Longitudinal extension of primary afferents is regulated by spingosine 1-phosphate receptors and tyrosine kinase receptor B in the embryonic spinal cord via a brain derived neurotrophic factor related mechanism

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LONGITUDINAL EXTENSION OF PRIMARY AFFERENTS IS REGULATED BY SPHINGOSINE 1-PHOSPHATE RECEPTORS AND TYROSINE KINASE RECEPTOR B IN THE EMBRYONIC SPINAL CORD VIA A BRAIN DERIVED NEUROTROPHIC FACTOR RELATED MECHANISM

A Dissertation Presented

by

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of

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ABSTRACT

Primary sensory afferent outgrowth within the developing longitudinal pathway of the spinal cord is important for intrasegmental and intersegmental communication that underlies coordination and development of reflexes and contributes to sensory perception. The endogenous mechanisms that regulate primary sensory afferent extension are the primary focus of this dissertation.

This dissertation tested the hypothesis that primary sensory afferent extension in the longitudinal pathway is regulated by sphingosine 1-phosphate type 1 receptor (S1P₁R) and tyrosine kinase receptor B (TrkB) through a brain derived neurotrophic factor (BDNF) related mechanism. To test this hypothesis we used embryonic day five (E5) chicken embryos, as this is the developmental time point when sensory afferents are growing along the longitudinal axis of the spinal cord but have not yet turned ventrally to make connections with the grey matter of the spinal cord. Chicken embryos were removed from their in ovo environment to allow for labeling of primary afferent neurons in the thoracic 3/4 (T3/4) dorsal root ganglia (DRG). Tissue was then put into culture with or without various pharmacological agents and subsequently assayed for length of growth of the labeled primary afferent axons along the longitudinal axis of the spinal cord.

Results showed both BDNF and fingolimod-p, an S1P₁R agonist known to increase BDNF mRNA and protein production/secrection in cortical neurons, increased primary axon extension along the longitudinal pathway. Further, fingolimod-p increased BDNF mRNA production in DRG in this system. Conversely, inhibition of BDNF or S1PRs attenuated primary afferent axon extension along the longitudinal pathway. We found BDNF signaling to be required for fingolimod-p’s effects as addition of αBDNF attenuated the effects of fingolimod-p on axon outgrowth.

TrkB, the high affinity receptor for BDNF, is expressed in chicken DRG during embryonic development. We hypothesized that TrkB activation by BDNF regulates DRG axon extension in the longitudinal pathway through the PLC-γ signaling pathway. We found inhibition of TrkB and/or PLC-γ signaling pathway attenuated DRG axon extension with or without BDNF stimulation. Additional pathways associated with TrkB activation: mitogen activated kinase (MAPK) and phosphoinositide 3-kinase (PI3K) appeared to either have no effect on DRG axon extension or were involved in DRG axon extension through a mechanism that is not related to TrkB.

Collectively, these studies suggest an endogenous mechanism for the regulation of DRG axon outgrowth within the longitudinal pathway. With this mechanism, DRG axon outgrowth may be enhanced or attenuated following manipulation of S1P₁R, BDNF and/or TrkB. Further, these findings suggest an action through BDNF on CNS axons as a potential therapeutic effect of fingolimod-p, a treatment for relapsing remitting forms of Multiple Sclerosis.
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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

INTRODUCTION

Our ability to form spinal reflexes is the result of an elaborate neuronal growth pattern that begins in early development and requires formation of neuronal connections between the peripheral nervous system (PNS) and multiple segments of the spinal cord. The developmental events leading up to the formation of these connections are the result of endogenous regulation and response to exogenous cues. Sensory axons must enter the cord, extend the appropriate length along the cord and innervate appropriate neurons within the cord. Understanding the endogenous mechanisms that control important developmental events such as axon extension, is important for our understanding of reflex formation. The following body of work sought to understand the endogenous regulation of primary sensory afferent extension in the developing longitudinal pathway of the embryonic chicken spinal cord.

THE CHICKEN EMBRYO

The chicken (Gallus gallus domesticus) embryo is an advantageous model for developmental studies as it has a well characterized anatomical development. Descriptions of chicken embryo development were published in 1951 when Victor Hamburger and Howard Hamilton published a paper entitled: “A series of normal stages in the development of the chicken embryo” (Hamburger and Hamilton, 1951). This document contained a comprehensive depiction of normal chicken development from the earliest HH st.1, prior to the formation of the primitive streak, until hatching at HH st.46. Hamburger and Hamilton’s document was the first of its kind to provide illustrations and
written descriptions for all stages of chicken embryo development, allowing for more accurate identification of embryonic age (Hamburger and Hamilton, 1951).

The chicken embryo has many advantages over mammalian models, with the most obvious advantage being that development occurs outside of a live animal. This system allows for greater access to the developing embryo, making surgical manipulation and neuronal labeling a less difficult endeavor than in mammalian embryos. In regards to the study of primary afferent growth, the chicken embryo is an advantageous model because the pattern in which afferents grow and establish connections with the CNS is well characterized (Eide and Glover, 1995, Coutinho-Budd et al., 2008).

Scrutiny of both the anatomy and cytoarchitecture of the chicken spinal cord reveals close resemblance to mammalian spinal cord. Both the chicken and the mammal contain grey matter consisting of motor neuron cell bodies, interneurons and various glial cells. Further, the grey matter is stratified into ten domains, each with unique cytoarchitecture known as Rexed lamina. Layers I-VI occupy the dorsal horn, layers VII-IX constitute the ventral horn and layer X surrounds the central canal of the spinal cord (Martin, 1979). Surrounding the grey matter is a layer of white matter containing ascending and descending myelinated axons that form ventral, lateral and dorsal funiculi.

A further parallel between these models lies within the somatosensory reflex system. Both mammals and chicken contain a reflex system in which stimulation of a peripheral receptor sends afferent signals to the spinal cord. The cell bodies of these afferents are located in dorsal root ganglia (DRG) located adjacent to each segmental level of the spinal cord. Axons may enter different laminae of the spinal cord and synapse directly onto motor neurons or interneurons, or may ascend and descend rostrally and
caudally within the ipsilateral dorsal funiculus to then innervate neurons in multiple segments of the spinal cord (Martin, 1979, Ozaki and Snider, 1997).

While there are many advantages to the chicken embryo model, the use of this model is tempered by a major disadvantage. Unlike the mouse, transgenic technologies have proven to be extremely difficult in the chicken. This is in part due to the reproductive system of the laying hen. The first 20-24 hours of embryonic development begins in the hen oviduct where a significant amount of cell division and growth occurs. By the time the egg is laid, the embryo consists of >60,000 undifferentiated cells. During this time, the embryo is inaccessible to manipulation making the use of many genetic techniques technically difficult (Menna et al., 2003). As a result, techniques using retroviral vectors and morpholinos to introduce exogenous genes have been developed (Michael et al., 1997). While these methods for exogenous gene expression have allowed for genetic manipulation within the chicken embryo model, there are limitations that makes using these methods difficult in early developmental studies. Both retroviral vectors and morpholinos have been used effectively to knockdown and/or knock in specific genes into the chicken embryo. However, use of these techniques require injection or electroporation of retrovirus at early stages of development which may alter the development of afferents prior to the time point in which afferents begin to extend within the longitudinal pathway (Krull, 2004). Electroporation at early stages of development can be problematic as injection of current puts stress on the developing embryo which could result in damage to the developing afferents and alteration of growth.
The chicken embryo is the most appropriate model system for this work for the following reasons. First, all experiments conducted in this work were done at an early developmental stage that is easily accessible in a chicken model, yet more difficult to access in a mammalian embryo. Second, we were interested in the developmental growth of DRG axons in the longitudinal pathway, an anatomical pathway that is easily labeled in situ in chicken. Thus, the following investigation makes use of the chicken embryo at embryonic day (E5), HH st.25. This is an ideal time to investigate longitudinal afferent extension within the longitudinal pathway because afferents have entered into the spinal cord, bifurcated and have begun to extend rostrally and caudally along the longitudinal axis of the spinal cord. At this time, afferents have not invested into the grey matter of the spinal cord and will not do so until E8.

**Experimental preparation: advantages and disadvantages**

The experimental preparation utilized in this work requires that an embryo be removed from its in ovo environment. Following injection of tracing materials and pharmacological manipulation, the embryo is placed into a culture dish containing a sterile cell culture insert known as a Millicell [Figure 1-1A,B]. The preparation is then placed into an incubator at 37°C for 5 hours. One important advantage of this preparation is that it allows for controlled drug delivery to the embryo. The in ovo environment contains many layers of material with various solubility characteristics, making the concentration of drug delivery to the embryo impossible to calculate (Menna et al., 2003). Isolation of the embryo from the in ovo environment and drug treatment in culture allows a more precise drug delivery to the embryonic tissues.
Additionally, maintenance of the substrate upon which these axons grow is essential, as the molecular composition of this substrate has yet to be characterized. Investigations using dissociated DRG neurons to study axon growth rates utilize different substrates. As a result, different growth rates and responses to pharmacological agents have been observed (Ketschek et al., 2007, Koch et al., 2012). Thus, it is essential that the endogenous substrate be maintained in order to accurately investigate endogenous regulation of growth.

Additionally, this preparation allows ease in injecting tracing materials into the DRG and maintenance of the integrity of the afferents and the substrate upon which DRG axons grow. Previous labeling experiments using DiI, a lipophilic cell tracing material demonstrate that neither dissection nor DiI injection damages the central or peripheral projecting afferent of the DRG [Figure 1-1C]. Maintenance of both the central and the peripheral projection is essential for normal growth, as it has been shown that damage to the peripheral projection enhances growth of the central projection (Yang and Yang, 2012). It is important that DRG axons are not damaged, as the preparation is designed to analyze growth of intact axons and not regenerating axons.

**DORSAL ROOT GANGLIA AND DORSAL ROOT ENTRY ZONE ANATOMY AND DEVELOPMENT**

**DRG origin and development**

Dorsal root ganglia are a heterogeneous population of neural crest derived cells. Their primary function is to provide somatosensation, nociception and proprioception to the entire body with the exception of the face.
Dorsal root ganglia development begins with neural tube closure and dorsal migration of neural crest cells (NCC) at every level of the spinal cord. In the chicken embryo, migration of NCCs destined to become DRG begins at embryonic day 2 (E2), Hamburger Hamilton stage 12 (HH st.12) and is complete by E3 (HH st.20) (Lallier and Bronner-Fraser, 1988, Rifkin et al., 2000). Chicken DRG neurogenesis occurs between E3 and E6. Experiments using $[^{3}\text{H}]$ thymidine labeling to label dividing cells in ovo found $[^{3}\text{H}]$ thymidine labeled DRG neurons at E3-5, with unlabeled neurons appearing at E4 and no label after E6. These data suggest neurons had withdrawn from the cell cycle by E6 and that DRG neurons originate between E3 and E6 (Scott, 1990).

Once DRG position is established, neurons extend peripheral and central afferents in a highly characterized manner. At E3 (HH st.19), DRG neurons extend afferents centrally toward the developing spinal cord and peripherally toward the developing muscles and skin (Eide and Glover, 1995). Sensory afferents enter the spinal cord via a region known as the dorsal root entry zone (DREZ) at each segmental level of the spinal cord [Figure 1-2A] (Sprague, 1958).

The DREZ is a unique anatomical region where primary sensory afferents from each segmental level enter into the spinal cord. Afferents extend central projections towards the DREZ and not to other areas of the spinal cord or notochord due to chemorepulsive cues from these regions (Masuda et al., 2009). Demarcating the developing DREZ is a unique population of multipotent stem cells called boundary cap cells. These cells are located at axon entry and exit points in the spinal cord. Their primary function is thought to be regulation of sensory axon bundle ingrowth into the spinal cord (Hjerling-Leffler et al., 2005). In the mature animal, the DREZ is surrounded by a thickened layer
of glia called the glia limitans. These structures form barriers with channels for myelinated and non-myelinated axon bundle penetration. The only structures permitted to transverse this region are the axons (Fraher, 1999).

Prior to afferent entry into the DREZ, DRG neurons maintain a bipolar morphology within the ganglia. As development progresses, the two opposing processes fuse to form a single extension of the cell body’s cytoplasm, thus giving DRG neurons pseudounipolar morphology (Takahashi and Ninomiya, 1987). By E4.5, in the developing chicken embryo, the central process of the DRG cells have entered the DREZ and have branched rostrally and caudally to establish the longitudinal pathway [Figure 1-2B]. In the in situ preparation used in this study, chicken DRG central afferents growing within the longitudinal pathway at E5, HH st.25, extend axons at a rate of 56.64µm/hr (McNamara et al., 2015; unpublished findings).

Organization of this pathway follows a somatotopic and modality based pattern (Brown et al., 1974). Following longitudinal extension (E8; HH st.34), the centrally projecting axons simultaneously invest into each segmental level of the grey matter of the spinal cord to establish intrasegmental and intersegmental connections with neurons and interneurons within the developing spinal cord [Figure 1-2C]. This pattern of development underlies the coordination of reflex formation (Davis et al., 1989). Following grey matter investment, some afferents continue to lengthen as a single bundle up until E10. Following E10, longitudinal axons segregate into either the dorsal funiculus or Lissauer’s tract (dorsolateral fasiculus) (Eide and Glover, 1995). Analysis of these axons at the electron microscopic level shows the number of axons within the dorsal funiculus is highest during embryonic development and significantly diminishes
following birth and postnatal development due most likely to cell death (Chung and Coggeshall, 1987). During development, programmed cell death occurs in all sensory ganglia. In the chicken DRG, programmed cell death occurs in 30% of neurons within the developmental time period of HH st. 25-38 (Hamburger and Levi-Montalcini, 1949, Wets and Vaughn, 1998).

In addition to the central projection, the peripheral projection of DRG neurons connect to a variety of sensory receptors in the periphery. The type of peripheral receptor afferents connect with is dependent upon the tyrosine kinase receptor (Trk) expressed by the cell body of the DRG. In adult rat DRG, TrkA expressing neurons contain little to no myelin, are small in diameter (<30µm), and innervate nociceptors. TrkB expressing neurons are myelinated, are variable in size and innervate high threshold receptors. TrkC expressing neurons are myelinated, large in diameter (>30µm) and innervate low threshold receptors (McMahon et al., 1994). The Trk profile of these cells changes throughout early development, however whether Trk receptor profile in the early embryo affects the future differentiation pattern or function is unknown (Rifkin et al., 2000).

THE AXON AND AXON OUTGROWTH

The theory that axons are a distinct, morphological component of nerve cells was first recognized by Wilhelm His in 1887. His observed continuity between post mitotic neurons and their growing axons. This observation was in direct contradiction with the previously held notion that the nervous system was composed of a continuous, reticulum of fused processes. In later studies, using a silver staining technique developed by Camillo Golgi, Santiago Ramon Y Cajal was able to identify the structure of the growth
cone in unmyelinated embryonic neurons. Using this technique, Cajal described the
growth cone as a rounded, enlargement at the distal end of the axon containing a
“neurofibrillary bundle” (de Castro et al., 2007). These structures would later be
identified as actin filaments and microtubules. Upon further investigation, Cajal observed
that axons follow what appeared to be a predefined direction. Cajal postulated that during
migration growth cones are guided by chemical signals. These early studies set precedent
for later analysis of the growth cone and axon growth during development (de Castro et
al., 2007).

Advances in immunoflourescent techniques have confirmed Cajal’s initial
observations of the growth cone, and further identified more specific components of this
structure. Within the growth cone are three structural domains: the peripheral (P) domain,
transition (T) domain and central (C) domain. Within the P-domain are two, distinct
structures: the lamellipodia and the filopodia. The lamellipodia consist of a mesh-like
network of actin fibers whereas the filopodia consist of filamentous actin (F-actin)
[Figure 1-3]. Time lapse microscopy reveals both of these structures are highly dynamic,
forming, extending and retracting processes over a second to minute time courses
(Steketee et al., 2001).

The T-domain contains arch like actin filaments that lie perpendicular to the actin
filaments of the P-domain. As the growth cone advances, actin within the T-domain
functions to compress microtubules within the central domain, an important process for
crosslinking microtubules into bundles and positioning microtubules into closer
proximity to microtubule associated proteins (MAPS). MAPS are proteins required for
stabilization of microtubules and coordinated interactions between actin and
microtubules. Previous investigations of Tau, a developmentally regulated MAP, found this protein to be important for neurite outgrowth and extension. In chicken DRG, microCALI ablation of Tau protein in vitro resulted in reduced number and length of neurites as well as a decrease in neurite extension rate (Liu et al., 1999).

The C domain of the growth cone consists of an unstable population of microtubules that do not associate with MAPs and thus remain de-fasciculated. These microtubules undergo dynamic instability, alternating between slow, positive end growth and rapid positive end disassembly. The result is microtubule penetration of the T and P domains where they interact with F-actin. This functions to produce a radial array of microtubules across the P domain, an important morphology for axon extension.

Also within the C-domain of the growth cone is a stable group of microtubules, organized into bundles by MAPs. These microtubules are oriented with their positively charged, polymerizing ends towards the T-domain. The role these microtubules play in the growth cone is not entirely understood, although it has been suggested that they may incorporate into the growing axon (Prokop, 2013).

Axon outgrowth requires the coordination of actin and microtubules. In one study using dissociated sympathetic neurons, the rate of growth cone advance was directly correlated with size and dynamics of lamellipodia and filopodia (Argiro et al., 1984). Another study using dissociated embryonic chicken DRG found lamellipodia and filopodia dynamics and extension rates were greatest at the leading edge of the growth cone, whereas at the base of the growth cone, filopodia had a greatest rate of retraction, often retracting into the axon/neurite (Bray and Chapman, 1985). These studies suggest
lamellipodia and filopodia formation and dynamics are important events required for axon/neurite extension.

Previous investigations on the rate of sensory afferent extension report a range of growth rates that are highly dependent upon whether observations are made in vivo or in vitro. Embryonic DRG axon growth rate in chicken is dependent upon the substratum upon which cells are plated. For instance, DRG plated on laminin grow at a rate of 54 µm h⁻¹ whereas axons grow at a rate of 36 µm h⁻¹ when plated on polylysine (Ketschek et al., 2007). Previous investigations of peripheral trigeminal axon extension in E10-13 chicken in ovo report a growth rate of 20µm h⁻¹ (Davies, 1987). While Coutinho-Budd et al., (2008) calculated the growth rate for chicken DRG afferents to be 22 µm h⁻¹ between E4-E6, the growth rate of E5 DRG axons in vivo or in the in situ preparation used in this work is yet to be characterized (Coutinho-Budd et al., 2008).

