THE ROLE OF NCK IN DCC-SENSITIVE SPINAL CORD CIRCUITS

by

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DEDICATION PAGE

I would like to dedicate this work to all of those who struggle in the pursuit of understanding the incredible complexity of nature.

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LIST OF ABBREVIATIONS USED

ANOVA	Analysis of Variance
ARP	Actin Related Protein
ARPC	Actin Related Protein Complex
ATP	Adenosine Triphosphate
BEN	Bursal Epithelium and Neurons
CDC42	Cell Division Control Protein 42 Homolog
CDNA	Complementary DNA
CRK	V-Crk Avian Sarcoma Virus CT10 Oncogene Homolog
CSK	C-Src Kinase
CST	Corticospinal Tract
DCC	Deleted in Colorectal Cancer
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DNA	Deoxyribonucleic Acid
DSCAM	Down Syndrome Cell Adhesion Molecule
E#	Embryonic Day
EDTA	Ethylenediaminetetraacetic Acid
F-ACTIN	Filamentous Actin
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FLX	Flanked by LoxP
FYN	FYN oncogene related to SRC, FGR, YES
G-ACTIN	Globular Actin
GEF	Guanine Nucleotide Exchange Factor
GST	Glutathione S-Transferase
HB9	Homeobox 9
HBSS	Hanks Balanced Salt Solution
HEK293	Human Embryonic Kidney 293

LIMK	LIM Domain Kinase
MEF	Mouse Embryonic Fibroblasts
MRNA	Messenger RNA
MSN	Mishapen
MTOC	Microtubule Organizing Centre
MUC18	Melanoma Cell Adhesion Molecule 18
NACL	Sodium Chloride
NCK	Non-Catalytic Region of Tyrosine Kinase Adaptor Protein
NIK	NCK Interacting Protein
NPF	Nucleation Promoting Factor
OCT	Optimal Cutting Temperature Compound
P#	Postnatal Day
P130CAS	Protein 130 Kilodaltons Cas
PAK	P21 Activated Kinase
PCR	Polymerase Chain Reaction
PEI	Polyethylenimine
PFA	Paraformaldehyde
PLCg	Phospholipase C g
PY	Phosphorylated Tyrosine
RAC1	Ras-related C3 Botulinum Toxin Substrate
ROBO	Roundabout Receptor
RP3	Receive-Poly 3
RT	Room Temperature
RT-PCR	Real Time – PCR
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS - Polyacrylamide gel electrophoresis
SH2	Src Homology 2
SH3	Src Homology 3
TAG1	Transient Axonal Glycoprotein
TRIO	Triple Functional Domain Protein
TRIS	tris(hydroxymethyl)aminomethane

VCA	Verpolin homology, Cofilin homology, Acidic region
WASP	Wiskott–Aldrich Syndrome Protein
WIP	WASP Interacting Protein
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ABSTRACT

Non-Catalytic region of tyrosine Kinase (NCK) adaptor proteins connect cell surface receptors to the actin cytoskeleton. In the central nervous system loss of NCK proteins leads to a hopping like phenotype. Since NCK proteins have been shown to regulate the development of the corticospinal tract, we were interested in examining the expression of these proteins in the spinal cord development and better defining their role in global processes of axon guidance. Further, since *in vitro* studies have shown that NCK associates with the netrin receptor Deleted in Colorectal Cancer (DCC) we set out to determine is loss of NCK affected DCC sensitive pathways *in vivo*. Thus, the purpose of our study was to better define the *in vivo* role for NCK in the developing nervous system. Developing cranial nerves were examined by whole mount immunohistochemistry (IHC) in NCK-deficient and control embryos (embryonic day 11.5) using anti-neurofilament. Guidance defects in the descending projections of the hindbrain and spinal accessory nerve were noted including misprojections into the dorsal tissue and axonal defasiculation; however most cranial nerves including the facial and trigeminal showed no obvious defect. This suggested a role for NCK in normal axon pathfinding. We next focused on the development of dorsally derived DCC positive commissural axons. Sections of E11.5 spinal cord revealed a reduction of commissural axons in the floor plate region of NCK-deficient mice. To more directly test the link between NCK and DCC, we have developed a primary dissociated spinal cord assay to examine the growth cone on the NCK-deficient spinal neurons. Here we found NCK-deficiency causes growth cone defects and increased axon length. Together, these studies reveal the importance of NCK proteins in neuronal development, and show that NCK is required for normal guidance of DCC positive axons.

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CHAPTER 1 INTRODUCTION

The developing nervous system requires the precise patterning of billions of neurons over a short period of time. Neurons have three main parts: a cell body, a single axon and multiple dendrites. Dendrites are responsible for receiving incoming signals, the cell body is responsible for the metabolic activity of the neuron, and the axon innervates a target cell and is necessary for the propagation of electrochemical signaling to the target cell.

During development, axons travel over long distances to reach their appropriate target. The process that guides axons to their appropriate targets is termed axon guidance. Complex signaling processes between the axon and the environment are facilitated by the axonal growth cone guide axons. The growth cone is an actin-rich structure at the tip of the developing axon, that responds to extracellular cues such as growth factors or other protein gradients. These cues interact with receptors on the tip of the developing axon leading to the activation of appropriate receptor complexes. This then leads to an intracellular signaling cascade that involves a number of protein-protein interactions. Intracellular signaling includes a wide range of protein-protein interactions that can initiate the activation of kinases, phosphatases, or simply allow two proteins to associate. In the growth cone many of the intracellular signaling cascades that are initiated affect the dynamics of the actin and microtubule cytoskeletons. Understanding how these intracellular signaling pathways are activated provides critical insight into how the nervous system develops and how neuronal circuits are formed.

This thesis focuses on identifying the role of an intracellular adaptor protein, non-catalytic region of tyrosine kinase (NCK) and how it connects with a membrane receptor, Deleted in Colorectal Cancer (DCC) receptor, to regulate the development of critical spinal circuits involved in movement. To properly explain the nature of these studies it is important to understand the existing molecular studies relating to the NCK proteins, how they have been connected to axon guidance, and what this means for the development of the complete nervous system.

1.1 NCK Proteins

1.1.1 NCK Gene Family

There are two *NCK* genes, *NCK1* and *NCK2*. The original complementary DNA (cDNA) clone encoding *NCK1* was isolated from a human melanoma library using monoclonal antibodies produced against melanoma-associated antigen, MUC18 (Lehmann, 1990). In the search of the mouse orthologue for *NCK1*, the first *NCK*2 gene was isolated. When screening mouse cell lines with a mRNA probe of human *NCK1*, the mouse *NCK2* mRNA was isolated by cross-reactivity. *NCK2* was also separately isolated in a screen for novel SH2 domain containing proteins (Margolis et al., 1992; Li . 1992). The human *NCK1* gene is located on chromosome 3, 3q21, while *NCK2* gene is located on chromosome 2, 2q12 (Vorobieva et al., 1995; Huebner et al., 1994; Chen et al., 1998).

1.1.2 NCK Protein Family

NCK genes are evolutionarily conserved from *Caenohabitidus elegans* to *Homo sapiens.* In mammals, including *Mus musculus* and *Homo sapiens,* there are two NCK genes that encode functional proteins. In non-mammals, including *Xenopus laevis, Caenohabitidus elegans* and *Drosophila melanogaster,* there is a single *NCK*-like gene (Figure 1.1). The conserved nature of the *NCK* gene suggests it arose early in evolution.

The human *NCK1* and *NCK2* genes encode proteins with 68% amino acid sequence identity (Figure 1.1; Li et al., 2001). However, the amino acid identity within the signaling domains is higher (Figure 1.1; Chen et al., 1998). Given this high degree of amino acid identity within the different domains of NCK1 and NCK2, many groups have suggested that these two proteins have functional and physiological redundancy (Frese et al., 2006; Fawcett et al., 2007; Jones et al., 2006; Bladt et al., 2003; Chen et al., 1998). However, structural studies have shown there to be differences in both the SH2 and SH3 domains (Arold et al., 1998; Kaneko et al., 2010; Chen et al., 1998) leading some to suggest that NCK2 has some divergent roles from NCK1 (Cowan et al., 2001; Bong et al., 2004; Pramatarova et al., 2003). Much of the debate of the redundancy of NCK1 and NCK2 comes from in vitro studies, and until careful analysis of the mutant NCK1 and NCK2 mice have been conducted this debate will remain unresolved. Nonetheless, both NCK1 and NCK2 have similar architectures and are implicated in a number of different cellular functions.

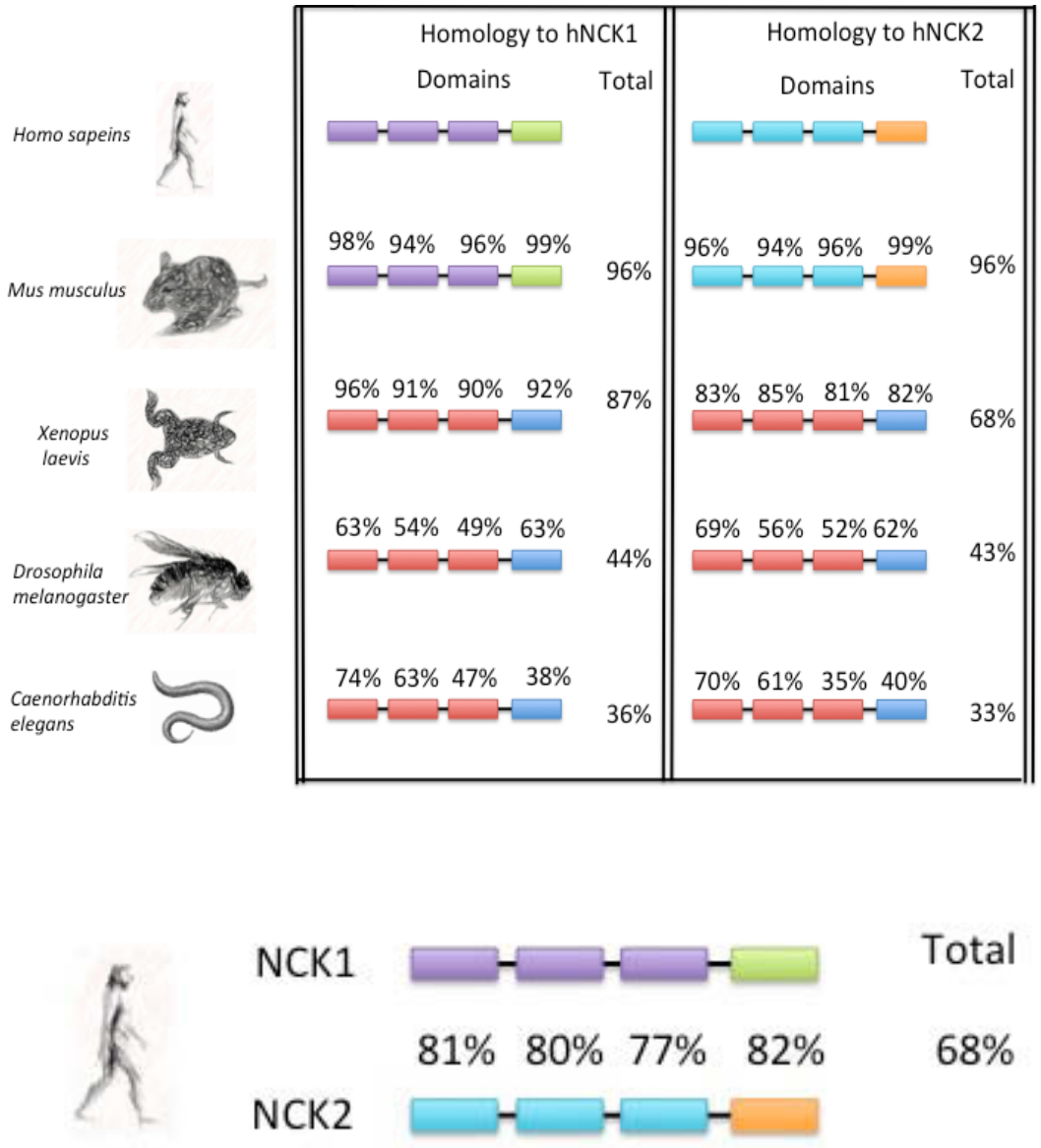

Figure 1.1: Evolutionary Homology of NCK Proteins, percentages are amino acid sequence homology (Percentages from Chen et al., 1998). *Xenopus laevis, Drosophila melanogaster*, and *Caenorhabditis elegans* have only a single *NCK*-like protein (red and blue boxes) where as *Mus musculus* and *Homo sapiens* have two NCK proteins (purple and green boxes and light blue and orange boxes respectively). The domains of NCK are conserved from *Caenorhabditis elegans* to *Homo sapiens* (percentages). Human NCK1 and NCK2 share a high degree of amino acid sequence homology between their domains.

1.1.3 Structure of NCK Proteins

NCK proteins are 48 kilodaltons and contain three amino terminal Src-homology 3 (SH3) domains and one carboxyl terminal Src-homology (SH2) domain. SH3 domains are known to bind to the consensus sequence PXXP (where P is proline and X is any amino acid), while SH2 domains interact with phosphorylated tyrosine (pY) residues (Ren et al., 1993; Pawson, 2001). Throughout the rest of my thesis, I will refer to the collective *NCK1* and *NCK2* genes as *NCK*, but will differentiate between the two where necessary. A complete understanding of the role of NCK can only begin with a clear discussion of the function of its individual domains.

1.1.4 NCK SH3 Domains

The discovery of the SH3 domain was made possible by the alignment of divergent signaling proteins. A 60 amino acid region between Crk, PLCϒ, and Src kinase had a high degree of amino acid similarity implicating this region as an important region that may serve similar functions (Mayer et al., 1988; Stahl et al., 1988). Indeed, it was shown that this region could bind to proteins containing a PXXP motif (Ren et al., 1993). The affinity and selectivity of a single SH3 domain for its target PXXP motif is low, indicating SH3 domains can have highly promiscuous interactions (Mayer, 2001).

NCK proteins contain three SH3 domains. Each individual SH3 domain of the NCK proteins has been shown to bind different target proteins (see Figure 1.2 for examples of binding partners); however, it has been shown that the multiple SH3 domains function to increase their selectivity for binding partners through the summation of multiple low

affinity interactions (Alder et al, 2000; Wunderlich et al., 1999). This allows NCK proteins to recruit other proteins to specific locations within a cell. The low affinity of the SH3 domain also implies that interactions throughout this domain have a strong off–rate. This property allows the NCK proteins to separate from binding partners quickly when it changes localization within the cell (Mayer et al., 2001).

1.1.5 NCK SH2 Domain

Similar to the SH3 domain, the SH2 domain was originally identified as a distinct sequence of 100 amino acids conserved among proteins found in distinct signaling cascades (Sadowski et al., 1986; Mayer et al., 1988; Stahl et al., 1988; Trahey M. 1988, Pawson et al., 2001). The SH2 domain was the first protein signaling domain identified and has been shown to bind to phosphorylated tyrosine residues (Pawson et al., 2001). The selectivity for the phosphate group is achieved through a positively charged pocket in the SH2 domain that specifically attracts the amino acid tyrosine when it is phosphorylated (Pawson et al., 2001). SH2 domains derive further binding specificity through their ability to recognize specific amino acids on the target protein carboxyl to the tyrosine residue (Pawson et al., 2001). Furthermore, not every SH2 domain has the same binding efficiency and may be selective for specific consensus target sequence. Mutating specific residues within the binding site of an SH2 domain alters the biological function of the SH2 domain and can even change the specificity of the domain. This indicates that the structure of the SH2 domain is directly responsible for the function of phosphotyrosine binding and that individual SH2 domains have specific binding partners (Kimber et al., 2000). For the SH2 domains contained in the NCK proteins this consensus

binding sequence has been identified as YDXV (where Y is tyrosine, D is aspartic acid, X is any amino acid, and V is valine; Blasutig et al., 2008).

Cell surface receptors are often the binding target of the SH2 domain in NCK (Li et al, 2001). Receptors containing tyrosine kinases become phosphorylated within tyrosine residues on their intracellular domain upon interaction with their ligand. This phosphorylation event of the receptor acts as a signal to recruit SH2 domain containing proteins such as NCK.

Taken together the unique combination of SH2 and SH3 domains in NCK has led to the hypothesis that NCK acts as molecular adaptor connecting signaling proteins, such as cell surface receptors, to their downstream targets.

1.1.6 Molecular Interactions

Proteins can form complex networks within a cell that lead to signaling processes required for different cell functions. In the case of NCK, it is useful to examine its binding partners to determine the context in which NCK is able to connect to different signaling cascades. A list of binding partners for NCK1 and NCK2, separated by their signaling domains, can be found in Figure 1.2. It is interesting to note that to date the majority of these binding partners are based on *in vitro* studies. Further *in vivo* studies could identify additional partners and provide a better understanding of the importance of these interactions, especially during development. Examination of known NCK binding partners reveals that NCK plays a role in a variety of cellular processes including: T-cell

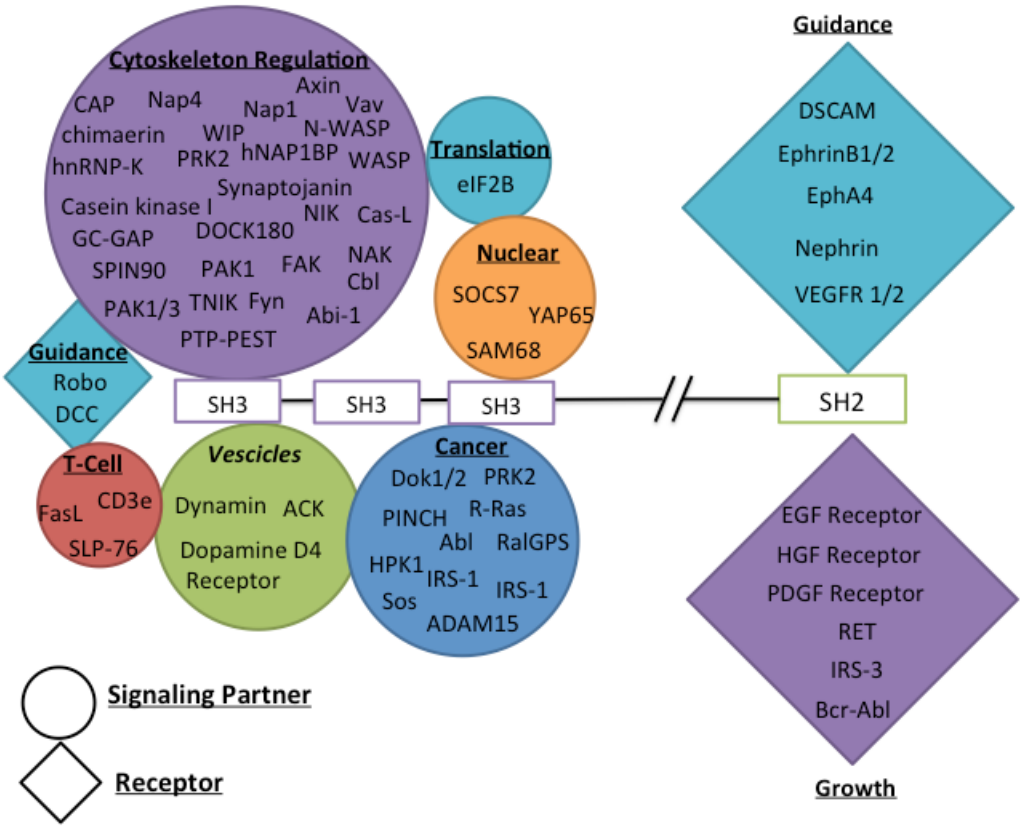

Figure 1.2: Summary of binding partners of NCK. The SH3 domains interact with proteins known to be involved in vesicle signaling, cancer signaling, T-cell receptor signaling, nuclear signaling, translation signaling, and actin signaling. The majority of SH3 binding partner in NCK are related to cytoskeletal regulation. The SH2 domain primarily binds to cell surface receptors involved in growth and guidance.

receptor signaling, vesicles dynamics, cancer progression, nuclear signaling, translational control, and regulation of the cytoskeleton. Since the majority of the SH3 binding partners for NCK are involved in actin regulation, it is logical to assume this represents a major role for the NCK proteins in cellular biology. Indeed, loss of NCK proteins has been shown to affect the structure of the actin cytoskeleton (Bladt et al., 2003).

