ENVIRONMENTAL MAINTENANCE OF SENSORY NEURON IDENTITY

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ABSTRACT

Cellular identity is conferred through complex genetic programs, extracellular cues, and environmental inputs. Though neuronal identity has been historically considered fixed and unchanging, it is increasingly evident that neuronal fate requires continued regulation, even after cells have become post-mitotic. In my thesis work I investigated signaling required for maintenance of a sensory identity in dorsal root ganglion neurons in the zebrafish (Danio rerio). Previous work implicated the neurotrophin Brain-Derived Neurotrophic Factor as the molecular cue required for maintenance of dorsal root ganglion neuron identity. I will demonstrate that while dorsal root ganglion neurons express two different receptors for BDNF, the Nerve Growth Factor Receptor (NGFR; formerly known as p75 neurotrophin receptor) and TrkB, it is only inhibition or genetic perturbation of TrkB signaling that promotes loss of sensory identity by DRG neurons. Interestingly, previous work suggested that the source of the BDNF signal is not dorsal root ganglion neurons themselves, but another sensory neuron type, the Rohon-Beard cells. I have found that Rohon-Beard cells indeed produce BDNF transcripts and protein, consistent with this cell type being the source of the relevant identity maintenance signal.

Additional findings will demonstrate that early environmental sensory inputs to Rohon-Beard cells serve as the source of activity that tunes the level of signaling by BDNF and TrkB. Thus one role of the mechanosensitive Rohon-Beard cell
population is to instruct the dorsal root ganglion neurons of the sensory requirements provided by the environment. Finally, I provide evidence that factors that govern the development and differentiation of another neuronal cell type, sympathetic ganglion neurons, exert influence on DRG neurons that are losing their sensory identity. Overall, my work elucidates a molecular mechanism by which environmental inputs instruct the maintenance of neuronal identity, and a manner in which cells lacking maintenance signals can assume an alternative identity.

The form and content of this abstract are approved. I recommend its publication.

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LIST OF ABBREVIATIONS

**BDNF**: Brain Derived Neurotrophic Factor

**bHLH**: basic helix-loop-helix

**CNS**: central nervous system

**CRISPR**: Clustered Regularly Interspaced Short Palindromic Repeats

**CyTXB**: CyclotraxinB

**DA**: dorsal aorta

**dpf**: days post fertilization

**DRG**: Dorsal root ganglion

**EM**: Embryo Media

**Fig.**: figure

**hpf**: hours post fertilization

**LTMR**: low threshold mechanoreceptor

**MO**: morpholino

**NC**: notochord

**NCC**: neural crest cell

**NGF**: Nerve Growth Factor

**NT-3**: neurotrophin-3

**NGFR**: Nerve Growth Factor Receptor

**PKC**: protein kinase C

**PLCγ**: phospholipase C gamma

**RB**: Rohon Beard

**SC**: Spinal cord
**SEM**: standard error of the mean

**SG**: sympathetic ganglia

**SH2**: Src homology 2

**TH**: tyrosine hydroxylase

**Trk**: tropomyosin-related kinase
CHAPTER I

INTRODUCTION

Introduction

My thesis work examined signaling mechanisms involved in the maintenance of sensory neuron identity. Specifically, I focused on molecular cues responsible for maintenance of a somatosensory identity in differentiated dorsal root ganglion (DRG) neurons in the developing zebrafish (*Danio rerio*). I will demonstrate that the TrkB neurotrophin receptor is necessary for differentiated DRG neurons to maintain their somatosensory identity. Further, I will show that the relevant ligand, the neurotrophin Brain-Derived Neurotrophic Factor (BDNF), is synthesized by another somatosensory cell type in zebrafish, the Rohon-Beard (RB) cells, and that environmental tactile inputs represent the endogenous mechanism by which this molecular factor is secreted from RBs to maintain DRG neuron identity. Finally, I will present evidence that neurons that leave their ganglion have a unique molecular identity, and that a subset of these cells assume markers of an alternative neuronal population. To provide background to my studies, I will review cell types that are responsible for somatosensation in zebrafish and other organisms, as well as the neurotrophin family of signaling molecules and their receptors, including in the zebrafish. Emphasis will be placed on BDNF and the TrkB receptor, given their relevance to my work. Finally, I will conclude by remarking on the strengths of the zebrafish model in studies of cellular identity, and provide specific hypotheses that guided my studies.
Somatosensation

Somatosensation in an encompassing term that describes the ways an organism is able to interpret sensory information such as pain, touch, temperature, and body position. Sensing one's environment bestows a diverse range of survival behaviors, including food acquisition, evasion of predators, and reproduction. Further, the sense of touch allows for not only sensing but also interacting with the environment and other organisms.

Defining the ways in which sensory systems develop and transduce cues from the environment continues to be of great scientific interest. Further, altered somatosensation is encountered in an incredible variety of disease settings, including diabetes, multiple sclerosis, infectious diseases (e.g., HIV) and developmental disabilities, and leads to considerable morbidity (Rapin, 1991; Franse et al., 2000; Davies et al., 2006; Battaglia, 2011; Sindic, 2012; Kaku and Simpson, 2014; Drulovic et al., 2015). Studying mechanisms that allow for proper somatosensation will lead to a better understanding of the pathophysiology of these diverse disease states, and could contribute to therapeutics.

