The coordination of netrin signal transduction via TUBB3 and JNK1 in axon guidance

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A Dissertation

Entitled

The Coordination of Netrin Signal Transduction via TUBB3 and JNK1 in Axon Guidance

by

Chao Qu

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biology

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Microtubule dynamics plays an important role in axon guidance, but much still remains to be learned about the specifics of the interaction. TUBB3 is a neuronal specific microtubule subunit and TUBB3 mutations perturb microtubule dynamics and axon pathfinding, suggesting that TUBB3 may be required for axon guidance in the developing nervous system. Here, we report that DCC, a receptor of netrin-1, interacts directly to TUBB3 both \textit{in vitro} and \textit{in vivo}. Importantly, netrin-1 increased TUBB3/DCC interactions and the inhibition of Src family kinase or disruption of microtubule dynamics abolished these interactions. TUBB3 siRNA or shRNA reduced netrin-1-induced axon outgrowth in both E3 chicken dorsal spinal cord explants and E15 mouse cortical neurons and chick commissural axon turning \textit{in vitro} and disrupted commissural axon projection \textit{in vivo}. These results suggest that netrin-1 mediated signaling through DCC is linked directly to microtubule dynamics and that this link may play an essential role in netrin-mediated axon guidance.
Microtubule dynamics is regulated by microtubule associated proteins (MAPs) and the JNK family of MAPKs, one of the major regulators of MAPs, plays a key role in neural survival, migration, and polarity as well as axon regeneration. However, no role for JNK activation in netrin-1 signaling had been identified previously. Here we show that JNK1 is required for netrin signaling in the developing nervous system. Netrin-1 acting through either DCC or DSCAM increased JNK1 activity in both HEK cells and primary neurons. DCC and DSCAM co-expression further enhanced netrin-1-induced JNK1 activity in vitro. In contrast, netrin-1 had no effect on JNK2 or JNK3 activity. Phospho-JNK is strongly expressed in commissural axons before and as they crossed the floor plate, and netrin-1 stimulation dramatically increased the level of endogenous phospho-JNK in commissural axon growth cones. As predicted, inhibition of JNK signaling either by JNK1 RNA interference (RNAi) or the JNK inhibitor suppressed netrin-1-induced neurite outgrowth and axon attraction and JNK1 knockdown in ovo interfered with spinal cord commissural axon projection and pathfinding. Together, these results suggest that JNK1 may be involved in the coordination of DCC and DSCAM in netrin-1-mediated attraction.
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List of Abbreviations

12-HPETE…………………………12-Hydroperoxy-5, 8, 10, 14-Eicosatetraenoic Acid
γ-TuRC………………………………γ-tubulin ring complex
ANOVA………………………….Analysis of variance
CNS………………………………Central nervous system
DBD……………………………….DCC binding domain
DCC………………………………Deleted in colorectal cancer
DD………………………………Death domain
DSCAM………………………….Down syndrome cell adhesion molecule
FAK………………………………Focal adhesion kinase
FBS………………………………Fetal bovine serum
FNIII…………………………….Fibronectin III repeats
GPI………………………………Glycophosphatidylinositol
HEK………………………………Human embryonic kidney
Ig…………………………………Immunoglobulin
LCC………………………………L-type calcium channels
MAPs………………………….Microtubule associate proteins
MLB………………………….Mild lysis buffer
MKK………………………….MAPK kinase
Pak1………………………….p21-activated kinase 1
PFA………………………….Paraformaldehyde
PITP…………………………Phosphatidylinositol transfer protein
PLC…………………………..Phospholipase C
PLL………………………….Poly-L-lysine
PNS…………………………..Peripheral nervous system
Tsp…………………………Thrombospondin
TRPC………………………….Transient receptor potential channels
TUBB3……………………….Tubulin beta-3 chain
UNC5………………………………..Uncoordinated-5
UNC-6………………………………..Uncoordinated-6

WT………………………………..Wild type
Chapter 1

Background

1.1 The Nervous System

The vertebrate nervous system consists of two parts, the central nervous system (CNS) and the peripheral nervous system (PNS). The central nervous system includes the brain and spinal cord, while the peripheral nervous system consists of sensory neurons, ganglia, and nerves that connect them with the central nervous system.

Neurons and glial cells are the main components of the nervous system. A typical neuron is comprised of one long axon, the cell body or soma, and multiple short dendrites. Neurons communicate with each other or other cells via synapses. It is estimated that there are 100 billion neurons and 100 trillion synapses in the human brain. Glial cells usually provide neurons with structural and metabolic support.

1.2 Axon guidance

Axons often follow very precise pathways to find their targets, and how they manage to do that is a matter of axon guidance. Growing axons contain a highly dynamic structure at the growing tip called the growth cone, which senses the extracellular environment for signals that instruct the axon in which direction to grow. These signals,
called guidance cues, can be fixed on the membrane or diffusible to attract or repel axons. Growth cones express receptors, referred as guidance receptors, which recognize these guidance cues and interpret the signal into a chemotropic response. The general theoretical framework is that when the growth cone comes across a gradient of guidance cues, the intracellular signaling in the growth cone takes place asymmetrically \cite{1}\cite{2}\cite{3} and cytoskeletal rearrangement follows so that the growth cone turns towards or away from the guidance cue (attraction versus repulsion).

Molecular and genetic studies of axon guidance have led to identification of several families of guidance cues in the past 20 years. Among which, four canonical guidance cues have been well studied, such as netrin-1 with its receptor Deleted in Colorectal Cancer (DCC)\cite{4}, Down Syndrome Cell Adhesion Molecule (DSCAM)\cite{5} and Uncoordinated 5 (UNC5)\cite{6}, slits with Robos\cite{7}, ephrins with Ephs, and semaphorins with plexins and neuropilins. In addition, many other classes of extracellular molecules are involved in growth cone navigation: developmental morphogens, such as, BMPs, Wnts, Sonic Hedgehog, and FGFs; extracellular matrix and adhesion molecules, such as, laminin, tenascins, proteoglycans, N-CAM, and L1; growth factors like NGF and BDNF; and neurotransmitters like GABA \cite{15}.

### 1.3 Netrins

Netrins, a family of guidance cues, are conserved from worms to mammals \cite{8}. Netrin is first identified in the nematode *Caenorhabditis elegans* in 1990\cite{8}, and named UNC-6, according to the standard *C. elegans* naming protocol. The first mammalian homologue of UNC-6 is discovered in 1994\cite{9} as a vital guidance cue for rodent
commissural axons in the spinal cord. Structurally, netrin resembles the N terminal of extracellular matrix protein laminin \(^9\).

The vertebrate netrin-1 and netrin-2 are discovered as proteins that are capable of promoting axon outgrowth and attracting the commissural axon projection in the neural tube. Netrin-1 mainly distributed near the floor plate, and netrin-2 largely located in the ventral half of the spinal cord \(^{80}\). Netrin-3 is shown with a reduced ability to bind with DCC when compared with netrin-1, which suggests that it mainly operates through other receptors \(^{79}\). Netrin-Gs are secreted but remain bound to the extracellular surface of the cell membrane through glycophosphatidylinositol (GPI) \(^{79}\). They bind to ligand NGL-1 instead of DCC or UNC5 \(^{79}\). Netrin-Gs are expressed predominantly in the central nervous system in places such as the thalamus and mitral cells of the olfactory bulb \(^{79}\). It is believed that netrin-G1 and netrin-G2 evolve independently of other netrins in order to facilitate the construction of the brain \(^{79}\). Netrin-4 is found to be responsible for neural, kidney, and vascular development \(^{10}\).

Netrin-1 secreted by the developing floor plate in mouse embryos plays an essential role in directing commissural axon projection \(^{77}\). Netrin-1 deficiency in mouse exhibits the disruption of the formation of major axon projections to the midline in the brain, including the corpus callosum (CC) and hippocampal commissure (HC) \(^{9}\). Other than assisting axons crossing the ventral midline, netrin-1 is also required for axons of the retinal ganglion cells to exit the retina and enter the optic nerve as well as the axon projections within the hippocampus \(^{77}\). Additionally, netrin-1 knockout mice reveals profound defects in the organization of many axonal tracts, elevated cell death in the developing brain stem, and abnormal neuronal migration activities \(^{11}\).
1.4 Netrin-1 receptors

1.4.1 Deleted in Colorectal Cancer (DCC)

DCC is encoded by the DCC gene in vertebrates. Since it is first discovered in a colorectal cancer study in 1990\cite{8}, DCC has been the focus of a significant amount of research. Although DCC is considered controversial as a tumor suppressor gene, it is well known as an axon guidance receptor that responds to netrin-1\cite{17}\cite{18}. The human DCC gene is located at 18q21.3, and has a total of 57 possible exons and 43 possible introns, which theoretically results in 13 sliced proteins\cite{81}. The typical DCC protein has one signal peptide motif and 11 domains, including six immunoglobulin-like domains, one transmembrane domain, three fibronectin type 3 domains, and one intracellular domain. The DCC intracellular domain contains several protein binding subdomains, such as P1, P2 and P3 domains. The P1 domain is a highly conserved 17 amino acid motif and the P2 domain rich in proline residues, while the P3 domain contains several phosphorylation sites\cite{7}.

The role of DCC in commissural axon projection has been extensively investigated. In the developing spinal cord, commissural neurons located dorsally extend axons ventrally towards a ventral midline structure, the floor plate\cite{20}. A gradient of netrin-1 generated from the floor plate guides orientation of the extending commissural axons, aiding the development of the dorsal-ventral axis of commissural axon projection\cite{21}. When membrane DCC in the GC of commissural axons is stimulated by netrin-1, it promotes axon projection towards the midline. In mouse, bath application of DCC functional blocking antibodies is able to block the netrin-1 induced axon outgrowth of the
spinal cord explants [20]. Also, DCC deficient mice exhibit phenotypes very similar to those generated by loss of netrin-1 function, including loss of spinal ventral commissure, corpus callosum and hippocampal commissure [4].

1.4.2 Down syndrome cell adhesion molecule (DSCAM)

The human DSCAM gene is originally identified as a gene associated with the Down syndrome [5]. It encodes a transmembrane protein containing ten Ig domains, six fibronectin type III (Fn III) domains, one transmembrane domain, and one intracellular domain [5]. The Drosophila homolog of DSCAM is isolated in a screening for Dock interacting proteins [3]. Interestingly, the fly Dscam1 could potentially have 38,016 alternative splicing forms from four variable exon clusters, and the diversity of isoforms from alternative splicing of the Dscam1 gene allows neurons in D. melanogaster to display different sets of Dscam proteins on its cell surface [23][24]. Interactions of Dscam isoforms stimulate self-avoidance mechanisms [25] that are essential for normal neural circuit development and neuronal wiring. Human DSCAM also plays an important role in neurite arborization [26], cell body spacing [27][28], and lamina-specific synaptic targeting in vertebrate retina [29]; although the vertebrate DSCAM only encodes few splicing isoforms [30].

DSCAM is shown to bind to p21-activated kinase 1 (Pak1) and stimulate Pak1 activity [31]. DSCAM functioned as a netrin receptor in collaboration with DCC involved in netrin-dependent commissural axon outgrowth and path finding [32][33]. DSCAM is also shown to coordinate with UNC5C in axon repulsion [33]. Our recent research also
indicates that DSCAM is able to associate with focal adhesion kinase (FAK) and Fyn in netrin signaling \[^{[33]}\].

1.4.3 Uncoordinated-5 (UNC5)

There are four vertebrate UNC5 homologs, UNC5A-D, compared to just one in *C. elegans* and *Drosophila*. The UNC5 extracellular domain contains two Ig domains and two thrombospondin (Tsp) domains, while the intracellular domain consists of a ZU-5, a DCC binding domain (DBD), and a death domain (DD) \[^{[34]}\]. The protein sequence of the ZU-5 domain is similar to the Zona Occludens-1 scaffolding protein found in tight junctions. In *C. elegans*, the cytoplasmic region between ZU-5 and DD is crucial in the downstream protein binding. The DCC binding domain is involved in netrin-1 long-range repulsion, while UNC5 alone can function in short-range netrin-1 repulsion \[^{[35]}\]. It is also reported that UNC5 may coordinate with either DCC or DSCAM in initiating the repulsive signaling \[^{[12]}\].

In mice, UNC5 mutants exhibit defects in dorsally directed migrations, away from the ventral midline source of netrin-1. Also neurons deficient in UNC5 tend to have their axons redirected along a dorsal trajectory \[^{[12]}\]. In addition, the UNC5B knockout mice exhibit impaired growth cone collapse response as well as background sensitive lethality during organogenesis \[^{[8]}\].

1.5 Netrin-1 signaling
Netrins are chemotropic and bifunctional, functioning as either a chemoattractant or chemorepellent depending on the receptors that they bind to\textsuperscript{(11)}. Notably, DCC or DSCAM alone induces attractive responses, whereas UNC5 or combination of UNC5 with DCC or DSCAM mediates repulsion\textsuperscript{(12)}.

