of single dendritic spines critically depends on the autocrine release of BDNF. The existence of postsynaptic BDNF has been controversial (Tanaka 2008; Dean 2009; Matsuda 2009; Jakawich 2010; Dieni 2012), but the single-cell knockout approach clearly demonstrates that spine structural plasticity requires BDNF that is synthesized in the postsynaptic cell. Furthermore, the BDNF-SEP construct reveals that glutamate uncaging can induce postsynaptic BDNF release with a rapid kinetic profile and high fidelity with uncaging pulses. Finally, we used electron microscopy to unambiguously demonstrate that endogenous BDNF can be found in both the dendrites and spines of CA1 pyramidal cells.

Additionally, we have developed a novel FRET-based sensor in order to image activation of a receptor tyrosine kinase – TrkB – in a living neuron in real time. This sensor revealed a rapid and prolonged activation of TrkB in a single dendritic spine undergoing sLTP that depends on NMDAR-CaMKII signalling. The fast onset of its activation together with its persistence sheds light on the requirement of TrkB for both the transient and sustained phases of sLTP, as shown here, as well as for both early and late LTP, as shown previously (Figurov 1996; Korte 1996; Patterson, SL 1996; Kang 1997; Korte 1997; Minichiello, L., 1999; Xu, B 2000; Kovalchuk 2002; Bekinschtein 2007; Minichiello, Liliana, 2009). Additionally, the dependence of TrkB activation on CaMKII...
raises two potentially important implications for CaMKII signalling within sLTP. First, persistent TrkB activation may provide a mechanism for relaying transient CaMKII activation (Lee, S-JRJ 2009) to signalling that lasts more than ~20 min, like small GTPase proteins (Murakoshi 2011) (Fig. 25). Second, since TrkB activation can initiate multiple signalling pathways in parallel, its activation during sLTP may serve as a source of signal divergence, thereby expanding the complexity of the signalling network initiated by CaMKII.

Finally, we illustrate that the activation of TrkB at a single spine depends, at least in part, on postsynaptic sources of BDNF, suggesting autocrine activation of the TrkB receptor during sLTP. In sum, we have described a novel autocrine signalling system within a single spine achieved by rapid release of BDNF from the stimulated spine and subsequent activation of TrkB on the same spine during single spine structural plasticity.
While CaMKII signaling converts millisecond Ca2+ influx into a signal lasting ~1-2 minutes, TrkB/GTPase signaling can transduce the CaMKII signal into one lasting >30 minutes. The combination of the temporal profiles of these signals thus fully overlaps with the expression of structural plasticity (‘Volume’; black curve).

Figure 25: Temporal model of the molecular components of sLTP
5. Dependence of Rho GTPases on postsynaptic, autocrine BDNF

5.1 Introduction

Given the requirement of the Rho GTPases for spine structural plasticity, it is likely that BDNF-TrkB signalling converges on these proteins to produce its effect on sLTP. Consistent with this possibility, several lines of evidence indicate that BDNF and/or TrkB can activate Rac1 or otherwise exert their signalling effects through this pathway (Lai 2012; De Rubeis 2013). As such, the observed effects of BDNF-TrkB signalling on sLTP described above is likely ultimately a result of control over the actin cytoskeleton by the Rho GTPases.

5.2 The requirement of BDNF-TrkB for the Rho GTPases

5.2.1 Rac1 and Cdc42 activation during sLTP requires extracellular BDNF

To probe the requirement of BDNF signalling in regulating the Rho GTPases, we first monitored the activity of Rac1, Cdc42, and RhoA during sLTP in the presence of the extracellular BDNF scavenger, TrkB-Ig (2 µg/mL). Interestingly, the activities of both Rac1 and Cdc42, but not RhoA, required extracellular BDNF, as their activities were significantly reduced in the presence of TrkB-Ig (Fig. 26-28). This suggests that BDNF-TrkB’s role in sLTP is via control of positive regulation of the actin cytoskeleton through Rac1 and Cdc42 specifically.
Figure 26: Extracellular scavenging of BDNF attenuates uncaging-evoked Rac1 activation

(a) Change in binding fraction (left) of the Rac1 sensor, and corresponding spine volume change, in the absence (black) and presence (blue/red) of the extracellular BDNF scavenger TrkB-Ig during sLTP. (b) Summary of data presented in (a). Asterisks represent a statistically significant difference as determined by an independent-samples t-test for the average of the indicated time windows. All data plotted as mean ± sem.
Figure 27: Extracellular scavenging of BDNF attenuates uncaging-evoked Cdc42 activation.

(a) Change in binding fraction (left) of the Cdc42 sensor, and corresponding spine volume change, in the absence (black) and presence (blue/red) of the extracellular BDNF scavenger TrkB-Ig during sLTP. (b) Summary of data presented in (a). Asterisks represent a statistically significant difference as determined by an independent-samples t-test for the average of the indicated time windows. All data plotted as mean ± sem.
Figure 28: Extracellular scavenging of BDNF does not significantly affect uncaging-evoked RhoA activation

(a) Change in binding fraction (left) of the RhoA sensor, and corresponding spine volume change, in the absence (black) and presence (blue/red) of the extracellular BDNF scavenger TrkB-Ig during sLTP. (b) Summary of data presented in (a). Asterisks represent a statistically significant difference as determined by an independent-samples t-test for the average of the indicated time windows. All data plotted as mean ± sem.
While the above data clearly delineates a requirement of BDNF-TrkB in Rac1 and Cdc42 activation, the cellular source of this BDNF is not clear. In order to address this, we next employed a single-cell knockout technique (Lu 2009) using hippocampal organotypic slices prepared from BDNF conditional knockout mice (BDNF<sup>fl/fl</sup>) coupled with biolistic transfection of Cre recombinase alongside a Rho GTPase sensor. Consistent with the findings detailed above, we found that removal of postsynaptically synthesized BDNF significantly reduced uncaging-evoked sLTP (Fig. 29-31). Notably, we also found that this condition significantly attenuated Rac1 and Cdc42 activation during sLTP (Fig. 29, 30) without affecting RhoA (Fig 31). This suggests that Rac1 and Cdc42 – but not RhoA – require postsynaptic, autocrine BDNF signalling for appropriate activation during sLTP.

Interestingly, these results also suggest that autocrine BDNF-TrkB signalling instructs two distinct spatial signalling domains: a spine-specific domain consisting of BDNF-TrkB-Cdc42, and a more diffuse domain consisting of BDNF-TrkB-Rac1. Since BDNF-TrkB signalling is largely restricted to stimulated spines, it may be that diffusion of Rac1 or of Rac1 activators downstream of TrkB (Miyamoto 2006; Lai 2012) causes the observed spreading of Rac1 activity.
Figure 29: Requirement of postsynaptic BDNF for Rac1 activation during sLTP

(a) Change in binding fraction of the Rac1 sensor in BDNF\textsuperscript{fl/fl} cells in the absence and presence of Cre recombinase to remove postsynaptic BDNF. (b) Summary of the data presented in (a). Bar graphs represent the average change in binding fraction of the respective sensor 1-2 minutes post stimulation. (c) Corresponding sLTP curves for the experiments presented in (a). (d) Summary of the data presented in (c). Bar graphs correspond to the average sustained change in spine volume (10+ minutes post stimulation). All data plotted as mean ± sem.
Figure 30: Requirement of postsynaptic BDNF for Cdc42 activation during sLTP

(a) Change in binding fraction of the Cdc42 sensor in BDNFfl/fl cells in the absence and presence of Cre recombinase to remove postsynaptic BDNF. (b) Summary of the data presented in (a). Bar graphs represent the average change in binding fraction of the respective sensor 1-2 minutes post stimulation. (c) Corresponding sLTP curves for the experiments presented in (a). (d) Summary of the data presented in (c). Bar graphs correspond to the average sustained change in spine volume (10+ minutes post stimulation). All data plotted as mean ± sem.
Figure 31: Requirement of postsynaptic BDNF for RhoA activation during sLTP

(a) Change in binding fraction of the RhoA sensor in BDNFfl/fl cells in the absence and presence of Cre recombinase to remove postsynaptic BDNF. (b) Summary of the data presented in (a). Bar graphs represent the average change in binding fraction of the respective sensor 1-2 minutes post stimulation. (c) Corresponding sLTP curves for the experiments presented in (a). (d) Summary of the data presented in (c). Bar graphs correspond to the average sustained change in spine volume (10+ minutes post stimulation). All data plotted as mean ± sem.
5.3 Dependence of Rho GTPases on postsynaptic TrkB

In previous sections, we demonstrated that postsynaptically synthesized BDNF functions, at least in part, via activation of TrkB on the postsynaptic dendritic spine. Nonetheless, the contribution of this source of BDNF to Rho GTPase activation could be due to activation of TrkB on other cellular sources, which might modify release of other factors that influence the GTPases (e.g. activation of presynaptic TrkB that induces or increases glutamate release). Thus, we performed a similar single-cell knockout approach to target postsynaptic TrkB and measured the effect of this perturbation on the activation of Rac1, RhoA, and Cdc42.