DRG Neurons

The primary afferent cells of the trunk somatosensory system are neurons of the dorsal root ganglia (DRG). While it has long been known that DRG neurons comprise a heterogeneous population, the remarkable degree of diversity continues to be elucidated (Usoskin et al., 2015).
DRG neurons are a derivative of the neural crest. Following neural crest induction and migration, a subset of neural crest cells (NCCs) give rise to the neurons of the DRG (for review: Dupin and Le Douarin, 2014). The cell bodies of these sensory neurons reside in ganglia adjacent to the spinal cord, as part of the peripheral nervous system. Their pseudounipolar axons project to second order neurons in the central nervous system (CNS), and to the peripheral targets. DRG neurons are able to detect and transduce a variety of sensory stimuli including pain, temperature, touch, and limb position (for reviews: Basbaum et al., 2009; Abraira and Ginty, 2013). These stimuli are detected by subtype specific DRG neurons, with the unique identity of a DRG neuron determined by many factors, including early specification and fate restriction as neural crest cells, expression of transcription factors and sensory receptors that grant a neuron the ability to detect a particular stimulus, the presence of myelination and axon diameter (i.e., conduction velocity), and location of their synapses on second order neurons in the CNS (Li et al., 2011; Liu and Ma, 2011; Lallemand and Ernfors, 2012).

For example, DRG neurons that convey the sense of touch constitute a unique sensory neuron subpopulation. Generally, gentle touch is transduced by low threshold mechanoreceptors (LTMRs; Abraira and Ginty, 2013; Walsh et al., 2015). However, even within the subpopulation of DRG neurons that transduce touch, there is an impressive amount of diversity; they differ based on conduction velocity (Aβ, Aδ, C fibers), their ability to detect continuous stimuli (slowly adapting vs. rapidly adapting), the structure and morphology of their peripheral endings, and the array of molecular markers (e.g., Piezo, Ret, VGLUT3) that they express (Marmigère and
One molecular family, the neurotrophin growth factors and their receptors (reviewed below), has long been linked with survival and identity of DRG neuron subtypes. For example, DRG neurons expressing the TrkB neurotrophin receptor are thought to comprise a mechanosensitive subpopulation (Lallemand and Ernfors, 2012).

A recent breakthrough in the field of touch and mechanosensation came with the discovery of the large transmembrane Piezo proteins (Coste et al., 2012; for review: Walsh et al., 2015). Piezo proteins comprise a family of ion channels that are activated by mechanosensitive inputs that deform the membrane (Coste et al., 2012). Disruption of Piezo channels results not only in loss of mechanosensitive inputs, but behaviorally results in diminished response to touch (Ranade et al., 2014). While the identification of the Piezo channels resulted initially from an *in vitro* tour de force, early vertebrate evidence supporting a role for the Piezo channels in touch and mechanotransduction arose in part from studies of the *piezo2b* gene in the zebrafish model organism (Faucherre et al., 2013). This is not surprising, as the strengths of the zebrafish model system (reviewed below) have allowed for many insights into the development and function of the somatosensory system.

**Zebrafish DRG Neurons**

Much has been learned about zebrafish DRG neurons, particularly their specification from neural crest precursor cells and their initial differentiation into
sensory neurons. Less is known about factors that regulate DRG neuron identity at later stages.

Zebrafish DRG neurons, as in other vertebrates, are a derivative of the neural crest. Specifically, NCCs that give rise to DRG neurons are those that migrate ventrally, as opposed to those that migrate between the somite and overlying ectoderm. In zebrafish this is often referred to as the “medial” pathway, and occurs in the earlier of two waves of neural crest migration. (Raible et al., 1992; Raible and Eisen, 1996; An et al., 2002). Zebrafish NCCs generate a diverse number of cell types, including peripheral nervous system components like DRG neurons and sympathetic ganglion (SG) neurons, in addition to Schwann cells, pigment producing cells and craniofacial cartilage and bone (Raible et al., 1992; Raible and Eisen, 1994). Interestingly, individual NCCs can give rise to multiple derivatives, such as a DRG neuron and a melanocyte, indicative of plasticity even within single NCCs (Raible and Eisen, 1994, 1996). Raible and Eisen (1994) also demonstrated that early migrating neural crest cells, those that produce DRG neurons, give rise to a larger number of derivatives.

A number of genes have been identified as necessary for NCC migration or specification as DRG neurons, and thus result in abnormal DRG neuron formation when knocked down or mutated. For example, sensory deprived fish harbor a mutation in the gene encoding the metalloproteinase inhibitor Reck. They display improper migration of neural crest DRG precursor cells, and absent DRG neurons (Prendergast et al., 2012). Zebrafish with a mutation in the sox10 gene also display a reduction in DRG neuron number; interestingly, DRG neurons that do develop are
often abnormally positioned (Kelsh and Eisen, 2000; Dutton et al., 2001; Carney et al., 2006). sox10 is transiently expressed in NCCs, and directs a DRG neuron fate by driving expression of the gene encoding the proneural basic helix-loop-helix transcription factor, Neurogenin1 (Carney et al., 2006). Consistent with these studies, knockdown of neurogenin1 also causes a reduction in DRG neuron number, and results in fish that are touch insensitive (Cornell and Eisen, 2002). Functional sonic hedgehog signaling is also required in neural crest precursor cells for the proper development of DRG neurons, again due its role in the expression of the Neurogenin1 transcription factor, as well as the transcription factor neurod (Ungos et al., 2003). Further, zebrafish with a mutated erbb3 gene, which encodes a receptor tyrosine kinase proto-oncogene that is an EGFR homologue, completely lack DRG neurons as a result of aberrant neural crest cell migration, as do ouchless fish which lack the gene encoding the scaffold protein Sorbs3 thought to integrate erbb3 signaling (Honjo et al., 2008; Malmquist et al., 2013).