Several signaling pathways have been characterized to address the mechanisms involved in the axonal attraction. Netrin-1 increased the homodimerization of DCC\textsuperscript{(4)} and recruitment of the focal adhesion kinase (FAK)\textsuperscript{(13)}\textsuperscript{(2)}, Fyn, Src, p130CAS and DOCK180 through DCC to form a multi-protein signal complex\textsuperscript{(14)} which is hypothesized to lead to activate small GTPases of Rho family such as Rac1 and Cdc42, thereby promoting axon outgrowth and turning. Phosphatidylinositol transfer protein α (PITP α) binds to phosphorylated DCC regulating phospholipase C (PLC) to increase the ratio of cAMP to cGMP, which in turn activates L-type Ca\textsuperscript{2+} and transient receptor potential channels (TRPC’s) leading to an influx of extracellular Ca\textsuperscript{2+}. It is reported that this increased calcium is responsible for initiating growth cone extension as well as the activation of Rho GTPases, Cdc42, Rac1 and the nuclear transcription factor NFAT\textsuperscript{(15)}. Additionally, netrin targets NcK and Wiskott–Aldrich syndrome protein (ISP) to trigger axonal growth\textsuperscript{(16)}.

For repulsion mechanisms, it is currently hypothesized that netrin-1 activates the downstream signaling cascades upon binding to UNC5 only or UNC5 with DCC or DSCAM\textsuperscript{(33)}. Netrin-1 increases the intracellular levels of 12-HPETE (12-Hydroperoxy-5, 8, 10, 14-Eicosatetraenoic Acid), which induces cGMP signaling and subsequently causes a decrease in the cAMP/cGMP ratio. This decrease contributes to the subdued cellular Ca\textsuperscript{2+}, which ultimately results in growth cone repulsion through a possible
activation of RhoA. Additionally, the recruiting of the tyrosine phosphatase Shp2 as well as FAK and Src to the netrin-1/UNC-5 complex may also trigger chemorepulsion through RhoA\[82\]. UNC5 is also shown to be able to collaborate with DSCAM in regulating repulsion through binding with FAK, Fyn, and Pak1 \[31\]\[33\].

1.6 Cytoskeleton

1.6.1 Microtubule

While it is generally believed that actins play an important role in growth cone steering, recent studies suggest that nervous system development is highly dependent on the microtubule cytoskeleton \[36\]\[37\]. For example, the inhibition of microtubule dynamics by taxol or nocodazole abolished the growth cone turning behavior \[38\] and mutations of α- or β-tubulin subunits caused defects in neuronal migration and axon projection in the developing nervous system \[38\].

Microtubule, rope-like polymers of tubulin, can grow as long as 25 micrometers and are highly dynamic \[39\]. The outer diameter of microtubule is about 25 nm \[39\]\[40\]. Microtubules are important for maintaining cell structure, providing platforms for intracellular transport \[41\], forming the spindle during mitosis \[42\], as well as other cellular processes. Many proteins that bind to the microtubule, including motor proteins such as kinesin and dynein, and severing proteins like katanin, play an important role in regulating microtubule dynamics \[42\]\[43\].

Microtubule dynamics refers to the coexistence of assembly and disassembly at the plus end of a microtubule. The microtubule can switch between the growing and
shrinking phases dynamically at this region\textsuperscript{[44]}. During polymerization, both the $\alpha$- and $\beta$- subunits of the tubulin dimer are bound to a molecule of GTP. While the GTP bound to $\alpha$-tubulin is stable, the GTP bound to $\beta$-tubulin hydrolyzed to GDP shortly after assembly. A GDP-bound tubulin subunit at the tip of a microtubule will fall off, though a GDP-bound tubulin in the middle of a microtubule is stable. A cap of GTP-bound tubulin at the tip of the microtubule is required for tubulin to add onto the end of the microtubule to protect it from disassembly. When hydrolysis catches up to the tip of the microtubule, it begins a rapid depolymerization and shrinkage. This switch from growth to shrinkage is called a catastrophe\textsuperscript{[83]}, while rescue is referred to the process that GTP-bound tubulins begin adding to the tip of the microtubule again, providing a new cap and protecting the microtubule from shrinking\textsuperscript{[84]}.

In the axon shaft, microtubules are bundled by microtubule-associated proteins (MAPs), whereas in the growth-cone C-domain (central domain), they are spread apart. In the growth cone, microtubules may extend through the T-zone (transit zone) as far as the filopodia in the P-domain (peripheral domain). Microtubules in growth cones exhibit dynamic instability, constantly converting between catastrophe and rescue\textsuperscript{[85]}.

\subsection*{1.6.2 \textit{Actin}}

Actin is one of the most highly-conserved proteins, differing by no more than 20\% in species as diverse as algae and humans. Actin is present as either F-actin (filamentous actin), a subunit of microfilaments (one of the main components of the cytoskeleton) or G-actin (globular actin), a monomer. Actin participates in many
important cellular processes including cell mobility, division, signaling, and the establishment and maintenance of cell junctions and cell shape by interacting with actin binding proteins \[^{45}\]. In vertebrates, three main groups of actin isoforms, alpha, beta, and gamma have been identified. The alpha actin is mainly located in muscle tissues and serves as a major constituent of the contractile apparatus. The beta and gamma actins co-exist in most cell types as components of the cytoskeleton \[^{45}\].

Nucleating factors are essential to stimulate actin polymerization. For example, the Arp2/3 complex functions as a barbed end of actin to stimulate the nucleation of G-actin. Also, actin filaments themselves bind to ATP, and hydrolysis of this ATP stimulates destabilization of the polymer. The growth of actin filaments can be regulated by thymosin and profilin. Thymosin binds to G-actin to buffer the polymerizing process while profilin binds to G-actin to exchange ADP for ATP promoting the monomeric addition to the plus end \[^{46}\].

Dynamic interaction between microtubules and actin filaments in the growth cone is an essential event that underlies neurogenesis, growth-cone advance and growth-cone turning, which are fundamental behaviors during axon guidance \[^{37}\].

### 1.7 Tubulin beta-3 chain (TUBB3)

Microtubule behavior varies according to isotype composition, suggesting each isotype may have properties necessary for specific cellular functions \[^{47}\][^48][^49]. β-tubulin isotype III (TUBB3) is one of the at least 6 tubulin subunits found in mammals \[^{50}\]. Purified microtubules enriched in TUBB3 are considerably more dynamic than those
composed from other β -tubulin isotypes\textsuperscript{[51],[52]}. Also, the expression of TUBB3 is primarily limited to neurons, and has been widely used as a neuronal marker to differentiate neurons from glial cells.

TUBB3 expression level is the highest during periods of axon guidance and maturation in the developing nervous system, while it decreases in the adult central nervous system (CNS) but remains high in the peripheral nervous system (PNS) \textsuperscript{[83]}. The unique dynamic properties and spatiotemporal expression patterns of TUBB3 suggest that it has a specific function for neuronal development and axon maintenance. It has also been reported that mutated TUBB3 could lead to a spectrum of human nervous system disorders including hypoplasia of oculomotor nerves, dysgenesis of the corpus callosum, anterior commissure, and corticospinal tracts\textsuperscript{[53]}.

Recent studies also show that TUBB3 is associated with various tumors, and has been recognized as a prominent mechanism of drug resistance\textsuperscript{[54]}.

1.8 c-Jun N-terminal kinase 1 (JNK1)

JNKs, one subfamily of the MAPKs, are involved in a variety of biological processes, including apoptosis, cell proliferation, cell migration, and cytokine production \textsuperscript{[65]}. JNKs are strongly expressed in the nervous system and play an important role in neuronal degeneration, development, migration, polarity, regeneration, as well as learning and memory \textsuperscript{[66]}. JNK activation is triggered by MAPK kinase 4 (MKK4) and MKK7 \textsuperscript{[67],[68]}. Mammalian JNKs are composed of JNK1, JNK2, and JNK3 with at least 10 different splice isoforms. Although initial studies indicated compensative roles for JNK1 and
JNK2 in neuronal survival and apoptosis during early brain development [71], and JNK2 and/or JNK3 knock-out mice have no obvious brain defects, JNK1-deficient mice display an abnormality in anterior commissure formation and axonal microtubule integrity, as well as altered dendritic architecture [69][70], suggesting that the JNK1 pathway may be involved in axon guidance in the developing nervous system.

JNK, by phosphorylation, modifies the activity of numerous proteins that reside at the mitochondria or act in the nucleus [73]. Downstream molecules that are activated by JNK include c-Jun, ATF2, ELK1, SMAD4, p53 and HSF1. The downstream molecules that are inhibit by JNK activation include NFAT4, NFATC1 and STAT3 [73]. JNKs are required for both the integrity and the function of the cytoskeleton and the reception of signal emanating from the cytoskeleton [74]. Several cytoskeletal components are JNK substrates such as neurofilaments and microtubule-associated proteins (MAPs) [74]. In cultured neurons, the distribution of JNKs reveals a predominant distribution within cytoskeleton-associated structures such as synapses, growth cones [75].
Chapter 2

Significance

During the nervous system development, differentiating neurons require dynamic populations of microtubules in order to appropriately respond to guidance cues [5]. TUBB3 is the most dynamic tubulin isotype [55], whose expression is restricted primarily to the CNS and PNS. Thus, it is possible that TUBB3 may be required for specific developmental process, such as axon guidance.

Notably, the dysgenesis of the corpus callosum, anterior commissure, and corticospinal tracts are observed in the TUBB3 mutated patients, suggesting TUBB3 is involved in axon guidance. Neuroimaging, clinical manifestations of cranial motor nerve misrouting, and the phenotypic analysis of a TUBB3 disease mouse model elucidate a critical role of TUBB3 in proper axon guidance. These phenotypes are similar to that of netrin-1 knockout mouse [53]. Thus, TUBB3 might be involved in netrin-1 induced axon guidance.

Recent studies have shown that DSCAM collaborates with DCC in netrin-1 induced axon outgrowth and projection [32], and the animal models of DCC knockout [19] or DSCAM knockdown also share similar phenotypes with those of netrin-1 knockout [11]. Although netrin-1 can bind to the receptors on the growth cone to initiate the growth cone steering, and several downstream signaling cascades have been identified in the regulation of the asymmetrical movement of the cytoskeleton [16], what links the
downstream signaling cascades with cytoskeleton dynamics remains unclear. Thus, we propose that TUBB3 may play a role in netrin-1 signaling by interacting with DCC or DSCAM.

The JNK family of MAPKs is involved in a large variety of physiological and pathological processes in brain development, such as neural survival, migration, and polarity as well as axon regeneration\textsuperscript{[65],[66]}. Given JNKs’ function as the regulator of the microtubule associated proteins, the clarification of its role in microtubule dynamics and axon guidance will provide us more insight into the neuronal circuit formation in the developing nervous system.
Chapter 3

Materials and methods

3.1 Plasmids and oligonucleotides

Full-length DSCAM tagged with a Flag tag at the C terminus was described previously [33]. The TUBB3-siRNA pool was purchased from Dharmacon. Plasmids encoding the full-length human DSCAM-FLAG, DSCAMΔN, DSCAMΔC, the full-length human DCC, and DCC truncation mutants (∆P1, ∆P2, and ∆P3) have been described previously. The full-length human JNK1 was PCR-amplified from a human brain cDNA library and subeloned into a pcDNA3 vector. PcDNA3 FLAG JNK2a2 and pcDNA3 FLAG JNK3a1 plasmids were provided by Roger Davis (Addgene, Cambridge, MA). The targeted sequences of control shRNA, DCC shRNA, DSCAM shRNA, control JNK1 shRNA, and JNK1 shRNA are as follows: 5′-AATGCATCTCTGCAAGAGGTA-3′ (control DCC shRNA); 5′-CATCCGATGTGCGACTGTA-3′ (DCC shRNA); 5′-AAAGAGTTTAGCTGAAATGCT-3′ (DSCAM shRNA); 5′-CCAGTCAGGCAAGGGATTT-3′ (control JNK1 shRNA), and 5′-CCTTCATTCTGCTGGAATT-3′ (JNK1 shRNA), respectively. The oligonucleotide templates were inserted into the mU6pro vector and verified by sequencing. Mouse JNK2 and JNK3 siRNAs were purchased from Santa Cruz Biotechnology (sc-39102 for JNK2
siRNAs and sc-39104 for JNK3 siRNAs). DCC-ICD-MBP and DSCAM-ICD-GST were generous gifts from Dr. Weiquan Li. TUBB3 control shRNA and TUBB3 shRNA were generous gifts from University of Michigan.