It should be pointed out that NCK is not always coupled to cell surface receptors by its SH2 domain, but may also bind through its SH3 domain, as is the case for the DCC and robo receptor (See 1.3.2.2 and 1.3.2.5).

1.2 Actin Regulation

1.2.1 The Cyotoskeleton

The cytoskeleton of a cell is composed of three components: actin, microtubules, and intermediate filaments. Actin is found at the leading edge of a cell, and is responsible for membrane protrusion and pathfinding. Microtubulues are anchored at the microtubulin organizing center (MTOC) and extend toward the membrane of the cell. Microtubules are responsible for the basic organization of the cell including the localization of organelles and polarization during mitosis. Intermediate filaments are minor components of the cytoskeleton, and their function is not fully understood. Microtubules, intermediate filaments and the actin cytoskeleton all have a major role in cellular structure, integrity, and movement. There is a great deal of cross talk and physical associations between the three components of the cytoskeleton. Since NCK mainly affects the actin cytoskeleton, I will focus the remainder of my introduction on the actin cytoskeleton.

1.2.2 Actin

Actin is a highly dynamic structure and consists of soluble actin monomers (g-actin) that can polymerize with other actin monomers to form filamentous actin (f-actin). Actin polymerizes linearly, with new actin monomers added in the same orientation as previous ones. This gives rise to filamentous actin having a plus end and a minus end. At the plus end, new monomeric actin is continuously added, while at the minus end, actin monomers are continuously removed through a process known as catastrophe. This polarization of actin allows regulatory proteins to recognize the leading end of actin from the trailing end. Actin regulatory proteins can either enhance actin polymerization or regulate the catastrophe of actin filament. These events in turn regulate the ability of a cell to become more or less motile. Thus, actin is a highly controlled component of the cytoskeleton and can be regulated in a number of different ways.

1.2.3 Actin Catastrophe

One way actin filaments are controlled is through the regulation of their catastrophe. Cofilin is a regulatory protein that binds to the minus end of f-actin. Upon binding, cofilin induces strain in polymerized actin causing actin monomers to unhinge from their binding partners through the process of catastrophe. Catastrophe of actin is an important process in the regulation of cell motility since it leads to an increase in the stores of monomeric actin that can be used for polymerization at the leading end. Thus, intracellular signaling cascades that affect the interaction of cofilin with actin will regulate cellular motility. For example, activation of LIM Kinase (LIMK) leads to the phosphorylation of serine 3 in cofilin that decreases the ability of cofilin to bind f-actin

(Moriyama et al., 1996; Agnew et al., 1995). Thus, inactivation of cofilin by phosphorylation stabilizes actin filaments allowing for greater extension and protrusion at the leading edge of a cell. Molecules that regulate the phosphorylation of cofilin thereby indirectly regulate actin dynamics.

1.2.4 Actin Related Proteins (ARP)

Another way to regulate the structure of actin is through addition of new branches of f-actin. Branching leads to increased complexity of the actin network and is required for lamellipodia formation that maintains directional cell migration (Chharba et al., 2007). The regulation of actin complexity can be controlled by a number of different branch nucleating proteins. For example, Wiskott-Aldrich syndrome protein (WASP) and Actin Related Protein 2/3 (ARP2/3) are two regulators that together are able to nucleate new actin filament branches (Chharba et al., 2007).

The ARP2/3 complex consists of seven proteins: two actin related proteins (ARP2 and ARP3) and five smaller subunits (ARPC1-5). The ARP2/3 complex can either nucleate the formation of new actin filaments or cross-link newly formed filaments into Y-branches (Welch et al., 2002; Mullins et al., 1998). ARP2 and ARP3 mimic the structure of f-actin and when associated with ARPC 1-5, bind directly to f-actin. The process of actin nucleation by the ARP2/3 complex involves ATP hydrolysis by the ARP2 subunit and activation by a member of the WASP family (Dayel et al., 2004). When activated, the ARP2/3 complex creates new f-actin filaments at a 70° angle from the preformed filaments (Mullins et al., 1998). This “dendritic model” of ARP2/3 activity outlines a

critical role for the ARP2/3 complex in lamellipodia formation in migrating cells since the lamellipodia contains highly branched actin (Bailly et al., 2001).

1.2.5 Wiskott-Aldridge Syndrome Protein (WASP)

Another regulator of actin polymerization is the WASP family of proteins. WASP and Neuronal WASP (N-WASP) are known as actin nucleation promoting factors (NPFs). WASP is able to bind monomeric actin and the ARP2/3 complex through its C-terminal Verpolin homology, Cofilin homology, Acidic region (VCA) domain. Binding of the VCA to ARP2/3 causes a conformational change and delivers the first actin monomer to the daughter filament (Padrick et al., 2011). When WASP binds ARP2/3 it creates a nucleation core for the addition of new actin filaments. This nucleation core recruits two g-actin monomers and a new actin filament at 70° from the side of another actin filament. In this way, the creation of new actin filaments introduces more complexity to the actin network and supports the membrane structure at the leading edge.

1.2.6 NCK Regulates WASP

Regulatory proteins such as ARP2/3 and cofilin control actin filaments, but upstream signaling networks also control these regulatory proteins. Many of NCK's binding partners are involved in the actin regulatory network and NCK plays an essential role in activating actin polymerization through multiple distinct pathways. Two of these pathways are described below.

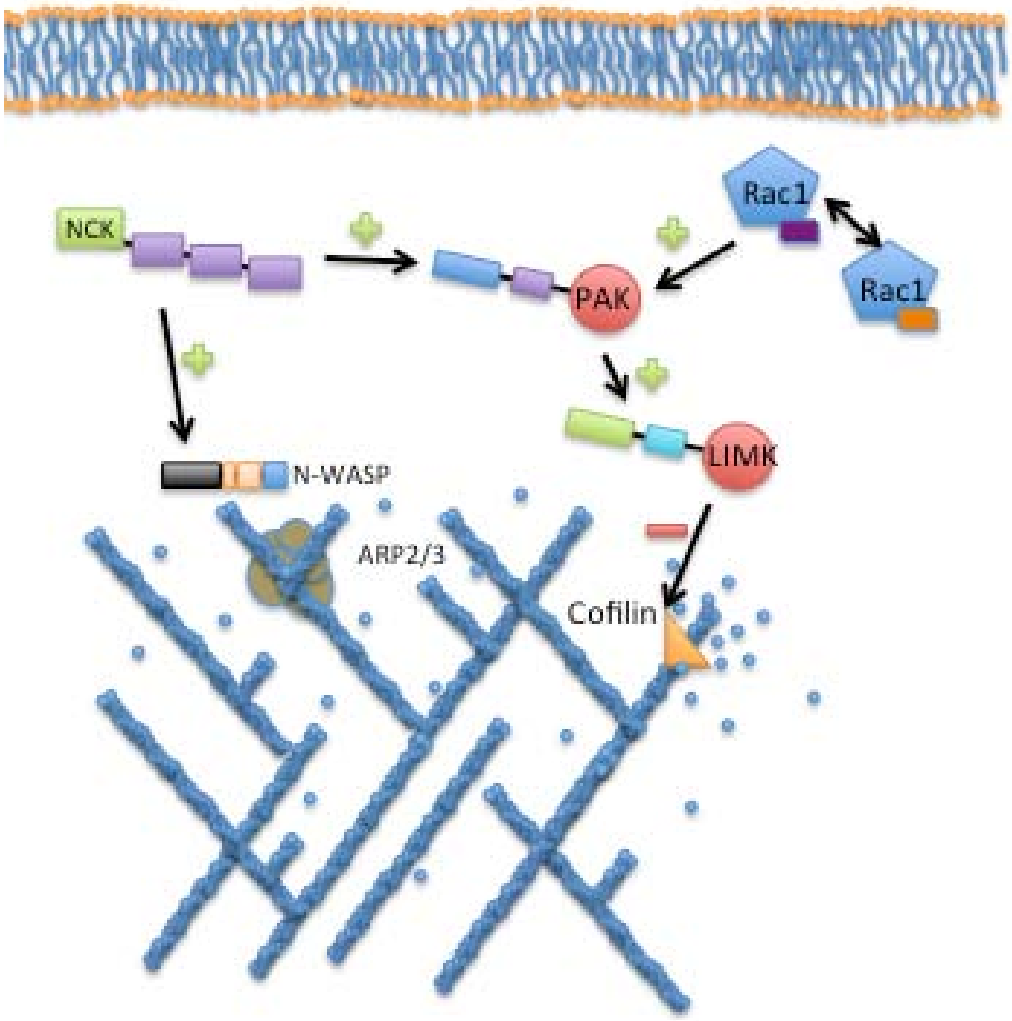

Figure 1.3: Mechanism of Actin Regulation by NCK. NCK activates downstream PAK activity including LIMK and Cofilin to stabilize actin filaments. NCK also activated N-WASP and ARP2/3 by a distinct mechanism to increase actin complexity by introducing new actin branches.

NCK is able to bind WASP at its proline rich region by the SH3 domains of NCK (Rivero-Lezcano et al., 1995). The binding of NCK to WASP activates the WASP and ARP2/3 nucleation core to create new actin filament branches and actin protrusions at the membrane. The mechanism by which NCK activates WASP has not been fully elucidated, as NCK binds both directly to WASP and through the common WASP Interacting Protein (WIP; Rohatgi et al., 2001). Nonetheless, NCK can recruit the WASP complex to NCK clusters at the membrane. These NCK clusters are also associated with tyrosine phosphorylated proteins, and actin polymerization (Rivera et al., 2009). Furthermore, quantitative and computational experiments that have proposed the possible ratio of NCK:WASP:ARP2/3 at 4:2:1 in the activation of actin polymerization, outlining the potency of this signaling pathway (Ditlev et al., 2012).

Taken together, these molecular experiments support a role for NCK in activating WASP and ARP2/3 to create new actin filament branches and increase the complexity of the actin network.

1.2.7 NCK Regulates PAK

In addition to NCK associating with proteins that directly bind actin, NCK can engage other signaling molecules that will indirectly regulate the actin cytoskeleton. For example, the second SH3 domain of NCK is able to bind directly to p21-activated kinase (PAK). Once bound, NCK is able to relocalize PAK to the membrane (Lu et al., 1997). At the membrane, PAK is activated by the Rho GTPases Rac1 and Cdc42 as well as the lipid phosphatidylinositol (4,5)-bisphosphate (Malecka et al., 2013). Once activated,

PAK in turn can phosphorylate one of its targets LIMK. LIMK is a serine kinase that can phosphorylate cofilin on serine 3 (Yang et al., 1998). As mentioned above the phosphorylation of cofilin on serine 3 reduces the affinity of cofilin for f-actin. Thus, NCK regulates PAK activity leading to actin filament stability by inhibiting the activity of cofilin.

Taken together, NCK adaptors are able to bind to important regulators of the actin cytoskeleton through their SH3 domains. Two of these pathways include the creation of new actin branches through the function of WASP and ARP2/3 and the regulation of actin catastrophe by the inactivation of cofilin through the PAK signaling pathway.

1.3 Axon Guidance

1.3.1 *Drosophila* Dock

1.3.1.1 Mutant Flies

Insight into the role of the NCK adaptor protein came from studies of the *Drosophila* NCK homologue, dreadlocks or dock (Garrity et al., 1996). Dock was originally identified in a genetic screen for novel effectors of axon guidance using the *Drosophila* photoreceptor as a model of axon connectivity. In this study, two mutants were identified that showed defects in axon fasciculation, targeting, and innervation patterning of the retina. The accumulation of axon guidance defects resembled the hairstyle dreadlocks, from which the gene obtains its name (Garrity et al., 1996). Both mutants turned out to have mutations in the same gene – dock.

The *Drosophila* dock gene encodes a protein with 44% amino acid homology to human NCK1 with 63% amino acid identity in the SH2 domain and the first SH3 domain. (Figure 1.1).

1.3.1.2 Domain Requirements

Since dock contains both SH2 and SH3 domains, the next important question was which domains were important for the defects observed in dock mutants. To examine the individual domains of dock in guidance, the Zurpinsky group developed a rescue experiment where dock was deleted from the embryo and exogenous dock was reintroduced by overexpression. In these rescue experiments, the guidance phenotype was fully rescued when wildtype dock was reintroduced, but not when some mutant dock transcripts (where specific domains are inactivated) were reintroduced (Rao et al., 1998). These studies suggested that the first and third SH3 domain and the SH2 domain are not essential for the photoreceptor guidance phenotype and that the first and third SH3 are functionally redundant with the SH2. Interestingly, wild type human *NCK1* was also able to rescue loss of dock phenotype suggesting that the role of these proteins in axon guidance was functionally conserved through evolution (Rao et al., 1998).

To determine if these domains were consistently important in all types of neurons, another region of the eye, the inner optic ganglia, was examined. Interestingly, these neurons showed a different domain requirement than those of the photoreceptor axons (Rao et al., 1998). Since the dock is an adaptor thought to connect receptors to downstream regulators, researchers concluded that dock is able to connect both receptor

tyrosine kinases and non-receptor tyrosine kinases to the actin cytoskeleton in a neuron specific manner (Rao et al., 1998). In fact, we now know that NCK is able to connect a number of different axon guidance receptors to the actin cytoskeleton through unique mechanisms.

1.3.1.3 Dock in Motoneurons

In addition to axon guidance phenotypes observed in the developing eye, others have begun to examine the role of dock in other neuronal systems including developing motoneurons (Desai et al., 1999). Using *Drosophila* embryos, Desai and colleagues demonstrated that dock was expressed in almost all regions of the developing central nervous system with highest expression in developing growth cones. They identified a specific phenotype in motor neurons where loss of dock caused a delay in synapse formation by the RP3 motoneuron, similar to that of netrin B fly mutants (Desai et al., 1999; Winberg et al., 1998). Furthermore, they identified defects in longitudinal axon guidance, however did not identify any motoneuron guidance defects in dock mutant embryos. Taken together they concluded that dock had an important role in growth cone cytoskeleton remodeling during synapse formation in motoneurons and axon guidance in interneurons in the *Drosophila* embryo (Desai et al. 1999). Dock mutants are unable to survive past their pupal stage so researchers were not able identify any behavioral phenotype from loss of dock.

1.3.1.4 Dock Interactions

Since Dock had been shown to be an important protein in axon guidance in the drosophilia and an important regulator of actin dynamics, a number of groups set out to understand how Dock was able to regulate axon guidance by looking for proteins that associated with Dock. Using yeast two hybrid dock was shown to interact with a protein tyrosine phosphatase dPTP61F and Ste20-like kinase Misshapen (Msn). Both of these proteins have similar functions in *Drosophila* R-cell axon guidance and were shown to directly bind dock and mimic the dreadlocks photoreceptor axon guidance phenotype (Ruan et al., 1999; Clemens et al., 1996). These studies in *Drosophila* were instrumental in showing that dock was an important regulatory protein connecting cell surface receptors and the actin cytoskeleton, and that this link was required for the molecular mechanism that allows neurons to precisely connect during development.

1.3.1.5 Dock and Pak

At the same time, another group was looking into the role of PAK in dock related photoreceptor (R-cell) axon guidance. It was established in vertebrates that NCK is able to interact with PAK, an important kinase in cell motility (Galisteo et al., 1996). In the *Drosophila* model system the homologues of PAK were identified as Mbt, Dpak2, and pak. Of these three homologues, pak was the only homologue shown to bind dock. *Drosophila* pak co-localized with dock in the R cell growth cone and loss-of-function mutants mirror those of dock mutants. Interestingly, membrane-bound pak is known to be constitutively active and dock is able to recruit pak to the membrane. This indicates that dock may be important for pak's activity at the membrane as previously shown (Becker

et al., 2000). Confirming this signaling pathway, the dock pak proteins were shown to be involved in the patterning of olfactory axons (Ang et al., 2003).

Similar to pak and dock phenotypes, a Guanine-Nucleotide exchange Factor (GEF) trio was able to phenocopy R-cell guidance phenotype seen both in pak and dock knock-out flies. Using genetic interactions in flies, they noticed a dose-dependent interaction in loss of function experiments between dock and trio. Newsome and colleagues propose that trio, pak, and dock act in a common pathway to control photoreceptor axon guidance during development (Newsome et al. 2000).

Taken together, these studies comprise the initial evidence for a signaling pathway in *Drosophila* retinal cells, through which neurons control and target their growth. We now know that there are many more proteins that have been implicated in axon guidance, often times intersecting pathways to create the complexity in wiring required for the developing nervous system.

