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Neuronal activity enhances Brain-Derived Neurotrophic Factor local signaling in axon through modifying its axonal transport

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by

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TABLE OF CONTENTS

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ABSTRACT OF THE DISSERTATION

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This dissertation, by Zheng Zeng, explores a role for neuronal activity in modulating brain-derived neurotrophic factor (BDNF) signaling in axons. BDNF

gene expression and release are modulated by activity and, correspondingly, BDNF serves to mediate several facets activity-dependent neuronal adaptation, including changes in neuronal structure and function. Remarkably little is known of what effect activity may have on BDNF signaling and function in axons. I employed microfluidic devices, *in vitro* neuronal activation, live imaging and biochemical methods to show that activation of neurons reduced the speed and net flux of BDNF retrograde transport in axons with a resulting increase in the robustness of BDNF signaling in this compartment. My findings set the stage for studies to explore the mechanism by which neuronal activity impacts BDNF transport and the biological consequences of increased signaling in axons.

Chapter 1 INTRODUCTION

1. Neuronal adaptation, the concept and its manifestation

1.1 The biological context and definition.

One of the biggest challenges facing a living being is how to adapt to the constantly changing environment, external and internal, and how to make changes to ensure survival and function. The nervous system plays an important role in meeting this challenge by lending its inherent flexibility, as expressed both structurally and functionally, to respond to environmental cues. Indeed, it is poised to quickly perceive changes, reliably assess their potential meaning, and induce changes in the structure and function of neurons and circuits to respond so as to maximize benefit and minimize threat. In so doing, the nervous system is equipped to act in real time to receive, process and act upon information critical to the wellbeing of the organism in which it resides.

1.2 The history of neuronal adaptation: discovery and studies exploring its underpinnings.

The first documented studies of neuronal adaptation can be traced back to the 18th century. In 1783, Swiss naturalist Charles Bonnet and anatomist Michele Vicenzo Malacarne tested experimentally whether mental exercise could induce growth of the brain (Rosenzweig, 2007). Malacarne chose pairs of littermate dogs and birds. He extensively trained one animal of each pair while

leaving the other unattended. After years of differential treatment, Malacarne compared the brains of each member of a pair and found that trained animals had bigger cerebellums than untrained ones. This discovery, though impressive, failed to inspire follow-up studies, at least as documented in published work. In part this may have been due to the inability at that time to envision how the outcome was achieved. Indeed, the work predated by more than a century the work of Santiago Ramón y Cajal and his proposal of the neural doctrine (1888) based on evidence that the nervous system is made up of discrete individual cells. As a speculation, had Cajal's insights been extant when Malacarne performed his studies it may have enabled follow-on studies, including work to explore the effect of activity on the structure of the cerebellum.

The next landmark in neuronal adaptation was contributed by Donald Hebb. In his renowned publication, *The Organization of Behavior* (Wiley, New York, 1949), he wrote:

"When one cell excites another repeatedly, a change takes place in one or both cells such that one cell becomes more efficient at firing the other."

Surprisingly, a decade after Hebb's hypothesis, neural adaptation had not been proved experimentally. In fact, the scientific community in the 1950s was not optimistic about finding the neuronal basis for learning and memory. For instance, Karl S. Lashley, Hebb's mentor, publically expressed his criticism of his pupil's hypothesis of neuronal adaptation.

A breakthrough was made by researchers at the University of California, Berkley. They were Mark R. Rosenzweig and David Krech, two psychology professors, Edward L. Bennett, a chemist, and Marian C. Diamond, a research anatomist. They systematically discovered that both formal training and enriched experience led to chemical and anatomical brain changes (Bennett et al., 1964). Interestingly, the authors started out by looking for the possible neuronal basis for individual differences among rodents in terms of problem solving, as measured by completing mazes in the lab setting. It is interesting that a key member of the research team, Marian Diamond, was among the few neuro-anatomists who examined the brain of Albert Einstein. Even though the research team observed the expected result, that of a significant correlation between maze scores and cortical AChE activity, they discovered unexpectedly that AChE activity was a function of whether animals had undergone maze testing. Specifically, there was a consistent trend for groups that went testing to have higher AChE activity than the group naïve to testing. Intrigued by this discovery, and to reduce the cost of actively running animals through mazes, the team explored the consequences of “informal learning” by placing rats for prolonged periods in cages that were either more enriched or more impoverished than the standard colony. Therefore, these investigators pointed to AChE activity a specific molecular manifestation of neuronal activity. As a consequence of this finding they created a new context for studies of activity, environmental enrichment. In the end, they found that environmental enrichment increased

brain weight, AChE activity and total protein. It was noteworthy regardless of the age of adult animals, those exposed to the enrichment environment responded.

Around the same time, Hubel and Wiesel carried out their famous monocular deprivation experiments(Wiesel and Hubel, 1963), where occluding the one eye of a kitten led to a reduction in the number of neurons in visual cortex that responded to that eye. They also discovered an important concept, the critical period for visual development. The latter is defined as is a developmental stage when nervous system is most susceptible to alteration by the environment.

Twenty years later studies re-explored the critical period theory. Merzenich and colleagues studied the representation of body parts in the somato- sensory cortex in monkeys. They amputated one finger in adult animals and showed that while a region of the somatosensory cortex was initially electrically silent, it later adapted and began to respond to neighboring fingers (Merzenich et al., 1984). Similar research explored “critical period-like” phenomena in other sensory systems and discovered a remarkable degree of ability of adult neuronal circuits to respond to changes in input. As a result, dynamic neuronal adaptation is well accepted and intensively studied. Re-evaluating the concept of critical periods thus pointed to a nervous system that is much more adaptable than initially appreciated.

1.3 Categorization of neuronal adaptation and its manifestations.

Neuronal adaptation can occur in two distinct developmental stages: postnatal/early development and adulthood. In developing brains, neuronal adaptation is robust. Classical examples include Lorenz's famous discovery of filial imprinting observed in fledglings, the establishment of ocular dominance and its plasticity in the mammalian visual system (Wiesel and Hubel, 1963), and a stereotypical time course for first language acquisition in human infants (Kuhl, 2010).

Though less robust in comparison to early postnatal life, neuronal adaptation has been consistently documented in adult animals, both human and non-human. Generally speaking, there are several different triggers of adult adaptation: exercise (van Praag, 2009), nutrition (Gómez-Pinilla, 2008), environmental enrichment (Gage et al., 2000), antidepressant drugs (Castrén and Antila, 2017), drugs that inhibit pathways that restrain changes in neuronal structure and function (Hübener and Bonhoeffer, 2014), and repetitive transcranial magnetic stimulation (rTMS), an intervention increasingly used clinically (Chang et al., 2014). In terms of mechanisms and consequences, adult adaptation manifests itself in adult neurogenesis, synaptic strengthening, improved cognitive performance, and delayed onset/progression of certain neurodegenerative diseases.

2. BDNF is involved in neuronal adaptation.

Once the phenomenon of neuronal adaptation was established, researchers explored underlying molecular and cellular mechanisms. Brain-derived neurotrophic factor (BDNF), a member of a family consisting of small polypeptide neurotrophic factors, is one of the most studied candidates.

2.1 BDNF is involved in neuronal adaptation in developing brains.

Carla Shatz and colleagues contributed significantly to the study of neuronal adaptation by documenting a role for BDNF in the formation of ocular dominance columns (ODCs) in visual cortex. In normal development of the visual circuit, geniculo-cortical axons project from lateral geniculate nucleus (LGN) to layer IV of the visual cortex. Axons originating from the left and right eye compete to form synapses with cortical neurons in visual cortex, eventually creating a pattern of alternating columns in which cortical neurons are predominantly responsive to one eye or the other. The Shatz group disrupted the normal formation of ODC by either supplying excessive of BDNF (Cabelli et al., 1995) or depleting BDNF (Cabelli et al., 1997). Specifically, infusing BDNF to the kitten visual cortex allowed inputs from both eyes to persist; in contrast, infusion of TrkB-IgG to competitively sequester BDNF and prevent binding to its TrkB receptors eliminated inputs from both eyes. Therefore, BDNF, produced in visual cortex(Castrén et al., 1992), attracts and supports geniculo-cortical axons projecting to and innervating visual cortex. To explore a role for neuronal activity in this process, Hans Thoenen, Wolf Singer and colleagues showed that infusing

BDNF in visual cortex during monocular deprivation (MD) prevented the effect of MD (Galuske et al., 1996). Similar results were observed in rat visual system (Lodovichi et al., 2000). However, how BDNF acted to induce these changes is not yet fully understood. While the most attractive interpretation is that BDNF substitutes for reduced activity from the deprived eye, other explanations are possible. However, BDNF unexpectedly suppressed the activity of neurons receiving inputs from the non-deprived eye (Galuske et al., 1996). It is noteworthy also that inhibiting TrkB activity failed to impact the effect of MD in mice (Kaneko et al., 2008). Additional studies will be needed to clarify the mechanisms by which BDNF secretion and action mediate neuronal adaptation in the developing visual cortex.

As introduced above, an important concept in postnatal neuronal adaptation is the critical period. The Tonegawa group showed that BDNF participates in defining the timing of termination of the critical period in visual cortex (Huang et al., 1999). The research paradigm employed a transgenic mouse line whose BDNF levels in postnatal brain increased more rapidly than wild type mice. These mice exhibited earlier termination of the critical period for ocular dominance shift in response to MD and concurrently an acceleration of GABA-mediated inhibition. Other studies showed that dark rearing, which results in lower BDNF expression, prolonged the critical period (Mandolesi et al., 2005). Taken together, the evidence suggests that BDNF modulates the duration of the

critical period, thus providing another manifestation of its role in postnatal neuronal adaptation.

2.2 BDNF is involved in neuronal adaptation in the adult brain.

Studies of BDNF have also examined effects in adult animals. BDNF infusion was used to explore the possibility of re-opening a “critical period-like” window in adulthood. Postnatal day 55 is the end of the classically defined critical period in rats; MD of the contralateral eye after this age did not change the representation of projections to visual cortex, resulting in sustained dominance by the contralateral eye in evoking population responses. However, intracortical infusion of BDNF plus MD of the contralateral eye in P100 rats resulted in a gain in the response of cortical neurons to the ipsilateral (open) eye (Maya Vetencourt et al., 2008). While the mechanism is yet to be fully defined, this finding suggests that BDNF continues to play a role in the synaptic biology of visual projections and that in doing so BDNF and neuronal activity interact to enable neuronal adaptation.

Studies of BDNF have also explored its role outside visual cortex. Environmental enrichment led to about 2-fold increase of neurogenesis in dentate gyrus of hippocampus; however, BDNF+/- mice exposed to the enriched environment failed to show increased neurogenesis (Rossi et al., 2006). Pointing to a BDNF effect in mediating another trigger for neurogenesis, TrkB appears to be involved in exercise-induced neurogenesis (Li et al., 2008). Investigators

discovered that selective TrkB ablation in neural progenitor cells (NPCs), but not mature dentate gyrus granular cells, blocked the impact of environmental enrichment on neurogenesis. BDNF has also been shown to mediate exercise-induced enhancement in learning and memory. Mice receiving TrkB-IgG while participating in voluntary exercise failed to show improved scores in the Morris Water Maze (Vaynman et al., 2004).

BDNF also regulates the motor system. The area of cortex that maps to the activation of a specific muscle was defined using TMS. After motor training the investigators found an increase in map area in those whose BDNF genotype was val/val but not in those who were met/met (Kleim et al., 2006). Thus, different BDNF isoforms were linked to different levels of cortical adaptation following training.

To summarize, a number of research groups have independently and consistently gather evidence showing that BDNF is a mediator of both postnatal and adult neuronal adaptation. The findings point to BDNF and its receptor TrkB as playing defining roles. They raise the possibility that local changes in BDNF signaling may intersect with neuronal activity to regulate synaptic connectivity.

3. Introduction of neurotrophin family

The neurotrophin family is a small family of secreted proteins whose activity is essential for neuronal survival, maturation, and differentiation. The first member of the neurotrophin family, nerve growth factor (NGF), was discovered in

1954 as a target-derived protein that promotes the survival and growth of sympathetic and sensory neurons (Cohen et al., 1954). Later, Thoenen and his group successfully purified and characterized another protein, brain-derived neurotrophic factor (BDNF), which exerted a similar neurotrophic effect on cultured sensory neurons (Barde et al., 1982). The protein sequence of the two proteins is highly conserved and defines a family that also contains neurotrophin-3 (NT-3) (Hohn et al., 1990) and neurotrophin-4 (NT-4) (Hallböök et al., 1991), discovered through sequence homology analysis.

All neurotrophins bind to a receptor called p75NTR and selectively to one of the three tropomyosin-related kinase (TRK) receptors — NGF binds to TRKA, BDNF and NT4 bind to TRKB, and NT3 binds to TRKC. Additionally, neurotrophins and the immature or pro- forms of these proteins interact with other receptors to execute a variety of functions in both neurons and non-neuronal cells (Chao, 2003).

BDNF, the most abundant neurotrophin family member most abundantly expressed in the central nervous system, has been extensively studied, both for its functions and with respect to how it is regulated.

4. BDNF is a molecular mediator of neuronal activity: evidence from molecular and cellular studies.

4.1 BDNF modulates the structure and function of central nervous system.

In order to serve as a mediator connecting neuronal activity and neuronal adaptation, an important requirement is a molecule that acts on the structure/morphology and/or biochemical/electrophysiological properties of neuronal circuits. BDNF meets this criterion.

4.1.1 BDNF promotes axon and dendritic growth and synapse formation

Cohen-Cory S. and Fraser S.E. used in vivo microscopy to trace optic nerve axon branching in living *Xenopus laevis* tadpoles (Cohen-Cory and Fraser, 1995). They discovered that by injecting BDNF into optic tectum, the target of the optic nerve, they increased arborization of optic axons; injecting BDNF neutralizing antibody led to the opposite effect. This study was among the first to directly show that BDNF engages in as a target-derived factor to promote axon branching. BDNF was also shown to boost maturation of dendrites. Lawrence C. Katz's team applied BDNF to organotypic slices of developing (P14) ferret visual cortex and found that BDNF has pronounced local effects on dendritic growth (McAllister et al., 1995)(Horch and Katz, 2002). By acting on both axons and dendrites BDNF may enhance physical contact between axons and dendrites and thereby increase the probability of synapse formation. Indeed, applying

BDNF to neonatal hippocampal slices increased synapses in the CA1 region. Conversely, blocking BDNF delayed synaptic maturation (Sallert et al., 2009).

4.1.2 BDNF promotes synaptic transmission and LTP.

BDNF has also been shown to modify the electrophysiological properties of neuronal circuits. The earliest findings came from studies of dissociated embryonic and postnatal rat hippocampal neurons. BDNF treatment increased the frequency of evoked miniature excitatory postsynaptic currents, evidence of presynaptic action (Lessmann et al., 1994). Independently, studies demonstrated that the postsynaptic NMDA receptor subunit NR2B was phosphorylated at Tyr^{1472} by BDNF(Alder et al., 2005). In addition, AMPA receptor subunit GluR1 was phosphorylated on Ser-831 by BDNF through activation of protein kinase C and Ca(2+)-calmodulin-dependent protein kinase II(Caldeira et al., 2007).