### 3.2 Antibodies and other reagents

Affinity-purified anti-DSCAM antibody was described previously [33]. Anti-Myc antibody (mouse, 9E10) was obtained from the Developmental Studies Hybridoma Bank. Other antibodies were obtained from commercial sources: anti-HA (Santa Cruz Biotechnology), anti-Flag, anti-TUBB3, and phosphor-tyrosine antibody 4G10 (Abcam), anti-DCC (BD Biosciences), anti-DCC functional blocking antibody (Calbiochem), Tuj1 (Covance), and rabbit anti-JNK3 (Upstate Biotechnology, Lake Placid, NY). Rabbit polyclonal antibodies (anti-p38, anti-phospho-p38, anti-JNK2, anti-ERK1/2, anti-phospho-ERK1/2, and anti-phospho-JNK) were obtained from Cell Signaling Technology (Danvers, MA). B27, SP600125, DAPI, Alexa Fluor® 555 phalloidin, Alexa Fluor® 488 donkey anti-mouse IgG, Alexa Fluor® 488 donkey anti-rabbit IgG, Alexa Fluor® 647 goat anti-rabbit IgG, and Alexa Fluor® 633 goat anti-mouse IgG were purchased from Invitrogen. Cy3-conjugated anti-mouse IgM was purchased from Jackson ImmunoResearch (West Grove, PA). Purified chick netrin-1 protein was either obtained from R&D or made from the conditioned media of HEK293 cells as described previously [1]. *Clostridium difficile* toxin B and EGF were purchased from Sigma, and KinaseSTAR™ JNK activity assay kit was from BioVision (Milpitas, CA). Other
3.3 Immunoprecipitation and western blot analysis

HEK cells were transiently transfected with the calcium phosphate method according to a protocol described previously [14]. To test protein-protein interaction, the cells were harvested and lysed 48 hours after transfection in MLB lysis buffer [1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 10mM MgCl2 and protease inhibitor mixture (Roche)]. Extract were centrifuged at 14,000 rpm for 20 min, and the supernatants were incubated with the primary antibody for 2 hours and then further incubated with protein A/G beads (Santa Cruz Technology) for one additional hour. The precipitates were separated by SDS-PAGE, transferred to PVDF membranes and analyzed with specific antibodies. To netrin-1 stimulation, transfected cells were starved for 6 hours in serum-free media and then incubated with netrin-1 protein (250 ng/ml) for 20 min before lysing. For the phosphotyrosine experiment, phosphatase inhibitors were added into MLB lysis buffer. Primary cortical neurons were lysed with RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% Na.Deoxycholate, 1% Nonidet P-40, protease inhibitors). Lysates were immunoprecipitated with specific antibodies and protein A/G beads for 2 hours.
3.4 Dissociated primary neuron culture and RNAi electroporation

The culture procedure for dissociated neurons was described previously\textsuperscript{[33]} with some modifications. Briefly, the E15 (E1, the day of vaginal plug; Charles River) mouse cortex, and the E13 mouse spinal cord were dissected in cold HBSS (invitrogen), and the meninges were removed. Tissues were dissociated at 37°C using 0.25% Trypsin for 15 mins and 20 U/ml DNaseI (Roche) for 5 mins. Cells were triturated with ice-cold DMEM culture medium containing 10% Fetal Bovine Serum (FBS), centrifuged at 1000 RPM, and washed 3 times in culture medium before plated at 4x10\textsuperscript{6} cells on PLL-coated (50 ug/ml) 35 mm tissue culture dishes for biochemical analysis or 1x10\textsuperscript{5} cells on PLL-coated glass coverslips for the immunostaining.

When RNAi was performed, dissociated neurons (4x10\textsuperscript{6}/group) were mixed with Venus-YFP plasmids or Venus-YFP plus control shRNAs (4 ug), shRNAs (4ug) or siRNA (4ug) and immediately placed into a Nucleofector (Amaxa Biosystems). After electroporation (program O-005), the cells were immediately transferred into warm media, and plated on the PLL-coated dishes or coverslips.

3.5 Immunocytochemistry

Primary neurons on coverslips were fixed with 4% prewarmed paraformaldehyde solution (127 mM NaCl, 5mM KCl, 1.1 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.4 mM KH\textsubscript{2}PO\textsubscript{4}, 2 mM MgCl\textsubscript{2}, 5.5 mM glucose, 1 mM EGTA, 10 mM PIPES), permeabilized with 0.1% TritonX-100
for 15 mins and blocked in 10% normal goat serum for 1 hr. Cells were incubated in the primary antibodies to DCC and p-JNK over night at 4°C, washed 3 times in PBS, and incubated with the secondary antibodies for 1 hr at 37°C. After being washed 3 times, coverslips were counterstained with DAPI, washed again, and mounted onto slides using Fluorogel. Images were taken under confocal microscope (Olympus IX70 Fluoview).

3.6 Protein purification

DCC-ICD-MBP/DSCAM-ICD-GST vectors are obtained from Dr. Weiquan Li, University of Michigan. Briefly, the vectors were transformed into BL21 cells, and plated on LB agar with ampicillin (100 mg/ml). A single colony was picked after 16 hours, and transferred to 250ml of LB broth with ampicillin (100 mg/ml) for overnight culture at 37 °C. The broth culture was induced by adding 0.1 mM IPTG for 2 hours after the OD reached 0.8. The culture was centrifuged at 7k rpm for 10 min at 4 °C, and the supernatant was discarded afterwards. The bacterial pellets were resuspended by 10 ml chilled lysis buffer (50 mM Tris, pH7.5, 150 mM NaCl, 0.05% NP-40), and incubated on ice for 20 minutes. Afterwards, the lysed bateria were sonicated at output of 30% for 140 seconds (20 seconds sonication with 40 seconds break). Then, the lysed bacteria were centrifuged at 15k rpm for 20 minutes, and the supernatant was subjected to a GST or MBP column for purification. The columns were washed by the wash buffer (50 mM Tris, pH 7.5) for 3 times, and the purified proteins were eluted by 2 ml of elution buffer (50 mM Tris, pH 7.5, Glutathione (GST) or maltose(MBP)) for 6 times. The eluted
proteins will be stained by coomassie blue staining to determine the purity, and snap frozen in the nitrogen tank for future use.

3.7 Microtubule cosedimentation assay

Cells were lysed at 4 °C for 10 minutes, and let sit for 20 minutes at 4 °C, then spun at 100,000 x g for 1 hour at 4 °C. The supernatant was collected, and placed on ice in a pre-chilled tube. The lysate was subsequently supplemented with 40 µM Taxol and 1 mM GTP. Afterwards, the samples were incubated at room temperature for 30 minutes, layered over a 10% sucrose cushion and centrifuged at 50,000 x g for 30 minutes at 4 °C. The supernatant was collected and frozen in liquid nitrogen, while the pellets were washed with wash buffer (50 mM HEPES, 1mM MgCl2, 1 mM EGTA, protease inhibitors, PMSF) and resuspended with tubulin buffer (50 mM HEPES, 1mM MgCl2, 1 mM EGTA, protease inhibitors, PMSF, 10% glycerol, 150 mM KCl, 40 uM taxol, 1 mM GTP, 5 mM Mg-ATP).

3.8 Axon outgrowth assay

To analyze neurite outgrowth of E15 cortical neurons, the dissociated neurons (4x10^6 cells per group) were nucleofected, and diluted in pre-warmed DMEM medium (100,000 cells per well). Neurons were cultured in growth media (DMEM, 10%FBS, penicillin/streptomycin) for 2 hours, and then transferred to serum free medium (DMEM, B27, penicillin/streptomycin) for 18-20 hours containing purified netrin-1 (250 ng/ml) or...
PBS. Cells were subsequently fixed with 4% PFA for 10 min at 37 °C, and stained with phalloidin (Invitrogen), while the nuclei were visualized with the DAPI dye.

For chicken spinal cord explants, white leghorn chicken embryos were collected and staged according to methods described by Hamburger and Hamilton [86]. Electroporation was done as described previously [33]. Briefly, plasmids or siRNA (4µg/µl) were injected into the neural tube of chicken embryo in ovo at stage 12-15. The electroporation was done on a BTX ECM830 instrument at 25 V, 5 ms, 5 pulses. The embryos were dissected out at stage 18-20 when commissural axon had not contacted the floor plate in vivo. The dorsal half of the spinal cord was dissected, and cut into explants of around 200 µm in size, and transferred into culture dishes. 35 µl of mix gel (3:2:1 collagen, matrigel, medium) was used to immobilize the explants. Following polymerization, the explants were cultured with conditioned media of either control HEK cells or netrin-1 secreting HEK cells. After 18 hours of culture, the explants were fixed with 4% PFA in PBS overnight at 4 °C, and the images of these explants were taken under a fluorescent microscope. The numbers of axons and the total axon length were measured using the National Institute of Health Image J software.

### 3.9 Chicken spinal cord axon turning assay

The electroporation was conducted as described above. At the stage 20-21 [56], embryos were dissected out and examined under the fluorescent microscope. The labeled half spinal cords were isolated and co-cultured alongside an aggregate of the control or netrin-1 secreting HEK cells for 40 hours. As described previously [33], an axon was
considered turning when the angle of turning towards the aggregate was above 5 degrees. The percentage of axon turning was calculated based on the numbers of turning axons divided by the total axons visualized within 300 um distance of the cell aggregates.

3.10 Analysis of commissural axon projection in vivo

Chicken embryos were collected at the stage 22-23 after electroporation when commissural axons in the lumbosacral region cross the midline. Samples expressing Venus-YFP were chosen for open-book preparation, and the lumbosacral region of the spinal cord was mounted in Gel Mount, and images were taken under a confocal microscope. Transverse sections (thickness at 200 µm) were collected after electroporation and mounted. Stacked images were collected through the same confocal microscope.

3.11 JNK activity assay

JNK activity assay was performed following the instructions on the kit (Biovision). Briefly, both transfected HEK293 cells and primary neurons were lysed on ice for 5 min, and the supernatant was immunoprecipitated with anti-JNK antibody. The protein A-Sepharose was mixed with each sample for 1 h at room temperature followed by incubating with c-Jun Protein/ATP mixture at 30°C for 2 h. The supernatant was collected after brief centrifugation, mixed with protein loading dye, and boiled for 3 min. Protein samples were separated with 7.5% SDS-PAGE, and the Western blot was probed
with the rabbit anti-phospho-c-Jun antibody. ERK activity was analyzed by incubating the precipitated kinase with the substrate (2 μg of GST-Elk) in the kinase assay buffer in the presence of 10 μCi of [γ-32P] ATP at 30 °C for 20 min. The kinase reaction was analyzed by SDS-PAGE. To examine other protein expression, western blots were analyzed using specific antibodies, such as anti-DCC, anti-DSCAM, anti-phospho-JNK, anti-phospho-p38, anti-phospho-ERK1/2, anti-p38, anti-ERK1/2, and anti-JNK antibodies. For examining the effect of RNAi knockdown, dissociated primary neurons were cultured on PLL-coated dishes for 2 days after nucleofection, and cell lysates were then analyzed by Western blotting.

### 3.12 Statistical methods

All experiments including biochemical and functional assays were repeated independently for at least three times. The relative intensity of the bands in western blots were acquired through NIH ImageJ, and normalized. In the neurite outgrowth and turning assay, the total cell number of each replicate was used in the quantification. All the data in each experiment was subject to one way ANOVA post-hoc analysis through SPSS.
Chapter 4

Results

4.1 TUBB3 interacts with DCC

To examine the potential interaction of TUBB3 with DCC in the developing brain, primary neurons from the cerebral cortex of embryonic day 15 (E15) mice were isolated, cultured and treated with conditioned media from control HEK cells or HEK cells stably expressing netrin-1. Cell extracts were immunoprecipitated with anti-DCC antibody before probing the blots with the anti-TUBB3 antibody. Anti-DCC antibody immunoprecipitated TUBB3 (Fig. 4-1A). In contrast, as predicted, TUBB3 was not present in the coIP when primary antibody was omitted (see the “IgG” control lanes in Fig. 4-1A). Treatment with netrin-1 conditioned media increased the interaction of TUBB3 with DCC (Fig. 4-1A, quantification in Fig. 4-1C, upper panel). Netrins play a crucial role in promoting commissural neuron axon outgrowth and pathfinding in the developing spinal cord. To determine whether endogenous TUBB3 interacted with DCC in spinal cord neurons, dissociated cells from the dorsal half of E13 mouse spinal cords were cultured and treated with netrin-1 conditioned media. DCC interacted with TUBB3 and netrin-1 dramatically increased this interaction (Fig. 4-1B, quantification in Fig. 4-1C, lower panel). Netrin-1 increased the interaction of TUBB3 with DCC within 5 min and the induction lasted up to 20 min after netrin-1 stimulation (Fig. 4-1D). To confirm
that netrin-1 was directly responsible for increasing the interaction of TUBB3 with DCC, we tested purified netrin-1 from two sources: chicken netrin-1 from a commercial source and human netrin-1 purified from a stable HEK cell line established in our lab. Both sources of netrin-1 increased the interaction of TUBB3 with DCC in E15 primary cortical neurons in a dose-dependent manner (Fig. 4-1E). In contrast, DCC did not co-immunoprecipitate with TUBB1 and TUBB2, two other β-tubulin subunits, in the absence or presence of purified netrin-1 (Fig. 4-1F). These results strongly suggest that TUBB3 specifically associates with DCC in primary neurons.

