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Characterizing the Role of Key Planar Cell Polarity Pathway Components in Axon Guidance

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Characterizing the Role of Key Planar Cell Polarity Pathway Components in Axon Guidance

A thesis submitted in partial fulfillment of the requirement for the degree of Masters of Science at Virginia Commonwealth University.

by

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Table of Contents

Acknowledgements........................................................................................................... ii

List of Figures.................................................................................................................. vi

List of Abbreviations...................................................................................................... vii

Abstract......................................................................................................................... ix

Introduction.................................................................................................................... 1

Axon Pathfinding and Neural Development................................................................. 1

Planar Cell Polarity Pathway......................................................................................... 2

Developmental Roles of the PCP Pathway................................................................. 3

Commissural Axons as a Model for Studying Axon Guidance................................. 6

PCP Signaling and Axon Pathfinding......................................................................... 7

Zebrafish CoPA Neurons............................................................................................ 8

CoPA neurons and the PCP pathway.......................................................................... 9

Prickle in the Nervous System.................................................................................... 9

ROR1 and ROR2 in the Nervous System.................................................................. 10

Significance and Aim of Research............................................................................ 11

Materials and Methods............................................................................................. 12

Fish Strains.................................................................................................................... 12
Table 4. In Situ Hybridization Probe DNA template Sequences……………………………...19

Results…………………………………………………………………………………………………...20

Prickle paralogs are expressed in the spinal cord.............................................................20

Prickle1a and Prickle2b are dispensable in axon guidance of CoPA Neurons..............20

prickle CRISPR/Cas9 experiments..................................................................................21

T7 endonuclease I assay confirms presence of prickle mutations..............................22

prickle genes have a role in CoPA neuron axon guidance..........................................23

ror2 is dispensible in axon guidance of CoPA Neurons...............................................23

ror1 and ror2 CRISPR/Cas9 experiments.....................................................................24

T7 endonuclease I assay confirms presence of ROR1 and ROR2 mutation..............24

ror1 and ror2 have a role in CoPA neuron axon guidance........................................25

Discussion......................................................................................................................26

Figures.........................................................................................................................33

Works Cited.................................................................................................................46

Vita...............................................................................................................................53
List of Figures

Table 1. Morpholino Sequences…………………………………………………………………18

Table 2. gRNA DNA template sequences………………………………………………………18

Table 3. T7 Assay gRNA target region primer sequences…………………………………….19

Table 4. In situ hybridization probe DNA template sequences……………………………19

Figure 1. Model of the Planar Cell Polarity Pathway in Axon Guidance.......................33

Figure 2. Model of Prickle protein structure…………………………………………………34

Figure 3. Phylogenetic tree of Prickle orthologues in humans, mice, and zebrafish……...35

Figure 4. Model of ROR1 and ROR2 protein structure……………………………………36

Figure 5. Phylogenetic tree of ROR1 and ROR2 orthologues in humans, mice, and zebrafish...37

Figure 6. mRNA Expression of prickle1a, prickle2a, prickle2b, prickle3……………………38

Figure 7. Prickle CRISPR/Cas9 mutant morphology……………………………………….39

Figure 8. T7 Assay gels of prickle CRISPR/Cas9 mutants………………………………….40

Figure 9. prickle loss of function mutation……………………………………………………41

Figure 10. Knockdown of ror2…………………………………………………………………42

Figure 11. ROR1 and ROR2 CRISPR/Cas9 mutant morphology…………………………43

Figure 12. T7 Assay gel of ror1 and ror2 CRISPR/Cas9 mutants………………………….44
List of Abbreviations

A-P: Anterior-posterior

Celsr: Cadherin EGF LAG seven-pass G-type receptor

CoPA: Commissural primary ascending

CRD: Cysteine rich domain

CRISPR: Clustered, Regularly Interspaced, Short Palindromic Repeat

Daam: Dishevelled-associated activator of morphogenesis

D-V: Dorsal-ventral

DCC: Deleted in colorectal cancer

DLF: Dorsal longitudinal fasciculus

Dsh, Dvl: Disheveled

FBMN: Facial branchiomotor neuron

FLNa: filamin A

Fzd: Frizzled

GFP: Green fluorescent protein

GDP: Guanosine Diphosphate
GTP: Guanosine Triphosphate

Ig: immunoglobulin-like

JNK: Jun-N-terminal kinase

MO: Oligonucleotide Morpholino

PBS: Phosphate-buffered saline

PBSTx: Phosphate-buffered saline Triton-X 100

PCP: Planar cell polarity

Pk: Prickle

PRD: proline rich domain

Ptk7: Protein tyrosine kinase 7

ROR: Receptor Tyrosine Kinase like orphan receptor

S/TRD: serine/threonine-rich domains

Scrib: Scribble

TK: tyrosine kinase

Vangl: Van Gogh like

Wnt: Wingless-type
Abstract

Characterizing the Role of Key Planar Cell Polarity Components in Axon Guidance

By Grayland W. Godfrey, B.S.

A thesis submitted in partial fulfillment of the requirement for the degree of Masters of Science at Virginia Commonwealth University.

Virginia Commonwealth University

Major Director: Dr. Gregory S. Walsh, Department of Biology

An essential process to the development of the neural network of the nervous system is axon guidance. The noncanonical Wnt/Planar Cell Polarity pathway has been identified as an integral component in controlling the projection of axons during axon guidance. Prickle, ROR1 and ROR2 are PCP related proteins that do not have clearly defined roles in the process. This study aims to use zebrafish CoPA neurons as a model to study the roles of Prickle, ROR1, and ROR2 in axon guidance. Using in situ hybridization, morpholino knockdown, and CRISPR/Cas9 loss of function experiments were able to identify ror1, ror2 and prickle as potential required components in CoPA neuron axon guidance. Elucidating the role of these protein in axon guidance not only will increase our knowledge of the PCP pathway but it will also increase our understanding of the development of the nervous system.
Introduction

Axon Pathfinding and Neural Development

The human nervous system is composed of a complex network of approximately 100 billion neurons that control both behavior and movement. Neurons utilize axons and dendrites to form a neural network of approximately 60 trillion connections that allow neurons to transmit and receive information throughout the body (Stiles et al., 2010). Axon pathfinding is a fundamental feature of neural development that allows for the assembly of neural circuits that control behavior. Axon guidance has been shown to be controlled by various secreted and contact-dependent attractive and repulsive cues (Battum et al., 2015). These cues are sensed by the axon growth cone, which is at the distal tip of the axon. The growth cone uses these cues to initiate axon extension in the direction of an attractive cue and away from a repulsive cue (O’Donnell, 2009). The structure of the axon growth cone includes finger like protrusions called filopodia and web like protrusions in between the filopodia called lamellipodia. The filopodia extend toward attractive cues through actin polymerization, which causes the axon to grow toward an attractive cue. Polymerization and depolymerization of actin filaments allows the axon to respond to both attractive and repulsive guidance cues. In addition to actin, microtubule polymerization and depolymerization in response to guidance cues may contribute to axon extension in the direction of the cue (Dent et al., 2010).

Disruption in axon guidance has been shown to cause disorders such as horizontal gaze palsy with progressive scoliosis, congenital mirror movements, and congenital fibrosis of the extraocular muscles (Nugent et al., 2012). Although limited evidence currently exists, autism spectrum disorders, epilepsy, and schizophrenia may also be the result of abnormal neural
connectivity caused by disruptions in axon guidance (Battum et al., 2015; Ruiz et al., 2013). Studying the factors that control axon guidance could be beneficial in developing treatments for many of these disorders caused by abnormal neural connectivity. The guidance cues that direct axons to a particular location in the nervous system have been well characterized. However, the molecular mechanisms that physically steer the axon in response to those cues have not been well defined (Dickson, 2002). The Planar Cell Polarity pathway has been identified as an integral signaling pathway involved in steering axons in a particular direction in response to guidance cues during axon pathfinding (Komiya and Habas, 2008; Tissir and Goffinet 2013).