Besides improving synaptic efficacy, Bai Lu's group showed that BDNF modulates synaptic plasticity. In their studies (Figurov et al., 1996), BDNF acted presynaptically to convert short term-potentiation (STP) to long term potentiation (LTP) in CA3-CA1 in response to tetanic stimulation. In contrast, in dentate gyrus, BDNF, paired with weak stimulation, induced LTP through upregulating calcium influx post-synaptically; calcium influx was mediated by NMDA receptors and voltage-gated calcium channels (Kovalchuk et al., 2002).

Genetic knock-out mice were also used to illustrate BDNF's role in LTP. In hippocampal slices from BDNF deleted mice, both LTP amplitude and the

probability of LTP induction rate were compromised (Korte et al., 1995). The LTP defect was rescued by re-expressing the BDNF gene in the CA1 region(Korte et al., 1996). In parallel, Eric Kandel's group also rescued LTP in BDNF mutant mice by applying BDNF (Patterson et al., 1996). BDNF's effect on synaptic transmission and LTP was also tested *in vivo* (Messaoudi et al., 1998). In urethane-anesthetized rats, infusion of BDNF above the dentate gyrus molecular layer contributed to a long-lived increase in the slope of field excitatory postsynaptic potentials and amplitude. In summary, BDNF has been repeatedly shown to promote synaptic strength and long-term potentiation *in vitro* and *in vivo*.

4.1.3 BDNF promotes neurotransmitter release.

BDNF increased the number of docked neurotransmitter vesicles at the active zone of presynaptic neurons in organotypic hippocampal slices (Tyler and Pozzo-Miller, 2001). The Ras-MAPK pathway downstream of BDNF signaling was found to promote glutamate release by phosphorylating synapsin I (Jovanovic et al., 2000) or the synaptic vesicle protein Rab3A (Alder et al., 2005).

4.1.4 BDNF is involved in adult neurogenesis linked to LTP.

Infusing BDNF into the hippocampus of adult rats for 2 weeks resulted in a significant increase in BrdU(+)/NeuN(+) double-labeled cells (Scharfman et al., 2005). Specifically deleting full-length TrkB in adult progenitors compromised

growth of dendrites and spines of adult-born neurons. Neurogenesis-dependent LTP and cell survival were also impaired(Bergami et al., 2008). Taken together, the data point to a role for the BDNF-TrkB pathway in positively regulating adult neurogenesis and its impact on synaptic plasticity.

4.1.5 BDNF is involved in learning and memory.

A cohort of 641 subjects were tested with a revised version of the Wechsler Memory Scale, which tests the capacity to recall information from two stories with 50 total elements after 30min. A BDNF polymorphism, a valine (val) to methionine (met) substitution in the 5′ pro-region of the human BDNF protein, was found to be strongly correlated with the memory score, with Met/Met carriers performing worse (Egan et al., 2003). The underlying mechanism by which different isoforms act differently is yet to be fully defined, but differences in BDNF trafficking and release have been suggested to modify learning and memory.

4.2 Different stages of BDNF life cycle are activity-dependent.

For BDNF to mediate neuronal activity, it is not enough to meet the criterion that BDNF exhibits circuit shaping and refinement properties. A second criterion is that aspects of BDNF availability or receptor activation be responsive to activity. Differences in production, trafficking and secretion, receptor binding and signaling would each satisfy this criterion.

4.2.1 BDNF transcription is regulated by activity.

The original analysis of the regulatory elements in the Bdnf gene revealed four distinct promoters upstream of the 5' untranslated region (UTR). A more recent, comprehensive study uncovered nine promoters (Aid et al., 2007). Among them, promoter IV received most attention because it appeared to be the key locus for activity-dependent regulation of Bdnf gene expression. Specifically, promoter IV activity is modified by increased calcium signaling, which is the downstream of neuronal activity. Michael Greenberg's group contributed significantly to identifying activity-dependent Bdnf gene expression. In 1998, his team discovered that the Bdnf gene has a cAMP responsive element (CRE) DNA sequence, to which the cAMP responsive element binding protein (CREB) binds (Tao et al., 1998). Depolarization of cortical neurons led to phosphorylation of CREB which binds to the CRE sequence with resulting transcription initiation. Blocking CRE or CREB significantly reduced Bdnf transcription. Four years later, the same group discovered a second mechanism through which neuronal activity could increase Bdnf transcription. Ca^{2+} influx activated a transcription factor, calcium response factor (CaRF), that also initiates transcription from Bdnf promoter IV (Tao et al., 2002). Evidence that activity-dependent regulation of Bdnf transcription is complex was also demonstrated by Greenberg and colleagues (Chen et al., 2003) by showing that activation of Ca2+/calmodulin kinase II (CaMKII), due to depolarization, phosphorylates MECP2. It is noteworthy that MECP2 is a transcriptional repressor of Bdnf. Its

phosphorylation resulted in release from promoter IV together with its interacting proteins histone deacetylase 1 (HDAC1) and SIN3 transcription regulator homolog A (SIN3A) (Martinowich et al., 2003). Thus, neuronal activity not only initiates Bdnf transcription, it also disinhibits transcriptional repressors.

The operation of these, and perhaps additional, mechanisms collectively contributes to an overall increase of Bdnf transcription in active neurons, as confirmed by the observation that LTP inducing stimuli increased BDNF mRNA in CA1 region of hippocampal slices (Patterson et al., 1992). On the macroscopic level, exercising on running wheels for just 7 nights significantly increased BDNF mRNA in rat hippocampus(Cotman et al., 1995). Similarly, environmental enrichment, which restored visual acuity in amblyopic rats, was accompanied by increased BDNF expression (Sale et al., 2007).

4.2.2 BDNF translation is activity-dependent.

After synthesis in neurons, Bdnf mRNA is transported by kinesin to sub-cellular compartments, notably dendrites. Dendritic BDNF mRNA is translated locally (Korte et al., 1996). Dendritic accumulation of Bdnf transcripts may be promoted by neuronal activity through the Ca2+ influx mediated by VGCCs and NMDARs (Tongiorgi, 2008). The exact mechanisms are as yet undefined, but several possibilities have been discussed. One is that Ca2+-dependent phosphorylation of kinesins regulate cargo–motor interactions, resulting in increased net delivery to dendrites (Hirokawa et al., 2010). Another is that

neuronal activity aggregates the protein complexes needed to support local translation. In this context, a role has been suggested for phosphorylation by metabotropic glutamate receptor- or NMDAR-dependent activation of kinases, such as mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR) and CaMKII (Besse and Ephrussi, 2008). Candidate proteins contributing to increased translation include ribosomes, and translation initiation factors, such as eukaryotic translation initiation factor 4 (EIF4), elongation factors, such as eukaryotic elongation factor 2 kinase (EEF2), S6 kinase, and cytoplasmic polyadenylation element-binding protein (CPEB).

4.2.3 BDNF secretion is activity-dependent.

A modified ELISA method was used to detect secreted endogenous BDNF in rat primary sensory neuron cultures (Balkowiec and Katz, 2000). The authors found that patterned electrical field stimulation (high frequency stimulation) effectively increased BDNF secretion. A less ideal detection method of BDNF secretion (overexpression of BDNF by adenoviral vectors) but using a more physiological stimulus, Theta Burst Stimulation (TBS), also demonstrated increased BDNF release following neuronal activation (Gärtner and Staiger, 2002).

The site(s) from which BDNF is secreted was investigated (Matsuda et al., 2009). Secretion events were visualized by fusing BDNF with pH-sensitive green

fluorescent protein. Interestingly, 10Hz electrical stimulation was sufficient to elicit dendritic secretion, yet axonal secretion required 100Hz stimulation.

Another form of secretion, re-exocytosis of formerly endocytosed BDNF was described recently (Wong et al., 2015). TBS of hippocampal neurons led to the release of an intracellular pool of exogenous QD-BDNF residing at postsynaptic sites. The biological meaning of this phenomenon is not clear yet, however, it is speculated to serve as a source of BDNF that can be quickly released to modify synapse transmission and strength.

4.2.4 The BDNF receptor, TrkB, is translocated to surface membranes in response to neuronal activity.

For secreted BDNF to exert its biological functions, it must bind to its receptors on the membrane of recipient neurons. Interestingly, neuronal activity also seems to play a role in trafficking of the TrkB receptor. Two studies explored TrkB surface levels in response to neuronal activation. In an early study (Meyer-Franke et al., 1998) using rat retinal ganglion cells (RGCs), depolarization was elicited either by KCl (50 mM) or by glutamate receptor activation (a combination of NMDA, 200 μM; kainate, 200 μM; and quisqualate, 200 μM) for one hour. Surface TrkB was measured by immunostaining in cells with intact membranes. Both depolarization methods increased the percentage of cells with positive TrkB signaling. A study from the Lu group showed that TBS for one hour increased

TrkB surface receptors in hippocampal neuronal cultures using additional methods including biotinylation of surface receptors (Du et al., 2000).

The possibility that activity would enhance BDNF transfer between neurons was also examined (Kohara et al., 2001). In these studies, the authors microinjected both GFP-BDNF and DsRed into the nucleus of one neuron. Transfer of GFP-BDNF to non-injected neurons visualized as the presence of GFP in non-injected - i.e. non-DsRed cells. It was reported that the intensity of GFP fluorescence was higher in non-DsRed neurons surrounded by DsRed positive axons. The increase in GFP was further enhanced when neuronal activation was increased by applying the GABAA receptor antagonist, Picrotoxin, and was abolished after applying TTX, which blocks action potentials.

5. BDNF functions are specific to the subcellular environment in which it signals.

Given activity-dependent features of BDNF production, processing, secretion, and uptake, a question that arises is whether BDNF's intracellular functions are also modulated by activity. Indeed, all of BDNF effects are ultimately registered as a result of signaling on or within the cell, raising the possibility that local signaling events may differ between subcellular domains.

Neurons are highly polarized cells whose somal and dendritic compartments differ greatly in size and structure from axons. Indeed, the linear extent and volumes of the somal and dendritic compartments are typically much

smaller than for axons, whose length can exceed 1000-fold the width of the cell body. As one manifestation of local differences in signaling, for BDNF to signal in support of survival it must signal within the somal compartment (Heerssen et al., 2004). Indeed, for dorsal root ganglia (DRG) neurons *in vitro* disrupting dynein in axons to which BDNF was applied distally abolished BDNF's survival function. Thus, while BDNF signaling in axons may play important roles, inhibiting retrograde transport of its signal to cell bodies is essential for survival.

Given evidence for the potential importance of the subcellular compartment for the effects of BDNF signaling, and evidence pointing to activity-dependent modulation of BDNF actions, I proposed herein to investigate whether activity regulates local BDNF signaling. I focused specifically on axons because very little is known of the role(s) for BDNF signaling plays in this compartment. Toward this end I created an experimental platform that allowed me to induce neuronal activation using a physiologically-consistent stimulus in a microfluidic device that allowed for studies to selectively examine BDNF signaling in axons. The results of my investigations point to an intersection between neuronal activity and axonal BDNF transport and signaling.

Chapter 1 is currently being prepared for submission for publication. The dissertation author was the primary investigator and author of this paper. Authors will be Zheng Zeng, Zhixia Shi, and William Mobley.

CHAPTER 2: ESTABLISHING A PLATFORM FOR *IN VITRO* NEURONAL ACTIVATION

Background

As yet unexplored is the possibility that, in addition to impacting other BDNF-mediated effects, neuronal activity modulates BDNF functions in axons. To address this question, two technical obstacles had to be overcome. The first was how to activate neurons effectively using physiologically meaningful stimuli *in vitro*. The second was how to create a system that allowed for examining BDNF function selectively in axons. To surmount the first obstacle, field stimulation was used. For the second, microfluidic chambers were employed.

Historically, a number of strategies have been used to activate neurons in vitro. They include increasing extracellular KCl, applying an excitatory neurotransmitter or antagonists for inhibitory neurotransmitter receptors, field stimulation, and optogenetics.

In one example, increasing extracellular potassium was used to activate neurons in an epilepsy model (Feng and Durand, 2006). Raising extracellular potassium to higher than 12mM (Xiong and Stringer, 1999) led to persistent firing in hippocampal slices, mimicking continuous epileptiform discharges in CA1 and CA3 neurons. While raising extracellular potassium is effective in inducing spiking and for increasing intracellular calcium, this stimulus produces a condition that is distinctly different from what is seen in the normal brain. Thus, instead of the normal typically phasic neuronal activity, repetitive firing typical of excessive

of neuronal activation follows application of increased levels of KCl. In addition to the concern that excessive neuronal activation may introduce non-physiological artifacts, applying KCl at high concentrations may illicit different activity-dependent biological processes. For example, patterned stimulation is essential for neurotrophin release (Balkowiec and Katz, 2000). Increasing KCl for a short time failed to stimulate primary sensory neurons to release BDNF, while patterned stimulation for the same period of time increased release 20-fold. Another study demonstrated that chronic depolarization by KCl acts to induce neuronal gene expression in a pattern different from that for patterned field stimulation. KCl was found to act through L- type calcium channels and employ the Erk pathway, while patterned electrical stimulation acted through N-type calcium channel and was independent of Erk activation (Brosenitsch and Katz, 2001).

Pharmacologically activating neurons through addition of neurotransmitters, though arguably more physiological than KCl addition, also suffers because tonic rather than phasic stimulation results. The latter is typical of neuronal circuits *in vivo*. Thus, addition to cultures of glutamate or the GABAA receptor antagonist picrotoxin while widely used (Yuste et al., 2011) induces bursting in neurons whose temporal features may not conform to the physiological context.

Field stimulation provides another way to activate neurons *in vitro*. This paradigm uses current delivered in a Theta Burst Stimulation (TBS) pattern

(Gärtner and Staiger, 2002)(Du et al., 2000)(Guo et al., 2014)(Brosenitsch and Katz, 2001)(Wong et al., 2015). TBS stimulation more closely conforms to the physiological situation in that it mimics two characteristics of neuronal physiology, especially as registered in hippocampus: the theta rhythm frequency of 5 Hz and the complex-spike burst of 100Hz (Larson and Munkácsy, 2015). Though different parameters for TBS have been used in the many studies in which it has been employed, a universal feature is that a minimum unit is one burst, which consists of 4-5 pulses delivered at 100Hz (i.e. 10ms interval between pulses), and that bursts are spaced by 200ms intervals (i.e. at a burst frequency of 5Hz). In examples, some studies (Gärtner and Staiger, 2002; Brosenitsch and Katz, 2001) used 25 bursts every 20s; while others employed 4-5 bursts every 5 s (Du et al., 2000)(Guo et al., 2014) and (Wong et al., 2015).