To determine whether TUBB3 interacted directly with DCC, cDNAs expressing HA-tagged human TUBB3 were co-transfected with DCC-Myc into HEK293 cells. Anti-HA antibody immunoprecipitated DCC (Fig. 4-1G), detected by probing the blots with anti-Myc antibody. This result suggests that TUBB3 may directly interact with DCC. To further characterize the interaction, an isolated intracellular domain of DCC (DCC-ICD-MBP) was purified and incubated with purified TUBB3. TUBB3 interacted directly with DCC-ICD-MBP (Fig. 4-1H). DCC has three conserved intracellular domains (P1, P2, and P3) that are required for netrin-mediated signal transduction. To identify the DCC domain(s) that interact with TUBB3, TUBB3 was co-transfected with three distinct DCC truncation mutants, ∆P1 (deletion of residues 1147-1171) or ∆P2 (deletion of residues 1335-1356) or ∆P3 (deletion of residues 1426-1447) into HEK293 cells (Fig. 5-1). TUBB3 co-immunoprecipitated with a truncated DCC lacking the P1 domain, but not truncated DCCs lacking either the P2 or P3 domains (Fig. 1I). These results suggest that the intracellular P2 and P3 domains of DCC may be required for its interaction with TUBB3.
Figure 4-1. Interaction of TUBB3 with DCC. (A-B) Interaction of endogenous TUBB3 with DCC in E15 cortical (A) and E13 dorsal spinal cord (B) neurons. (C) Quantification of A and B from three independent experiments. * indicates p< 0.05 (two-tailed Student’s t-test). (D) Netrin-1 increased the interaction of endogenous TUBB3 with DCC in a time-
dependent manner. Lysates of dissociated neurons from E13 mouse spinal cords were
immunoprecipitated with anti-DCC and analyzed with anti-TUBB3. (E) Netrin-1
increased the interaction of endogenous TUBB3 with DCC in a dose-dependent manner.
E15 primary cortical neurons were treated with purified netrin-1 from 10, 50, to 200
ng/ml. (F) Endogenous DCC interacted with TUBB3, not TUBB1 and TUBB2, in E15
cortical neurons with or without netrin-1 treatment. Cell lysates of dissociated neurons
from E15 mouse cortexes were immunoprecipitated with anti-DCC and followed by
probing with anti-TUBB1, anti-TUBB2 or anti-TUBB3. (G) Interaction of TUBB3 with
DCC in HEK293 cells. TUBB3-HA was co-transfected with DCC-Myc into HEK293
cells. Anti-HA (TUBB3) precipitated DCC-Myc. (H) Direct interaction of TUBB3 with
DCC. Purified GST-TUBB3 was incubated with purified intracellular domain of DCC
tagged with MBP in vitro. The anti-GST antibody was used to immunoprecipitate
TUBB3 proteins and the blot was analyzed with anti-MBP. (I) P2 and P3 domains in
DCC are required for the interaction of DCC and TUBB3. TUBB3-HA was co-
transfected with different truncated DCCs (DCC-ΔP1, ΔP2 and ΔP3) tagged with Myc in
HEK293 cells.

4.2 MT dynamics modulates the interaction of TUBB3 with DCC

4.2.1 The netrin-1 induction of TUBB3/DCC interaction requires MT
dynamics
MT dynamics in growth cones plays a crucial role in axon guidance. To investigate whether MT dynamics is required for the binding of TUBB3 to DCC, primary E15 cortical neurons were treated with paclitaxel (taxol) or nocodazole, drugs that disrupt MT dynamics. As expected, netrin-1 increased the interaction of endogenous TUBB3 with DCC (Fig. 4-2A-C). The netrin-1-induced interaction was inhibited by both the MT-stabilizing drug taxol and the MT-destabilizing drug nocodazole (Fig. 4-2A-C), indicating that MT dynamics is required for the netrin-1 dependent interaction of TUBB3 with DCC. To further determine whether monomeric or polymeric TUBB3 binds to DCC, a MT cosedimentation assay was performed on E15 cortical neuron cell lysates. MT in cell lysates become unstable in vitro and constantly depolymerize at lower temperatures. Incubation of the lysate on ice induced MT depolymerization and yielded monomeric and/or dimerized TUBB3 subunits in the soluble supernatant and polymerized TUBB3 in the pellet after centrifugation (Fig. 4-2D and F). Most of the endogenous DCC also remained in the supernatants with or without netrin-1 stimulation (Fig. 4-2D-E). In contrast, in the presence of taxol in cell lysate that prevented MT depolymerization during cold treatment, DCC cosedimented with polymerized MT, with a large quantity of DCC and TUBB3 in the pellet (Fig. 4-2D and quantification in Fig. 4-2E-F). As predicted, in the presence of taxol, netrin-1 further increased DCC and TUBB3 in the pellets (Fig. 4-2D and quantification in Fig. 4-2E-F). These results indicate that netrin-1 induces the interaction of endogenous DCC with polymerized TUBB3 in primary neurons.
Figure 4-2: The induction of the interaction of TUBB3 with DCC by netrin-1 depends on MT dynamics. (A-C) Taxol and nocodazole (Noc) inhibited netrin-1-induced interaction of endogenous TUBB3 with DCC. E15 cortical neurons were treated with purified netrin-1 in the presence of different concentrations of taxol or nocodazole (1µM taxol and 3 µM nocodazole in A). (B) Quantification of (A) from three independent experiments showing relative binding of DCC and TUBB3. (D-F) E15 cortical neurons were stimulated with netrin-1 and the cosedimentation assay of cell lysates was performed.
in the absence or presence of taxol. DCC and TUBB3 in the pellet (P) and supernatant (S) fractions were examined by western blot analysis using anti-DCC and anti-TUBB3, respectively. (E and F) Quantification of three independent experiments showing P/S ratio of DCC (E) and TUBB3 (F), respectively. ns, not significant ; ***, p<0.001 (two-tailed Student’s t-test). Netrin-1 increased the cosedimentation of DCC and TUBB3 with polymerized MTs in primary neurons.

4.2.2 The activity of Src family kinases is required for netrin-1 induction of TUBB3 tyrosine phosphorylation and the interaction of TUBB3 with DCC.

Tyrosine phosphorylation of signaling molecules such as FAK and Src is required for netrin signaling [2]. Tyrosine phosphorylation of MT subunits is crucial for intracellular MT organization and dynamics [2][59]. To investigate whether the tyrosine phosphorylation of TUBB3 is involved in netrin signaling, primary neurons from E13 spinal cords and E15 cortices were dissociated and examined for the TUBB3 tyrosine phosphorylation in the presence of or absence of netrin-1 (Fig. 4-3A-C). TUBB3 tyrosine phosphorylation was increased by netrin-1 in a dose- and time-dependent manner (Fig. 4-3A-C). Netrin-1-induced TUBB3 tyrosine phosphorylation was inhibited by PP2, an inhibitor of the Src family kinases, but not PP3, a non-functional control for PP2 (Fig. 4-3D). PP2 also inhibited he netrin-1-dependent induction of the endogenous interaction of TUBB3 with DCC in E15 neurons (Fig. 4-3E-F). These findings indicate that Src family kinases are required for the netrin-1-induced tyrosine phosphorylation of TUBB3 and the interaction of TUBB3 with DCC. To further examine the role of Src family kinases in the
netrin-1-induced binding of endogenous DCC to dynamic TUBB3, these key proteins were cosedimented from lysates of dissociated E15 primary cortical neurons after netrin-1 stimulation (Fig. 4-3G-H). As expected, netrin-1 increased the amount of DCC in the MT-sedimented pellets (Fig. 4-3G-H) and PP2, not PP3, inhibited the netrin-1-induced cosedimentation of DCC and MTs (Fig. 4-3G-H). These results suggest that Src family kinases play an important role in regulating the dynamic interaction of TUBB3 with DCC in primary neurons.
Figure 4-3: Src family kinase activity is required for the induction of TUBB3 tyrosine phosphorylation and the interaction of TUBB3 with DCC by netrin-1. (A-B) Induction of TUBB3 tyrosine phosphorylation in dissociated cortical neurons by netrin-1. E15 cortical neurons were treated with purified netrin-1 from 5 to 20 min. The anti-pY antibody was used to immunoprecipitate proteins and the immunoblot was analyzed with anti-
TUBB3. (C) Induction of TUBB3 tyrosine phosphorylation in dissociated E13 dorsal spinal cord neurons by netrin-1. Primary neurons were treated with purified netrin-1 (200 ng/ml). (D) Src family kinase-specific inhibitor PP2 inhibited netrin-1-induced TUBB3 tyrosine phosphorylation. E15 cortical cells were stimulated with netrin-1 in the presence of PP2 or PP3. Quantification was shown in the lower panel. * indicates $p<0.05$ (Student’s $t$-test). (E) PP2, but not PP3, blocked netrin-1-induced interaction of endogenous TUBB3 with DCC in primary neurons. (F) Quantification from three independent experiments showing relative binding of DCC and TUBB3 in (E). (G-H) Netrin-1-stimulated cosedimentation of DCC with MTs was inhibited by PP2, not PP3. E15 cortical neurons were stimulated with netrin-1 in the presence of PP2 or PP3. DCC in the pellet and supernatant fractions was examined by Western blot. Quantification of (G) was shown in (H). *** indicates $p<0.001$ (One-way ANOVA and Fisher LSD post-hoc comparisons).

4.3 TUBB3 is required for netrin-1-induced neurite outgrowth

4.3.1 The knockdown of TUBB3 inhibits netrin-1-induced axon outgrowth in cortical neurons.

To study the function of TUBB3 in netrin-1 signaling, a TUBB3 siRNA pool (Dharmacon) or short hairpin-based RNA interference constructs (TUBB3 shRNAs, gift from Dr. David L. Turner, University of Michigan, targeting a sequence common to mouse and chicken TUBB3) were transfected into E15 cortical neurons (Fig. 4-4K). The
siRNA pool and one shRNA construct significantly reduced the level of endogenous TUBB3 in these neurons (Fig. 4-4K) and were used in subsequent experiments.

To examine whether TUBB3 is involved in netrin-1-induced neurite outgrowth, primary cortical neurons from E15 mice were dissociated and transfected with a construct expressing Venus yellow fluorescent protein (Venus YFP) only (Fig. 4-4A-B) or Venus YFP together with the TUBB3 siRNA pool (Fig. 4-4C-D), the control shRNA (Fig. 4-4E-F), the TUBB3 shRNA (Fig. 4G-H) or the TUBB3 shRNA plus the shRNA-resistant rescue constructs (Fig. 4-4I-J), respectively, as we have described previously [33]. These neurons were stimulated with netrin-1 and cultured for 20 hrs. In neurons transfected with the Venus YFP only, neurite outgrowth was stimulated by netrin-1 (Fig. 4-4A-B, quantification in Fig. 4L). As predicted, either the TUBB3 siRNA pool (Fig. 4-4C-D) or TUBB3 shRNA (Fig. 4-4G-H), but not control TUBB3 shRNA (Fig. 4-4E-F), inhibited netrin-1-induced neurite outgrowth (quantification in Fig. 4-4L). Importantly, the expression of the wild-type human shRNA-resistant TUBB3 rescued netrin-1-promoted neurite outgrowth in neurons treated with TUBB3 siRNA (Fig. 4-4I-J, quantification in Fig. 4-4L). Together, these results suggest that the knockdown of TUBB3 inhibits netrin-1 induced axon outgrowth in cortical neurons.
Figure 4-4: Inhibition of netrin-1-induced axon outgrowth of cortical neurons by TUBB3 knockdown. E15 mouse cortical neurons were transfected with Venus YFP only (A-B), Venus YFP plus the TUBB3 siRNA pool (C-D), Venus YFP plus control shRNA (E-F), Venus YFP plus TUBB3 shRNA (G-H), and Venus YFP plus shRNA and the wild-type human RNAi-resistant TUBB3 (I-J), respectively. Neurite outgrowth from YFP-positive neurons was assessed in the presence of purified netrin-1 (B, D, F, H and J) and in
the sham-purified control (A, C, E, G and I). Scale bar, 10 μm. (K) Both TUBB3 siRNA pool and shRNA (#3) reduced endogenous TUBB3 protein levels in E15 cortical neurons. ***, p<0.001 (Student’s t-test). (L) Quantification of netrin-1-induced neurite outgrowth. Only the neurites of YFP-positive neurons not in contact with other cells were measured and used in the statistical analyses. Data are mean ± s.e.m. from three separate experiments. The numbers on the top of each bar indicate the numbers of neurons tested in the corresponding groups. ***, p<0.001 (One-way ANOVA with Fischer LSD for post-hoc comparisons)

4.3.2 TUBB3 does not bind to endogenous TrkB, a BDNF receptor, and is not involved in BDNF-promoted neurite outgrowth

To test the specificity of the binding of TUBB3 and DCC, BDNF and its receptor TrkB were used as controls. TUBB3 did not bind to TrkB (Fig. 4-5A-B) and TUBB3 knockdown did not affect BDNF induced axon outgrowth (Fig. 4-5C-I), suggesting the TUBB3 was not involved in BDNF signaling.
Figure 4-5: TUBB3 does not bind to endogenous TrkB, a BDNF receptor, and is not involved in BDNF-promoted neurite outgrowth. (A-B) TrkB did not form protein-protein interaction complexes with TUBB1, TUBB2 and TUBB3 in primary cortical neurons with or without purified BDNF treatment. In contrast, the interaction of endogenous TrkB with Fyn was increased by BDNF stimulation. The anti-TrkB antibody was used to immunoprecipitate proteins and the blot was analyzed with anti-TUBB1, anti-TUBB2, anti-TUBB3 or anti-Fyn. Equivalent amounts of input TrkB, TUBB1, TUBB2, TUBB3 and Fyn in cortical lysates (B). (C-I) Cortical neurons from E15 mouse embryos were transfected with either Venus YFP only (C-D), Venus YFP plus TUBB3 control siRNA (E-F) or TUBB3 shRNA (G-H), respectively, and then cultured on coverslips coated with PLL. Neurons were cultured after 18 h and stained with the Alexa Fluor® 555 phalloidin and DAPI. Purified BDNF (50 ng/ml) induced the neurite outgrowth from YFP-positive neurons transfected with Venus YFP only (C and D) and Venus YFP plus control siRNA (E and F). Expression of TUBB3 shRNA did not affect BDNF-induced neurite outgrowth (G-H). (I) Quantification of the length of the longest neurite from individual neuron. Scale bar, 10 µm. The neurites of YFP-positive neurons not in contact with other neurons were quantified and used in the statistical analyses. Data are reported as mean ± s.e.m. ***, p<0.001 (One-way ANOVA with Fischer LSD for post-hoc comparisons).