After NCCs arrive at the stereotypical location of zebrafish DRG neurons, the spinal cord:notochord (SC:NC) boundary, DRG neurons begin to differentiate both molecularly and morphologically. They begin to express the post-mitotic marker Elavl3 beginning at approximately 36 hours post fertilization (hpf), in a rostral to caudal sequence (Kim et al., 1996; An et al., 2002). Zebrafish DRG neurons begin to sprout bipolar axons at approximately 2 days post fertilization (dpf; Williams et al., 2000; An et al., 2002). These axons extend peripherally to targets of innervation such as the skin, and centrally to the hindbrain via the dorsal longitudinal fasciculus. Interestingly, DRG neuron peripheral axons reach their targets during a time in
which the peripheral axons from the transient somatosensory RB cell population (reviewed below) are still present at the periphery (Wright and Ribera, 2010). Originally there are 1-3 DRG neurons present per hemisection – this number increases as development proceeds (Raible et al., 1992; An et al., 2002; Honjo et al., 2011). In their characterization of zebrafish DRG development, An et al. (2002) noted that a subset of DRG neurons were located in an “ectopic” position, ventral to the regular position of DRG neurons at the SC:NC boundary. However, whether these cells represented a unique population and their functional consequences were unknown.

Several genes are required for the proper development of zebrafish DRG neurons. Knockdown of foxd3 results in a reduction in DRG neuron numbers; neural crest induction and early migration is normal in these fish, suggesting foxd3 is involved in early specification of a sensory neuron lineage (Lister et al., 2006). The basic helix-loop-helix transcription factor Neurogenin1 is also required for neural crest cells to assume a sensory DRG neuron fate (Blader et al., 1997; Cornell and Eisen, 2002; Lister et al., 2006; McGraw et al., 2008). Intriguingly, when neurogenin1 is knocked down or mutated, DRG neuron precursors are able to adopt characteristics of other cellular identities, e.g. glial cells, suggesting that it functions as a key regulator of cell fate for those neural crest precursors that give rise to the DRG (McGraw et al., 2008).

Less is known about DRG neurons in zebrafish past their initial differentiation. As in early stages, the number of neurons within each ganglion continues to increase in larval and juvenile stages; An et al. (2002) demonstrated that 14 dpf fish
have ~10-15 neurons, a number that increases to approximately 100 by 28 dpf. A population of progenitors that reside within the ganglia gives rise to at least a subset of these new neurons at later stages, in a process mediated by Notch signaling (McGraw et al., 2012). However, whether these cells being added represent specific sensory modalities and the mechanisms by which this diversity may arise are unknown. Studies using markers of neuronal subtypes and pharmacologic agents have demonstrated that zebrafish DRG neurons at later stages comprise a heterogeneous population, as in other species and to be expected given the diversity of stimuli these cells transduce (Won et al., 2012; Stil and Drapeau, 2015). For example, the TrkB neurotrophin receptor is expressed by only a subset of DRG neurons in juvenile zebrafish (Honjo et al., 2011). Interestingly, maintenance of post-mitotic DRG neuron identity requires activity of the sodium channel Na$_V$1.6 and the neurotrophin BDNF (Wright et al., 2010; Wright and Ribera, 2010); without these factors, differentiated DRG neurons undergo a secondary migration away from the ganglia, not related to the original migration of NCC DRG precursors.

There have been many studies of the genes and signaling pathways that direct DRG neuron specification and differentiation from neural crest precursor cells. Further, studies have shown that zebrafish DRG neurons present at later stages comprise a heterogeneous population. However, mechanisms by which DRG neuron identity is maintained over the course of development, likely critical for maintenance of sensory neuronal subtype, are incompletely understood. Surprisingly, clues about the maintenance of a sensory neuron identity in DRG neurons have arisen from studies of another sensory cell found in zebrafish, the RB cells.
Rohon-Beard Cells

Rohon-Beard cells are a transient somatosensory cell type in the CNS of amphibians and teleost fish. They were originally identified near the turn of the 20th century and are named for the scientists that described the cell type in teleost fish, the Austrian neuroanatomist Joseph Victor Rohon and the Scottish embryologist John Beard (Rohon, 1884; Beard, 1889; Reyes et al., 2004). RB cells are thought to function primarily as mechanosensitive neurons during embryonic and early larval phases, and are the primary neuron in the escape response circuit of the embryonic zebrafish. Subsequently, at least some RB cells display signs of apoptosis, as the permanent population of somatosensory neurons, the DRG neurons, are established. Much has been learned about the molecular cues that contribute to the formation and function of RB cells. However, RB cells are increasingly understood to comprise a heterogeneous population, raising the possibility that this transient cell type has additional functions beyond mediating early mechanosensitive inputs.

RB cells arise during gastrulation, and are derived from cells at the lateral neural plate (Lamborghini, 1980; Cornell and Eisen, 2000). Their specification involves signaling by BMP4, a member of the bone morphogenetic protein family, in addition to Delta-Notch signaling (Appel and Eisen, 1998; Cornell and Eisen, 2000; Cornell and Eisen, 2002; Rossi et al., 2008). RB cells can be identified by their dorsal position in the spinal cord, their large soma size, and their expression of genes encoding several transcription factors that control their development, including the LIM homeodomain transcription factor gene islet1 and the bHLH
transcription factor gene *neurogenin1* (Korzh et al., 1993; Cornell and Eisen, 2002; Dyer et al., 2014).

By 24 hpf, RB cells have elaborate, peripherally projecting arbors (Svoboda et al., 2001; Reyes et al., 2004). By 27 hpf, RB cells respond to mechanosensitive inputs in the trunk, which can elicit stereotypical behavioral responses such as the escape response (Clarke et al., 1984; Kimmel et al., 1995; Granato et al., 1996; Ribera and Nuslein-Volhard, 1998). The molecular components underlying RB cell activity have been dissected. Specifically, the sodium channel alpha subunits Na\(_{v}1.6\), and to a lesser extent Na\(_{v}1.1\), contribute to the ability of RB cells to serve as the primary cell in the touch response behavior (Pineda et al., 2005). Further, several mutants have been identified in which fish that can swim normally do not respond to these mechanosensitive inputs, such as the *macho* fish, which carries a mutation in the gene *pigk* and has reduced sodium currents in RB cells (Granato et al., 1996; Ribera and Nuslein-Volhard, 1998; Carmean et al., 2015). More recently, *piezo2b* transcripts that encode the stretch-activated, mechanosensitive Piezo2 channel have been identified in RB cells and are required for response to light touch (Faucherre et al., 2013).