**Planar Cell Polarity Pathway**

The Planar Cell Polarity (PCP) signaling pathway, a noncanonical Wnt pathway is an important signaling pathway involved in the regulation of the actin cytoskeleton (Zou, 2004). PCP components have context specific roles in the development of different parts of the body through regulation of morphogenesis and tissue organization (Butler et al., 2017). Wnts are secreted cysteine-rich proteins that have multiple roles in development. Wnts are integral components of the canonical wnt-β-catenin pathway, the PCP pathway, and the Wnt-Ca$^{2+}$ pathway. These proteins are involved in developmental processes such as cell fate determination, cell proliferation, tissue polarity, and synaptogenesis. The PCP pathway includes both transmembrane and cytoplasmic proteins. Core transmembrane vertebrate PCP proteins include Frizzled, Celsr, and Van Gogh-like (Vangl). Core cytoplasmic PCP proteins include Dishevelled (Dsh), Prickle (Pk), and Scribble (Scrib). Proteins that act as Wnt co-receptors such as Glypican4 (GPC4), Protein tyrosine kinase(PTK)7, and Receptor tyrosine kinase like orphan receptor (ROR)2 have also been implicated as accessory PCP proteins (Minami et al., 2010 and Figure 1).
Wnt/Fz signaling directly results in activation of Rho and Rac in independent parallel pathways. Activation of RhoA requires Dsh and Daam1 (Dishevelled-associated activator of morphogenesis) whereas activation of Rac does not require Daam1 (Habas et al., 2001; Habas et al., 2003). Daam1 binds to both Dsh and RhoA, which mediates Wnt-induced Dsh-RhoA complex formation (Habas et al., 2001). Wnt/ Frizzled activation of Rac and not Rho activates JNK (Figure 1). Both Rac and Rho are required for convergence extension during Xenopus gastrulation (Habas et al., 2001; Habas et al., 2003).

ROR2 is a PCP related protein found to mediate Wnt5a signaling to regulate polarized cell migration. Wnt5a binds to the Cysteine rich domain (CRD). The binding of Wnt5a to the ROR2 CRD activates C-Jun N-terminal kinase (JNK) through being activated by actin binding filamin A (FLNa), which is activated by the ROR2 proline-rich domain (PRD) and serine/threonine-rich domains (S/TRD1 and 2). Activation of JNK affects polarized cell migration. In addition to cell migration, ROR2 may also be an integral component to aspects of neural development such as axon guidance (Minami et al. 2010).

**Developmental Roles of the PCP pathway**

Most epithelial cells in the body are polarized along several axes. Columnar epithelia have apical-basal polarity that is perpendicular to the plane of the epithelium, organized through the functions of polarity complexes such as the Par complex, Scribble complex, and Crumbs complex. On the other hand, epithelial cells are polarized within the plane of the epithelium through the function of the PCP signaling pathway (Jussilla et al., 2017). This was first described in Drosophila in which actin–based hairs called trichomes, are positioned and oriented in the distal side of each wing cuticle cell. In vertebrates, the PCP pathway has also been shown to
control the orientation of cilia in inner ear hair cells, orientation of skin hair follicles, positioning of cilia in the node that controls left-right asymmetry, as well as asymmetric localization of cilia on floorplate cells in the nervous system that are responsible for moving cerebrospinal fluid through the nervous system (Vinson and Adler, 1987; Komiya and Habas 2008; Jusilla et al., 2017).

In addition to controlling asymmetric localization of structures in epithelial cells, accumulating evidence indicates that PCP signaling pathway controls directional migration of various cell types during development. Perhaps the best-known example is the regulation of convergence-extension cell movements during gastrulation of vertebrate embryos (Jusilla et al., 2017). First described in Xenopus, convergence extension involves a combination of collective cell movement and cell intercalations that trigger the narrowing of the body axis along its medial-lateral axis and elongation along its anterior-posterior axis. The mechanisms of PCP related convergence extension have not been well defined (Keller et al., 1985; Tada and Hesisenberg, 2012; Butler et al., 2017). Currently there are two potentially related models that attempt to explain the mechanism of convergence extension. The first model describes convergence extension as occurring due to the formation of cellular protrusions that exert traction force on neighboring cells that causes the cells to intercalate (Shih and Keller, 1992). The second model asserts that junctions between opposing cells contract to compel cells to converge together (Shindo et al., 2014). Since PCP proteins are involved in both the polarity of cell protrusions and contraction of cell junctions the role of the PCP pathway in convergence extension is supported by both models (Butler and Wallingford, 2017). In mammals, PCP-mediated convergence extension movements in the developing neuroectoderm layer are required for proper neurulation, or neural tube closure. In the absence of PCP-mediated convergence, the
floorplate is wider than normal, and the neural tube fails to fuse dorsally, leaving an open neural tube in mouse PCP mutants (Ybot-Gonzales et al., 2007).

PCP has also been shown to regulate the directional migration of other cell types, including the collective migration of neural crest cells, and the migration of facial branchiomotor neurons in the developing hindbrain. For facial neurons, PCP components are required both within the neuron (cell autonomously) as well as in the surrounding environment (non-cell autonomously) to regulate sustained posteriorly directed migration (Walsh et al., 2011; Davey et al., 2016).

Wnts are integral components of the canonical wnt-β-catenin pathway, the PCP pathway, and the Wnt-Ca^{2+} pathway. Wnt signaling in the PCP pathway can have both attractive and repulsive effects on the axon (Lyuksyutova et al. 2003). Wnt4, Wnt5a, and Wnt11 are the forms of Wnt that are involved in the PCP pathway (Komiya and Habas, 2008). The PCP pathway was first described in studies that showed that mutations in Wnt signaling components including Frizzled and Dishevelled in Drosophila changed the orientation of epithelial structures such as cuticle hairs and sensory bristles (Komiya and Habas, 2008). Within the nervous system, the PCP components have significant roles in neuron migration, dendrite formation, and axon guidance (Tissir and Goffinet, 2013). During axon guidance attraction is believed to be controlled by a Frizzled dependent pathway, whereas repulsion may be controlled by the receptor tyrosine kinase like protein Derailed (Lyuksyutova et al., 2003).

**Commissural Axons as a Model for Studying Axon Guidance**

Due to their consistent navigation pattern along both axes in the developing nervous system, commissural neurons in the spinal cord are a well-represented model for studying axon
guidance (Stoekli, 2017). Long range guidance cues guide the axons to the midline of the floorplate, and short range cues ensure the axon crosses the midline before turning away from the floorplate. Netrin/DCC signaling attracts commissural axons ventrally towards the floor plate. Netrin regulates Rho GTPases which cycle between an inactive, GDP-bound state and an active, GTP-bound state. Activation of Rho negatively regulates axon extension (Serafini et al., 1996; Bashaw et al. 2010; Hall et al. 2010). Slit/Robo signaling is required to expel commissural axons from the floorplate midline, and then be repelled away from the floorplate. Different forms of Robo have specialized roles to direct the axon once it reaches the floorplate. Robo 3.1 promotes midline crossing of commissural axons through suppressing repulsion Robo 1 and Robo 2 mediated slit repulsion. Then Robo 1 prevents the axons from stopping its projection and, Robo 2 is required for axons to project away from the floor plate. Robo3.2 is expressed in the post midline crossing axons and it mediates repulsion of the axon from the midline. After guidance across the midline of the spinal cord, commissural axons turn in a rostral direction along the border of the contralateral floor-plate to project towards the hindbrain (Jaworski et al., 2010; Sabatier et al. 2004; Chen et al. 2008).
PCP and Axon Pathfinding

