ROLE OF RACK1 IN AXONAL OUTGROWTH OF DEVELOPING NEURONS

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by

Joel Serre

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Thesis written by

Joel Serre

Approved by

___________________________________________, Advisor

___________________________________________, Chair, Department of Biological Sciences

Accepted by

___________________________________________, Dean Honors College
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CHAPTER I

ABSTRACT

The complexity of the nervous system is generated during development. During neural development, neurons must extend axons and make synapses with correct target regions. One mechanism that is critical to the appropriate growth and guidance of the axon to its target region is local translation. Local translation of a subset of mRNAs takes place within the growth cone, which is a pathfinding structure found at the tips of developing axons. Receptor for activated C kinase 1 (RACK1) is a ribosomal scaffolding protein found at point contacts, which are integrin-dependent adhesion sites and likely a region of local translation in the growth cone. In line with this, RACK1 is necessary for appropriate local translation of β-actin mRNA in the growth cone. Thus, this project seeks to elucidate the role of RACK1 in the extension and branching of axons. A combination of primary cortical cell culture, lentiviral transduction, growth factor starvation-stimulation, and fluorescent microscopy techniques were used to examine the role of RACK1 in regulating axonal outgrowth. We find that axon length and branching decrease when RACK1 wt is overexpressed. However, axon length and branching is not affected when RACK1 cannot be phosphorylated at the 246th amino acid site (RACK1 Y246F). These data suggest that RACK1 is critical to the formation of point contacts, which are known to be involved in the extension rate of axons. Overexpression of RACK1 wt may increase the size or number of point contacts, thus resulting in decreased axon extension. These data provide further insight into how axon growth and guidance is regulated, and leads to appropriate connectivity formation in the developing nervous system.
Nervous system development is a complicated process and encompasses a number of stages, including neurogenesis, migration, differentiation, neurite growth and guidance, and synaptogenesis. Following the commitment of a precursor cell to become a neuron, the cell body extends neurites, which differentiate into axons and dendrites. Next, axons must pathfind to and identify their targets, which is followed by the process of synaptogenesis. The complex organization of the nervous system requires that each neuron form a specific synapse on its proper cellular target. This process of neural development ultimately defines the connectivity that is established in the nervous system. If proper neural development is disrupted at any step, this can lead to a defect in connectivity and a disease state. Therefore, it is of great importance to understand the mechanisms underlying the formation of connectivity in the developing nervous system. Although many of the guidance cues that direct axons to their appropriate targets have been identified, the intracellular molecular mechanisms that underlie the axonal guidance process are still not well understood. Thus, studying the mechanisms directing the process of axon guidance is necessary to further our understanding of how the nervous system functions in both health and disease.

**Axon Growth and Guidance**

A growth cone, which is located at the anterior of the axon in a developing neuron, is a sensory and motor structure that responds to guidance cues in its
environment to find the region in which its target is located. There are three major regions of the growth cone: the central domain, lamellipodia, and filopodia. The central domain is composed mainly of microtubules. Actin is found in both the lamellipodia and filopodia. The lamellipodial region of the growth cone surrounds the central domain and contains actin in a meshwork, while filopodia are the fingerlike-extension protruding from the growth cone and contain actin in bundles and chains (Sanes et al., 2011). There are two forms of actin found in the growth cone, γ-actin and β-actin. Whereas γ-actin protein is distributed evenly throughout the developing neuron, β-actin protein is found in especially high concentrations in the growth cone; furthermore, only β-actin mRNA is localized to the growth cone (Bassell et al., 1998). It has also been shown that β-actin localization to the growth cone is essential for directional polarization and movement of the cell (Shestakova et al., 2001). The suppression of motility following decreased β-actin localization is speculated to be a result of decreased rates in actin polymerization or the decrease of actin filament nucleation sites (Condeelis et al., 2005). Numerous studies have demonstrated that β-actin protein must rapidly polymerize or depolymerize asymmetrically in growth cones in response to the various guidance cues prevalent in the early nervous system (Dent et al., 2003). If actin polymerization is inhibited, then axon guidance is disrupted (Bentley et al., 1986; Forscher, 1988).

Axon extension occurs through a series of steps. First, the filopodia and lamellipodia of the growth cone extend forward when β-actin polymerizes and pulls the growth cone in the direction that the filopodia are extending. Microtubules will then extend by polymerizing onto the anterior end (the end nearest to the growth cone). As this
happens, the rear of the growth cone will collapse around the microtubules. Repetition of this process leads to increased axonal length (Sanes et al., 2011).

There are four categories of guidance cues that influence growth cone guidance: substrate bound and attractive, substrate bound and repulsive, diffusible and attractive, and diffusible and repulsive. The substrate bound cues are those found on cell surfaces or in extracellular matrices. Cell adhesion molecules (CAMs) are an example of substrate bound cues. In the case of homophilic CAMs like fasciclin, similar cell adhesion molecules will bind to each other and hold cells together. This causes growth cones to grow along axons that are expressing similar surface proteins. While this example is an attractive cue, there are also repulsive ligand cues that will affect the growth cone upon contact (e.g. Semaphorins). Diffusible cues are ones that are released from other cells and dissolved in the extracellular fluid. Thus, diffusible cues are able to signal from further distances. Similarly, diffusible cues can be repulsive (e.g. bone morphogenic proteins) or attractive (e.g. brain derived neurotrophic factor) to pathfinding growth cones (Sanes et al., 2011).

Growth cones contain receptors for these guidance cues, and receptor binding results in the activation of intracellular signaling pathways. Although numerous signaling pathways have been identified within growth cones, they all ultimately converge on the cytoskeleton of the growth cone. Specifically, these signaling pathways act on either actin or microtubules, and can cause polymerization (attraction) or depolymerization (repulsion or collapse). In summary, guidance cues influence axonal pathfinding via intracellular signaling pathways that act on the growth cone cytoskeleton.
The growth cone cytoskeleton interacts with the extracellular matrix or substrate through point contacts, which function similarly to focal adhesion sites in fibroblasts, and aid the growth cone in pathfinding (Robles et al., 2006). Point contacts are composed of many proteins, including paxillin, vinculin, β1-integrin, focal adhesion kinase (FAK), Rac1, and RhoA (Renaudin et al., 1999). We have recently discovered that RACK1 is also localized to point contacts (Kershner and Welshhans, personal communication). Previous studies have demonstrated that these adhesion sites are necessary to create the force that allows the growth cone to move forward (Renaudin et al., 1999; Robles et al., 2006; Myers et al., 2011). The mechanisms underlying the maintenance and stabilization of point contacts in the growth cone is not well understood, but inhibition or overactivation of members of the point contact complex has been shown to modulate neurite outgrowth (Woo et al., 2006). Taken together, these studies demonstrate that the regulation of point contacts has significant implications for axon outgrowth.