4.3.3 TUBB3 knockdown Inhibits netrin-1-induced axon outgrowth of chick dorsal spinal cord explants.
To examine the role of TUBB3 in netrin-1-induced commissural axon outgrowth, we examined cultured chick dorsal spinal cord explants, as described previously. The TUBB3 siRNA pool or shRNA together with Venus YFP plasmids were introduced into chicken neural tubes at stage 12–15, and the YFP-labeled segments were dissected at stage 18–20. Axon outgrowth was quantified by measuring the number of axon bundles and the total axon length per explant. In explants transfected with Venus YFP only (Fig. 4-6A-B) or with Venus YFP plus control shRNA (Fig. 4-6E-F), netrin-1 significantly induced axon outgrowth (quantification in Fig. 4-6L-M). TUBB3 siRNA (Fig. 4-6C-D) or shRNA (Fig. 4-6G-H), but not control shRNA (Fig. 4-6E-F), significantly inhibited netrin-1-induced axon outgrowth (quantification in Fig. 4-6L-M). The expression of wild-type human TUBB3, which is resistant to TUBB3 shRNA, rescued the effect of TUBB3 knockdown on netrin-1-induced axon outgrowth (Fig. 4-6I-J, quantification in Fig. 4-6L-M). In contrast, basal axon outgrowth of either dissociated E15 mouse cortical neurons or chick spinal cord commissural neurons was not affected by TUBB3 knockdown. These results indicate that TUBB3 is required for netrin-1-induced commissural axon outgrowth \textit{in vitro}. 

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Figure 4-6: Inhibition of netrin-1-induced axon outgrowth of chick dorsal spinal cord explants by TUBB3 knockdown. The chick neural tubes were electroporated in ovo at stage 12-15 by Venus YFP only (A-B), Venus YFP plus the TUBB3 siRNA pool (C-D), Venus YFP plus control shRNA (E-F), Venus YFP plus TUBB3 shRNA (G-H), and Venus YFP plus TUBB3 shRNA and the wild-type human RNAi-resistant TUBB3 (I-J),
respectively. Netrin-1 increased commissural axon outgrowth with longer and more axon bundles in the netrin-1 group (B and F) than the control group (A and E). Netrin-1-induced axon outgrowth was inhibited either by the TUBB3 siRNA pool (C-D) or shRNA (G-H). The expression of the wild-type human RNAi-resistant TUBB3 plasmids reversed the effect of TUBB3 shRNA (I-J). Scale bar is 100 µm. (K) Both TUBB3 siRNA pool and shRNA efficiently knocked down endogenous TUBB3 in chick spinal cords. (L-M) Quantification of netrin-1-induced commissural axon outgrowth. Only YFP-positive axon bundles were measured and used in the statistical analyses. The numbers on the top of each bar indicate the numbers of explants tested in the corresponding groups. Data are mean ± s.e.m. from three separate experiments. ***, p<0.001 (One-way ANOVA with Fisher LSD for post-hoc comparisons).

4.4 TUBB3 is required for axon attraction by netrin-1

4.4.1 TUBB3 is required for netrin-1 attraction of spinal commissural axons

Netrin-1 plays a crucial role in attracting the commissural axon projection in the developing neural tube. To determine whether TUBB3 is required for commissural axon turning towards netrin-1, we used the open-book assay with commissural axons from chick embryos as illustrated in A. The Venus YFP construct was electroporated either alone or with the TUBB3 siRNA pool, TUBB3 control shRNA, TUBB3 shRNA, and TUBB3 shRNA plus TUBB3 shRNA-resistant constructs, respectively, into the chick
neural tube at stages 12 to 15. The electroporated neural tube (visualized by green fluorescence) was then isolated and laid out as an “open book” at stage 18 - 20 (Fig. 4-7A). A rectangle of neural tube explant containing the floor plate was prepared and cocultured with an aggregate of control HEK293 cells or HEK293 cells that stably secreted netrin-1 for 40 h (Fig. 4-7A).

When the neural tube was electroporated with Venus YFP alone, commissural axons turned towards netrin-1 secreting cell aggregates (Fig. 4-7C-C’ and quantification in Fig. 4-7L), whereas most axons projected straight towards the floor plate when the neural tube explants were co-cultured with control cell aggregates not secreting netrin-1 (Fig. 4-7B-B’ and L). The TUBB3 siRNA pool significantly inhibited commissural axon turning toward netrin-1 (Fig. 4-7C-C’, E-E’ and quantification in L). To confirm the role of TUBB3 in netrin-1-induced axon turning, TUBB3 shRNA or the control shRNA were introduced into the neural tube by electroporation. When the control shRNA was electroporated together with Venus YFP, commissural axons turned towards netrin-1 (Fig. 4-7F-G’ and L). In contrast, the co-tranfection of TUBB3 shRNA with Venus YFP significantly inhibited axon turning towards netrin-1 (Fig. 4-7H-I’ and L). To further determine the specificity of shRNA knockdown, TUBB3 shRNA was electroporated with plasmids encoding a wild-type human siRNA-resistant TUBB3 into the chick neural tube. The wild-type RNAi-resistant TUBB3 transgene rescued the netrin-1 dependent phenotype (Fig. 4-7J-L). These data suggest that TUBB3 is required for netrin-1 attraction of spinal commissural axons.
**Figure 4-7: Requirement of TUBB3 for netrin-1 attraction of spinal commissural axons.** (A) Schematic diagram of the *in ovo* electroporation and the coculture assay with the open-book preparation. Electroporation of Venus YFP into the neural tube of chick embryos allowed visualization of axons. In the left panels (B, B’, D, D’, F, F’, H, H’, J, J’), neural tube explants were co-cultured with control HEK293 cells and commissural axons projected straight toward the floor plate. In the right panels (C, C’, E, E’, G, G’, I, I’, K, K’), neural tube explants were co-cultured with aggregates of HEK293 cells secreting netrin-1. The neural tube was electroporated with Venus YFP alone (B-B’ and C-C’); Venus YFP together with the TUBB3 siRNA pool (D-D’ and E-E’); Venus YFP together with the control shRNA (F-F’ and G-G’); Venus YFP with the TUBB3 shRNA (H-H’ and I-I’) and Venus YFP with the TUBB3 shRNA plus the wild-type human RNAi-resistant TUBB3 (J-J’ and K-K’). Expression of either TUBB3 siRNAs or shRNA inhibited commissural axon turning towards the netrin-1 source. The commissural axon turning defect of RNAi knock-down could be rescued by expressing RNAi-resistant TUBB3. The scale bar is 100 μm. (L) Quantification of axon turning. The numbers on the top of each bar indicate the numbers of explants tested in the corresponding groups. Data are mean ± s.e.m. from Group I-VI. *** indicates $p<0.001$ (One-way ANOVA and Fisher LSD post-hoc comparisons). NS, not significant.

### 4.4.2 The intracellular P2-3 domain of DCC inhibits the interaction of TUBB3 with full-length wild-type DCC, as well as netrin-1-induced neurite outgrowth and commissural axon attraction.
DCC P2-3 domains are responsible for the binding of TUBB3 and DCC \[^4\]. The expression DCC P2-3 domain not only inhibited the interaction of TUBB3 with full-length DCC \textit{in vitro} (Fig. 4-8A-B), but also netrin-1 induced axon outgrowth of E15 cortical neurons (Fig. 4-8C-G) as well as the commissural axon attraction induced by netrin-1 (Fig. 4-8H-I). These experiments reaffirm the functional role of the interaction of DCC and TUBB3 in netrin signaling.
Figure 4-8: The DCC intracellular P2-3 domain inhibits the interaction of TUBB3 with full-length wild-type DCC as well as netrin-1-induced neurite outgrowth and commissural axon attraction. (A-B) TUBB3-V5 were co-transfected with either wild-type full-length DCC-Myc, DCC P2-3-HA or full-length DCC-Myc and DCC P2-3-HA together into HEK293 cells. Cell lysates were immunoprecipitated using either anti-Myc or anti-HA and the membrane was blotted for TUBB3 using anti-V5. (B) Equivalent amounts of input TUBB3-V5, DCC P2-3-HA and DCC-Myc. (C-G) Expression of DCC P2-3 domain inhibited netrin-1-induced neurite outgrowth of cortical neurons. E15 mouse cortical neurons were transfected with either Venus YFP only (C-D) or Venus YFP plus DCC P2-3 (E-F). Primary neurons were treated with either purified netrin-1 (D and F) or the sham-purified control (C and E). Scale bar, 10 μm. (G) Quantification of netrin-1-induced neurite outgrowth. Data are mean ± s.e.m. NS, not significant; ***, p<0.001 (One-way ANOVA with Fischer LSD for post-hoc comparisons). (G-H) Expression of DCC intracellular P2-3 domain blocked netrin-1 attraction of chick spinal commissural axons. Axons expressing Venus YFP and DCC P2-3 domain were not attracted by the aggregates of HEK293 cells secreting netrin-1 (quantification in Fig. 4-7, group I).

4.5 TUBB3 is required for spinal commissural axon projection in vivo
4.5.1 TUBB3 knockdown inhibits of commissural axon projection \textit{in vivo}

The results discussed above suggest that TUBB3 is required for netrin-1 induced neurite outgrowth and axon attraction \textit{in vitro}. To examine the role of TUBB3 in axon projection \textit{in vivo}, the effects of TUBB3 siRNA and TUBB3 shRNA were examined on commissural axon projection in the developing chicken spinal cord. Venus YFP was introduced by electroporation into the neural tube of stage 12 chick embryos \textit{in ovo} and the embryos were allowed to develop until stages 23 (Fig. 4-9A). The YFP-labeled lumbosacral segments of the spinal cord were isolated and laid out as an “open book” (Fig. 4-9A). By stage 23, 81.1 ± 3.3% of the commissural axons expressing Venus YFP alone reached the floor plate (Fig. 9B, quantification in Fig. 4-9G). In contrast, only 15.5 ± 2.6% of the commissural axons transfected with the TUBB3 siRNA pool reached the floor plate (Fig. 4-9C and G). Similarly, TUBB3 shRNA also significantly inhibited the projection of commissural axons towards the floor plate, while TUBB3 control shRNA had no effect on the projection of commissural axons (Fig. 4-9D-E and G). The percentage of the YFP-labeled commissural axons per embryo reaching the floor plate was decreased from 79.1 ± 5.3% in the control shRNA group to 24.3 ± 1.3% in the TUBB3 shRNA group. The effect of TUBB3 shRNA on commissural axon projection was reversed by co-transfecting the wild-type siRNA-resistant human TUBB3 plasmid (Fig. 4-9F-G) with 82.6 ± 4.7% of the YFP-labeled commissural axons per embryo reaching the floor plate. These data suggest TUBB3 knockdown inhibits of commissural axon projection \textit{in vivo}.
Figure 4-9: Inhibition of commissural axon projection *in vivo* by TUBB3 RNAi. (A) Schematic diagram showing the experimental design. Different combinations of plasmids and siRNAs were electroporated into the chick neural tube *in ovo* at stage 12 and the lumbosacral region of the spinal cord was isolated at stages 23. (B) Neurons electroporated with Venus YFP only. (C) Neurons electroporated with Venus YFP plus the TUBB3 siRNA pool. (D) Neurons with Venus YFP plus control shRNA. (E) Neurons with Venus YFP plus TUBB3 shRNA. (F) Neurons with Venus YFP plus shRNA and wild-type human
RNAi-resistant TUBB3. The red arrowheads point to shortened axons. Scale bar, 100 μm.

(G) Quantification of the percentage of axons reaching the floor plate. The numbers on the top of each bar indicate the numbers of embryos tested in the corresponding groups. ***,

\[ p<0.001 \] (One-way ANOVA with Fischer LSD for post-hoc comparisons).