More recent studies using video enhanced contrast differential interference contrast (VEC-DIC) microscopy demonstrate axon growth requires three distinct morphological stages. First, the axon protrudes F-actin containing filopodia and lamellipodia, second axon engorgement occurs when vesicles and organelles invade the protruding lamellipodia and third, consolidation of the axon results when F-actin depolymerizes in the T-domain, membrane shrinking and the formation of an axon shaft. The end result is axon outgrowth (Dent and Gertler, 2003) [Figure 1-4].

Also required for axon outgrowth and extension is an increase in cell surface area. To accomplish this, growing neurons must undergo membrane insertion. This membrane is translocated from intracellular locations and inserted into the neuron cell membrane. One point of controversy is where in the neuron this membrane is inserted. One approach
to study this question is to analyze the overall movement of the neuronal cell surface relative to stationary objects within the cell surface (Bray, 1979). Using this approach, Dai and Sheetz (1995) demonstrated membrane insertion occurs at the growth cone. Using antibody coated beads that bind to membrane glycoproteins and lipids, Dai and Sheetz demonstrated that this bead moved towards the cell body at a rate five times greater than that of axon extension in these neurons. From these studies, Dai and Sheetz suggest that membrane insertion occurs at the growth cone and that a gradient of membrane tension, with highest tension in the cell body and lowest tension in the growth cone, permits membrane addition at the growth cone and retrograde flow of membrane to the axon (Dai and Sheetz, 1995).

In contrast to these findings, Popov et al., (1992) suggest membrane insertion occurs throughout the entire neurite and cell body of the neuron. Using cultured spinal neurons from Xenopus, Popov et al., (1992) used fluorescent labeling techniques to analyze the rate of membrane flow in different areas of the neurite relative to fixed reference points within the neurite. The result was evidence that membrane addition occurs throughout the neurite and cell body, with increased membrane addition at locations further from the soma (Popov et al., 1993).

**Molecular factors controlling afferent growth and axon guidance**

Throughout development, afferent axon growth and guidance is regulated by complex molecular events that mediate attraction, repulsion and growth of axons. Within the in situ environment, axons encounter multiple guidance cues and respond by extending and/or retracting axons or directional turning. The influence of these factors allows for axon pathfinding within the longitudinal pathway.
Netrins are a family of long range diffusible molecules that through interactions with receptor deleted in colorectal cancer (DCC) receptor mediate chemoattractive effects or UNC receptors mediate chemorepulsive effects. During early development (E10-11; mouse), netrins are expressed in the floor plate of the ventral spinal cord of the developing neural tube where via interactions with UNC receptors prevent afferent growth towards the ventral spinal cord (Masuda et al., 2009).

Later in development, netrins are secreted from the dorsal spinal cord. Interaction with the Unc5c receptor is one of the receptor ligand interactions shown to regulate pathfinding of DRG axons in the developmental time period following bifurcation of afferents but before investment into the grey matter. Wantanabe et al., (2006) found netrin-1 is expressed and secreted from the dorsal spinal cord in a restricted developmental window when afferents have yet to turn centrally into the grey matter of the spinal cord (E12.5; mouse). This study found netrin-1 Unc5c interactions prevented DRG axon investment into the ventral spinal cord (Watanabe et al., 2006).

After DRG axons enter into the DREZ at E3 (chicken), they must then bifurcate to establish the longitudinal pathway. This bifurcation is regulated by Cardiac Natriuetic Peptide (CNP), a member of the natriuetic peptide family. While CNP is classically thought to regulate homeostatic control of body water and salt, it has also been shown to act as a cue for control of axon branching, extension and guidance during the development of the longitudinal pathway (Zhao and Ma, 2009).

Bifurcation of DRG afferents is followed by rostral and caudal extension within the longitudinal pathway. Recent studies of DRG axon extension in the longitudinal pathway point to the involvement of many different factors in the regulation of axon
extension within this pathway. For instance, previous investigations suggest G protein coupled receptors (GPCRs) are important for DRG axon growth within the longitudinal pathway. In these studies, pharmacological manipulation of cyclic adenosine monophosphate (cAMP) signaling resulted in attenuated or enhanced axon extension. Specifically, activation of adenylate cyclase attenuated afferent extension whereas inhibition of cAMP substrate, PKA enhanced afferent extension (Coutinho-Budd et al., 2008). Additionally, inhibition of myosin II, a mechanoenzyme known to be involved in generating contractile forces necessary for cell motility, attenuates afferent extension in the developing longitudinal pathway (Robinson et al., 2015).

There are many molecular factors known to control DRG afferent pathfinding and guidance including: Semaphorins, Sonic hedgehog (SHH) and neurotrophins. A summary of these factors and their influence on afferent pathfinding and guidance is depicted in Figure 1-5.

Semaphorins are a family of long-range, diffusible, guidance factors known to influence DRG pathfinding in situ via their interactions with plexin A1 and A3 receptors. In chicken, Sema5B is expressed in the dorsal horn of the spinal cord at E5, when axons are extending along the longitudinal axis of the cord. Sema5B expression is attenuated by E8, the developmental time point when axons invest within the grey matter of the developing spinal cord. In vivo investigations of the role of Sema5B found knockdown of Sema5B using short hairpin RNA (shRNA) resulted in premature investment of DRG axons into the grey matter of the developing spinal cord suggesting Sema5B is an important factor for pathfinding for developing DRG (Liu et al., 2014). Thus, both
semaphorins and netrins are secreted at E5, when afferents are extending within the longitudinal pathway of the developing spinal cord.

Sonic hedgehog signaling is also known to be important for DRG axon guidance in E13.5 rat. Through its canonical signaling pathway Patched (Ptc), SHH relieves inhibition of a transmembrane bound protein known as Smoothened (Smo). Activation of Smo leads to subsequent activation of Gli transcription factors which have been shown to promote turning of afferents towards SHH sources via an unknown mechanism. SHH also acts through a non-canonical pathway in which it activates Src family kinases (SFKs). Investigations of non-canonical SHH signaling revealed inhibition of SRK activity in the presence of SHH prevented dissociated embryonic commissural axons from turning towards SHH sources (Yam et al., 2009). Thus, these findings would suggest SHH signaling is important for axon turning. Whether these signaling pathways are important for afferent extension and pathfinding within the longitudinal pathway has yet to be investigated.

Neurotrophic factors are known to act as axon guidance factors for sensory axons as well. Previous studies show NT4 and BDNF are important factors for geniculate ganglion axon guidance towards fungiform papilla epithelium, the tissue that will differentiate into taste buds (Runge et al., 2012). Additionally, zebrafish spinal neurons grown on laminin were shown to turn in response to exogenous application of BDNF delivered to an in vitro assay via pulsatile injection from a micropipette (Chen et al., 2013). These results suggest neurotrophins, potentially through their high affinity tyrosine kinase receptors (Trk) act as chemoattractant axon guidance factors [Figure 1-5].
NEUROTROPHINS

Neurotrophins are a family of structurally homologous proteins consisting of: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (Chu-Wang and Oppenheim, 1978). NGF was the first member of this family to be identified and isolated from mouse salivary gland (Levi-Montalcini and Hamburger, 1951, Levi-Montalcini and Angeletti, 1964). To uncover NGF’s function, an antiserum was developed and injected into newborn rodents. The result was rodents with a severely deficient to absent sympathetic nervous system and sensory ganglia (Levi-Montalcini and Booker, 1960). These results were the first evidence of a molecular factor regulating neuronal survival during development. In mouse, genetic knockdown of either NGF or TrkA results in a loss of 70-80% of DRG neurons (Farinas, 1999).

The sequencing of NGF provided a platform for the preparation of synthetic oligonucleotides, complementary DNA and the cloning of human, mouse, bovine and chicken genes, all with a high degree of interspecies sequence homology (Scott et al., 1983, Large et al., 1989). The remainder of the neurotrophin family (BDNF, NT3, NT4/5) were found to contain the ability to support growth, differentiation and survival in both the developing and mature nervous system (Misko et al., 1987).

Each member of the neurotrophin family is synthesized as a pre-pro-protein. Removal of the N-terminal pre signal results in a pro-neurotrophin that may act on the low affinity P75NTR subsequently inducing P75NTR signaling (Berg et al., 1991). In terms of neurite outgrowth, pro-neurotrophins have been shown to activate neurite outgrowth through activation of RhoGTPases (Lee et al., 2001, Teng et al., 2005).
Processing of the pro-neurotrophin into the mature neurotrophin occurs in the Trans-Golgi network. Here, pro-forms of neurotrophins encounter serine proteinases known as convertases. Of the convertases, Pace4, PC5/6 and furin have been shown to participate in the processing of neurotrophins (Seidah et al., 1996a, Seidah et al., 1996b). Once neurotrophins are cleaved into their mature form they may be shuttled to the plasma membrane and released into the extracellular space via exocytosis (Lim et al., 2007). The mature forms of neurotrophins contain similar motifs and structures as well as the ability to form high affinity homodimers and low affinity heterodimers with NT3. (McDonald and Hendrickson, 1993, Narhi et al., 1993, Jungbluth et al., 1994).

**Brain derived neurotrophic factor (BDNF) overview**

In 1982, Barde et al. (1982) isolated 2 μg of an unknown factor from 3 kg of homogenized pig brain (Barde et al., 1982). In this seminal work Barde demonstrated that this factor, hence forth known as BDNF, could support survival, neurogenesis and neurite extension in cultured embryonic chicken neurons (Lindsay et al., 1985, Ockel et al., 1996). Since its discovery, BDNF has taken the lead as the most influential and thoroughly characterized member of the neurotrophin family.

**BDNF: Roles in developing DRG**

BDNF was first appreciated for its roles in promoting survival and neurite outgrowth in embryonic sensory neurons in vitro. For instance, BDNF enhances neurite number and outgrowth in dissociated sensory neurons of the nodose ganglion and DRG during the embryonic period of E6-12 (Lindsay et al., 1985). Explant cultures of chicken
DRG and all additional sensory ganglia respond to BDNF during early development as well (Davies et al., 1986). In vivo, BDNF has been shown to promote target innervation and axon arborization (Cohen-Cory and Fraser, 1995, Huang et al., 2007). Further, mouse genetic knockouts for BDNF results in smaller stature, defective coordination, movement and balance, degeneration of sensory ganglia and death in the second postnatal week (Ernfors et al., 1995b, Liebl et al., 1997). Thus, these studies set the precedent for our investigations into the potential role of BDNF in afferent extension in the developing chicken embryo.

**BDNF: Sources and signaling**

Although BDNF is known to promote survival and neurite extension in embryonic neurons, the location of BDNF sources within the embryo is not well understood. The process of finding these sources in embryonic tissue has been an arduous task due to the low concentration and low molecular weight of this protein (MW: 27kDa) (Barakat-Walter, 1996). Further, competing evidence regarding whether BDNF acts locally in an autocrine/paracrine manner or via long range signaling further complicates this issue.

BDNF is known to act as a target derived factor as well as in an autocrine/paracrine manner. Evidence that BDNF affects neurons via long range signaling stems from observations that sensory axons grow towards targets containing BDNF. Huang et al., (2007) found that trigeminal nerves in Xenopus embryos extend long distances towards the cement gland, a transient, embryonic structure known to secret BDNF (Huang et al., 2007). Additionally, previous investigations suggest BDNF is
released from the developing spinal cord where it can be taken up by E7-14 DRG neurons and retrogradely transported within the cell (Ba et al., 2010). These findings suggest BDNF signals to neurons from a distal source.

In contrast, it has been suggested that BDNF signals in an autocrine/paracrine manner. For instance, using E4.5 chicken sensory neurons in low density cultures, Wright et al., (1992) found DRG neurons synthesize their own BDNF and signal in an autocrine and paracrine manner (Wright et al., 1992). Further evidence for BDNF autocrine/paracrine signaling derives from BDNF’s short plasma half-life of 0.92 minutes. This short half-life limits the ability of BDNF to act in locations distant from the BDNF source (Poduslo and Curran, 1996, Robinson et al., 1996).

Further evidence for BDNF autocrine/paracrine signaling comes from investigations of anterograde transport of BDNF. Anterograde transport of BDNF has been observed in rat DRG, optic nerve and hippocampus (Menna et al., 2003, Ng et al., 2007). In one investigation, immunoreactivity for BDNF was observed in the axon terminals of adult rat DRG central and peripheral processes. Further immunohistochemical analysis of cervical spinal cord with attached DRG found BDNF protein accumulation in the dorsal horn of the spinal cord suggesting that BDNF is anterogradely transported within the DRG central process and released into the dorsal spinal cord (Michael et al., 1997). Additionally, the rate of anterograde transport for BDNF has been determined in E18 rat hippocampal neurons has been calculated to be $1.49 \mu m s^{-1}$ (Lo et al., 2011).

While compelling evidence exists in support of both target derived BDNF signaling and autocrine/paracrine signaling, it seems likely that autocrine/paracrine
signaling regulates afferent extension within the longitudinal pathway. This is because afferents are extending both rostrally and caudally within the longitudinal pathway. Although possible, it is unlikely that an afferent would branch to grow in opposite directions along a target derived BDNF gradient.

**FINGOLIMOD-P AND SPHINOSINE-1 PHOSPHATE RECEPTORS**

Fingolimod-phosphate (fingolimod-P), is a novel immune-modulator previously shown to increase production of BDNF mRNA and protein in embryonic neurons (Deogracias et al., 2012, Doi et al., 2012). Originally synthesized in 1992 through modification of a fungal metabolite isolated from the culture media of *Isaria sinclairii*, the effects of this compound on the CNS were largely unknown. (Fujita et al., 1994).

Fingolimod is phosphorylated by sphingosine kinase 1/2 (Sphk1/ Sphk2) to its bioactive form, fingolimod-p. A linear relationship is observed between dose administration and blood concentrations of fingolimod and fingolimod-p (Brinkmann et al., 2002). Once active, fingolimod-p binds competitively to sphingosine 1-phosphate receptors type 1,3,4,5 (S1P₁, S1P₃, S1P₄ and S1P₅) with sub-nanomolar affinities (Brinkmann et al., 2002). Once fingolimod-p is bound, the S1P receptor undergoes endocytosis, ubiquitination and degradation (Oo et al., 2007).

S1PRs are a family of G-protein coupled receptors (GPCRs) found in nearly every tissue in the body. Within this family there are five known S1P receptor subtypes, S1P₁-5 all of which bind with high affinity to S1P, a potent, sphingolipid metabolite. A concentration gradient for S1P is found within the body with high S1P concentrations in the plasma (0.1-0.6µm) and blood (400-900nm) and lower concentrations within tissue
(0.5-75 pmol/mg) (Berdyshev et al., 2005). The purpose of this concentration gradient is thought to allow for S1P1R mediated migration of T lymphocytes from secondary lymphoid tissue to the circulation (Edsall and Spiegel, 1999).

Recent analysis of the crystal structure of the S1P1 receptor has led to important insight into the selectivity of all S1P receptors. The S1P receptor family contains a GPCR morphology with seven transmembrane domains. A particularly important feature of these receptors is a two looped structure located on the extracellular surface. When these loops come together they form a cap that prevents binding of amphiphatic extracellular ligands. As a result, S1P is thought to bind to S1P receptors via lateral diffusion within the plasma membrane (Parrill et al., 2012).

Uncovering the S1P1R crystal structure has allowed for the development of two classes of selective agonists. Class I agonists are lipid like and mimic S1P ligands. A prototypical example, fingolimod-p binds to the S1P1R causing receptor internalization, and degradation. The other form of S1P agonists within class I are non-lipid, small molecules. A classic example of these agonists is SEW2817. Computational modeling and mutational studies support the idea that this agonist receptor pair utilizes hydrophobic interactions between R residues within the S1P1R binding pocket and the negatively charged trifluoromethyl ring on SEW2817 to mediate the agonist response (Jo et al., 2005). The second class of agonist consists of small molecules that do not require interactions with the polar head groups of the S1P1R binding pocket but rather, require ionic bonds and aromatic interactions with residues within S1P1R binding pocket. The result is heightened selectivity to S1P subtype (Hanson et al., 2012).
**Sphingosine 1-phosphate (S1P)**

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite involved in cell survival, cytokine production, signal transduction, cell motility/migration and lymphocyte trafficking (Goetz et al., 1999, Price et al., 2008, Meng et al., 2011, Kong et al., 2014). Synthesis of S1P occurs via two means: in the ER from ceramide or in the plasma membrane in response to stimulus induced cleavage of glycoproteins. Thus, levels of S1P are regulated not only by biosynthesis but by degradation pathways mediated by S1P phosphatases and S1P lyase (Mandala et al., 2000, Vogel et al., 2009). Balance between S1P synthesis and degradation is critical, as its regulation is essential for the operation of nearly every system in the body.

Development is profoundly affected by the operation of S1P. Inhibition of Sphk1/2 during embryonic development results in cranial neural tube deficits, vascular malformations and 100% lethality by E12.5 in mouse (Mizugishi et al., 2005). Additionally, pathfinding errors in Sphk mutants have been observed in developing axons (Strochlic et al., 2008).

**Fingolimod-p mechanism of action**

Fingolimod-p is a structural analogue of S1P and has been shown to inhibit lymphocyte egress from lymphoid tissue without inducing generalized immunosuppression. There are two theories regarding the mechanism used by fingolimod-p to prevent lymphocyte egress from lymphoid tissue. First, fingolimod-p has been shown to induce receptor internalization on lymphocytes. This effectively prevents signaling and function of the S1P1 receptor (Pham et al., 2008). Second, there is evidence
to suggest that fingolimod-p acts upon S1P1 receptors on the endothelium to close egress portals (Wei et al., 2005, Sanna et al., 2006). Thus, while compelling evidence exists for both theories, a clear mechanism of Fingolimod-p-S1P1 mediated inhibition of lymphocyte egress has yet to be clearly defined.

In theory, fingolimod-p would have been an ideal drug treatment for individuals who had undergone organ transplantation and were being treated with cyclosporine, a conventional immunosuppressive agent (Budde et al., 2002, Tedesco-Silva et al., 2005). However, despite early positive results, fingolimod-p was later found to induce adverse side effects such as bradycardia, lymphopenia/leukopenia and nephrotoxicity in healthy and transplant patients (Mulgaonkar et al., 2006, Schmouder et al., 2006). Further, long term studies demonstrated that fingolimod-p in combination with cyclosporine failed to decrease organ rejection rates. (Salvadori et al., 2006). As a result, clinical trials were terminated.