Another recently employed method for neuronal activation uses optogenetics. After introducing into neurons constructs encoding channel-rhodopsin, a cation channel that opens in response to light stimulation, neurons can be activated by pulses of blue light (470nm).This tool has enjoyed widespread use and has made it possible to activate neurons using well-defined and controlled stimulation paradigms in vitro and in vivo (Deisseroth, 2011).

A second important technical hurdle was creating a platform that allowed us to selectively examine the impact of neuronal activation on BDNF function in axons. For this, we turned to microfluidic chambers. Taylor et al. described a PDMS-based culture platform that allows for the growth of CNS axons into an

environment in which they are fluidically isolated from neuronal somas and dendrites (Taylor et al., 2005). The dimensions of a chamber are as follows: compartments (100um in height X 1500um in width X 7mm in length) are connected by microgrooves (3um in height X 10um in width X 450um in length). Given our lab's experience using such chambers, and the ability using them to engage in biochemical, morphological and real-time imaging, this platform was chosen for my studies.

Methods

Neuronal dissection and culture. E18 embryonic rat cortices were isolated, dissociated with Trypsin, and titrated with fire-polished Pasteur pipettes. Dissociated neurons solution ($3X10^7$ cells/ml) was added to one compartment of microfluidic chambers. Neurons were cultured in plating medium (10% bovine serum, B27 (Gibco, 17504-044), GlutaMax (Invitrogen, 35050-061) and Neurobasal (Gibco, 21103-049) on the first day. From the second day, neurons grew in maintenance medium, which was identical with plating medium except omitting serum. The medium was replaced, 2/3 volume per change, every other day until experiments. By about 3-4 days, neurons whose cell bodies lie close to microgrooves extend their axons through the grooves to reach the other side of compartment, which I refer to herein as the axon compartment.

The system used to stimulate neurons was as follows: a LabView-DAQ board sends electrical signals whose pattern conforms to TBS. Since the initial

signal has a constant voltage at 5V, it is connected with an isoflex isolator (A.M.P.I.) with which the signal can be adjusted. The output of the isolator is delivered to the cell body compartment of a chamber; this is followed in series by a resistor (15k Ω). An oscilloscope is connected in parallel to the resistor to measure voltage in real-time (Figure 1a). This approach, using series circuit design, allows one to monitor current flow through the chamber by calculating current through the resistor (Figure 1b). The bath electrodes that go into the culture media of chambers are Ag/AgCl pellets (World Precision Instruments, EP2). The TBS paradigm chosen was the same as for an earlier study (Wong et al., 2015): 5 bursts were delivered over 1s (i.e. burst frequency of 5Hz) followed by a 4 s silence. In several experiments, this pattern was repeated for a total 12 times over 1 min.

Since the somas and processes of neurons are covered in PDMS chambers, they are not accessible for direct electrophysiological recordings. Calcium imaging was therefore used to detect the changes of intracellular calcium as a measure of neuronal activity (Smetters et al., 1999). Fluo-4 from Molecular Probes® was used for calcium imaging. The binding of calcium ions causes fluo-4 to undergo an intramolecular conformational change leading to a change in fluorescence properties with increased light emission. Neuron cultures in microfluidic chambers were incubated with Fluo-4 at 37C for 30min and at room temperature (21C) in the dark for 5-10min. After multiple rounds of gentle washing with maintenance medium, chambers were imaged. Calcium imaging

was carried out using an inverted fluorescent microscope equipped with an EMCCD camera. Chambers were placed in a live imaging unit controlled for temperature (37C) and 5% CO. X-cite Arclight (set at 2-5% intensity), FITC filter cube, and an oil-immersed 100X objective were used. Fluorescent signals were recorded in Stream Acquisition in Metamorph software throughout imaging sessions: 30sec pre-, 1min during-, and 30sec post TBS. The acquisition rate was 31fps, and exposure time was 10ms. The digitizer was 10MHz with 50 EM gain.

Results

The first objective was to demonstrate that well-defined conditions could be developed to activate neurons in microfluidic chambers. After defining conditions for calcium dye loading and imaging, we defined the minimum current needed to activate neurons in microfluidic chambers. A graded series of currents was applied to the cell body compartment while calcium imaging proximal axon segments (i.e. those at the entrance of the microgrooves) were recorded. The goal was a current that consistently elicited a calcium response; 9-16 chambers were tested to define optimal current values. I found that 150uA was optimal in reproducibly eliciting responses across multiple chambers. A typical successful activation is shown in Figure 1c-e. For neurons that responded to every train of stimulation over 1 min, 12 calcium signal peaks would be detected (figure 1c). Note in Figure 1c and d the presence of repeated spikes of calcium signal

corresponding to individual trains of delivered current. Note also in Figure 1d the “sawtooth” appearance was created by individual bursts of current. Thus individual bursts and trains of stimulation were faithfully captured by the increase in fluo-4 signal (Figure 1c, d,e) and the responses were tightly aligned with the temporal pattern of TBS. In contrast, 100uA evoked full responses in some chambers but only partial responses in others. While the average response rate was ~35% (e.g. 5 responses out of 12 trains of TBS stimuli; Figure 2a), variability in responsiveness resulted in a large standard deviation (SD; error bar) for responses to 100uA (Figure 2a). Similarly, delivering a 300uA current failed to consistently activate neurons, resulting in response rates significantly lower than for 150uA.

In the cases where stimulation elicited calcium responses, the intensity of the increases in signal was quantified. After normalization to baseline fluorescence, the increase in signals was approximately 5%; no significant differences were found between different currents (Figure 2b). Therefore, the difference between 150uA and 100 or 300uA is explained not by the size of induced calcium influx, but possibly by one or more other properties under which neurons responded to different current densities, as reflected in calcium influx. Taken together, 150uA was found to reliably excite cortical neurons in the microfluidic chambers.

It is interesting to compare the results of these studies with theoretical values. Current density threshold is used to calculate the minimum current

needed to excite neurons. The literature shows (Tehovnik et al., 2006) that a range of current densities (600-1000uA/mm^2) is effective in exciting cortical neurons. In the microfluidic chambers, the area of the cell body compartment into which current was introduced is 0.1mm (by height) by 1.5mm (by width) = 0.15mm^2. Using established values, the calculated minimum current needed for exciting cortical neurons in my system was calculated to be ~ 90-150uA, a value consistent with my findings. Thus, the 150uA value discovered to reproducibly activate neurons in my studies is fully consistent with predictions for cortical neurons provided by others.

An important question is whether current injection introduces damage to neurons. To address this question, chambers were repeatedly stimulated, and calcium responses across trials were compared. As one manifestation of damage, neurons may demonstrate less robust calcium responses after repeated trials. No such evidence was discovered under the conditions used in these studies. Neurons continued to demonstrate robust responses after repeated 150uA stimulation. In trials in which 150uA was repeatedly delivered, neurons continued to show 100% response rates (i.e. 12 calcium peaks in response to 12 trains of TBS). Moreover, the increase in the amplitude of calcium signals was relatively constant across trials (Figure 2c,d).

After establishing 150uA as the stimulating current to be used, I next asked if depolarizing cell bodies would evoke current changes in distal axons, again as measured by increased calcium influx. Accordingly, the calcium

response was imaged at different locations along axons. The first location imaged was where axons exited microgrooves (i.e. at least 450um distant from those cell bodies adjacent to the entrance of microgrooves). The responses in axons were registered as transient increases in fluorescence with the same characteristics as those seen in proximal axons. Next, the imaging field was moved consequently and one field at a time (512X512 pixel, as imaged at 100X lens, results in a distance of 80um) to more distal portions of the axon. The increases in calcium signal were of the same size as in proximal axons (figure 3c). As for proximal axons, distal axons responded to TBS with changes in calcium flux that tightly mirrored in time the timing of stimulus application.

It is worth noting, that as the field of view was moved distally it was more difficult to detect calcium signal responses. Two factors may contribute. The first is that it was easier to define a relevant field in which many axons were present, sometimes bundled and overlapping. For example, in locations close to microgrooves, even a small region of interest (ROI) area included multiple axons. In locations distant from the microgroove, axons were more spread out and the ROI contained one or very few axons. Considering the fact that not every neuron and its axon were activated by field stimulation, the chance of failing to see a response would be higher in locations containing fewer axons within an ROI. Another possible reason as to why imaging distally showed less frequent responses is that direct current-induced depolarization is less in distal region. The two compartments of microfluidic chambers are fluidically connected via a

conducting salt containing solution, so that current delivered to the cell body compartment will unavoidably spread to axon compartment. Because passive spread degrades significantly over distance, the direct effect of current on neuronal membranes distant from the site of current delivery will be negligible. As a result, it is reasonable to assume that the calcium response imaged from proximal axons is a combination of direct current-induced depolarization and propagating action potentials. The response in the most distal aspect of axon chambers would result only from propagated action potentials. In any case, positive responses with similar deltaF/F values were observed in axons at least 2mm away from cell bodies, proving that delivering current to the cell body compartment elicited activation of axons in the axon chamber, including regions relatively distant from cell bodies.

Conclusion

I established an *in vitro* platform for neuronal activation that was compatible with a device that enabled me to fluidically isolate axons from their cell somas. Empirically testing a series of current amplitudes, it was discovered that a 150uA current effectively activated neurons, as measured by calcium imaging. The resulting current was found to be in good agreement with earlier studies defining the range of current densities that effectively activate the cortical neurons, as are used in these studies. Evidence that the current delivered was consistent with maintenance of neuronal function was that repeatedly stimulating

with 150uA resulted in no decrease in the response rate or change in calcium flux. Finally, it was demonstrated that the stimulating parameters employed enabled activation of axons distant from cell bodies. I conclude that the experimental platform thus established is well suited to examine what effect neuronal activation may have on BDNF signaling in axons.

Chapter 2 is currently being prepared for submission for publication. The dissertation author was the primary investigator and author of this paper. Authors will be Zheng Zeng, Zhixia Shi, and William Mobley.

CHAPTER 3: NEURONAL ACTIVATION ENHANCES AXONAL SIGNALING OF BDNF

Background

With an experimental platform in place that enables studies of neuronal activity on BDNF signaling in axons, I asked if Theta Burst Stimulation (TBS) measurably impacted axonal signaling of BDNF. BDNF binding elicits dimerization of TrkB receptors, followed by autophosphorylation in the catalytic domains at two key amino acids: Tyrosine 515 (Y515) and Y816. This enables recruitment of effectors that induce activation of downstream signaling pathways, thus translating BDNF/TrkB binding into a complex series of commands that influence cellular events ranging from changes in gene expression to neuronal structure and function. Three classical signaling cascades are activated by BDNF binding to TrkB . Y515 mediates both the Erk and AKT pathways; Y816 engages the PLC gamma pathway. Y515 phosphorylation serves as a docking site for recruitment of several different adaptor proteins, including Shc. Directly and indirectly, Shc initiates the activation of Erk pathway (extracellular signal-regulated kinases, which belongs to family of mitogen-activated protein kinases, MAPK). Ras activates protein kinase Raf, which in turn phosphorylates Mek1 and/or Mek2, which then phosphorylate and activate Erk1 and Erk2. The Erk pathway is involved in neuronal differentiation, in part through phosphorylation of Elk-1(Besnard et al., 2011). Once phosphorylated, Elk-1 translocates from

cytoplasm to nucleus, thereby initiating transcription of target genes. Other facets of Erk activation also contribute to neuronal structure and function.

The second classical BDNF signaling pathway is that mediated by AKT through phosphatidyl inositol-3 (PI3)-kinase (PI-3K). In addition to other effects, the AKT pathway is involved in neuronal survival through at least three distinct mechanisms. Firstly, AKT degrades IkB, which frees NFkB, thereby promoting gene transcription for promoting sensory neuron survival (Hamanoue et al., 1999). Secondly, AKT inhibits a pro-apoptosis messenger, BAD, by sequestering it with 14-3-3 proteins (Yuan and Yankner, 2000). Thirdly, AKT phosphorylates and restricts the retention of some pro-apoptosis transcription factors, such as FKHRL1, in cytoplasm (Brunet et al., 2001). Taken together, PI-3K/AKT pathway serves as one means by which BDNF enhances neuronal survival.

The third classical BDNF signaling pathway is PLC gamma pathway. This pathway generates inositol tris-phosphate (IP3) and diacylglycerol (DAG) that results in release of calcium from internal stores and activation of protein kinases C (PKC) respectively (Reichardt, 2006). As for Erk and AKT, activation of PLC gamma appears to support several neuronal functions.

The elucidation of BDNF signaling pathways creates the opportunity to define signaling in sub-cellular compartments. NGF, another member of the neurotrophin family, has been extensively studied in this regard. Studies of NGF speak to its role as target-derived neurotrophic factor for sensory and sympathetic neurons. That NGF is synthesized and released in targets of

innervation imposes the requirement that activation of its receptors on presynaptic terminals is somehow communicated retrogradely to distant cell bodies. Indeed, very different effects have been registered for NGF located in presynaptic terminals versus cell bodies, pointing to possibly unique signaling events defined by the local context in which they are created (Campenot, 1977). NGF acts through two distinct mechanisms to activate Erk, one through TrkA internalization-dependent engagement of Rap1, the other is through Ras that is independent of TrkA internalization (York et al., 2000). In PC12 cells, NGF promotes differentiation and cell survival. Inhibiting internalization of the NGF-TrkA complex inhibited neurite formation but promoted cell survival effects (Zhang et al., 2000). Therefore, at least in PC12 cells, the location of NGF-TrkA complex determines which subset of signaling pathways is activated and which end effect of NGF is registered at the macroscopic level. A similar theme can be found in DRG neurons. NGF-induced axon growth is dependent on the co-localization of AKT and GSK3-beta in the tip of distal axons (Zhou et al., 2004). While there is much to learn about the local effects of NGF signaling, very few studies address the impact of local – i.e. subcellular BDNF signaling.

Among the numerous downstream targets of the above-mentioned pathways, CREB has served as a prominent focus. The CAMP response element-binding protein (CREB) is a transcription factor that once phosphorylated binds to CRE, a conserved DNA sequence present in the promotor/enhancer region of a number of genes whose products contribute to

neuronal structure and function. CREB binding initiates these transcriptions. The Greenberg group identified two pathways involved in NGF-induced CREB phosphorylation: 1) NGF—Erk—RSK (pp90 ribosomal S6 kinases)—CREB; 2) NGF—P38 MAPK—CREB (Xing et al., 1998). On the cellular level, Ginty and colleagues established that after NGF application to distal axons, activation of TrkA in cell bodies is necessary for phosphorylation of nuclear CREB (Riccio et al., 1997). This suggests that pCREB can serve as a read-out for active transport of NGF-TrkA from axon terminals to cell bodies. Interestingly, Cox and colleagues (2008) showed that CREB was locally synthesized in axons in response to NGF and that it was actively transported together with NGF-TrkA complex to nucleus. Based on their measurements, ~75% of NGF-induced pCREB signal in nucleus was attributed to the axonally translated CREB. This suggests that that nuclear arrival of pCREB signal after NGF application to axons is a function of NGF-TrkA complex formation, local synthesis of CREB, and retrograde axonal transport of pCREB. Given these findings, the arrival of nuclear pCREB following axonal exposure to neurotrophins may serve to define the timing of axonally-based BDNF signaling. By extension this measure may be helpful in understanding how BDNF signaling in axons is transported to the nucleus.