The magnitude of literature implicating *Drosophila* dock in axon guidance, and the ability of human NCK to rescue this phenotype in the *Drosophila* system provides the first evidence for a biological role of these adaptor proteins. What is conserved throughout all of the studies to date on NCK, is its ability to interact with the PAK protein kinase and its ability to connect cell surface receptors to the actin cytoskeleton in a cell type, temporal, and signaling pathway specific manner. These studies have

provided the foundation for my thesis, where I aim to understand the biological significance of NCK in the context of murine axon guidance.

1.3.2 NCK Interacts with Multiple Axon Guidance Receptors

To understand the mechanisms of axon guidance and neural development, it important to understand the molecular signaling by which each receptor connects to downstream effectors. Since the discovery that the NCK homologue dock controls the development of R cell photoreceptors, NCK has been connected to multiple axon guidance receptors through *in vitro* and limited *in vivo* studies (Figure 1.3). Axon guidance receptors interact with the NCK adaptors by unique mechanisms and can bind to both SH3 and SH2 domains. What remains unknown for many of these interactions is how they are involved in vertebrate development and which neurons require the function of these receptors for their normal development. Answering these questions is the first step in understanding how higher order neural circuits develop and how they can be altered in diseased states. What follows is an outline of the current understanding of the role of NCK in axon guidance.

1.3.2.1 Down Syndrome Cell Adhesion Molecule (DSCAM)

As indicated above, the first receptor shown to interact with *Drosophila* dock was the *Drosophila* DSCAM receptor. *DSCAM* is so named due to its chromosomal location on chromosome 21 in the Down syndrome critical region. When *DSCAM* is overexpressed in the fetal nervous system it leads to Down syndrome.

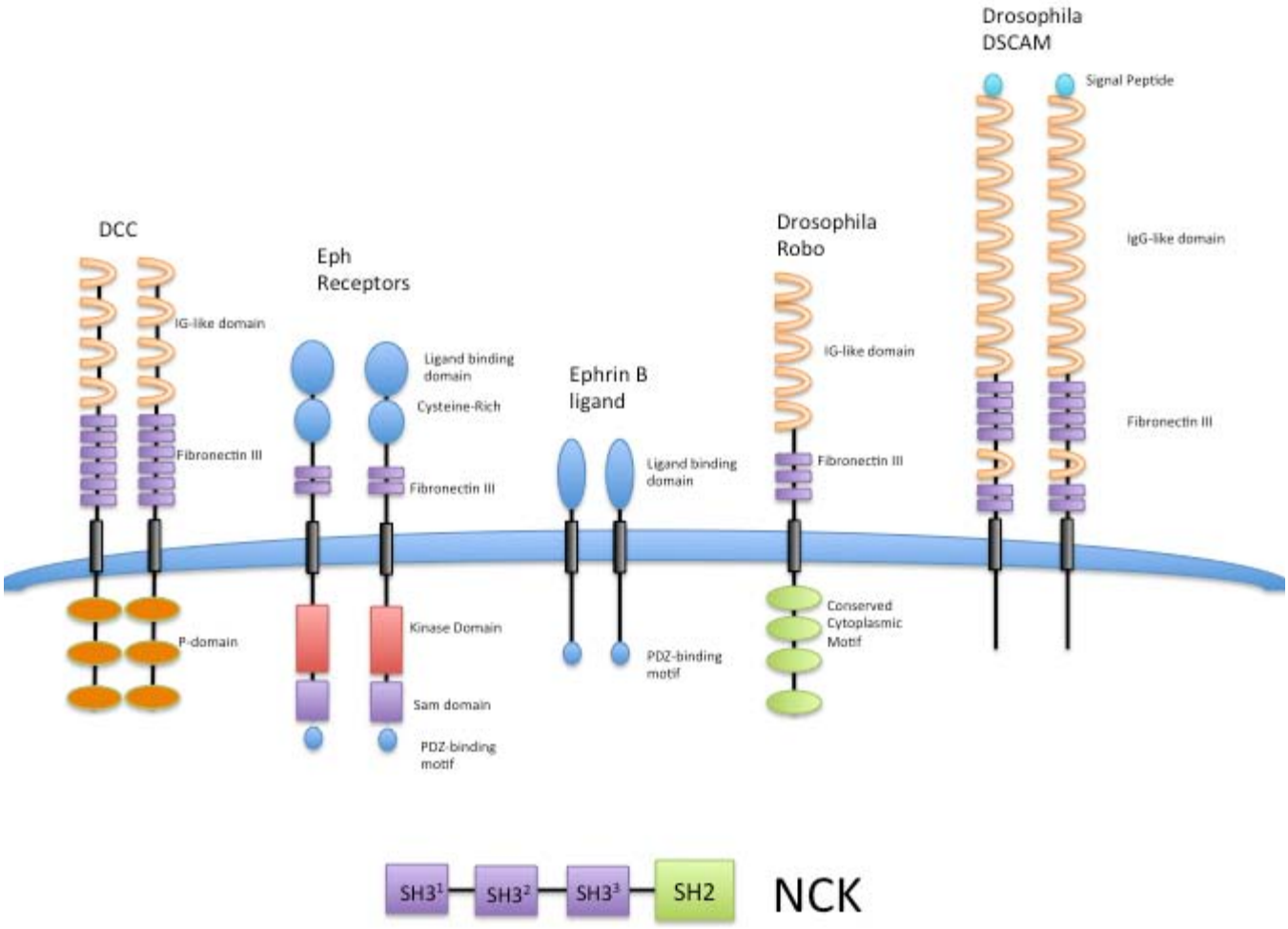

Figure 1.4: Axon Guidance Receptors Shown to Interact with NCK. Each of these guidance receptors contains unique intracellular domains that are able to interact with either the SH3 or SH2 domains of the NCK adaptor protein. This diversity of protein binding interactions has led to the idea that NCK links multiple different types of axon guidance receptors to the actin cytoskeleton**.**

Since the initial discovery that dock interacts with *Drosophila* DSCAM and that this connects DSCAM to PAK, little more has been uncovered. This is partially due to the fact that vertebrate DSCAM and *Drosophila* DSCAM do not share a similar intracellular domain (Schmucker et al., 2000). One group reported that vertebrate DSCAM was able to bind PAK by overexpression in HEK293 cells, dissimilar to that of the *Drosophila* mechanism (Li and Guan, 2004). Thus, it seems unlikely that NCK proteins function as adaptors for the DSCAM receptor in a biologically significant context. Further studies must be done to confirm that vertebrate DSCAM acts independently of NCK in a biological context.

1.3.2.2 Roundabout Receptors (Robo)

The roundabout receptor (robo) was named after the *Drosophila* mutant that had developing axons that resemble the British road junctions of the same name. This was especially apparent at the embryonic midline where axons, which cross the midline, would circle around after crossing. The ligand for the robo receptor is slit, which regulates robo activity in a ligand dependent manner.

In *Drosophila* dock was connected to robo by its ability to transduce signals to the actin cytoskeleton. Robo and dock are both expressed in the developing nervous system with regions of overlapping expression. Robo is expressed in longitudinal axons. Dock is also expressed both in longitudinal axon and in the embryonic neural commissure bundles. Using genetics to determine the interactions of robo, it was shown that dock and robo interact upon slit ligand stimulation through the first and second SH3 domain of dock and the intracellular proline rich regions of the CC2 and CC3 robo receptor. This type of

interaction was relatively new for dock, as it is usually connected through its SH2 cell surface receptors. Yet, conserved in this mechanism is the ability of dock to recruit pak to the signaling complex activates RAC1 activity (Fan et al., 2003).

Long and colleagues proposed an alternate mechanism of downstream signaling from the robo receptor. They agreed that dock is able to bind the robo receptor, but in their study they focused on the interaction of dock with downstream sos guanine nucleotide exchange factor (GEF). Sos is able to form a ternary complex with dock and robo which activates downstream cytoskeletal proteins (Yang et al. 2006).

Taken together these studies establish dock as an important intracellular adaptor for the robo receptor in mediating axon guidance in the *Drosophila*. What has yet to be discovered is the significance of these interactions in a mammalian context.

1.3.2.3 Ephrin B Type

Ephrin B1-3's are the ligands for eph receptors found on opposing cell and mediate bidirectional signaling via direct cell-cell interactions. This interaction is especially notable at the embryonic midline where eph-ephrin signaling regulates midline crossing.

NCK2, but not NCK1 has been connected to the Ephrin B receptors through its interaction with the critical pY residue 298, or pY 304 for Ephrin B1 or Ephrin B2 respectively. (Bong et al., 2004) NCK2 connects Ephrin B's to the intracellular actin regulators through its SH3 domains (Cowan et al., 2001). Using transgenic mice for

Ephrin B3, NCK2 was shown to mediate axon pruning in hippocampal neurons by regulating signaling downstream of Ephrin B3. In this context the 2nd SH3 domain on NCK2 connects Ephrin B3 to the downstream regulators dock180 and PAK. (Xu et al., 2009, Xu et al., 2011) It would be interesting to determine if NCK2 and Ephrin B3 have any other biologically relevant roles during development, and also if they are connected to any cases of human neurological disorders.

1.3.2.4 Eph Receptors

Binding their ephrin ligands activates Eph receptors. These receptors compose the largest family of receptor tyrosine kinases. As above, Eph receptors are critical for axon guidance processes relating to midline boundaries. These receptors are divided into two main classes based on sequence homology EphA's and EphB's. In total 16 Eph receptors have been identified in humans, reflecting the diversity of axon guidance events controlled by Eph receptors.

NCK was shown to interact with EphB1/2 and EphA2/3/4 in a similar way, by binding intracellular phosphotyrosine residues on the intracellular domain of Eph receptors. Eph receptors are receptor tyrosine kinases and are capable of autophosphorylation of their intracellular domain. (Holland et al., 1997, Stein et al., 1998; Hock et al., 1998; Bisson et al., 2007; Mohamed et al, 2012) The biological relevance of this binding was nicely outlined in a landmark paper by Fawcett and colleagues where NCK was shown to be important in the development of EphA4 corticospinal tract neurons (Fawcett et al., 2007). This study made use of a genetically modified mouse model discussed in detail below to

study the effect of the loss of NCK. This model displayed similarities to the EphA4 loss of function phenotype where the descending corticospinal tract neurons aberrantly recross the midline. To date this study provides the clearest evidence for a biologically important role for NCK proteins in axon guidance. What was still unclear from this study was the contribution of local spinal cord circuitry to the development of the hopping phenotype observed in NCK-deficient mice

1.3.5 Deleted In Colorectal Cancer

1.3.2.5.1 DCC Axon Guidance Receptor

The Deleted in Colorectal Cancer (DCC) protein was originally named after it was pulled from a screen of colorectal cancers (Fearon et al., 1990). Since then it has held a controversial role in cancer progression, but has established itself as one of the most studied guidance receptors in a mammalian context.

DCC is a receptor that is found in the growth cone of developing neurons. The ligand for DCC is the netrin molecule. The interaction of netrin with the receptor DCC causes an expansion of the growth cone and targets the axon toward increasing netrin gradients. In this way netrin can attract developing axons through its interaction with the DCC receptor.

In the body, specific cells secrete netrin in order to create gradients of netrin during development. These signaling gradients guide axons to their appropriate targets through

the process of axon guidance. Netrin can act as a guidance cue and a growth cue through its action on the DCC receptor (Bashaw et al., 2010).

Through this process, along with other axon guidance cues, the precise patterning of neural circuits is possible during development. What remained relatively unknown until recently are the connections between the DCC receptor and the downstream signaling components that activate changes in the actin cytoskeleton.

1.3.2.5.2 DCC Signaling

In search of the downstream targets of the DCC receptor, the Kennedy group found that NCK1 was able to bind DCC through its first and third SH3 domain in a GST-binding assay. They demonstrated that using a dominant negative NCK1, RAC1 activity was reduced, indicating that NCK1 was a functional adaptor for DCC (Li et al., 2002). DCC signaling was more clearly explained in a follow-up paper where NCK1 was connected to PAK and that this interaction was required for the RAC1 activity induced by DCC receptor activation (Shekarabi et al., 2005).

At the same time, two other groups showed that netrin activation of DCC induced FAK kinase and DCC phosphorylation, and that these steps were required for the activation of the DCC receptors. In these studies SRC and FYN kinases were also shown complex with DCC (Ren et al., 2004; Li et al., 2004). Another group outlined that FYN kinase, but not SRC kinase is responsible for phosphorylation of the intracellular domain of DCC (Meraine et al., 2004). More specifically, pY1418 on the intracellular domain of DCC,

near the P3 domain, was required for the phosphorylation of DCC. The pY1418 was a critical residue for all future phosphorylation of DCC and thus its signaling effects (Meriane et al., 2004). Interestingly, the P3 is important for dimerization of the receptor, however no direct connection has been made between phosphorylation and dimerization (Stein et al., 2001).

Upon netrin binding, an intracellular complex including PAK kinase, TRIO GEF, and NCK is recruited to DCC. In this context NCK is believed to act to bridge the interaction of TRIO to DCC, or possibly also through PAK (Briancon-Marjollet et al., 2008). Interestingly, DCC is also able to recruit CDC42, RAC1, and WASP upon ligand stimulation. Considering the constitutive interaction of NCK and DCC, and the number of FYN binding partners in this activated complex, it was proposed that NCK acts to bridge PAK and WASP complexes to the intracellular domain of DCC (Shekarabi et al., 2005).

Alongside this study, p130cas was shown to be required for netrin dependent signaling in DCC neurons, although it is not clear how p130cas fits in with the current molecular mechanism (Qu et al., 2013). A full description of DCC signaling is found in figure 1.5.

1.3.2.5.3 Future Directions of DCC Signaling

A number of questions have yet to be answered with regards to the activation of these factors. For example, how does FAK kinases become phosphorylated and activated upon ligand stimulation? Which of the components implicated in DCC signaling are required

and which are bystanders? What distinct signaling cascades are produced from DCC activation and in what context are these important? And finally, what is the role of NCK in the recruitment of PAK and WASP to the intracellular domain of DCC?

At this point, we may speculate as to the mechanistic role of NCK in DCC related signaling. A recent study on the role of NCK in nephrin receptor, the kidney guidance receptor, activation described a mechanism where NCK was required and sufficient to recruit FYN kinase to the intracellular domain of the nephrin. (New et al., 2013) Considering that FYN is an essential component of DCC activation it may be that NCK is also acting to recruit NCK to the intracellular domain of DCC leading to complex formation. From a NCK-centric point of view, the best way to test NCK's relationship with the DCC signaling complex is to use a genetic knock-out model of NCK1 and NCK2 and look at the function of DCC signaling.

1.3.2.5.4 Clinical Relevance of DCC

Congenital mirror movement disorder is a neurological disorder that is characterized by a voluntary movement on one side of the body leading to involuntary movement of the opposite side of the body. For example, a patient presenting with mirror movement disorder may have twitching in his left hand when he writes with his right hand. Using genetic sequence analysis a group of patients with mirror movement were shown to have a similar mutation in the gene encoding the DCC receptor. This mutation was in the extracellular domain of DCC and the function of the mutation was to abolish netrin's ability to bind to DCC (Srour et al., 2010).

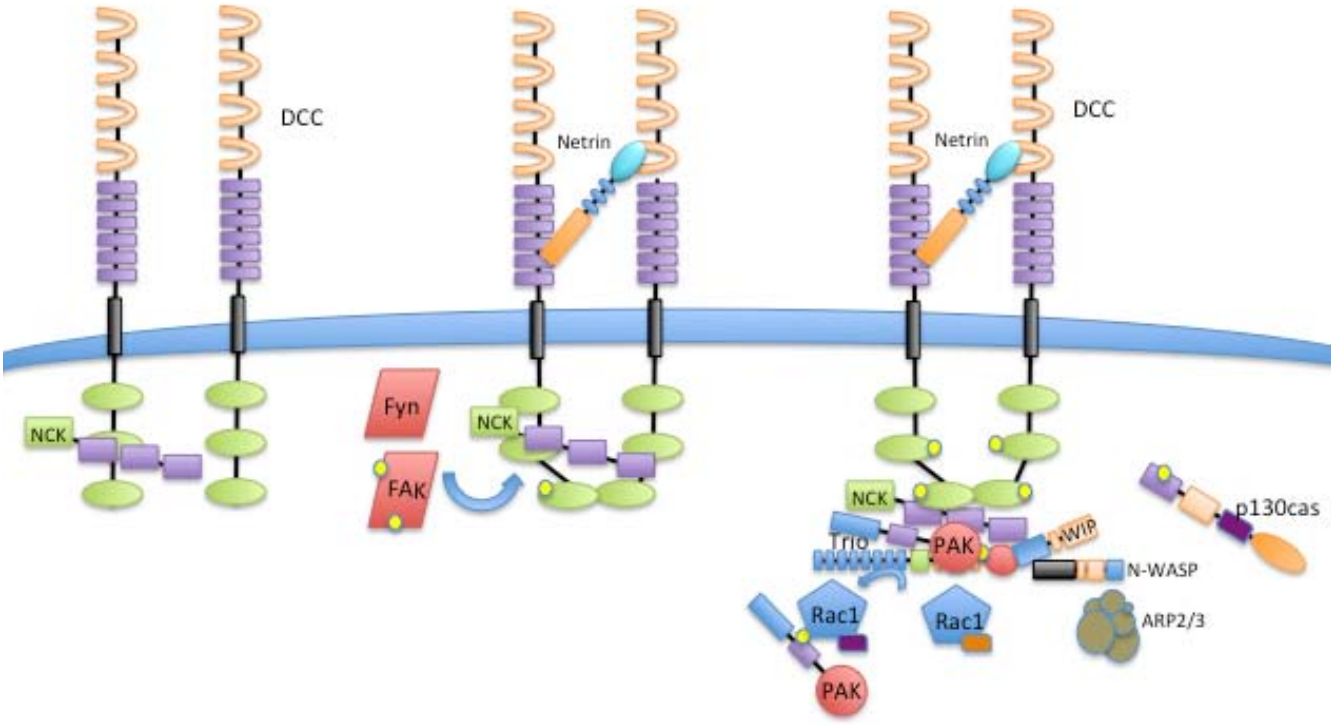

Figure 1.5: Summary of the Signaling Mechanism of DCC. NCK is constitutively bound to the intracellular domain of DCC by its SH3 domains. Netrin ligand binding leads to activation of both FYN and FAK kinases. These kinases are both able to phosphorylate DCC and induce dimerization of the intracellular domain. One activated, DCC is able to recruit a complex of signaling proteins including NCK, PAK, WIP, p130cas, N-WASP, ARP2/3, and Rac1.