Despite early failures in transplantation studies, fingolimod-p was found to be a promising disease modifying treatment (DMT) in experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS), a progressive demyelinating disease of the CNS affecting an estimated 2.5 million individuals (Fujino et al., 2003). Clinically, disease presentation occurs in two patterns: the more prevalent RRMS (80% of all cases) and less common primary progressive (PPMS) (20%). RRMS is characterized by phases of symptom relapse followed by full recovery or recovery with persistent deficit leading to a secondary progressive pattern. (Confavreux et al., 2000, Williams et al., 2014). Clinical trials with fingolimod-p showed treatment with fingolimod-p resulted in decreased gadolinium enhanced lesions on MRI analysis and
decreased number of symptom relapses than those treated with other DMTs (O'Connor et al., 2009, Comi et al., 2010, Khatri et al., 2011). Currently, fingolimod-p is considered to be a highly efficacious DMT for the treatment of RRMS.

In 2007, Foster et al., (2007) demonstrated that fingolimod-p could penetrate the blood brain barrier, localize to the white matter and influence glial repair mechanisms, opening the door for investigations into fingolimod-p’s effects on central neurons (Foster et al., 2007). Fingolimod-p has been shown to have a direct effect on neurons. Previous studies in vitro and in vivo demonstrate that fingolimod-p acts on neurons to increase production of BDNF mRNA and protein (Deogracias et al., 2012, Doi et al., 2012). This effect appears to be specific for BDNF and does not affect the mRNA levels of other members of the neurotrophic factor family (Doi et al., 2012). Despite compelling evidence for a relationship between fingolimod-p and BDNF, the exact mechanism is not entirely understood. Deogracias et al., (2012) suggests fingolimod-p activates the MAPK signaling pathway resulting in phosphorylation of CREB and enhanced transcription of BDNF (Deogracias et al., 2012).

**S1PR in axons and neurite extension**

While S1PRs are found in many tissues types, only S1P₁R, S1P₂R, S1P₃R and S1P₅R are found in neurons where they have been shown to be involved in cellular processes such as survival, migration, differentiation and process extension/retraction (Soliven et al., 2011). The S1P₁R is important for neurite extension and retraction. In terms of neurite extension and retraction, previous investigations show S1P₁Rs are important for translocation of SphK1 to the plasma membrane where its substrate, S1P is
S1P can then activate the S1P₁R, initiating Gᵢ signaling. The interaction of these signaling pathways has been shown to regulate the activation of Rac, a small GTPase known to interact with the cytoskeleton and promote neurite outgrowth. These results were further supported by studies in which NGF induced neurite outgrowth was enhanced in response to S1P₁R overexpression. Thus, there is evidence to suggest S1P₁R is involved in neurite outgrowth (Toman et al., 2004)

S1PR signaling

S1PRs are G-protein coupled receptors that upon activation initiate canonical signaling pathways associated with Gi/0, G₉ and G12/13 proteins. The S1P₁R is associated with the Gi/0 proteins and downstream signaling pathways involving activation of PI3K, Ras and PLC activation of PKC and release of Ca²⁺ from IP₃ sensitive stores. Activation of this signaling pathway is known to be associated with neurite extension in TrkA positive neurons (Toman et al., 2004, Sanchez et al., 2007, Yoon et al., 2008) [Figure 1-6].

There is evidence for cross talk between S1PRs and tyrosine kinase receptors. However the exact mechanism by which this occurs is unknown. Investigations using PC12 cells containing S1P₁Rs or cultured DRG cells support a relationship between S1P₁R, TrkA and Rho GTPases; Rac and Rho, both of which have been implicated in cytoskeletal reorganization and neurite extension/retraction. In this model S1P₁R containing cells exposed to NGF undergo enhanced neurite outgrowth via TrkA activation and subsequent stimulation of Sphk1. The result is activation of Rac, decreased Rho activity and enhanced neurite outgrowth (Toman et al., 2004).
TYROSINE KINASE RECEPTORS

General characteristics of the Trk receptors

Trk receptors were originally discovered from a colon carcinoma derived oncogene. Studies by Mitra et al. (1987), identified P70TRK as the gene product of the TRK oncogene. Further investigation found that this gene product is phosphorylated at tyrosine residues, a canonical characteristic of tyrosine kinase receptors (Mitra et al., 1987).

Further investigations of Trk receptors reveal vertebrates express three homologous Trk receptors: TrkA, TrkB and TrkC. Each Trk receptor has a similar structure containing the following components: 1) an extracellular, ligand binding domain containing two immunoglobulin-like C2 domains, 2) a single pass transmembrane domain and 3) a cytoplasmic domain containing tyrosine kinase activity that functions as an autoregulatory loop. Additional tyrosine residues surrounding the kinase domain act as binding sites for adaptor proteins or enzymes (Ultsch et al., 1999). Activation of Trk receptors occurs in a two-step process. First, binding of the neurotrophin to the Trk receptor induces receptor dimerization. Second, the receptor autophosphorylates tyrosine residues. This allows the receptor to bind to enzymes and adaptor proteins important for the elicitation of intracellular signaling pathways (Ibanez et al., 1993).
Trk expression profiles in chicken DRG

The expression profile of Trk receptors in chicken embryo, DRG neurons varies throughout development. Early DRG development (E2.5; HH15-16) is characterized by a small population of migrating NCC expressing only TrkC. TrkA and TrkB do not reach detectable levels until E2.75 (HH st. 18) (Rifkin et al., 2000). As development progresses over the next few days, the molecular profile changes such that TrkC becomes down-regulated and TrkA and TrkB become upregulated (Rifkin et al., 2000).

At E4.5 (HH st. 24-25) chicken DRG express Trk receptors in the following proportions: 23% TrkA, 27% TrkB and 63% TrkC. This time in development coincides with a peak time for neurogenesis as well as apoptosis. In one study Rifkin et al., (2000) attempted to quantify the numbers of mitotically active Trk positive neurons in DRG at E4.5. Analysis of one interior region of the DRG found no actively dividing TrkA or TrkB positive neurons but found 20% of TrkC positive neurons were actively dividing (Rifkin et al., 2000). Comprehensive quantitative investigations of mitotically active, Trk expressing DRG neurons has not been accomplished.

By E6, all Trk positive neurons have segregated into subpopulations based on Trk expression type and function. TrkA expressing DRG are involved in nociception and are restricted to small diameter neurons in the dorsal medial region of DRG. TrkB expressing DRG neurons are involved in mechanoreception and are restricted to medium diameter neurons in the ventral lateral region of the DRG, and TrkC expressing DRG neurons are restricted to large diameter neurons in the ventral lateral region of the DRG (Mu et al., 1993, Rifkin et al., 2000).
TrkB overview

TrkB is involved in many developmental and physiological processes. The majority of these actions are dependent upon the binding of the mature form of brain derived neurotrophic factor (BDNF) to the membrane proximal, immunoglobulin like, C2 domain (Schneider and Schweiger, 1991, Urfer et al., 1995). This binding permits receptor dimerization and trans-autophosphorylation of tyrosine (Y) residues within the auto-regulatory loop (Peng et al., 1995). Phosphorylation of Y-residues outside of the auto-regulatory domain allows for docking of adaptor proteins which may then elicit the activation of three major intracellular signaling cascades: MAP-kinase pathway, PI3k pathway and PLC-γ pathway (Qian et al., 1998).

Regulation of neurite outgrowth by TrkB

TrkB is an important regulator of sensory neurite outgrowth. Previous work with neuroblastoma SH-SY5Y cells expressing TrkB exhibit enhanced neurite outgrowth in response to BDNF or synthetic TrkB antibodies designed to selectively activate TrkB receptors (Itoh et al., 2011). Further, DRG neurons in both developing systems and injury models exhibit enhanced neurite outgrowth in response to TrkB activation (Mu et al., 1993, Runyan and Phelps, 2009).

Additionally, negative modulation of TrkB has been shown to suppress axon outgrowth and arborization. Protein tyrosine phosphatase Receptor Type O (PTPRO), a phosphatase known to act on Trk receptors suppresses axon arborization and outgrowth in TrkB positive sensory neurons via inhibition of BDNF induced autophosphorylation of TrkB (Garris et al., 2013).
**TrkB splicing and expression patterns**

TrkB is a large transmembrane protein with complex splicing and expression patterns. Variations of the TrkB receptor include: the full length receptor, two isoforms with intact kinase domains lacking exon 16, two kinase deleted isoforms TrkB-T1 that lacks SHC binding sites and TrkB-SHC containing SHC binding sites (Eide et al., 1996, Ninkina et al., 1997).

All isoforms of the TrkB receptor are expressed in the CNS, PNS and leptomeninges of the developing chicken embryo; however, the exact role each of these isoforms play in development is poorly understood. Competing theories suggest truncated TrkB receptors regulate neuronal responsiveness to neurotrophins or may serve a role in cell metabolism (Baxter et al., 1997). Still other studies suggest truncated forms of the TrkB receptor may act as a barrier to prevent diffusion of BDNF between developing networks of neurons (Biffo et al., 1995).

The full length TrkB receptor is known to elicit the activation of multiple intracellular signaling pathways. The truncated isoforms lack much of the intracellular domain and thus are prevented from activating the intracellular signaling pathways classically associated with the full length TrkB receptor. Truncated TrkB receptors have been shown to regulate cytoskeletal changes in neurons independent of BDNF and to elicit the activation of Rho GTPases (Fenner, 2012). Whether truncated isoforms of TrkB are involved in axon outgrowth or extension is unknown.
TRKB SIGNALING

TrkB signaling overview

TrkB activation with BDNF results in receptor dimerization and trans-auto-phosphorylation of tyrosine residues. This allows for docking of proteins on phosphorylated tyrosines and activation of three canonical intracellular signaling pathways: PLC-γ, MAPK and PI3K (Qian et al., 1998). The downstream effectors activated by these pathways include: 1) PLC-γ: release of intracellular Ca^{2+} from IP_3 sensitive stores and diacylglycerol (DAG)/ protein kinase C (PKC) signaling, 2) MAPK: activation of Ras, 3) PI3K: activation of AKT (Middlemas et al., 1994, Atwal et al., 2000). The contribution of these signaling pathways to DRG axon extension in the developing longitudinal pathway is a major focus of this dissertation.

Phospholipase C- γ (PLC-γ) signaling pathway

Activation of TrkB with BDNF results in phosphorylation of Y816 and recruitment of PLC-γ to bind directly to the TrkB receptor (Huang and Reichardt, 2003). Activation of PLC-γ hydrolyzes Phosphoinositide (4,5)P_2, resulting in the production of inositol tris-phosphate (IP_3) and diacylglycerol (DAG). IP_3 interacts with IP_3 receptors on the endoplasmic reticulum (ER) to promote release of Ca^{2+} from internal stores. The result is activation of Ca^{2+} dependent enzymes such as Ca^{2+}-calmodulin regulated protein kinase (CamkII) and cAMP response element binding protein (CREB) (Minichiello et al., 2002) (Figure 1-7A). Previous studies suggest neurons depend upon activation of the PLC-γ signaling pathway for various cellular processes including axon outgrowth in model cell lines (Minase et al., 2010).
The importance of the PLC-γ pathway to neurite outgrowth is further highlighted by two lines of evidence. First, that in chicken embryo, DRG axons exhibit IP$_3$R enrichment in axon growth cones (Itoh et al., 2011). Second, inhibition of downstream effectors such as IP$_3$ receptor induced Ca$^{2+}$ release and Ca$^{2+}$ induced Ca$^{2+}$ release from intracellular stores attenuates neurite outgrowth (Stephens et al., 1994, Arie et al., 2009).

**Mitogen activated protein kinase (MAPK) signaling pathway**

The MAPK signaling pathway is a canonical pathway for the Trk receptor family and has been implicated in many developmental events including neurite outgrowth (Minichiello et al., 1998). Activation of TrkB with BDNF results in phosphorylation of Y490, a recruitment site for adaptor protein Shc. Upon binding to Y490 at its PTB domain, Shc auto-phosphorylates and recruits an additional adaptor protein Grb2. This adaptor is complexed with son of sevenless (SOS), a guanine nucleotide exchange factor that catalyzes the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) on Ras and Rac (Nimnual et al., 1998). Activation of Ras can then activate additional signaling pathways including: PI3K signaling and the MAPK signaling leading to activation of ERK1/2 by Mek1 and Mek2. Erk1/2 have been shown to phosphorylate CREB (Huang and Reichardt, 2003) [Figure 1-7B].

The MAPK signaling pathway is activated at early stages in chicken embryo development. Lunn et al, 2007 found ERK activity in the primitive streak and presomatic mesoderm in chicken (HH4-10) (Lunn et al., 2007). As development progresses, the MAPK pathway becomes abundantly expressed in the PNS where it is involved in development of glia, myelination and various stages of axon development (Newbern et
al., 2011). The exact role this signaling pathway plays in the development of DRG in vivo is not entirely understood.

**Phosphoinositide 3-kinase (PI3K) signaling pathway**

Endogenous activation of the PI3K pathway occurs in three major ways. First, PI3K signaling is initiated by Trk receptor activation and recruitment of adaptor proteins Shc, Grb-2 and Gab-1 (Liebl et al., 1997). Second, PI3K signaling is initiated independent of Trk receptors via Ras proteins (Holgado-Madruga et al., 1997). Finally, more recent findings suggest Neuronal tYrosine-phosphorylated Adaptor for PI 3-kinase (NYAP) activates PI3K signaling in developing neurons (Chu-Wang and Oppenheim, 1978) [Figure 1-7C].

Activation of PI3K signaling through Trk receptors has been shown to regulate sensory neurite outgrowth in vitro. Mullen et al., (2012) show PI3K signaling through Trk receptors regulates neurite outgrowth in cochlear spiral ganglion explants (Ernfors et al., 1995a). Ras, a family of GTPases with close ties to the actin and microtubule cytoskeleton has been shown to activate PI3K signaling and neurite outgrowth (Hamburger and Levi-Montalcini, 1949). Finally, activation of PI3K signaling through NYAP activates Rac, a member of the Rho family of GTPases known to relay signals to the cytoskeleton to promote actin polymerization. Additionally, NYAP interacts with Wiskott-Aldrich syndrome protein family verprolin homologous proteins (WAVE), a family of proteins known to influence the actin cytoskeleton through interaction with the Actin related protein 2/3 (ARP2/3) complex, a protein complex consisting of actin binding protein that associates with the actin cytoskeleton and initiate actin polymerization (Chu-Wang and Oppenheim, 1978, Wetts and Vaughn, 1998). Thus, there
is compelling evidence for the involvement of PI3K signaling in neurite outgrowth; however, the means by which PI3K signaling is initiated appears to be dependent upon cell type.

**P75 neurotrophin receptor**

The P75 neurotrophin receptor (P75NTR) is a member of the tumor necrosis factor (TNF) receptor superfamily. There are two isoforms of this receptor. The full length receptor, containing an extracellular, cysteine rich domain, a signal transmembrane domain and an intracellular death domain known to be involved in mediating an apoptosis signal. In contrast, the short isoform contains an extracellular domain lacking a cysteine rich domain, and thus lacks the ability to bind neurotrophins.

The function of P75 neurotrophin receptor (P75NTR) in the developing nervous system is less well understood than the Trk receptors. Previous investigations suggest P75NTR regulates responsiveness to neurotrophins in neurons by acting as a co-receptor with other Trk receptors. These interactions may increase receptor binding affinity and enhance neurotrophin specificity (Hempstead et al., 1991, Benedetti et al., 1993, Verdi et al., 1994).

Upon neurotrophin binding, the full length P75NTR may elicit intracellular signaling pathways associated with cell death, survival and cell cycle regulation such as: the MAPK signaling pathway. Additionally, full length P75NTR signaling has been shown to be important for axon elongation through interactions with RhoA, a subgroup of the Ras superfamily GTP binding proteins known to control actin organization. Yamashita et al., 1999 found activation of P75NTR with NGF decreased activation of Rho-A and increased axon elongation in chicken retinal ganglia neurons (Yamashita et
al., 1999). These findings suggest P75NRT may contribute to axon extension in other ganglia as well.

CALCIUM IN AXON OUTGROWTH

Competing theories exist for the role Ca\(^{2+}\) in axon outgrowth. One theory suggests elevation in cytoplasmic Ca\(^{2+}\) levels results in cytoskeletal remodeling in the growth cone and neurite outgrowth (Lankford and Letourneau, 1989, Ohbayashi et al., 1998). Evidence in support of this theory stems from the observation that intracellular Ca\(^{2+}\) levels are significantly higher in growing neurites than in retracting or stopped neurites (Connor, 1986).

The source from which this Ca\(^{2+}\) elevation is derived has been debated as well. Both release from intracellular stores and influx through voltage gated Ca\(^{2+}\) channels (VGCC) have been shown to contribute to increases in intracellular Ca\(^{2+}\) (Anglister et al., 1982, Doherty et al., 1991). Further evidence for the involvement of both of these sources comes from inhibition studies in which either inositol triphosphate receptor (IP\(_3\)R) or VGCC were pharmacologically inhibited. Inhibition of Ca\(^{2+}\) release from IP\(_3\) sensitive stores significantly attenuates neurite outgrowth in dissociated E10 DRG (Takei et al., 1998). In addition, a similar effect can be seen with pharmacological inhibition of VGCC (Harper et al., 1994). While both of these mechanisms have been shown to contribute to transient elevations in intracellular Ca\(^{2+}\), there is compelling evidence suggesting that neurite outgrowth in early embryonic development relies more heavily upon IP\(_3\)R mediated Ca\(^{2+}\) release, whereas later development may depend upon VGCC and ryanodine receptors (RYR) (Arie et al., 2009).
In contrast, competing evidence suggests that wide scale changes in intracellular Ca\textsuperscript{2+} do not affect axon morphology or outgrowth, but rather a Ca\textsuperscript{2+} set point must be maintained in order for neurite outgrowth to occur (Fields et al., 1993). Yet, additional theories suggest that small, localized changes and/or increases in Ca\textsuperscript{2+} cycling are required for axon outgrowth (Runyan and Phelps, 2009).

Many of the discrepancies in the literature stem from the fact that work has been performed in different cell types which may contain different Ca\textsuperscript{2+} channel profiles both on the plasma membrane and intracellularly. The exact mechanisms used by developing afferents in the chicken embryo has yet to be determined.