Local Translation in Axonal Guidance

Local translation is the mechanism by which mRNAs are localized and locally translated in subcellular regions of a cell. Although discovered relatively recently, local translation has been shown to be a critical molecular mechanism underlying axonal guidance in the developing nervous system. However, it has been well established that local translation underlies a number of developmental processes; for example, the localization of bicoid and nanos mRNAs to the poles of developing Drosophila embryos are necessary for the formation of the anterior-posterior axis (Martin, 2009; Ephrussi et al., 1991).
Localization and local translation of mRNAs requires multiple steps. After RNA is synthesized and spliced into mRNA, it enters the cytosol. mRNA binding proteins mediate the process of nuclear export and localization to the appropriate subcellular region. The binding of mRNAs to mRNA binding proteins is specific, and dependent on specific sequences located within the 3’ and 5’ untranslated regions (UTRs) of the mRNAs, which are termed “zipcode” sequences. This complex that is formed by the binding of the mRNA by its mRNA binding protein is a ribonucleoprotein (RNP). Following its nuclear export, a number of these RNPs come together to form granules, which are shuttled to their final subcellular location along microtubules and/or actin. In addition to playing a role in localization, mRNA binding proteins usually also repress mRNA translation. After the RNP reaches its final destination, the mRNA binding protein must release the mRNA so that local translation can occur (Abaza et al., 2008; Wells, 2006).

Recently it has become evident that local translation is necessary for appropriate axon growth and guidance in the developing nervous system. For example, when a growth cone encounters the negative cue Semaphorin 3A, translation of RhoA mediates collapse of the growth cone through cytoskeletal depolymerization (Wu et al., 2005). To respond to the attractive cue, netrin-1, β-actin is rapidly translated and polymerized toward the source (higher concentration) of the cue (Leung et al., 2006). High rates of β-actin local translation immediately following an attractive guidance cue, and on the side closest to the cue, are the impetus for growth cone turning (Leung et al., 2006). β-Actin is a major component of the growth cone cytoskeleton. This means that the pathfinding capabilities of the growth cone are dependent upon the availability of β-actin. Previous
findings demonstrate that much of the β-actin found in the growth cone is generated through local translation. In fact, preventing β-actin mRNA from localizing in the growth cone by changing the 3’ UTR zipcode sequence of β-actin mRNA results in decreased levels of β-actin protein in the growth cone, suggesting that β-actin protein levels in the growth cone are dependent upon local translation (Zhang et al., 2001). Furthermore, the pathfinding properties of growth cones are highly dependent on local translation. Guidance cues, such as netrin-1, cause a rise in β-actin protein levels that will not take place when translation is blocked (Leung et al., 2006). Another guidance cue, brain-derived neurotrophic factor (BDNF), also stimulates the localization of β-actin mRNA to the growth cone (Zhang et al., 1999). The localization and translation of β-actin mRNA allows the growth cone to turn toward the guidance cue (Leung et al., 2006). It follows that most growth cone turning studied thus far is dependent upon local protein synthesis (Campbell & Holt, 2001; Jung et al., 2012). However, it is important to note that in some cases axon extension can occur without guidance cues and local translation (Lin et al., 2007).

*Zipcode binding protein 1 (ZBP1)*

In order to be locally translated, β-actin mRNA must first be transported to the growth cone. ZBP1 is an mRNA binding protein that facilitates the localization of β-actin mRNA to growth cones by binding a zipcode sequence located in its 3’UTR (Shestakova et al., 2001). This binding prevents the translation of the mRNA, and enables the complex to be transported along the microtubules to the growth cone. ZBP1 will remain
bound to β-actin mRNA until it is phosphorylated; after ZBP1 is phosphorylated at tyrosine 396 by Src kinase, then β-actin mRNA is released and can be translated (Hüttelmaier et al., 2005). It has been shown that β-actin mRNA and ZBP1 both localize to the growth cone following stimulation with the guidance cue, BDNF (Yao et al., 2006). Specifically, BDNF stimulation results in Src phosphorylation of ZBP1 at tyrosine 396 in growth cones, and subsequent local translation of β-actin mRNA (Sasaki et al., 2010). Previous studies have demonstrated that this process is critical to axon guidance, because an interruption of the β-actin mRNA/ZBP1 interaction will inhibit turning responses, due to a lack of β-actin mRNA localization to the growth cone (Yao et al., 2006). Furthermore, appropriate axonal guidance responses to BDNF and netrin-1 are lost in neurons cultured from ZBP1 knockout mice, or neurons overexpressing ZBP1Y396F (mutant that cannot be phosphorylated, thus β-actin mRNA cannot be released). However, loss of ZBP1 does not affect axonal outgrowth in basal conditions (Welshhans et al., 2011). Taken together, these data demonstrate that ZBP1 is necessary for local translation of β-actin mRNA and attractive growth cone guidance in response to BDNF or netrin-1 stimulation.

Receptor for activated C kinase 1 (RACK1)

Receptor for activated C kinase 1 (RACK1) is another critical player in the local translation of β-actin mRNA. RACK1 was originally discovered due to its ability to bind and stabilize PKC (Ron et al., 1994), but it is also a ribosomal scaffolding protein (Nilsson et al., 2004). Specifically, RACK1 is located on the 40S ribosomal subunit (Rabl et al., 2011). RACK1 can initiate translation by recruiting PKC to the 40S subunit;
PKC phosphorylates and releases eIF6 (Nilsson et al., 2004). The release of eIF6 allows for the assembly of the large and small subunits of the ribosome (Ceci et al., 2003).

The ability of RACK1 to recruit multiple proteins has important implications for cell motility. RACK1 is known to bind and regulate Src family kinases, which play a major role in cell adhesion and motility. Studies in CHO-K1 cells demonstrate that RACK1 maintains and organizes focal adhesions through its Src binding site. Furthermore, overexpression of RACK1 in CHO-K1 cells results in decreased random migration, suggesting that the level of RACK1 regulates cell motility (Cox et al., 2003; Doan et al., 2007).