Similar to postsynaptic BDNF removal, we found that removal of postsynaptic TrkB mitigated the activation of Rac1 (Fig. 32) and Cdc42 (Fig. 33), but not RhoA (Fig. 34). It should be noted, however, that we failed to observe a spine volume phenotype when overexpressing RhoA, thereby limiting our power of interpretation on the dependence of RhoA on postsynaptic TrkB. Overall, however, these data suggest that the effect of postsynaptic BDNF on sLTP and the Rho GTPases is by autocrine activation of its receptor, TrkB, on the postsynaptic cell. Importantly, this also suggests that RhoA is completely independent of BDNF in its activation during sLTP.
Figure 32: Dependence of uncaging-evoked Rac1 activation on postsynaptic TrkB

(a) Change in binding fraction (left) of the Rac1 sensor, and corresponding spine volume change, in the absence (black) and presence (green/blue) of Cre recombinase in TrkB^{fl/fl} mouse slices. (b) Summary of data presented in (a). Asterisks represent a statistically significant difference as determined by an independent-samples t-test for the average of the indicated time windows. All data plotted as mean ± sem.
Figure 33: Dependence of uncaging-evoked Cdc42 activation on postsynaptic TrkB

(a) Change in binding fraction (left) of the Cdc42 sensor, and corresponding spine volume change, in the absence (black) and presence (green/blue) of Cre recombinase in TrkB<sup>fl/fl</sup> mouse slices. (b) Summary of data presented in (a). Asterisks represent a statistically significant difference as determined by an independent-samples t-test for the average of the indicated time windows. All data plotted as mean ± sem.
Figure 34: Dependence of uncaging-evoked Rac1 activation on postsynaptic TrkB

(a) Change in binding fraction (left) of the RhoA sensor, and corresponding spine volume change, in the absence (black) and presence (green/blue) of Cre recombinase in TrkB<sup>-/-</sup> mouse slices. (b) Summary of data presented in (a). All data plotted as mean ± sem.
5.4 BDNF is instructive of GTPase activation

The previous sections clearly illustrate that postsynaptically synthesized BDNF is necessary for the activation of the Rho family GTPases, Rac1 and Cdc42. However, the relationship between BDNF and subsequent activation of the GTPases is not clear from this data. More specifically, it was still not clear whether BDNF directly activates the GTPases, or if BDNF serves as a permissive signal for their activation (e.g. through disinhibition).

In order to test whether the Rho GTPases are directly activated by BDNF, we bath applied 20ng/mL of exogenous BDNF to cells expression the Rac1, Cdc42, or RhoA sensors, then imaged for 20 minutes. In this context, both Rac1 and Cdc42 were modestly but significantly activated by BDNF alone, while RhoA showed no activation (and in fact, deviated slightly below baseline) (Fig. 35).

These data suggest that BDNF can directly activate Rac1 and Cdc42, leaving this as a possible mechanism by which release of postsynaptically synthesized BDNF gives rise to activation of these GTPases.
Figure 35: BDNF is sufficient to activate Rac1 and Cdc42, but not RhoA

Rac1 (blue), Cdc42 (orange), and RhoA (green)-expressing neurons in rat slices were exposed to 20ng/mL BDNF at t = 0 minutes. Rac1 and Cdc42 were activated by this stimulus, whereas RhoA was not (and possibly exhibited some inactivation). All data plotted as mean ± sem.
Importantly, application of exogenous BDNF was not sufficient to cause significant changes in spine volume (Fig. 36), suggesting that this stimulus alone is not sufficient for sLTP. Furthermore, this suggests that activation of only a subset of the Rho GTPases is also not sufficient for sLTP. Since all three of these GTPases are activated during uncaging-evoked sLTP, and since all three are necessary for sLTP, it is possible that only the simultaneous activation of all three is sufficient for lasting spine volume changes.
Figure 36: BDNF application alone is not sufficient to induce spine volume changes

Application of 20ng/mL exogenous BDNF caused no significant changes in volume of spines in Rac1 (blue), Cdc42 (orange), or RhoA (green) sensor-expressing neurons.
5.5 Discussion

In this section, we have demonstrated that postsynaptic BDNF exerts control over the Rho GTPases, Rac1 and Cdc42, highlighting a potential molecular substrate through which BDNF-TrkB controls sLTP. Importantly, RhoA showed complete independence from postsynaptic BDNF, suggesting that a portion of the actin-modification pathway is maintained through other pathways during sLTP. The potential function of this signal divergence is unclear, but given the proposed role of RhoA in the negative regulation of actin (Sin 2002), it is reasonable that the positive and negative regulation of the cytoskeleton would be controlled by separate signals. Nonetheless, since all three of the Rho GTPases require NMDAR-Ca$^{2+}$-CaMKII, and are necessary for the full expression of sLTP, it is clear that they share many of the same induction mechanisms and functional consequences, and thus are primed to be simultaneously recruited in a variety of contexts. Still, the separation of Rac1 and Cdc42 from RhoA in terms of BDNF dependence indicates that the system maintains the ability to segregate these signals, if necessary. The potential functional advantage of this ability remains unclear.

Interestingly, the control that postsynaptic BDNF exerts over Cdc42 and Rac1 also suggests that its release conveys two different spatial profiles of signals: a synapse-specific domain involving TrkB-Cdc42, and a heterosynaptic domain involving TrkB-Rac1. As such, the spatial domain of influence exerted by BDNF release from spines
might be significantly larger than spine-specific sLTP would suggest.
6. Heterosynaptic GTPase signalling and heterosynaptic metaplasticity

6.1 Introduction

While the volumetric changes resulting from uncaging-evoked sLTP induction are restricted to the stimulated spine, both Rac1 and RhoA signals nonetheless spread into nearby spines during this phenomenon. This activity is pronounced and, in the case of Rac1, is sustained for long periods of time. As Rac1 and RhoA are canonically signals of actin modification, yet the stability of surrounding structures is maintained, what is the role of such diffusive signals during sLTP? And further, how is the synapse-specificity of sLTP maintained when actin-modification signals are diffuse?

Recent evidence has revealed that, while the manifestation of sLTP in terms of actin polymerization and spine enlargement are indeed spine-specific, the specificity of sLTP belies a truly complex system of heterosynaptic communication that occurs as a result of glutamatergic stimulation. Most notably, the induction of LTP at a single spine has been shown to confer threshold-modifying signals to surrounding spines, increasing the likelihood that those synapses will undergo sLTP themselves. This process, referred to as synaptic crosstalk (Harvey, CD 2007) is a form of heterosynaptic metaplasticity, or threshold modification conferred between synapses that is restricted to within ~5-10um on the parent dendrite of the stimulated spine. Further, this effect decays after ~5 minutes. While similar processes have been described that require protein synthesis, and thus serve as a form of synaptic tagging (Govindarajan 2011), synaptic crosstalk is...
independent of protein synthesis, and thus likely requires more immediate signalling effects from nearby spines.