**Calcium and BDNF**

BDNF is the only neurotrophin capable of elevating cytoplasmic Ca\textsuperscript{2+} and mediating Ca\textsuperscript{2+} transients in neurons (Berninger et al., 1993, Lang et al., 2007). BDNF transcription can be regulated by influx of Ca\textsuperscript{2+} through L-type VGCCs. Elevated levels of intracellular Ca\textsuperscript{2+} mediate phosphorylation and activation of cAMP response element binding protein (CREB). When inactive, CREB prevents BDNF transcription by binding to DNA. Phosphorylation of CREB relieves DNA binding allowing for recruitment of transcriptional machinery and the transcription of BDNF (Finkbeiner et al., 1997). How BDNF and Ca\textsuperscript{2+} then work to promote axon outgrowth during neurodevelopment in vivo is poorly understood.

**SUMMARY AND DISSERTATION AIMS**

A simple reflex is the result of an elaborate set of events that begin during early development. Underlying the formation of reflexes is communication between multiple
segments of the spinal cord. This is made possible by primary sensory afferent extension in the longitudinal pathway driven by signaling that occurs at the growth cone of these axons.

In this work, DRG axon growth is studied in situ and is therefore influenced by the intrinsic and extrinsic factors and guidance cues that are endogenously affecting afferent growth. The overarching goal of this work was to understand the endogenous regulation of afferent extension in the longitudinal pathway. To address this question we focused on the contributions of BDNF and S1P1Rs to afferent extension in the longitudinal pathway. Our hypothesis was that fingolimod-p, an S1P1R agonist, enhances afferent extension through a BDNF related mechanism. Given our observations that fingolimod-p enhances afferent extension in the longitudinal pathway through BDNF, we then sought to investigate the contributions of BDNF’s receptor, TrkB, and the endogenous signaling pathways associated with TrkB. Our hypothesis was that TrkB regulates DRG afferent extension through activation of the PLC-γ signaling pathway.

Ultimately, this work provides evidence for a mechanism that regulates afferent extension in the longitudinal pathway. This work proposes a mechanism involving S1P1R and TrkB. We propose fingolimod-p, through activation of the S1P1R, promotes BDNF release from neurons. BDNF may then act via an autocrine mechanism to enhance afferent extension in the longitudinal pathway via activation of TrkB and release of Ca^{2+} from IP3 sensitive stores via activation of the PLC-γ signaling pathway. Unlike other studies, this body of work provides a mechanism for growth that is occurring in situ, and is thus in the context of other endogenous factors and signals that affect axon extension.
References


Garris CS, Wu L, Acharya S, Arac A, Blaho VA, Huang Y, Moon BS, Axtell RC, Ho PP, Steinberg GK, Lewis DB, Sobel RA, Han DK, Steinman L, Snyder MP, Hla T,


Wright EM, Vogel KS, Davies AM (1992) Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. Neuron 9:139-150.


Figure 1-1. DiI label in DRG in in situ preparation. A. E5 (HH st. 25) chicken embryo from the dorsal perspective. T3/T4 DRGs have been labeled with DiI. Scale bar = 200µm. Picture provided by Danielle Robinson. B. Multiphoton image of E5 (HH st.25) chicken embryo with T3/T4 DRG labeled with DiI. Primary sensory afferents can be seen growing in the longitudinal pathway. Scale bar = 200µm. Picture provided by Danielle Robinson. C. Transverse section of E5 (HH st.25) chicken embryo. DiI label in the DRG (right) labels both the central and peripheral branches of the DRG neuron. DiI label placed distally to the DRG shows that the extent of the peripheral process is contained within the preparation and thus has not been damaged. Scale bar = 200µm. Picture provided by Dr. Betsy Ezerman.
Figure 1-2. Primary sensory afferent growth in the chicken embryo from the longitudinal perspective. A. E3 (HH st. 19; 72 hours) primary afferents extend towards the developing spinal cord. B. E5 (HH st.25; 120 hours) afferents have entered the DREZ and bifurcated along the longitudinal axis of the spinal cord to establish the longitudinal pathway. C. E8 (HH st.34; 192 hours) primary afferents branch ventrally to invest into the grey matter of the spinal cord.
**Figure 1-3. Organization of the axon growth cone.** Contained within the axon shaft are microtubules organized in a parallel arrangement by association with MAPs. The growth cone is located at the distal most aspect of the axon. Within the growth cone are three domains: the central (C) domain, the transition (T) domain and the peripheral (P) domain. The C-domain contains de-fasciculated microtubules that may extend into the T-domain. The T domain, contains arc-actin arranged in a perpendicular orientation to the filopodia of the P-domain. The primary purpose of the T-domain is to corral de-fasciculated microtubules. The P domain contains two distinct features: the filopodia containing parallel arrangements of F-actin and a mesh-like network of actin known as lamellipodia.
B

Engorgement

C

Consolidation of growth cone into axon

New Axon

- Green: Microtubules
- Black: Actin
- Purple: Arc-Actin
- Purple dot: MAPs
Figure 1-4. Cytoskeletal dynamics during axon growth. A. Axon outgrowth begins with protrusion of F-actin containing filopodia and lamellar structures located between adjacent filopodia. Microtubules (green) are coralled together in parallel arrangements by MAP (purple circles). B. Following protrusion, microtubules infiltrate the P-domain. During the engorgement period, microtubules translocate vesicles to the P-domain. C. Consolidation is marked by F-actin depolymerization and membrane shrinkage away from the proximal portion of the growth cone. The result is consolidation of the axon shaft.
Factors that prevent axon extension/promote retraction:
- Activation of adenylate cyclase
- Decrease in $[Ca^{2+}]_{i}$

Factors promoting axon attraction:
- Netrins via DCC receptors
- Sonic hedgehog signaling via Patched/smoothed

Factors that promote axon extension:
- Neurotrophic factors: BDNF, NGF
- Inhibition of cAMP substrate PKA
- Elevation $[Ca^{2+}]_{i}$

Factors that promote axon repulsion:
- Netrins via Unc receptors
- Semaphorins via plexin receptors
Figure 1-5. Summary of molecular factors influencing afferent axon extension, retraction, attraction and repulsion. Factors influencing axon extension include: neurotrophic factors: BDNF and NGF and elevation of intracellular Ca$^{2+}$. In contrast, factors that prevent axon extension and/or promote axon retraction include: activation of adenylate cyclase and decreases in intracellular Ca$^{2+}$. In terms of directional growth, factors that contribute to axon attraction include Netrin interactions with DCC receptors, SHH interactions with Patched receptors and smoothened and increases in intracellular Ca$^{2+}$. In contrast, factors contributing to axon repulsion include: Netrin interactions with Unc receptors, Semaphorin interactions with Plexin receptors and decreases in intracellular Ca$^{2+}$. 
Figure 1-6. S1P₁R signaling. Endogenous activation of S1P₁R occurs through interaction with S1P. Exposure to S1P₁R agonists, fingolimod-p or SEW2871 activate the receptor. Activation of S1P₁R elicits the activation of PI3K signaling, activation of RAS and activation of PLC-β, leading to cleavage of PIP2 into DAG and IP₃ leading to release of Ca²⁺ from IP₃ sensitive stores.
Figure 1-7. Intracellular signaling pathways elicited by activation of TrkB with BDNF. Activation of TrkB with BDNF leads to trans-autophosphorylation of the TrkB receptor resulting in the activation of three canonical signaling pathways. 1-7A.

Activation of the PLC-γ begins with phosphorylation on tyrosine (Y) residues on TrkB. This results in recruitment of PLC-γ binding to phosphorylated Y. Activation of PLC-γ leads to hydrolysis of PIP$_2$ into DAG and IP$_3$. IP$_3$ activates IP$_3$Rs on the ER, mediating release of Ca$^{2+}$ from IP$_3$ sensitive stores. Ca$^{2+}$ may then activate kinases such as CAM, CAMK and CAMKK, ultimately leading to phosphorylation of CREB and alteration in gene transcription 1-7B. Activation of the MAPK signaling pathway begins with phosphorylation of Y516 on TrkB. This leads to recruitment of adaptor proteins Shc, Gab1/2 and Grb2. Grb2 is associated with a GTP exchange factor, SOS which removes GDP from Ras, activating it with GTP. Ras then activates the remainder of the MAPK signaling pathway, including Raf, MEK1/2, ERK1,2 and Rsk. Rsk phosphorylates CREB within the nucleus to alter gene transcription. 1-7C. Activation of the PI3K signaling pathway occurs through three means. Phosphorylation of Y residues leads to recruitment of adaptor proteins that activate PI3K which in turn, may phosphorylate AKT. P-AKT can then alter gene transcription in the nucleus. Secondly, PI3K may be activated by GTP bound Ras and NYAP. Third, PI3K may be activated by GTP bound Ras.
CHAPTER 2

Fingolimod-p enhances primary afferent axon extension by embryonic sensory neurons through a BDNF related mechanism

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Abstract

Primary sensory afferents extend along the longitudinal axis of the developing spinal cord between embryonic day 4-8 (E4-8) to establish the longitudinal pathway. An understanding of the endogenous factors that regulate afferent extension in the developing longitudinal pathway is important for understanding the mechanisms that regulate development of the intersegmental communication that underlies reflex formation.

In this study we used the chicken embryo to look at the effects of brain derived neurotrophic factor (BDNF), a neurotrophic factor known to enhance neurite outgrowth in vitro, on afferent extension in the longitudinal pathway. We then investigated the effects of fingolimod-p, a sphingosine 1-phosphate receptor (S1PR) agonist known to enhance BDNF mRNA and protein production in embryonic systems.

We found BDNF enhances DRG axon extension whereas inhibition of BDNF attenuates axon extension. Additionally, fingolimod-p increases BDNF mRNA production two fold and enhances primary afferent axon extension through the S1P1R. Collectively, these results reveal an endogenous mechanism for DRG axon extension within the developing longitudinal pathway that requires BDNF and S1P1Rs. Additionally, fingolimod-p enhances afferent extension through a BDNF related mechanism.
Introduction

Fingolimod-p is a sphingosine 1-phosphate receptor (S1P₁R) agonist previously shown to be highly efficacious in ameliorating the symptoms of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, and in the treatment of relapsing remitting forms of multiple sclerosis (RRMS) (Fujino et al., 2003, Agius et al., 2014). In clinical trials, patients with RRMS treated with fingolimod-p (0.5mg or 1.25mg) had fewer inflammatory brain lesions as assessed by Gadolinium (Gd) enhanced lesions on T2 MRI, slower rates of brain volume loss and decreased Extended Disability Status Scale (EDSS) score (Kappos et al., 2006, Kappos et al., 2010, Radue et al., 2012).

The exact mechanism by which fingolimod-p ameliorates RRMS is not entirely understood. Within the immune system, two mechanisms of action have been proposed. First, that fingolimod-p binds to S1P₁Rs resulting in receptor internalization, ubiquitination and degradation. The result is inhibition of S1P₁R dependent lymphocyte egress from lymphoid tissues (Pham et al., 2008). A second proposed mechanism of action is that fingolimod-p binds to S1P₁Rs on endothelium resulting in closure of lymphocyte egress portals (Wei et al., 2005).

Other studies indicate fingolimod-p has direct effects on neurons. Previous work suggests fingolimod-p has neuroprotective qualities mediated by activation of the S1P₁R and production of trophic factors (Meng and Lee, 2009, Deogracias et al., 2012, Doi et al., 2013). A relationship between fingolimod-p and brain derived neurotrophic factor (BDNF) within neurons has been established. Deogracias et al., (2012) showed that fingolimod-p increases BDNF mRNA and protein production within E16.5 mouse
cortical neurons but not glial cells (Deogracias et al., 2012). Additionally, Doi et al., (2013) found fingolimod-p exerts a neuroprotective effect on cortical neurons through enhancement of BDNF and TrkB-ERK signaling (Doi et al., 2013). Both of these studies examined the neuroprotective effects of fingolimod-p in the context of neurodegenerative disease.

BDNF is expressed in chicken during early development (Baig and Khan, 1996). While originally identified as a survival factor, it has since been shown to have many functions throughout development including neurite outgrowth in dorsal root ganglia (DRG) cells in vitro (Barde et al., 1982, Lindsay et al., 1985a, Davies et al., 1986, Wright et al., 1992).

In this study we used an embryonic system to assess the effect of fingolimod-p on primary afferent neuron axon extension in the longitudinal pathway. In the chicken embryo, primary sensory afferents develop in a three step process that begins at embryonic day (E3) with afferents extending towards the spinal cord. At E4 axons enter the dorsal root entry zone (DREZ) and bifurcate to extend axons in the rostral to caudal direction to establish a longitudinal pathway before turning ventrally to invest within the grey matter of the spinal cord at E8 (Coutinho-Budd et al., 2008, Robinson et al., 2015). This developmental pathway is essential for intersegmental and intrasegmental communication between multiple segments of the spinal cord and is essential for the development of reflexes. Importantly, at this stage in development, glia are not present in the DREZ or longitudinal pathway and DRG cell bodies and axons are unmyelinated. This is because gliogenesis does not begin until E6 and myelin formation does not begin
until E13 (Keirstead et al., 1992, Zhou et al., 2001). Thus, the effects of fingolimod-p on axons can be directly addressed.

In this study, we investigate the effects of fingolimod-p on DRG neuron axon extension within the longitudinal pathway of the developing spinal cord. These results show fingolimod-p enhances axon extension via an S1P1R and BDNF dependent mechanism.

Materials and Methods

Animals

Fertilized eggs (Charles River Sunrise Farms; Premium SPF standard fertile eggs) were stored at 15°C until incubation at 37.5°C. Developmental stage was determined by criteria established by Hamburger and Hamilton (Hamburger and Hamilton, 1951). Embryos were removed from their embryonic sac, decapitated and eviscerated in cold artificial cerebral spinal fluid (ACSF: 124 mM NaCl, 2.5 mM KCl, 1.3 mM MgCl2, 1.25 mM KH2PO4, 26mM NaHCO3, dH2O, 2.5 mM CaCl2, 20mM D-glucose). ACSF was bubbled with 95% O2/5% CO2 to maintain pH: 7.2-7.4).

Immunohistochemistry

Antibody and Drug concentrations

Rabbit α S1P1R antibody (Cayman chemical) and sheep α BDNF antibody (Millipore) were diluted in phosphate buffered saline (PBS) to 1:500 and 1:1000, respectively. Specificity for the rabbit α S1P1R in chicken was established by (Crousilliac et al., 2009). Specificity of sheep α BDNF was previously established for chicken (Taylor
et al., 2012). Additionally, a mouse α Neurofilament SMI 31 antibody (Covance) was used to stain medium and heavy weight neurofilaments and a rabbit α neurofilament-M C-terminal (Millipore) for medium sized neurofilaments. Secondary antibodies used were goat α rabbit IgG alexa 488 (Invitrogen), rabbit α sheep IgG 488 (Millipore), goat α mouse IgG 488 (Millipore) and goat α rabbit IgG cy3 (Jackson laboratories). No primary controls were performed with all immunohistochemistry assays.

BDNF function blocking activity was established for the sheep α BDNF antibody at 0.5µg/µl (Taylor et al., 2012). Sheep IgG (Sigma Aldrich) was used in control experiments at 0.5 µg/µl. Human recombinant BDNF (R&D) was dissolved in dH2O to a 500ng/ml stock solution. Fingolimod-p phosphate (Echelon) was dissolved in 100% MeOH to make a 2.5mM stock solution. The S1P1R agonist, SEW2871 (Tocaris), was dissolved in 100% DMSO to make a 10mM stock solution. The S1P1R antagonist, W123 (Cayman chemical), was dissolved in 100% DMSO to 1mM stock solution. On day of use, all drugs were diluted in ACSF to make a final concentrations along with 1% DMSO.

Every experiment contained an internal control containing a group of chicken embryos that were exposed for 5 hours to a vehicle control (VC) solution consisting of ACSF and a final concentration of 1% DMSO. The length of the axons from each group of embryos exposed to a drug treatment was compared to its own unique VC treatment group. This was done to control for potential minor fluctuations in incubator temperature and batches of fertilized eggs.
**Tissue preparation and immunohistochemistry**

Dissected tissue was immersed in 4% paraformaldehyde overnight and equilibrated in 30% sucrose in phosphate-buffered saline. Tissues were then frozen in optimal cutting temperature compound (O.C.T) (Tissue Tek) and cryo-sectioned into 20µm transverse sections. Sections were incubated in primary antibody overnight at 4°C followed by incubation with secondary antibodies for 1hr at room temperature. Sections were rinsed and cover slipped with Ctiifluor AFI (Ted Pella Inc.). Tissues were imaged with a Microphot-SA (Nikon) fluorescent microscope.

**Afferent labeling, imaging and axon measurement**

120 hour old embryos (Embryonic day 5 (E5), HH st.25) were sacrificed, decapitated and eviscerated. A capillary pipet containing 1,1’dioctadecyl-3,3,3’3’-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) was placed into DRG at thoracic level 3 or 4 (T3 or T4) on both sides of the spinal cord (Robinson et al., 2015). DiI was applied using a picospritzer III with compressed nitrogen pressure (1ms; 40pA). Immediately after labeling, tissue was rinsed in sterile ACSF and transferred to a 0.4µm Millicell in a 35mm sterile culture dish and treated with a drug solution or a control solution (ACSF and 1% DMSO). Tissue was then incubated for 5 hrs at 37°C in 5% CO₂. Following incubation, tissue was fixed in 4% paraformaldehyde. Tissue was imaged with a BioRad Multiphoton Microscope. DRG axon length was measured using Volocity software (PerkinElmer). Measurements were made from the middle of the DREZ where
axons bifurcate to the most distal end of the axon in both the rostral and caudal directions. The z series images were flattened using Image J.

**RNA extraction**

T2 through T5 DRG from HH st.25 chicken embryos were isolated following 5 hour treatment with either ACSF (1% DMSO) or fingolimod-p [250µm] (1% DMSO). DRG were flash frozen in dry ice and frozen overnight at -80°C. Total RNA was extracted using TRI reagent (Sigma Aldrich).

**Quantitative real time-PCR**

For each sample, 1µg was reverse transcribed using Multiscribe reagent containing d-NTPs and random hexamers. E12 chicken brain served as a positive control. The cDNA (1µl) was amplified using TaqMan fast advanced master mix reagents (Applied Biosystems) using primers for BDNF from a TaqMan gene expression assay (Applied Biosystems). Chicken S17 ribosomal binding protein [CHRPS] was used as the constitutive control gene. Primers for CHRPS were forward 5’-AACGACTTCCACACCAACAA-3’ and backwards 3’-AAATTGCATCCGGCTATGA-5’ with probe 5’-CGCCATCATCCCCAGCAAGA-3’. After a 20 second 95°C heat activation step, cycling parameters were as follows: 95°C for 3 seconds, 60°C for 30 seconds and 72°C for 30 seconds. This was repeated 40 times.