RB cells begin to show signs of apoptosis as early as 34 hpf, though evidence of their persistence can be seen in juvenile stages, another hint of heterogeneity amongst RB cells (Williams et al., 2000; Svoboda et al., 2001; Reyes et al., 2004; Won et al., 2011; Palanca et al., 2013). RB cell apoptosis involves shrinkage of the soma and degeneration of its peripheral arbors, and in some cases is caspase-dependent (Williams et al., 2000; Reyes et al., 2004). Several factors that contribute
to the apoptosis of RB cells in zebrafish have been identified. For instance, mutation of the gene pigk, which diminishes sodium channel current in RBs, or blockade of sodium channel activity with tricaine, reduce the number of RB cells that undergo apoptosis, suggesting that activity in these cells may drive their ability to undergo programmed cell death (Svoboda et al., 2001). In addition, a subset of RB cells express transcripts of the neurotrophin receptor ntrk3a (formerly trkC1). At least some RB cells seem to require functional TrkC for survival, as ntrk3a negative RB cells are more likely to undergo apoptosis than those expressing the transcripts, and antibodies that deplete NT-3, the ligand for the TrkC receptor, cause an increase in cell death in the RB population (Martin et al., 1998; Williams et al., 2000).

The finding that not all RB cells express ntrk3a indicates a degree of heterogeneity within the RB cell population. Indeed, other markers are expressed by only some RBs, or in a temporally distinct manner. For example, members of the ATP-gated P2x ion channels are expressed in only some RB cells (Appelbaum et al., 2007), as is the protein kinase C alpha isoform (Slatter et al., 2005; Patten et al., 2007). Similarly, RNA in situ hybridization studies show members of the transient receptor potential potential (TRP) channels present in some, but not all RBs (trpa1b: Faucherre et al., 2013; trpcb4: Von Niederhäusern et al., 2013); the same is true for transcripts of the homeobox domain gene drg11 (McCormick et al., 2007). This heterogeneity in RB cells hints at the possibility of roles beyond mediating embryonic mechanosensation.

Intriguingly, though the disappearance of RB cells does not appear to depend on proper DRG development (Reyes et al., 2004), Wright and Ribera (2010)
demonstrated that RB cell activity, mediated by the sodium channel Na\textsubscript{v}1.6, is required for maintenance of the position of differentiated DRG neurons. This RB-mediated DRG positioning also requires BDNF, a member of the neurotrophin family of growth factors, which has a long history in the sensory neuron field.

**Neurotrophins**

In a reflection several decades after the discovery of the nerve growth factor (NGF), a molecule originally characterized as a survival factor for sensory and sympathetic neurons, Rita Levi-Montalcini described her efforts under the guidance of Viktor Hamburger and working with Stanley Cohen. These efforts led to her receiving the Nobel Prize for Physiology/Medicine in 1986, alongside Stanley Cohen. The piece, written in 1987 for the Nobel Foundation and remarking on the already long history of NGF to that point, could barely have anticipated the explosion of neurotrophin (and trk receptor) research that was on the horizon. To her credit, Dr. Levi-Montalcini speculated on “predictions of the unpredictable,” remarking that the circuitous path to discovery of NGF suggested that unknown forms and functions could still be in hiding, waiting to be discovered (Levi-Montalcini, 1987).

Neurotrophins are a family of secreted growth factors. Together with their receptors, they shape many aspects of neuronal identity, including survival, differentiation, and function (Zweifel et al., 2005; Harrington and Ginty, 2013; Park and Poo, 2013).

There are four common neurotrophins – nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-
4). Additionally, there are two neurotrophins, neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7), that are so far known only to exist in fish. Neurotrophins are produced as a “pro” form of the protein, e.g. Pro-BDNF. These pro-domains can then be cleaved to yield mature neurotrophins, which dimerize to activate their receptors. The two canonical receptors for neurotrophins are the tropomyosin-related tyrosine kinase (trk) receptors and the Nerve Growth Factor Receptor (NGFR). Each of the neurotrophins binds with high affinity to a particular trk receptor (NGF – TrkA, BDNF/NT-4 – TrkB, NT-3 – TrkC), and with a lower affinity to the NGFR. Following binding of the neurotrophins to their receptors and autophosphorylation of key residues, a deluge of responses can be elicited depending on many factors, including second messengers and effectors acting downstream of the NT-trk pair, the cellular context in which place the signaling is taking place, or the timing of NT activity (for review of neurotrophin/trk signaling pathways, see: Reichardt, 2006).

**Neurotrophin and Trk Function**

Neurotrophins were originally identified as survival factors for certain neuronal populations, including sensory neurons of the spinal, or dorsal root, ganglia. Targeted mutation of the genes encoding the neurotrophins and their receptors further refined our knowledge of the dependence of subpopulations of neurons on particular NT-trk activity for survival. Decades of work following the identification of the various NTs and their receptors have revealed a seemingly endless number of
roles these factors play in a neuronal lifecycle, including migration, neurite outgrowth, and differentiation.