Methods

Axon lysates were collected at ~ DIV 8. For this step, culture medium was added to fill the cell body side, then PBS was used to quickly wash the axon compartment. After removing PBS, the contents of axon compartment were lysed with 35ul lysis buffer (1% Triton, 150mM NaCl, 50mM Tris-HCl, cOmplete™ proteinase inhibitor cocktail). By directing the pipette tip at the openings of the axon compartment and pipetting up and down, axons were efficiently lifted off the plate and were then dissolved in lysis buffer. Standard methods were used for SDS/PAGE and western blotting. For immunoblotting I used the following steps: 1) primary antibody was diluted 1:200 in a commercial enhancer reagent 1(Nacalai USA cat.NU00102); 2) the HRP second antibody was diluted 1:2000 in enhancer reagent 2 from the same kit. Extensive washing with Tris-buffered saline with Tween 20 (TBST) was used after removing both the primary and secondary antibodies. For detection, Radiance Plus, a sensitive HRP substrate, was used for chemiluminescent detection (Azure Biosystems, SKU: AC2103). Antibodies used included pErk (rabbit monoclonal antibody from Epitomics, discontinued); total Erk (Cell Signaling, 4695s); pAKT (Cell Signaling, 4060s); and total AKT (Cell Signaling, 2920s).

Immunostaining of nuclear pCREB was also carried out at ~ DIV 8. As in other studies, BDNF was applied exclusively to the axon compartment. Somas were fixed at different time points by adding 4% freshly made paraformaldehyde (PFA) in 4% sucrose for 30min at 37C. Fixed cells were permeablized using 0.1% Triton X, and were blocked by 3% BSA plus 5% goat serum. The pCREB

antibody was against Ser 133 (1:500, Cell signaling, 9191)(Cohen et al., 2016); DAPI staining was employed to define nuclei.

Results

The design of microfluidic chambers makes it possible to selectively collect axonal protein. By keeping the fluidic level of the cell body compartment higher than the axon compartment, lysis buffer will be present in only the axon compartment in which it acts to dissolve axons and collect axonal proteins. That this was the case was confirmed by testing with a dye solution with physical properties similar to the lysis buffer; applied precisely as for lysis studies, there was no evidence for the dye entering the cell body side (figure 4a,b).

An important requirement was that I needed to collect sufficient axonal material to allow for immunodetection. Ideally, the protein present in a single chamber would be sufficient for visualization via western blotting. After many trials, a protocol was developed that allowed for this, as detailed in Methods. To confirm that this protocol allowed for reproducible results, dose response and time course studies were carried out to explore the responses to BDNF. As expected, neurons starved (i.e. cultured without B-27/GlutaMax for 2 hours) showed very weak or absent pErk or pAKT signals. This was evidence that a low background signal could be achieved and that no false positive signals arising from the high concentration of antibody use and etc. Using a series of final concentrations of BDNF (1, 3.3, 10, 33, and 100ng/ml), I found that pErk or

pAKT, as normalized by total total Erk or total AKT, increased in proportion to BDNF concentration. The EC50 for both pErk and pAKT approximated 3.3ng/ml (figure 4c,d,e,f); BDNF at 3.3ng/ml is ~127pM, pointing to the participation of TrkB-containing high affinity receptors in the responses, as expected. To avoid saturating the response of neurons to BDNF, 3.3ng/ml was chosen for all later studies. The time course study showed that signals peaked at around 15min, with lessening over the next 30min and beyond. This time course differs in two respects from those for mass cultures; the peak response is delayed by about 10min and the decline in signal is slower (unpublished data from Xuqiao Chen in our laboratory). It is noteworthy that in mass cultures BDNF accesses receptors and signaling pathways in all subcellular compartments and that all cellular protein is collected. Differences in the number and distribution of TrkB receptors and in the local processes that act to degrade BDNF/TrkB signals may be responsible for the differences seen. Indeed, a typical pyramidal cell in cortex, TrkB receptors can be found in both soma and processes, both axons and dendrites (Gomes et al., 2006).

Once the dose- and time-dependencies of BDNF responses were established, I designed a paradigm that would allow me to explore the impact of neuronal activity. I chose to apply 3.3ng/ml BDNF at time 0 and then at 15 min to apply a 1min period of TBS, a time at which BDNF signaling was maximal. The intent was to see if TBS would increase the response to a non-saturating concentration of BDNF. To explore possible differences in BDNF signaling due to

TBS, axons were harvested at a series of time points following either TBS or no activation, the control condition. AKT signaling in the control condition remained relatively high at 30min (75% of the peak), falling to ~ 50% at 60min and to ~40% at 120 min. Following TBS, there was increase in AKT signaling such that there was little if any decrement between 30 and 60 min (Figure 5 a,b). For Erk signaling, though TBS was associated with a trend to increased signaling through 120 min, the differences failed to reach statistical significance.

More samples will be tested before final conclusions can be reached as to whether or not TBS induces differences for Akt versus Erk signaling, but earlier findings may help understand the potential differences. BDNF-induced AKT signaling was dependent on endocytosis (Zheng et al., 2008), but Erk signaling was only partially impacted. Therefore, AKT activation may occur when BDNF-TrkB complex has been internalized, while the extent of Erk activation is less dependent on this step. Therefore, it is possible that in my studies, in which TBS was applied 15min after addition of BDNF, that signaling measures susceptible to TBS were most reflective of those originating from internalized receptors. More observations will be needed to test this idea and to explore differences in signaling that might be induced by applying TBS prior to the addition of BDNF.

In parallel to measuring BDNF axonal signaling, I assessed the time of arrival of a detectable signal for nuclear pCREB. The goal was to ask whether or not TBS impacted the timing of delivery of retrogradely transported BDNF signals. Using immunostaining, nuclear pCREB was rarely detected up to 30min

after applying BDNF to axons. Thereafter, the signal increased significantly such that a definite signal was present by 45min, when ~ 80% of nuclei were labelled. Because not every neuron sent an axon to the axonal compartment, it was not surprising that the maximum percentage of pCREB positive cells was only 80%. I noted that when TBS was injected 15min after BDNF application to axons that the presence of nuclear pCREB signaling was significantly later. While only 20% cells were nuclear pCREB positive at 45min, this value did increase to near control levels at 60 min (Figure 6). Given earlier findings (Riccio et al., 1997), the delay in nuclear pCREB points to a delay in the arrival of the BDNF/TrkB signaling complex to the soma.

Conclusion

In this series of studies I showed that neuronal activation, as achieved using a brief period of TBS enhances signaling of BDNF in axons through prolonged activation of AKT. Moreover, TBS resulted in a significant delay in the detection of nuclear pCREB, pointing to a change in both BDNF signaling in axons and to a delay in transmitting axonal signals to the neuron cell body. To our knowledge these findings are the first to define an effect of neuronal activation on BDNF signaling in axons.

Chapter 3 is currently being prepared for submission for publication. The dissertation author was the primary investigator and author of this paper. Authors will be Zheng Zeng, Zhixia Shi, and William Mobley.

CHAPTER 4: ACTIVITY-INDUCED INHIBITION OF BDNF AXONAL TRANSPORT CONTRIBUTES TO THE ENHANCED BDNF SIGNALING

Background

Evidence that neuronal activation increased BDNF signaling in axons, and delayed the BDNF-mediated increase in nuclear pCREB suggested that one means by which activity influenced BDNF signaling in axons could be a change in BDNF axonal transport. That this topic had not been examined previously, and would represent an unexpected finding, prompted studies of TBS on BDNF transport in axons. Because of the highly polarized morphology of neurons, the transport of organelles including mitochondria, lysosomes, autophagosomes, and endosomes between axonal terminals and somas to support neuronal function is an important biological theme (Maday et al., 2014). This active process is referred to as axonal transport. The transport machinery includes microtubules, motors (and their adaptor and scaffolding proteins) and cargoes. Each of these elements contributes fundamentally to the characteristic features of transport. Theoretically, changes in any component could lead to changes in the transport of axonal cargoes destined for the soma.

Axonal transport employs microtubules. In axons, parallel microtubules form a unipolar array with plus-ends oriented away from the soma. Modifications may directly target microtubules or microtubule-associated proteins (MAPs), such as Tau, which modulates the stability of microtubules and interaction of motors with the microtubule (Dixit et al., 2008).

Another component of transport machinery subject to modification is the dynein motor and its many subunits and interacting proteins. Dynein is a complex of several different chains of different sequences and sizes whose function is to move cargoes toward the minus end of the microtubule. Dynein subunits include two heavy chains (HC), two intermediate chains (IC), two light intermediate chains (LIC), and three light chains. The heavy chain interacts with six AAA+ ATPase modules that provides the chemical energy for the mechanical movement of motors. All dynein subunits are subject to post translational modification. Additionally, some modifications are mediated through dynactin and other effectors of dynein. One example is BICD1, which was shown to control the trafficking of activated neurotrophin receptors for degradation to balance neuronal responses to neurotrophin stimulation (Terenzio et al., 2014). Another dynein effector Lis1 binds directly to the dynein motor domain, to uncouple ATP hydrolysis from force production, leading to sustained attachment of the motor to the microtubule (Huang et al., 2012).

Kinesin, and anterograde motor, may also serve as a target for modifying axonal transport, since many axonal cargos have both motor types bound simultaneously. The kinesin superfamily members expressed in human brain total 38 in number; motors from the kinesin-1, kinesin-2, and kinesin-3 subfamilies contribute to axonal transport. Kinesin-1 motors are formed by a dimer of kinesin heavy chains (encoded by three mammalian genes, KIF5A, B and C) (Maday et al., 2014). Switching between kinesins and dynein, and

differences in the relative number of kinesin and dynein motors attached to a cargo, would directly impact transport kinetics.

To study axonal transport, it was necessary to visualize cargo. Traditionally, this has been achieved by plasmid-transfecting cells with a fusion between a target protein and GFP or mCherry. There are two limitations for this methodology in studying BDNF axonal transport. The first is that BDNF is produced by cortical neurons; thus, movement of a labeled BDNF in axons is both secretory and endosomal. In the former case, the compartment labeled would be devoid of TrkB binding and activation. For this reason, movement of BDNF labeled in this fashion would not serve as a proxy for movement of BDNF/TrkB signaling endosomes. Another limitation is the size of the signal emitted by either GFP or mCherry. While initially not well appreciated, our lab found that signaling endosomes may carry a single neurotrophin dimer, thus enforcing the requirement for a label with a very strong fluorescence signal to noise ratio. For these reasons, I added exogenous BDNF molecules attached to Quantum dots (Qdots) to the axon compartment to mark retrogradely transported BDNF.

Methods

Visualization of BDNF was achieved by attaching Qdot® nanocrystal to BDNF through biotin-streptavidin binding. Biotinylated BDNF production followed an established protocol (Zhao et al., 2014). Briefly, HEK293 cells were co-

transfected with two plasmids expressing BDNF-Avitag (a biotin receiving site) and a biotin ligase enzyme, BirA. The supernatant of culture media was collected and used to purify biotinylated BDNF. This biotinylated BDNF allows for addition and high affinity binding of streptavidin-conjugated Qdots to BDNF without loss of activity. Qdots demonstrate long term photostability. By adjusting reaction conditions, it is possible to attach an average of one Qdot to each BDNF dimer.

Dissociated neurons were seeded in microfluidic chambers as described above. Within about a week *in vitro*, neurons sent axons through the microgrooves to reach distal axon compartment. 5-10ul biotinylated BDNF and 0.5ul Qdot 655–Streptavidin (Q10121MP, Thermo Fisher) were incubated on ice overnight to form QDot-BDNF, and the mixture was applied to the axonal compartment. After waiting 2-3 hours to allow for binding and endocytosis of QDot-BDNF, retrograde axonal transport was imaged in the microgrooves of the compartmented chamber using an inverted fluorescent microscope (LEICA DMI6000B), with a 100X oil-immersed objective lens and HCRed filter cube set (Chroma 41043, 575nm ex/640nm em). Images were acquired by Metamorph ® Software (Molecular Devices) at 1fps with 200ms exposure. In order to prove the signals observed in microgrooves are results of specific BDNF binding and endocytosis, Cui at al. did multiple controls: streptavidin-Qdots alone, and Qdot-NGF plus a 1000-fold excess of unlabeled NGF (Cui et al., 2007), none of the conditions yielded any Qdot signals in cells. Kymographs and videos were automatically generated with a custom-coded macro program in Image J (NIH).

Velocity were calculated with Kymo-Toolbox plugin in Image J by dividing distance projected to X axis of kymograph with total time on Y axis. The percentage of Qdots that were stationary (stalled) and moving was calculating by manually tracking the number of signals in kymographs during each 2min imaging session. In the single-groove-imaging experiment, two data were collected for each QD-BDNF: the time when the QD-BDNF first entered the observed segment of microgrooves and its velocity.

Results

BDNF axonal transport was examined in two different sets of experiments.

In the first set of experiments, sampling first measured baseline movement in a set 10 to 15 grooves. Then after TBS current was injected, imaging resumed in the same grooves. The goal was to sample a consistent set of axons and to data to best represent the overall result, thus limiting the variability in responses between grooves.

The first outcome of these studies was that TBS for 1 min significantly reduced the speed of retrograde BDNF transport. “Average Velocity’ refers to the average speed of movement of individual Qdots over the 2 minute period of observation. As in other studies examining Qdot BDNF, the data show differences in speed that vary greatly from Qdot to Qdot in both the control and TBS conditions. Following TBS, the speed decreased by about 30%. In addition, the percentage of stalled BDNF-containing endosomes was increased by

stimulation (Figure 7b,c). Stalling refers to QD-dots that failed to move during the 2min imaging session, an event which results in vertical lines in kymographs. Note that the velocity measurement excluded dots that stalled. Thus, the two measures are independent, possibly reflecting different molecular events impacting distinct subsets of QD-BDNF.

Even though the groove sampling procedure just referenced allowed me to examine a large population of axons, it made it difficult to collect temporal information. Thus, it was unclear how quickly the effect of TBS was registered and how long its effects persisted. To collect these data, a single groove per chamber was examined throughout a course of 40 min. I first imaged the groove for ~10min to measure baseline movement. Then I then delivered TBS for 1min (i.e. 12 trains). At the same time, images were captured. Quantification of average velocity was about 1.75um/sec before TBS, dropped by about 30% in the first 7 min after applying TBS, and decreased by another 20% to 1um/sec for the next 13min. At approximately 20min after TBS, I noted that average velocity showed increases, but not to the initial, pre-TBS value. Similarly, by comparing the same four periods for flux, average flux decreased to almost zero 7min after TBS; 20min after TBS, flux partially recovered. It will be important to further pursue the time course of changes induced by TBS. Nevertheless, these findings are evidence that a brief period of TBS is sufficient to reduce both the velocity and flux of retrograde transport of BDNF-containing endosomes and that the

changes were present during the time for measurements of BDNF axonal signaling reported in Chapter 3.