**Statistical analysis**

Groups were compared using a one-way analysis of variance (ANOVA) or a student’s T test (two tailed). In the event of detected significance, Tukey’s Post-hoc-Test
was used to determine groups significantly different from each other. All calculations were made using PRISM scientific graphing software from Graphpad (GraphPad Software, San Diego). Statistical significance was set at p<0.05.

Results

The current study sought to assess the role of fingolimod-p on DRG axon extension within the developing longitudinal pathway of the spinal cord. To address this question, all embryos were studied at HH st.25, a time point at which thoracic primary sensory afferents have bifurcated and are extending processes along the longitudinal axis of the developing spinal cord (Coutinho-Budd et al., 2008). At this time point, these axons have not yet turned ventrally to invest into the grey matter of the cord, thus making HH st.25 an ideal point to investigate regulation of extension within the longitudinal pathway (Eide and Glover, 1995, Coutinho-Budd et al., 2008) (Figure 2-1 A,B).

Expression of S1P1R and BDNF in DRG and DREZ in HH st.25 chick embryo

We first sought to determine if S1P1R and BDNF were expressed at HH st.25. Immunoreactivity for S1P1R and BDNF was observed in DRG and DREZ at HH st.25 [Figure 2-2A-D]. Immunoreactivity for BDNF and heavy and medium chain neurofilament, a marker for axons, demonstrates considerable overlap of the two labels at HH st.25 (Figure 2-2E-G). Additionally, immunoreactivity for S1P1R and neurofilament both appear in the DRG axons [Figure 2-2H-L].
**S1P1R agonists enhance DRG axon extension while S1P1R antagonists inhibit axon extension.**

To determine whether S1P1R agonists affected afferent extension we exposed our preparation to S1P1R agonists fingolimod-p and SEW2817. Afferent extension was enhanced in fingolimod-p [250µm] treated embryos and in SEW2871 [100µm] treated embryos [Figure 2-3A, B]. Additionally, S1P1R antagonist W123 [25µm] attenuated afferent extension [Figure 2-3B]. Given that both fingolimod-p and SEW2871 enhance afferent extension, we then asked whether combined exposure to fingolimod-p and SEW2817 or fingolimod-p and W123 affected afferent extension. We found fingolimod-p [250µm] and SEW2817 [100µm] enhanced afferent extension, but the extension was not different for either agonist alone whereas addition of fingolimod-p [250µm] and W123 [25µm] together attenuated afferent extension to levels of W123 alone [Figure 2-3C].

**Addition of exogenous BDNF enhances afferent extension whereas blocking BDNF attenuates afferent extension.**

To determine if BDNF regulates primary afferent extension, we tested the effects of treatment with BDNF or a function blocking antibody against BDNF. Treatment with exogenous BDNF (75-100 ng/ml) resulted in an increase in DRG axon extension [Figure 2-4A]. Conversely, inhibition of endogenous BDNF with αBDNF function blocking antibody [5ng/ml] attenuated afferent extension [Figure 2-4B].
Fingolimod-p’s effect on afferent axon extension requires BDNF

To determine whether fingolimod-p regulated BDNF mRNA production in DRG neurons we performed qPCR analysis on DRG from E5 chicken embryos following fingolimod-p treatment. We found a two fold increase in BDNF mRNA levels in fingolimod-p [250µm] treated embryos [Figure 2-5A]. Given the finding that fingolimod-p enhances BDNF mRNA production, we then asked whether BDNF was required for fingolimod-p enhanced afferent extension. Combined treatments with fingolimod-p [250µm] and a function blocking antibody to BDNF (5ng/ml) resulted in significant attenuation of afferent extension in the longitudinal pathway [Figure 2-5B].

Discussion

In this study we investigated the regulation of primary sensory afferent axon extension in the longitudinal pathway of the developing spinal cord. The primary finding of this investigation is that DRG afferent extension is enhanced by a mechanism requiring S1P₁R activation and BDNF.

There are many chemoattractive and chemorepellant signals present within the developing embryo. For instance, netrins and semaphorins have been shown to be expressed in developing spinal cord grey matter between E5-7.5 when afferents are extending within the longitudinal pathway. Here, both netrins and semaphorins act as repulsive guidance cues, preventing afferents from entering into the grey matter of the developing spinal cord (Watanabe et al., 2006, Liu et al., 2014). It is likely that S1P₁R activation and subsequent augmentation of BDNF signaling influences parallel signaling
mechanisms. Interestingly, afferents were never observed to deviate outside of the longitudinal pathway or prematurely turn towards the grey matter of the cord in response to any of the drug treatments in this investigation.

Precedent for this work derives from previous investigations of fingolimod-p, an S1P$_1$R agonist shown to enhance BDNF mRNA and protein production in embryonic cortical neurons (Deogracias et al., 2012, Doi et al., 2013). A functional role for the S1P$_1$R has been investigated, and previous studies find that activation of S1P$_1$R with its endogenous agonist, sphingosine 1-phosphate (S1P), influences neuronal morphology, neurite retraction and neurite extension (Sato et al., 1997, Toman et al., 2004). Our findings support previous studies, as we show activation of the S1P$_1$R with fingolimod-p or SEW2871 enhances axon extension. Agonists, fingolimod-p and SEW2871 activate the S1P$_1$R through different mechanisms. Fingolimod-p is known to bind to S1P$_1$Rs and induce receptor internalization and degradation whereas SEW2871 promotes S1P$_1$R internalization and recycling back to the cell membrane (Jo et al., 2005, Ng et al., 2007). Despite the difference in mechanism, a similar effect was observed with application of either pharmacological agent. Additionally, we show inhibition of S1P$_1$R with W123 attenuates DRG axon extension. These findings suggest that S1P$_1$R acts as a regulator of DRG axon extension within the developing longitudinal pathway.

A significant relationship has been established between fingolimod-p and BDNF, a neurotrophic factor shown previously to promote neurite outgrowth in developing chicken DRG when used at [50ng/ml] (Lindsay et al., 1985b). We found higher concentrations of BDNF [75-100 ng/ml] were required to enhance DRG axon extension in our preparation. This discrepancy is most likely due to the additional tissue present
within the experimental preparation. Additionally, previous work by Davies et al., (1986) found neurite outgrowth was enhanced in E5-6 DRG explants with [100ng/ml] of BDNF. An explant preparation is a closer representation of our in situ preparation and is more consistent with our findings. A functional role for endogenous BDNF in DRG axon extension in the longitudinal pathway was further supported by the attenuating effect on axon extension in response to αBDNF function blocking antibody.

The mechanism by which BDNF promotes afferent extension within the longitudinal pathway was not investigated. One possibility is that BDNF is secreted from the DRG afferent and through autocrine and paracrine signaling activates its high affinity receptor, tyrosine kinase receptor B (TrkB). This is a likely possibility as TrkB mRNA and protein are known to be expressed in primary sensory afferents in E5 HH st.25 chicken (Dechant et al., 1993, Straub et al., 2007).

Previous investigations found pM-nM concentrations of fingolimod-p increased BDNF mRNA and protein production in embryonic cortical neurons in mouse (Deogracias et al., 2012, Doi et al., 2013). In line with these findings we found fingolimod-p [250µm] increased BDNF mRNA levels in our system by two fold. This led us to assess whether BDNF protein levels were elevated in response to exposure to fingolimod-p. However, ELISA assays were inconclusive as BDNF concentrations detected from control and fingolimod-p treated embryos were below the level of detection for available ELISA kits (data not shown).

Whether BDNF is being transcribed and translated in the growth cone or the cell body in response to fingolimod-p exposure could not be determined in this investigation. Previous investigations suggest transcription and translation occur locally within the
growth cone (Jung et al., 2014). Whether BDNF is transcribed and translated within the growth cone is unknown. A more likely possibility is that fingolimod-p acts on S1P1Rs on the cell body and BDNF is transcribed and translated within the cell body. Anterograde transport can then move BDNF from the cell body to the afferent growth cone. Anterograde transport of BDNF has been previously calculated in E18 rat hippocampal neurons to be 1.49µm s⁻¹ (Lo et al., 2011). In this investigation, afferents grew between 500-1000µm along the longitudinal pathway. The distance from the DRG cell body to the longitudinal pathway is 80-100µm. Thus, in a 5 hour incubation period, BDNF could readily be anterogradely transported to the growth cone at the most distal end of the growing axon.

Given both S1P1R agonists and BDNF enhance afferent extension and that the S1P1R agonist, fingolimod-p enhances BDNF mRNA, we next sought to investigate whether enhancement of afferent extension by exposure to fingolimod-p requires BDNF. Our findings suggest that the fingolimod-p effect on afferent extension is the result of its effect on BDNF production.

Ultimately, these findings provide evidence for a mechanism that enhances DRG axon extension in the developing longitudinal pathway [Figure 2-6]. This mechanism can be pharmacologically manipulated within a live in situ preparation. Additionally, these findings suggest the disease modifying effects of fingolimod-p in RRMS may be the result of the ability of fingolimod-p to enhance BDNF in neurons and promote axon outgrowth.
References


Wright EM, Vogel KS, Davies AM (1992) Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. Neuron 9:139-150.

Figure 2-1. DRG axons extend within the longitudinal pathway of the developing chicken spinal cord at E5, HH st.25. A. Multiphoton image of Dil labeled primary sensory afferents from HH st.25 chicken embryo. Longitudinal extension of primary sensory afferents is observed and measured from the point of axon bifurcation at the DREZ to the most distal axon tip. Scale bar =100μm. B. Schematic representation of longitudinal extension of primary sensory afferents. Red denotes labeled DRG following injection of DiI.
**Figure 2-2. BDNF and S1P1R expression in E5 HH st.25 chicken embryos.**

Transverse sections (20µm). 

**A.** BDNF immunoreactivity in DRG, DREZ and SC  

**B.** S1P1R immunoreactivity in DRG, DREZ and spinal cord (SC)  

**C.** Neurofilament (Covance) immunoreactivity in DRG axons and DREZ.  

**D.** No primary control.  

**E-G.** BDNF immunoreactivity (green) and neurofilament (red) immunoreactivity are coexpressed (yellow) in DRG.  

**H-K.** S1P1R (green) and neurofilament (red) immunoreactivity expressed in DRG and spinal cord.  

**K.** Higher magnification insert from S1P1R- neurofilament merge. Individual cell bodies contain immunoreactivity for S1P1R (green) and neurofilament (red).  

**L.** No primary control. Scale bar in figure 2D=50µm and pertains to figures 2A-D. Scale bar figure 2L=50µm and pertains to figures 2E-J (scale bar is 2K=10µm). SC= spinal cord.  

NF1= mouse α Neurofilament SMI 31(Covance) was used to stain medium and heavy weight neurofilaments. NF2= rabbit α neurofilament-M C-terminal (Millipore) for medium sized neurofilaments.
Figure 2-3. S1P₁R agonists enhance afferent extension whereas S1P₁R inhibitor attenuates afferent extension. A. Afferent extension was enhanced following treatment with fingolimod-p [200-250μM] (n>6). Each fingolimod-p treatment was completed as a separate experiment and compared to a control group of embryos treated with ACSF+ 1% DMSO (student’s t-test, two tailed; p<0.05). B. S1P₁R agonist, SEW2871 [100μM] (n>4) promotes DRG axon extension, whereas S1P₁R antagonist W123 [25μM] decreases DRG axon extension (n>9). Experiment comparing VC to SEW2817 exposure was done separately from experiment comparing VC to W123 exposure. C. Addition of both agonists fingolimod-p [250μM] and SEW2817 [100μM] enhances DRG axon extension (n>5) while addition of fingolimod-p [250μM] and W123 [25μM] together decrease axon extension (n>6). Experiments comparing VC to fingolimod-p [250μM] and SEW2817 [100μM] was done separate from experiments comparing VC to fingolimod-p [250μM] and SEW2817 [100μM]. Significance was determined using a student’s t test (two tailed). p<0.05*, p<0.01**, p<0.005***.
Figure 2-4. BDNF enhanced afferent extension whereas blocking BDNF attenuated afferent extension. A. Primary afferent extension is enhanced with exposure to BDNF [75ng/ml] and BDNF [100ng/ml] treatment (n>4). Each BDNF treatment was completed as a separate experiment and compared to a separate group of embryos treated with ACSF + 1% DMSO. Significance was determined using a student’s t-test (two tailed). B. αBDNF [5µg/ml] function blocking antibody attenuate axon extension compared to VC or IgG control [5µg/ml] (n>6). Significance was determined using an ANOVA. p<0.05*, p<0.01**
Figure 2-5. Fingolimod-p requires BDNF to enhance afferent extension. A. BDNF mRNA is elevated two fold in response to fingolimod-p [250µm] treatment compared to vehicle control (n>8). BDNF mRNA was compared relative to housekeeping gene CHRPS. Significance was determined using a student’s t-test (two tailed). B. DRG axon extension was attenuated with fingolimod-p [250µM] and αBDNF[5µg/ml] treatment (n>6). Significance was determined using an ANOVA. $p<0.05^*, p<0.01^{**}, p<0.005^{***}, p<0.001^{****}$
Figure 2-6. Model for enhanced afferent extension following exposure to fingolimod-p. Fingolimod-p activates the S1P1R in the cell body of the DRG neuron. Through an unknown mechanism, S1P1R activation enhances BDNF mRNA transcription and translation of BDNF protein within the cell body. BDNF may then be anterogradely transported to the growth cone where it is released to act on TrkB on the same or neighboring growth cone. The result is enhancement of afferent extension in the longitudinal pathway.

*Occurs via an unknown mechanism
**Occurs in axon growth cone
***Occurs in cell body
CHAPTER 3

TrkB regulates DRG axon extension through PLC-γ signaling

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Number of Figures: 7
Number of Tables: 2

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Abstract

The anatomical pathway taken by developing dorsal root ganglia (DRG) axons to make connections with the central nervous system has been previously characterized. However, the endogenous mechanisms utilized by these axons to extend along the longitudinal axis through multiple segments of the developing spinal cord is poorly understood. This study investigates the role of TrkB in the growth of DRG axons along the longitudinal axis of the developing spinal cord. DRG axons growing along this axis are confined within a longitudinal pathway until they turn ventrally to make connections with neurons and interneurons within the grey matter of the spinal cord. It is this growth pattern that underlies the intersegmental communication required for reflex formation. In this investigation we found TrkB regulates DRG axon extension by the activation of phospholipase C-gamma (PLC-γ) signaling pathway and subsequent release of Ca$^{2+}$ from IP$_3$ sensitive stores stores. This work provides evidence for a mechanism for endogenous regulation of DRG axon extension that can be enhanced or attenuated in situ.
Introduction

Primary sensory afferents establish connections to the grey matter of the spinal cord in a three step process. In the chicken thoracic spinal cord, at embryonic day 3 (E3) (Hamburger and Hamilton (HH) stage (st) 19), dorsal root ganglia (DRG) neurons extend processes centrally into the developing spinal cord. At E4.5, these axons bifurcate to form a T shape. Between E4.5 and E8, axons extend along the rostral to caudal axis to establish the longitudinal pathway. Finally, at E8, DRG axons simultaneously invest into the grey matter of the spinal cord. The result is the establishment of intrasegmental and intersegmental connections with neurons and interneurons within the developing spinal cord (Eide and Glover, 1995, Coutinho-Budd et al., 2008). Longitudinal extension of DRG axons along multiple segments of the spinal cord is essential for the establishment of intersegmental communication and subsequent coordination of reflexes (Sprague, 1958). Previous studies suggest an endogenous growth factor, brain derived neurotrophic factor (BDNF) regulates axon growth in the longitudinal pathway (McNamara et al., 2015).

Tropomyosin receptor kinase B (TrkB), the high affinity receptor for BDNF, is a good candidate for the regulation of DRG axon extension in the longitudinal pathway. TrkB mRNA has been detected within chicken DRG beginning at E4.5 and protein immunoreactivity has been detected in neurons at developmental stages that coincide with longitudinal extension of DRG axons (Dechant et al., 1993, Baig and Khan, 1996, Straub et al., 2007).
Activation of TrkB with BDNF elicits three canonical signaling pathways: PLC-γ dependent generation of IP$_3$ and diacylglycerol (DAG), mitogen activated protein (MAP) kinase cascade leading to activation of Ras and the phosphatidyl inositol-3 (PI3)-kinase stimulation of Akt (Middlemas et al., 1994).

The following study investigates an endogenous mechanism regulating primary sensory afferent extension within the longitudinal pathway of the developing spinal cord. In this work, we demonstrate stimulation of TrkB with BDNF regulates DRG axon extension by PLC-γ signaling.

**Materials and Methods**

**Animals**

Fertilized eggs (Charles River Sunrise Farms; premium SPF standard fertile eggs) were stored at 15°C. Incubation time was 120 hours at 37.5°C. Developmental stage was determined by criteria established by Hamburger and Hamilton 1951 (Hamburger and Hamilton, 1951). At 120 hours embryos were removed from their in ovo environment where they were decapitated and eviscerated in cold artificial cerebral spinal fluid (ACSF: 124 mM NaCl, 2.5 mM KCl, 1.3 mM MgCl$_2$, 1.25 mM KH$_2$PO$_4$, 26 mM NaHCO$_3$, 2.5 mM CaCl$_2$, 20 mM D-glucose dissolved in double distilled H$_2$O). ACSF was bubbled with 95% O$_2$/5% CO$_2$ until a pH =7.2-7.4 was established.
Antibody and Drug concentrations

Table 3-1. Antibodies used in this work:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit αTrkB IgG</td>
<td>Dr. Louis Reichardt; UCSF</td>
<td>[1:2000]</td>
</tr>
<tr>
<td></td>
<td>(von Bartheld et al., 1996)</td>
<td></td>
</tr>
<tr>
<td>Goat α rabbit Alexa Fluor 488</td>
<td>Invitrogen</td>
<td>[1:500]</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Sigma Aldrich</td>
<td>100ng/ml</td>
</tr>
</tbody>
</table>

Table 3-2. Drugs used in this work:

<table>
<thead>
<tr>
<th>Drug</th>
<th>Company</th>
<th>Stock solution/solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>K252a</td>
<td>Cayman Chemical</td>
<td>100mM (DMSO)</td>
</tr>
<tr>
<td>Human recombinant BDNF</td>
<td>R&amp;D</td>
<td>500ng/ml (dH2O)</td>
</tr>
<tr>
<td>U73122</td>
<td>Tocaris</td>
<td>2mM (DMSO)</td>
</tr>
<tr>
<td>Xestospongin-C (Xe-C)</td>
<td>Tocaris</td>
<td>2mM (DMSO)</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>Cayman Chemical</td>
<td>50mM (DMSO)</td>
</tr>
<tr>
<td>1-oleoyl-2-acetyl-sn-glycerol (OAG)</td>
<td>Sigma Aldrich</td>
<td>50mM (DMSO)</td>
</tr>
<tr>
<td>Bisindolylmaleimide-1 (Bim-1)</td>
<td>Cayman Chemical</td>
<td>50mM (DMSO)</td>
</tr>
<tr>
<td>LY294002 (Cell Signaling)</td>
<td>Cell Signaling</td>
<td>50mM (DMSO)</td>
</tr>
<tr>
<td>U0126</td>
<td>Cell Signaling</td>
<td>50mM (DMSO)</td>
</tr>
<tr>
<td>PD98059</td>
<td>Cell Signaling</td>
<td>10mM (DMSO)</td>
</tr>
<tr>
<td>------------</td>
<td>----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>Sigma Aldrich</td>
<td>100mM dH₂O</td>
</tr>
</tbody>
</table>

All drugs were diluted to final concentrations in ACSF on the day of experimentation. Final treatment solutions were adjusted to contain 1% DMSO. Additionally, all drug treatments began immediately after tissue dissection. Exposure to a drug and BDNF were begun simultaneously. Total exposure time was 5 hours. A vehicle control (ACSF + 1% DMSO) was included with each drug treatment.