Regarding somatosensation, the neurotrophin ligands and trk receptors are linked to subpopulations of DRG neurons with stimulus specificity, and have even been shown to modulate the receptive properties of sensory neurons. Early work showing the loss of specific sensory neuron populations when a ligand or receptor was knocked out indicated that each neurotrophin-trk pair seemed to support particular DRG subtypes (Carroll et al., 1992; Lee et al., 1992; Buchman and Davies, 1993; Klein et al., 1993; Crowley et al., 1994; Ernfors et al., 1994; Jones et al., 1994; for review of early neurotrophin/trk KO mice, see: Snider, 1994). Further, many of these knockouts displayed sensory deficits (Fariñas et al., 1994; Smeyne et al., 1994; Liu et al., 1995; Perez-Pinera et al., 2008). Nociceptive, or pain sensing neurons, are thought to comprise a group of TrkA expressing DRG neurons, while TrkB and TrkC expressing neurons are typically associated with mechanosensitive and proprioceptive subpopulations, respectively (McMahon et al., 1994; Marmigère and Ernfors, 2007; Lallemend and Ernfors, 2012). The expression of trk receptors within DRG neurons is therefore carefully controlled, and in turn can themselves regulate the expression of other modality specific markers (Ma et al., 1999; Kramer et al., 2006; Inoue et al., 2007; Luo et al., 2007; Marmigère and Ernfors, 2007; Lallemend and Ernfors, 2012). Further, neurotrophins can alter the receptive properties of sensory DRG neurons (Snider, 1998). For example, sustained BDNF signaling is required for normal mechanotransduction in post-natal mice (Carroll et al., 1998). More recently, Rutlin et al. (2014) demonstrated that BDNF signaling
underlies the tuning and directional selectivity of LTMRs. While many early studies focused on the role of neurotrophins and their receptors in survival of neuronal subpopulations, sustained expression of neurotrophins and their receptors hinted at other, post-natal roles.

Interestingly, neurotrophins are known to be secreted in an activity-dependent manner, a feature suggestive of their role in synaptic plasticity (Balkowiec and Katz, 2000; Bruno and Cuello, 2006; Hong et al., 2008; Greenberg et al., 2009; Kuczewski et al., 2009). For example, BDNF/TrkB signaling is thought to contribute to the development of long term potentiation in the hippocampus (Korte et al., 1995; Minichiello et al., 2002). Another role for activity-dependent neurotrophin signaling is in experience-driven modulation of sensory systems, including the auditory, visual, and somatosensory systems (Domenici et al., 1994; Galuske et al., 1996; Caleo et al., 1999; Huang et al., 1999; Rossi et al., 1999; Pizzorusso et al., 2000; Gianfranceschi et al., 2003; Katz and Meiri, 2006; Liu et al., 2007; Kaneko et al., 2008; Wright and Ribera, 2010; Anomal et al., 2013).

Given the diverse roles neurotrophins have in the development and function of the nervous system, it is not surprising that a variety of disease states result from mutation of neurotrophin genes or perturbed neurotrophin signaling (Chao et al., 2006). For example, mutation of the bdnf gene or changes in BDNF signaling are thought to contribute to the pathogenesis of schizophrenia, eating disorders, depression, and developmental disorders (e.g. autism spectrum disorders, Rett syndrome; Nelson et al., 2001; Katoh-Semba et al., 2007; Rosas-Vargas et al., 2011; Autry and Monteggia, 2012; Manners et al., 2015; Notaras et al., 2015;
Theoharides et al., 2015; Bjorkholm and Monteggia, 2016). It is interesting that many of these diseases share components of altered somatosensation, given the role NTs play in the survival and function of sensory neurons (Kern et al., 2006; Devarakonda et al., 2009; Downs et al., 2010; Battaglia, 2011). The trk receptors are also implicated in a variety of disease states. Human patients in which the gene encoding TrkB is mutated suffer from a spectrum of morbidities, including hyperphagia, deficits in learning and memory, and notably, impaired somatosensation (Yeo et al., 2004; Gray et al., 2007).

Despite their relevance to human disease and diverse functions, much remains to be studied about neurotrophin / trk influence on the nervous system. The zebrafish represents a unique model system in which to investigate neurotrophin activity in vivo. Given the historical link between neurotrophins and zebrafish, it is surprising how little is known about neurotrophin and trk functions in the zebrafish.

**Zebrafish Neurotrophins and Trk Receptors**

Prior to the discovery of the full family of mammalian neurotrophins or their receptors, Judith Weis demonstrated the presence of NGF-like factors in teleost fish, and studied the effects of these factors in the adult zebrafish spinal cord (Weis, 1968c). Specifically, Weis demonstrated that application of NGF purified from mouse submandibular gland increased the number of neurons in zebrafish DRG, and conversely that introduction of anti-serum raised against NGF decreased the number of neurons in zebrafish DRG (Weis, 1968b). Intriguingly, though anti-serum against NGF decreased the size of zebrafish DRG, Weis did not detect dying
neurons. Further, it is noteworthy that these studies were done in adult fish, perhaps an early hint of the many roles that would be attributed to neurotrophins outside of a developmental context. Finally, Weis demonstrated that exogenous NGF altered complex behaviors in fish, including swimming, schooling behavior, and responses to startling stimuli (Weis and Weis, 1972).

After the identification of the other neurotrophins and their respective trk receptors in mammalian systems, they were identified in zebrafish using homology-based strategies (For review: Heinrich and Lum, 2000). Genes encoding each of the trk receptors exist in zebrafish, as with their respective ligands. As mentioned, neurotrophin ligands that exist only in fish have also been identified, termed NT-6 and NT-7 (Gotz et al., 1994; Lai et al., 1998; Nilsson et al., 1998).

The zebrafish neurotrophins and trk receptors are homologous to their mammalian counterparts. For example the zebrafish bdnf gene encodes a protein that has 98% identity with the human BDNF amino acid sequence (Hashimoto and Heinrich, 1997). Not unexpectedly given their homology to mammalian neurotrophins, fish NTs are capable of activating trk receptors from non-teleost species (Gotz et al., 1992).