Consistent with this possibility, it was subsequently shown that synaptic crosstalk requires intact Ras-MEK signalling, a signalling system also shown to have a diffusive activity pattern after the induction of single-spine sLTP (Harvey, C 2008). What relevant signaling features might be conferred by Ras-MEK activation in nearby spines? Much of the classical understanding of MAPK signaling (like MEK) involves an endpoint at modification of gene transcription. Given that crosstalk is independent of protein synthesis, however, the mechanisms by which Ras-MEK signalling modified the threshold of surrounding spines is unclear. One possibility is that extrasynaptic delivery of AMPARs after induction of sLTP, which is also Ras-MEK-dependent (Patterson, M 2010), confers this metaplasticity. This possibility is further supported by the finding that AMPAR insertion is not entirely restricted to the stimulated spine. It is important to note, however, that single-spine sLTP does not increase the glutamate-sensitive current in surrounding spines (Matsuzaki 2004), suggesting that these receptors are not delivered to the synaptic surface at nearby spines, though might be primed to do so. Furthermore, sLTP is largely independent of AMPAR current, and thus the modification of sLTP threshold at nearby spines is likely not explained by this mechanism alone (though increased AMPAR current might provide additional depolarization, helping to unblock NMDARs).
6.2 Synaptic crosstalk requires BDNF-TrkB-Rac1 signalling

6.2.1 Induction of synaptic crosstalk

Notably, the length scale of BDNF-TrkB-mediated Rac1 spreading is similar to that of synaptic crosstalk. Thus, we next addressed whether BDNF-TrkB-Rac1 signalling contributes to this heterosynaptic phenomenon.

First, we confirmed that we could induce synaptic crosstalk in hippocampal CA1 neurons. Consistent with previous studies (Harvey, C 2007; Harvey, C 2008), we found that the induction of sLTP at a single spine allowed a subsequent subthreshold stimulus – normally incapable of causing sLTP (~1/4 – 1/6 in strength) – to reliably induce normal sLTP in a nearby (~5 µm) spine (Fig. 36). The sustained phase of crosstalk was comparable to the sustained phase of normal sLTP, suggesting common core mechanisms between the two phenomena. Notably, however, the transient phase of crosstalk, while larger than the subthreshold stimulus alone (suggesting heterosynaptic potentiation of this phase), was significantly smaller than the stimulated spine, suggesting the signalling is not likely not identical between these two processes.
Figure 37: Synaptic crosstalk

(Top left) Schematic of experimental approach. In the unpaired condition, a single spine on a single dendrite is targeted with a weak (1ms dwell) uncaging stimulus (‘sub’). In the paired condition, the subthreshold stimulus is preceded by a strong, LTP-inducing stimulus at a nearby spine on the same dendrite. (Top right) Representative images of both unpaired (top row) and paired (bottom row) paradigms. (Bottom) Quantification of spine volume change during both the unpaired (left) and paired (right) conditions. An unpaired LTP spine (without an accompanying subthreshold stimulus) is included on the left graph for comparison. All data plotted as mean ± sem.
6.2.2 Synaptic crosstalk requires BDNF-TrkB-Rac1 signaling

While strong inhibition of BDNF signalling has been shown to significantly affect sLTP expression, we found that partial inhibition of this pathway with a low concentration (0.25 µg/mL) of TrkB-Ig preserved sLTP in response to the first stimulus, but abolished sLTP in response to the second, subthreshold stimulus, suggesting that BDNF is required for synaptic crosstalk (Fig. 38). To ensure that the effects of TrkB-Ig were due to BDNF signalling through TrkB, we utilized mice containing a point mutation in the TrkB kinase domain (F616A) (Chen, X 2005). This mutation renders the mutant TrkB uniquely susceptible to inhibition by the small molecule 1NMPP1. Using a similar strategy of a low concentration (0.125 µM) of 1NMPP1, crosstalk was selectively abolished without affecting sLTP in the first spine (Fig. 38). Likewise, while a large concentration of the Rac1 inhibitor NSC-23766 significantly reduced sLTP (Fig. 12), a small concentration (15 µM) inhibited facilitation of the second spine without significantly affecting sLTP in the first spine (Fig. 38).
Figure 38: Synaptic crosstalk requires BDNF-TrkB-Rac1 signalling

Effect of application of a low concentration of (a) NSC-23766 (120µM), (b) TrkB-Ig (0.25mg/mL), and 1NMPP1 (0.25mg/mL in TrkB616A mice) during synaptic crosstalk. (c) Summary of data presented in a-d in comparison to normal crosstalk controls (see Fig. 29). All data plotted as mean ± sem.
In order to begin to test the hypothesis that TrkB-Rac1 signal spreading during sLTP is required for synaptic crosstalk, when next measured the effects of these low-concentration pharmacological perturbations on Rac1 signal spreading. Consistent with our hypothesis, we found that both TrkB-Ig and 1NMPP1 were capable of reducing Rac1 signal spreading. However, we found no significant changes when using the Rac1 inhibitor NSC-23766 (Fig. 39). One potential explanation for this is that this drug blocks the Rac1-GEF interface, and as such, could be heavily affected by the overexpression of Rac1.

It should also be noted that these pharmacological perturbations trended towards inhibiting Rac1 activity in the spine, suggesting that, while the dendritic activity is more sensitive to these perturbations, this approach is not specific to dendritic activity, thus complicating the interpretation of these data.
Figure 39: Effect of weak pharmacological inhibition of BDNF-TrkB-Rac1 signaling on Rac1 signal spreading

(a). Effects of 0.25mg/mL TrkB-Ig on the spatial profile of Rac1 activation during sLTP (black = ctrl dendrite; green = TrkB-Ig dendrite). Data is presented as the binding fraction of Rac1 in the parent dendrite of a stimulated spine as a function of distance from that spine. Stimulated spine data is plotted on the y-axis (red = ctrl spine; blue = TrkB-Ig spine). (b) Summary of the effects of 0.25mg/mL TrkB-Ig on the stimulated spine (ctrl: red; TrkB-Ig: blue) and the dendrite (within 5µm of the stimulated spine; black = ctrl; green = TrkB-Ig) during the first two minutes after stimulation. Asterisks indicate statistical significance as determined by an independent-samples t-test. (c-f) Same as (a-b), but for 0.125µM 1NMPP1 (c-d) and 15µM NSC-23766 (e-f), respectively. All data plotted as mean ± sem.
6.2.3 BDNF is sufficient to modify the threshold of sLTP

The requirement of BDNF for the induction of synaptic crosstalk suggest that BDNF initiates a signalling cascade capable of lowering the threshold of sLTP at affected areas. This notion is consistent with previous reports implicating BDNF in altering the threshold of LTP induction in the visual cortex (Huber 1998). Indeed, when BDNF (20 ng/mL) was included in the perfusion for ~10-15 min, a weak stimulus alone was sufficient to induce sLTP (Fig. 40), suggesting that BDNF is sufficient to modify the threshold for structural plasticity. Taken together, these results suggest that the BDNF-TrkB–mediated activation of Rac1 facilitates sLTP in nearby spines after sLTP induction, allowing synaptic crosstalk.

These data, taken together with the observation that BDNF alone is not sufficient for sLTP (Fig. 36), suggests that the effects of BDNF can be described largely as a priming mechanism, preparing the system for structural plasticity without itself inducing it. In this sense, BDNF and crosstalk subserve sLTP in similar ways. An intriguing possibility is that both BDNF and crosstalk modify the threshold for sLTP through a similar mechanism: namely, the partial activation of Rho GTPase signaling.
Figure 40: BDNF is sufficient to modify the threshold of sLTP

(left) Change in spine volume in response to a subthreshold stimulus (‘Sub’) in the absence and presence of exogenous, bath-applied BDNF. (right) Summary of data presented at left, with the addition of a crosstalk (‘Paired’) spine for comparison of the effects of BDNF application and crosstalk induction. All data plotted as mean ± sem.