Many studies have illustrated the importance of PCP signaling in axon guidance. PCP components are essential for precise anterior turning of commissural axon growth cones after midline crossing in the spinal cord (Lyuksyutova et al., 2003). The importance of the PCP pathway in commissural axon guidance has been shown in both mouse and chick embryos (Lyuksyutova et al., 2003; Avilés and Stoeckli, 2016). PCP proteins such as Fzd3, Celsr3, and Vangl2 have all been found to be essential in for anterior turning of commissural axons (Tissir and Goffinet, 2013). Instead of turning anteriorly after midline crossing, post-crossing commissural axons turn either randomly (anteriorly or posteriorly) along the A-P axis, or stall in the contralateral spinal cord (Tissir and Goffinet, 2013). Exogenous Wnts can attract commissural axons. The expression of some Wnts, like Wnt4, was found to be high anteriorly and low posteriorly. This suggests a model whereby Wnt ligands secreted in a gradient attract commissural axons anteriorly after midline crossing. (Zou, 2004). In the midbrain and hindbrain, PCP components are also necessary for anterior-posterior guidance of dopaminergic and serotonergic axons (Zou, 2004). Other PCP proteins including Prickle, Celsr, and Daam1a have been shown to regulate axon guidance in other systems. For example, Prickle has been shown to affect axon advance in Drosophila through interactions with flamingo/Celsr (Mrkusich et al., 2011). A knockdown study in zebrafish has revealed that Prickle1a is required for both retinal inner plexiform layer organization and neurite outgrowth (Mei et al., 2013). Daam1a knockdown in zebrafish causes individual habenular neurons to display pruned dendritic arbors and incomplete axon terminal patterns (Colombo et al., 2013).
Zebrafish CoPA Neurons

Zebrafish (Danio rerio) commissural primary ascending (CoPA) neurons are commissural neuron found in the dorsolateral region of the spinal cord. They are located just ventral to touch-sensitive Rohon-Beard sensory neurons in the dorsal-most portion of the zebrafish spinal cord. CoPA neurons are important in the formation of neural circuits with Rohon-Beard cells to control a touch-evoked withdrawal response in zebrafish (Bernhardt et al. 1990; Roberts et al., 2000). CoPA neurons receive glutamate signaling through AMPA receptors in response to touch stimuli (Pietri et al., 2009; Knogler & Drapeau, 2014). These neurons have a single unbranching axon that projects ventrally and crosses the midline in the floor of the spinal cord. After midline crossing, CoPA axons extend simultaneously dorsally away from the midline and anteriorly towards the head. In this last stage, CoPA axons grow in a dorso-anterior direction, ascending at an oblique angle to the dorsal spinal cord where it joins other CoPA axons from more caudal segments in the dorsal longitudinal fasciculus (DLF).

CoPA neurons form two distinct dendrites that emerge from the rostral and caudal poles of the cell body projecting longitudinally up to four spinal segments (Bernhardt et al., 1990). These neurons are one of the earliest developed neurons in the zebrafish embryonic spinal cord which indicates that their axons act as pioneer axons that establish the commissure (Kuwada et. al., 1990). Since it is an early developed neuron it can provide valuable information on the early development of neural connectivity. The molecular mechanisms that control axon pathfinding in CoPA neurons could be applied to other neurons in the nervous system to further study the mechanisms that control the organization of the nervous system.
CoPA Neurons and the PCP pathway

Similar to commissural neurons in other vertebrates, PCP signaling pathway components are required for anterior guidance of CoPA axons (Tissir and Goffinet, 2013; Sun et al., 2016). Frizzled3a, Vangl2, and Scribble are all are required for proper axon guidance of zebrafish spinal CoPA neurons. Analysis of *fzd3a*, *vangl2*, and *scrib* homozygous mutant fish revealed that CoPA axons randomly turn either anteriorly or posteriorly into the contralateral spinal cord. Importantly, loss of PCP had no effect on the dorso-ventral guidance of CoPA axons (Sun et al., 2016). In addition, CoPA axons do not stall but instead project for long distances along the A-P axis regardless of the directional they are travelling. This indicates that the PCP pathway is not important for axon extension, but is required either for the guidance or the interpretation of other guidance cues along the A-P axis (Sun et al., 2016).

Prickle in the Nervous System

Although many recent studies have sought to define the roles of PCP components involved in axon guidance, the roles for many potential components have not been well established. Prickle, ROR1 and ROR2 are PCP related components that require additional study to better understand their role in axon guidance (Minami et al., 2010). Prickle is a protein that contains three cysteine-rich zinc finger-like LIM (Linl-1, Isl-1, and Mec3) domains, a highly-conserved PET (Prickle, Espinas, and Testin) domain, a nuclear localization signal (NLS) and a farnesylation motif (CAAX) (Sweede et al., 2008; Shimojo & Hersh, 2003; Jenny et al., 2003; Mapp et al., 2011 and Figure 2). Currently, human paralogs of Prickle include Prickle1, Prickle2, Prickle3, and Prickle4 (Figure 3). Prickle forms in zebrafish that have been identified include
prickle 1a, prickle 1b, prickle 2a, prickle 2b, and prickle 3 (Figure 3). Zebrafish have additional forms of Prickle due to an ancient genome duplication event (Kent et al., 2002; Jussilla and Ciruna, 2017). Within the zebrafish nervous system prickle1a is expressed in distinct neuronal and glial cell populations during development (Liu et al., 2013). Prickle is involved in axonal and dendrite extension, neurite outgrowth in the retina and, facial branchiomotor neuron migration (Liu et al., 2013; Mei et al., 2013; Mapp et al., 2011; Mrkusich et al., 2011).

**ROR1 and ROR2 in the Nervous System**

ROR1 and ROR2 are transmembrane proteins that contain one immunoglobulin-like (Ig-like) domain, one Frizzled (FZD)-like cysteine-rich domain (CRD), and one Kringle domain in their extracellular region. In its cytoplasmic region, it contains a tyrosine kinase(TK) domain, two serine threonine rich domains (S/TRD1 and S/TRD2) and a proline rich domain (PRD) (Stricker et al., 2017 and Figure 4). In vertebrates ROR1 and ROR2 are highly expressed in areas such as the heart, lungs, and central nervous system during embryonic development. Although the expression and function of ROR2 in zebrafish has been determined to be similar to other vertebrates, the expression and function of ROR1 in zebrafish has not been well defined (Stricker et al., 2017 and Figure 5). ROR2 associates with Wnt5a through its Cysteine Rich Domain which is able to activate JNK (Oishi et al., 2003; Jussilla & Ciruna, 2017). Similar to other core PCP components, loss of ROR2 in zebrafish leads to convergence extension defects resulting in a shorter wider embryo (Oishi et al., 2003; Bai et al., 2014). In mice, loss of both ROR1 and ROR1 leads to more severe morphogenetic movement abnormalities that cause developmental disorders such as shorter limbs and facial structure, than ROR1 or ROR2 mutants alone (Ho et al., 2012). Expression analysis and knockdown of ROR1 has not been reported. Current research has suggested that ROR1 and ROR2 are involved in cell differentiation into neurons, synapse
formation, and dendrite development (Endo et al., 2012; Alfaro et al., 2015; Pagononi et al., 2010).