One can speculate that loss of netrin's ability to bind DCC would lead to improper development of DCC sensitive circuits in the human nervous system. This axon guidance defect would manifest itself through the inappropriate activation of circuits controlling movement. A clear understanding of any neurological disorder is important when formulating options for treatment. Thus, it is critical to understand the downstream effects of DCC activation to better understand the disorders associated with its mutation.

1.4 NCK Mouse Models

Since the creation of the first transgenic mouse model in 1974, mice have quickly become the model of choice for studying disease progression and gene function (Rudolf et al., 1974). The ability to alter the genetic code of an entire model organism has allowed researchers to gain biologically significant access to the study of protein function. Genetically altered model organisms circumvent one of the biggest problems in proteomics research, that is the potentially hundreds of protein isoforms that can be produced from a single gene transcript. Since the initial discovery of mouse transgenic manipulation using a virus, the field of mouse genetics has expanded considerably to include transgenic, knock-in, knock-out, and conditional gene modifications.

1.4.1 NCK Knockout Mouse

Much of the work on the NCK adaptor proteins has focused on elucidating *in vitro* binding partners and signaling pathways. What has become clear from these studies is that many of the binding partners for NCK are involved in actin regulation including

WASP which acts to activate ARP2/3 mediated actin branching and PAK which acts on downstream effectors of actin catastrophe (Eden et al., 2002, Rohatgi et al., 2001).

In order to better study the function of vertebrate *NCK1* and *NCK2,* two strains of knock-out mice were generated. These knock-out mice have β-Galactosidase reporters incorporated into the *NCK1* or *NCK2* loci (Bladt et al., 2003). In this study, Bladt and colleagues first examined the expression pattern of *NCK1* and *NCK2* by knocking out either gene and examining adult tissue extracts by western blot for the levels of the other gene that was not knocked-out. They found that both *NCK1* and *NCK2* were widely expressed during adulthood, during which *NCK1* had a fairly even distribution throughout the body while *NCK2* was found to have a varying expression with particularly high expression levels in the thymus. This indicated that *NCK1* and *NCK2* have mostly overlapping roles, but may also possess some unique biological function. When a *NCK1* knock-out strain or a *NCK2* knock-out strain was created, no gross functional or morphological abnormalities were noted. This indicated as previously supposed, that NCK1 and NCK2 might be functionally redundant and that they could compensate for each other's loss (Bladt et al., 2003).

To uncover the *in vivo* function of NCK, $NCK1^{+/LacZ}$ was crossed with $NCK2^{+/-}$ to create $NCK1^{LacZ/LacZ}$;$NCK2^{-/-}$ mice. In the absence of any functional NCK, this genotype was embryonic lethal at embryonic day 9.5 (E9.5) due to three main defects: inability to undergo the turning stage, defective chorion-allantoic fusion, and impaired closure of the cephalic neural folds. The combination of defects led researchers to conclude NCK was

required for generation of mesoderm structures and was essential for life (Bladt et al., 2003).

Using the $NCK1^{LacZ}$ and $NCK2^{LacZ}$ reporters, this group next examined the expression of the two genes during the embryonic developmental stages E8.5 and E10.5. As in the adult mice, they found a strongly overlapping expression pattern, and the wide anatomical distribution of each gene included the ganglia, stomach, gut, arteries, and somites that matched the defects observed in NCK knock-out embryos (Bladt et al., 2003).

When comparing other knock-out mice phenotypes, $NCK1^{-/-}$;$NCK2^{-/-}$ have similarity to $CSK^{-/-}$, $NIK^{-/-}$, $WASP^{-/-}$ and FAK -/- (Imamoto et al., 1993, Furuta et al., 1995, Xue et al. 2001, Snapper et al., 2001). On the protein level all of these proteins are binding partners of NCK, intractably involved in similar signaling pathways. Interestingly, both FAK and WASP have been implicated in DCC regulated axon guidance.

To confirm that NCK was required for normal actin structures, platinum electron microscopy was performed on either $NCK1^{+/-}$ $NCK2^{+/-}$ or $NCK1^{-/-}$ $NCK2^{-/-}$ mouse embryonic fibroblasts (MEFs). $NCK1^{-/-}$ $NCK2^{-/-}$ knock-out MEFS show deficiencies in their lamellipodia compared to control and this can be partially rescued by reintroducing exogenous NCK1. Thus, NCK has been shown to be a critical signaling partner for the maintenance of the actin cytoskeleton (Bladt et al., 2003).

Taken together, this study by Bladt and colleagues highlights the physiological significance of the mammalian NCK proteins during embryonic development. It solidifies many of the *in vitro* studies that have implicated NCK in actin dynamics and the embryonic lethal phenotype of the double knock-out highlights the physiological requirement of the NCK proteins in vertebrate development. However, this study is not able to address the potential contribution of NCK during organogenesis and fails to outline a continued role for NCK in mature animals (Bladt et al., 2003).

In order to address the contribution of NCK to individual tissues, it is necessary to create conditional deficient mice where NCK is conditionally deleted. Building on the knowledge that NCK1-/- or NCK2-/- mice have no gross physiological defects, it is only necessary to conditionally mutant one of the genes.

1.4.2 NCK Conditionally Deficient Mouse

To address the contribution of NCK proteins to the development of individual tissues, a conditional genetics approach using the Cre-lox system was employed to specifically target the *NCK2* gene. One advantage of this approach is the ability to combine $NCK2^{flx/flx}$ mice with mice knocked-in for multiple tissue specific cre-promotors. This allows the promoter specific deletion of *NCK2* and therefore the genetic loss of NCK proteins in an NCK1-/- background. As outlined above, NCK adaptors are able to link a number of axon guidance receptors to the actin cytoskeleton. Thus, it was logical to test the function of NCK-deficiency in the mouse nervous system.

By combining the promoter region of *nestin* with a knock-in for cre-recombinase a system is created where *NCK2* is deleted in the time and place that *nestin* becomes active. Nestin is an intermediate filament protein produced by the developing nervous system and is used as a marker for neural stem cells (Dahlstrand et al., 1995). In mice, *nestin* expression turns on around E8.5 and is expressed throughout the nervous system, when combined with lox-p flanked coding region of *NCK2*, this allows for the selective deletion of *NCK2* from the nervous system (Dubois, 2006; Tronche et al., 1999). Thus, to examine the function of NCK proteins in the nervous system, NCK-deficient mice were created.

$NCK1^{-/-}$;$NCK2^{flx/flx}$;Nestin-Cre (NCK-deficient) animal were viable near the Mendelian frequency and survived into adulthood, but had defects in gait patterning, termed "hopping" (Fawcett et al. 2007). These "hopping" mice had a synchronous gait characterized by simultaneous movement of the left and right fore or hind limbs (Figure 1.6). Gait defects are indicative of a fundamental "mis-wiring" within the local lumbar spinal cord circuits. The correct patterning of the nervous system is dependent on the process of axon guidance. NCK is able to connect a number of different axon guidance receptors to the actin cytoskeleton, thus it is likely the inability of these axon guidance receptors to transduce their signals that led to the inappropriate development of the nervous system in the NCK-deficient mice. The next question was which neurons were affected by NCK-deficiency and by which mechanism NCK was acting. Pursuing this

avenue required the systematic examination of circuits in the nervous system that are known to be effected by the loss or malfunction of axon guidance receptors.

A clue toward the nature of the axon guidance receptor(s) comes from the hopping phenotype itself. To date a number of mice have been reported with similar phenotypes including the EphA4 and Ephrin-B3 mutant mice (Kullander et al., 2003). Ephrin-B3 and EphA4 proteins create a signaling barrier at the embryonic spinal cord midline where descending contralateral corticospinal tract (CST) neurons are not able to re-cross the midline. CST neurons normally descend from the motor cortex of the brain and activate interneuron and motor neurons in the lumbar region of the spinal cord. CST neurons are mostly contralateral and cross the midline at the point of decussation. In either EphA4 mice or Ephrin-B3 mice this midline barrier is no longer functioning and neurons are able to aberrantly re-cross the midline. The functional consequence of aberrant CST axons is the disorganization between the left and right side of the spinal cord. Since NCK proteins have been shown to bind to Ephrin-B and EphA4 receptors it raised the possibility that these receptors were unable to transduce their signals in the absence of NCK.

Examination of the corticospinal tract (CST) in NCK-deficient mice shows axon guidance defects in the CST similar to that of Ephrin B3 or EphA4 mutant mice. In fact the dorsal faniculus, a concentrated region of CST neurons, is reduced in size in the NCK-deficient mice. Yet, loss of NCK had no effect on the localization of Ephrin-B3 or

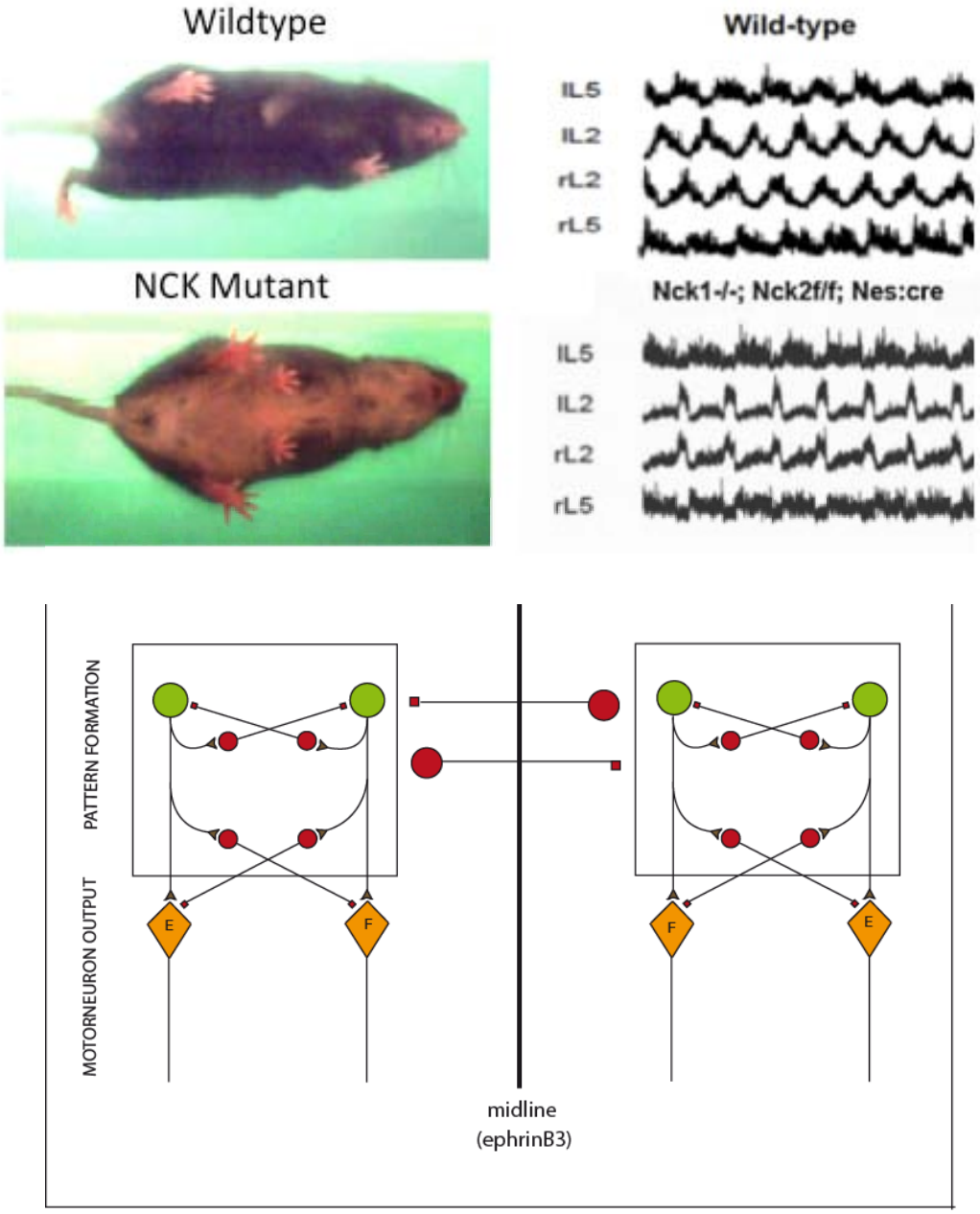

Figure 1.6: NCK-Deficient Mice have a Hopping Phenotype. Wildtype mice have alternating gait pattern, while NCK-deficient mice have synchronous gait. This is due to a problem in the local spinal cord circuitry shown in electrophysiological experiments where the rL2 and lL2 have synchronous firing (Adapted from Fawcett et al. 2007). A circuit diagram of the local spinal cord circuitry identifies the commissural neurons as responsible for cross-repressive interactions with the left and right sides of the spinal cord.

he total expression of EphA4 (Fawcett et al., 2007). Together these results outline a role for NCK in EphA4-EphrinB3 regulated axon guidance in the mouse nervous system.

1.4.3 NCK in the Spinal Cord

In pursuit of the underlying mechanism of the hopping phenotype, it was noticed that NCK-deficient mice have an altered gait at neonatal stages, before CST neurons are able to innervate the lumbar spinal cord. Electrophysiological experiment on neonatal mice revealed that indeed these NCK-deficient pups had electrophysiological synchrony between the left and right L2 segments of the spinal cord, but did not possess any ipsilateral electrophysiological defects between L2 and L5 (Fawcett et al., 2007; Figure 1.6; unpublished data). Since CST axons have not yet developed to the lumbar spinal cord at this stage, this raised the possibility that lumbar spinal cord circuits were "miswired" leading to disorganization in the local circuitry that controls movement.

EphA4 is also expressed in local spinal cord interneurons and is thought to be mostly excitatory in nature, thus it is possible that aberrant crossing of the midline of interneurons in the lumbar region led to synchrony as in the EphA4 mutant mice (Butt et al., 2005). To determine the contribution of aberrant crossing of excitatory neurons, NCK-deficient spinal cord preparations were treated with sacrosine, a glycine uptake inhibitor. Treatment with sacrosine restores normal ventral root activity in Ephrin-B3 and EphA4 mutant mice, but was unable to completely restore NCK-deficient spinal cords to alternating gate (Rabe et al., 2003; Fawcett et al., 2007). A potential explanation to the differences observed between EphA4 and EphrinB3 mice, and NCK-deficient mice is the

presence of additional defects in the NCK-deficient mice not present in the EphA4 or Ephrin-B3 mice.

NCK proteins are known to couple multiple axon guidance receptors in vitro including the DCC receptor. Furthermore, DCC is known to guide commissural neurons that are both inhibitory and excitatory in nature. Since sacrosine is not able to fully restore the walking pattern in NCK-deficient mice, additional defects are likely in inhibitory commissural neurons. Thus, a loss of inhibitory neurons would explain the NCK-deficient phenotype. NCK-deficient mice also show defects in the anterior commissure, thought to be important in pain sensation. Interestingly, both DCC and the DCC ligand netrin mutant mice, and an intracellular signaling GEF Trio mutant mouse model, show defects in this region of the brain (Briancon-Marjollet et al., 2008; Fazeli at el., 1997; Serafini et al, 1996).

The ligand for the DCC receptor is the netrin signaling molecule. In the developing spinal cord netrin is secreted from the floor plate cells and creates a gradient toward which DCC axons are sensitive. These DCC axons are able to target toward the floor plate where they cross the midline at the ventral commissure and extend rostral up the spinal cord. Building the case for a genetic interaction between NCK and DCC, mutant mice for either netrin or DCC exhibit similar disorganization in the local lumbar spinal circuitry that control movement (Rabe et al., 2009; Bernhardt et al., 2012). In either DCC or netrin mutant mice, commissural neurons are unable to appropriately target toward the floor plate and cross the midline leading to a loss of communication between the left and right

side of the spinal cord (Matsumoto et al., 2007). Taken together the evidence that NCK may be acting in DCC sensitive pathways warrants investigation.

1.5 Rationale and Specific Objectives

Studies on the *in vitro* function of the NCK adaptor proteins have outlined their role as adaptor proteins that connect cell surface receptors to the actin cytoskeleton. The process of axon guidance during nervous system development makes use of such cell signaling processes when guidance molecular directs the development toward a target source. NCK-deficient mice have abnormal gait due to improper development of the spinal cord circuits important for walking. Mistargeted or lost neurons lead to a synchronous gait in NCK-deficient mice compared to control. Gait defects are observed before the development of corticospinal tract neurons, indicating there is a "miswiring" of the local spinal cord circuitry. DCC and netrin mutant mice have similar gait defects, thus are good potential targets for an axon guidance pathway by which the NCK proteins function. Thus, since NCK proteins are still poorly understood in development of the nervous system, we establish the following hypothesis and objectives:

Hypothesis*: NCK Functions in a DCC Sensitive Pathway to Control the Development of Spinal Cord Circuits by Regulating the Actin Cytoskeleton.*

Objectives:

1) Establish the distribution of NCK proteins in the developing embryo and spinal cord

2) Define the role of NCK proteins in global processes of axon guidance

3) Examine the role of NCK proteins in pathways known to be sensitive to DCC signaling

CHAPTER 2 MATERIALS AND METHODS

2.1 Reagents

All reagents were purchased from Sigma Aldrich unless otherwise noted. Cell culture reagents were purchased from Wisnet. Antibodies reagents are described in 2.4

2.2 Constructs

Full length human DCC was purchased from Addgene (plasmid #16459). cDNA for NCK1, and NCK2 were obtained from lab stock.