If one compares the two different sets of BDNF axonal transport experiments, the “same groove imaging” proves to be more sensitive for uncovering changes in transport. The reason is mainly because in pooling grooves one combines grooves with different axons, with different kinetics and possibly with somewhat different responses to TBS. Serial imaging of individual grooves may be useful for the enhanced temporal resolution needed for studies to explore mechanisms through which activity interacts with transport. Nonetheless, a consistent phenomenon was observed: by slowing and in some cases stalling endosomal movement, TBS reduced the flux of BDNF retrograde transport.

To explore the link between TBS effects on BDNF transport and signaling, it was important to ask if the two findings were related. One way of doing so was to test whether an independent manipulation that inhibits transport of BDNF would enhance axonal BDNF signaling. The criteria for the proposed manipulation were that it: 1) should be relatively specific to retrograde transport; and 2) should act relatively quickly, aligning with the latency of the TBS effect. I first considered a pharmacological intervention, nocodazole. This drug inhibits axonal transport by interfering with tubulin assembly (Samson et al., 1979). However, its specificity is low and effects take 2-4 hours to be registered. A

similar or even longer (days) delay in onset of effects would attend plasmid-based overexpression of a protein targeting transport machinery.

After reviewing a panel of reagents, Ciliobrevin D was chosen. Firstly, it inhibits dynein. Firestone and colleagues (Firestone et al., 2012) showed that Ciliobrevin D effectively collapsed spindle formation in NIH3T3 cells, inhibited melanosome aggregation in *Xenopus Melanophores*, and reduced peroxisome motility in *Drosophila* S2 cells. These biological processes are heavily dependent on dynein. Importantly, Ciliobrevin D was also successfully used in chicken DRG primary neurons (Sainath and Gallo, 2015) in which it inhibited transport of mitochondria, lysosomes, and Golgi-derived vesicles in axons. Additionally, it has been shown to abolish NGF-induced formation of axonal filopodia and branching. These independent lines of evidence collectively support its role in inhibiting dynein and utility for studies in neurons. A second reason for choosing Ciliobrevin D was specificity. Ciliobrevin D did not perturb cellular mechanisms independent of dynein function, including actin cytoskeleton dynamics and the mitogen-activated protein kinase and phosphoinositol-3- kinase signaling pathways. In addition, Ciliobrevin D did not broadly target other members of the AAA+ ATPase family, as it had no effect on p97-dependent degradation of endoplasmic reticulum-associated proteins or Mcm2-7-mediated DNA unwinding. The third reason for this choice is the rapid onset of action (Firestone et al., 2012). The drug is cell membrane permeable and acts on the scale of minutes. The fourth reason is reversibility of effects. In NIH3T3 cells, Ciliobrevin D effects

could be reversed 7 minutes after washing off the drug. This property also aligns with the TBS effect. Finally, it is a small molecule (393g/mol, $C_{17}H_8Cl_3N_3O_2$), which makes it practical to apply in microfluidic chambers, including the need for it to diffuse into microgrooves.

Before testing Ciliobrevin D's effects on axonal signaling of BDNF, I tested its effects on transport. Using microfluidic chambers, the level of the solution on soma side must be higher than that on the axon side to prevent QD-BDNF from passively diffusing into microgrooves. To ensure that Ciliobrevin D reached the microgrooves, the drug was applied on the soma side. To test how long it takes for the soma side solution to diffuse into the microgrooves, Trypan blue was used to visualize diffusion because of its similar molecular weight (873g/mol) to Ciliobrevin D (393g/mol). It was observed to penetrate the entire 450um segment of microgrooves, thus reaching the axon compartment within 30sec. Next, QD-BDNF movement was tracked in individual grooves before, during and after adding 20uM Ciliobrevin D. This confirmed the drug's effectiveness in inhibiting transport; indeed, essentially all movement of QD-BDNF ceased minutes after applying Ciliobrevin D (figure 9).

Next, I asked if the drug replicated TBS effect in enhancing axonal BDNF signaling. Similar to the timing of TBS, Ciliobrevin D was added 15min after applying BDNF on the axon side and axonal lysates were collected at 30, 60, and 120min. Both pErk and pAKT (normalized to total Erk and AKT) increased under the drug treated condition, thus phenocopying the TBS effect (figure 9).

It is worth noting that increased Erk activation at 30min was seen with Ciliobrevin D but not TBS. A possible explanation is that TBS inhibited BDNF transport to a lesser degree than Ciliobrevin D; indeed traffic stalled much more dramatically with Ciliobrevin D (figure 7,8 and 9). Another observation is that enhanced signaling was not sustained 120min after applying BDNF (105min after adding Ciliobrevin D). This surprising finding suggested that intra-axonal mechanisms exist by which to reduce BDNF signaling. One speculation is a feedback mechanism is engaged when the BDNF-TrkB complex stalls for prolonged period. Future studies will be needed to pursue the means by which intra-axonal downregulation of BDNF signaling occurs.

Conclusion

A number of potential mechanisms can be envisioned for how neuronal activity impacts BDNF signaling in axons. Herein we explored the possibility that changes in the retrograde transport of BDNF/TrkB complexes played a role. Our findings support this mechanism and invite additional studies into how neuronal activation impacts BDNF axonal transport and signaling.

Chapter 4 is currently being prepared for submission for publication. The dissertation author was the primary investigator and author of this paper. Authors will be Zheng Zeng, Zhixia Shi, and William Mobley.

CHAPTER 5 DISCUSSION

Is the discovery that activity inhibits axonal transport novel?

I found that neuronal activation inhibits BDNF axonal transport. However, another research group observed an increase of transport in response to activation (Wang et al., 2016). How can we explain this discrepancy? There are several key differences between the studies. Firstly, we used different probes to trace the BDNF-TrkB complex. I used labeled BDNF, while Wang and colleagues used the Cholera toxin B subunit (CTB). CTB is believed to enter intestinal cells by binding to ganglioside GM1(Holmgren et al., 1975), but how it enters neurons is unknown. Their immunostaining data showed that only 50% of CTB-labeled vesicles were TrkB positive, and that only 30% of TrkB positive puncta were co-labelled with CTB. Therefore, one may question whether CTB is an appropriate probe for TrkB-containing endosomes. Secondly, I used BDNF as a proxy for BDNF-TrkB signaling endosomes. This is a well-accepted marker because BDNF continues to bind to TrkB during endosomal transport. In addition, I showed that BDNF was necessary for the changes in signaling induced by TBS because the latter alone failed to induce any signaling changes in the absence of BDNF. In contrast, Wang and colleagues did not show that CBT co-localized activated TrkB. Raising further concerns for CBT labeling of signaling endosomes, the authors showed that blocking endogenous BDNF did not affect activity-induced increases in CBT flux. Taken together, it is unclear that CBT labeled transport is a reliable marker for signaling endosomes.

Even though my result is the first to directly show that neuronal activity modifies BDNF transport, other cargos have been investigated. For example, axonal mitochondria were less mobile in response to neuronal activity (Chen and Sheng, 2013). Therefore, activity may have consequences for movement of a number of organelles in axons. As an interesting side note, neuronal activity also inhibits dendritic spine motility (Oray et al., 2006) a morphological change defined as the change in spine length versus time. Thus, neuronal activity may be important for changes in structure, some of which run counter to expectations.

Given the impact of activity on BDNF signaling demonstrated herein, future studies of BNDF transport may benefit by insights from mechanistic studies for activity-dependent regulation of transport of other organelles. The best studied case is mitochondria transport. Mitochondrial Rho GTPase (Miro) (Fransson et al., 2003) has Ca2+-binding domains, and binds to the kinesin-1 adaptors. Elevated calcium levels resulting from higher neuronal activity cause the dissociation of kinesin-1 from mitochondria and Miro resulting in decreased transport of mitochondria (MacAskill et al., 2009). Independently, calcium also promotes the binding of another protein syntaphilin to both microtubules and mitochondria, thereby acting as a brake for immobile mitochondria following dissociation from kinesin (Chen and Sheng, 2013). Consequently, it will be important to test for a role for calcium in future studies. Experiments would employ removing extracellular calcium as well as use of specific inhibitors of voltage-gated calcium channels (general calcium channel blockers: Cd2+; N-type

Ca2+ channel blocker: ω-conotoxin GVIA; L type Ca2+ channel blocker: nifedipine; P/Q type Ca2+ channel blocker: agatoxin). If calcium is involved, one would expect to see that one or more of the above manipulations will abolish activity-induced inhibition of BDNF transport. If so, and since transport was inhibited within minutes of TBS (figure 8), and in view of the fact that Ciliobrevin D mimicked the effect of TBS, I hypothesize that dynein and/or its adaptor/effector/scaffolding proteins are downstream targets of calcium. Future studies to define the responsible proteins will be essential. One could also explore microtubule gliding assays wherein dynein is fixed onto a glass surface and supplied with microtubules and ATP (Tao et al., 2006). Changes in dynein-based motility could then be studied in the presence or absence of calcium.

Do other mechanisms contribute to the enhanced local BDNF signaling?

To date, I investigated only the contribution of transport to changes in BDNF signaling. Other mechanisms are readily envisioned. One candidate is activity-induced changes in TrkB surface expression. In (Meyer-Franke et al., 1998) and (Du et al., 2000) studies of activity-induced increases in surface TrkB receptors were reported. In both, KCl or TBS were applied to neuronal cultures for an hour before TrkB expression was measured. The latter, while similar to my study, differs considerably in that stimulation was for a significantly longer period. Accordingly, it is not clear to what extent changes in surface TrkB would

contribute to my findings. Nonetheless studies to explore a role for surface TrkB are indicated. A pilot experiment was used to test the feasibility of surface biotinylation of axonal TrkB in microfluidic chambers. Additional troubleshooting is needed to finalize the procedure. Another experiment involves removing free BDNF by adding excess BDNF antibodies prior to TBS. If extracellular BDNF is sequestered before applying TBS, then a potential contribution by TBS-induced TrkB insertion would be eliminated. This may thus allow me to distinguish and evaluate contributions for surface TrkB and transport.

How does slower transport lead to the enhanced local signaling?

Apart from further detailing the upstream events of activity-induced inhibition of transport, its downstream consequences should also be explored. The signaling endosome theory may help explain how slower transport leads to the enhanced local signaling. The signaling endosome theory says that endosomes containing ligand–receptor complexes remain active and continue to transduce cytoplasmic signals as they are transported (Grimes et al., 1997) (Delcroix et al., 2003) (Ye et al., 2003) (Wu et al., 2001). There is a good reason to believe that the signaling endosome theory applies to the BDNF/TrkB complex, as well as many other internalized ligand/TrK complexes. Slowing the transport within axons of the BDNF/TrkB signaling endosome would explain the higher level of axonal BDNF signaling seen in my studies. As indicated, whether this is the only manifestation of neuronal activation is yet to be defined.

What are the biological consequences of the enhanced local signaling?

It will now be essential to explore the downstream events that follow enhanced local signaling. It is well accepted that local signaling regulates growth cone motility, while signaling in the cell soma controls cell survival and gene expression (Reichardt, 2006). I intend to look into what axonal events are impacted by local signaling and which would benefit by the enhanced local signaling I observed. Some prior research may serve as a guide. For example, the Rho family of GTPase, Cdc42 and Rac (Yuan et al., 2003) mediates growth cone turning in response to neurotrophin gradients. In addition, my laboratory colleague Orlangie Natera has discovered a role for local BDNF signaling in axons on growth. The studies to be performed will benefit by very recent studies demonstrating that pausing of endosomal traffic appears to occur at actin patches (Sood et al., 2017), suggesting a role for local controls on activity-induced changes in transport that could mediate the structure and function of axons

Clinical implications

Efforts to develop drugs that stimulate BDNF signaling are aimed at increasing the viability and function of BDNF responsive neurons (Josephy-Hernandez et al., 2017). That activity may contribute to the efficacy of such

reagents must be considered. On a final note, it will be especially important to consider the impact of neuronal activity in the context of neurodegenerative and other neurological disorders. It is intriguing that exercise and enrichment/training may interact with BDNF-mimicking treatments to enhance the structure and function of vulnerable neurons and circuits.

Chapter 5 is currently being prepared for submission for publication. The dissertation author was the primary investigator and author of this paper. Authors will be Zheng Zeng, Zhixia Shi, and William Mobley.

CHAPTER 6 SUPPLEMENTAL OBSERVATIONS AND COMMENTS

I adopted optogenetic methods to microfluidic chambers with mixed success. Detailed accounts of trials are documented below to guide possible future efforts.

Methods and results

Thanks to Prof. Brenda Bloodgood's generous sharing of mice lines, a cre-dependent reporter line Ai32 expressing ChR2(H134R)-EYFP, was crossed with a cortical excitatory neurons labeling line, Emx-1 cre(Gorski et al., 2002). Embryos with YFP expression were selected out for dissection and seeding.

Two different types of light sources were tested: one from a built-in Arclight coupled with 470 excitation filter, the other light source is a stand-alone LED light from Thorlab (M470L3-C4: Blue (470 nm) Collimated LED, for Zeiss Axioskop, 1000 mA).

The next step is to show whether neurons could be activated. Calcium imaging was done with a different calcium dye to avoid exciting ChR2 (peak excitation at 470nm) when visualizing the calcium dye. A red-shifted calcium dye, Rhod-2 (552nm,ex/581nm,em), was used. Rhod-2 imaging parameters followed the user manual (Molecular Probes®), and tested positive after increasing extracellular KCl from 5mM to 15mM in wild type neurons. The combo light source, Arclight and 470nm filter allowed me to see an increased fluorescence in Rhod-2 in CHR2-expressing neurons. The multi-dimensional acquisition function

of Metamorph software was used to control the filter wheel between two cubes: 1) using the Rod cube (542ex/580em) I collected 100 frames to image Rhod-2 signals: 2) at selected frames, Metamorph commanded the filter wheel to rotate to GFP cube position to provide the 470nm stimulus for durations ranging from 1 to 500ms; and 3) then, Metamorph switched to the Rod cube to allow for Rhod-2 imaging. The 500 ms application of blue light resulted in a 12% delta F/F increase (data not shown). In spite of success in creating this system, I abandoned it because using this paradigm I was unable to simultaneously activate ChR and image calcium flux. Therefore, an independent source for 470nm was tested. After troubleshooting, the Thorland 470nm LED light turned out to emit light with wider range of wavelengths, so that it "leaked" through the cube set (542 Longpass/580) for Rhod-2 visualization. After filtering the LED light through a 470/40 filter, the leak problem was solved, but without seeing a Rod-2 response. Troubleshooting revealed that this was due to low light intensity. Less than 1mW/mm^2 of light was delivered to ChR expressing neurons in the imaging chamber containing the microfluidic chamber.