**Significance and Aim of Research**

In addition to their potential role in axon guidance, Prickle and ROR are involved in a variety of neurological and developmental disorders. An autosomal recessive mutation in Prickle 1 has been linked to progressive myoclonus epilepsy-ataxia syndrome. This mutation is the result of a single missense nucleotide in the Prickle coding region (Bassuk et al. 2010). The role of Prickle in epilepsy has been demonstrated through experiments generating seizures in zebrafish using PTZ (Pentylenetetrazol) treatment. Zebrafish with a prickle1a knockdown were more sensitive to PTZ treatment resulting in increased seizure activity (Mei et al., 2013). Mutations in ROR2 gene causes Robinow Syndrome and dominant Brachydactyly type B1. Robinow affects the development of multiple organs and Brachydactyly type B1 causes shortening of the distal phalanges of fingers and toes (Stricker et al., 2017). Inhibition of ROR2 causes mice to display dwarfism, facial abnormalities, and short limbs (Oishi et al., 2003).

Learning more about the role of core PCP proteins such as ROR1, ROR2, and Prickle will not only increase our knowledge of neural development but it will also allow us to understand the mechanisms that cause neurological disorders such as epilepsy and developmental disorders such as Robinow syndrome and Brachydactyly type B1. In this current study, I aim to use zebrafish CoPA neurons as a model to determine the role of Prickle, ROR1, and ROR2 in axon guidance. This research will provide valuable information that could be used to not only better understand axon guidance, but also to understand how these two proteins may function in developmental and neurological disorders.
Materials and Methods

Fish Strains

Zebrafish (Danio rerio) were maintained according to standard procedures and were staged as previously described (C. B. Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). Wild type embryos were obtained through mating AB fish. Tg(tbx16:GFP) fish were obtained from the Lardelli lab (Wells et al., 2011).

Microinjections

Injection plates were made at a concentration of 1.2% agarose using fish water as a solvent. A mold was used to create triangular wells at a 45 degree angle to allow embryos to be supported against the back of the wells for injection. Zebrafish were placed in a breeding cage dividing males and females the night before injections. Dividers were pulled the morning of injections to allow the zebrafish to breed. Embryos were collected from the cages and placed in injection dishes. Injection reagents were loaded into glass capillary needles pulled using a needle puller. Embryos were injected at the one-cell stage using an ASI MPPI-3 (Applied Scientific Instrumentation) pressure injector. Once they were injected they were placed in an incubator at 28.5°C.

Morpholino Microinjections

Tg (tbx16: GFP) zebrafish were mated to collect embryos for microinjection. Antisense morpholino oligonucleotides (MOs) were obtained from Gene Tools, LLC (Table 1). Embryos were initially injected with 1 nL of prickle 2b translation blocking MO at a concentration of
5ng/nL. For the next prickle 2b MO injection, embryos were injected with 1 nL of a 5ng/nl Prickle 2b MO and p53 MO (4ng/nl). For prickle 2b and prickle 1a translation blocking MO co-injections, embryos were injected with 1 nl of prickle 2b morpholino at concentrations consisting of 2ng/nl, 3ng/nl, and 4ng/nl, each combined with prickle 1a morpholino (4ng/nl) and p53(4ng/nl). Separate injections were performed for each concentration of prickle 2b used in the Co-injections. For the ror2 MO knockdown experiment, embryos were injected with 1 nl of ror2 E111 splice blocking morpholino at a concentration of 7ng/nl alone. Then embryos were injected with the ror2 splice blocking morpholino combined with p53(4ng/nl) (Table 3).

**CRISPR/Cas9 gRNA template synthesis**

CRISPR/Cas9 gRNA was prepared following a zebrafish specific CRISPR/Cas9 protocol (Shah et al., 2015). The guide RNA (gRNA) template sequence for CRISPR/Cas9 genome editing was designed through obtaining target sequence from UCSC genome browser and submitting it to the CRISPR design website crispr.mit.edu (Table 2). The gRNA template was synthesized using an Oligonucleotide scaffold and a gene target specific oligonucleotide sequence containing a T7 RNA polymerase binding site. gRNA templates were designed for prickle 1a, prickle 2a, prickle 2b, prickle 3, ror1, and ror2 (Table 1). An overlap PCR reaction was performed using the oligonucleotide scaffold and gene target oligonucleotide template containing a T7 binding site to synthesize the gRNA template DNA. The reaction utilized a PCR protocol that included 2.5µl of water, 12.5µl 2 X Phusion master mix (New England Bio Labs, M0531L), 5µl Scaffold (Invitrogen), and 5 µl of gRNA (Invitrogen). The thermocycler program used for the reaction was as follows: 95 degrees Celsius for 30sec; 40 cycles of 95 degrees Celsius for 10 sec. 60 degrees Celsius for 10 secs, 72 degrees Celsius for 10 secs; then 72
degrees Celsius for 5 mins. PCR products were purified using DNA purification columns (Zymo Research DNA Clean and Concentrator).

**CRISPR/Cas9 Guide RNA transcription**

gRNA was made from the purified PCR-amplified DNA template. The RNA was transcribed using a T7 in vitro transcription reaction that utilized between 0.1 and 0.3 µg of PCR product as template (T7 Megascript, Ambion). The transcribed RNA was purified using either a phenol-chloroform and isopropanol precipitation or using a RNA purification column (Zymo Research RNA Clean and Concentrator).

**CRISPR/Cas9 Microinjections**

Cas9 mRNA at a concentration of 984 ng/ul was combined with guide RNA at a concentration of 200 ng/µl was combined in a 1:1 ratio to yield a final concentration of Cas9 mRNA (492 ng/ul) and gRNA (100 ng/ul). The embryos were injected with a volume of 1 nL. For ROR1 and ROR2 duplex gRNA injections, 1µl of ROR1 gRNA (200 ng/µl) and 1µl of ROR2 gRNA (200 ng/µl) was combined with 2µl of Cas9 mRNA to yield final injection concentration of (50 ng/µl of each gRNA). For the Prickle multiplex CRISPR/Cas9 injections 1µl of each prickle gRNA was combined for a total of 4µl. 2µl of this solution was combined with 2µl of Cas9 mRNA to yield a final injection concentration of 20 ng/µl of each gRNA.

**T7 Assay**

DNA was extracted through placing embryos in 1x Base solution (25Mm NaOH, 0.2 Mm EDTA) in a 96-well plate and incubated 95ºC for 30 minutes. They were then allowed to cool to room temperature before adding 1x Neutralization solution (40Mm Tris-HCL Ph5. Extracted
DNA was mixed through pipetting solution repeatedly in and out of the tube. Primers were designed to target the region containing the gRNA target sequence using UCSC genome browser (Table 3). PCR reaction was performed to produce DNA product for each gene target region. DNA samples used in PCR reactions for each prickle gene were from the same set of embryos. PCR products for each gRNA targeted region were generated from these DNA samples. DNA products were denatured and re-annealed in a thermocycler to generate heteroduplexes from mismatches in the DNA. T7 endonuclease was added to the DNA products to cleave the DNA at the mismatched locations. Then the DNA products were incubated at 37°C. Results of the T7 assay were visualized using gel electrophoresis.

**Immunohistochemistry**

Embryos were fixed at 30hpf using 4% Paraformaldehyde (in 1 x phosphate-buffered saline (PBS)) overnight at 4°C. The embryos were washed in PBSTx (1x PBS with 0.25% Triton X-100) and permeabilized using Acetone. After washing out the acetone using PBST the embryos were blocked with PBST+10% Goat Serum + 10% BSA at room temperature for an hour. The embryos were then stained with 3A10 (mouse IgG1) primary antibody (neurofilament antibody) overnight at 4°C. The embryos were then treated with a 1:200 concentration of Alexa Fluor 568 Goat Anti-Mouse IgG (H+L) Secondary Antibody (catalog number A11031, Life Technologies) overnight at 4°C. After secondary antibody treatment, the embryos were washed 5 x 30 mins using PBSTx (1 x PBS with 0.25% Triton X-100). Embryos were then dehydrated in 25%, 50%, and 75% glycerol in sequence.
Embryo Mounting

In order to analyze CoPA neuron axon guidance, immunostained embryos were dehydrated in 75% glycerol. The tails of the dehydrated embryos were removed through microdissection in order to image the CoPA neurons in the spinal cord. The tails were mounted on their sides in 75% glycerol between two microscope coverslips to image the lateral region of their spinal cord.