Five genes encoding trk receptors were identified in zebrafish and named according to homology to the mammalian trk receptors - ntrk1 (encodes TrkA), ntrk2a and ntrk2b (duplicated ntrk2 paralogues), and ntr3a and ntrk3b (duplicated ntrk3 paralogues). The zebrafish trk receptor genes share homology with their mammalian counterparts, with some regions being particularly highly conserved. Martin et al. (1995) reported that zebrafish trk genes share 68-81% identity with
vertebrate trk receptor genes, and 69-82% identity with each other (Martin et al., 1995). Each of the predicted zebrafish trk receptor extracellular domains contain regions found in the mammalian trks, including cysteine rich clusters, leucine rich motifs, and immunoglobulin domains. The second immunoglobulin domain, for example, has a high degree of similarity in amino acid sequence to the mammalian trks (Martin et al., 1995). The predicted intracellular regions of the zebrafish trk receptors show the highest degree of homology with the mammalian counterparts; for instance, the ntrk2a and ntrk2b encode intracellular domains that have 92% and 90% homology to rat TrkB protein, respectively. Further, several residues important for docking of downstream effectors are present in the zebrafish trks, including PLCγ1 and SH2 sites (Martin et al., 1995).

Many of the studies of zebrafish BDNF and TrkB have been devoted to the timing of their expression and localization in various tissues. BDNF transcripts are detectable during all of embryonic development and increase approximately 12-fold over the first seven days post-fertilization (Hashimoto and Heinrich, 1997; Lum et al., 2001). Early localization studies using an RNA in situ hybridization protocol detected BDNF transcripts in a number of locations, such as the brain, dorsal spinal cord, skin, and pectoral fin, and in several developing sensory organs, including the retina, otic vesicle, and neuromast hair cells (Hashimoto and Heinrich, 1997). ntrk2a/b transcripts are also present throughout zebrafish embryonic development, suggesting some maternal contribution. However, full length transcripts that include a kinase domain are present only from the beginning of neurogenesis (Lum et al., 2001). RNA in situ hybridization studies also suggest that the two ntrk2 paralogues,
*ntrk2a* and *ntrk2b*, are differentially expressed. For example, *ntrk2b* transcript expression in the trunk seems to be developmentally restricted in a caudal to rostral manner, with most of the transcripts present in the rostral hemisegments of embryonic fish (Thisse et al., 2008). The distribution of BDNF and TrkB protein has been examined in depth in several zebrafish tissues, including the lateral line, retina, and hypothalamus (Germanà, et al., 2010a,b; Sánchez-Ramos, et al., 2010; De Felice et al., 2014). It is noteworthy that these studies found expression of BDNF/TrkB in juvenile and adult fish, suggesting that their function may be required beyond developmental stages. Finally, TrkB expression has been demonstrated in a subset of DRG neurons in juvenile zebrafish, though its expression in embryonic and larval DRG neurons has not been studied (Honjo et al., 2011).

Relatively little is known about the function of neurotrophins and their receptors in zebrafish. However, several existing studies highlight the importance of neurotrophin signaling in the development of the zebrafish nervous system. For instance, knockdown of the zebrafish gene *huntingtin*, an orthologue of the human gene that accumulates CAG repeats in Huntington’s disease, results in significant neuronal death at 24 hpf and a reduction of BDNF – this neuronal loss can be mimicked by knockdown of *bdnf* transcripts, and rescued by introduction of exogenous BDNF (Diekmann et al., 2009). Another study showed that a subset of the transient sensory RB cells (reviewed above) depend on intact NT-3/TrkC signaling for survival (Williams et al., 2000). Additionally, BDNF influences Schwann cell myelination, as well as proper development of the zebrafish sensory lateral line organ (Tep et al., 2012; Gasanov et al., 2015).
In early larval stages, BDNF does not seem to be required for survival of early DRG neurons, but rather for maintenance of DRG neuron position and identity following proper differentiation as sensory neurons (Wright et al., 2010; Wright and Ribera, 2010). Without BDNF, properly differentiated DRG neurons migrate away from their stereotypical position at the SC:NC boundary, and begin expressing markers of another cell type, sympathetic ganglion neurons.

Another neurotrophin receptor, NGFR (ngfra), is also present in zebrafish. ngfra is expressed in several tissues in the developing embryo and larva, including neural crest cells, lateral line primordium, RB cells, and DRG neurons. NGFR, as part of the Nogo receptor complex, regulates both peripheral nervous system axon outgrowth and development of the lateral line sensory organ (Brösamle and Halpern, 2009; Han et al., 2014). Further, knockdown of a NGFR signaling partner, pard3, results in aberrant Schwann cell myelination of motor neuron axons (Tep et al., 2012).

Though the fish neurotrophins and their receptors were identified some time ago, relatively few studies have investigated their functionality in zebrafish, which is surprising given the many strengths of the zebrafish model system.

**Zebrafish Model**

Importantly, development of the nervous system in zebrafish is similar to that in other vertebrates, including the existence of the neural crest and its derivatives (Raible et al., 1992; Raible and Eisen, 1994, 1996; An et al., 2002; Halloran and Berndt, 2003).
Of particular relevance for my dissertation work, the transparency of zebrafish embryos and larvae make it an ideal system for tracking cellular movements and studying changes in cellular identity over time (Kirby et al., 2006; McGraw et al., 2008; Wright et al., 2010). Further, a large number of transgenic lines are available that express fluorescent proteins in specific cellular populations (Blader et al., 2003; Nechiporuk et al., 2007; Obholzer et al., 2008; Drerup and Nechiporuk, 2013).