6.3 Preventing the spreading of Rac1 signaling: dendritic inhibition of Rac1 signaling

6.3.1 Design of a dendrite-specific inhibitor

If indeed the spreading of Rac1 activation is the critical feature to conferring the effects of synaptic crosstalk, then restricting this spreading should be sufficient to prevent crosstalk from occurring. This assumption led us to devise a genetic tool to prevent the activation of Rac1 outside of the stimulated spine. In order to achieve this, it
would be necessary to target an inhibitor with subcellular specificity, such that dendritic spines were free from the inhibitory influence, but dendritic segments were subject to it. To this end, we reasoned that molecular targeting of an inhibitory peptide to microtubules, which are enriched in dendrites and largely excluded from dendritic spines (Korobova 2009), might be able to halt the activity spread of Rac1 after it leaves the spine. To do this, we fused the Rac1 inhibitory peptide W56 (Gao, Y., 2001) to the N-terminus of an mCherry fluorophore, which was then C-terminally fused to the microtubule binding domain of human MAP2c (MTBD; Fig. 40)(Zhong 2009). The primary domains were then connected by glycine-rich linkers (see Methods).
Figure 41: Design of dendritic Rac1 inhibitor construct

(a) Schematic of dendritic inhibitor design. (b) Representative images of the expression pattern of the dendritic inhibitor in HEK293T cells (top) and CA1 pyramidal neurons (bottom two rows). In HEK293T cells, W56-mCh-MTBD forms thick, filamentous structures. In neurons, localization is largely restricted to the dendrites, and absent in spines.
6.3.2 Confirming microtubule localization of the dendritic inhibitor construct

Expression of the W56-mCherry-MTBD construct in HEK293T cells yielded enrichment of W56-mCh-MTBD on thick, filamentous structures throughout the cell (Fig. 40), a pattern consistent with localization to microtubules. Since microtubules are enriched in the dendrites (Korobova 2009; Zhong 2009) and largely excluded from spines, this construct would presumably allow the effect of the Rac1 inhibitory peptide to be exerted preferentially in the dendrite. Consistent with the localization of microtubules in dendrites, W56-mCherry-MTBD was accumulated in the dendritic shaft (Fig. 40), and excluded from dendritic spines.

6.3.3 Subcellular inhibition of Rac1 signaling

In order to test whether this construct yielded any subcellular specificity of Rac1 inhibition, we measured the activation of the Rac1 sensor during glutamate uncaging in the presence of W56-mCherry-MTBD and a scrambled-peptide version of the construct, scr-MTBD. Consistent with our hypothesis, W56-mCherry-MTBD expression largely preserved Rac1 activation in the stimulated spine, while significantly reduced its activation in the dendrite (S.I. = 0.20) (Fig 41). In contrast, W56-mCherry without MTBD resulted in a more uniform reduction in Rac1 activation (Fig. 42).

These data suggest that dendritic localization of a Rac1 inhibitor (W56) is sufficient to prevent the activity spread of Rac1 activation after sLTP induction.
Importantly, this offers some mechanistic insight into the nature of Rac1 activity diffusion: since the W56 peptide mimics a portion of Rac1 that binds to Rac1 GEFs, it effectively acts as a dominant negative peptide that prevents activation (as opposed to causing inactivation), suggesting that Rac1 signal spreading requires Rac1 activation in the dendrite. In other words, diffusion of the molecules that are initially activated in the spine is likely insufficient to explain the subsequent signal diffusion. As such, either de novo activation by an upstream source or positive feedback from activated Rac1 acting through Rac1 GEFs is likely a necessary component of this signal spreading.
c) Representative images of Rac1 activation in the presence of scr-mCh-MTBD (top) and W56-mCh-MTBD (bottom). Note that the scrambled variant of the dendritic inhibitor allows invasion of Rac1 activity into nearby spines, while W56-mCh-MTBD
largely prevents this. d) (left) Quantification of the conditions described in (c). Rac1 activity is plotted as a function of distance from the stimulated spine. The Rac1 activation in the stimulated spines is plotted on the y-axis. (right) Summary of the spreading indices of Rac1 in the conditions described in (c). All data plotted as mean ± sem.

In order to confirm that the effects of W56-mCh-MTBD on Rac1 signal spreading were conferred by localization to microtubules, we next compared the activation of Rac1 in both the presence of W56-mCh-MTBD and W56-mCh lacking MTBD. This construct should be soluble and freely diffusible, and as such, should confer a more global inhibitory effect on Rac1. Consistent with this idea, we found that expression of W56-mCh caused a significant reduction in Rac1 activation in the stimulated spine (Fig. 43) as well as the dendrite (Fig. 44). Thus, dendritic localization of W56 via MTBD is a critical component of selectively preventing Rac1 activation in the dendrite.
Figure 43: Spine activation of Rac1 is preserved in the presence of microtubule-restricted W56-MTBD, but not soluble W56-mCh.

Rac1 activation in the presence of W56-mCh-MTBD (listed here as W56-MTBD) is normal in dendritic spines, as compared to Rac1 sensor controls (black curve). W56-mCh that is not localized to microtubules, on the other hand, significantly reduces Rac1 activation in spines.
Figure 44: Comparison of the effect of microtubule-targeted vs. non-targeted inhibitor expression on Rac1 signal spreading

Microtubule-targeted (MTBD) expression preferentially reduces Rac1 activation in the dendrite, while non-targeted/soluble W56 (W56-mCh; right) has a similar effect on both the spine and dendritic activity of the Rac1 sensor. All data plotted as mean ± sem.

6.3.4 Testing the target specificity of the dendritic Rac1 inhibitor

Although W56 essentially comprising a small portion of the Rac1 protein that should specifically bind to Rac1-specific binding partners, there is significant sequence homology between Rho GTPases, and as such, it is possible that this peptide could interfere with the signaling of other GTPase proteins. In order to test the specificity of W56-mCh-MTBD for Rac1, we co expressed this construct along with RhoA, a Rho
family GTPase that shares many signaling features with Rac1, including its rapid activation and signaling spreading during sLTP. Because of the overlap of Rac1 and RhoA signaling in the dendrite, it is possible that W56-mCh-MTBD could interfere with both of these signaling cascades. Nonetheless, we found that co-expression of W56-mCh-MTBD with the RhoA sensor caused no significant change in RhoA activation in the spine or dendrite (Fig. 45), suggesting that this manipulation is relatively selective for Rac1.

Figure 45: Expression of W56-mCh-MTBD does not affect RhoA signaling

Co-expression of the dendritic Rac1 inhibitor W56-mCh-MTBD had no significant effect on RhoA sensor activity (as measured by the RhoA sensor). While there was a trend towards an increase, this was not a significant change.
6.3.5 Dendritic inhibition of Rac1 selectively blocks synaptic crosstalk

We next asked whether reducing the signal spreading of Rac1 during sLTP induction was sufficient to block synaptic crosstalk. Consistent with this hypothesis, expression of W56-mCherry-MTBD significantly reduced sLTP of the crosstalk spine without significantly affecting sLTP at the first (compared to a scrambled peptide control) during synaptic crosstalk (Fig. 46). Thus, extra-spineous activation of Rac1, which depends on BDNF-TrkB signalling, is necessary for synaptic crosstalk.
Figure 46: Dendritic inhibition of Rac1 selectively blocks crosstalk

Expression of W56-mCherry-MTBD (W56-MTBD) prevents the expression of crosstalk, but does not affect sLTP. The expression of a scrambled version of W56 (scr) tethered to microtubules allows the expression of both sLTP and crosstalk. All data plotted as mean ± sem.
6.3.6 An alternate strategy for dendritic inhibition: GAP-MTBD

In order to provide additional evidence that the effect of the dendritic Rac1 inhibitor described above is specific to Rac1 signaling, we next devised an alternate dendritic inhibitor construct that worked through a separate mechanism. Whereas W56 acts as a dominant negative and prevents activation, GTPase accelerating proteins (GAPs) function by assisting the small GTPase in GTP hydrolysis, moving it from an active to an inactive state more quickly. We thus replaced W56 with a Rac1 GAP, ARHGAP15, in the dendritic inhibitor construct. This construct would thus rapidly inactivate Rac1 specifically in the dendrite.

Similar to W56, the expression of GAP-mCherry-MTBD resulted in a reduction of Rac1 activation in the dendrite, without showing a significant effect in the stimulated spine. (Fig. 47). Additionally, the presence of GAP-mCherry-MTBD also prevented the expression of synaptic crosstalk without affecting the expression of sLTP in the stimulated spine (Fig. 48).

Thus, either the prevention of activation of Rac1 (through W56) or the inactivation of Rac1 (through ARHGAP15) in the dendrite also prevents the expression of synaptic crosstalk.
Figure 47: An alternative strategy for dendritic inhibition of Rac1 signaling recapitulates the effects of W56-MTBD.