Conclusion

Several steps will be needed to effectively use optogenetic methods to activate neurons for studies of BDNF signaling in axons. First, TBS can be used as positive control to confirm the Rhod-2 incubation protocol and microscope setup. Second, the 470nm LED light source should be used to illuminate from the

bottom of the sample – i.e. through a thin coverslip. Third, the parameter settings for frequency and pulse width will benefit by referencing similar studies (Hedrick et al., 2016)(Renault et al., 2015).

CHAPTER 7 AGE-DEPENDENT MODIFICATION OF AN OLFACTORY RECEPTOR ON INNATE BEHAVIOR OF *DROSOPHILA*

The research shown in this chapter was carried out in Professor Jing Wang's lab. I discovered that an olfactory receptor-engaged behavior is age dependent, which resulted in one publication (Lin et al., 2016).

Background

Mating is a multisensory behavior, it requires input from vision, gustation, audition and olfaction, among which gustation and olfaction mediate pheromone sensing between two sexes. For male pheromones, it has been well established that Or67d (Kurtovic et al., 2007) and Gr32a (Wang et al., 2011) sense the male volatile pheromone cis vaccenyl acetate and non-volatile pheromone 7-tricosene, respectively. For female pheromones, all the known chemicals, 7,11-Heptacosadiene and 7,11-Nonacosadiene, are non-volatile. The chemical identity of volatile female pheromones and their receptors remain to be determined. One way to address this question is to identify olfactory receptors which are involved in mating behavior, and use these receptors, which presumably detect air born chemicals, as a guide, to identify possible volatile female pheromones.

Among a family of 60 olfactory receptors expressed in Drosophila, Or47b is indicated to be involved in male-female interaction. This is supported by behavior results. Root at. al. showed that when $GABA_BR$ expression is knock

downed by RNAi in Or47b ORNs, males need longer time to locate females in the dark (Root et al., 2008). Electrophysiology results also suggest Or47b responses to female extract (van der Goes van Naters and Carlson, 2007). Furthermore, an optical imaging study (Masuyama et al., 2012) using a reporter gene expression system—called CaLexA—to mark activated neurons in intact flies to show that after exposing a testing male carrying the reporter system with 10 virgin females (two sexes were separated by a mesh screen), both Or47b ORNs and the projection neurons which Or47b ORN project to, were labeled.

Methods and results

Based on the above literatures, we hypothesized that Or47b is involved in mating behavior, and its ligand is a candidate volatile female pheromone.

In order to prove that Or47b participates in mating behavior in *Drosophila*, a loss of function experiment was performed: to test whether male flies have defect in mating behavior when Or47b gene is mutated. Mating is a complex behavior, on one hand, besides olfaction, there are other sensory inputs assist males. In order to reduce gustation contribution, I designed a larger mating chamber (figure 11) compared to the commonly used ones in previous studies, so that testing males have to depend more on long distance detection of females. In order to eliminate visual contribution, the behavior was done under red light, which is not visible to flies. On other hand, female receptivity is another confounding issue to reveal the contribution of female pheromone. In order to

bypass this issue, two males were set to compete for a single female

Firstly, I tested whether this behavior assay is sensitive to olfaction defect. Or83b is a co- receptor that is required for the normal function of most ORNs. Or83b knockout males show mating defect in this behavior setting (figure 12).

After confirming the sensitivity of this behavior assay, Or47b mutants were tested (Figure 13a)

In order to further prove that mutants' defect is indeed due to loss of Or47b, using Gal4- UAS binary system, I generated flies with certain genotype in which Or47b receptor was restored in Or47b neurons in mutant background. When these males competed with unrescued males, the rescued males won over them (Figure 13b)

Conclusion

I designed a novel competition mating assay to assess the contribution of an olfactory receptor to Drosophila male flies' mating behavior. I found that Or47b is necessary for the mating success in mature male flies, but not in young male flies. A rescue experiment done in mature flies proved Or47b is sufficient to grant male flies advantages in mating behavior.

APPENDIX: FIGURES

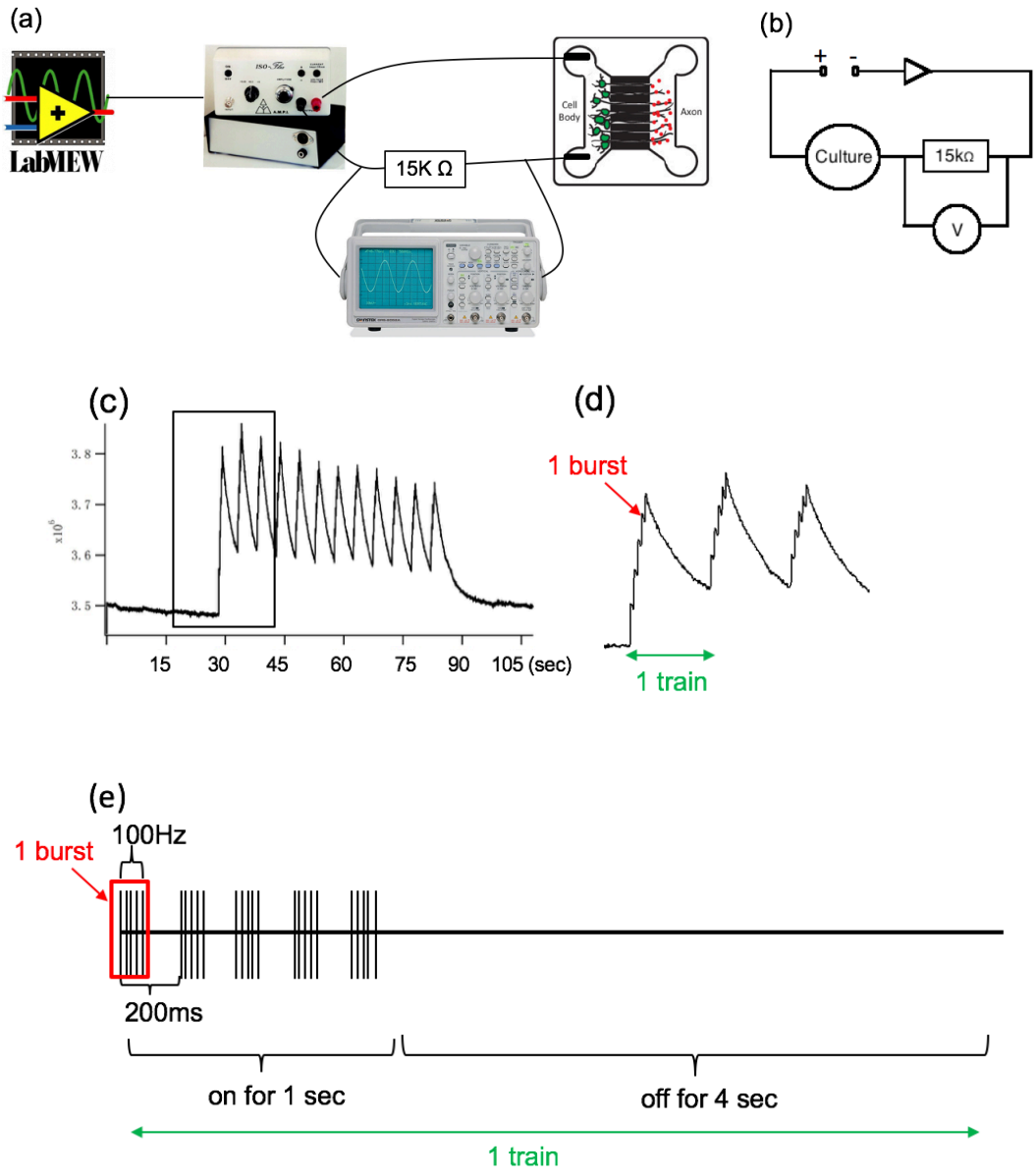

Figure 1 Diagrams of electrical circuit used for neuronal stimulation and a representation trace of a positive response. (a,b) Illustration and simplified circuit diagram of the setup; (c) a representation trace of calcium imaging; (d) is obtained after zooming in the boxed area in c; (e) is a diagram of Theta Burst Stimulation. The strict correspondence between (d) and (e) showed calcium imaging with 31fps acquisition was able to capture a single burst of stimulation.

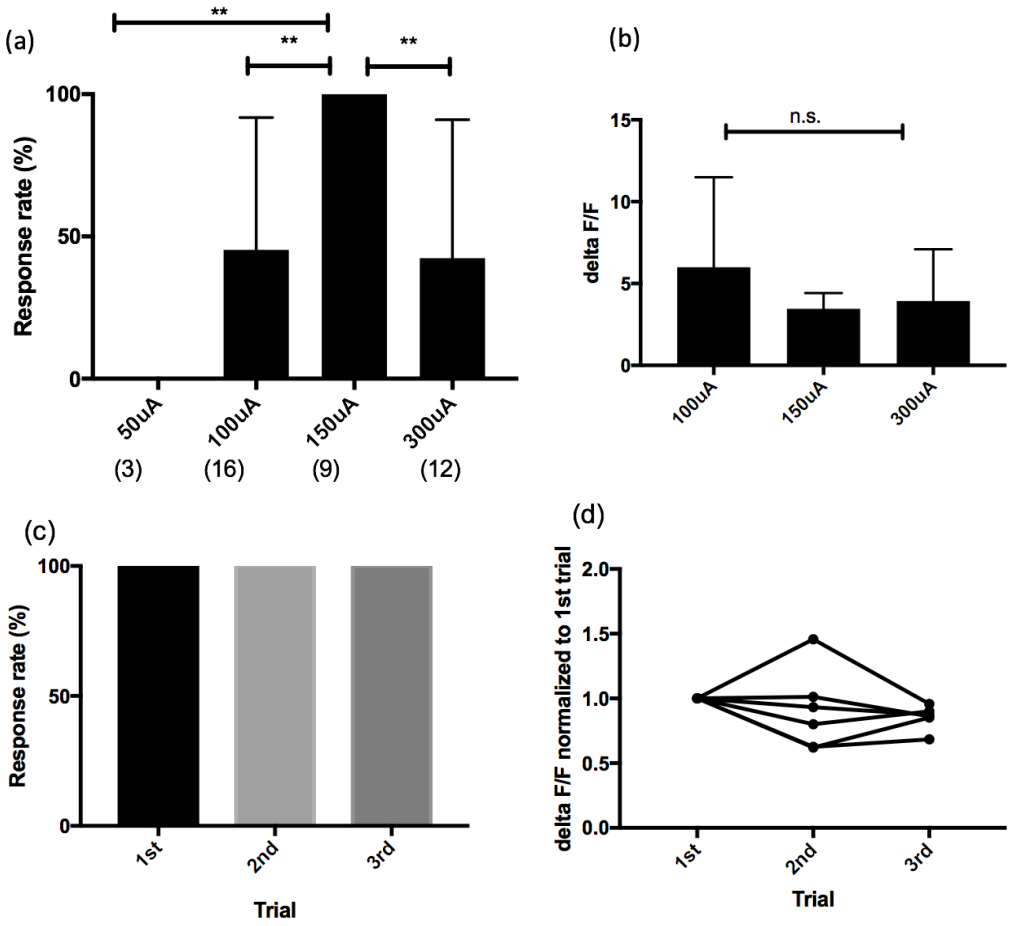

Figure 2 150uA reliably and sustainably excited neurons. Currents with different amplitudes were injected in the cell body compartment of microfluidic chambers. Fluorescence from calcium dye in proximal axons were recorded and measured. (a) Response rate is defined as the percentage of positive response out of total 12 stimulation trains; (b) Quantification of the calcium signal strength was illustrated in deltaF normalized by basal fluorescence; (c,d) repeated activation using 150uA didn't affect the response rate or signal strength of later trials, thus neuronal excitability remained after repeated field stimulation. One-way ANOVA was used in (a,b)

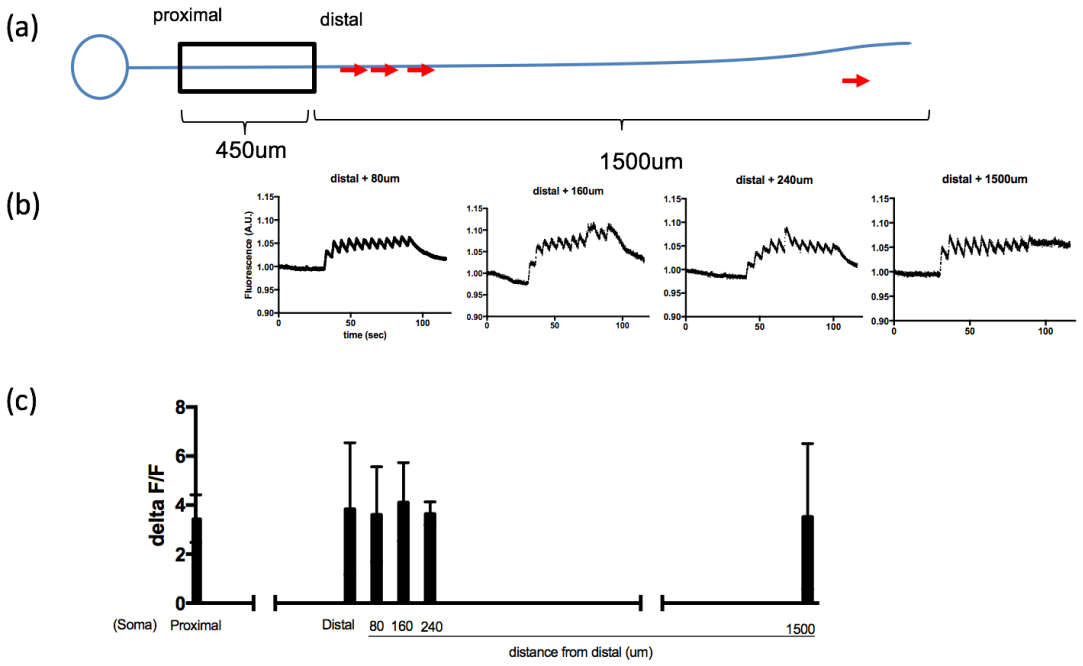

Figure 3 TBS patterned response could be found in axons further away from soma. (a) a diagram shows a simplified situation: one neuron extending one axon through a microgroove, represented by a rectangle box. Red arrows indicate the locations of imaging beyond microgrooves; (b) representative traces of calcium response at different locations; (c) quantification of calcium response intensity over distance from soma. No significant differences were found, one-way ANOVA was used