In addition to the significant advantages afforded by the zebrafish for *in vivo* imaging, development of zebrafish embryos occurs external to the mother, allowing for relative ease of pharmacologic and genetic manipulation. Drug treatments can be injected into the yolk sac of the developing embryo or applied directly to the embryo media (Wright et al., 2010; Wright and Ribera, 2010; Hines et al., 2015). Gene products can be knocked down using antisense morpholino oligonucleotides (Pineda et al., 2006; Fein et al., 2008; Gasanov et al., 2015, for review: Eisen and Smith, 2008). More recently, the ability to target genes of interest for mutation using technologies such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) has made zebrafish a powerful model for reverse genetic studies (Hwang et al., 2013; Varshney et al., 2015; for review: Blackburn et al., 2013).

Finally, zebrafish can be used to study behavioral paradigms, which themselves can be linked to molecular components using the aforementioned tools (Friedmann et al., 2015; Naderi et al., 2016). In particular, the ability of a gentle touch stimulus to evoke a stereotyped swim response in embryonic and larval zebrafish has provided valuable insights in my studies of the zebrafish somatosensory system (Pineda et al., 2005).
Establishment and maintenance of a cellular identity is a coordinated interplay of autonomous genetic programs and signals from the environment, both extracellular and external to an organism. My thesis work addresses two specific hypotheses. First, Chapter II tests the hypothesis that environmental stimuli interpreted by the transient somatosensory RB population are communicated to a subset of DRG neurons via BDNF/TrkB signaling to match the permanent sensory pool size to the environment experienced by the developing embryo. Chapter III tests the hypothesis that loss of expression of sensory neuron transcription factors and a response to signals directing development of the sympathetic ganglion neurons allow for ectopic DRG neurons to assume alternative identities. These studies further our knowledge of the mechanisms by which neuronal identity is maintained, and the plasticity that is retained by some neurons in a differentiated state.
CHAPTER II

BDNF-TRK SIGNALING MEDIATES EXPERIENCE-DEPENDENT
PLASTICITY OF SENSORY NEURON FATE

Introduction

Sensory systems enable an organism to interpret and respond to diverse external stimuli. Touch, the first sense to develop (Marx and Nagy, 2015), also allows for interaction with the environment and other organisms. Several human diseases, including Rett Syndrome and those on the autism spectrum, comprise deficits in sensory function leading to increased morbidity (Biersdorff, 1994; Kern et al., 2006; Devarakonda et al., 2009; Downs et al., 2010). Further, clinical evidence suggests that sensory experience in early human life influences later somatosensory function (Cermak and Daunhauer, 1997; Lin et al., 2005). Classic studies have demonstrated that sensory experience induces changes that occur at the level of the somatosensory cortex (e.g., Simons and Land, 1987). Less attention has been paid to plasticity that might occur at other levels of the somatosensory pathway.

Specification of sensory neurons in the peripheral nervous system requires the function of members of the Neurogenin transcription factor family (Ma et al., 1999; Marmigère and Ernfors, 2007). Additional transcription factors, such as the runt-related (RUNX) family, subsequently generate diversity within the sensory neuron lineage (Levanon et al., 2002; Marmigère et al., 2006; Inoue et al., 2007). In addition to transcriptional control, environmental conditions also regulate sensory systems, an effect typically mediated by neurotrophins and their receptors (Katz and Shatz, 1996; Lush et al., 2005; Liu et al., 2007; Anomal et al., 2013).
In mammals, dorsal root ganglion (DRG) neurons mediate and discriminate amongst diverse somatosensory inputs to the trunk. DRG neurons are derived from the neural crest (Le Douarin and Kalcheim, 1999). After neural crest-DRG precursors migrate and arrive at the ganglion location, they differentiate into a diverse population of somatosensory neurons. Time of migration, degree of multipotency, and marker expression support the view that neural crest-DRG precursors themselves already constitute a heterogeneous population (Raible and Eisen, 1994, 1996; Henion and Weston, 1997; Carney et al., 2006; McGraw et al., 2008). Differentiated DRG neurons subsequently manifest their heterogeneity on the basis of several characteristics, including peripheral and central projections, soma size, extent of axon myelination, expression of molecular markers (e.g., trk neurotrophin receptors), and sensory modality (Marmigère and Ernfors, 2007; Lallemend and Ernfors, 2012). Although we are acquiring information about the factors that govern specification and differentiation of dorsal root ganglion neurons, less is known about mechanisms that allow differentiated sensory neurons to maintain their identity once acquired (Sun et al., 2008; Wright and Ribera, 2010; Serrano-Saiz et al., 2013; for review, see Deneris and Hobert, 2014).

In amphibia and teleosts, an early differentiating sensory neuron known as the Rohon-Beard (RB) cell mediates somatosensory function for the developing embryo and young larva. Subsequently, as the RB cell population disappears, DRG neurons mediate somatosensation for larvae and adults. In zebrafish, a subset of larval DRG neurons requires activity in RB cells and Brain-Derived Neurotrophic Factor (BDNF) to maintain a position within the ganglia at the spinal cord:notochord
Upon knockdown of the sodium channel Na\(_{\text{v}1.6}\) in RB cells, some differentiated DRG neurons leave the ganglia and initiate expression of morphological and molecular markers indicative of a new identity (Wright et al., 2010).

Here, we show that maintenance of a sensory fate by a subset of DRG neurons requires an environment providing appropriate tactile stimulation. Further, exogenous BDNF rescues DRG neurons from loss of sensory identity produced by exposure to reduced levels of sensory stimulation. Moreover, inhibition of TrkB, but not NGFR (formerly, p75NTR), signaling prevents maintenance of a sensory fate by a subset of DRG neurons. Overall, the results indicate that BDNF-TrkB signaling mediates experience-dependent plasticity that targets the first neuron in the somatosensory pathway.