In neurons, RACK1 is essential to the interaction between ZBP1, the ribosome, and Src kinase. Tyrosine 246 on RACK1 can be phosphorylated, and this phosphorylation is critical to its scaffolding function (Ceci et al., 2012). If the tyrosine at the 246th site is replaced with the non-phosphorylatable amino acid phenylalanine (RACK1Y246F), RACK1 will no longer be able to facilitate the recruitment of the ZBP1/β-actin mRNA complex and Src to the ribosome. Consequently, the growth cone will experience decreased levels of β-actin protein, but increased levels of β-actin mRNA (Ceci et al., 2012). Because RACK1Y246F has a decreased ability to bring these proteins together, it is reasonable to assume that fewer ZBP1 proteins become phosphorylated, and thus fewer β-actin mRNAs are translated (Figure 1). Furthermore, overexpression of RACK1wt results in increased dendritic branching, which is likely due to increased translation of β-actin mRNA (Ceci et al., 2012). Because local translation of β-actin is required for appropriate axon guidance, these studies therefore suggest that
overexpression of RACK1\textsubscript{wt}, or expression of the non-phosphorylatable RACK1 mutant (RACK1\textsubscript{Y246F}) will affect axon growth and guidance.
**Figure 1.** Model of interactions between RACK1, Src kinase, and the ZBP1/β-actin mRNA complex (A) RACK1\textsubscript{wt} can recruit both BDNF-activated Src and the ZBP1/β-actin mRNA complex to the ribosome. Following recruitment, RACK1\textsubscript{wt} facilitates the phosphorylation of ZBP1 and the release of β-actin mRNA so that it can be translated. (B) RACK1\textsubscript{Y246F} is incapable of facilitating the phosphorylation of ZBP1 because it cannot recruit BDNF-activated Src or the ZBP1/β-actin mRNA complex.
Aims

We hypothesize that RACK1 regulates axon growth of developing neurons. Because BDNF is known to activate the local translation of β-actin mRNA, as well as affect axonal outgrowth of various neuronal cell types, we first examined how BDNF affects cortical axon outgrowth and branching. After we had determined the effect of BDNF, we directly manipulated RACK1, in combination with BDNF stimulation, to examine its role in axonal outgrowth and branching. We considered that there might be two mechanisms through which RACK1 affects axon outgrowth. One, RACK1 is known to regulate focal adhesions in non-neuronal cell types; thus, the regulation of point contacts in growth cones by RACK1 may affect axon growth. Two, RACK1 regulates the local translation of β-actin mRNA; thus, a change in local translation of β-actin could affect axon growth.

Hypothesis 1: BDNF stimulation of mouse cortical neurons will change growth cone motility which will then have an effect on axon length and branching.

Experiments 1 and 2 Expected Results: To test this hypothesis, E17 mouse cortical neurons were treated with either 100ng/ml or 40ng/ml BDNF, or vehicle (control). We predicted that incubation with BDNF would increase axon length and branching. Previous studies have shown that BDNF binds to the TrkB receptor, leading to Src kinase activation in growth cones, and an increase in axon length (Cheng et al., 1994; Robles et al., 2005). One potential pathway through which an increase in axon length could occur is via the phosphorylation of ZBPI by Src kinase, resulting in the release and translation of the cytoskeletal protein β-actin mRNA. The increase in translation of β-
actin mRNA will then result in a difference in the rate of axon extension or number of axonal branches between the control group and the BDNF treated group.

**Hypothesis 2:** RACK1 regulates BDNF-stimulated axon growth and branching of developing mouse cortical neurons.

**Experiment 3 and 4 Expected Results:** To test this hypothesis, we overexpressed GFP-RACK1<sub>wt</sub>, GFP-RACK1<sub>Y246F</sub>, or GFP in E17 mouse cortical neurons, in combination with BDNF treatment, and examined axon length and branching. We predicted that GFP expression would not cause a difference in axon morphology. However, both RACK1<sub>wt</sub> and RACK1<sub>Y246F</sub> are expected to change the rate of β-actin translation and/or point contact formation (RACK1<sub>wt</sub> is expected to increase both; RACK1<sub>Y246F</sub> is expected to decrease both). We expect that the change of β-actin translation and/or point contact formation, in conjunction with BDNF or vehicle treatment, will result in significant differences in axon length and branching.
CHAPTER III

MATERIALS AND METHODS

Animals

C57BL/6J mice (Jackson Laboratory) were used in all studies. Mice were housed in plastic cages, and food and water were available *ad libitum*. Mice were maintained on 12:12 light-dark cycle. To obtain timed pregnant female mice, two female mice were placed in the cage of a single male mouse overnight. The following morning, the female mice were removed from the cage and checked for sperm plugs. All mice mated in these studies were 6 weeks or older. If pregnant, females were euthanized at E17 (E0 is the day of the plug), and the embryos of either sex were removed and immediately decapitated before dissection. Kent State University’s Institutional Animal Care and Use Committee approved all animal protocols.

Buffer and Media Compositions

*Primary Neuronal Cell Culture Media*

HBSS (Cellgro) was supplemented with 0.24% HEPES (Sigma) and adjusted to a pH of 7.3. MEM (Cellgro) was supplemented with 10% FBS (Sigma). Neurobasal (Life Technologies) was supplemented with 1% glutaMAX (Life Technologies) and 2% B27 (Life Technologies). Borate Buffer is an aqueous solution consisting of 0.24% boric acid (Sigma) and 0.38% Borax (Sigma). Borate buffer was adjusted to a pH of 8.5.
**Lentiviral Buffers & Media**

The CaCl₂ solution was prepared by making 2.5 M CaCl₂·2H₂O (Amresco) in distilled H₂O. The CaCl₂ solution was sterilized by filtration through a 0.22µm filter. 2X HEPES-buffered saline (2X HBS) consisted of 50mM HEPES (Sigma), 140mM NaCl (Sigma), and 1.5 mM Na₂HPO₄·2H₂O (Sigma-Aldrich) solution in water. Calcium Phosphate-DNA coprecipitate for six 150mm plates consists of 135µg lentiviral transfer plasmid, 88µg pLP1 plasmid, 34µg pLP2 plasmid, 47.5µg VSVG plasmid, and 0.25M CaCl₂·2H₂O (Amresco) in 0.1X TE buffer. 0.1X TE consisted of 1mM Trizma-HCl (Sigma) and 0.1mM EDTA; this solution was adjusted to a pH of 7.6. The DMEM/High Glucose (Hyclone) was supplemented with 10% FBS (Sigma).