Confocal Microscopy

CoPA neurons were imaged using a Carl Zeiss Spinning Disk Laser Confocal Observer.Z1 (Virginia Commonwealth University, Biology Department.). In order to obtain images of CoPa axon pathfinding confocal projections were made through imaging from CoPA neurons on one side of the spinal cord to the cell bodies on the other side of the spinal cord.

Microscopy

Embryos were placed in porcelain or glass dishes with circular wells to complete imaging. Embryo morphology from morpholino, CRISPR, and in situ hybridization experiments was imaged at 30 hours post fertilization using a Carl Zeiss Stereo microscope Discovery.V8.

In Situ Hybridization

In Situ hybridization was performed following a zebra fish specific protocol (Moens, 2008). Zebrafish embryos were fixed at 30hpf and incubated in RNA probes overnight.
**PCR template synthesis**

Forward and reverse Primers were designed for target gene mRNA expression using UCSC genome browser (Table 4). A PCR reaction was performed using target primers to amplify the DNA template sequence. DNA template for antisense probes were produced using forward primers without a T7 sequence and reverse primers with a T7 sequence. Sense probes were made using forward primers with a T7 sequence and reverse primers without a T7 sequence.

**In Vitro Transcription**

DNA template for target sequences was used in a 20µl in vitro transcription reaction. The reaction was performed using an in vitro transcription reaction protocol that included 5x Trans buffer, 100Mm DTT, 10x Dig Trans Mix (Roche), RNAsin, and T7 RNA polymerase.

**RNA purification**

The RNA produced from the reaction was purified using an Illustra probe quant G-50 microcolumn (GE healthcare illustra probe quant G-50 microcolumn). The RNA concentration was measured using a Nanodrop spectrometer. Purified RNA was re-suspended in 50µl of Hybridization mix without tRNA.
### Table 1. Morpholino Sequences

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<thead>
<tr>
<th>Target Protein</th>
<th>Sequence (5’ to 3’)</th>
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<tr>
<td>Prickle 1a</td>
<td>GCCCACCCTGATTTCTCCAGCTCCAT</td>
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<tr>
<td>Prickle 2b</td>
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<tr>
<td>ROR2</td>
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</tr>
<tr>
<td>P53</td>
<td>GCGCCATTTGCTTTGCAAGAATTG</td>
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### Table 2. gRNA DNA Template Sequences

<table>
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<th>gRNA Gene Target</th>
<th>gRNA Template Sequence</th>
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<tr>
<td>ror1</td>
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<td>ror2</td>
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<td>prickle2b</td>
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<td>prickle3</td>
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Table 3. T7 Assay gRNA target region Primer Sequences

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<th>Primer Sequence</th>
<th>Product Size(bp)</th>
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Table 4. In Situ Hybridization Probe DNA template Sequences

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<td>pk2a Right1</td>
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</tr>
<tr>
<td>pk2b Left1</td>
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<td>pk2b Right1</td>
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<td>pk3 Right 3</td>
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Results

Prickle paralogs are expressed in the spinal cord

We first sought to determine whether prickle genes are required for CoPA axon pathfinding. In order to determine which prickle paralog might be expressed in the spinal cord at the right time (24 hpf) during development, we performed in situ hybridization for prickle 1a, prickle 2a, prickle 2b, and prickle3. Previous work has shown that prickle 1b is not expressed in the spinal cord (Mapp et al., 2011). We found that prickle 1a, prickle 2a, prickle 2b, and prickle 3 all exhibited ubiquitous staining throughout their body (Figure 6). Importantly, all four prickle paralogs were expressed in the spinal cord at 24 hpf, suggesting that multiple prickle proteins may function to steer CoPA growth cones. prickle 3 had additional expression at somite boundaries.

Prickle1a and Prickle2b are dispensable in axon guidance of CoPA Neurons

To determine whether prickle 2b is essential for CoPA guidance, we performed loss-of-function experiments using antisense morpholino oligonucleotides (MOs). Previous unpublished work from the Walsh Lab had shown that embryos injected with prickle 1a MOs caused a convergence extension phenotype resulting in shorter and wider embryos as previously reported (Carreira-Barbosa, 2003). However, no defect in anterior turning of CoPA axons was observed, indicating that prickle1a alone is dispensable for commissural axon guidance. We therefore turned our attention to prickle 2b. Embryos injected with prickle 2b MO alone exhibited convergence extension defects and cell death in the brain. The CoPA neurons of these embryos could not be imaged due to the cell death. This cell death was likely due to morpholino toxicity, and not related to loss of prickle 2b. To get around this problem, we co-injected with p53 MOs,
which has been previously shown to block morpholino-toxicity induced cell death (Bedell et al., 2000). Once again, embryos injected with prickle 2b morpholino and p53 MO displayed a convergent extension phenotype. Importantly, these embryos did not exhibit cell death in their brains. We imaged CoPA neurons in co-injected embryos and observed that all CoPA axons projected their axons in an anterior direction, despite other morphological defects. This suggested that prickle 2b alone is dispensable for CoPA axon pathfinding.

We therefore sought to determine whether loss of both prickle 1a and prickle 2b would lead to CoPA defects. Embryos were injected with a combination of a standard amount of prickle1a MO and varying amounts of prickle 2b MO. We observed that embryos co-injected with both prick1a and prickle 2b MO also displayed a convergence extension phenotype, as described earlier. The percentage of embryos displaying a convergence extension phenotype increased from approximately 20% to 40% in embryos that were injected with higher concentrations of prickle 2b morpholino. However, in all combinations of prickle 2b and prickle 1a morphants imaged CoPA neurons underwent proper midline crossing followed by correct turning in an anterior direction.

**prickle CRISPR/Cas9 experiments**

We decided to investigate whether mutations in all prickle paralogs (with the exception of prickle 1b) would lead to axon guidance defects. To accomplish this, we decided to use CRISPR/Cas9 genome editing to induce insertion/deletion mutations in all of the prickle genes simultaneously. Previous work had demonstrated that injection of multiple CRISPR guide RNAs (gRNAs) are capable of inducing multiple mutations in a single founder (F0) embryo. It should
be noted that injection of CRISPR gRNA and Cas9 into one-cell stage embryos leads to founder fish carrying mosaic mutations. At high doses (that do not cause toxicity) CRISPR/Cas9 can cause bi-allelic mutations allowing one to screen for recessive phenotypes in founder fish (Ablain et al., 2015). As proof of principle, the Walsh lab generated a single CRISPR gRNA that targeted frizzled 3a (fzd3a), that had already been shown to cause a randomized CoPA turning defect in fzd3a-/- mutants (Sun et al., 2016). Injection of fzd3a gRNA + Cas9 mRNA led to a defect in anterior turning in CoPA axons. However, only one CoPA neuron per embryo exhibited a defect, likely due to the mosaic nature of CRISPR-induced mutations in founder fish. This suggests that CRISPR genome editing is sufficient to induce axon pathfinding defects in this system, with the caveat that they will be rare events due to mosaicism.