2.3 Mice Genotyping

The generation of Nestin-Cre NCK1$^{-/-}$; NCK2$^{flx/flx}$ mice has been previously described (Fawcett et al., 2007). For genotyping mouse embryos (E10 – E14), the embryos were dissected and the embryonic sac was separated from the placenta. The embryonic sacs were then frozen at -20^{o}C or processed immediately. To isolate DNA 600μL to 1mL of Lysis solution (0.1M Tris pH 8.0, 5mM EDTA, 0.2% SDS, and 0.2M NaCl) containing 100 μg/mL Proteinase K (New England Biolabs, #P8102S) was added to each sac digested at 55^{o}C overnight with constant agitation. The following day digested samples were centrifuged at 20,000xg for 10 minutes, and the supernatant was transferred to a new Eppendorff tube. DNA was precipitated by adding an equal volume of isopropanol (Fischer, #bp2618) mixed and centrifuged for 10 minutes at 13,000 rpm. The resulting pellet was washed with 70% ethanol, air-dried and suspended in 100ul MilliQ water. PCR was preformed at standard conditions for 30 cycles using the following primers: Cre 5'-GTTATAAGCAATCCCCAGAAATG; reverse, 5-

GGCAGTAAAAACTATCCAGACA; floxed *NCK2* 5′-GGATACCACATTGGCATTAGTAG; reverse, 5′-GTGCTCATTTGACAAGTGACAC; NCK1-Ires 5-GCATGTAGACAATTACACTTCAGCACC-, 5′-ATTCATGGAATTTCGAACTCGCCACC, 5′-CTGATTGAAGCAGAAGCCTGC GATG, and 5′-TATTGGCTTCATCCACCACATACAGG. (Bladt et al., 2003; Fawcett et al., 2007)

2.4 Antibodies

TAG1 (4D7) antibody and neurofilament (3A10) were purchased from Developmental Studies Hybridoma Bank and used at 1:20 or 1:100 dilution respectively for immunohistochemistry. Anti-myc (sc-40) was purchased from Santa Cruz and used at 1:1,000 dilution. Anti-HA antibody was purchased from Cell Signaling Technology and used at 1;5,000. DCC (A-20) was purchased from Santa Cruz and used at 1:1,000 for western blot while anti-human DCC (1:1,000) was purchased from Millipore was used for immunohistochemistry. Anti-β-Galactosidase was a generous donation from Dr. Kishore Pasumarthi and used at 1:1,000. Anti-phosphotyrosine 4G10 was from purified hybridoma cell line lab stock and used at 1:1,000 dilution.

2.5 Cell Culture And Transfections

Human Embryonic Kidney (HEK) 293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Wisent Cat # 319-005-CL) with 10% heat inactivated fetal bovine serum (FBS) (Wisent Cat# 090150, Lot #112662) without antibiotics. For transfections, cells were plated on 10cm dishes and allowed to reach a density ~70%.

Cells were transfected by mixing 30μl polyethyleneimine (PEI) transfection reagent with 15μl DNA at 2:1 ratio in 1000ml DMEM, vortexed, left or 5 minutes, then added dropwise to cells in serum-containing media.

2.6 Immunoprecipitation

Following appropriate transfection conditions, cultured HEK 293T cells were washed twice with PBS (Wisent; Cat # 311-010-CL) followed by lysis in 500μl of NP-40 lysis buffer containing 20mM tris pH 8.0, 37.5mM NaCl, 10% glycerol, 1% NP-40 with fresh (1x) complete protease inhibitor cocktail (Roche). Cells were scraped, collected, and transferred to eppindorff tubes. Lysate was cleared of nuclei and debris by centrifugation at 13,000 rpm for 10 minutes at 4^{o}C. Cleared lysates were then incubated with 2μg anti-DCC antibody (A20) overnight at 4^{o}C with constant agitation. The following day, 20μl of a 10% Protein A beads slurry was added to the mixture and further agitated for 2 hours at 4^{o}C. The beads were then washed by spinning the mixture at 3,000 rpm for 4 minutes, removing the supernatant and adding 1 ml fresh lysis buffer. This was repeated three times. Following the final wash, the sample was spun, the supernatant removed, and 35ml of 2X SDS samples buffer was added. Samples were eluted from beads by boiling at 100^{o}C for five minutes prior to loading and running through SDS-PAGE gels.

2.7 Commissural Spinal Cord Neuron Culture

Commissural neurons were isolated from E13.5 mouse embryos in a protocol modified from Langlois et al., 2007. Briefly E13.5 pregnancy-staged mice (E0.5 = first day following mating day) were euthanized using 0.2mL euthanol overdose injection

according to Dalhousie Animal Care guidelines. Uterine horns were isolated and placed in ice-cold L-15 media supplemented with 10% horse serum. Each individual embryo was isolated, and the embryonic sac from each animal was saved for genotyping. To dissect the embryonic spinal cord, heads were removed and the remaining body was placed dorsal side up on agar dissection plates containing ice-cold L-15 media. Spinal cords were dissected by tearing along either side of the spinal column and open-book preparation was used to isolate the spinal cord strips dorsal to the dorsal root ganglion. The dorsal embryonic spinal cord tissue preparations were kept on ice until all embryos were dissected. Following dissection, spinal cord preparation were centrifuged at 300xg for 5 minutes and washed 2 times with warm Hanks Balanced Salt Solution (HBSS; Invitrogen Cat #14175). Spinal preparation was then incubated with 0.25% trypsin containing HBSS for 7 minutes at 37°C and washed twice with 1ml HBSS as above. Cells were then triturated by passing the preparation through P200 10 times with gentle force and counted using trypan blue staining on haemocytometer. Four chambers were counted to give an average density and only preparations containing greater than 90% cell viability were used. Dissociated cells were plated onto either 12mm (Cedar lane; Cat #72196-12) or 18mm coverslips coated with Poly-L-lysine (PLL; Peptides International, Louisville, KY Cat #OKK3056) at a density of either 15,000 or 30,000 cells. Coverslips were coated with 150µl of 400mg/mL PLL solution for 2 hours, washed 2x with milliQ water and allowed to equilibrate with plating media in 5% CO2 incubator for 1 hour prior to plating. Cells were suspended in 0.5mL plating media; Neurobasal media containing 10% FBS, 2mM L-Glutamine (Wisent; Cat #609-065-EL), and Penicillin/Streptomycin (Wisent; Cat #450-201-EL; half normal concentration). The following day media was

changed to growth media, Neurobasal containing 2% B27 supplement, 2mM L-Glutamine, and Penicillin/Streptomycin. Cells were grown for 48 hours from time of plating at 37C 5% CO2 and fixed as described below.

2.8 Cell Culture Immunohistochemistry

Dissociated spinal cord commissural neurons were washed once with 1mL PBS, then fixed with 1mL PBS containing 4% paraformaldehyde (PFA), 4% sucrose for 10 minutes at room temperature. The PFA solution was removed and cells were washed three subsequent times with 1 mL PBS. Cells were then blocked and permeablized with a solution of PBS containing 5% FBS and 0.3% triton X-100 (PHT) for 2 hours at room temperature. Cells were then incubated overnight at 4^{o}C in 0.5mL PHT containing appropriate antibody on rotator. The following day cells were washed 3 times with PHT and incubated with Donkey anti-goat Alexa 488 (Invitrogen; Cat #A11005) secondary antibody at 1:2,000 dilution in PHT for 2 hours at room temperature. Cells were then washed two times in 0.5 mL PBS. BisBenzamide Hoescht – 33258 (Sigma Cat #B1155) was used as a nuclear stain at 1:10,000 in PBS. Following staining procedures, the dissociated spinal neurons were then washes three times with 0.5 mL PBS followed by dipping the slides in milliQ water and then mounted in 20μl fluoromount mounting media (Sigma Cat #F4680) on glass microscope slides (Fischer Scientific Frosted). Cells were imaged using a Zeiss Axiovert microscope, and images were captured using Slidebook software. Images were imported into Photoshop for further manipulations.

2.9 Spinal Cord Slice Immunohistochemistry

For spinal cord immunohistochemistry, pregnancy-staged mice were euthanized as described above and embryos were isolated, and embryonic sacs were saved for genotyping. For E11.5 embryo's the lumbar region of the embryo was kept intact and allowed to fix in 4% PFA for one hour at room temperature, while for >E12.5 embryo's the spinal cord was isolated before fixation by eviscerating the organs, removing the spinal vertebrae and extracting the entire spinal cord. These isolated spinal cords were then fixed for one hour at room temperature in 4% PFA. Post-fixation both intact embryo and spinal cords were cryoprotected overnight in 20% sucrose in PBS at 4C. The following day either intact embryo or spinal cord was sunk in OCT and flash-frozen using dry-ice and ethanol. Either preparation was then sectioned at 25μm with -14^{o}C chamber temperature and -16^{o}C sample temperature in a Leica brand CM 1650 cryostat onto microscope slides. These slides were allowed to dry at room temperature for one hour and used immediately or stored long term at -20^{o}C. For immunohistochemistry slides were equilibrated in PBS and blocked in humidified slide chambers at room temperature for one hour in blocking solution (PHT; 10% goat-serum in PBS containing 0.1% Triton X-100). Slides were then washed three times for 5 minutes in PHT and incubated overnight at room temperature with primary antibody diluted in blocking solution in humidified slide box. The following day slides were washed three times for ten minutes and incubated with secondary antibody diluted in blocking solution (1:1,000). Again slides were washed three times for five minutes in PHT before mounting with fluoromount as above. Spinal cord slices were imaged the same day using an

inverted fluorescent wide field microscope. Imaged were cropped and quantified using Image J image analysis program.

2.10 Whole Mount Immunohistochemistry

For whole mount immunohistochemistry was preformed as per Mouse Embryology Wood's Hole Module (Angelo Iulianella). E10.5-E12.5 embryos were dissected from pregnancy-staged mothers according to animal care guidelines. Embryonic sacs were kept for genotyping. Following dissection, embryos were fixed in 4% PFA for 2 hours at room temperature then washed three times for five minutes in PBS. Embryos were transferred to eppendorff tubes for all further incubations. PBS was replaced with blocking solution (PHT; 3% goat serum in PBS +0.1% Triton X-100) and left to rock for 1 hour at room temperature. Anti-neurofilament antibody was diluted at 1:1,000 in blocking solution and added to the embryos left to rock overnight at 4C. The following day embryos were rinsed in PHT for three times five minutes and left to rock for 1 hour at room temperature in PHT. Fluorescent secondary antibody (anti-mouse Alexa Fluor 1:500) was diluted in blocking solution and incubated with rocking for 1 hour at room temperature. Embryos were again washed three times for five minutes with PBS and incubated with Hoescht (1:5,000) for 20 minutes. Embryos were then washed three times for five minutes in PBS and cleared by placed in 25% glycerol/PBS and rocked at room temperature (RT) for 1 hour. Glycerol solution was increased to 50% glycerol/PBS and left for 1 hour on rocking at RT. Embryos were imaged in custom made agar containers to position embryo with Zeiss Stereo Discovery V8 (Angelo Iulianella).

2.11 X-Gal Staining

For X-gal staining tissue was isolated from embryos and fixed with a 3% gluteraldehyde solution for 1 hour at room temperature. Following fixation tissues were rinsed three times with PBS for five minutes and cryoprotected in PBS containing 20% sucrose overnight at 4^{o}C. The following day the tissues were immersed in OCT, and flash frozen using an ethanol dry-ice mixture. 25μm sections were collected using a Leica cryostat, sections were then air dried for 1 hour at RT. Following drying, slides containing the sectioned tissue were equilibrated in PBS and stained overnight at 37^{0}C protected from light in a humidified slide chamber box. Staining solution was preheated to 37^{0}C and prepared fresh from stock solutions of 5mM potassium ferricyanide and 5mM potassium ferrocyanide mixed 1:40 with X-gal from 25 mg/mL stock in DMF stock. Following color development, slides were washed with PBS, dipped in MilliQ water and mounted with three drops of fluoromount mounting media (Sigma, F4680-25ML).

2.12 Quantitative PCR

Total RNA was extracted using Trizol reagent (Invitrogen,Carlsbad, CA) according to the manufacturer's instructions. For RT-PCR amplification of DCC, an initial amplification using DCC primers was done with a denaturation step at 95°C for 10 min, followed by 28 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 30 s, and primer extension at 72°C for 45s. Upon completion of the cycling steps, a final extension at 72°C for 5 min was done and then the reaction was stored at 4°C. Reactions were run in duplicate and compared to internal cyclosporin control. Expression data were normalized

to the internal control cyclosporine gene to control the variability in expression levels and were analyzed using the 2 -ΔΔCT method.

2.13 Quantification And Statistics

For spinal cord slices, four lumbar level spinal cord sections per embryo were used for quantifications. Thickness of the ventral commissure was measured using NIH Image J. N (indicated in figure) was defined as the number of animals and each N was the average of four separate measurements. Statistical analysis was done with Graphpad Prism. *Post hoc* analysis of wild-type, *NCK1* $^{-/-}$*, NCK2* $^{-/-}$*, NCK1*$^{-/-}$*;NCK2*$^{flx/flx}$*;Nestin-Cre* mutant commissure thickness was done using Holm's test for multiple comparisons as previously reported (Jaworski et al., 2010).

For cultured commissural neurons, approx. 15 neurons were counted for each coverslip, where four coverslips were used per condition for a total >50 neurons. Images were imported to Image J for analysis. Axon length was defined as the distance between the edge of the cell body and the center of the growth cone. One-way ANOVA test was used to test for statistical significance. Growth cones were scored double blind for presence or absence of a growth cone and scored in duplicate.

CHAPTER 3 RESULTS

3.1 NCK1 and NCK2 are Expressed in the Spinal Cord

NCK proteins have been shown to be important for gait (Fawcett et al., 2007). To better define the role of the NCK proteins in spinal cord development and thus the contribution of these proteins to gait, I first wanted to focus on the distribution of NCK1 and NCK2 proteins in early development. Previously, Bladt and colleagues had shown that *NCK1* and *NCK2* were widely distributed in the E10.5 developing spinal cord (Bladt et al., 2003). Since we have yet to characterize an NCK antibody that can selectively discriminate NCK1 from NCK2, we took advantage of the fact that the $NCK1^{-/-}$ mice had a LacZ reporter fused into the NCK1 locus and used b-Galactosidase staining (herein X-Gal) to define NCK1 expression in $NCK1^{+/LacZ}$ embryos (Bladt et al., 2003). For the detection of NCK2, we turned to *in situ* hybridization for detection, since LacZ was not fused into the NCK2 locus in the $NCK2^{-/-}$ mice. NCK1, as determined by X-gal staining, is expressed throughout the embryo at E13.5 with strong staining in the spinal cord (Figure 3.1A). In addition to the expression in the spinal cord, and in agreement with previous results, we found NCK1 expression in non-neuronal tissue including developing bone, skin, limb, lung, and mesenophros (Figure 3.1B; Bladt et al., 2003).

Our *NCK2 in situ* at E11.5 revealed NCK2 mRNA expression in limb buds and body tissues, with concentrated staining at the embryonic spinal cord midline (Figure 3.1A). Since both NCK1 and NCK2 showed high expression within the spinal cord, we next examined the distribution of NCK1 in the lumbar spinal cord at two developmental

stages, E11.5 and E13.5. At 11.5, X-Gal staining revealed strong staining in the mantle layer of the spinal cord and dorsal root ganglion cells (Figure 3.1C, top left). The mantle region of the developing spinal cord contains neuroblasts that will undergo terminal differentiation giving rise to different neuronal populations in the spinal cord. The dorsal root ganglion is a collection of neurons that relay sensory information to the spinal cord. At E13.5, distinct motor neurons and a population near the midline where spinal interneuron populations arise were labeled with X-gal (Figure 3.1C, top right).

To confirm the staining we saw using the X-Gal protocol, we stained E11.5 and E13.5 sections with a b-Gal antibody (Figure 3.1C, bottom panels). We believe the b-Gal staining (Figure 3.1C, bottom panels) is positive, since a non-primary control showed no staining (Figure 3.1C, bottom right panel). At E11.5 strong NCK1 expression was seen in the mantle layer similar to the X-Gal staining; however, higher expression was found in the dorsal spinal cord compared to X-Gal at the same stage. (Figure 3.1C top and bottom left panels). Interestingly, at E13.5 the b-Gal staining revealed high NCK1 expression throughout the spinal cord, and consistent with X-Gal staining, expression was highest in the developing motor neurons and interneuronal region near the midline (Figure 3.1C, middle panels). Taken together, these studies reveal that NCK1 is expressed in all regions of the spinal cord during early stages of spinal cord development with discrete localization of NCK1 protein in motoneurons and post-mitotic neurons.

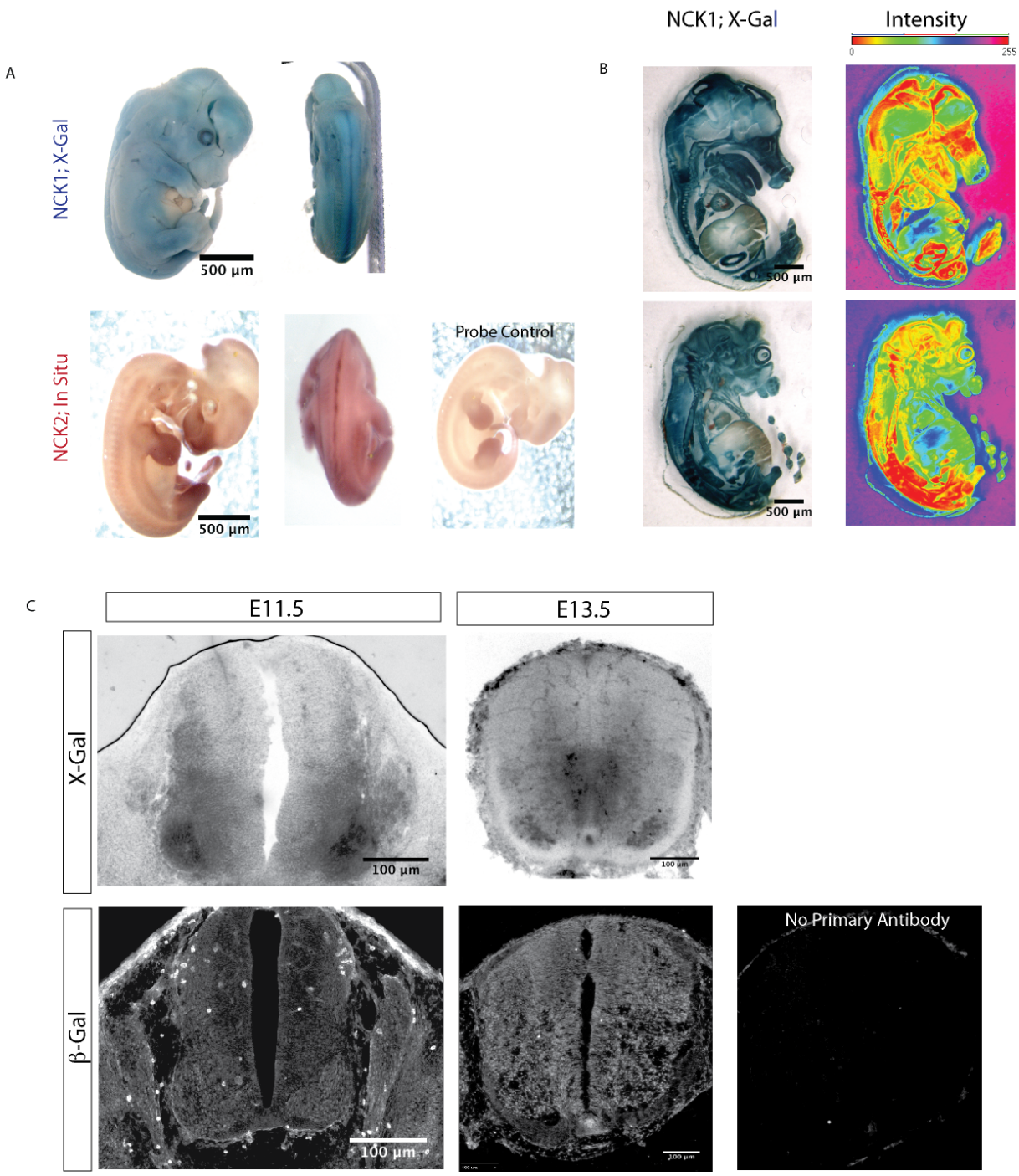

Figure 3.1: NCK1 and NCK2 are widely expressed during development, with highest expression in the spinal cord (A). Sagittal sections of E14.5 NCK1$^{+/LacZ}$ are expressed in hindbrain, spinal cord, muscles, and developing organs (B). NCK1 expression is concentrated in developing motor neurons at E11.5 and E.13.5 (C, top panels). NCK1 is also found in other parts of the spinal cord and dorsal root ganglion (Figure C, bottom two panels).