**Immunofluorescence Buffers**

TBS₅₀ is an aqueous solution consisting of 50mM Trizma-HCl (Sigma) and 150mM NaCl (Sigma). Immunofluorescence (IF) buffer consisted of 2% BSA Fraction V Heat Shock (Roche) and 0.1% Triton X-100 (Sigma) in TBS₅₀ solution. Paraformaldehyde (Sigma-Aldrich) was prepared at a 4% concentration in 1X PBS with 5mM MgCl₂ (Sigma). The solution was adjusted to a pH of 7.4. Paraformaldehyde rinses were done in PBS with 5mM MgCl₂ (Sigma). 1X PBS consists of 137mM NaCl (Sigma), 2.7mM KCl (Sigma-Aldrich), 10mM Na₂HPO₄·2H₂O (Sigma-Aldrich), and 1.8mM KH₂PO₄ (Sigma-Aldrich) in nanopure water. For immunocytochemistry, the permeabilization buffer consisted of 0.3% Triton X-100 (Sigma) in TBS₅₀. Permeabilization buffer for phalloidin staining consisted of 0.1% TritonX-100 (Sigma) in 1X PBS.
Coverslip Preparation

Glass coverslips (Carolina Biological) were placed in porcelain racks and immersed in 70% nitric acid for 48 hours. After nitric acid treatment, they were rinsed in nanopure water (4 times for 30 minutes each time) on a rocker. Then the coverslips were baked at 200°C for 2 hours, allowed to cool, and stored in sterile dishes sealed with parafilm.

For experiments 1 and 2: Coverslips were treated with 70,000-150,000 MW poly-L-lysine (30µg poly-L-lysine/1ml borate buffer) and incubated at 37.5°C/5% CO₂ overnight. The following day the coverslips were rinsed with autoclaved water, 4 times for 30 minutes each time. Following the rinses, the coverslips were placed in MEM with FBS and incubated at 37.5°C/5% CO₂ until cells were plated.

For experiments 3 and 4: The day prior to dissection, the coverslips were transferred to sterile 12-well plates (1 coverslip per well). The coverslips were treated with 70,000-150,000 MW poly-L-lysine (Sigma-Aldrich; 100µg poly-L-lysine/1ml borate buffer). The 12-well plates were sealed with parafilm, and the coverslips were incubated overnight at room temperature. The following day, the poly-L-lysine was aspirated off and the coverslips were rinsed with autoclaved nanopure water (4 times for 30 minutes each time). The coverslips were then treated with laminin (Life Technologies; 10µg laminin/1ml HBSS with HEPES) and incubated at 37.5°C/5% CO₂ for 2 hours. The laminin was aspirated off of the coverslips after 2 hours and the coverslips were rinsed with HBSS (2 times for 30 seconds each time). MEM with FBS was then placed in the
wells with the coverslips and incubated at 37.5°C/5% CO₂ until cells were ready to be plated.

Dissection, Cell Culture, and Experiments

Embryonic day 17 mice were extracted from a pregnant mouse and promptly decapitated. After decapitation, the brains were removed and placed into a small dish filled with HBSS with HEPES. The cortices were separated from other neural tissue and cut into smaller pieces. The cortical masses were transferred from the HBSS with HEPES via a transfer pipette (special care was taken to transfer as little HBSS with HEPES as possible) into 0.25% trypsin (Life Technologies). The cortical tissue in the trypsin was placed in a 37.5°C water bath for 5-8 minutes. After the trypsin treatment, the cortical tissue was transferred to HBSS with HEPES with a transfer pipette. The tissue and HBSS with HEPES were placed in a 37.5°C water bath for 5 minutes. The tissue was transferred to another tube containing HBSS with HEPES and placed in a 37.5°C water bath for 5 minutes. The tissue was then transferred to a tube containing MEM with FBS. With a fire-polished pipette, the cells were dissociated by pipetting the tissue up into the fire-polished pipette and then pipetting the tissue down along the side of the tube containing MEM with FBS; the cells were pipetted up and down no more than 15 times. Neurons were then counted by making a dilution with trypan blue. 10μl of this cell/trypan blue mixture was put in a hemocytometer and cells were counted. Following counting, the appropriate volume of cortical cells in MEM with FBS was added to each well of the 12-well plates, such that 30,000 neurons were plated on each coverslip. The cells were
incubated for a 2 hour interval in MEM with FBS, to allow for adherence to the coverslip. After the cells adhered to the coverslip, the media was changed to neurobasal with glutaMAX and B27.

Experiment 1 (Does 100ng/ml BDNF affect axon length and branching?): When media was switched to neurobasal with glutaMAX and B27, either BDNF (Preprotech) or vehicle control was added to the cells. BDNF (100ng/ml) was dissolved in PBS with BSA. Vehicle control solution consisted of an equal volume of PBS with BSA. After 2 days in vitro (DIV) at 37.5°C/5% CO₂, the cells were fixed and stained with Alexa Fluor 488 phalloidin. The axon length and number of branches per axon was measured.

Experiment 2 (Does 40ng/ml BDNF affect axon length and branching?): When media was switched to neurobasal with glutaMAX and B27, either BDNF or vehicle control was added to the cells. BDNF (40ng/ml) was dissolved in PBS with BSA. Vehicle control solution consisted of an equal volume of PBS with BSA. After 2 DIV at 37.5°C/5% CO₂, the cells were fixed and stained with Alexa Fluor 488 phalloidin. The axon length and number of branches per axon was measured.

Experiment 3 (Does expression of RACK1wt, or a non-phosphorylatable mutant of RACK (RACK1Y246F) affect BDNF-stimulated axon length and branching?): When the media was changed to neurobasal with glutaMAX and B27 the cells were infected with
lentivirus encoding GFP-RACK1<sub>Y246F</sub>, GFP-RACK1<sub>wt</sub>, or GFP. The cells were then incubated at 37.5°C/5% CO<sub>2</sub>. When neurons were expressing GFP, after 2-3 DIV, they were treated with 40ng/ml of BDNF (in PBS with BSA) or vehicle control. After 2 hours, the cells were fixed. ICC was performed using monoclonal mouse anti-GFP (DSHB) at a 1:19 dilution as the primary antibody and goat anti-mouse 488 (Jackson ImmunoResearch; catalog# 115-545-003) at a 1:1000 dilution as the secondary antibody, in conjunction with rhodamine phalloidin (Life Technologies; catalog# R415) staining (administered with the secondary antibody). The axon length and number of branches per axon was measured.

Experiment 4 (Does expression of RACK1<sub>wt</sub> or a non-phosphorylatable mutant of RACK (RACK1<sub>Y246F</sub>) affect BDNF-stimulated axon length and branching?): When the media was changed to neurobasal with glutaMAX and B27, the cells were infected with lentivirus encoding GFP-RACK1<sub>Y246F</sub>, GFP-RACK1<sub>wt</sub>, or GFP. The cells were incubated at 37.5°C/5% CO<sub>2</sub>. Once neurons were expressing GFP, after 2-3 DIV, they were starved in neurobasal without B27 for 3 hours, and then treated with either 100ng/ml BDNF in phosphate buffered saline (PBS) with BSA for 30 minutes or an equal volume of PBS with BSA (vehicle control). Immediately following the 30 minute BDNF treatment, the cells were fixed using paraformaldehyde. ICC was performed using monoclonal mouse anti-paxillin1 monoclonal antibodies (1:500 dilution in IF buffer; catalog #610051 BD Transduction Laboratories) in IF buffer as the 1º antibody, with goat anti-mouse Cy3 (1:1000 dilution in IF buffer; catalog #115-165-146; Jackson Immuno Research) as the 2º
antibody. GFP was still able to be visualized following fixation, even without the use of a GFP antibody.