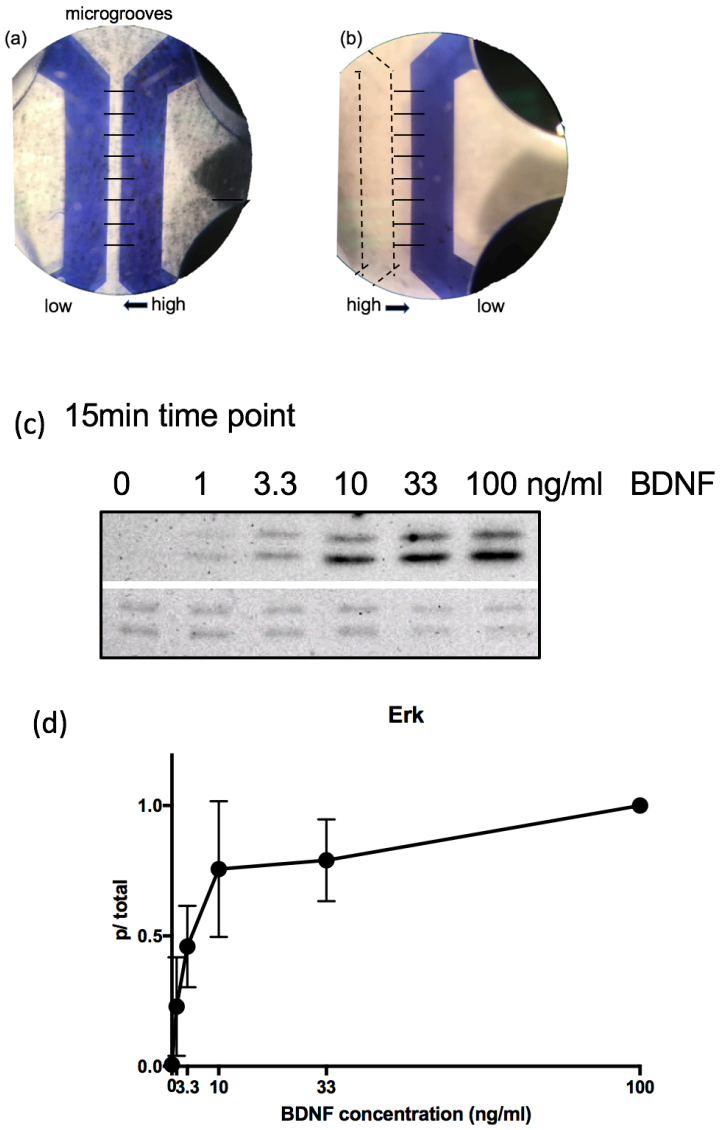

Figure 4 BDNF Erk and AKT signaling in axons was dose dependent and decayed over time. (a,b) testing the fluidic diffusion and isolation property of the chambers. In both cases a blue dye was added on the right compartment, (a) right side was added higher than the left (originally with water), so the dye diffused to the left chamber, picture was taken at the end of diffusion; or (b) right side was lower than left (water was added) so the dye was well contained on the right; (c,d) representative western blotting gel and quantification for experiments of applying BDNF with different concentrations on axon side. Signaling intensity of 100ng/ml was set as 1 in each trial before pooling data. 3.3ng/ml BDNF is EC50; (e,f) the same procedure but for AKT; (g,h) representative western blotting gel and quantification for experiments of harvesting axon lysate at different time points after applying 3.3ng/ml BDNF to axons. 15min was the signal peak.

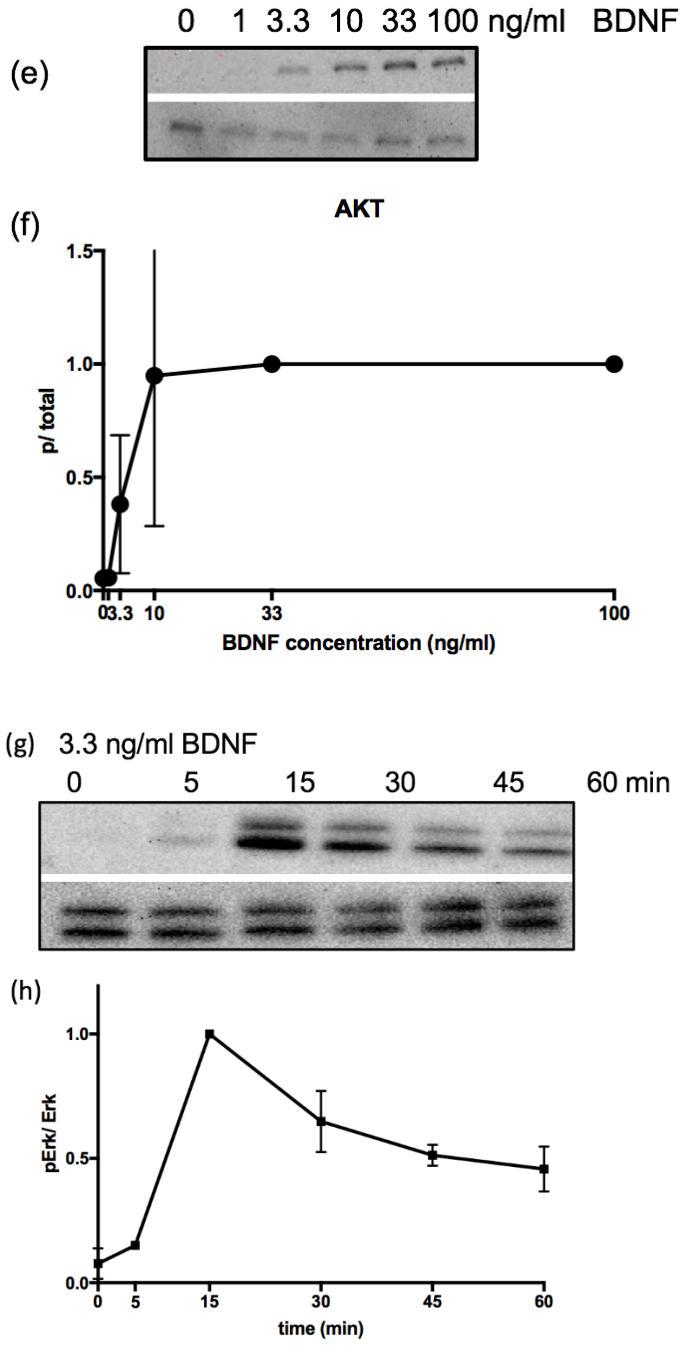

Figure 4 BDNF Erk and AKT signaling in axons was dose dependent and decayed over time (continue).

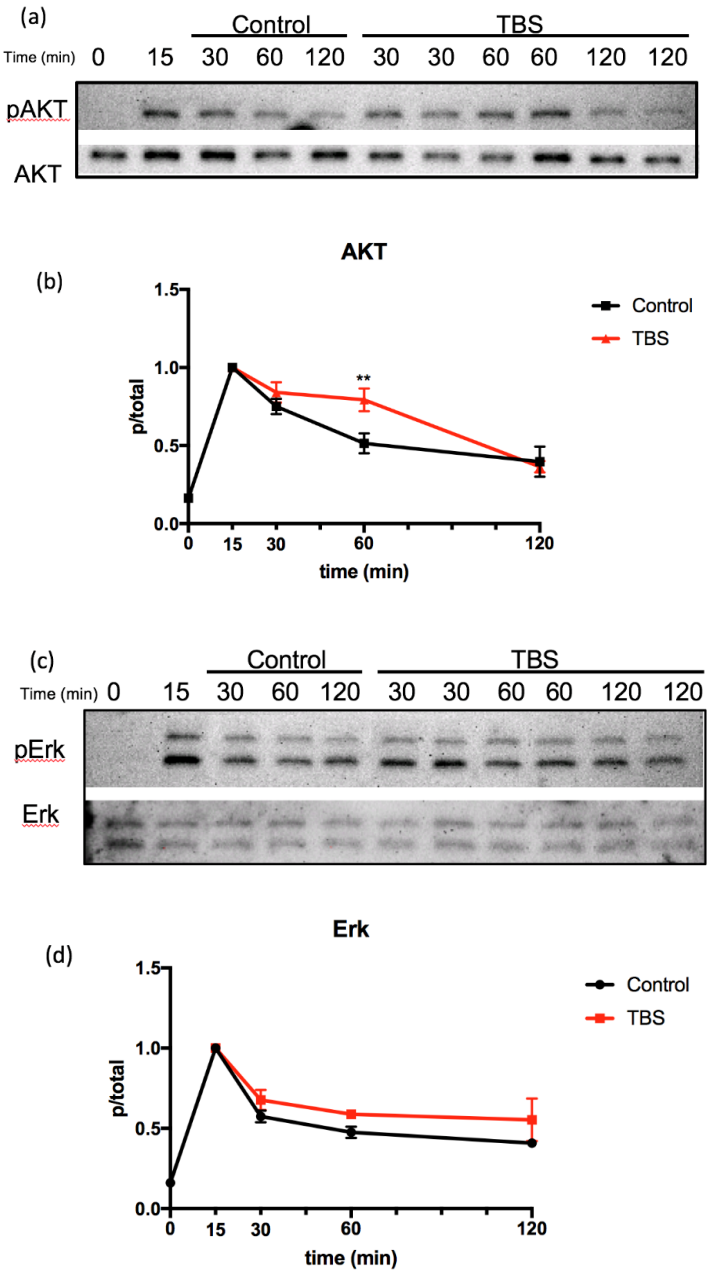

Figure 5 TBS enhanced BDNF axonal signaling. Representative western blotting gel and quantification for (a,b) AKT and (c,d) Erk were shown. Time point 0 all chambers were added BDNF on axons, axon lysate was harvested at each time point, for TBS treated group, TBS current was injected to cell body side at time point 15min. Within each trial, intensity of blots were normalized to the 15min one. Each time point control group and TBS group were compared with student T test

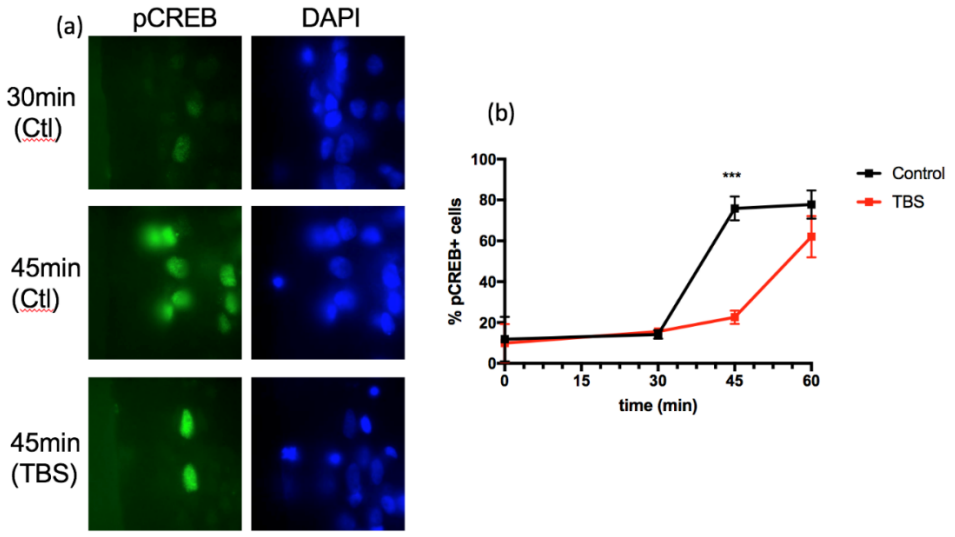

Figure 6 TBS delayed the CREB activation in soma. (a) representative images; (b) quantification of the percentage of pCREB positive cells over time, at time 0 BDNF was added only to axon side, in TBS group, TBS was added at 15min. Student T test was used to compare between two groups at the same time point

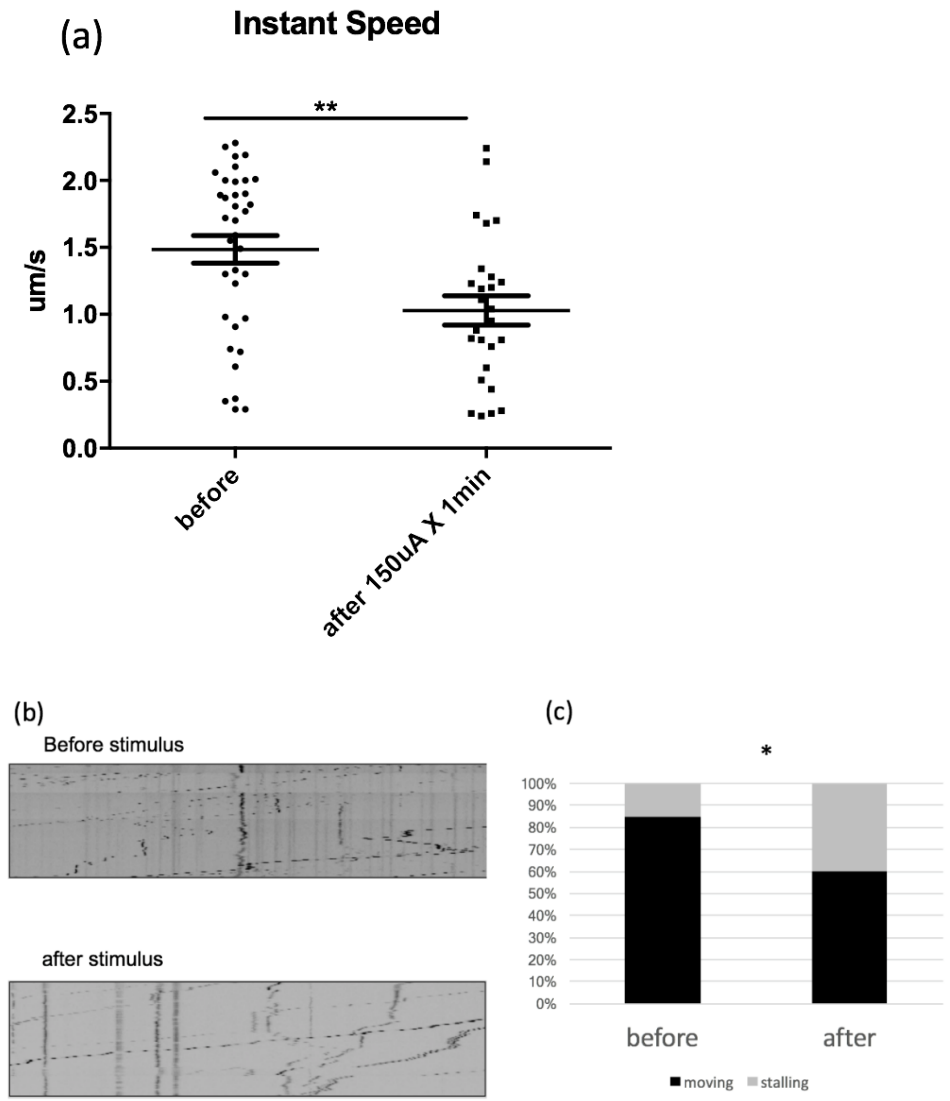

Figure 7 TBS slowed down QD-BDNF movement in axons (sampling different grooves per chamber) Both speed (a) and percentage of moving dots (b,c) were reduced after TBS. Student T test was used in (a), Chi square was used in (c). (b) representative kymographs were shown for before and after TBS. Kymographs were generated by plotting location of dots against time, so vertical lines represent dots that didn't move throughout the imaging sessions.