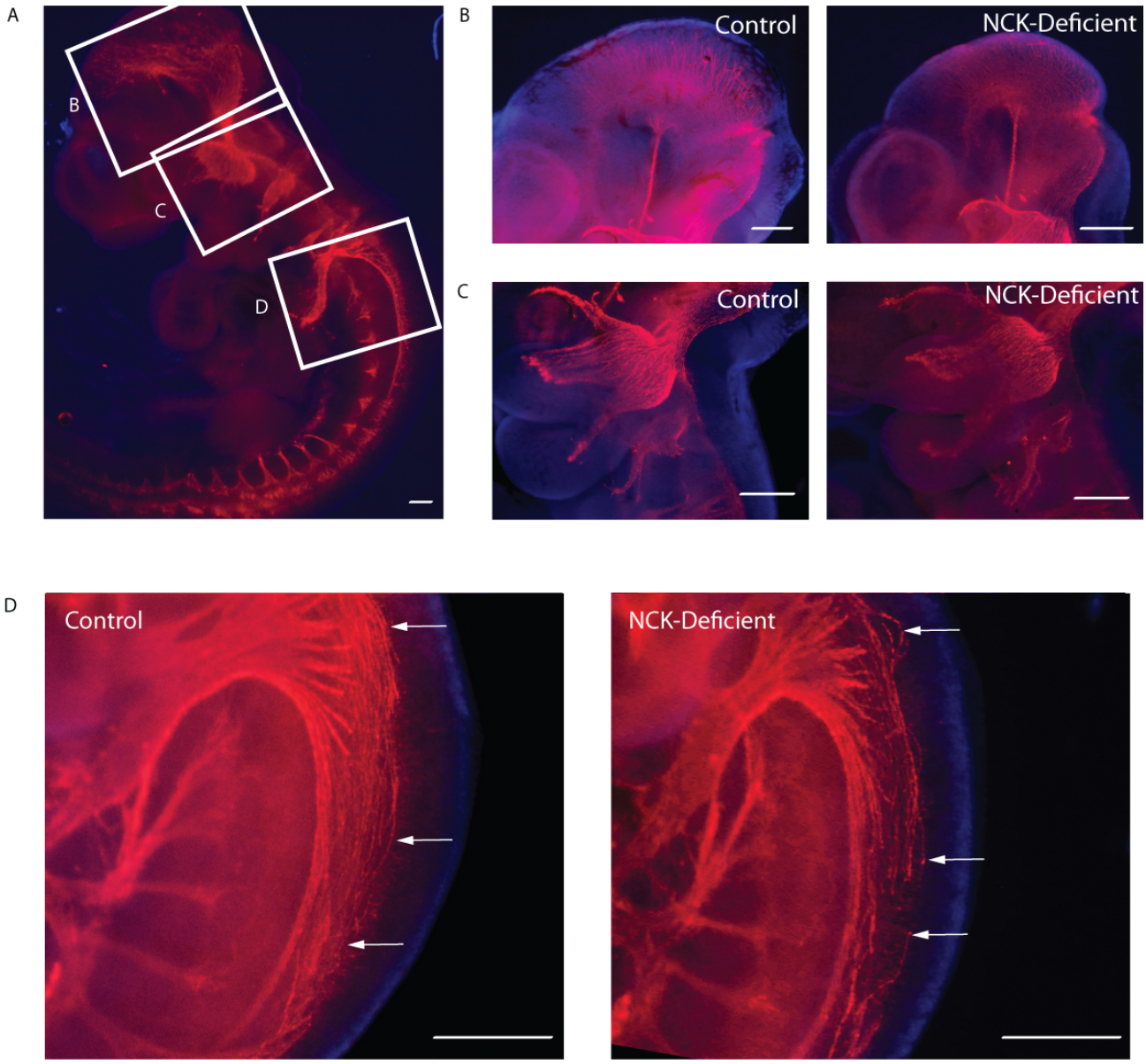

Figure 3.2: Whole mount cranial nerve screen using Anti-Neurofilament reveals no obvious defects in facial, trigeminal, or cortex neurons, however descending projections of the spinal accessory nerve show defasiculation and aberrant targeting in NCK mutant embryos. Representative embryo using whole mount immunohistochemistry (Panel A). NCK-deficient mice have no obvious defects in facial, trigeminal, or cortex neurons compared to control embryos (Panel B and C). Descending projections of the hindbrain and spinal accessory nerve show defasiculation and misprojections into the dorsal tissue in NCK-deficient mice compared to control (white arrows, Panel D). Scale bar is 100μm.

3.2 NCK-Deficient Mice have Axon Guidance Defects

Since NCK is widely distributed throughout the central nervous system including the developing spinal cord and NCK proteins have been implicated in axon guidance downstream of multiple guidance receptors including EphA4, DCC, and robo to name a few, we next asked if there were any obvious axon guidance defects in the developing embryo. Since many of these guidance receptors affect cranial nerve development we decided to focus on the development of the cranial nerves in the NCK-deficient mice (ie. $NCK1^{-/-}$;$NCK2^{flx/flx}$;Nestin-Cre; Guthrie, 2007). To do this, we stained control and mutant embryos at either E10.5 or E11.5 with a neurofilament antibody to mark developing neurons, but not glia (Figure 3.2A). No obvious defects were seen in the developing cortex in the NCK-deficient mice compared to controls (Figures 3.2B). Similarly, no obvious defects were noted in the axonal projections arising from the trigeminal or facial nerves (Figure 3.2C). However, guidance defects, including defasiculation and aberrant projections, were noted in a number of descending projections from the hindbrain and spinal accessory nerve (white arrow; Figure 3.2D). Similar defects have been identified in both the robo-slit and DCC pathways (Wright et al, 2012, Ratcliffe et al., 2008). Furthermore, studies examining the NCK homologue in *Drosophila*, dock, have shown a genetic link to unc5 and robo, the *Drosophila* homologues of DCC and robo respectively (Fan et al., 2003). Taken together, this suggests that loss of NCK proteins may affect the development of axons that are dependent on appropriate signaling from DCC or robo, or both. Since, loss of DCC, or its ligand netrin leads to the bilateral firing of paired ventral roots, similar to loss of NCK, I focused my analysis on DCC for the remainder of the thesis (Bernhardt et al., 2007; Rabe et al., 2009).

3.3 DCC interacts with NCK1 and NCK2

NCK1 directly interacts with the DCC receptor *in vitro* (Li et al., 2002). The first and third SH3 domains of NCK1 and the cytoplasmic tail of DCC mediate this interaction. Further, the association between NCK and DCC has been shown to be independent of netrin stimulation, suggesting the possibility of a constitutive association (Li et al., 2002). Previous work from multiple groups, suggested that NCK1 and NCK2 are functionally redundant; however, others have shown *in vitro* that NCK1 and NCK2 associate with different signaling pathways (Jones et al., 2006; Bladt et al., 2003; Fawcett et al., 2007; Cowan et al., 2001). Since NCK1 has been shown to associate with DCC, we next asked whether NCK2 could also associate with DCC. In the presence of DCC, either NCK1 or NCK2 were able to associate (Figure 3.3A, lane 2-3). Taken together this suggests that both NCK1 and NCK2 can associate with DCC *in vitro*.

Since NCK is able to recruit the soluble tyrosine kinase FYN to receptors, and FYN has been shown to phosphorylate DCC, we next addressed whether NCK would affect DCC tyrosine phosphorylation (New et al., 2013; Meriane et al., 2004). Here DCC was expressed either alone, or in the presence of NCK1 or NCK2. Interestingly very low levels of DCC tyrosine phosphorylation were detected in the absence of NCK1 or NCK2 overexpression; however in the presence of NCK1 or NCK2, DCC tyrosine phosphorylation was detected (Figure 3.3B). Further, the level of DCC tyrosine phosphorylation was higher in the presence of NCK2 vs. NCK1. These results suggest that in the presence of either NCK1 or NCK2, these adaptors are able to recruit a tyrosine

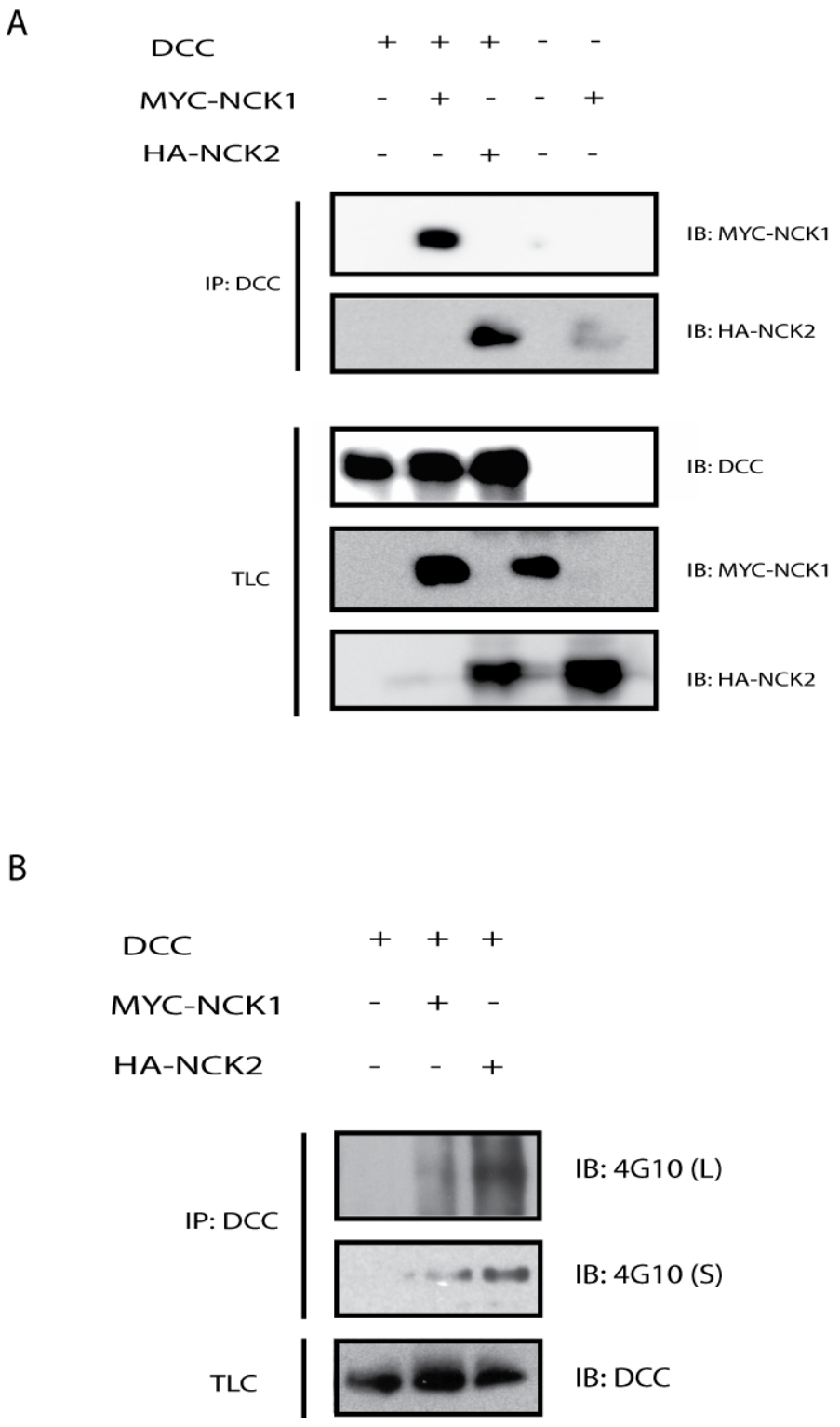

Figure 3.3: DCC associated with myc-NCK1 and HA-NCK2 when overexpressed in HEK293T cells. NCK1 and NCK2 immunoprecipitate with DCC, but not its absence (A top two panels). DCC, NCK1, and NCK2 are properly expressed in HEK293T cells (A, bottom three panels). DCC is tyrosine phosphorylated in the presence of either NCK1 or NCK2 (B).

kinase and affect DCC phosphorylation. Whether this phosphorylation is affected by netrin stimulation remains to be tested.

3. 4 NCK-Deficient Embryos have Defects in DCC-Dependent Commissural Neurons

We have been able to show that both NCK1 and NCK2 associate with DCC *in vitro*, and that this association can affect the phosphorylation state of DCC. To more directly test the significance of this interaction *in vivo*, we next tested effects of reducing NCK protein on the development of DCC sensitive circuits. In the spinal cord, DCC-positive neurons are born in the dorsal part of the spinal cord and project axons toward the floor plate through the attraction of the netrin guidance molecule (Figure 3.4A). Loss of the DCC ligand netrin-1 or the DCC receptor leads to aberrant projections of these commissural axons into the surrounding tissues and ultimately a reduction in the number of axons able to cross the spinal cord at the ventral commissure (Matsumoto, 2007; Serafini, 1996). Furthermore, loss of DCC, or its ligand netrin, leads to the bilateral firing of paired ventral roots similar to our NCK-deficient mice (Bernhardt et al., 2007; Rabe et al., 2009). Therefore, we tested if NCK is required for the guidance of DCC^+ commissural neurons. Since DCC^+ axons are $TAG1^+$, we followed the expression of TAG1+ axons in the NCK-deficient mice.

In NCK-deficient embryos $TAG1^+$ axons are defasiculated and failed to project directly to the floor plate (Figure 3.4B). Tracing these axons shows the loss of directed trajectory of the TAG1+ fibers (Figure 3.4D). In addition, there was a reduction in the thickness of

TAG1^{+} fibers crossing at the floor plate (Figure 3.4C). To more directly quantify the loss of commissural neurons at floor plate, we measured the thickness of the ventral commissure (VC), defined as the TAG1^{+} region directly below the central canal. Since both NCK1 and NCK2 were shown to interact with DCC, we tested if TAG1^{+} axons were affected in the NCK1 or NCK2 mutant mice. Compared to control animals (NCK1$^{+/-}$;NCK2$^{flx/flx}$), the NCK1 mutant mice (NCK1$^{-/-}$ NCK2$^{flx/flx}$) showed a 6.6 μm reduction in the commissural axon thickness (Figure 3.4E, first and second panel). Next we tested whether there were defects in the DCC sensitive axon projections in the NCK2$^{-/-}$ mice. Indeed loss of NCK2 protein leads to a decrease of 6.1μm in commissural thickness compared to control animals (Figure 3.4E, third panel). However, despite a trend towards a reduction compared to control mice, neither of these groups reached statistical significance. This may be due to the fact that the control mice were heterozygous for NCK1; statistical significance may result if a wild-type mouse were to be used as a control. Next, since both NCK proteins can associate with DCC, we next tested the effect on TAG1^{+} thickness in the NCK-deficient mice. Similar to the NCK1 mutant and NCK2 mutant mice, there was a decrease in commissural thickness; however, in the NCK-deficient mice (NCK1$^{-/-}$NCK2$^{flx/flx}$:Nestin-Cre) there was a statistically significant difference in the thickness of the TAG1^{+} fibers at the floor plate with 8.8μm reduction compared to control (Figure 3.4E, bottom panel). Taken together, these data suggest that NCK proteins contribute to the guidance of TAG1+ commissural axons. Note, we monitored whether loss of NCK protein led to a reduction in the thickness of the ventral floor plate, no change in overall thickness was detected in any of the mouse lines tested.