**Virus Production**

Dishes were prepared for transfection by pipetting 10ml of 0.1% bovine gelatin (Sigma) into six 150mm diameter dishes and then swirling the dishes to maximize the gelatin-coated area. After a 5 minute period, excess gelatin was aspirated off of the dishes. Once the gelatin dried, 20ml of DMEM with FBS was added to each dish. The dishes were then incubated at 37.5°C/5% CO₂ to prepare the media. HEK 293T cells that had previously been grown in DMEM with FBS were plated into the dishes such that 10 million cells were in each dish. The dishes were swirled to evenly distribute the cells. The dishes were then incubated at 37.5°C/5% CO₂.

Once the cells were 70% confluent, the media was pipetted off and replaced with 20ml of new DMEM with FBS. The dishes were incubated at 37.5°C/5% CO₂ for 2 hours. After the two hour incubation, 0.1ml of the calcium phosphate-DNA coprecipitate was added for each 1 ml of media on 150mm dishes (2ml per plate). The following DNA vectors were transfected: pLP1, pLP2, pVSVG, and the FUGW lentiviral backbone containing the genes of interest (either GFP, GFP-RACK1<sub>wt</sub>, or GFP-RACK1<sub>Y246F</sub>). The dishes were rocked gently to mix the media with the coprecipitate, and then placed back into the incubator at 37.5°C/5% CO₂ for 16 hours. The media was then removed gently and replaced with pre-warmed DMEM with FBS (18ml per dish).
Viral particles were harvested 48 hours post transfection by removing the media and storing it at 4°C in sterile conical tubes. The media was immediately replaced with pre-warmed DMEM with FBS (18ml per dish). More viral particles were harvested 72 hours after the transfection by removing the media and from the dishes and placing it into sterile conical tubes.

All of the collected supernatant was centrifuged at 500 x g for 5 minutes at 4°C to pellet detached cells. The supernatant containing the virus was filtered through a 0.45µm sterile filter. The filtrate was then centrifuged with 70,000 x g (19,400 RPM on an SW28 rotor) for 2 hours at 4°C. The supernatant was immediately discarded and the tubes were inverted to allow the pellets to partially dry. 30µl of PBS was added to the first tube, and 15µl was added to each of the remaining tubes. The tubes were covered with parafilm and chilled on ice for 30 minutes. In the first tube, the PBS was pipetted gently up and down and used to wash the walls in a manner that prevented foaming. 15µl was transferred to a sterile tube, and the remaining 15µl was transferred to the next tube. This process was repeated until all of the pellets had been resuspended. Following a 5 second centrifugation at top speed, the virus was separated into 3µl aliquots and stored at -80°C.

**Fixation, Immunocytochemistry, and Phalloidin Staining**

Following incubation with either BDNF or vehicle, the media was aspirated off of the cells and replaced by 4% paraformaldehyde. The paraformaldehyde was removed from the cells after 17 minutes and the cells were rinsed 3 times with PBS with MgCl₂ for 5 minutes each time.
After the rinses, TBS$_{50}$ was placed on the cells for 5 minutes. The TBS$_{50}$ was removed from the cells, and they were permeabilized in permeabilization buffer for 5 minutes. After permeabilization, the cells were treated with IF buffer for 5 minutes. To prevent nonspecific antibody binding, the cells were blocked in blocking buffer for 1-2 hours. Coverslips were then incubated facedown onto a 30µl drop of 1º antibodies for 1 hour at room temperature. After incubation the coverslips were washed in IF buffer (4 times for 5 minutes each time) on a rocker. Coverslips were then incubated at room temperature, facedown on a 30µl drop of 2º antibodies for 30 minutes at room temperature. Special care was taken to keep the antibodies, and incubation chamber in a dark environment. The coverslips were then rinsed 4 times for 5 minutes with IF buffer on a rocker (exposure to light was minimized as much as possible). The coverslips were then mounted facedown onto a slide with mounting media (Probes Molecular) and allowed to dry in a cool, dark, and dry environment.

Phalloidin Staining: After the rinses, the cells were permeabilized in permeabilization buffer for 5 minutes. After permeabilization, the cells washed with PBS (3 times for 3 minutes each time). The coverslips were then incubated facedown on a 30 µl drop 1:40 dilution of rhodamine phalloidin (Invitrogen) in PBS for 20 minutes at room temperature. Exposure to light was minimized as much as possible. Coverslips were washed 3 times for 5 minutes each time using PBS. The coverslips were mounted facedown onto a slide with mounting media (Probes Molecular) and allowed to dry in a cool, dark, and dry environment.
Imaging and Measuring Axons and Branching

Once the coverslips were securely mounted onto the slides, they were imaged on an Olympus IX70 fluorescent microscope at 20x magnification with a high speed SensiCamQE camera (The Cooke Corporation). Images of neurons were only captured if the entire axon was visible, no overlap of axons was observed, the neurons appeared healthy, and it was evident that the neuron was expressing the transgene from the lentivirus. Axon length and branching was measured using ImageJ analysis software. To measure total axon length, the longest branch was measured. 1º and 2º branches were only counted if they were over 5μm in length.

Statistics

Each experiment was repeated at least three independent times, except for experiment 3. Significance was set at \( p \leq 0.05 \). Data are graphed as mean ± SEM. Statistical tests were performed using SPSS and included student t-tests and two-way ANOVAs, as appropriate.
CHAPTER IV

RESULTS

Experiment 1: BDNF (100ng/ml) stimulation results in decreased axon length, but has no effect on axon branching

Previous research had shown that stimulation of E18 hippocampal rat neurons with BDNF for 48 hours results in increased axon length (Labelle et al., 2000). BDNF is known to bind to the TrkB receptor, which is present in mouse cortical growth cones (Cheng et al., 1994). This results in the activation of Src, a kinase that phosphorylates both RACK1 and ZBP1 (Ceci et al., 2012; Chang et al., 2003). While the Labelle et al. (2000) experiments used a 40ng/ml treatment of BDNF, more recent research had demonstrated that stimulation of rat cortical neurons with 100ng/ml BDNF for 1 hour also resulted in an increase in axon growth, as measured with live cell imaging (Welshhans et al., 2011). Therefore, the first experiment conducted was intended to verify that BDNF stimulation would cause a change in the rate of axon outgrowth and/or axon branching in developing mouse neurons.