To determine whether prickle is involved in axon guidance, we generated guide RNAs for each of the prickle genes. Zebrafish embryos were then injected with a multiplex cocktail of prickle1a, prickle2a, prickle2b, and prickle3 gRNA + Cas9 mRNA. Approximately 30% of embryos injected with the prickle multiplex gRNAs displayed a convergence extension defect phenotype. Morphologically, embryos ranged in size from wild type appearance to less than half the size of a wild type embryo. (Figure 7).

**T7 endonuclease I assay confirms presence of prickle mutations**

To validate that our gRNAs were capable of generating mutations at all prickle loci, we extracted genomic DNA from individual embryos displaying a convergence extension phenotype and performed PCR to amplify the genomic region around each guide RNA mutation site. T7 endonuclease I cleaved mismatches in PCR products of prickle 1a, prickle 2a, prickle 2b, and prickle 3 gRNA targeted regions creating additional bands on the gel (Figure 8). DNA samples
from the same embryo injected with the prickle multiplex CRISPR/Cas9 construct exhibited cleaving DNA mismatches suggesting successful genome editing of each gene within the same individual embryos. Wild type embryos treated with T7 endonuclease did not result in additional bands caused by cleaved DNA mismatches, suggesting that mutations were caused by the prickle multiplex CRISPR/Cas9 construct (Figure 8).

**prickle genes have a role in CoPA neuron axon guidance**

We focused our imaging on CoPA axons in embryos displaying a convergence extension defect. Of the 27 embryos imaged, 3 of them had at least one CoPA axon project in a posterior direction after crossing the midline of the floor plate (Figure 9). At least 5 neurons were imaged in each embryo. We never observed any defect in the dorsal-ventral guidance of CoPA axons in prickle gRNA multiplex injections. Taken together, this suggests that prickle genes are essential for anterior turning of commissural axons.

**ror2 is dispensible in axon guidance of CoPA Neurons**

To determine whether ror2 is essential for CoPA guidance, we performed loss-of-function experiments using antisense morpholino oligonucleotides (MOs). Zebra fish embryo injected with ROR2 morpholino alone exhibited both convergence extension defects and cell death in the brain. This cell death was likely due to morpholino toxicity, and not related to loss of ror2. To get around this problem, we co-injected with p53 MOs, which has been previously shown to block morpholino-toxicity induced cell death (Bedell et al., 2011). The CoPA neurons of these embryos could not be imaged due to the cell death. Zebrafish embryos injected with ror2 morpholino and p53 MO exhibited both a wild type and convergence extension defect
phenotype. These embryos did not exhibit cell death in their brains. None of the eight embryos that were imaged exhibited disruption in CoPA anterior-posterior axon guidance (Figure 10).

**ror1 and ror2 CRISPR/Cas9 experiments**

We decided to investigate whether mutations in both *ror1* and *ror2* would lead to axon guidance defects. To accomplish this, we used CRISPR/Cas9 genome editing to induce insertion/deletion mutations *ror1* and *ror2* simultaneously. Zebrafish embryos injected with a *ror1* and *ror2* duplex CRISPR/Cas9 construct exhibited a convergence extension defect phenotype (Figure 11). Approximately 30% of embryos injected with the CRISPR/Cas9 construct displayed the convergence extension defect phenotype. Since inhibition of *ror2* causes disruption in convergent extension during development, the convergent extension phenotype exhibited by the loss of function of both *ror1* and *ror2* in this present experiment is consistent with the current established role of *ror2* in the early development (Jussilla & Ciruna, 2007).

**T7 endonuclease I assay confirms presence of *ror1* and *ror2* mutation**

Genomic DNA was extracted from individual embryos displaying a convergence extension phenotype. PCR was performed on this DNA to amplify the genomic region around each guide RNA mutation site. T7E1 cleaved mismatches in PCR products of *ror1* and *ror2* gRNA targeted regions creating additional bands on the gel (Figure 12). DNA samples from the same embryo injected with the *ror1* and *ror2* duplex CRISPR/Cas9 construct exhibited cleaving DNA mismatches suggesting successful genome editing of each gene within the same individual embryo. Wild type embryos treated with T7 endonuclease did not produce additional bands on the gel from cleaved mismatches suggesting that mutations were caused by the *ror1* and *ror2* CRISPR/Cas9 construct (Figure 12).
**ror1** and **ror2** have a role in CoPA neuron axon guidance

We focused our imaging on CoPA axons in embryos displaying a convergence extension defect. Embryos size ranged from a wild type appearance to less than half the size of a wild type embryo. Of the 100 embryos imaged, 6 of them had at least one CoPA axon project in a posterior direction after crossing the midline of the floorplate (Figure 13). At least 5 neurons were imaged per embryo. One of the 6 embryos had 4 neurons out of 7 projecting in a posterior direction after crossing the floorplate midline. We did not observe any defect in the dorsal-ventral guidance of CoPA axons in **ror1** and **ror2** gRNA duplex injections. These results suggest that **ror1** and **ror2** genes may be required for anterior turning of commissural axons.
Discussion

Axon guidance is a fundamental component of neural development that is controlled by a series of chemo attractive and chemo repulsive cues that guide an axon to its appropriate target (O’Donnell et al. 2009). Axon guidance allows the nervous system to form an integral network of connections that control human behavior and physiology (Stiles et al. 2010). Disruption in neural connectivity can cause disorders such as epilepsy and schizophrenia (Battum et al. 2015; Ruiz et al. 2013). Although the role of the molecular cues that guide axons to their target location has been well described, the molecular mechanisms that physically steer axons toward their target location is not well understood (Dickson, 2002; Dent et al., 2011).

The noncanonical wnt pathway otherwise known as the Planar Cell Polarity (PCP) pathway is thought to be an integral component to steering axons during axon guidance (Zou et al., 2004). In commissural neurons, the Wnt-Frizzled pathway is specifically required for A-P axon guidance after midline crossing in vivo (Lyuksyutova et al., 2003). Wnt5a exposure to commissural axons increases Frizzled3 and Vangl2 concentration in axon growth cones. Vangl2 reduces Frizzled3 phosphorylation which reduces the concentration of Frizzled3 on the cell surface due to increased endocytosis of the protein. Vangl2 reduces Frizzled3 phosphorylation whereas Dishevelled1 promotes increased Frizzled3 phosphorylation (Shafer et al., 2011). Although both Dishevelled 1 and Dishevelled 3 increase Frizzled phosphorylation. Dishevelled2 suppresses Dishevelled1-induced Frizzled3 hyper phosphorylation (Onishi et al., 2013). Ultimately, Frizzled activation of Rho GTPases initiates JNK regulation of the actin cytoskeleton that allows the axon to turn in response to guidance cues (Habas et al., 2003; Dent et al. 2010).
Although significant research has been completed to describe the roles of major components of the PCP pathway involved in axon guidance, the roles of PCP related proteins such as Prickle, ROR1, and ROR2 have not been clearly defined. The purpose of this present study was to further describe the roles of Prickle, ROR1, and ROR2 in commissural neuron axon guidance. In situ hybridization of prickle1a, prickle 2a, prickle2b, and prickle3 exhibited nearly ubiquitous expression (Figure 6). Expression of each of these prickles in the spinal cord region of the zebrafish embryo at the time that CoPA axons are actively pathfinding suggests that these genes may be involved in CoPA axon guidance. prickle knockdown experiments were performed using morpholinos to determine if these prickles are required for CoPA neuron axon guidance. All morpholino injections included p53 MO to reduce the risk of off target effects that may have caused cell death (Bedell et al., 2011). Morpholino Injection of prickle 2b and prickle 1a resulted in embryos that displayed convergence extension disruption phenotypes. Convergence extension disruption phenotype is consistent with research that suggests that prickle 1 is involved in convergence extension through its interaction with wnt (Carreira-Barbosa et al., 2003).