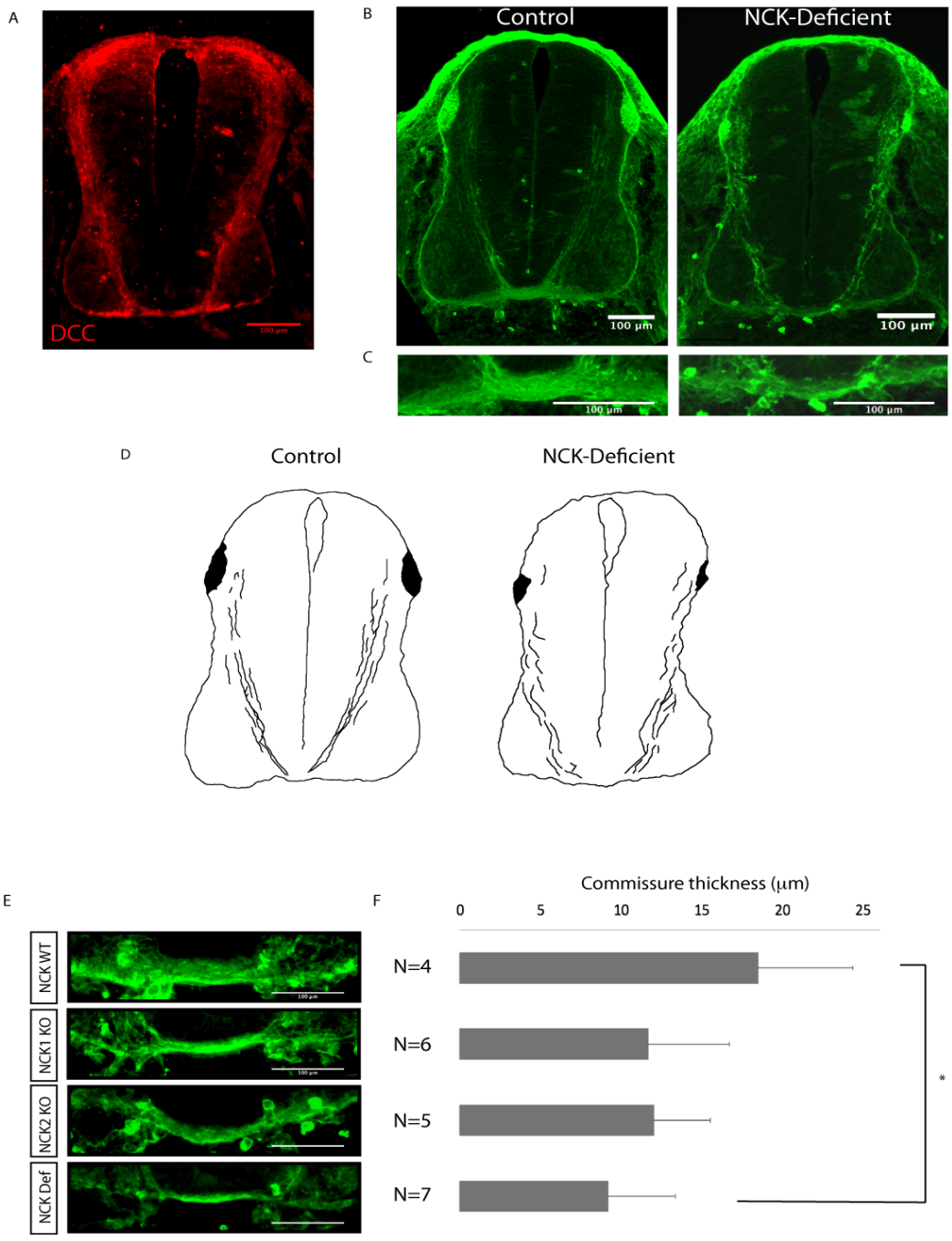

Figure 3.4: NCK-deficient mice have a reduction in the thickness of the ventral commissure and aberrant projections in commissural neurons. DCC positive neurons project axons toward the floor plate (A). TAG1 positive axons project linearly in control mice, but are less organized in NCK-deficient mice cumulating in a loss of axons at the ventral commissure (B,C). Camera Lucida drawing of control and NCK-deficient mice outlines axon trajectories (D). Quantification of ventral commissure thickness in control, NCK1 knock out, NCK2 deficient, and NCK-deficient embryonic lumbar spinal cords (E and F).

3.5 NCK-Deficient Spinal Cords have a Reduction in DCC mRNA at P5

Since loss of NCK proteins ($NCK1^{-/-}$;$NCK2^{flx/flx}$;Nestin-Cre) leads to a decrease in the thickness of $TAG1^{+}$ commissural axons crossing at the floor plate, this suggested the possibility that there may be fewer DCC^{+} neurons in the dorsal spinal cord in the NCK-deficient animals. Support for this comes from previous studies showing that loss of Netrin-1/DCC signaling leads to a loss of DCC positive cells in the dorsal spinal cord (Furne et al., 2008). To assess the integrity of the DCC^{+} cells in the spinal cord, we dissected the lumbar spinal cord from postnatal day 5, control or NCK-deficient animals and performed RT-PCR reactions and measured the level of DCC mRNA using quantitative PCR. Interestingly, the NCK-deficient spinal cords had a 50% reduction in the amount of DCC mRNA (Figure 3.5). It will be interesting to follow these studies up with *in situ* studies to quantify more directly whether this is due to a loss of DCC^{+} neurons or a reduction in mRNA expression in the individual neurons.

3.6 NCK and DCC Colocalize in Commissural Neuron Growth Cones

Our results suggest that NCK proteins are important adaptor proteins necessary for the directed guidance of DCC positive axons in the developing spinal cord. To more directly test the role of NCK in DCC positive commissural neurons we developed dissociated cultures from the dorsal spinal cord. 48 hours post plating the cultures were fixed and stained for DCC and NCK (Figure 3.6A). Roughly 90% of all cells were NCK positive while approximately 70% were DCC positive. Importantly, all NCK positive cells were DCC positive.

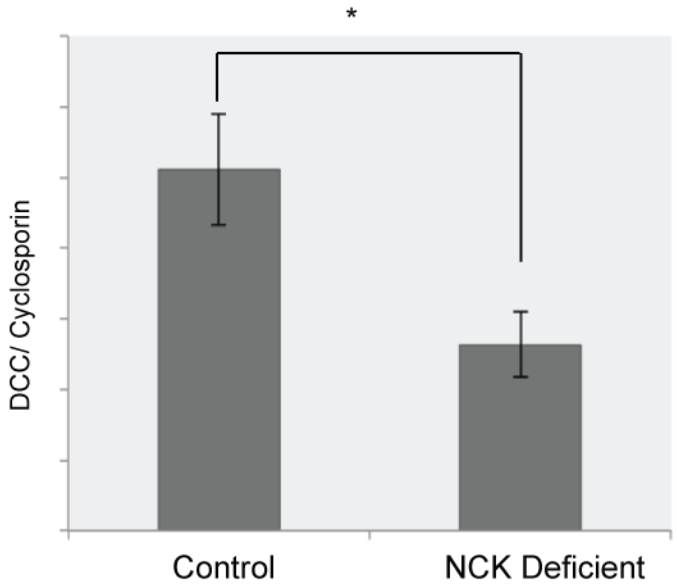

Figure 3.5: DCC mRNA is reduced in NCK-deficient mice compared to control. DCC levels are given as a ratio of total DCC to internal control cyclosporine mRNA. Statistical analysis was done using students T-test at the 95% confidence interval ($p<0.05$).

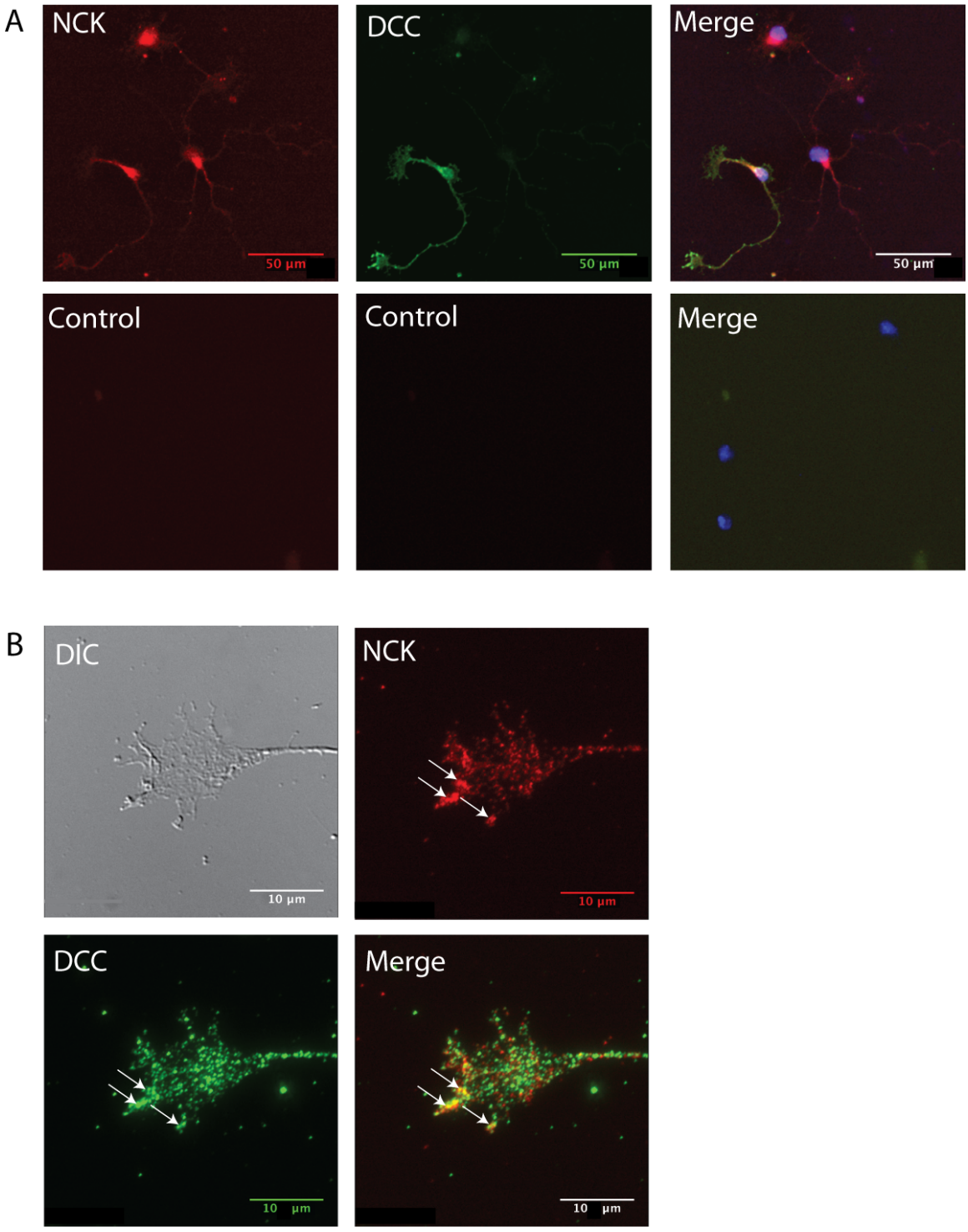

Figure 3.6: NCK and DCC are coexpressed in commissural neurons. NCK is expressed in all cultured cells. DCC is cultured in ~70% of growth cones. All DCC expressing cells also have NCK expression (A). NCK and DCC are coexpressed in the growth cone and their expression overlaps at the leading edge of the growth cone (B).

Since DCC has been reported in the developing growth cones of commissural interneurons, we next looked at the localization of NCK and DCC in the growth cone of neurons cultured from wild-type neurons. Interestingly, NCK and DCC were colocalized at the leading edge of the developing growth cones in filopodia extensions (Figure 3.6B; white arrows). Taken together this suggests that all DCC positive neurons in the dorsal spinal cord contain NCK protein, and that in the developing growth cone both proteins concentrate at the leading edge.

3.7 NCK Deficiency Leads to a Disruption in Growth Cone Structure

Since NCK is an important adaptor protein that regulates the actin cytoskeleton, we next asked what effect reducing endogenous NCK proteins would have of the morphology of DCC^{+} commissural neurons. To assess any possible effect we characterized two distinct parameters: growth cone complexity and axon length.

Growth cones were scored as either complex or simple. A growth cone was scored complex if the shape of the growth cone was fan-like and contained well-defined lamellipodia and filopodia (see, Figure 3.7A'). A simple growth cone lacked these features. Of the DCC^{+} neurons from the control animals ($NCK1^{+/-};NCK2^{flx/flx}$), approximately 75% showed a complex growth cone phenotype (Figure 3.7B). Similarly, over 68% of the NCK1 mutant mice ($NCK1^{-/-};NCK2^{flx/flx}$) and 65% of the NCK2-deficient ($NCK1^{+/-};NCK2^{flx/flx}$;Nestin-Cre; Figure 3.7B' and 3.7B'') contained complex growth cones. Interestingly, only 40% of the neurons isolated from the NCK-deficient

(NCK1$^{-/-}$;NCK2$^{flx/flx}$;Nestin-Cre; Figure 3.7B’’’) mice contained complex growth cones (Figure 3.7D).

Since the actin cytoskeleton is a major structural protein involved in growth cone dynamics and cellular mobility, we next asked what effects reducing endogenous NCK proteins would have on axonal outgrowth. In the neurons isolated from control, NCK1$^{-/-}$, and NCK2-deficient (ie. NCK1$^{+/-}$;NCK$^{flx/flx}$;Nestin-Cre) mice, there was no significant difference in total axonal length 48 hours following plating (Figure 3.7C). However, the axonal lengths of neurons isolated from the NCK-deficient mice was 1.5 times longer compared to neurons isolated from control mice (Figure 3.7C; $p<0.0001$) Taken together these results suggest that NCK proteins play an important role in growth cone structure and axonal growth.

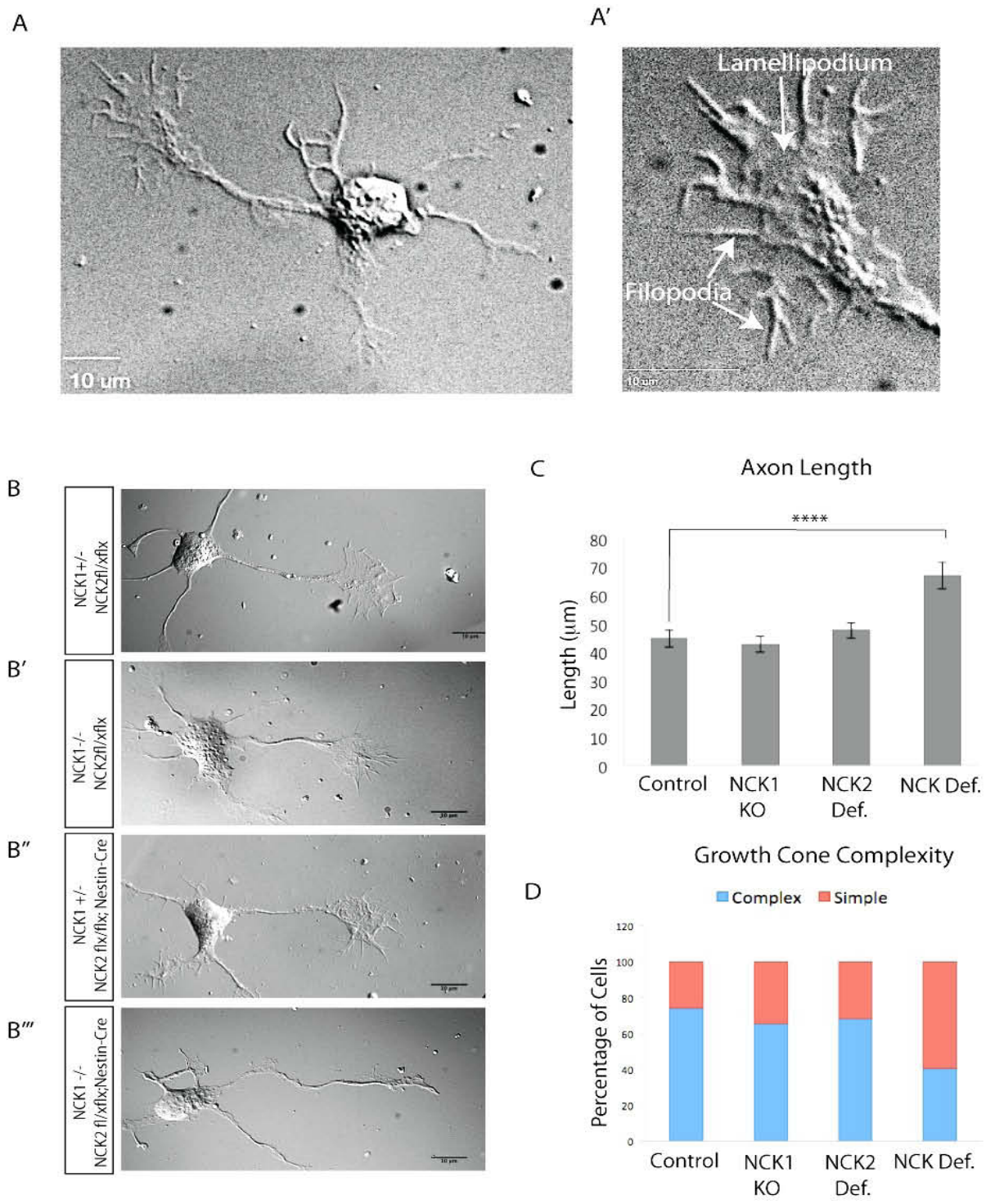

Figure 3.7: NCK-deficient spinal cord commissural neurons show morphological defects in their axon and growth cone complexity. Wildtype neurons growth cones have specific features (A). NCK-deficient spinal cord commissural neurons have longer axons and less complex growth cones compared to control (B-B’’’). Quantification of axon length and growth cone complexity (C and D; $p<0.001$).

CHAPTER 4 DISCUSSION

4.1 Summary of Findings

My project set out to better define the role of NCK proteins in the developing central nervous system. Previously, Fawcett and colleagues have shown a role for NCK proteins in the development of spinal circuits important for walking. Despite these findings, it was unclear how NCK was acting to affect changes in the circuitry to lead to the hopping phenotype. My project focused on the expression profiles of NCK1 and NCK2 during development and whether global guidance defects that might help give insight into the underlying cause of the hopping phenotype.

I found that both NCK1 and NCK2 are expressed in the developing spinal cord during a time when spinal circuits are being established. Specifically, NCK1 is expressed in motor neurons and interneurons, while NCK2 is expressed in the midline. Using while mount neurofilament staining, I was able to characterize defects in the development of axonal projections descending from the hindbrain and spinal accessory nerve. This is similar to previous studies showing defects in the spinal accessory nerve of DCC mutant mice (Dillon et al., 2005). Since DCC has been shown to directly associate with NCK1 in vitro, I next addressed whether DCC sensitive circuits are affected in the NCK-deficient mice. Indeed, this was the case. I found a 50% decrease in the thickness of TAG1+ neurons in the ventral floor plate of E11.5 mice. Consistent with this defect, I noted a 50% loss of DCC mRNA suggesting that the DCC was downregulated in the absence of NCK. We then looked directly at cultured commissural neurons to more closely examine

the loss of NCK phenotype. We found that NCK and DCC colocalize at the leading edge of the growth cone. When NCK was reduced in these cultured neurons it resulted in a more simple growth cone compared to control. We also examined the axon length of cultured commissural neurons and found that NCK-deficient neurons had significantly longer axons compared to control. Taken together, these studies outline a specific role for NCK in DCC-sensitive spinal cord circuits.