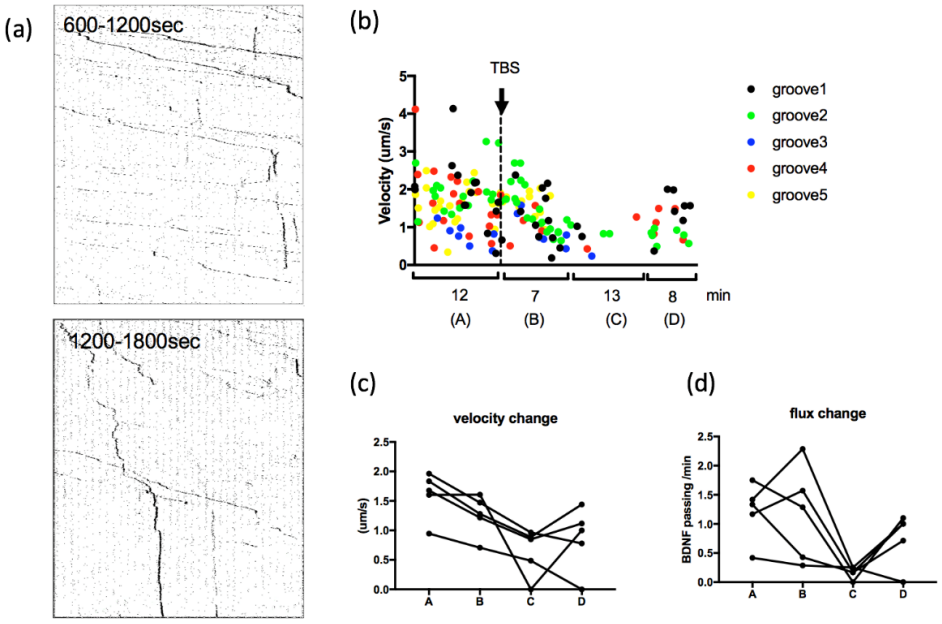

Figure 8 TBS slowed down QD-BDNF movement in axons (one groove per chamber) One groove was imaged for 40min (4X10min sessions). (a) kymograph showing the sessions during and after TBS; (b) velocity changes over imaging time. Every dot in the figure represents one QD-BDNF, whose coordinators are its time when it entered the imaging segment of axons and its average velocity before leaving the view. 4 periods of time were arbitrarily picked for best grouping dots showing similar kinetics; (c) average velocity of QDots in each period of time, the 4 data points from the same groove were connected with one line; (d)number of QD-BDNF moving per minute (flux) were calculated in each of 4 periods, similarly, the 4 data points from the same groove were connected with one line.

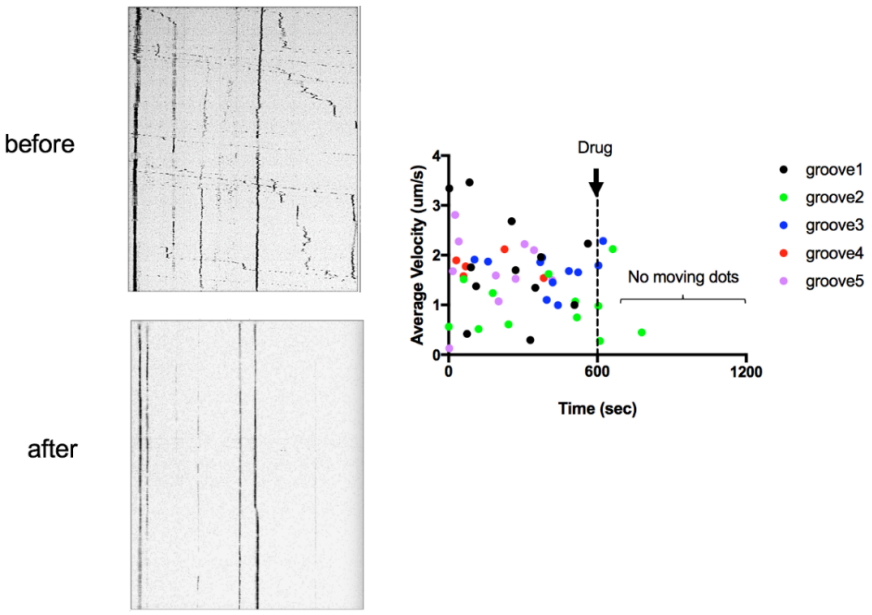

Figure 9 Ciliobrevin D inhibited QD-BDNF axonal transport. Kymographs and quantification of velocity were shown. Less than 2min after applying Ciliobrevin D, no QD-BDNF could be seen.

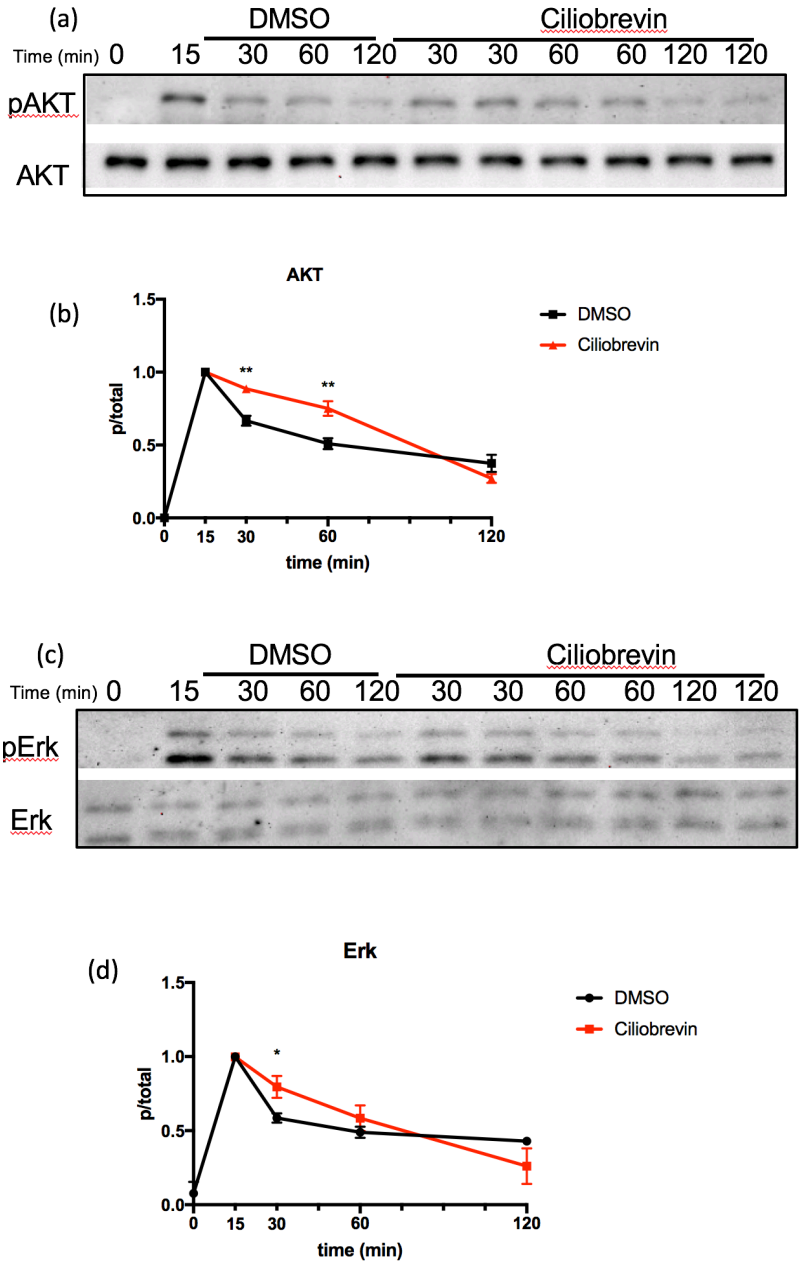

Figure 10 Ciliobrevin D enhanced BDNF axonal signaling. BDNF was added only to axons at 0 time point, Ciliobrevin D was added to axon compartment at 15min. Axon lysate was collected at the labeled time point. Student T test was used to compare DMSO group and Ciliobrevin D group at each time point

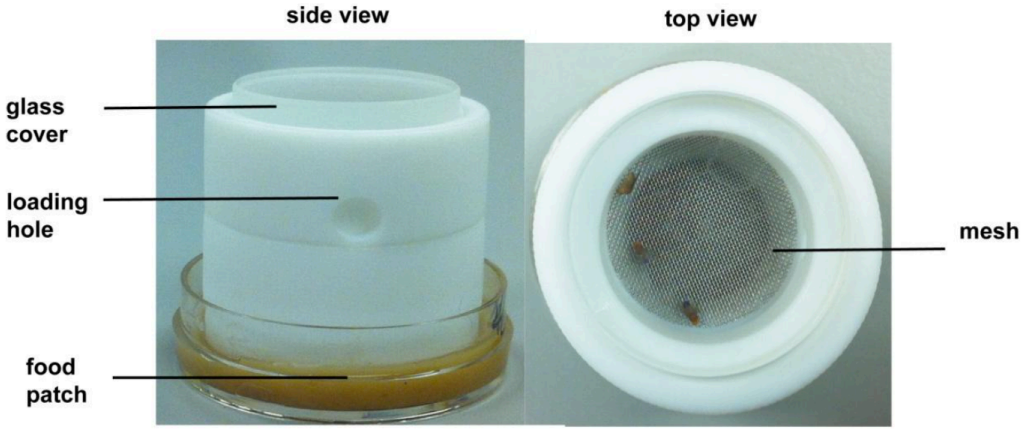

Figure 11 Behavior chamber used in competition mating assay. The top of the chamber has a glass cover to allow observation, and the bottom of the flies moving space is mesh sieve, designed to avoid odors accumulated inside the chamber over time and allow food odor to fill in to mimic a more natural environment. Two males and one virgin female were loaded into one chamber, and given 120min observation time, if there is copulation occurring between the female and one of the two males, the latency was recorded, and the genotype of the winning male was identified by the fluorescent dust, which was added one night before the experiment.

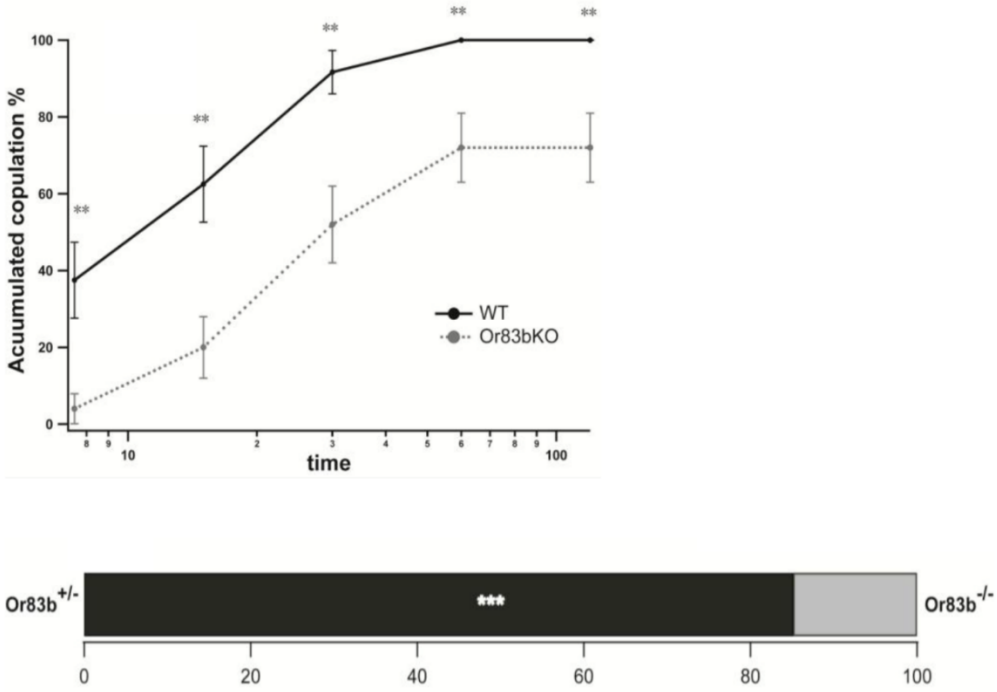

Figure 12 Olfaction is important for mating behavior. (a) how soon copulation occurs in 2 WT ♂1 WT ♀ and 2 Or83b KO ♂with 1 WT ♀. The absence of Or83b significantly lengthens copulation latency (Z test, n=24-25). (b) when 1 Or83b heterozygote knock out and 1 Or83b homozygote knock out compete for 1 WT female, heterozygotes won over homozygotes in most cases. (chi square test, n=74)

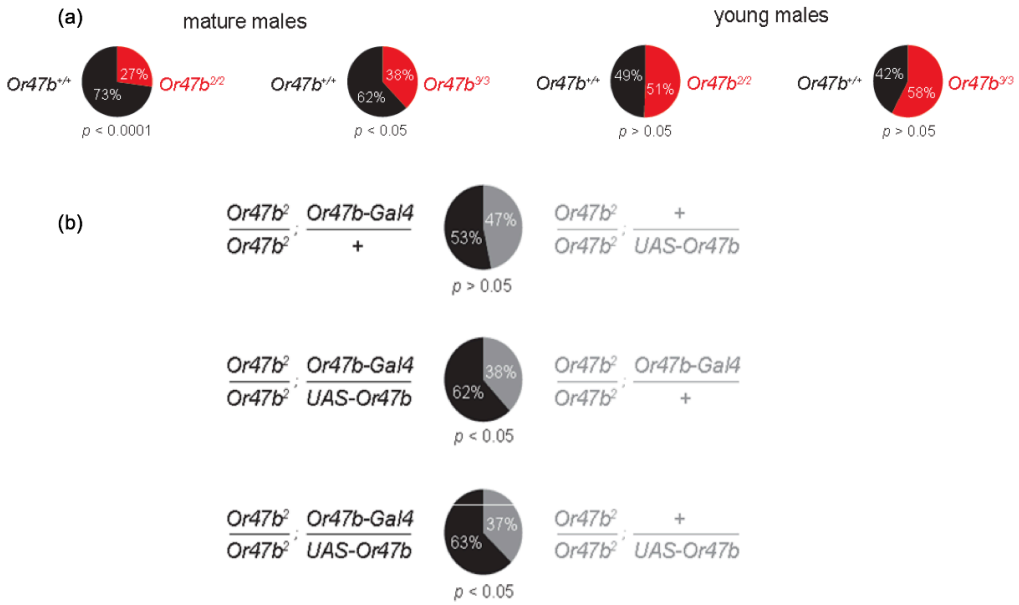

Figure 13 Or47b effect is age dependent, and the deficit in null mutant is a result of the absence of Or47b. (a) mature male exhibits mating deficit when competing with control males, young males didn't show this disadvantage. (b) males rescued with Or47b in Or47b expressing neurons rescued the mating deficit

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