4.5.2 TUBB3 is essential for spinal cord commissural axon pathfinding

*in vivo.*

Although the open-book preparation demonstrated obvious defects in commissural axon projection *in vivo*, it was difficult to effectively assess effects on axon turning. To examine whether knockdown of TUBB3 disrupted commissural axon pathfinding in addition to inhibiting axon extension *in vivo*, either Venus YFP alone or Venus YFP with the TUBB3 siRNA pool were electroporated into chick spinal cord and transverse sections of the chick spinal cord at stage 23 were prepared (Fig. 4-10A-C). In addition to the inhibition of axon extension, some commissural axons transfected with TUBB3 siRNAs were misguided (Fig. 4-10C and quantification in G-I) instead of projecting normally towards the floor plate (Fig. 4-10B and G-I). These phenotypes were further confirmed by expressing Venus YFP and the TUBB3 shRNA in commissural axons that also exhibited shortened and misguided axons *in vivo* (Fig. 4-10E and G-I). In contrast, the expression of the TUBB3 control shRNA had no effect on commissural axons projection (Fig. 4-10D and G-I). As predicted, the expression of the wild-type siRNA-resistant human TUBB3 rescued the effects of TUBB3 RNAi knock-down on commissural axon extension and turning (Fig. 4-10F-I). These results indicate that
TUBB3 is required for both the projection and pathfinding of commissural axons in vivo in the developing spinal cord.
Figure 4-10: TUBB3 is essential for spinal cord commissural axon pathfinding in vivo. (A) Schematic diagram showing the transverse section of the chick spinal cord after electroporation. (B-F) The chick neural tube was electroporated with Venus YFP only (B), Venus YFP plus the TUBB3 siRNA pool (C), Venus YFP plus control shRNA (D), Venus YFP plus TUBB3 shRNA (E), or Venus YFP plus shRNA and wild-type human RNAi-
resistant TUBB3 (F). Expression of TUBB3 siRNAs or shRNA not only inhibited the comissural axon extension but also caused aberrant pathfinding (C, E, G-I). The wild-type RNAi-resistant TUBB3 rescued the defect of TUBB3 shRNA knockdown on comissural axon projection and turning (F and G-I). The red arrows point to misguided axons. Scale bar, 100 μm. (G) Quantification of the percentage of axons reaching the midline of the chick spinal cord. (H) Quantification of the average distance of axons away from the midline. ***, p<0.001 (One-way ANOVA with Fischer LSD for post-hoc comparisons). (I) The percentage of embryos with misguided axons. The numbers of embryos tested were: 17 for the Venus YFP group; 14 for the siRNA pool group; 12 for the control shRNA group; 15 for the TUBB3 shRNA group; 14 for the rescue group.

4.6 Netrin-1 increases JNK1 activity

The activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38, two subgroups of MAPKs, is required for netrin/DCC signaling\[67\]. To test whether JNK is also activated by netrin-1, plasmids expressing full-length human JNK1 were co-transfected with either full-length human DCC (DCC-Myc) or DSCAM (DSCAM-FLAG) into HEK293 cells. JNK1 was immunoprecipitated from cell lysates, and JNK activity was accessed as phospho JNK by immunoblotting with a phospho-c-Jun-specific antibody. Netrin-1 increased JNK1 activity in the presence of DCC (Fig. 4-11A) or DSCAM (Fig. 4-11B). The induction of JNK1 activity by netrin-1 appeared within 5 min, and was sustained up to 20 min in both DCC (Fig. 4-11C) and DSCAM transfected cells (Fig. 4-11D). To further determine whether DCC collaborates with DSCAM in this process, we co-transfected JNK1 either with DCC only, DSCAM only, or DCC and
DSCAM together into HEK293 cells and JNK activation was assessed. As expected, JNK1 activity was increased in the presence of either DCC or DSCAM after netrin-1 stimulation (Fig. 4-11E and F). Expression of both DCC and DSCAM further increased netrin-1-induced JNK1 activity (Fig. 4-11E and F). Together, these data suggest that netrin-1 increases JNK1 activity through its receptors.
Figure 4-11: Induction of JNK1 activity by netrin-1. A and B, netrin-1 increased JNK1 activity in the presence of DCC (A) and DSCAM (B). Human full-length JNK1 was co-transfected with either DCC-Myc (A) or DSCAM-Flag (B) in HEK293 cells. JNK1 was immunoprecipitated with anti-JNK and JNK activity performed following the JNK activity assay kit instructions. C and D, time-dependent induction of JNK1 activity by netrin-1 in HEK293 cells expressing DCC (C) or DSCAM (D). Netrin-1 increased JNK1 activity within 5 min, and this induction was persistent over 20
min. E and F, additive effect of DCC and DSCAM on netrin-1-induced JNK1 activation. Human full-length JNK1 was co-transfected with either DCC only, DSCAM only, or DCC plus DSCAM in HEK293 cells. Cells were stimulated with purified netrin-1 (250 ng/ml) for 20 min. F, quantification of the relative induction of JNK1 activity of E. Netrin-1 increased JNK1 activity in HEK293 cells expressing DCC or DSCAM only, and expression of both DCC and DSCAM together led to an even more pronounced netrin-1-induced JNK1 activity. The relative induction of JNK activity was examined by the ratio of band intensity of netrin-1 group versus the control. Data are mean ± S.D. from three separate experiments. One-way ANOVA and Fisher's LSD post hoc comparisons were performed. ***, p < 0.001.

4.7 Netrin-1-induced JNK1 activation requires the intracellular domains of DCC and DSCAM and is inhibited by JNK inhibitors

Both DCC and DSCAM are transmembrane proteins belonging to the immunoglobulin (Ig) superfamily [3][33]. DCC has three conserved intracellular domains (P1, P2, and P3)[3]. To identify which DCC domains are required for netrin-1-induced JNK1 activity, JNK1 was co-transfected into HEK293 cells with either the wild-type human DCC-Myc or three distinct DCC truncation mutants, ΔP1 or ΔP2, or ΔP3. Netrin-1 increased JNK1 activity in the presence of the wild-type DCC or truncated DCCs lacking the P1 or P3 domain. In contrast, deletion of the DCC P2 domain eliminated netrin-1 induction (Fig. 4-12A). DSCAM truncation mutants (DSCAMΔC and
DSCAMΔN) co-transfected with JNK1 in HEK293 cells also revealed the DSCAM intracellular domain was necessary for the netrin-1-induced JNK1 activity (Fig. 4-12B). These results suggest that the intracellular domain of DSCAM and the P2 domain of DCC may be required for netrin-1-mediated JNK1 activation. Expression of a dominant negative form of MKK4, a specific JNK activator, inhibited netrin-1-induced JNK1 activation in DCC-transfected HEK293 cells (Fig. 4-12C). The JNK inhibitor SP600125 also blocked netrin-1 effect on JNK1 activation in the presence of DCC (Fig. 4-12D) and DSCAM (Fig. 4-12E), although it did not affect EGF-induced ERK activity (Fig. 4-12F).
Figure 4-12: Netrin-1-induced JNK1 activation depends on the intracellular domains of DCC and DSCAM and was inhibited by JNK inhibitor and MKK4 (Lys/Arg). A and B, Mapping the domains in DCC (A) and DSCAM (B) responsible for JNK1 activation. DCC-ΔP1, -ΔP2, and -ΔP3 correspond to deletions of residues 1147–1171, 1335–1356, and 1412–1447, respectively. JNK1 was co-transfected with the full-length wild-type DCC (DCC WT), different truncated mutants of DCC tagged by Myc or the full-length DSCAM, DSCAM ΔN, or DSCAM ΔC tagged by FLAG into HEK293
cells. The P2 domain in DCC was essential for DCC-dependent JNK1 activation (A). Netrin-1-induced JNK1 activation relied on both intracellular and extracellular domains of DSCAM in transfected HEK293 cells. C, expression of MKK4 (Lys/Arg), a kinase-inactive form of MKK4, inhibited the induction of JNK1 activation by netrin-1 in the presence of DCC. D and E, SP600125, a JNK inhibitor, blocked both DCC-dependent (D) and DSCAM-dependent (E) induction of JNK1 activity by netrin-1. F, SP600125 did not affect EGF-induced ERK activity in transfected HEK293 cells.

To test if JNK1 is specifically involved in netrin-1 signaling, JNK2 or JNK3 was co-transfected with JNK1 together with DCC or DSCAM in HEK cells. The expression of JNK1 alone is able to induce JNK activity in presence of DCC or DSCAM, and netrin-1 stimulation further increases the JNK1 activity (Fig. 4-13A-D). However, netrin-1 could not induce JNK2 or JNK3 activity in presence or absence of DCC (Fig. 4-13A-B) or DSCAM (Fig. 4-13C-D). These results suggest that JNK1 is specifically involved in netrin-1 signaling.
Figure 4-13: Netrin-1 increases JNK1, not JNK2 or JNK3, activity in presence of DCC and DSCAM. Human full-length JNK1 (A-D), JNK2 (A and C) and JNK3 (B and D) were co-transfected with either DCC-Myc (A-B) or DSCAM-Flag (C-D) in HEK 293T cells. Cells were stimulated with purified netrin-1 (250 ng/ml) for 20 minutes.

4.8 Netrin-1 induces endogenous JNK activity

To determine whether netrin-1 increases endogenous JNK activity, primary neurons from E15 cortices were used because these neurons express both DCC and DSCAM and respond to netrin-1 stimulation. We examined JNK activation by probing the Western blots with the specific anti-phospho-JNK antibody. JNK phosphorylation was induced by netrin-1 within 3 min and the induction was sustained up to 20 min (Fig. 4-14A). Although netrin-1 had no effect on ERK1/2 activation, p38 phosphorylation also was increased by netrin-1 stimulation (Fig. 4-14A). Netrin-1-induced JNK activation was
blocked by either the addition of the JNK inhibitor SP600125 (Fig. 4-14B) or the anti-DCC function-blocking antibody (Fig. 4-14C). Toxin B, a Rho family small GTPase inhibitor, blocked netrin-1-induced Rac1 and JNK activities, but not p38 phosphorylation (Fig. 4-14D). To determine whether DSCAM is required for endogenous JNK activation, DSCAM shRNA and DSCAM control shRNA constructs were nucleofected into dissociated E15 cortical neurons. Knockdown of DSCAM partially inhibited netrin-1-induced JNK activation, whereas expression of the control DSCAM shRNA had no effect on netrin-1 induction (Fig. 4-14E and F). The addition of the anti-DCC function-blocking antibody with the expression of DSCAM shRNA in primary neurons together totally abolished netrin-1 induction (Fig. 4-14E and F), suggesting that DCC cooperates with DSCAM in this process. Simultaneous knockdown of both DCC and DSCAM also blocked netrin-1-induced JNK1 activity significantly greater than either DCC or DSCAM alone (Fig. 4-14G), further supporting this hypothesis.
Figure 4-14: Induction of endogenous JNK1 activity by netrin-1. A, Netrin-1 increased the levels of phospho-JNK and phospho-p38, but not phospho-p44/42 MAPK (ERK1/2), in primary E13 spinal cord neurons. Neurons were stimulated with purified netrin-1 (250 ng/ml) for 3–20 min. The cell lysates were blotted with anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-phospho-ERK1/2, and anti-ERK1/2,
respectively. B, Netrin-1-induced endogenous JNK activity was blocked by SP600125. E15 cortical neurons were stimulated with purified netrin-1 (250 ng/ml) for 20 min in the presence or absence of SP600125. C, anti-DCC function-blocking antibody inhibited the increase of phospho-JNK levels by netrin-1 in E15 cortical neurons. D, toxin B inhibited netrin-1-stimulated levels of endogenous phospho-JNK and GTP-Rac1 without affecting phospho-p38 levels in primary E15 cortical neurons. E, coordination of endogenous DCC and DSCAM in netrin-1-induced JNK activation. Primary neurons from E13 dorsal spinal cords were transfected with Venus only, Venus YFP plus control shRNA, and Venus YFP plus DSCAM shRNA. Neurons were stimulated with purified netrin-1 or the control in the presence or absence of the functional blocking DCC antibody. F, quantification of the relative induction of JNK1 activity in E. The relative induction of JNK activity was examined by the ratio of band intensity of netrin-1 group versus the control (without netrin-1), compared with the normalized basal JNK activity change from the control shRNA-transfected versus mock transfection group. Data are mean ± S.D. from three separate experiments. One-way ANOVA and Fisher LSD post hoc comparisons were performed. *, p < 0.05; ***, p < 0.001. G, double knockdown of endogenous DCC and DSCAM abolished netrin-1-induced JNK activity in primary E13 spinal cord neurons.

4.9 JNK activation in the developing spinal cord

To examine whether JNK activation specifically occurs in commissural axons, transverse cryosections of E11 mouse spinal cords were immunostained with specific anti-phospho-JNK and DCC or TAG1. Phospho-JNK was strongly expressed in
commissural axons before and as they crossed the floor plate (Fig. 4-15A–F).

Interestingly, high levels of phospho-JNK and DCC were also detected in post-crossing commissural axons, such as the ventral and lateral funiculi as well as motor columns and the dorsal root entry zone (Fig. 4-15D–F). To further determine whether JNK activation in developing commissural neurons relies on netrin-1, primary neurons from E11 mouse dorsal spinal cords were dissociated and cultured. JNK activity in the growth cone of commissural neurons was assessed using immunocytochemistry with the anti-phospho-JNK and anti-DCC antibodies. In the absence of netrin-1, low levels of phospho-JNK were detected in the growth cone (Fig. 4-15G–I, quantification in P). Netrin-1 stimulation dramatically increased the level of phospho-JNK in the growth cone (Fig. 4-15J–L, and quantification in P). The addition of JNK inhibitor (1 μM) abolished netrin-1 induction (Fig. 4-15M–O, quantification in P). These results suggest that netrin-1-dependent JNK activation occurs in developing spinal commissural axons.
Figure 4-15: JNK activation in the developing spinal cord. A–C, JNK activation occurs in TAG1-positive precrossing commissural axons of E11 mouse spinal cord. Phospho-JNK and TAG1 were detected by specific anti-phospho-JNK (A) and anti-TAG1 (B) antibodies. C, merged picture of A and B. D–F, co-expression of phospho-JNK
and DCC in the developing spinal cord. Transverse sections of E11 mouse spinal cords were double-immunostained with anti-phospho-JNK (D) and anti-DCC (E). F, superimposed images of D and E. Both phospho-JNK and DCC were strongly expressed in the DREZ, precrossing commissural axons, motor column, and postcrossing commissural axons (ventral funiculus and lateral funiculus). Scale bar, 100 μm. G–P, Netrin-1 induced phospho-JNK levels in the growth cone of spinal cord commissural axons. Primary neurons from E11 mouse dorsal spinal cords were dissociated and stimulated with purified netrin-1 (250 ng/ml) (J–P) or the control (G–I and P) in the presence (M–P) or absence (G–L and P) of 1 μM SP600125. Neurons were double-immunostained with anti-phospho-JNK (H, K, and N) and anti-DCC (G, J, and M). I, L, and O are the superimposed images of G, H, J, K, M, and N, respectively. P, quantification of relative induction of JNK activity by netrin-1 in commissural axon growth cones. Data are mean ± S.D. ***, p < 0.001 (one-way ANOVA and Fisher LSD post hoc comparisons). Scale bar, 10 μm. Inh, inhibitor.