Live imaging of embryos injected with prickle 2b and prickle 1a combined morpholino did not reveal any CoPA anterior-posterior axon guidance defects. In order to determine if any of the prickle genes are required for axon guidance, we used CRISPR/Cas 9 genome editing to create loss of function mutations in prickle1a, prickle2a, prickle2b, and prickle3. We used CRISPR/Cas9 because morpholino injection of all of these genes may cause too much convergence extension disruption in the embryos or cause too much lethality. CRISPR/Cas9 is an effective method of genome editing in zebrafish with low off target effects (Hruscha et al., 2013). Prickle1b was not included due to its low expression in the spinal cord region (Thisse and Thisse, 2005). Embryos injected with prickle CRISPR/Cas9 resulted in 3 of 27 imaged
embryos that displayed CoPA axon guidance defects (Figure 9). Since wildtype zebrafish CoPA neurons always project in an anterior direction after crossing the floorplate midline, even a small percentage of embryos with aberrant axon guidance suggests that *prickle* may have a role in CoPA axon guidance. The low percentage of defects may be the result of CRISPR/Cas9 mosaic expression (Ablain et al. 2015). This could result in two reasons for inefficient expression of the guidance phenotype. First, mutations may occur more often in cells outside the spinal cord where CoPA neurons develop. Secondly, we used a multiplex strategy of CRISPR gRNAs to generate mutations in all prickle genes. Although we confirmed that we were able to generate mutations in all prickle genes, we are unsure whether these reside in the same clones or different clones of cells scattered in the embryo. Thus, we predict that the proportion of cells in the embryo that have mutations in all prickle genes is low. This would contribute to the low efficiency of generating a CoPA neuron with axon guidance defects in founder embryos. However, the demonstration that, although rare, a defect in anterior guidance is observed in some prickle CRISPR-injected embryos is consistent with the idea that prickle is required for A-P guidance of commissural axons.

Current research on Prickle suggests that it may have multiple roles in the developing nervous system. Prickle interacts with the PCP protein Flamingo to promote sensory neuron axon growth in Drosophila (Mrkusich et al. 2011). Loss of Prickle1 results in too many neurites and Prickle1 overexpression results in too few neurites. Prickle overexpression is sufficient to suppress neurite formation in VC4 and VC5 (peripheral motor neurons) in *C. elegans* (Sanchez-Alvarez et al., 2011). Prickle1a knockdown induces neurite outgrowth in the retina (Mei et al., 2013). Knockdown of *prickle1a* in zebrafish causes them to be more sensitive to PTZ treatment which causes the zebrafish to exhibit seizure like behavior. Since Prickle1 has been linked to
seizure inducing epilepsy, Prickle and the PCP pathway may have a critical role in the disorder (Mei et al., 2013). The results of this study and the role of Prickle in many different aspects of neural development suggests that the it should have some role in CoPA neuron axon guidance.

Our *ror2* morpholino experiments as well as our *ror1/ror2* CRISPR/Cas9 experiments all resulted in some embryos displaying convergence extension defects (Figure 11). This phenotype is consistent with studies that show that the Ror2 CRD domain interacts with Wnt to regulate convergence extension (Oishi et al., 2003; Bai et al. 2014; Jusilla et al., 2017). Mutations in *ror2* gene causes disorders that affect development of various body parts such as Robinow Syndrome and dominant Brachydactyly type B1 (Stricker 2017). Zebrafish embryos injected with *ror2* morpholino did not reveal any anterior-posterior CoPA axon guidance disruption. Since knockdown of *ror2* did not cause any anterior posterior axon guidance defects alone, *ror2* may not be required for proper CoPA axon guidance (Figure 10). One possibility is that *ror1* plays a redundant role in axon guidance with *ror2*.

In order to further study the role of ROR1 and ROR2 in axon guidance we decided to use CRISPR/Cas9 to analyze the effects of loss of function mutations of both ROR1 and ROR2 to determine if these proteins are required for CoPA axon guidance. We used CRISPR/Cas 9 instead of morpholino because a combined *ror1* and *ror2* morpholino injection may have increased embryo convergence extension disruption or even embryo cell death which would have hindered CoPA analysis. *ror1/ror2* CRISPR/Cas9 treated embryos displayed convergence extension defects and anterior-posterior axon guidance disruptions in 6 of the 100 embryos imaged (Figure 11 and Figure 12). Since wildtype CoPA neurons should always project in an anterior direction after crossing the midline, the observation of rare CoPA neurons with axon guidance defects in the *ror1* and *ror2* CRISPR/Cas9 injected embryos suggests that *ror1* and
ror2 may have a role in CoPA neuron axon guidance. The low percentage of embryos with axon guidance defects could be due to the mosaic expression pattern of CRISPR/Cas 9 injected embryos (Ablain et al., 2015). In mouse limb bud development Ror2 is required to regulate Vangl2 phosphorylation (Gao et al., 2011). Vangl2 phosphorylation promotes an increase in JNK and Rho phosphorylation which regulate the actin cytoskeleton (Gao et al., 2011; Habas et al., 2003; Dent et al., 2010). Since Vangl2 also regulates Frizzled3 phosphorylation which increases endocytosis of Frizzled3, regulation of Vangl2 by Ror2 may be an integral component of PCP pathway regulation of JNK through two distinct signaling mechanisms or a combination of these mechanisms. Since Ror2 has an important role in PCP signaling in the limb bud, it may exhibit a similar function in axon guidance.

Current research shows that ROR1 and ROR2 both seem to have integral roles in the neural development. ROR1, ROR2, and Wnt5a interact to regulate neural progenitor cell differentiation (Endo et al. 2012). In addition to neural progenitor cell differentiation, interactions between ROR1, ROR2, and Wnt5a also are required for synaptogenesis between hippocampal neurons (Paganoni et al., 2010). High expression of ROR2 in the dendritic spines of hippocampal neurons and increased dendritic spine growth in response to ROR2 overexpression suggests that ROR2 has an important role in dendrite development (Alfaro et al. 2015). Our results indicate for the first time that ROR receptors may play a role in axon guidance. Future studies will be required to uncover the crosstalk between ROR1/2 and Wnt-Frizzled in mediating commissural axon guidance.

In order to better elucidate the function of Prickle, ROR1, and ROR2 in axon guidance a greater variety of experiments need to be completed. CRISPR/Cas9 loss of function genome editing needs to be performed on each of the prickle genes separately, and then in different
combinations. For instance, although we observed rare defects in axon guidance, it remains unclear whether this defect was a result of losing one, two, three, or four prickle genes. Similarly, CRISPR genome editing also needs to be performed on ROR1 separately. Our morpholino data indicates that ROR2 is dispensable. However, we have not performed the same experiment on ROR1 alone. Performing loss of function experiments on each gene separately will allow us to determine which specific proteins are required for proper CoPA axon guidance. In some cellular contexts, overexpression of PCP proteins causes similar defects as loss-of-function. We also would like to determine whether overexpression of either ROR or Prickle genes is sufficient to elicit a CoPA axon pathfinding defect. In addition to performing loss of function and overexpression experiments to manipulate gene expression throughout the entire embryo we could use CRISPR/Cas9 to target neurons specifically.