**Tissue preparation and immunohistochemistry**

Following dissection, tissue was immersed in 4% paraformaldehyde at 4°C overnight, rinsed in phosphate-buffered saline (PBS) and equilibrated in 30% sucrose in PBS at 4°C overnight. Tissue was frozen in Optimal Cutting Temperature media (OCT) (Tissue Tek) and cryo-sectioned into 20µm transverse sections. Sections were incubated in normal goat serum (NGS) for 1 hour, rinsed with PBS and incubated in primary antibody at 4°C overnight. Secondary antibodies were applied for 1 hour at room temperature. Tissues were imaged with a fluorescent microscope or an Andor CSU W1 spinning disk confocal microscope. No primary controls were done with each experiment to insure there was no non-specific binding of the secondary antibody.

**Afferent labeling and multiphoton imaging**

120 hour old embryos (E5; HH st.25) were inspected to determine embryonic stage. Embryos were removed from their egg sac, decapitated, eviscerated and pinned
into a dissection dish dorsal side up. A capillary pipet containing 1,1’dioctadecyl-3,3,3’3’-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) was transiently placed into thoracic level 3 or 4 (T3 or T4) DRG on one or both sides of the spinal cord. DiI was delivered to the tissue via nitrogen gas pressure supplied by a Picospritzer III (1ms pulse, 40pA). Manipulation of the capillary pipet was controlled by a micromanipulator. This allowed for direct injection of DiI into the tissue and labeling of DRG.

Following DRG labeling, the tissue was rinsed in ACSF and transferred to a 0.4 μm Millicell (Millipore) in a 35mm sterile culture dish to allow a controlled delivery of either drug solution or control solution (ACSF+1% DMSO). Tissue was incubated for 5 hours at 37°C in 5% CO₂ incubator and then fixed in 4% PF for >24 hours. Tissue was then washed in PBS and imaged with a BioRad Multiphoton Microscope. Axon length measurements were made with Volocity software. The z series images were flattened using ImageJ.

**Statistical analysis**

All statistical analysis was completed using PRISM scientific graphing software from Graphpad (GraphPad Software, San Diego). A one-way analysis of variance (ANOVA) was used to compare mean axon length of ≥3 treatment groups. Tukey’s Post-hoc-test (two tailed) was used to compare mean axon length of 2 treatment groups. Statistical significance was set at p<0.05.
Results

Inhibition of TrkB signaling attenuates DRG axon extension

At HH st.25 DRG axons form the longitudinal pathway by extending rostrally and caudally along the longitudinal axis of the spinal cord. Previous studies indicate BDNF, the endogenous ligand for TrkB, increases neurite outgrowth in dissociated chicken DRG in vitro (Lindsay et al., 1985). A similar enhancement in DRG axon extension has been observed in response to BDNF in the developing longitudinal pathway (McNamara et al., 2015). Therefore, we asked whether activation of TrkB by its endogenous ligand, BDNF, was involved in the regulation of DRG axon extension in the longitudinal pathway.

Using immunohistochemistry, we determined TrkB was present within DRG cell bodies, axons and dorsal root entry zone (DREZ) [Figure 3-1A, B]. We then asked whether inhibition of TrkB affected DRG axon extension in the longitudinal pathway. Exposure to the kinase inhibitor K252a [200nM] significantly attenuated DRG axon extension. However, K252a is known to inhibit tyrosine phosphorylation and kinase activity on all Trk receptors (Hashimoto, 1988). Therefore, to specifically inhibit TrkB, preparations were treated with rabbit α TrkB IgG function blocking antibody. Exposure to this blocking antibody attenuated DRG axon extension. DRG axon extension was not affected by treatment with vehicle control solution or rabbit IgG [Figure 3-1C].

As blocking TrkB attenuated axon extension, we then focused on the contributions of TrkB activated intracellular signaling pathways: PLC-γ, MAPK and PI3K. We investigated whether these signaling pathways contributed to the endogenous extension of DRG axons. In the first set of experiments, we found inhibition of PLC-γ signaling attenuated DRG axon extension. Specifically, we found exposure to the PLC-γ
inhibitor U73122 [1µM, 3µM] significantly attenuated DRG axon extension [Figure 3-2A].

Next, we investigated the contributions of the MAPK signaling pathway to DRG axon extension. We tested the contribution of this pathway with the inhibitors U0126 [10 µM], a selective inhibitor of MEK1 and MEK2 and PD8059 [2µM], an inhibitor of MEK1 activation. Neither inhibitor had an effect on DRG axon extension [Figure 3-2B]. We then investigated the potential contribution of the PI3K signaling pathway on DRG axon extension. Exposure to the PI3K inhibitor LY294002 at 10µM had no effect on DRG axon extension whereas at 20µM, LY294002 significantly attenuated DRG axon extension [Figure 3-2C].

**Inhibition of TrkB stimulated PLC-γ signaling attenuates DRG axon extension**

BDNF, the high affinity ligand for TrkB has been shown to promote DRG axon extension in the developing longitudinal pathway (McNamara et al., 2015). We next asked whether the presence of a PLC-γ inhibitor would attenuate BDNF stimulated DRG axon extension when applied along with the neurotrophic factor BDNF. We found DRG axon extension was attenuated when exposed to both U73122 [3µM] and BDNF [100ng/ml] [Figure 3-3A].

As activation of TrkB is known to elicit the PI3K and MAPK signaling pathways, we then asked whether exposure to LY294002, an inhibitor of PI3K signaling, or U0126, an inhibitor of MAPK signaling, would affect BDNF stimulated axon extension when the inhibitors were added along with BDNF. Following exposure to either inhibitor along with BDNF (U0126 [100µM] + BDNF [100ng/ml] or LY294002 [20µM] + BDNF
[100ng/ml]) DRG axon extension was comparable to that produced by BDNF [100ng/ml] alone [Figure 3-3B, C].

We used immunohistochemistry to confirm that U0126 inhibited MAPK signaling in the chicken preparation. Embryos were exposed to U0126 [100μM] alone or during application of BDNF [100ng/ml] and stained for phosphorylated ERK (pERK). Less pERK immunoreactivity was evident in embryos exposed to U0126 + BDNF than in embryos exposed to vehicle control solution or BDNF alone [Figure 3-4A-C]. Thus, U0126 blunted pERK levels with and without BDNF stimulation in this preparation.

**IP₃ induced Ca²⁺ release, but not DAG generation contributes to the BDNF stimulation of DRG axon extension**

BDNF activation of TrkB can elicit PLC-γ signaling, the generation of IP₃ and release of Ca²⁺ from IP₃ sensitive intracellular Ca²⁺ stores (Yoshii and Constantine-Paton, 2010). As inhibition of PLC-γ attenuated DRG axon extension, we tested whether a BDNF induced release of Ca²⁺ from IP₃ sensitive intracellular Ca²⁺ stores was a factor stimulating DRG axon extension. To determine this, embryos were treated with Xestospongin-C (Xe-C), an allosteric IP₃R inhibitor (Gafni et al., 1997). Xe-C [0.5μM, 1μM] treatment significantly attenuated DRG axon extension [Figure 3-5A]. This inhibition by Xe-C implicated a rise in intracellular Ca²⁺ as a mechanism stimulating axon extension. To test this possibility further, we exposed preparations to the cell permeable Ca²⁺ chelator BAPTA-AM [5mM]. Exposure to BAPTA-AM significantly attenuated DRG axon extension [Figure 3-6B], providing additional support for the hypothesis that a rise in intracellular Ca²⁺ is a factor regulating axon extension.
We then investigated the effects of simultaneous exposure to Xe-C and BDNF on DRG axon extension. Co-treatment with Xe-C caused a significant attenuation of BDNF stimulated DRG axon extension. However, axon length was not different from that noted for embryos exposed to vehicle control solutions [Figure 3-6A].

As activation of PLC-γ also leads to activation of the DAG/ PKC signaling cascade we tested whether activation of DAG signaling was also a factor, which contributed to DRG axon extension. Exposure to the DAG analogue OAG [10 µM] or the PKC inhibitor Bim1 [50 µM or 100 µM] had no effect on DRG axon extension [Figure 3-5B], indicating that DAG/PKC signaling did not contribute to axon extension.

**Inhibition of Ca\(^{2+}\) influx through voltage gated Ca\(^{2+}\) channels (VGCC) attenuated afferent extension, but is not required for BDNF stimulation of afferent extension.**

Embryonic chicken DRG are electrically active cells with a diverse expression profile of VGCCs (Nowycky et al., 1985). As VGCCs are known to be present at this stage of development, we tested whether an elevation of intracellular Ca\(^{2+}\) due to Ca\(^{2+}\) influx through VGCCs also stimulates DRG axon extension. Exposure to CdCl\(_2\) [100µM], an inhibitor of VGCCs attenuated DRG axon extension [Figure 3-7A].

Given the observation that exposure to CdCl\(_2\) suppressed DRG afferent extension in naïve preparations, we investigated whether the addition of CdCl\(_2\) along with BDNF affected the BDNF stimulation of axon extension. BDNF still enhanced axon extension during co-treatment with CdCl\(_2\) [100µM] and BDNF [100ng/ml]. Further, with co-treatment, the magnitude of the trophic factor enhancement was comparable to that seen with BDNF [100ng/ml] alone [Figure 3-7B].
Discussion

Extension of DRG axons in the developing longitudinal pathway allows for communication between multiple spinal segments, a process essential for the coordination of reflexes. The molecular mechanisms regulating this growth pattern are largely uncharacterized. Our findings provide evidence for an endogenous mechanism that regulates DRG axon extension within the developing longitudinal pathway. Here, we show endogenous activation of the PLC-γ signaling pathway by stimulation of TrkB with BDNF regulates DRG axon extension within the developing longitudinal pathway.

Precedent for this study derives from the following observations. First, TrkB mRNA and protein have been detected in chicken DRG at a time when DRG axons are extending within the longitudinal pathway (Rifkin et al., 2000, Straub et al., 2007). Second, stimulation of DRG neurons with BDNF enhances DRG axon extension in the developing longitudinal pathway (McNamara et al., 2015). In line with these findings we found TrkB protein expression in chicken DRG and DREZ at HH st.25, a time when DRG axons are extending within the longitudinal pathway (Coutinho-Budd et al., 2008). The coincident expression of TrkB with the developmental time point at which DRG axons are extending within the longitudinal pathway prompted us to explore a potential role for TrkB in DRG axon extension. We found attenuated DRG axon length in response to TrkB inhibition and enhanced axon length in response to BDNF stimulation. These findings suggest TrkB activation with BDNF contributes to the regulation of axon extension within the longitudinal pathway.
As TrkB has the capacity to regulate DRG axon extension in the longitudinal pathway, we next sought to understand which of the endogenous intracellular signaling pathways utilized by TrkB affect DRG axon extension within the longitudinal pathway. We hypothesized endogenous PLC-γ signaling regulates DRG axon extension within the longitudinal pathway. Precedent for this hypothesis derives from in vitro investigations showing pharmacological stimulation of PLC-γ and IP₃Rs enhances neurite outgrowth in model cell lines (Itoh et al., 2011). In line with these findings, we show inhibition of PLC-γ signaling attenuates DRG axon extension in the longitudinal pathway.

To further test this hypothesis we then asked if either the inhibition of MAPK or PI3K signaling pathways had an effect on DRG axon extension. We found treatment with MAPK signaling inhibitor, U0126 had no effect on DRG axon extension. In contrast, treatment with the PI3K inhibitor LY294002 did blunt axon extension. Previous investigations of sensory neurite outgrowth show PI3K signaling is involved in sensory neurite outgrowth (Ernfors et al., 1995). Thus, consistent with earlier studies, our findings suggest PI3K signaling also contributes to afferent extension in the chicken embryo in situ.

To further examine the effects of endogenous PLC-γ signaling on DRG axon extension, we focused on the effects of PLC-γ to activate 1) Ca²⁺ influx from IP₃ sensitive intracellular stores and 2) activation of DAG/PKC signaling. Our results suggest IP₃ induced release of Ca²⁺ from intracellular stores contributes to the regulation of DRG axon extension, whereas activation of the DAG/PKC signaling cascade does not. Prior studies indicate that PKC signaling can have opposite or similar effects. For instance, activation of PKC signaling has been shown to enhance neurite outgrowth in chicken
ciliary ganglia, whereas it has been observed to inhibit neurite outgrowth in NG108-15 cells (Sprague, 1958, Davies et al., 1986, Mu et al., 1993). The different responses suggest that the contribution of DAG/PKC signaling is cell type and substrate dependent. Our results suggest activation of the DAG/PKC signaling cascade is not required for afferent extension in the developing longitudinal pathway in the chicken embryo in situ.

With evidence that PLC-γ signaling contributes to the endogenous regulation of DRG axon extension, we then asked whether BDNF activation of TrkB regulates DRG axon extension through PLC-γ signaling. DRG axons did not respond to TrkB stimulation following inhibition of PLC-γ signaling. These findings suggest activation of TrkB and subsequent PLC-γ signaling contribute to DRG axon extension in the longitudinal pathway.

We then asked whether MAPK and/or PI3K signaling pathways had an effect on DRG axon extension in response to TrkB stimulation. Enhanced extension was observed in response to TrkB stimulation following inhibition of MAPK and/or PI3K inhibition. These findings suggest that while PI3K signaling contributes to the endogenous regulation of DRG axon extension in the longitudinal pathway, this signaling is not entirely dependent on TrkB activation. These findings are not surprising, as PI3K signaling occurs in response to not only tyrosine kinase receptors, but by Ras, G- protein coupled receptors- Gβγ subunits and recently by neuronal tyrosine phosphorylated Adaptor for PI3K (NYAP) (Chu-Wang and Oppenheim, 1978, Mack and Eickholt, 2011). An alternative explanation for these findings could be that LY294002 inhibits PI3K signaling at a slower rate than BDNF activates TrkB to promote DRG axon extension. These results would suggest that while PI3K signaling is important for regulation of DRG
axon extension, and that inhibition of this signaling can be overcome by addition of exogenous BDNF. This study required all embryos to be incubated for approximately 5 hours. This was to ensure DRG axons were given the same opportunity to extend within the longitudinal pathway. Thus, studies to explore the timing of the effects of different drug treatments were not feasible under the experimental paradigm.

We also examined the role of TrkB activated release of Ca^{2+} from IP_3 sensitive intracellular stores on DRG axon extension. We hypothesized that inhibition of IP_3 induced release of Ca^{2+} from intracellular stores during TrkB stimulation would attenuate DRG axon extension. We observed inhibition of IP_3 induced release of Ca^{2+} stores blunted the enhanced extension mediated by TrkB stimulation. These results are consistent with previous observations that neurite extension in chicken embryos during early development (≤E10) requires elevation of intracellular Ca^{2+} via release of IP_3 sensitive Ca^{2+} stores (Arie et al., 2009). While TrkB activation in the presence of inhibition of Ca^{2+} release from IP_3 sensitive stores attenuated DRG axon extension relative to TrkB stimulation alone, there was no significant difference in DRG axon extension relative to treatment with vehicle control. These results suggest TrkB activation may be operating via an additional mechanism to enhance intracellular Ca^{2+} levels and promote DRG axon extension. To investigate other mechanisms of Ca^{2+} entry into the intracellular environment, we focused on VGCCs. Previous investigations demonstrate that neuronal depolarization opens VGCCs, allowing for Ca^{2+} influx and elevation of intracellular Ca^{2+}. Neuronal depolarization and subsequent influx of Ca^{2+} has been shown to increase the expression of TrkB in cultured cortical neurons (Nowycky et al., 1985, Kingsbury et al., 2003). Our findings that inhibition of VGCCs attenuates afferent
extension are consistent with previous investigations that VGCC activity is required for endogenous regulation of afferent extension (Gomez et al., 1995).

As blocking VGCCs attenuated axon extension in the absence of exogenous BDNF, we next asked whether co-treatment with BDNF and CdCl₂ affected DRG axon extension.

The observation that TrkB stimulation still enhanced axon growth when VGCC were blocked, suggests release of IP₃ sensitive intracellular stores may be the primary mechanism for the rise in intracellular Ca²⁺ that contributes to the regulation of afferent extension in the longitudinal pathway. These findings were consistent with investigations by Arie et al. (2009) showing neurite outgrowth depends upon IP₃ sensitive intracellular Ca²⁺ store release during early development and ryanodine receptors at later stages in development (Arie et al., 2009).

Collectively, these findings provide evidence for an endogenous mechanism that regulates DRG axon extension within the developing longitudinal pathway. Importantly, this mechanism can be manipulated to enhance or attenuate axon extension in developing tissue. Ultimately, an understanding of the mechanism driving axon extension is important not only for our understanding of how axons grow during development, but how we might manipulate axon extension to mediate pathological states.
References


McNamara M, Clason T, Forehand CJ (2015) Fingolimod through a BDNF related mechanism increases axon extension of primary sensory neurons. "Unpublished manuscript".