4.10 JNK1 inhibition blocks netrin-1-induced neurite outgrowth

To examine whether JNK activation plays a functional role in netrin-1 signaling, dorsal spinal cord explants from E13 mouse embryos were dissected and cultured in the collagen gel in the presence or absence of netrin-1. Netrin-1 enhanced commissural axon outgrowth (Fig. 4-16A, B, and E). The JNK inhibitor SP600125 inhibited netrin-1-induced axon extension in a dose-dependent manner (Fig. 4-16C–E). As expected, netrin-1 increased cortical neurite extension (Fig. 4-16F and G, quantification in N) and
SP600125 blocked the netrin-1 effect (Fig. 4-16H, I, and N). These results indicate that JNK activation is required for netrin-1-induced axon outgrowth. To further determine the functional role of JNK1 in netrin signaling, several short hairpin-based RNAi constructs (shRNAs) targeting a sequence common to chick and mouse JNK1 were designed and transfected in both HEK293 cells (Fig. 4-16O) and dissociated E15 cortical neurons (Fig. 4-16P). One shRNA construct (construct 5) significantly knocked down the level of JNK1 protein, whereas others could not (Fig. 16O and P). We used these RNAi constructs as a JNK1 shRNA and JNK1 control shRNA, respectively. Netrin-1-induced neurite outgrowth was assessed with primary E15 cortical neurons transfected either with Venus yellow fluorescent protein (Venus YFP) only or together with the control shRNA or the JNK1 shRNA. These neurons were stimulated with netrin-1 and cultured for 20 h. In neurons transfected with Venus YFP or Venus YFP plus the control shRNA, neurite extension was stimulated by netrin-1 (Fig. 4-16J, K, and N). In contrast, JNK1 shRNA inhibited netrin-1-induced neurite outgrowth (Fig. 4-16L and N). The expression of wild-type human JNK1 rescued the defects in netrin-1-induced neurite outgrowth caused by JNK1 knockdown (Fig. 4-16M and N). These data suggest JNK1 inhibition blocked netrin-1-induced neurite outgrowth.
Figure 4-16: JNK1 inhibition blocks netrin-1-induced neurite outgrowth. A–D, dorsal spinal cord explants from E13 embryos were cultured in the presence or absence of JNK inhibitor SP600125. Scale bar, 100 μm. Explants without netrin-1 (A) showed fewer axon bundles than did explants with netrin-1 (B). Netrin-1-promoted axon outgrowth was inhibited by 1 μM (C) and 5 μM (D) SP600125. E, quantification of axon outgrowth (the number of axon bundles). The numbers of explants tested are as follows: 17 (control group), 20 (netrin-1 group), 18 (netrin-1 plus 1 μM SP600125 group), and 16 (netrin-1 plus 5 μM SP600125 group). Data are mean ± S.E. ***, p < 0.001 (Student’s t test).

F–I, neurite outgrowth from dissociated neurons. E15 cortical neurons were cultured 18 h in the presence (G–I) or absence (F) of purified netrin-1 with (H and I) or without (F and G) SP600125. Neurons were stained with TUJ1 (green), phalloidin (red), and DAPI (blue). J–M, Venus YFP was co-transfected with control shRNA (J and K), JNK1 shRNA (L), or JNK1 shRNA plus the wild-type human JNK1 (M) into E15 cortical neurons. Primary neurons were incubated with (K–M) or without (J) purified netrin-1 (100 ng/ml) and stained with Alexa Fluor® 555 phalloidin (red) and DAPI (blue). Scale bar, 10 μm. N, quantification of netrin-1-induced neurite outgrowth of cortical neurons. The y axis is the longest neurite length. Data are mean ± S.E. from three separate experiments with 80 neurons in each group. ***, p < 0.001 (one-way ANOVA and Fisher LSD post hoc comparisons); ns, not significant. O, JNK1 shRNA (lane 5) significantly reduced JNK1 protein levels in HEK293 cells. P, endogenous JNK1 protein levels were reduced in dissociated E15 cortical neurons after shRNA transfection. Inh, inhibitor.
To test if JNK1 is specifically required for netrin-1-induced axon outgrowth, either JNK2 siRNA or JNK3 siRNA was transfected into E15 cortical neurons and JNK2 and JNK3 knocked down examined by the Western blotting. The expression of either JNK2 siRNA or JNK3 siRNA did not inhibit netrin-1 induced JNK activity (Fig. 4-17A) or axon outgrowth (Fig. 4-17B-H), suggesting that JNK1, but not JNK2 or JNK3, is involved in netrin-1 signaling.
Figure 4-17: Netrin-1-induced endogenous JNK activity and neurite outgrowth of primary neurons are not affected by knockdown of JNK2 or JNK3. A. JNK2 or JNK3 siRNAs were transfected into E15 cortical neurons by nucleofection. Primary neurons were stimulated with purified netrin-1 (250 ng/ml) for 20 min. The cell lysates were blotted with anti-JNK, anti-JNK2 and anti-JNK3, respectively. B-G. Netrin-1-induced neurite outgrowth of dissociated E15 cortical neurons. Primary neurons (8 X 10^6 neurons per group) were transfected with either Venus YFP only (1 µg, B and C), Venus YFP (1 µg) plus 4 µg of JNK2 siRNAs (D and E) or Venus YFP (1 µg) plus 4 µg of JNK3 siRNAs (F and G) in the absence (A, D and F) or presence of purified netrin-1 (C, E and G). Neurons were labeled with Alexa Fluor® 555 phalloidin (red) and DAPI (blue). Scale bar, 10 µm. H. Quantification of netrin-1-induced neurite outgrowth of cortical neurons from B-G with 80 neurons in each group. The Y axis is the longest neurite length. Data are mean ± S.E. ***: p < 0.001 (One-way ANOVA and Fischer LSD post-hoc comparisons); ns: not significant.

4.11 JNK1 is required for netrin-1-mediated commissural axon attraction

The open book turning assay with commissural axons from the neural tube of chick embryos is a model for studying netrin-1 attraction. To determine whether JNK activation is required for commissural axon turning toward netrin-1, the Venus YFP construct only or Venus YFP plus the control shRNA or JNK1 shRNA was electroporated into the chick neural tube at stages 12–15. An explant of the neural tube was isolated, and co-cultured with an aggregate of HEK cells. As expected, most
commissural axons turned toward HEK cells stably secreting netrin-1 in explants transfected Venus YFP only (Fig. 4-18B and I) or Venus YFP together with the control shRNA (Fig. 4-18F and I), whereas control HEK293 cells did not attract commissural axons, with most axons projecting straight toward the floor plate (Fig. 4-18E and I). As predicted, SP600125 inhibited netrin-1-induced commissural axon turning (Fig. 4-18C, D and I). Co-transfection of Venus YFP with JNK1 shRNA significantly inhibited axon turning towards netrin-1 (Fig. 4-18G and I). To confirm the role of JNK1, we introduced wild-type human JNK1 into the neural tube together with JNK1 shRNA and Venus YFP by electroporation. Wild-type JNK1 significantly rescued the netrin-1 dependent axon turning after JNK1 knockdown (Fig. 4-18H and I). Together, these results demonstrated a requirement for JNK1 in netrin-1 mediated axon attraction.
Figure 4-18: JNK1 is required for netrin-1-mediated commissural axon attraction.
A, schematic diagram of the commissural axon turning assay. Venus YFP was electroporated into the neural tube of chick embryos for visualizing commissural axon projection. B–D, netrin-1-induced axon turning was inhibited by SP600125. The neural tube was electroporated with Venus YFP alone and co-cultured with a cell aggregate secreting netrin-1 in the absence (B) or presence of 1 μM (C) and 5 μM (D) SP600125. E and F, neural tube explants expressing the control JNK1 shRNA were co-cultured either with control HEK293 cells (E) or HEK293 cells secreting netrin-1 (F). G and H, Venus YFP together with JNK1 shRNA or with JNK1 shRNA plus wild-type JNK1 were electroporated into chick neural tubes and explants co-cultured with netrin-1 cells. Scale bar, 100 μm. I, quantification of axon turning. Data are mean ± S.E. *** indicates $p < 0.001$ (one-way ANOVA and Fisher LSD post hoc comparisons). Inh, inhibitor.

4.12 JNK1 is essential for spinal cord commissural axon projection and pathfinding in vivo.

To determine the functional role of JNK1 in vivo, we examined the effect of JNK1 knockdown on commissural axon projection in chick embryos. Venus YFP only or Venus YFP plus vector, Venus YFP plus the control shRNA, or Venus YFP plus JNK1 shRNA were introduced into the neural tube of stage 12 chick embryos by in ovo electroporation. The lumbosacral segments of the spinal cord at stage 23 after electroporation were isolated and transverse sections prepared. Commissural axons projected normally toward the floor plate with most axons reaching the floor plate in the
neural tube transfected with Venus YFP alone, Venus YFP plus the vector, or Venus YFP with the control shRNA (Fig. 4-19B–D and G–I). In contrast, the expression of JNK1 shRNA not only inhibited commissural axon projection, but also caused axon misguidance (Fig. 4-19E and G–I). Co-expression of wild-type human JNK1 with JNK1 shRNA rescued the defects in commissural axon projection caused by the JNK1 shRNA (Fig. 4-19F–I). These findings demonstrate that JNK1 is required for commissural axon projection and pathfinding in the developing spinal cord.
Figure 4-19: JNK1 is essential for spinal cord commissural axon projection and pathfinding in vivo. A, chick spinal cord diagram showing commissural axon projection after electroporation. Venus YFP only or combinations of Venus YFP with other
plasmids were electroporated into the chick neural tube in ovo, and the lumbosacral region of the spinal cord was collected as described previously. B, spinal cord electroporated with Venus YFP only. C, commissural neurons electroporated with Venus YFP plus the empty vector. D, neurons with Venus YFP plus control shRNA. E, commissural neurons with Venus YFP plus JNK1 shRNA. JNK1 shRNA not only inhibited the commissural axon extension but also caused aberrant pathfinding. The red arrows point to misguided axons. F, neurons expressing Venus YFP plus JNK1 shRNA and wild-type human JNK1. Scale bar, 100 μm. G, percentage of axons reaching the midline. H. Quantification of the average distance of commissural axons away from the midline. Data are presented as the mean ± S.E. in G and H. ***, p < 0.001 (one-way ANOVA with Fisher LSD for post hoc comparisons). I, percentage of embryos with misguided axons. n = 33 (Venus YFP), 20 (vector group), 15 (control shRNA group), 16 (JNK1 shRNA group), and 18 (JNK1 shRNA + wild-type JNK1).
Chapter 5

Discussion

5.1 TUBB3 is an essential signaling component in netrin-1-mediated axon outgrowth/guidance.

TUBB3 is a neuronal specific microtubule subunit \[^{48}\]. Missense mutations in TUBB3 result in dysgenesis of the corpus callosum, anterior commissure, and corticospinal tracts in the ocular motility disorder (CFEOM3) \[^{53}\]. These TUBB3 mutations appear to impair tubulin heterodimer formation and alter their dynamic instability, which is essential for axon growth cone cytoskeleton redistribution, based on modeling each mutation in yeast tubulin \textit{in vitro} \[^{42}\]. These results suggest that endogenous TUBB3 may be required for axon guidance and maintenance in the developing nervous system.

Netrin-1, one of four classic guidance cues, plays an important role in axon guidance, especially in commissure axon projection in both the brain and spinal cord \[^{17}\]. Netrin-1 secreted from the floor plate attracts commissural axons to project towards the floor plate and cross the midline by binding to netrin-1 receptors, DCC and DSCAM on the axon growth cone \[^{32}\]. As predicted, most, if not all, axons fail to cross the midline in the corpus callosum in either netrin-1 or DCC knockout mice \[^{4}\]. Interestingly, a knock-in
TUBB3 mutation exhibits a similar phenotype [53]. The phenotypic similarities between the TUBB3 mutation knock-in and the DCC and netrin-1 knockout mice propelled us to hypothesize that TUBB3 could be involved in netrin-1 signaling. Indeed, our data have demonstrated that TUBB3 knockdown in primary neurons and chick spinal cords totally abolishes netrin-1 induced axon outgrowth, and this loss of function phenotype is rescued by the re-introduction of wild type TUBB3. Similarly, TUBB3 knockdown in chicken embryos impairs the ability of axons in the growth cone to turn toward netrin-1 source. Finally, the TUBB3 knockdown limits the ability of commissural axons of the chick spinal cord to cross the midline, and most importantly, causes commissural axon guidance defects, including stalling, misguided, or turning back in most embryos. These findings support the hypothesis that TUBB3 is required for netrin-mediated axon outgrowth and pathfinding. Future work will be designed to identify the TUBB3 domains responsible for netrin-1 mediated axon guidance and that how TUBB3 mutations in CFEOM3 modulate netrin-1 signaling.