(a) Schematic for the design of an alternate dendritic Rac1 inhibitor. Full-length ARHGAP16 (GAP) replaced W56 in the general dendritic inhibitor construct. 

(b) Representative 2pFLIM images of Rac1 activation in the presence of GAP-mCherry-MTBD. 

(c) Quantification of Rac1 signal spreading in the presence of GAP-mCherry-MTBD plotted as a function of distance from the stimulated spine. Spine data plotted on the y-axis. 

(d) Summary of the data presented in (c). Data from the scr-MTBD construct is included for comparison. All data plotted as mean ± sem.
Figure 48: Effect of expression of GAP-MTBD on synaptic crosstalk

(e) Effect of the expression of GAP-mCherry-MTBD on synaptic crosstalk. (f) Summary of the data presented in (e) compared to crosstalk from GFP and scr-MTBD controls. All data plotted as mean ± sem.
6.4 Extending crosstalk to other diffusive signals: dendritic inhibition of RhoA

Since RhoA activation also spreads into nearby spines after sLTP induction, we next tested whether this BDNF-independent signal is also required for synaptic crosstalk. To do this, we employed a similar strategy as that described above for Rac1: a dominant negative version of RhoA was tethered to MTBD, presumably preventing its extra-spineous activation. Like W56-mCherry-MTBD, DNRhoA-mCherry-MTBD significantly reduced RhoA activation in the dendrite without affecting its activation in the spine during sLTP (Fig. 49). It should be noted, however, that a significant portion of RhoA activation in the dendrite was still apparent even under dendritic inhibition. Since the RhoA dendritic inhibitor construct works by prevent activation of RhoA, this would suggest that the remaining portion of RhoA activity in the dendrite in this context is due to spreading of the RhoA molecules that were initially activated in the spine, then diffuse out into the dendrite. This is in contrast to the Rac1 dendritic inhibitor, which left very little residual activity in the dendrite. This might suggest that the mechanisms by which RhoA and Rac1 activity diffuse into the dendrite are distinct. This data is supported by the differential time courses of spreading of the two GTPases.
Figure 49: Dendritic inhibition of RhoA signaling
(a) Schematic of the design of dendritic inhibitor constructs for other GTPases. Full-length, dominant negative (DN) GTpases replace W56 in the general dendritic inhibitor design. (b) Representative 2pFLIM images of RhoA activation in the absence (top) and presence (bottom) of the dendritic RhoA inhibitor, DNRhoA-mCh-MTBD. Warmer colors represent a shorter lifetime/more activation. (c) Quantification of the spatial spreading of RhoA activation in the presence of a scr-MTBD control (left) vs. the dendritic RhoA inhibitor. (d) Summary of the spreading data presented in (c). Binding fraction data is binned into distinct spatial bins to illustrate region of maximal effect. Asterisk represents a statistically significant difference as determined by an independent-samples t-test. All data plotted as mean ± sem.

Consistent with the hypothesis that the signal spreading of RhoA after sLTP is necessary for synaptic crosstalk, DNRhoA-mCherry-MTBD blocked crosstalk without significantly affecting sLTP, similar to the performance of both W56-MTBD and GAP-MTBD (Fig. 50). These data suggest that both BDNF-dependent Rac1 signalling and BDNF-independent RhoA signalling must converge at nearby spines to achieve threshold modification at these sites.

To test whether the blockade of crosstalk by this approach is limited to proteins whose activity profile is diffusive after sLTP induction, we next employed a similar strategy against Cdc42, which shows very restricted activity in response to this stimulus. Unlike W56-mCh-MTBD and DNRhoA-mCh-MTBD, DNCdc42-mCh-MTBD had no significant effect on either sLTP or crosstalk (Fig. 47). Thus, the effect of the -MTBD-containing constructs is not due to inhibition of GTPase signalling in general, but is specific to those whose activity diffuses into the dendrite.
Figure 50: Dendritic inhibition of diffusive, but not restricted, Rho GTPases inhibits synaptic crosstalk

(a) Expression of a RhoA dendritic inhibitor (DNRhoA-mCh-MTBD, left), but not a Cdc42 dendritic inhibitor (DNCdc42-mCh-MTBD, right) blocks synaptic crosstalk. LTP is not perturbed in either case. (b) Summary of the effects of various dendritic inhibitor constructs on LTP (left) and crosstalk (CT, right). Asterisks indicate statistical significance as determined by an ANOVA followed by a post-hoc test using the least significant difference. All data plotted as mean ± sem.
6.5 Discussion

Taken together, the data in this section suggest that diffusive GTPase activation after the induction of sLTP serves to modify the threshold of nearby synapses, without being sufficient for sLTP expression. Such diffusive signaling has both a BDNF-TrkB-dependent (Rac1) and independent (RhoA) component, suggesting a requirement of multiple converging signaling cascades to achieve this form of heterosynaptic metaplasticity.

By reducing the threshold for LTP induction at nearby synapses, a phenomenon like synaptic crosstalk favors the selective strengthening (and thus stabilization) of synapses within a local region of dendrite surrounding an LTP event. Over time, the repeated occurrence of such a phenomenon would likely lead to a clustered arrangement of spines along the dendritic arbor, an arrangement which has been described numerous times in previous studies. Clustered synapses are thought to produce nonlinear integration of inputs when those synapses are concurrently active, thus leading to a higher likelihood of action potential output than the same input strength on dispersed synapses. Thus, the threshold-modifying properties of diffusive Rho GTPase signaling after LTP might favor the clustering of synapses, and thus an arrangement of synapses representing a significant contribution to the cell’s output behavior.
In order to probe the effects of diffusive GTPase signaling, we developed novel genetic tools targeted at the inhibition of specific proteins in specific subcellular compartments. The precision of such tools allowed inferences that were not possible with current pharmacological or genetic means of perturbing signaling cascades. To our knowledge, this is the first demonstration of such an approach.

Additionally, the mechanistic insights revealed by the dendritic inhibitor constructs (namely, that signal spreading requires activation of the GTPases in the dendritic compartment) may provide clues about certain neurological disorders that involve dysregulation of these signaling pathways. Since there are several such disorders associated with mutations in GEFs, it may be that the inappropriate spatial regulation of Rac1 during LTP-like phenomena is an important underlying cause of these disease states.
7. Signal spreading of GTPases overcomes high activation thresholds and facilitates a heterosynaptic coincidence mechanism

7.1 Introduction

The spreading of signals like Rac1, RhoA (Fig. 1d), and Ras implies that, after LTP induction, nearby spines are supplied with an active pool of these molecules (Murakoshi 2011). This collection of signals seems to be insufficient to induce LTP, as nearby spines on average show no change in volume (Harvey, C 2007; Murakoshi 2011) (Fig. 8). Notably missing from this pool of active molecules is Cdc42, a Rho GTPase whose activity is largely restricted to the stimulated spine after sLTP induction. Since Cdc42 activity is necessary for sLTP (Murakoshi 2011), Cdc42 activity must somehow be supplied to the second spine during synaptic crosstalk. Because Cdc42 is highly spine-specific, we hypothesized that Cdc42 must be activated by the weak stimulus during crosstalk, which would provide the second spine with Rho GTPase activity complementing that provided by signal spreading (i.e. Rac1 and RhoA).

7.2 Rho GTPases show different thresholds of activation

To test this possibility, we measured the activation of Rho GTPases in response to both an unpaired and a paired (crosstalk) sub-threshold stimulus (Fig. 51-53), and then compared these responses to the activation during normal sLTP. Consistent with our hypothesis, Cdc42 was fully activated by the subthreshold stimulus in both the paired and unpaired conditions, thus explaining how nearby spines achieve the required
levels of Cdc42 activation during crosstalk (Fig. 50a,c). In contrast, both RhoA and Rac1, which show diffusive activity profiles, were much less sensitive to weak stimuli, as the subthreshold stimulus only weakly activated Rac1 and did not activate RhoA (Fig. 48 & 49a,c). However, when paired with sLTP, signal spreading supplied the crosstalk spines with additional Rac1 and RhoA activation (Fig. 48 & 49b,c). Thus, during synaptic crosstalk, through a combination of low-threshold, spine-specific Cdc42 activation and signal spreading of RhoA/Rac1 provided by nearby sLTP, a paired sub-threshold stimulus can provide the necessary components for inducing sLTP.
Figure 51: Rac1 is only weakly activated by a subthreshold stimulus, but this can be overcome by signal spreading.