Mouse cortical neurons were dissected, dissociated and plated. Two hours following plating, neurons were either treated with 100ng/ml BDNF or an equal volume of vehicle in neurobasal media. Neurons were then cultured for 2DIV (48 hours). Following the 2 day treatment, the cells were fixed and stained with Alexa Fluor 488 phalloidin. The length of axons was measured, and the number of branches (≥5μm) was counted. Surprisingly, the average axonal length of the vehicle treated neurons (149.845 ± 7.353μm) was significantly longer than that of BDNF treated neurons (125.640 ±
4.931μm; p =0.007; Figure 2). However, this experiment did not demonstrate a significant difference in axon branching between the two groups (vehicle 1.467 ± 0.200 branches; BDNF 1.267 ± 0.193 branches; p=0.473; Figure 3).

Experiment 2: BDNF (40ng/ml) stimulation results in decreased axon length, but has no effect on axon branching

To further investigate the effects of BDNF on axonal outgrowth, we repeated experiment 1, but decreased the concentration of BDNF to 40ng/ml, which more closely replicated the experiments of Labelle et al. (2000). Mouse cortical neurons were dissected, dissociated and plated. Two hours following plating, neurons were either treated with 40ng/ml BDNF or an equal volume of vehicle. Neurons were then cultured for 2DIV (48 hours) in neurobasal. Following the 2 day treatment, the cells were fixed and stained with Alexa Fluor 488 phalloidin. The length of axons was measured, and the number of branches (≥ 5μm) was counted. As in experiment 1, the axonal length of the neurons receiving vehicle treatment (154.424 ± 7.970μm) were significantly longer than the neurons that had been stimulated with BDNF (133.563 ± 6.592μm; p= 0.046; Figure 4). However, as seen in experiment 1, there was no significant difference in the axon branching of the two groups (vehicle 2.083 ± 0.214 branches; BDNF 1.833 ± 0.180 branches; p=0.373; Figure 5).
Experiments 3 and 4: Overexpression of RACK1<sub>wt</sub> results in decreased axon length and branching

The goals of experiments 3 and 4 were to determine if expression of GFP-RACK1<sub>wt</sub> or GFP-RACK1<sub>Y246F</sub> would affect axon length and branching, as compared to control expression of GFP. In addition, these experiments compared neurons that were stimulated with BDNF to those treated with vehicle, to determine if BDNF stimulation in combination with RACK1 expression resulted in a change in axon length and/or branching that would not be evident under basal conditions (no BDNF stimulation).

Experiment 3 was carried out simultaneously with experiment 4, in order to determine if starvation of the neurons prior to BDNF stimulation (Experiment 4) would have a greater effect on axon length and branching, as compared to stimulation alone (Experiment 3).

We used the starvation and stimulation paradigm because previous studies have demonstrated that starvation and stimulation results in enhanced motility, as well as increased β-actin mRNA localization and translation (Kislauskis <i>et al.</i>, 1997; Welshhans <i>et al.</i>, 2011; Zhang <i>et al.</i>, 2001). In these experiments, we also greatly reduced the time of BDNF stimulation because of other studies that had found significant results with a shorter treatment period (Welshhans <i>et al.</i>, 2011; Dajas-Bailador <i>et al.</i>, 2012).

In experiment 3, neurons were infected with 1 of 3 lentiviruses (encoding either GFP, GFP-RACK1<sub>wt</sub>, or GFP-RACK1<sub>Y246F</sub>). The three groups were cultured until the virus was expressed for at least 24 hours (expression was determined by the fluorescence of GFP), and then treated with BDNF (40ng/ml) or an equal volume of vehicle. Cells were stained using ICC with antibodies for GFP (to differentiate virus-expressing cells from those cells that were not expressing virus) and rhodamine phalloidin, to label the
entire actin cytoskeleton of the cell. The axon length of RACK1_{wt}-expressing cells that were treated with BDNF were, on average, shorter than the rest of the groups (Figures 6 and 7). An analysis of axon branching did not demonstrate any striking differences, although all RACK1_{Y246F}-expressing cells appeared to have fewer branches than the rest of the groups (Figure 8). We completed experiments 3 and 4 simultaneously, and following analysis of preliminary data found a much stronger effect of BDNF on all groups in experiment 4, as compared to experiment 3. As such, we completed experiment 3 only once (n=1).

In experiment 4, neurons were infected with 1 of 3 lentiviruses (encoding either GFP, GFP-RACK1_{wt}, or GFP-RACK1_{Y246F}). The three groups were cultured until the virus was expressed for at least 24 hours (expression was determined by the fluorescence of GFP). Then, the neurons were starved for 3 hours in neurobasal without B27, followed by treatment with BDNF (100ng/ml) or an equal volume of vehicle for 30 minutes. ICC was used to stain cells for paxillin protein. GFP staining, indicating expression of the lentiviruses, was still evident following fixation.