4.2 NCK1 and NCK2 Expression

Our study of the NCK1 and NCK2 adaptor proteins indicate that they are both expressed in the developing spinal cord. This is in agreement with a previous report by Bladt and colleagues in which they identify NCK1 and NCK2 expression in the motor neurons and dorsal root ganglion at E10.5 (Bladt et al., 2003). Also in agreement with previous reports, we identify NCK1 in multiple other tissues during development, albeit at lower concentrations. Many of these tissues are later stages of mesoderm-derived structures, which have been outlined in the study by Bladt et al. Further work needs to be done to improve the sensitivity of the NCK2 mRNA probe and to identify its localization within the spinal cord.

4.3 NCK1 Distribution

At E11.5, in the lumbar spinal cord NCK1 is found in the mantle layer of the developing spinal cord. The ventral part of the mantle layer also known as the basal plates forms the motor areas of the spinal cord while the dorsal part, the alar plates, form the sensory areas. At this stage, NCK was not detected in the ventricular zone, a region where mitosis

occurs; supporting the previous suggestion that NCK has a role in guidance, but not in cell fate determination and proliferation (Rao et al., 1998).

In the lumbar spinal cord at E13.5, NCK is expressed throughout the spinal cord. In the E13.5 spinal cord many neurons are migrating to their destination, thus NCK may have a role in directed migration of neurons during development.

Using X-Gal, NCK1 is highly expressed in developing motor neurons and in the ventral neural progenitor domains. The motor neurons of the spinal cord are responsible for activating flexor and extensor muscle groups responsible for coordinated movement of the hind limbs. Of interest are the guidance cues that motoneurons use to appropriately reach their target muscles. Previously, dock, the *Drosophila* homologue of NCK, has been reported to be important for the development and appropriate formation of *Drosophila* motor neurons synapses. Thus, the high expression of NCK in motor neurons indicates a potential role for this adaptor in mouse motor neuron development.

The ventral progenitor domains of the lumbar spinal cord are responsible for the development of distinct classes of interneurons known to regulate walking behavior. Two of these interneuron populations, the V0 and V3 interneurons are responsible for coordinating the movement between the left and right side of the spinal cord. We also know that NCK-deficient mice have problems in gait coordination between the left and right side of the spinal cord (Fawcett et al., 2007). It may be that NCK is important for the development of these V0 and V3 interneuron populations and in its absence they are not able to form appropriate connections during development.

4.4 X-Gal Stain and Immunohistochemistry

Using immunohistochemistry, we see that NCK is expressed in a wider region of the spinal cord. Immunohistochemistry identifies specific molecules of NCK and provides higher sensitivity compared to the X-Gal method, which relies on the enzymatic activity of β-Galactosidase. Since we do not observe any signal in our no-primary antibody control, we conclude that immunohistochemistry provides a more accurate depiction of the expression of NCK1. Thus, NCK is likely expressed throughout the spinal cord and is involved in the development of multiple neuronal populations. Since NCK is known to connect to regulate actin dynamics through its interactions with WASP and PAK and since actin is a major component of migrating growth cones in developing neurons, NCK likely plays a widespread role in axon guidance.

4.5 NCK in Embryonic Axon Guidance

In our screen of the developing cranial nerves, we identified axon guidance defects in the descending projections of the hindbrain and spinal accessory nerve, supporting the hypothesis that NCK is an important regulator of axon guidance (Fawcett et al., 2007). Furthermore, we did not identify major defects in many of the other cranial nerves including the trigeminal, facial, and developing brain. Since NCK is thought to be important downstream of multiple axon guidance receptors, such as B type Ephrins and EphA4, one might expect to see similar phenotypes to these receptors when NCK is reduced from the nervous system (Guthrie et al., 2007). However, we did not detect any major defects in facial nerves affected by the Ephrin B or EphA families of proteins, possibly due to the sensitivity of our technique (See Limitations 4.10).

Regardless, here we identify NCK as important for the development and guidance of descending projections from the hindbrain. The defects noted phenocopies another genetic model, the β-1,3-N-Acetylglucosaminyltransferase 1 (B3gnt1) mutant mouse, in which exon 1 of the B3gnt1 gene has a point mutation, and those of the DCC and Netrin mutant mice (Wright et al. 2012; Dillon et al., 2005). This group identifies B3gnt1 as an important regulator of slit, the ligand for the robo receptor. Interestingly, they also identify B3gnt1 as an important regulator of commissural axon guidance. (Wright et al., 2012) Further similarities between NCK-deficient mice and B3gnt1 mutant mice are in the defects observed in the dorsal faniculus (Fawcett et al., 2007; Wright et al., 2012). This may indicate a common pathway in development between these two proteins, since we know dock (NCK homologue) is able to interact with the robo receptor in *Drosophila* (Fan et al, 2003). Alternatively, B3gnt1 may interact directly with NCK in a pathway independent of slit-robo signaling to regulate the development of these circuits.

DCC and Netrin mutant mice have defects in their spinal accessory motor neurons which make up the spinal accessory nerve (Dillon et al., 2005). Here we identify similar defects in NCK-deficient mice. It would be interesting to use the BEN label as done in this study to examine a spinal accessory specific marker.

4.6 Molecular Interaction between NCK2 and DCC

NCK1 binds directly to DCC by its first and third SH3 domain (Li et al., 2002) and here we identify that NCK2 is also able to bind DCC (Figure 3.3). Previous reports have

indicated NCK is able to bind DCC endogenously in cultured commissural neurons, but since there exists no good antibody to distinguish between NCK1 and NCK2, it is unclear which protein was responsible for endogenous binding (Li et al., 2002). We use overexpression of individual tagged NCK1 or NCK2 transcripts to identify NCK2 as well as NCK1 as binding partners of DCC. NCK1 and NCK2 are homologous genes that share 68% sequence identity at the amino acid level (Chen et al. 2001). This adds to increasing evidence that NCK1 and NCK2 have redundant function during development (Jones et al., 2006; Bladt et al., 2003).

4.7 NCK is Sufficient to Phosphorylate DCC

When overexpressed in HEK293 cells, either NCK1 or NCK2 is sufficient to cause a phosphorylation event on DCC. NCK recruits the FYN kinase to other guidance receptors such as nephrin (New et al., 2013). FYN is required for the activation of DCC receptor in commissural axons (Meriane et al., 2004). Similar to experiments on the nephrin receptor, NCK2 has an increased effect on the phosphorylation of DCC compared to NCK1. However, both NCK1 and NCK2 show increased phosphorylation compared to control, indicating each alone is sufficient to cause activation. Netrin binding is also able to cause phosphorylation of DCC. Phosphorylation acts as a signal to recruit signaling proteins to the intracellular domain. It would be of interest to test if NCK is required for the phosphorylation of endogenous DCC in the presence of netrin. To test this, we could immunoprecipitate DCC from the lysate of NCK-deficient commissural neuron cultures in the presence or absence of netrin and compare it to control mice.

4.8 NCK in DCC-Sensitive Circuits

4.8.1 *In vivo* Commissural Neurons

Since we were able to show that both NCK1 and NCK2 could interact with DCC, we wanted to determine the *in vivo* effects of reducing NCK protein on the developing DCC sensitive circuits. In order to test the hypothesis that NCK acts downstream of the DCC receptor, we looked at a population of circuits known to be sensitive to DCC. Commissural neurons express DCC and are sensitive to netrin gradients produced from the floor plate of the spinal cord. In both netrin and DCC mutant mice, commissural neurons show guidance defects and a reduction or complete loss of axons crossing the embryonic midline (Matsumoto et al., 2007; Serafini, 1996). In our studies, we find that loss of both NCK1 and NCK2 leads to a reduction in the size of the $TAG1^+$ region crossing at the floor plate. When either NCK1 or NCK2 alone is removed, there is a reduction in ventral commissure thickness that is trending toward significance. It will be necessary to complete more replicates of these mice to determine if NCK1 or NCK2 alone can contribute to the crossing phenotype. Nonetheless, our results clearly show DCC positive axons require NCK for appropriate guidance and crossing at the ventral floor plate.

In vitro data from Li et al. outlined a role for NCK in the outgrowth of NIE-115 neuroblastoma cells in relation to the receptor DCC (Li et al., 2002). Here we confirm that NCK has a role in the development of DCC-sensitive commissural neuron circuits. Considering the direct molecular interaction, it is tempting to speculate that NCK acts to recruit downstream regulators of actin dynamics to the DCC receptor both *in vivo* and *in*

vitro (Shekarabi et al., 2005). Considering the not fully explained hopping phenotype of NCK-deficient mice, these commissural neurons may represent a population of neurons required for normal gait.

NCK is required for normal commissural axon guidance in the E11.5 spinal cord. These dorsal commissural neurons express the guidance receptor DCC. If commissural DCC positive circuits do not correctly form during development, the DCC receptor has an alternate function in apoptosis (Forcet et al., 2001). In fact, NCK-deficient spinal cord mRNA preparation from P5 mice show a 50% reduction in total DCC mRNA compared to control. However it is also possible that the guidance phenotype observed in NCK-deficient embryos results from surrounding factors that influence the development of commissural axons, for example the netrin-gradient.

4.8.2 *In vitro* Commissural Neurons

To simplify the guidance assay and more directly test the function of NCK in DCC-positive commissural neurons, I developed a technique to culture these commissural neurons *in vitro* (Moore et al., 2008). Cultured NCK-deficient commissural neurons have differences in growth cone complexity and axon length compared to control. The growth cone of a neuron is an actin-rich structure responsible for receiving guidance cues and directing the growth of the axon (Purves et al., 2001). DCC is the major receptor involved in the guidance of commissural neurons and when stimulated by its ligand, netrin, it activates cytoskeleton dynamics that result in expansion of the growth cone (Shekarabi et al., 2005). The simplicity of the growth cone in NCK-deficient mice may represent a role

for NCK in normal maintenance of the cytoskeleton, but does not necessarily relate to the receptor DCC. However, taken together with previous reports and *in vivo* data, this supports a role for NCK in DCC signaling pathways.

What remains largely unknown is how the two major components of a developing axon, microtubulin and actin, connect to one another to regulate the normal development of an axon to its appropriate target. Both actin and tubulin are important components of the growth cone. Interestingly, the size of the growth cone has been inversely correlated with an increase in axon length (Dent et al., 2003; Dent et al., 2011). Using f-actin depolymerizing agents it was shown that loss of f-actin has no effect on axon outgrowth, indicating that tubulin plays a major role in axon propulsion (Lefont et al., 1993; Dent and Kalil, 2001). Furthermore, growth cones with disrupted f-actin have guidance defects (Chein et al., 1993; Kaufmann et al., 1998). It is interesting to note that one downstream effector of NCK, ARP2/3, shows similar defects in axon guidance and growth cone structure (Norris et al., 2009; Strasser et al., 2004). Taken together, we speculate that NCK acts to connect the DCC receptor to the downstream effectors of the actin-cytoskeleton, such as ARP2/3 to mediate the guidance of commissural neurons. In the absence of NCK, DCC is not able to transduce guidance signals and commissural neurons do not appropriately respond to guidance cues (Figure 4.1).

4.9 Future Directions

4.9.1 Downstream Effectors of NCK

NCK has been shown to be sufficient to connect a number of receptors, including DCC, to their downstream targets (Shekarabi et al., 2005). We have shown that NCK1 and NCK2 can interact with the DCC receptor. In future work, it will be important to determine if this interaction is required for the connection of DCC to its downstream molecular targets. In order to test this we can use NCK-deficient and control commissural spinal cord neurons and immunoprecipitate endogenous DCC to see if we can pull out downstream signaling partners such as PAK, WASP, and Trio.

4.9.2 Open-Book Spinal Cord DiI Labeling

In this study, NCK-deficient commissural neurons are not appropriately guided across the midline. Since NCK is able to act downstream of multiple axon guidance receptors, such as robo, it will be of interest to see what effect NCK-deficiency has on axons which are able to cross the midline. Robo receptors act to repel commissural axons from the midline after crossing and are involved in longitudinal axon guidance. Using diI-labeling of open book spinal cords will establish whether NCK is required for maintaining axons trajectory after crossing.

4.9.3 Netrin Stimulation

We have shown that NCK-deficient spinal cord commissural neurons have longer axons and more simple growth cones compared to control. It will be important to determine if this effect is dependent or independent of the DCC receptor. In order to test this

hypothesis, we can preform dorsal spinal cord explant culture and look at the total axon outgrowth in netrin-stimulated conditions. Alternatively, we can examine the change in growth cone morphology of single neurons upon perfusion of netrin into the growth chamber. This will be useful to determine if NCK is acting in a netrin dependent manner to signal with DCC.

4.9.4 NCK Role in Spinal Cord Progenitor Domains

NCK-Deficient Mice have gait abnormalities termed “hopping”. The lumbar spinal cord neurons that control walking are collectively known as the central pattern generator. NCK-deficiency affects the development of commissural neuron guidance, but may have additional roles in specific populations of interneurons. Of special interest are the HB9 progenitor neurons that encompass the motor neurons. NCK is highly expressed in developing motor neurons and it would be interesting to examine the development of these neurons in normal and NCK-deficient mice. Furthermore, appropriate synapse formation may require NCK proteins. To examine the role of NCK in motor neurons we could test the electrophysiological and structural properties of motor neurons and synapses respectively, in a co-culture model.

4.10 Limitations

Two caveats to our genetic model may explain the mild defects observed in the cranial nerve screen. The first caveat is the NCK-deficient mouse model does not have a complete loss of NCK2 from the nervous system. NCK-deficient mice have a >95% reduction of NCK2 mRNA in the cortex of P1 animals (Fawcett et al., 2007). However,

the nestin promotor is active at E8.5 and may not completely excise the NCK2 gene at our experimental age of E11.5 (Dubois et al., 2006). The net effect of incomplete excision is that we may have higher levels of NCK2 (Tronche et al., 1999). Secondly, the stability of NCK2 protein is unknown. Since the nestin-cre promotor would be active as early as E8.5 with higher activity by E11.5 there would presumably be a residual NCK2 protein that may contribute to the normal development period between E11.5 and even up to E13.5 Further studies would need to adequately determine the protein expression at these stages. Further analysis using the $NCK2^{flx/-}$ mutant will help clarify these issues.

Finally, we are using a model organism to study the function of NCK in spinal cord circuits. It remains a possibility that these studies are not translatable to human subjects. Further studies using primary patient samples will clarify the role of NCK in the human spinal cord and in human neurons.

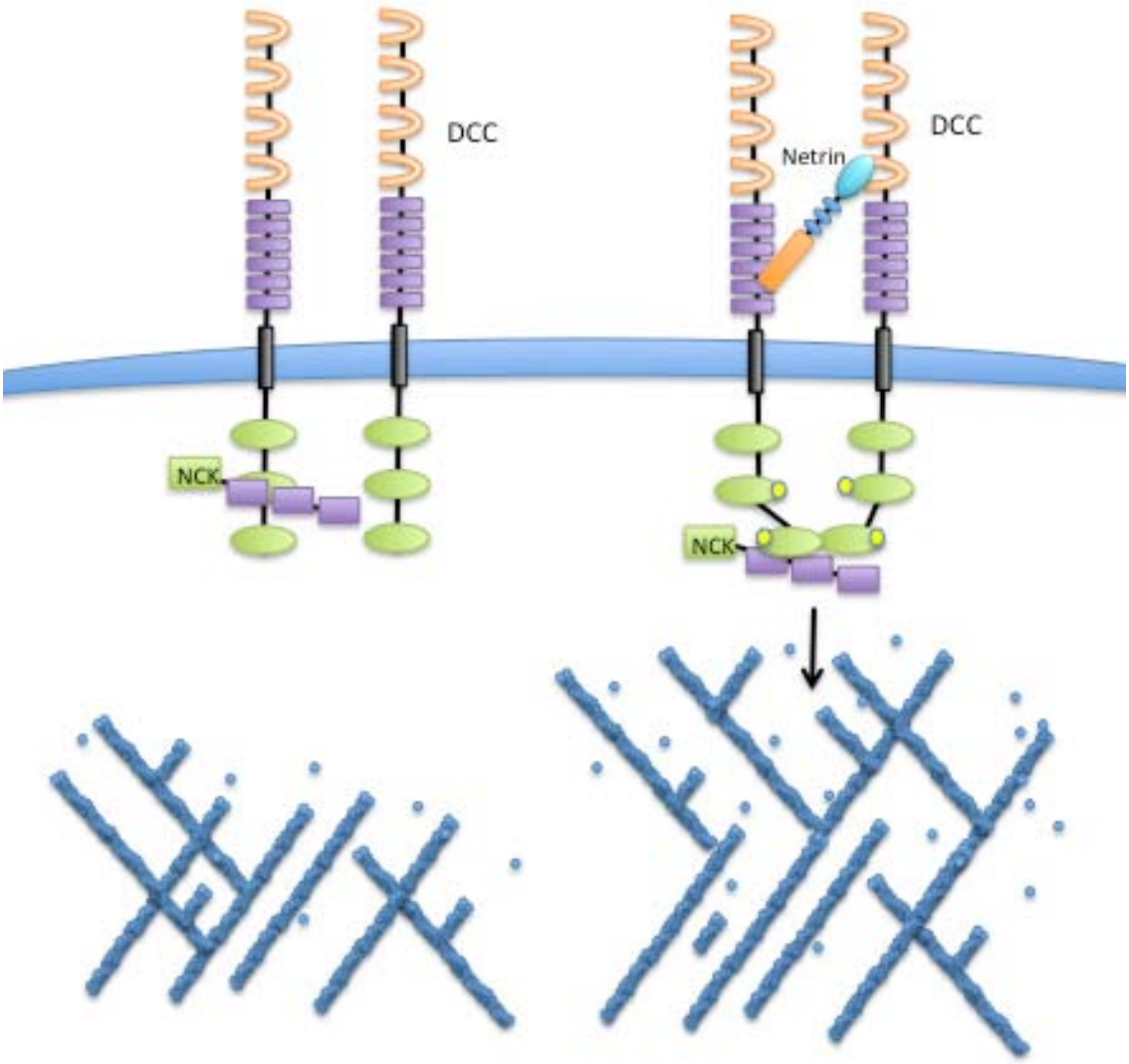

Figure 4.1: Proposed Mechanism of the Role of NCK in DCC Spinal Cord Circuits. NCK connects DCC to phosphorylation signals and to the downstream activation of actin dynamics through NCK signaling pathways.

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