(a) A weak stimulus (red curve) elicits a correspondingly weak level of Rac1 activation in response to sLTP induction as compared to a normal strength stimulus (black curve). (b) Rac1 activation during crosstalk (‘paired’) reveals that the combination of signal spreading and a weak stimulus yields LTP-like levels of Rac1 activation in the weakly-stimulated crosstalk spine (red curve). ‘Unpaired’ experiments were always performed on different dendrites, and were not performed simultaneously. ‘Paired’ experiments followed the crosstalk paradigm described previously. (c) Summary of data presented in (a) and (b). Asterisks indicate a statistically significant difference as determined by an ANOVA and post-hoc test using the least significant difference. All data plotted as mean ± sem.
Figure 52: RhoA is minimally activated by a subthreshold stimulus, but can be supplemented by signal spreading.

(a) A weak stimulus (red curve) elicits a correspondingly weak level of RhoA activation in response to sLTP induction as compared to a normal strength stimulus (black curve). (b) RhoA activation during crosstalk ('paired') reveals that the combination of signal spreading and a weak stimulus yields LTP-like levels of RhoA activation in the weakly-stimulated crosstalk spine (red curve). ‘Unpaired’ experiments were always performed on different dendrites, and were not performed simultaneously. ‘Paired’ experiments followed the crosstalk paradigm described previously. (c) Summary of data presented in (a) and (b). Asterisks indicate a statistically significant difference as determined by an ANOVA and post-hoc test using the least significant difference. All data plotted as mean ± sem.
Figure 53: Cdc42 is fully activated by a weak stimulus

(a) A weak stimulus (red curve) elicits a statistically normal level of Cdc42 activation in response to sLTP induction as compared to a normal strength stimulus (black curve). (b) Cdc42 activation during crosstalk (‘paired’) does not change the response to a subthreshold stimulus (red curve). ‘Unpaired’ experiments were always performed on different dendrites, and were not performed simultaneously. ‘Paired’ experiments followed the crosstalk paradigm described previously. (c) Summary of data presented in (a) and (b). All data plotted as mean ± sem.
7.3 Response of BDNF release and TrkB activity to a weak stimulus

7.3.1 BDNF release

Since Cdc42 activation in response to a subthreshold stimulus is similar to LTP-like levels of activation, and since normal Cdc42 activation requires BDNF-TrkB signaling, it stands to reason that a weak stimulus produces at least some BDNF-TrkB signaling. In order to test this possibility, we first measured the change in BDNF-SEP fluorescence in response to a subthreshold stimulus. In this context, the increase in BDNF-SEP fluorescence upon glutamate uncaging was minimal, and dominated by noise, but the uncaging triggered average revealed an average increase that scaled with the decrease in stimulus intensity (ΔF = 1.6 for 4ms control, ΔF = 0.4 for 1ms subthreshold) (Fig. 5).

7.3.2 TrkB activation

Next, we utilized the TrkB FRET-based sensor to visualize TrkB activation in response to a subthreshold stimulus. In this context, there was a much more gradual increase in TrkB activity, with significantly attenuated activity during the typical peak phase (1-2 min post stimulation) (Fig. 5). However, the activation was comparable to a threshold stimulus in the sustained phase of activity (>5 min post stimulation). Taken together with the small signal observed with BDNF-SEP, these data suggest that TrkB is weakly activated by a subthreshold stimulus.
The TrkB activation profile observed in response to a weak stimulation was similar to that observed for Rac1, which showed only a weak increase in response to this stimulus. This is consistent with the previously described requirement of TrkB signaling for normal Rac1 activation. However, it is important to note that Cdc42, which also requires TrkB activity, was fully activated by a weak stimulus, and thus does not scale with the reduced TrkB activation in this context. This might be due to a different sensitivity of Cdc42 to BDNF-TrkB signaling, or perhaps due to the subthreshold stimulus activating a different subset of signaling components.
Figure 54: Effect of a subthreshold stimulus on BDNF-TrkB signaling

(a) (left) Change in fluorescence of BDNF-SEP in response to a strong/LTP-inducing stimulus vs. a weak/subthreshold stimulus. (right) Uncaging-triggered average of the response of BDNF-SEP to a single glutamate uncaging pulse (quantified from data at left). (b) Response of the TrkB sensor to a strong/LTP-inducing stimulus vs. a weak/subthreshold stimulus. (right) Corresponding sLTP curves for the data presented at right. All data plotted as mean ± sem.
7.4 BDNF application does not modify the threshold of GTPase activation

One potential mechanism by which the GTPases might show differential thresholds for activation is through disinhibition via BDNF-mediated signaling. However, since the overall levels of GTPase activation do not seem to change during synaptic crosstalk, we hypothesized that this would not be the case.

In order to test whether BDNF can modify the activation of the thresholds for the Rho GTPases, we bath applied 20ng/mL of exogenous BDNF for 10-20 minutes, then performed a subthreshold uncaging stimulation on GTPase sensor-expressing neurons. We found that pre-incubation of BDNF did not increase the activation of any of the GTPase sensors, suggesting that BDNF does not regulate the GTPases via disinhibition (Fig. 5). In fact, the activation of Cdc42 in response to a subthreshold stimulus actually decreased slightly, perhaps due to prior activation upon application of BDNF (Fig. 35).

Since BDNF alone is sufficient to activate Rac1 and Cdc42 (Fig. 35), these data collectively suggest that the primary means of GTPase activation by BDNF is through direct activation through BDNF-TrkB, and not through disinhibition. As such, the differential thresholds of activation of the GTPases are likely not explained by a disinhibition mechanism. Finally, this suggests that the BDNF-mediated component of synaptic crosstalk is due to the modification of the threshold for plasticity itself, and not for the activation of plasticity-associated proteins such as the Rho GTPases.
Figure 55: Effect of pre-incubation of BDNF on GTPase response to a subthreshold stimulus

Pre-incubation with BDNF (red curves) does not increase the activation of Rac1 (top left), Cdc42 (bottom left), or RhoA (top right) in response to a subthreshold stimulus. All data plotted as mean ± sem.

7.5 Removing the spatial specificity of Cdc42

Taken together, our data suggest that the spatiotemporal alignment of Rac1, Cdc42, and RhoA activation predicts the occurrence of sLTP. The localised nature of Cdc42 within spines would thus serve to prevent nonspecific structural plasticity at synapses proximal to a site of sLTP induction. To provide further evidence for this model, we next asked whether it would be possible to degrade the spine-specificity of
sLTP by artificially decreasing the spine-specificity of Cdc42 signalling. To do this, we first over-expressed constitutively active Cdc42 (CA-Cdc42) under the hypothesis that global Cdc42 activation would allow Rac1 and RhoA signal spreading after sLTP induction to become sufficient for sLTP. The expression of CA-Cdc42 in this context shows no obvious phenotypic effect on spine number, size, or morphology, suggesting that hyper-activation of Cdc42 alone is not sufficient to induce gross morphological rearrangements of spines. However, consistent with our hypothesis, we find that sLTP induction in the presence of CA-Cdc42 allows nearby spines to show a significant increase in size (Fig. 56). This is in sharp contrast to control conditions, in which nearby spines show no average change in volume. Thus, removing the spine-specificity of Cdc42 activation also degrades the spine-specificity of sLTP induction, providing further evidence that the coincidence of Cdc42 activation with Rac1 and RhoA activation is predictive of sLTP. Importantly, this effect was almost completely eliminated when simultaneously expressing W56-mCh-MTBD, suggesting that the reduction of the signal of even one of the Rho GTPases is sufficient to eliminate heterosynaptic plasticity across a dendrite.
Figure 56: Effect of CA-Cdc42 expression on the spine specificity of sLTP induction at a single spine

(a) Representative 2p images of the effects of the expression of CACdc42 on nearby spines during sLTP induction. Normally, uncaging at a targeted spine (indicated by a red circle) shows effects only at that spine (bottom row). The expression of CACdc42, however, causes generalized enlargement of nearby spines as
well (top row; yellow arrows indicate spines that have enlarged since the previous image. Green arrows indicate apparent appearance of new spines). (b) Quantification of the effect of CACdc42 on nearby spine enlargement. Data corresponds to the averaging of all nearby spines within the imaging window after sLTP induction at a single spine. Also shown is data corresponding to the co-expression of CACDc42 and the dendritic Rac1 inhibitor, W56-MTBD. (c) Summary of data presented in (b). Asterisks represent a statistically significant difference as determined by an ANOVA followed by a post-hoc test using the least significant difference. All data plotted as mean ± sem.