5.2 Netrin/DCC signaling couples directly to MT dynamics

The functional neuronal cytoskeleton is essential for proper axon outgrowth and pathfinding [54]. For instance, 1) dynamic MTs become oriented and stabilized preferentially in the direction of the growth cone turn [37], 2) the local stabilization of MTs in one side of the growth cone causes the growth cone to turn towards that side [63], 3) the local disruption of MT stabilization on one side of a growth cone is sufficient to induce growth cone turning away from that side [62], 4) taxol enhances axon outgrowth in vitro and in vivo via MT stabilization, promoting MT polymerization at plus ends [63].
These results suggest that MT dynamics plays an important role in axon guidance. However, it is still unclear if MT dynamics are directly regulated by guidance cues.

In this study, we have found that TUBB3 interacts directly with DCC, and netrin-1 further stimulates TUBB3/DCC interactions. Disruption of MT dynamics, either by taxol or nocodazole, abolishes netrin-1 induced TUBB3/DCC interactions, suggesting that MT dynamics are required. DCC co-sediments with stabilized MTs, and netrin-1 increases the ratio of DCC in the cell pellet versus supernatant fraction, presumably associated with polymerized MTs. As predicted, netrin-1 stimulation also results in more polymerized TUBB3 in the pellet. These results lead to a generalizable model that netrin-1 signaling directly regulates MT dynamics through the coupling the netrin-1 receptor, DCC, to TUBB3 and polymerized MTs. In this model (Fig. 5-1) polymerized MTs are ‘captured’ by DCC in the growth cone in response to netrin-1, stabilizing filopodia against retraction and promoting axon outgrowth and turning (Fig. 5-1B).

Our model that netrin-1 signaling stabilizes filopodia against retraction and promotes axon turning generates a number of interesting questions.

Firstly, does this model apply to other netrin-1 receptors such as UNC5-mediated repulsion, i.e., does netrin-1 signaling disrupt MT dynamics and destabilize filopodia in the growth cone? On a more general level, does this model apply to other guidance pathway? For example, in addition to netrin-1, other guidance cues also play key roles in commissural axon guidance, such as BMP, sonic hedgehog (SHH), Slits and Draxin. BMPs and Draxin repel commissural axons. In contrast, SHH is a chemoattractant made in the floor plate [6][15]. Slit repels commissural axon projection from the midline and silences netrin attraction in the commissural axon midline crossing [7]. It will be important
to examine if TUBB3 interacts with these additional guidance receptors or their downstream signal components.

Secondly, are other signaling molecules involved in TUBB3/DCC interaction? Our data indicate that the interaction of DCC with TUBB3 requires the intracellular P2 and P3 domains of DCC. The domains could potentially form a signaling complex with Src [2]. The inhibition of Src family kinases blocks netrin-1 induced TUBB3 tyrosine phosphorylation and the interaction of DCC with TUBB3 and polymerized MTs in primary neurons. These results suggest that Src family kinases are essential in regulating MT dynamics in netrin-1 signaling and may function as a key downstream signaling component of the DCC/TUBB3 complex. Indeed, Src family kinases are involved in modulating growth cone steering via MT dynamics and are required for netrin-1-mediated axon guidance [76]. Therefore, these results suggest a model in which DCC serves as a signaling platform for recruitment of a multi-protein complex, including TUBB3, Src family kinases and other key signal molecules to modulate MT dynamics in netrin-1-induced axon outgrowth and turning.

Many other signaling molecules appear to be involved in netrin-1 signaling, including FAK, p130CAS, DOCK180, etc. The knockdown of either FAK, p130CAS or DOCK180 interferes with netrin-1 mediated axon guidance, i.e., axons fail to turn upon netrin-1 stimulation [1][2][3]. Netrin-1, upon binding to DCC, activates FAK, which in turn activates Src family kinases and p130CAS [1][2]. Since our data demonstrate that Src family kinases are involved in netrin-1 signaling, it will be important to determine whether these additional signal molecules play a role in modulating MT dynamics via the TUBB3/DCC interaction. More importantly, how MT dynamics are incorporated in the process? Since
our data suggest that the netrin-1 induced binding of DCC and TUBB3 or polymerized microtubule is reduced by the inhibition of Src family kinases, Src family kinases probably function upstream of MT dynamics. However, further studies will be required to determine how these different signaling molecules are oriented in the complex to modulate MT dynamics by netrin-1.

Finally, do actin dynamics play a role in coupling netrin signaling to MT dynamics? Actin dynamics are implicated in netrin-1 signaling. For example, netrin-1 regulates actin dynamics by locally increasing actin polymerization or decreasing the retrograde actin flow [96]. Meanwhile, retrograde flow of filopodia actin bundles also moves MT backwards, indicating that MTs are structurally linked to actin [98]. Also, contractile ‘arc’ associated MT are less dynamic than those associated with filopodia [97]. The local disruption of MT stabilization on one side of a growth cone is sufficient to induce growth cone turning away from that side [62]. These findings indicate that actin dynamics coordinate with MT dynamics in netrin-1 signaling. Interestingly, disruption of actin dynamics by cytochalasin D blocks growth cone turning without affecting axon outgrowth [45], suggesting that actin dynamics is involved in axon turning, but not axon extension. Further research will be needed to address if MT dynamics is upstream, downstream or paralleled to actin dynamics.
Figure 5-1: A simple model to explain direct involvement of MT dynamics in netrin-1-promoted growth cone turning. (A) The peripheral region of the growth cone contains unpolymerized tubulins and 'pioneer polymerized MTs'. (B) Binding of netrin-1 to DCC results in recruiting tyrosine phosphorylated TUBB3, Src family kinases and other key signaling molecules to form a 'molecular clutch' on the side of growth cone.
close to netrin-1 gradient. Netrin-1-induced MT polymerization/stabilization occurs in the clutch site to polarize the growth cone and further maneuver growth cone steering.

5.4 The JNK1 pathway is required for commissural axon projection and pathfinding in the developing spinal cord.

JNK activity is essential in axon formation and regeneration [66]. We have demonstrated that JNK1 is required for netrin-1 mediated axon outgrowth from both cortical neurons and mouse dorsal spinal cords. For example, either a JNK inhibitor or JNK1 shRNA inhibits netrin-1 induced axon outgrowth induced by netrin-1. In addition, tract formation in the anterior commissure is disrupted in \textit{jnk1-/-} mice further supporting a role for JNK1 in commissural axon projection [72]. JNK signaling often interacts with that of another MAPK, p38, as they share several upstream regulators and p38 often negatively regulate JNK signaling [91]. Interestingly, previous studies have demonstrated that netrin-1 increases ERK1/2 activity, but has no effect on p38 or JNK activation [67]. In contrast, the present study has demonstrated that netrin-1 signaling activates endogenous JNK1 and p38, but not ERK1/2. Similarly, netrin-1 also activates p38 in \textit{Xenopus} retinal growth cones [68]. The reasons for these different results are unclear, but may reflect different cell types, non-neuronal cells vs primary mouse cortical and commissural neurons. Certainly, additional studies are warranted to sort out these differences.

In addition to netrin-1, many other guidance cues have been implicated in commissural axon guidance. These include sonic hedgehog that acts as a chemoattractant
in the floor plate, Wnt4 that guides post crossing commissural axons along the rostral-caudal axis, and bone morphogenetic proteins that act as chemorepellants involved in the initial guidance of commissural axons \[87\][88][89]. Unfortunately, it is still unclear if any of these guidance cues modulate JNK signaling. In fact, many of the upstream regulators of JNK signaling still remain to be identified. However, it is clear that MAP kinase kinase 7 (MKK7) acts upstream of JNK1 and that MKK7 knockdown decreases the number of TAG-1 expressing neurons and disrupts contralateral axon projection layer 2/3 neurons in the developing cerebellum \[67\]. This data also support our hypothesis that JNK1 plays an important role in commissural axon projection. JNK1 activity is also regulated by dual zipper kinase (DLK), a MAP kinase kinase kinase (MAPKKK), suggesting that DLK may also be involved in netrin-1 signaling \[68\]. However, no role for DLK in netrin-1 signaling has yet been identified. Together, these studies highlight the potential complexity of both netrin-1 and JNK1 signaling and how much still remains to be learned about these key interactions.

### 5.5 JNK1 coordinates netrin/DCC, netrin/DSCAM, and netrin/UNC5 signaling.

Both DCC and DSCAM belong to the same immunoglobulin subfamily and share similar protein structures \[28\]. DSCAM functions as a netrin-1 receptor, collaborating with DCC in netrin signaling \[33\]. We have demonstrated that DCC cooperates with DSCAM through regulating JNK activity in netrin signaling. For example, knockdown of DSCAM partially inhibit netrin-1-induced JNK activation, and combination of the anti-DCC
function-blocking antibody with expression of DSCAM shRNA in primary neurons together totally abolishes the netrin-1 effect. In addition, DCC knock-out mice exhibit aberrant commissural axon pathfinding [17]. Surprisingly, mice carrying a deletion of DSCAM exon 1 have no detectable abnormal spinal commissural axon projection, whereas DSCAM knockdown in chick and mice as well as in Drosophila show that DSCAM is essential for the netrin-1 dependent promotion of axon outgrowth and midline crossing [23]. This disparity may be caused by compensatory mechanisms involving other related guidance receptors and/or signal molecules downstream of DCC and DSCAM, suggesting that DSCAM may play more complex roles than we expected in netrin signaling.

In addition to the coordination of DCC and DSCAM, other netrin-1 receptors have also been implicated in axon guidance. For instance, the interaction of DCC and UNC5 mediates netrin-1 repulsion, and DSCAM functions as a repulsive receptor cooperating with UNC5C in netrin signaling [90]. These results suggest that combination of DSCAM with DCC or UNC5C may be of importance in netrin-1-mediated axon attraction or repulsion. Unfortunately, the signaling mechanisms downstream of netrin/DSCAM/UNC-5-mediated repulsion are less understood than those of netrin/DCC-induced attraction. However, it is clear that several signal molecules, such as FAK and Src family kinases, the tyrosine phosphatase SHP2 (PTPN11), MAX-2, a PAK family member, and MAX-1, an adaptor protein, are involved in netrin/UNC5 repulsion [76]. PAK1 interacts with DSCAM and netrin-1 induces phosphorylation of PAK1 and Fyn in the presence of DSCAM [31]. In the netrin repulsive signaling, DSCAM cooperates with UNC5C through regulation of endogenous protein kinases activities, such as Src family kinases, FAK, and PAK1 [33]. Because expression of DSCAM induces JNK1 kinase activity in mammalian cells, it will
be interesting to determine whether JNK1 activation is involved in coordinating netrin/DSCAM and netrin/UNC5 repulsion.

5.6 Coordination of JNK1 and MT/actin dynamics in axon guidance

Microtubule associated proteins (MAPs) play an important role in modulating MT dynamics. MAPs include MT motor proteins as well as the MT polymerization promoting proteins, Tau, MAP1b, and MAP2 \(^\text{[45]}\). JNK1, a key regulator of certain MAPs, is involved in the regulation of MT dynamics. For example, JNK1 can phosphorylate certain MAPs, such as MAP1b and MAP2, which may potentially modulate microtubule dynamics \(^\text{[74]}\). JNK1 deficient mice exhibit a loss of MT integrity in dendrites that is associated with hypophosphorylation of MAP1b and a reduced ability to promote tubulin polymerization \(^\text{[75]}\). JNK1 is also involved in the phosphorylation of Stathmin and SCG10 \(^\text{[95]}\). Stathmin is an important MT destabilizing protein, whose activity is modulated by its phosphorylation sites, and SCG is a key regulator of neurite extension through regulation of MT dynamics \(^\text{[93][94]}\). Our data indicate that both MT dynamics and JNK1 are implicated in netrin-1 signaling. These results suggest that the JNK1 pathway may modulate MT dynamics through MAPs in netrin-1-mediated axon guidance.

Actin dynamics are regulated by small GTPases such as RhoA \(^\text{[82]}\). For example, mutants of Rho GTPases, when introduced into 3T3 cells, cause drastic actin cytoskeletal
rearrangement \cite{92}. The downstream targets of RhoA include ISp family proteins, which have direct effect on actin dynamics pertinent to motility \cite{92}. Small GTPases function upstream of MAPKKK \cite{91}. Interestingly, ToxinB, an inhibitor of the small GTPases Rho, Rac, and Cdc42, not only block netrin-1-induced Rac1 activity, but also inhibit the induction of phospho-JNK1 by netrin-1 without affecting phospho-p38 \cite{82}. These results suggest that JNK1 may function downstream of small GTPases in netrin signaling. Further investigation is necessary to untangle how small GTPases regulate JNK activity in netrin-mediated attractive signaling through modulating actin dynamics. In this case, JNK1 may serve as the link between the actin and microtubule dynamics.

In summary, MT dynamics and JNK1 are involved in netrin-1 signaling through netrin-1 receptors, DCC and DSCAM. JNK1 may regulate MT dynamics through MT associate proteins such as MAP1b. MT and actin dynamics may coordinate each other to regulate netrin-1-mediated growth cone turning.
Reference


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