Figure 3-1. Inhibition by Trk inhibitor K252a and TrkB function blocking antibody attenuates DRG axon extension. A. Epi-fluorescent images of transverse sections (20µm) of spinal cord (SC) and DRG TrkB immunoreactivity in DRG, DREZ and SC. B. No primary control. Scale bar = 100µm. C. Exposure to K252a [200nM] (n= 9) to inhibit TrkB attenuated DRG axon extension relative to VC (n=8). Inhibition of TrkB with a rabbit αTrkB IgG function blocking antibody [11ng/ml] (n=8) attenuated axon extension relative to VC (n=8) and/or rabbit IgG [11ng/ml] (n=14). **p<0.01, ****p<0.001.
A

Axon length (µm)

VC [0.5 µM] U73122 VC [1 µM] U73122 VC [3 µM] U73122

B

Axon length (µm)

VC PD8059 [2 µM] VC U0126 [10 µM]

C

Axon Length (µm)

VC [10 µM] [20 µM] LYS294002
Figure 3-2. Inhibition of PLC-γ and PI3K but not MAPK signaling, attenuated DRG axon extension. 

A. U73122 attenuated DRG axon extension at 1μM [U73122 (n=19), VC (n=10)] and 3μM [U73122 (n=4), VC (n=5)], but not at 0.5μM [U73122 (n=4), VC (n=5)]. 

B. Exposure to 2μM PD8059 (n=6) had no effect on axon extension relative to VC (n= 5). Exposure to 10μM U0126 (n=8) had no effect on DRG axon extension relative to VC (n=5). 

C. Exposure to 10μM LY294002 had no effect on DRG axon extension (n=9) whereas 20μM LY294002 (n=9) significantly attenuated DRG axon extension relative to VC (n=6). 

***p<0.005, ****p<0.001
Figure 3-3. Inhibition of TrkB stimulated PLC-γ but not MAPK or PI3K signaling attenuates DRG axon extension. A. U73122 [3µM] (n=4) attenuates axon extension induced by BDNF [100ng/ml] (n=7). BDNF [100ng/ml] (n=6) enhances axon extension relative to VC treatment (n=7). B. U0126 [100µM] (n=6) had no effect on axon extension relative to VC (n=7). Exposure to either BDNF [100ng/ml] or BDNF [100ng/ml] +U0126 [100µM] (n=6) enhanced axon extension. C. LY294002 [20µM] (n=5) attenuates DRG axon extension induced by BDNF [100ng/ml] (n=5). Exposure to BDNF ([100ng/ml] (n=5) enhances axon extension relative to VC treatment (n=6). *p<0.05, **p<0.01, ****p<0.0001.
Figure 3-4. Exposure to U0126 [100µM] inhibits phosphorylation of ERK. (A-E)

Immunoreactivity for pERK following a 5 hour exposure with one of the following conditions A. VC (ACSF+1% DMSO) exposure. B. BDNF [100ng/ml] exposure C. Simultaneous exposure of BDNF [100ng/ml] + U0126 [100µM] exposure. Scale bar = 100µm (4A) and refers to all panels.
Figure 3-5. Role of PLC-γ related signaling events in DRG axon extension. A.
Inhibition of Ca$^{2+}$ release from IP$_3$ sensitive intracellular Ca$^{2+}$ stores attenuates DRG axon extension. Exposure to Xe-C at 0.1µM (n=6) had no effect on axon extension compared to VC (n=4), whereas, exposure to Xe-C at 0.5µM (n=8) and [1µM] (n=10) attenuated axon extension compared to VC exposure (n=13). B. Exposure to a DAG analogue or a PKC inhibitor did not attenuate DRG axon extension. Treatment with OAG [10µM] (n=6) had no effect on DRG axon extension as compared to VC exposure (n=5). Exposure to Bim1 at 50 µM (n=6) or 100 µM (n=9) had no effect on DRG axon extension compared to VC exposure (n=5). **p<0.005
Figure 3-6. Exposure to Xe-C or BAPTA-AM blunted axon extension in the absences and presence of added BDNF. A. Exposure to Xe-C [1µM, (n=6)] suppressed axon extension. The inhibition of Xe-C was not reversed when Xe-C and BDNF were applied together [Xe-C 1 µM, BDNF 100ng/ml (n=5)]. B. Exposure to the cell permeable Ca²⁺ chelating agent BAPTA-AM attenuated DRG axon extension [BAPTA-AM 5mM (n=6), VC (n=8)]. The addition of BAPTA-AM along with BDNF did not reverse the effect of the treatment with the cell permeable Ca²⁺ chelator [BAPTA-AM 5mM and BDNF 100ng/ml (n=5), VC (n=8), BDNF 100ng/ml (n=5)].

p<0.05, ***p<0.005, ****p<0.001.
Figure 3-7. CdCl2 treatment attenuated axon extension when applied alone, but did not blunt BDNF stimulated axon extension. A. Exposure to CdCl2 [100 μM] (n=4) attenuated DRG axon extension compared to VC (n=6). B. When BDNF [100ng/ml] and CdCl2 [100μM] were applied together (n=6), axon extension was similar to BDNF alone (n=7) and greater than with VC treatment (n=5). **p<0.01, ***p<0.005, ****p<0.001.
Chapter 4

Summary, discussion, interpretations and future directions

Summary of findings

The purpose of this dissertation was to investigate the endogenous regulation of dorsal root ganglia (DRG) primary sensory afferent extension within the developing longitudinal pathway of the spinal cord, an important growth pattern for the intersegmental coordination underlying reflex formation. In this work, I have manipulated afferent extension within the longitudinal pathway by pharmacologically manipulating BDNF-TrkB signaling. Using an in situ preparation, I found BDNF enhances afferent extension whereas disrupting BDNF signaling attenuates afferent extension in the longitudinal pathway in situ. BDNF binds with high affinity to TrkB, a tyrosine kinase receptor known to promote growth and survival in DRG neurons in vitro. Disruption of TrkB or TrkB dependent PLC-γ signaling attenuates afferent extension in the longitudinal pathway. In contrast, blocking TrkB dependent PI3K or MAPK signaling had no effect on afferent extension within the longitudinal pathway. Thus, I propose that DRG axon extension in vivo is regulated by release of Ca^{2+} from IP_3 sensitive stores following BDNF activation of TrkB and initiation of PLC-γ signaling.

As BDNF was shown to enhance afferent extension in the longitudinal pathway, I then sought to investigate the effects of fingolimod-p, an S1P_{1}R agonist known to enhance BDNF mRNA and protein production in mouse embryonic cortical neurons (Deogracias et al., 2012, Doi et al., 2012). I found fingolimod-p enhances BDNF mRNA production and enhances afferent extension via a BDNF related mechanism. Thus, I
propose S1P₁R activation by fingolimod-p enhances DRG axon extension in the longitudinal pathway through a BDNF related mechanism. A summary of the results in this dissertation is shown table 4-1. Other experiments from our laboratory are summarized in table 4-2 and will be integrated into this discussion.

**Discussion and interpretation**

The molecular mechanisms that regulate DRG afferent extension in the longitudinal pathway in situ are poorly understood. Previous studies have investigated the molecular events leading up to longitudinal extension. Following coalescence of DRG from migrating neural crest cells, afferents extend towards the developing spinal cord. The growth of these afferents towards the dorsal root entry zone (DREZ) is controlled by repulsive cues mediated by netrins, a family of diffusible factors that act as either attractive or repulsive cues depending upon the receptor they interact with. Netrin expression can be found in the floor plate and an expression gradient with high netrin expression in the ventral spinal cord and low netrin expression in the dorsal spinal cord can be observed throughout early embryonic chicken development. At E3 HH st.22, netrins act on the Unc5c receptor to mediate chemorepulsive actions away from the floor plate and ventral spinal cord. Previous investigations of netrin signaling in the chicken embryo find that siRNA knockdown of netrin-1 results in aberrant growth towards the floor plate and notochord, further supporting netrins as a repulsive factor that controls afferent pathfinding in the developing chicken embryo (Masuda et al., 2008).

How netrins interact with BDNF-TrkB signaling in developing afferents extending within the longitudinal pathway is not entirely understood. One investigation using Xenopus spinal neurons found netrins and BDNF operate together to promote
chemotaxis. In this study, a netrin gradient was found to control the sensitivity of the growth cone to BDNF by modulating the number of TrkB receptors in the growth cone (Ming et al., 2002). It is possible that afferent extension within the longitudinal pathway is controlled by both the chemoattractive forces of BDNF-TrkB signaling and the chemorepulsive forces of netrins. Previous investigations in E12.5 mouse (equivalent to E5 in chicken) find netrin release from the dorsal spinal cord. Netrins then activate Unc receptors on DRG axons resulting in repulsion away from the spinal cord grey matter and thus keeping them within the longitudinal pathway (Masuda et al., 2009). It is likely that netrins are also secreted from E5 st. 25 chicken dorsal spinal cord in situ, that they repulse afferents away from the dorsal spinal grey matter and maintain afferent growth within the longitudinal pathway.

Once afferents penetrate the DREZ, their rostral to caudal bifurcation is controlled by cGMP signaling. Exposure to a cGMP analogue, Br-cGMP or YC-1, a soluble guanylyl cyclase (sGC) activator, increases the number of branch points of E14 rat DRG in vitro. In contrast, an inhibitor of protein kinase cGMP-1 (PrkG1), a downstream effector of cGMP signaling, prevents axon bifurcation (Zhao et al., 2009). Further studies of DRG axon bifurcation suggest interactions between cardiac naturitic protein (CNP) and its receptor natriuretic peptide receptor 2 (NPR2) regulate afferent bifurcation through cGMP signaling. Similar to experiments in which PrkG1 was blocked, CNP knockouts produced afferents that fail to bifurcate but turn to extend in only one direction. Whether these afferents turn rostrally or caudally is random (Zhao and Ma, 2009).
BDNF is known to enhance levels of cAMP and support axon pathfinding and turning. Whether activation of TrkB with BDNF or the intracellular signaling pathways elicited by TrkB activation are important for axon bifurcation is unknown. One possible scenario is that cAMP is elevated during axon bifurcation and attenuated when afferents begin to extend within the longitudinal pathway. While this has not been directly tested, cAMP has been shown to initiate a turning response in the growth cone of Xenopus spinal neurons in vitro (Lohof et al., 1992).

S1P1Rs are G protein coupled receptors activated by sphingosine-1 phosphate, a sphingolipid derivative of Sphingomyelin (Bigaud et al., 2014). Fingolimod-p is a structural analogue of S1P and an agonist for S1PRs. Activation of S1PRs allows for the elicitation of various intracellular signaling pathways that affect cAMP signaling. Specifically, activation of the S1P1R inhibits adenylate cyclase and subsequently decreases levels of cAMP (Means and Brown, 2009).

Previous studies of afferent extension within the longitudinal pathway find extension can be manipulated pharmacologically (Coutinho-Budd et al., 2008) [Table 4-2]. In that study, using an intact chicken embryo in ovo preparation (E6), exposure to forskolin, an adenylate cyclase activator, attenuates afferent extension within the developing longitudinal pathway. In contrast, exposure to H89, a PKA inhibitor, enhances afferent extension (Coutinho-Budd et al., 2008). These findings are contrary to studies done in adult neurons where elevation of cAMP enhances neuronal responsiveness to growth factors such as BDNF, and promotes regeneration of axotomized neurites (Spencer and Filbin, 2004). In vivo in adult animals, elevation of cAMP by addition of cell permeable cAMP analogues enhances regeneration of DRG
axons whereas inhibition of PKA prevents regeneration in lesioned spinal axons in the dorsal columns of 5 week old rats (Qiu et al., 2002). In E5 HH st.25 chicken embryo preparation, and consistent with in ovo studies by Coutinho-Budd et al., (2008), blocking cAMP signaling enhances afferent extension and activating adenylate cyclase attenuates afferent extension (preliminary studies, data not shown).

There are many potential explanations for these findings. One possibility is that in vitro investigations of the involvement of cAMP in axon growth do not take into account the possibility that additional signaling mechanisms and/or substrates may influence cAMP signaling within the intact embryo. For instance, the intact embryonic chicken spinal cord contains sonic hedgehog (Shh) signaling, an important developmental signaling event required for axon guidance in longitudinal spinal cord tracts. Shh acts through the receptors Patched and Smoothened to attenuate levels of cAMP and reduce PKA signaling (Bovolenta and Sanchez-Arrones, 2012). Thus, it is possible that in situ Shh signaling is an important mechanism for blocking cAMP signaling so that afferents may extend within the longitudinal pathway. This effect would not be seen in dissociated DRG neurons grown in vitro, and therefore may account for the discrepancy in the results obtained from in vitro and in situ preparations.

Another potential explanation for these findings is that S1P1R may be transported to the cell membrane during the developmental time period when afferents are extending within the longitudinal pathway. Activation of S1P1Rs would inhibit adenylate cyclase and subsequently attenuate cAMP levels in the developing axons. A model proposed by Forbes et al., (2012) using rat sympathetic neurons in vitro shows that when levels of baseline Ca^{2+} are high, reducing cAMP levels promotes growth cone attraction (Forbes et
Previous investigations suggest elevation of intracellular Ca\(^{2+}\) is essential for axon extension (Connor, 1986a, Arie et al., 2009a). Further, we show chelation of intracellular Ca\(^{2+}\) attenuates afferent extension within the longitudinal pathway. Thus, it is likely that high intracellular Ca\(^{2+}\) levels and low cAMP levels promote DRG afferent extension within the longitudinal pathway.

Another potential contributing factor to the effect of cAMP on afferent extension is the substrate upon which afferents are grown. Differences in substrate composition affect afferent rate of extension in vitro. Koch et al., (2012) found changes in substrate tension affect DRG neurite extension such that as tension increases neurite extension increased (Koch et al., 2012). Additionally, E7 chicken DRG express lower levels of cAMP when plated on a substratum composed of low laminin concentrations, whereas high levels of cAMP are seen in response to being plated on high laminin substrates (Lemons and Condic, 2006). Additionally, integrin activation significantly decreases intracellular cAMP on both low and high laminin (Lemons and Condic, 2006). The substrate upon which DRG afferents grow within the longitudinal pathway is uncharacterized. Thus, low levels of laminin within the endogenous substrate could be one possible explanation as to why inhibition of the downstream effector of cAMP enhances afferent extension in situ.

Another potential explanation for the differential effects of cAMP signaling in vitro and in situ may be the result of the difference between intact and regenerating systems. In embryonic systems in which neurons have been dissociated, regenerative capacity can be prevented by blocking cAMP signaling with PKA inhibitors or cAMP antagonists (Spencer and Filbin, 2004). Another study found dissociated, embryonic
DRG and retinal ganglia axon extension was enhanced following inhibition of PKG or addition of cAMP analogue, db-cAMP (Cai et al., 2001). While there are many similarities between developmental growth and regenerative growth, such as the requirement for the advancement of the growth cone and the assembly of the cytoskeleton, there are distinct differences that make studies using dissociated neurons distinctly different from intact, developing neurons (Zhou and Snider, 2006). One important difference is the requirement of the growth cone of the regenerating axon to travel through variable terrain where it may encounter extracellular factors that affect its ability to assemble the axon. This is distinctly different from developing neurons that are known to be guided by repulsive and attractive cues that guide them along specific pathways to their targets (Zhou and Snider, 2006).

Additional studies on extension of primary afferents in the longitudinal pathway have found pharmacological manipulation of the neuronal cytoskeleton affects afferent extension [Table 4-2]. For example, exposure of E5 (HH st.25) chicken in situ preparations to Nocodzol, a pharmacological agent known to interfere with polymerization of microtubules, attenuates afferent extension, whereas exposure to Jasplakinolide, an actin polymerizing and stabilizing agent, enhances afferent extension in the longitudinal pathway (Mariani et al., 2011). BDNF has been shown to induce translation of β-actin in the axon and growth cone of rat cortical neurons in vitro (Sasaki et al., 2010). Thus, it is likely that BDNF also regulates actin polymerization of longitudinally extending afferents in the developing chicken embryo. One potential mechanism for the regulation of actin polymerization by BDNF could be through activation of the TrkB receptor and release of Ca^{2+} from IP_{3} sensitive stores via elicitation...
of the PLC-γ signaling pathway. Release of Ca\(^{2+}\) from these stores may activate Ca\(^{2+}\) dependent kinases such as Ca\(^{2+}\)-calmodulin-dependent kinases (CAMK), a family of signaling molecules known to be important for cytoskeletal remodeling, including actin polymerization and axon elongation in rat hippocampal and cerebellar neurons through regulation of growth cone motility (Wayman et al., 2004, Rosenberg and Spitzer, 2011).

Given a potential model for afferent extension that requires activation of TrkB with BDNF and release of Ca\(^{2+}\) from IP\(_3\) sensitive stores via activation of PLC-γ signaling, another critical question is: what is the location of BDNF in this preparation? In vivo, BDNF can be secreted in an autocrine/paracrine manner or from a distal target to promote target innervation of sensory neurons. In the chicken embryo, afferents growing within the longitudinal pathway have multiple targets in the spinal cord and developing brain stem. At E5 (HH st.25), afferents have not made contact with any of their potential targets. Further, individual afferents within the longitudinal pathway have targets in both the rostral and caudal direction. It is possible that there are two sources of BDNF that guide afferents in the rostral or caudal direction, although it is unclear how a gradient could set up in opposing directions along multiple segments that would result in rostral and caudal extension of these axons. A more likely scenario is that BDNF signals in an autocrine/paracrine manner. This is supported by the fact that BDNF mRNA and protein are found in DRG neurons during chicken development.

It is also possible that BDNF and TrkB are locally translated within the growth cone. To explore this possibility in an E5 chicken in situ preparation, afferents could be labeled with DiI and incubated for 15 minutes to allow for DiI to label the growing afferent. DRG cell bodies could then be removed prior to the 5 hour incubation growth
assay. Persistence of afferent extension within the longitudinal pathway would be a strong indication that local protein synthesis is required for afferent extension. To confirm local axon synthesis of BDNF is required for afferent extension, afferents could be labeled with DiI and incubated for 15 minutes to allow DiI to label the afferents. Afferents could then be disconnected from their cell bodies. The afferents, which remain within the preparation could be treated with fingolimod-p or fingolimod-p and αBDNF function blocking antibody. Enhanced afferents extension with fingolimod-p treatment and attenuated afferent extension with fingolimod-p and αBDNF function blocking treatment following removal of the DRG cell bodies would suggest S1P1Rs and TrkB receptors are not required in the cell body to regulate axon extension within the longitudinal pathway.