Importantly, the effect of the co-expression of W56-mCherry-MTBD with CACdc42 was specific to nearby spines, as the stimulated spine showed no difference in the expression of sLTP (Fig. 57). Indeed, neither CA-Cdc42 nor W56-MTBD showed significant effects on the sustained volume changes observed in sLTP. Thus, the observed effects on heterosynaptic spine enlargement in these experiments were not due to a general increase or decrease in the capacity for structural plasticity.
Figure 57: Co-expression of W56-mCherry-MTBD with CA-Cdc42 does not reduce sLTP at the stimulated spine.

(a) Quantification of the effect of CA-Cdc42 and co-expression of CA-Cdc42+W56-MTBD on sLTP of the targeted spines (solid lines) as compared to nearby spines (dotted lines). (b) Neither CA-Cdc42 nor W56-MTBD significantly affects the expression of sustained volume change of sLTP (10+min) in the stimulated spine, suggesting that its effects are specifically heterosynaptic. Right bar graph shows effects on nearby spines for comparison. All data plotted as mean ± sem.
8. Discussion

In this study, we have revealed a mechanism of both homosynaptic plasticity and heterosynaptic metaplasticity via the spatiotemporal coordination of the Rho GTPases Rac1, Cdc42, and RhoA. Using a combination of 2pFLIM, 2p glutamate uncaging, as well as novel biosensors, we have shown that the activation of Cdc42 and Rac1 during single-spine sLTP is dependent upon postsynaptic, autocrine BDNF released from single dendritic spines, and that this signalling cascade conveys the occurrence of sLTP over both spine-specific (Cdc42) and heterosynaptic (Rac1) signalling domains. While the existence of postsynaptic sources of BDNF has been controversial, this study clearly demonstrates its presence with a variety of converging pieces of evidence, and further identifies a functional role for this particular locus of BDNF release.

8.1 Autocrine BDNF release from spines: Purpose and other functional consequences

While this study clearly demonstrates the presence of postsynaptic BDNF, and further suggests that this BDNF can be released from individual spines, many questions remain. Notably, the very nature of such an autocrine loop suggests that the cell externalizes a signal that has already been conveyed across a synapse and internalized within the postsynaptic cell. Since this comes with a particular energy cost, there is presumably an advantage to handling synaptic activity in this manner. What could be
the purpose of re-externalization of the signal of synaptic activity? One possibility is that postsynaptically released ‘autocrine’ BDNF also serves as a paracrine signal to nearby cells, informing the presynaptic neuron – as well as invading glia – of the occurrence of synaptic activity. If true, what types of signals can this source of BDNF convey that presynaptically released BDNF cannot (if any)? Additionally, what function might this paracrine BDNF have at other synaptic cells? Both astrocytes and microglia have been suggested to invade synapses (Vladimir 1994; Hiroaki 2009), and have mechanisms of monitoring synaptic activity (such as glutamate receptors), and indeed can respond to synaptic activity. Might similar mechanisms come into play with postsynaptically released BDNF? These questions are beyond the scope of this study, but provide a springboard for several new venues of research that will further elucidate how the complex synaptic assembly of cells function so as to interpret and store information.

8.2 Signal separation of the Rho GTPases: BDNF-dependendent and -independent mechanisms of sLTP

This study also revealed a surprising division of synaptic signalling wherein postsynaptic BDNF was responsible for activating the Rho GTPases Rac1 and Cdc42, but not the other family member, RhoA. Both Cdc42 and Rac1 are suggested to promote actin polymerization (Ridley 1992; Kozma 1995; Sin 2002) and are necessary for the maintenance of sLTP (Murakoshi 2011) and LTP (Martinez 2010; Il Hwan 2014), thus providing an attractive substrate for the downstream targets of BDNF that might
regulate the diverse expressions of plasticity associated with this trophic factor. In contrast, RhoA, a purported actin depolymerisation signal that often negatively regulates spine structure (Tashiro, A., 2000; Sin 2002) and is particularly important for the initial phase of sLTP, did not show a BDNF-dependency. Thus, while both the positive and negative regulation of actin during sLTP require upstream NMDAR-Ca2+-CaMKII, actin polymerization signals then diverge by virtue of initiation of a spine-autonomous autocrine BDNF loop. The functional significance of this divergence of signal regulation is unclear, but is likely critical for maintaining a balance of actin regulation – and thus spine architecture – in different physiological and pathological contexts.

8.3 Diffusive synaptic signals and synaptic crosstalk

The spreading of BDNF-TrkB–mediated Rac1 signalling out of the stimulated spine was found to be necessary for facilitating sLTP in the surrounding region of the parent dendrite, consistent with a number of studies supporting the pro-plasticity properties of BDNF (Korte 1995; Huber 1998; Tanaka 2008; Bosch 2014), and further suggesting a role of BDNF in favoring the clustered strengthening of synapses along a dendrite. Such strengthening could potentially lead to preferential maintenance and longevity of synapses that exist within these clusters, thus providing a potential cellular and molecular mechanism by which synaptic clusters emerge.
Interestingly, RhoA, a BDNF-independent factor whose activity also spreads into nearby spines, was also found to be required for synaptic crosstalk. The activity of RhoA has been associated with both negative (Tashiro, A., 2000; Tashiro, Ayumu 2004) and positive (Murakoshi 2011) regulation of spine structural plasticity, and thus its role at nearby spines is unclear. One interesting possibility is that the negative and positive regulation of the actin cytoskeleton by Rac1 and RhoA, respectively, causes a balance of signals that results in no net change. The classical expression of sLTP might be possible due, in part, to the positive regulation of the actin cytoskeleton imparted by spine-specific Cdc42, which throws off the Rac1/RhoA balance. Thus, the spine specificity of Cdc42 might be especially advantageous in that it simultaneously allows heterosynaptic signaling while also maintaining input specificity of sLTP.

A critical question from these data is how signal spreading imparts threshold modification to nearby synapses. One possibility is that heterosynaptic signaling domains like that observed for Rac1 and RhoA might serve to lower the threshold for sLTP at nearby spines by activating signaling pathways in these regions that would otherwise require an additional strong stimulus for initiation. Consistent with this possibility, our results reveal that diffusive signals like Rac1 and RhoA possess a relatively high threshold of activation, and therefore require signal spreading to achieve LTP-like levels of activity during crosstalk. On the other hand, spine-restricted signaling domains, like that of Cdc42, show a lower activation threshold, and therefore do not
require signal spreading to achieve LTP-like levels of activity during crosstalk. Thus, we describe a mechanism whereby the diffusive nature of signals like Rac1 and RhoA activation after sLTP grant nearby spines access to high-threshold, plasticity-associated factors, thus relaying the context of plasticity to these regions. When these signals are combined with the activation of low-threshold, spine-specific factors like Cdc42, plasticity can be achieved from an otherwise insufficient stimulus (Fig. 5). Such heterosynaptic coincidence detection provides mechanistic insight into how the induction of plasticity might favor the potentiation of several nearby synapses while at the same time maintaining specificity for only the active ones, and could serve as a molecular strategy for the selective strengthening of neuronal inputs.
Figure 58: Signalling Model of Rho GTPase involvement in sLTP and synaptic crosstalk

(top) During sLTP, Ca^{2+} through NMDARs signals the activation of the Rho GTPases Rac1, Cdc42, and RhoA in both autocrine-BDNF-dependent and -
independent ways. The overlap of activity of all three Rho GTPases is conducive to sLTP expression, and the spine enlarges (indicated by red shading). Meanwhile, the diffusive activity profiles of RhoA and Rac1 invades nearby spines, modifying their thresholds for sLTP induction, but not reaching sufficiency for the development of sLTP. (bottom) With nearby spines being supplied with high-threshold Rac1 and RhoA activation, they are now primed for the induction of sLTP. When even a weak stimulus (depicted here by smaller Ca2+ entry and BDNF release) acts on a nearby spine, Cdc42 is fully activated, completing the tripartite Rho GTPase requirement for sLTP and allowing the induction of sLTP in an otherwise impermissible context.