Neurons that were overexpressing RACK1_{wt} (vehicle: 124.948 ± 4.822μm; BDNF: 125.115 ± 5.452μm) were significantly shorter than neurons that were overexpressing either GFP (vehicle: 147.057 ± 7.471μm; BDNF: 154.368 ± 11.657μm; p=0.001) or RACK1_{Y246F} (vehicle: 136.293 ± 7.085; BDNF: 144.181 ± 7.066; p= 0.024; Figures 9 and 10). There was no significant different in axon length between cells expressing GFP or RACK1_{Y246F} (p=0.162). Our preliminary data showing a strong effect of BDNF on axon length did not hold up when replicates were performed. In fact, BDNF had no significant effect on axon length (Figure 10; p=0.385). In terms of branching, a
similar result was obtained. Cells overexpressing RACK1_{Y246F} (vehicle: 1.350 ± 0.174 branches; BDNF: 1.083 ± 0.165 branches; p=0.046) or GFP (vehicle: 1.475 ± 0.224; BDNF: 1.200 ± 0.221; p=0.014) had significantly more branches than RACK1_{wt} expressing cells (vehicle: 0.917 ± 0.145; BDNF: 0.867 ± 0.131; Figure 11). However, BDNF had no effect on axon branching (p=0.168).
Figure 2. BDNF stimulation results in a significant decrease in axon length. (A, B) Neurons were treated with vehicle (A) or 100ng/ml BDNF (B) for 2 DIV and stained with phalloidin 488. (C) Average axon length measured following treatment for 2 days with 100ng/ml BDNF or equal volume of vehicle. Vehicle treated neurons (149.845 ± 7.353μm) were significantly longer than BDNF treated neurons (125.640 ± 4.931μm; Student’s t-test, *p ≤ 0.05; n=60 for both vehicle and BDNF).
Figure 3. BDNF stimulation does not affect axon branching. Average number of axon branches following treatment for 2 days with 100ng/ml BDNF or equal volume of vehicle. Branches are defined as extensions from the axon that were ≥ 5μm. There was no significant difference between the groups (Student’s t-test, p=0.473; n=60 for both vehicle and BDNF).
Figure 4. BDNF stimulation results in a significant decrease in axon length. (A) Neurons were treated with vehicle (A) or 40ng/ml BDNF (B) for 2 DIV and stained with phalloidin 488. (C) Neurons were treated with 40ng/ml BDNF or an equal volume of vehicle and cultured for 2 DIV. Vehicle-treated neurons (154.424 ± 7.970μm) had significantly longer axons than the neurons stimulated with BDNF (133.563 ± 6.592μm; Student’s t-test, *p ≤ 0.05; n=60 for both groups).
Figure 5. BDNF stimulation does not affect axon branching. Average number of axon branches following treatment for 2DIV with 40ng/ml BDNF or equal volume of vehicle. Branches are defined as extensions from the axon that were $\geq 5\mu$m. There was no significant difference between the groups (Student’s t-test, $p=0.373$; $n=60$ for both vehicle and BDNF).
Figure 6. Role of RACK1 in BDNF stimulated axon length and branching. GFP (A, B), GFP-RACK1\textsubscript{wt} (C, D), or GFP-RACK1\textsubscript{Y246F} (E, F), was expressed in neurons. Following expression, neurons were stimulated with either 40ng/ml BDNF (B, D, F) or vehicle (A, C, E) for 2 hours. Neurons were then fixed and stained with rhodamine phalloidin.
Figure 7. Role of RACK1 in BDNF stimulated axon length. Neurons were infected with lentivirus expressing GFP, GFP-RACK1_{wt}, or GFP-RACK1_{Y246F} and cultured until they showed expression of the virus. After expression was observed, the cells were treated with either 40ng/ml BDNF or an equal volume of vehicle for 2 hours. The axon length of cells expressing RACK1_{wt} and treated with BDNF is decreased as compared to all of the other groups.
Figure 8. Role of RACK1 in BDNF stimulated axon branching. Neurons were infected with lentivirus expressing GFP, GFP-RACK1_wt, or GFP-RACK1_Y246F and cultured until they showed expression of the virus. After expression was observed, the cells were treated with either 40ng/ml BDNF or an equal volume of vehicle for 2 hours. Branches are defined as extensions from the axon that were ≥5μm. Branching in GFP-RACK1_Y246F expressing neurons is decreased as compared to all other groups.
Figure 9. Overexpression of GFP-RACK1<sub>wt</sub> results in significantly reduced axon length. GFP (A, B), GFP-RACK1<sub>wt</sub> (C, D), or GFP-RACK1<sub>Y246F</sub> (E, F), was expressed in neurons. Following expression, neurons were starved for 3 hours and then stimulated with either 100ng/ml BDNF (B, D, F) or vehicle (A, C, E) for 30 minutes. Neurons were then fixed and stained for paxillin.
Figure 10. Overexpression of GFP-RACK1<sub>wt</sub> results in significantly reduced axon length. Neurons were infected with lentivirus expressing GFP, GFP-RACK1<sub>wt</sub>, or GFP-RACK1<sub>Y246F</sub> and cultured until they showed expression of the virus. After expression was observed, the cells were starved for 3 hours and then treated with either 100ng/ml BDNF or an equal volume of vehicle for 30 minutes. There was no significant effect of BDNF on axon length (p=0.385). However, overexpression of RACK1<sub>wt</sub> resulted in significantly decreased axon length as compared to either GFP or RACK1<sub>Y246F</sub>. Neurons that were overexpressing RACK1<sub>wt</sub> (vehicle: 124.948 ± 4.822μm; BDNF: 125.115 ± 5.452μm) were significantly shorter than neurons that were overexpressing either GFP (vehicle: 147.057 ± 7.471μm; BDNF: 154.368 ± 11.657μm; p=0.001) or RACK1<sub>Y246F</sub> (vehicle: 136.293 ± 7.085; BDNF: 144.181 ± 7.066; p= 0.024). Two-way ANOVA and Tukey’s post-hoc, *p ≤ 0.05. n=60 for all RACK1<sub>wt</sub> and RACK1<sub>Y246F</sub> groups, n=40 for both GFP groups.
Figure 11. Overexpression of GFP-RACK1<sub>wt</sub> results in significantly reduced axon branching. Neurons were infected with lentivirus expressing GFP, GFP-RACK1<sub>wt</sub>, or GFP-RACK1<sub>Y246F</sub> and cultured until they showed expression of the virus. After expression was observed, the cells were starved for 3 hours and then treated with either 100ng/ml BDNF or an equal volume of vehicle for 30 minutes. Branches are defined as extensions from the axon that were ≥ 5μm. There was no significant effect of BDNF on axon branching (p=0.168). However, overexpression of RACK1<sub>wt</sub> resulted in significantly decreased axon branching as compared to either GFP or RACK1<sub>Y246F</sub>. Cells overexpressing RACK1<sub>Y246F</sub> (vehicle: 1.350 ± 0.174 branches; BDNF: 1.083 ± 0.165 branches; p=0.046) or GFP (vehicle: 1.475 ± 0.224; BDNF: 1.200 ± 0.221; p=0.014) had significantly more branches than RACK1<sub>wt</sub> expressing cells (vehicle: 0.917 ± 0.145; BDNF: 0.867 ± 0.131). Two-way ANOVA and Tukey’s post-hoc, *p ≤ 0.05. n=60 for all RACK1<sub>wt</sub> and RACK1<sub>Y246F</sub> groups, n=40 for both GFP groups.
CHAPTER V

DISCUSSION

RACK1 is a ribosomal scaffolding protein found at point contacts, and known to regulate the motility of fibroblasts, as well as be necessary for appropriate local translation of β-actin mRNA in the growth cone. These studies examined the hypothesis that RACK1 regulates the extension of axons. In order to test this hypothesis, we conducted 4 experiments. Experiments 1 and 2 were conducted as a platform for Experiments 3 and 4. Experiments 1 and 2 examined how BDNF, which is known to affect axon outgrowth of various neuronal types and activate local translation in the growth cone, affects axon length and branching. Experiments 3 and 4 examined how RACK1, in combination with BDNF stimulation, regulates axon growth and branching.