Targeting neurons specifically would allow us to better study the role of Prickle and ROR in axon guidance without confounding influences of convergence extension defects. Since both Ror2 and Prickle1 are involved in the regulation of convergence extension, disrupting the function of these proteins often create short embryos (Carreira-Barbosa et al., 2003; Oishi et al., 2003; Bai et al.2014; Jusilla et al., 2017). CoPA neurons are located in the spinal cord of the embryo so if an embryo is shorter it may have fewer CoPA neurons or no CoPA neurons at all. Convergence extension defects limit our ability to analyze CoPA axon guidance in embryos that are exhibiting successful manipulation of Prickle, ROR1 or ROR2. Tissue specific CRISPR/Cas9 is an efficient method of genome editing that would allow us to analyze a neuron-specific role for Prickle, ROR1, and ROR2 in CoPA neuron pathfinding (Ablain et al., 2015).

The molecular mechanisms within Planar Cell Polarity pathway that control axon guidance have not been completely defined (Dickson, 2002; Dent et al., 2011). In order further
elaborate on the mechanisms behind the PCP pathway the interaction between the individual components require additional study. Studying PCP components such as Prickle, ROR1, and ROR2 is essential to better understanding how to manipulate this signaling to promote guided growth of injured axons. Increasing our comprehension of the molecular mechanisms of axon guidance will help us to gain a greater insight into the overall development of the nervous system. If we know how individual components of the nervous system develop, then we will be better able to develop solutions to treat people with neurological disorders, and insure that people maintain proper brain development and function.
Figure 1. Model of the Planar Cell Polarity Pathway in Axon Guidance. Core transmembrane Planar Cell Polarity (PCP) proteins include Frizzled (FZ), Celsr, and Van Gogh-like (Vangl). Core cytoplasmic PCP proteins include Dishevelled (DVL), Prickle (Pk), and Scribble (Scrib). Proteins that act as Wnt co-receptors such as Glypican4 (GPC4), Protein tyrosine kinase (PTK)7, and Receptor tyrosine kinase like orphan receptor (ROR)2 act as Wnt co-receptors. Daam activates RhoA and may be a PCP protein involved in axon guidance. The binding of Wnt to FZD activates Rho and Rac which regulate JNK (Minami et al., 2010; Habas et al., 2001; Habas et al., 2003; Mrkusich et al., 2011).
Figure 2. Model of Prickle protein structure. Prickle protein structure includes PET (Prickle, Espinas, and Testin) domain, three LIM (Lin1-1, Isl-1, and Mec-3) domains, nuclear localization signal (NLS), and Caax motif. (Sweede et al., 2008; Shimojo & Hersh, 2003; Jenny et al., 2003; Mapp et al., 2011).
Figure 3. Phylogenetic tree of Prickle orthologues in humans, mice, and zebrafish.
Sequences for Prickle orthologues were obtained from the USCS Genome Browser (Kent et al., 2002). Trees were generated using genious bioinformatic software (http://www.geneious.com, Kearse et al., 2012).
Figure 4. Model of ROR1 and ROR2 protein structure. ROR1 and ROR2 have one immunoglobulin-like (Ig-like) domain, one Frizzled (FZD)-like cysteine-rich domain (CRD), and one Kringle domain in their extracellular region. In their cytoplasmic region, they contain a tyrosine kinase (TK) domain, two serine threonine rich domains (S/TRD1 and S/TRD2) and a proline rich domain (PRD) (Stricker et al., 2017).
Figure 5. Phylogenetic tree of ROR1 and ROR2 orthologues in humans, mice, and zebrafish. Sequences for ROR1 and ROR2 were obtained from the USCS Genome Browser (Kent et al., 2002). Trees were generated using Genious (http://www.geneious.com, Kearse et al., 2012)
Figure 6. mRNA Expression of prickle 1a, prickle 2a, prickle 2b, prickle 3. Lateral view stereomicroscope image of whole mount in situ hybridization of zebrafish embryos at 24 hpf. A. prickle 1a mRNA expression is displayed in the zebrafish spinal cord region. B. prickle 2a mRNA expression is displayed throughout the zebrafish spinal cord region. C. prickle 2b expression is displayed throughout the zebrafish spinal cord region. D. prickle 3 mRNA expression is displayed throughout the zebrafish spinal cord region.
Figure 7. Prickle CRISPR/Cas9 mutant morphology. prickle CRISPR/Cas9 zebrafish mutants exhibit shorter and wider bodies.
Figure 8. T7 Assay gels of *prickle* CRISPR/Cas9 mutants. Each gel contains PCR products of each prickle region targeted by the CRISPR/Cas9 system. Lanes containing DNA PCR products from wild type embryos do not exhibit T7 enzyme cleaving of DNA mismatches. Lanes containing DNA PCR products from *prickle1a, prickle 2a, prickle 2b, and prickle 3* CRISPR/Cas9 mutants all show T7 enzyme cleaving of DNA product mismatches resulting in additional bands.
**Figure 9. prickle loss of function mutation.** Lateral view confocal image of zebrafish immunostained CoPA neurons in wild type embryo (30hpf) and prickle CRISPR/Cas9 mutant embryo (30hpf). **Top.** Representative of 10 Wild type CoPA neurons project in an anterior direction after crossing the floorplate midline. **Bottom.** CoPA neurons in representative of 27 imaged embryos Co-injected with Cas9 mRNA and *prickle 1a, prickle 2a, prickle 2b,* and *prickle 3* gRNA. One CRISPR/Cas9 prickle mutant CoPA neuron projects in a posterior direction after crossing the floorplate midline.
Figure 10. Knockdown of *ror2*. Lateral view confocal image of immunostained CoPA neurons in 1 of 10 imaged wild type embryo and GFP expressing CoPA neurons in *ror2* morphant Tbx16:GFP embryos. **Top.** Representative of 10 imaged wild type embryo CoPA neurons. All CoPA axons project in an anterior direction after crossing the floorplate midline. **Bottom.** Representative of 6 imaged *ror2* morpholino injected embryos. *ror2* morphant CoPA neuron projects in an anterior direction after crossing the floorplate midline.
Figure 11. *ror1* and *ror2* CRISPR/Cas9 mutant morphology. Top. Lateral view of wild type morphology. Bottom. Lateral view of *ror1* and *ror2* CRISPR/Cas9 mutants exhibit shorter and wider bodies.
Figure 12. T7 Assay gel of ror1 and ror2 CRISPR/Cas9 mutants. The gel contains PCR products of each prickle region targeted by the CRISPR/Cas9 system. These products were each treated with the T7 endonuclease. Lanes containing DNA PCR products from wild type embryos do not exhibit T7 endonuclease cleaving of DNA mismatches. Lanes containing DNA PCR products from ror1 and ror2 CRISPR/Cas9 mutants all show T7 endonuclease cleaving of DNA product mismatches resulting in additional bands.
**Figure 13. ror1 and ror2 loss of function mutation.** Lateral view of confocal Image of zebrafish immunostained CoPA neurons in wild type embryo and ror1 and ror2 CRISPR/Cas9 mutant embryo. **Top.** Representative of 10 wild type embryos CoPA neurons project in an anterior direction after crossing the floorplate midline. **Bottom.** Representative of CoPA neurons in 1 of 100 imaged embryos co-injected with Cas9 mRNA and ror1 and ror2 gRNA. Two ror1 and ror2 CRISPR/Cas9 mutant CoPA neurons project axons in a posterior direction after crossing the floorplate midline.
Works Cited


Vita

Grayland Wyndell Godfrey II was born on February 9th, 1993 in Norfolk, Va to Grayland and Diane Godfrey. He received his Bachelors of Science in Biology with a minor in History from Hampden-Sydney College in Hampden Sydney, Va on May 10th, 2015. He graduated from Hampden Sydney College with the Honors designation of Cum Laude. He will receive his Masters of Science in Biology from Virginia Commonwealth University. After graduating from Virginia Commonwealth University, he will attend Howard University College of Medicine’s Doctor of Medicine program in Washington D.C. starting in July of 2017.