8.4 Synaptic crosstalk and clustered plasticity

As mentioned previously, the occurrence of synaptic crosstalk might favor the clustered strengthening of synapses along a dendritic branch. While many models of clustered plasticity invoke the formation of new synapses to account for clustering (Fu, M 2012), this particular phenomenon does not (necessarily) include this feature (though it should be noted that similar proximity-based priming mechanisms have also been described for lowering the threshold for de novo spinogenesis along the length of a dendrite (Kwon 2011)). Nonetheless, it does provide a potential mechanism for selecting particular spines for survival based on their strengthening and maintenance due to sLTP (Fig. 56). Which of these processes (if any) is the primary contributor to the clustering of synapses is unclear. However, it is also possible that these two phenomena work in concert to produce synaptic clusters.

One interesting consequence of synaptic clustering is that the close proximity of co-active synapses can lead to their non-linear integration (Poirazi, P., 2001; Poirazi,
Panayiota 2003, 2003; Gasparini 2004; Polsky 2004; Gasparini 2006; Losonczy, Attila 2006). As such, it is more likely that spatially clustered (vs. dispersed) synapses produce a sufficient depolarization of the postsynaptic cell to produce action potential output. The consequence of this is that the co-activation of pre-synaptic cells that converge on a synaptic cluster has a disproportionate contribution to the output of the postsynaptic cell. In this way, the co-activation of these inputs becomes a higher-order feature that postsynaptic output is now capable of representing. This feature not only has the property of binding less salient information into the same representation as stronger, more ‘relevant’ information (Govindarajan 2006), but it also increases the total number of distinct representations that a neuron is capable of differentiating by means of adding another variable (spatial distribution) to the list of determinants of postsynaptic output (Poirazi, P., 2001)
Figure 59: Proposed model for the formation of a synaptic cluster as a result of synaptic crosstalk

(a) Along a given length of dendrite, there are a number of spines at various stages of maturity. Strong synaptic activity at one of these sites might lead to
potentiation of a single synapse (b; blue halo). The occurrence of sLTP at this site is spine-specific, but causes the threshold modification of nearby spines (green halo) as a result of the mechanisms described in previous sections (see Fig. 55). These nearby spines are thus ‘primed’ to be potentiated by even weak synaptic activity. If such activity occurs (indicated by the visibility of axonal boutons), then these synapses will also be potentiated. Such potentiation can potentially protect these synapses from some constant level of synaptic pruning (red halos), allowing their survival for longer periods of time. In this way, synaptic clusters arise in an area corresponding to the length constant of the heterosynaptic effects of crosstalk, and the co-activation of their inputs will lead to a nonlinear integration for postsynaptic output.

8.5 Degrading the synapse specificity of sLTP

Informed by the differential spatial profiles of the Rho GTPases, we found that global activation of Cdc42 via the expression of CACdc42 was sufficient to degrade the spine-specificity of uncaging-evoked sLTP, leading to significant increases in the volume of nearby synapses. This effect required extrasynaptic Rac1 activity, as nearby spine volume changes were blocked in the presence of W56-mCherry-MTBD. This data is consistent with a model of sLTP in which the three Rho GTPases described in this study (Rac1, Cdc42, and RhoA) are all required for the expression of sLTP, and further that their coincident activation is highly predictive of – and perhaps sufficient for – the occurrence of sLTP. Thus, heterosynaptic plasticity can be bidirectionally controlled with either the global activation of Cdc42 (to overcome its lack of signal diffusion) or by the dendritic inhibition of either Rac1 or RhoA. As such, the tools described in this study
provide a unique opportunity for exploring the functional consequences of the up- or down-regulation of heterosynaptic plasticity in a living animal.

### 8.6 Future directions

Taken together, this study provides a novel description of the molecular mechanisms by which structural plasticity is executed and controlled. Notably, tools for manipulating the spatial regulation of that plasticity have been revealed here, and will likely be of use in exploring the potential function of phenomena such as heterosynaptic plasticity as it relates to learning.

For example, as synaptic clustering has been shown to occur in response to learning (Fu, M 2012), then elucidating the functional purpose (if any) of such clustering is of significant interest. However, tools to specifically address such questions have not been available. The dendritic Rac1/RhoA inhibitors, which prevent the expression of synaptic crosstalk without affecting sLTP, could potentially be the first tools that could address such questions.

It should be noted, however, that while the dendritic inhibitors of Rac1 and RhoA signalling are capable of blocking synaptic crosstalk, they potentially have a host of other cellular effects that are not described in this study. As such, the interpretation of a behavioural – and even cellular – phenotype on a living animal would be extremely complicated. Nonetheless, this study has described a strategy for both the down- and
up-regulation of the occurrence of heterosynaptic plasticity, which would greatly aid in the interpretation of such results. For instance, if the expression of CACdc42 could exaggerate the spine clustering observed in a relevant behavioural paradigm, and the expression of W56-mCherry-MTBD could reverse this phenotype, then the interpretation of the effects of these constructs would be significantly easier. Furthermore, if the effects of DNRhoA-mCherry-MTBD mimicked these effects, then the case for these tools affecting a crosstalk-like phenomenon in vivo so as to modify synaptic clustering (and potentially learning behaviour) would be greatly strengthened.

Further adding to the specificity of the dendritic inhibitor constructs would be the ability to selectively target them to active areas of dendrite, as opposed to relying on their global expression throughout the cell. In this context, it would be significantly easier to discount potential cellular effects of the inhibitors that are remote to the areas of interest (a synaptically active region of dendrite). In this regard, recent studies have shown that the dendritic targeting element of Arc mRNA can target mRNA for transcription at dendritic compartments (or even individual spines) that have recently undergone plasticity induction (Akiko 2015). Through such targeting, the effects of the dendritic inhibitor constructs could be limited to only the dendrites (and not the soma), and further, to only highly active dendrites. Finally, as spine clustering on a particular dendrite is task-specific (Fu, M 2012), inducible expression systems along with temporal separation of behavioural tasks could be utilized to target the dendritic inhibitor
constructs to active dendrites associated with a particular task to potentially selectively block clustering and behavioural improvement in a task-specific manner.

Finally, our work highlights the potential of using basic biophysical properties of proteins to inform higher-order functions of cells, and even circuits. Using primarily information gleaned from observing the behavior of proteins in response to synaptic activity, we have identified neuronal functions that likely have expansive ramifications for how circuits in the brain are organized. Further, only by virtue of a molecular understanding of the processes underpinning these phenomena were we able to devise tools that could block them. As such, this work underscores the power of multi-level approach to understanding the many complicated functions of the brain.
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Biography

Nathan Gray Hedrick was born in High Point, North Carolina to parents Marty Lynn and Cathy Carpenter Hedrick. After playing Zelda for 20 years, he attended the University of North Carolina at Chapel Hill, where he majored in Biology, and minored in Chemistry, Japanese, and Spanish. He graduated with distinction from UNC Chapel Hill in 2009. He then enrolled in a PhD program in the Department of Neurobiology at Duke University Medical Center. He is scheduled to complete his Doctorate of Philosophy in November of 2015.

Posters

2013 Duke University Department Retreat. Wilmington, NC.

“Diffusive Rac1 signaling during single-spine sLTP contributes to synaptic crosstalk”

2014 Society for Neuroscience (SfN) Annual Conference. Washington, D.C.

“Autocrine BDNF simultaneously conveys homo- and heterosynaptic signals via modulation of the Rho GTPases”

2015 Dendrites Gordon Conference, Ventura Beach, CA.

“Autocrine BDNF simultaneously conveys homo- and heterosynaptic signals via modulation of the Rho GTPases”
Presentations

2011 Duke University Department Retreat, Durham, NC

“Imaging Rac1 spatiotemporal dynamics during LTP”

2014 Duke University Department Retreat, Wrightsville Beach, NC.

“Autocrine BDNF simultaneously conveys homo- and heterosynaptic signals via the Rho GTPases”