Experiments 1 and 2

In experiments 1 and 2, we found that both 100ng/ml and 40ng/ml BDNF significantly decreased axonal outgrowth, but had no effect on branching. Thus, two-day BDNF treatment of E17 mouse cortical neurons led to a decrease in axon length at both 100 and 40 ng/ml concentrations. These data suggest that 2 day-stimulation with BDNF at a constant concentration slows axon growth in mouse cortical neurons. However, similar experiments have shown that BDNF increases the length of axons in rat E18 hippocampal neurons, but does not increase β-actin protein (Labelle et al., 2000). The most likely explanation for the lack of β-actin protein increase is due to their measurement of β-actin protein throughout the cell, using western blot, instead of just within the growth cone. We have previously found that BDNF stimulation results in an
increase in β-actin protein specifically within developing axons, through the use of both western blot (in axons and growth cones) and quantitative immunocytochemistry (in growth cones) (Sasaki et al., 2010). Another study, which focused on mouse P4 corticospinal motor neurons, found that application of 25 ng/ml BDNF for 2 DIV decreases axon length (Özdinler et al., 2006).

There are many potential reasons for the discrepancy, in relation to axon length, between these studies. It may be due to the species that is used or the type of neuron that is being cultured. It could also be due to the length of time of exposure to the cue. Previous studies have demonstrated that cue-dependent guidance relies on the ligand, type and density of receptors expressed, and the stage of development of the neuron (Sanes et al., 2011). BDNF binds to the TrkB receptor, which is present in mouse cortical growth cones (Cheng et al., 1994). However, a point of note is the extremely long axon length found by Labelle et al. (2000); the average axon length that this study measured, even under basal conditions, is 3 times the average length of our axons. This would suggest that the different cell types have different growth rates—and perhaps even mechanisms—regardless of treatment.

**Experiments 3 and 4**

Experiment 3 was carried out simultaneously with experiment 4, in order to determine if starvation of the neurons prior to BDNF stimulation (Experiment 4) would have a greater effect on axon length and branching, as compared to stimulation alone (Experiment 3). We completed experiments 3 and 4 simultaneously, and following
analysis of preliminary data found a much stronger effect of BDNF in experiment 4, as compared to experiment 3. As such, we completed experiment 3 only once and were therefore incapable of conducting a significance test.

The significance found in experiment 4 suggests that overexpression of RACK1 results in a reduction in axon outgrowth. Specifically, overexpression of RACK1<sub>wt</sub> results in a decrease in axon length as compared to both RACK1<sub>Y246F</sub> and GFP overexpression. In previous research, the alteration of Rac1 (a Rho GTPase and member of the point contact complex) to either increase or decrease the number or size of point contacts resulted in a decrease in growth cone motility (Woo et al., 2006). In line with this research, we speculate that there is an optimal number of point contacts per area of cell, or optimal size of point contacts, for maximum axonal outgrowth; either increasing or decreasing point contacts from that optimal number or size will slow axon growth. Our data suggest that culturing neurons in an environment in which RACK1 is overexpressed creates an abundance of point contacts or an increase in point contact size, slowing the growth cone as it extends forward and thus decrease axon length.

However, the overexpression of either RACK1<sub>wt</sub> or RACK1<sub>Y246F</sub> may lead to an increased number of point contacts within the growth cone. In RACK1<sub>wt</sub>, all of these point contacts are functional, and therefore will decrease the motility of the growth cone. In neurons in which the non-phosphorylatable mutant version of RACK1 is expressed, it is expected that the non-phosphorylatable mutant will be out-competing the endogenous RACK1 produced by the cells, but a small portion of ribosomes will be bound to this endogenous and functional RACK1. We speculate that this small amount of endogenous RACK1 could increase the number of functional point contacts back to the optimal
threshold. This may be especially true if the lentiviral expression of RACK1\textsubscript{Y246F} was not optimal (\textit{i.e.} as high as it could be). Taken together, this is a possible explanation for why the axons of RACK1\textsubscript{Y246F} over-expressing cells are longer than the RACK1\textsubscript{wt} over-expressing cells, but not significantly different from expression of GFP (control). In any case, our results are in line with a previous study in CHO-K1 cells which demonstrated that the random cell migration of these cells was decreased following overexpression of RACK1\textsubscript{wt}, but not affected by overexpression of RACK1\textsubscript{Y246F} (Cox \textit{et al.}, 2003). In addition, this study also showed that overexpression of either RACK1\textsubscript{wt} or RACK1\textsubscript{Y246F} resulted in decreased chemotactic migration (in response to guidance cues). We did not test the role of RACK1 in chemotactic migration in the current study. However, future studies will address whether RACK1 regulates axon guidance in response to chemoattractive stimuli.

We did not find a change in axon length or branching following BDNF stimulation in GFP expressing cells in experiments 3 and 4. This is in contrast to experiments 1 and 2, where a significant decrease in axon length following BDNF stimulation was evident. However, the time of treatment with BDNF was strikingly different between these experiments. In experiments 1 and 2, neurons were treated for 48 hours with BDNF, whereas in experiments 3 and 4, neurons were treated for only 120 or 30 minutes, respectively. It is possible that neither the 2 hour BDNF stimulation (experiment 3) nor the 3 hour starvation followed by the half-hour BDNF stimulation (experiment 4) was enough time to see an effect of BDNF on axon length. Axon growth is slow (5-20 \( \mu \text{m/hour} \)) and 1-2 hours may not be enough time to measure a significant change in total axon length or branching. If we had measured axon growth rates (\( \mu \text{m/hr} \))
over 1-2 hours using live cell imaging, instead of total axon length (μm), a change in the axon growth rate may have been evident, as has been suggested by previous studies (Welshhans et al., 2011). However, our findings do suggest that the over-expression of RACK1<sub>wt</sub> or RACK1<sub>Y246F</sub> may change the number of point contacts enough to shift the number of point contacts out of or into the optimal range, regardless of BDNF treatment.

To better understand the dynamics by which RACK1 regulates axon growth, future studies will examine how overexpression of RACK1<sub>wt</sub> or RACK1<sub>Y246F</sub> affects point contact number and formation in growth cones. We will also further examine the necessity of RACK1 and point contacts for local translation in growth cones. This study focused on the role of RACK1 in axon growth and branching, but future studies will examine the role of RACK1 in directional axon guidance to specific cues, such as BDNF and netrin-1. Together, these studies will provide a deeper understanding into the mechanisms underlying the formation of neuronal connectivity in the developing brain.
REFERENCES