Another compelling question is: how does BDNF arrive at the growth cone? Various lines of evidence suggest axonally localized transcripts can be translated within the growth cone. Specifically, proteins known to be involved in axon elongation and responses to guidance cues have been shown to be translated locally within axon growth cones. In one study using severed axons from E14 rat DRG found NGF enhanced expression of Par3 protein, a regulator of cytoskeletal dynamics (Hengst et al., 2009). These data suggest translation of proteins involved in axon elongation occurs locally within the growth cone. To date, there is no evidence that BDNF is transcribed and translated in the developing growth cone. Rather, it is more likely that BDNF is transcribed and translated in the neuronal cell body, and anterogradely transported to the axon and growth cone, as is seen in the rat DRG, optic nerve, and hippocampus (Menna et al., 2003, Ng et al., 2007). Rate of anterograde transport for BDNF was determined in
E18 rat hippocampus to be 1.49 µm s\(^{-1}\) (Lo et al., 2011). Thus, over the course of a five hour incubation, BDNF has the potential to be transported >26,000 µm. As the average length of a vehicle control treated DRG afferent in an E5 HH st. 25 chicken in situ preparation is between 80-100 µm from cell body to the DREZ and 550-600 µm from the location of bifurcation to the most distal end of the growth cone, it is likely that BDNF is anterogradely transported from the cell body to the DRG growth cone during the five hour incubation period utilized in this work. TrkB is also known to be anterogradely transported to the axonal growth cone. Huang et al., (2011) found both full length and truncated isoforms of TrkB are anterogradely transported in hippocampal axons to the axonal growth cone via interactions with kinesin-1 motor protein (Huang et al., 2011). Thus, it is likely that BDNF and TrkB are transcribed and translated in the neuronal cell body and transported to the distal axon and growth cone via anterograde transport mediated by kinesins. Once at the growth cone, BDNF may be secreted allowing it to act in an autocrine and paracrine manner on TrkB expressing neurons.

In addition to autocrine signaling, BDNF is likely to signal via a paracrine mechanism as well. Previous investigations show TrkB mRNA in 47% of DRG cell bodies in E5 chicken embryos (Rifkin et al., 2000). Additionally, Straub et al., (2007) show TrkB positive afferents within the DREZ at HH st. 25. (Straub et al., 2007). However, the number of TrkB positive afferents within the longitudinal pathway has yet to be quantified at HH st. 25. Previous investigations and the immunohistochemistry for TrkB done in this dissertation suggest TrkB is expressed in many of the developing afferents within the longitudinal pathway. Further experiments using quantitative immunohistochemistry would need to be done to determine the percentage of afferents
within the longitudinal pathway that are TrkB positive. There are two possible outcomes for quantitative immunohistochemistry using an E5 HH st.25 chicken embryo in situ preparation. The first is that all afferents within the longitudinal pathway are TrkB positive. This would suggest that TrkB is required for growth within this pathway. This is a possibility as the afferents growing within this anatomical pathway will become the dorsal columns, a longitudinal pathway associated with fine touch, two point discrimination and conscious proprioception from the skin and joints in the adult organism. At HH st. 25 afferents have not made contact with their peripheral sensory receptor (Guan et al., 2003). Thus, it may be that TrkB is regulating growth within the longitudinal pathway until the peripherally extending process makes contact with its receptor at HH st. 30 (Guan et al., 2003). Whether TrkB expression in centrally projecting DRG afferents is affected by peripheral projecting afferents’ contact with its receptors is unknown. Another possibility is that a subpopulation of afferents growing within the longitudinal pathway express TrkB. This is a possibility as some of the afferents within the longitudinal pathway are destined to different central targets. Additionally, these afferents may make connections with peripheral receptors that transduce different sensory modalities such as nociception, typically associated with TrkA expressing DRG or proprioception, typically associated with TrkC expressing DRG. These results would suggest afferent extension in the longitudinal pathway may be regulated by the activation of multiple Trk receptors. With both scenarios, BDNF likely signals via both autocrine and paracrine mechanisms.

If BDNF is secreted from neurons and BDNF enhances afferent extension in the longitudinal pathway, then exposure to a pharmacological agent that enhances BDNF
mRNA and protein production should enhance afferent extension. Fingolimod-p is a sphingosine 1-phosphate analogue and an sphingosine-1 phosphate receptor (S1PR) agonist (Kong et al., 2014). While the effects of fingolimod-p as an S1PR agonist were originally studied in the immune system, as this drug was found to be a highly efficacious disease modifying treatment option for individuals suffering from relapsing-remitting forms of multiple sclerosis (RRMS), it has more recently been shown to enhance BDNF mRNA and protein production in mouse, embryonic, cortical neurons (Deogracias et al., 2012, Doi et al., 2012). While BDNF mRNA transcription was increased in response to treatment with fingolimod-p in our preparation, we could not demonstrate BDNF protein secretion into the media of our preparation following treatment with fingolimod-p. Previous investigations observe enhanced BDNF production in response to treatment with fingolimod-p. The primary difference between these studies and ours is the location from which BDNF protein was assayed. Both Deogracias et al., 2012 and Doi et al., 2013 assessed BDNF protein levels from cell lysates, whereas we attempted to quantify BDNF protein release into the media of our preparation (Deogracias et al., 2012, Doi et al., 2012). Our rationale for assessing BDNF protein in the media was as follows. We were primarily interested in BDNF secretion from primary sensory afferents growing in the longitudinal pathway. As isolation of these afferents would have damaged the connection to the cell body, we chose to leave the spinal cord and DRG intact and assess release of BDNF into the media. The inability to detect BDNF secretion using this methodology may be the result of one or more of the following possibilities: 1) the amount of BDNF secreted from our preparation is below the limit of detection for the ELISA assay used (10-0.06ng/ml) (MyBiosource), 2) BDNF levels were unable to be detected due to the
inability of BDNF to diffuse through neuronal tissue and into the cell media, thus preventing its detection (Djordjevic and Driscoll, 2002) or 3) BDNF mRNA may be upregulated but BDNF protein may not. Exploration of these possibilities is needed to further understand how BDNF secretion may or may not occur in response to fingolimod-p treatment.

Once BDNF activates TrkB, the receptor trans-autophosphorylates on tyrosine residues resulting in the activation of three intracellular signaling pathways: the PLC-γ, PI3K and MAPK signaling pathways, each of which has been shown to mediate important effects on the developing nervous system. Activation of the PLC-γ signaling pathway allows for the release of Ca^{2+} from IP_{3} sensitive stores, an important process for elevating intracellular Ca^{2+} and neurite extension in early chicken DRG in vitro (Connor, 1986b, Arie et al., 2009b). Arie et al., (2009) found chicken DRG neurons (≤ E10) expressed IP_{3}Rs in their developing growth cones in vitro. Following E10, IP_{3}R immunoreactivity is severely attenuated and blocking release of Ca^{2+} from IP_{3} sensitive stores had no effect on DRG neurite extension. This study suggests neurite outgrowth is differentially regulated throughout development by the means in which Ca^{2+} enters into the intracellular environment (Arie et al., 2009b). In this dissertation, release of Ca^{2+} from IP_{3} sensitive stores via activation of the TrkB activated PLC-γ signaling pathway was found to be important for afferent extension within the longitudinal pathway. However, unlike Arie et al., (2009), we found inhibition of voltage gated Ca^{2+} channels during early development attenuated afferent extension as well. It is possible that release of Ca^{2+} from IP_{3} sensitive stores and from the extracellular environment contribute to afferent extension within the longitudinal pathway. The difference seen in previous
studies and this work may be due to different model systems and/or that previous investigations used dissociated DRG neurons grown in vitro (Conner et al., 1997, Arie et al., 2009a). Damage to afferents resulting from the process of dissociation may alter the Ca\(^{2+}\) channels within the early developing DRG neuron thus altering the means by which Ca\(^{2+}\) enters into the developing neuron.

How Ca\(^{2+}\) promotes afferent extension in the longitudinal pathway was not explored in this dissertation, however, there is compelling evidence to suggest differences in intracellular Ca\(^{2+}\) levels regulate axon growth. Singh and Miller in 2005 support this idea in experiments using neonatal mouse sympathetic neurons. In this work, membrane depolarization and enhanced intracellular Ca\(^{2+}\) signaling resulted in longer neurites in the presence of NGF. Additionally, these experiments found Ca\(^{2+}\) acts through Calcium-calmodulin kinase II (CAMII), a kinase known to phosphorylate and activate additional members of the Ca\(^{2+}\) calmodulin family in the presence of Ca\(^{2+}\). Additional investigations of CaMK signaling on axon extension suggest that Ca\(^{2+}\) activated CamK signaling enhances afferent extension via interactions with the actin cytoskeleton. In these investigations CaMK1 localized to the axonal growth cone where it promotes actin polymerization and de-polymerization via interactions with substrates such as members of the Rho family of GTPases, which are known to regulate actin dynamics in filopodia and lamellipodia (Wayman et al., 2004). Additionally, CamKI phosphorylates myosin II, a mechanoenzyme known to be important for the regulation of E5 HH st.25 chicken DRG afferent extension within the longitudinal pathway (Wayman et al., 2004, Robinson et al., 2015). Thus, it is likely that in E5 HH st.25 chicken DRG elevation of intracellular Ca\(^{2+}\) due to PLC-\(\gamma\) dependent release of IP\(_3\) sensitive intracellular stores results in activation of
CaMKII which then phosphorylates CaMKI. CaMKI may then phosphorylate Rho GTPases to promote actin polymerization and organization of actin into F-actin. Additionally, CamKI may also phosphorylate and activate myosin II to promote axon extension within the developing longitudinal pathway.

Activation of the PI3K and MAPK signaling pathways have been implicated in axon outgrowth as well. Activation of the PI3K signaling pathway leading to activation of AKT has been implicated in many aspects of neurite outgrowth such as: elongation, branching and survival. Markus et al., (2002) found treatment of dissociated, E13 mouse DRG with NGF increased terminal branching of sensory neurons by activation of PI3K-AKT signaling and potentially through subsequent phosphorylation of AKT substrates known to interact with the actin cytoskeleton (Markus et al., 2002). While inhibition of PI3K in E5 HH st.25 chicken embryo DRG resulted in attenuation of afferent extension, addition of BDNF appeared to reverse the attenuating effect of exposure to the PI3K inhibitor. Thus, it is possible that BDNF acts on additional receptors to enhance extension in the longitudinal pathway. For instance, BDNF is known to activate P75NTR. While not explored in this study, P75NTR may become activated by BDNF and compensate for the loss of function due to inhibition of PI3K signaling. Previous investigations using retinal ganglia neurons from embryonic chicken suggest P75NTR contributes to axon extension through deactivation of Rho-A, a cytoskeletal binding protein (Yamashita et al., 1999). Thus, it is likely that P75NTR is activated by BDNF and its signaling contributes to afferent extension in the longitudinal pathway in this preparation, potentially via interactions with the axon cytoskeleton.
The MAPK signaling pathway has also been shown to contribute to neurite outgrowth in embryonic sensory neurons. Markus et al., (2002) found inhibition of Raf-1 or Ras prevents NGF induced axon growth in E13 mouse embryonic sensory neurons in vitro (Markus et al., 2002). Pharmacological inhibition of the MAPK signaling pathway with MEK inhibitors did not appear to have an effect on DRG afferent extension in E5 HH st.25 chicken embryo with or without BDNF stimulation. One explanation for these findings is that endogenous MAPK signaling does not influence axon extension in this developmental time period. This may be possible if the downstream effects of MAPK signaling such as regulation of transcription, occurs prior to E5 HH st. 25. Another potential explanation for these findings is that MAPK signaling is required for afferent extension within the longitudinal pathway, and that blocking MAPK signaling in the five hour treatment window had no effect on transcription and/or translation of proteins, two events known to be regulated by MAPK signaling.

Conclusions and Future directions

In summary, the following model is proposed for the regulation of afferent extension in the longitudinal pathway. BDNF is anterogradely transported from the cell body to the axon growth cone where it is secreted, allowing it to act upon TrkB receptors in an autocrine and paracrine manner. Activation of TrkB with BDNF results in transautophosphorylation of the TrkB receptor on tyrosine residues resulting in the elicitation of intracellular signaling pathways: PLC-γ, PI3K and MAPK pathways. TrkB activation of the PLC-γ signaling pathway allows for release of Ca$^{2+}$ from IP$_3$ sensitive Ca$^{2+}$ stores. This release of Ca$^{2+}$ activates Ca$^{2+}$ dependent kinases such as CAMKII and
Ca\textsuperscript{2+} dependent actin binding proteins known to interface with the axonal cytoskeleton and promote growth cone motility and remodeling. Additionally, release of IP\textsubscript{3} sensitive Ca\textsuperscript{2+} stores may also induce a Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release thereby promoting opening ryanodine sensitive channels on the ER membrane resulting in additional elevation of intracellular Ca\textsuperscript{2+} and activation of Ca\textsuperscript{2+} dependent kinases and Ca\textsuperscript{2+} activated actin binding proteins [Figure 4-1]. Fingolimod-p, through activation of the S1P\textsubscript{1}R increases BDNF transcription and translation through activation of the MAPK signaling pathway. Activation of the MAPK signaling pathway through this means results in the phosphorylation of CREB, a protein known to regulate the transcription of BDNF. CREB-P then promotes BDNF transcription resulting in increased translation of BDNF protein. BDNF can then be anterogradely transported from the cell body to the growth cone where it is secreted so that it may act on TrkB and promote TrkB signaling [Figure 4-1].

Interestingly, inhibition of the MAPK signaling pathway does not affect afferent extension within the longitudinal pathway. It is possible that fingolimod-p activates MAPK signaling and enhances BDNF mRNA and protein production before U0126 and PD98059, the MAPK inhibitors used in this dissertation, effectively supresses MAPK signaling. Another possibility is that fingolimod-p may activate CREB through PLC dependent release of Ca\textsuperscript{2+} from IP\textsubscript{3} sensitive stores and activation of CAMK signaling. CAMK signaling may then phosphorylate CREB and enhance BDNF transcription (Yoon et al., 2008). Future investigations could look at the effect of blocking MAPK signaling in the presence of fingolimod-p.
One intriguing question that remains unanswered in these studies is: what is driving axons to grow within the longitudinal pathway? One possible answer to this question is a combination of repulsive and attractive guidance cues as well as the trophic factors. The preparation used in this study is an excellent model system for an investigation of how different signaling mechanisms interact to affect axon growth in a specific anatomical pathway. Future investigations could investigate the interactions of repulsive or attractive guidance factors such as netrins, semaphorins and SHH signaling, and their interactions with BDNF-TrkB signaling.
References


Rosenberg SS, Spitzer NC (2011) Calcium signaling in neuronal development. CSH persp biol 3:a004259.


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<td>BDNF+ Xe-C</td>
<td>Stimulates TrkB and blocks release of Ca&lt;sup&gt;2+&lt;/sup&gt; from IP&lt;sub&gt;3&lt;/sub&gt; sensitive stores</td>
<td>Attenuate*</td>
</tr>
<tr>
<td>BDNF + BAPTA-AM</td>
<td>Stimulates TrkB and chelates intracellular Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Attenuate*</td>
</tr>
<tr>
<td>BDNF + CdCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Stimulates TrkB and blocks VGCCs</td>
<td>No change*</td>
</tr>
</tbody>
</table>
**Table 4-1. Summary of results.** Primary sensory afferent extension can be manipulated pharmacologically. Afferents extension is enhanced or attenuated when exposed to the above described pharmacological agents relative to vehicle control treatment (artificial cerebral spinal fluid + 1% DMSO). Both pharmacological treatment and vehicle control treatment were for five hours and under identical culture conditions.

*Denotes afferent extension was enhanced, attenuated or not affected relative to BDNF treatment alone.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Proposed action</th>
<th>Effect on afferent extension in the longitudinal pathway</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blebbistatin</td>
<td>Blocks myosin II</td>
<td>Attenuates</td>
<td>Robinson et al., (2015)(^a)</td>
</tr>
<tr>
<td>Anti β-1 integrin function blocking antibody</td>
<td>Blocks Beta-integrin receptors</td>
<td>Attenuates</td>
<td>Robinson et al., (2015)(^a)</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>Interferes with microtubule polymerization</td>
<td>Attenuates</td>
<td>Mariani et al., (2015)(^a)</td>
</tr>
<tr>
<td>Latrunculin</td>
<td>Actin stabilizer</td>
<td>Enhances</td>
<td>Mariani et al., (2015)(^a)</td>
</tr>
<tr>
<td>Jasplakinolide</td>
<td>Promotes actin polymerization</td>
<td>Enhances</td>
<td>Mariani et al., (2015)(^a)</td>
</tr>
<tr>
<td>H89</td>
<td>Blocks cAMP substrate PKA</td>
<td>Enhances</td>
<td>Coutinho-Budd et al., (2008)(^b)</td>
</tr>
<tr>
<td>Forskolin</td>
<td>Activates adenylate cyclase</td>
<td>Attenuates</td>
<td>Coutinho-Budd et al., (2008)(^b)</td>
</tr>
</tbody>
</table>
Table 4-2. DRG afferent extension in longitudinal pathway of embryonic chicken.  

Summary of results from other investigations of afferent extension in embryonic chicken E5 HH st. 25 with an in situ preparation unless otherwise noted. Afferents extension is enhanced or attenuated when exposed to the above described pharmacological agents relative to vehicle control treatment (artificial cerebral spinal fluid + 1% DMSO). Both pharmacological treatment and vehicle control treatment were for five hours and under identical culture conditions.

a Unpublished findings.

b Experiments performed in E6 chicken DRG. Pharmacological exposure was done in ovo.
**Figure 4-1. Model for regulation of afferent extension in the longitudinal pathway of E5 HH st.25 chicken DRG neuron at the growth cone.** The following events are thought to occur in the growth cone of the DRG afferent unless otherwise stated. BDNF binds to and activates TrkB. Activation of TrkB then promotes release of IP₃ sensitive Ca²⁺ stores via activation of the PLC-γ signaling pathway. Elevation of intracellular Ca²⁺ activates CaMKII phosphorylation of CaMKI and subsequent phosphorylation and activation of RhoGTPases, proteins known to interact with the actin cytoskeleton and promote growth cone motility via actin polymerization and de-polymerization.

Additionally, CaMKI phosphorylates Myosin II, a mechanoenzyme important for neuron substrate adhesion and afferent axon extension within the longitudinal pathway. Additionally, exposure to fingolimod-p enhances BDNF mRNA transcription via activation of S1P₁R, subsequent elicitation of MAPK signaling and phosphorylation of CREB. P-CREB binds to DNA and promotes transcription of BDNF mRNA which then becomes translated to protein and released from the axon growth cone. BDNF then acts in an autocrine manner on TrkB receptors through the above described mechanism.

*Occurs in the cell body.

- Ca²⁺


McNamara M, Clason T, Forehand CJ (2015) Fingolimod through a BDNF related mechanism increases axon extension of primary sensory neurons. "Unpublished manuscript".


Wright EM, Vogel KS, Davies AM (1992) Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. Neuron 9:139-150.