**Materials and Methods**

*Animal care*

The University of Colorado Committee on Use and Care of Animals approved all animal protocols. Adult zebrafish (*Danio rerio*) were maintained at 28.5°C on a 10/14h dark/light cycle in the Center for Comparative Medicine at the University of Colorado Anschutz Medical Campus and bred according to established protocols (Westerfield, 1995). Fish were raised in embryo media (EM: 130 mM NaCl, 0.5 mM KCl, 0.02 mM Na\(_2\)HPO\(_4\), 0.04 mM KH\(_2\)PO\(_4\), 1.3 mM CaCl\(_2\), 1.0 mM MgSO\(_4\), 0.4 mM NaH\(_2\)CO\(_3\)) and staged according to external morphology (Kimmel et al., 1995). For the developmental stages studied, it is not possible to determine sex.

*Transgenic lines*
Dr. Uwe Strähle (University of Heidelberg; Blader et al., 1997) provided the \( \text{Tg}(-3.4\text{neurogenin}:\text{GFP})\text{sb4} \) transgenic zebrafish line. The \( \text{Tg}(\text{CREST3}:\text{scn8aa}) \) transgenic line was engineered using standard Tol2 methods (Kwan et al., 2007) to create a transgene containing the CREST3 promoter (Uemura et al., 2005), followed by the zebrafish \( \text{scn8aa} \) coding sequence (Fein et al., 2008) and a polyA tail. The \( \text{Tg(olig2:EGFP)} \) and \( \text{Tg(sox10:mRFP)} \) transgenic lines were provided by Dr. Bruce Appel (University of Colorado, Anschutz Medical Campus).

**Manipulation of sensory environment**

The protocol for decreased sensory stimulation has been described previously (Wright and Ribera, 2010). The protocol for increased sensory stimulation entailed continual rocking, using a nutator, of embryos/larvae maintained at high density (~200/60 mm petri dish). Petri dishes and the nutator were maintained at 28.5°C within an environmental chamber (Lab-Line Instruments, Inc. Orbit-Environ Shaker). At 24 hours post fertilization (hpf), embryos were manually dechorionated and then returned to the environmental chamber until larvae were 96 hpf. EM was replaced three times per day.

**BDNF and NGFR antibody injections**

BDNF (~5 nl, 100 ng/ml; B-250, Alomone Labs) was injected into 48 hpf \( \text{Tg}(-3.4\text{neurogen1}:\text{GFP}) \) embryos, as described previously (Wright and Ribera, 2010). The anti-NGFR antibody, referred to as ChEX, was developed by Weskamp and Reichardt (1991; kindly provided by Dr. Lou Reichardt, UCSF), and raised against
the purified extracellular portion of chick NGFR. ChEX (~3 nl at 1 mg/ml) was injected into 48 hpf 
Tg(−3.4neurogenin:GFP) or double Tg(Olig2:EGFP); Tg(Sox10:mRFP) embryos for experiments assessing DRG neuron position or peripheral motor nerve myelination, respectively.

**Analysis of DRG position**

Analysis of DRG neuron position was performed blinded to group identity/experimental condition. Larvae were mounted laterally in 1.0% low melting point agarose containing 0.01% tricaine (Sigma-Aldrich, A5040). DRG position was analyzed in the 10 most rostral trunk segments of each embryo. DRG neurons were identified on the basis of either GFP expression or Elavl3 immunoreactivity in either live Tg(−3.4neurogenin:GFP) or fixed Tg(CREST3:scn8aa) 4 days post fertilization (dpf) larvae, respectively. DRG neurons were considered to be ectopic if they were located ventrally more than one cell body diameter from the SC:NC boundary (Wright et al., 2010).

**Confocal microscopy**

Confocal images were acquired as z-stacks using a Zeiss LSM5 Pascal Confocal Upright Microscope, equipped with a 10x, 40x, or 63x water-immersion objective. The pinhole was set to one Airy unit. Individual confocal slices ranged between 2-4 µm in thickness, depending upon the objective used. Data are presented as projections of a subset of a z-stack and typically include those slices
that captured DRG neurons on the left side of the larva within the ten most rostral trunk segments (10-30 slices, depending upon objective used and mounting).

**Touch sensitivity**

After confirming that larvae were motile, we assayed tactile sensitivity by touching a larva with a tungsten needle (Pineda et al., 2005). Ten trials were performed and scored according to the following scale; 1 = normal (swimming) response; 0.5 = abnormal (twitching, not swimming) response; 0 = no response.

**RNA in situ hybridization**

A template for *in vitro* synthesis of antisense RNA *in situ* hybridization probes for *bdnf* was generated by PCR using the following primers: Forward: 5'CGTGATCGAGGGCTGTTGG3', Reverse: 5'CTGCTTCAGTTGGCCTTTCG3'. RNA *in situ* hybridization was performed using a standard protocol (Novak and Ribera, 2003).

**Immunocytochemistry**

Whole mount immunocytochemistry was performed as described by Svoboda et al. (2001) with minor modifications. Embryos were fixed overnight at 4°C in 4% paraformaldehyde in phosphate buffered saline (PBS: 137mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.8mM KH2PO4, pH 7.4) solution containing 0.1% Tween-20 (PBSTw). Fixed embryos were permeabilized, blocked and incubated with antibodies. For cryostat sections, whole embryos were fixed (4% paraformaldehyde),
cryoprotected (30% sucrose), embedded, and frozen. 15 μm transverse sections were cut using a Leica 1950 cryostat. Sections were mounted on poly-L-lysine slides, blocked and then incubated overnight first with primary antibody followed by the species-appropriate secondary antibody (1:1000 goat anti-mouse Alexa 488 or 1:1000 goat anti-rabbit Alexa 568; Molecular Probes-Invitrogen). Sections were cover-slipped with Fluoromount-G (Southern Biotechnology Associates). The primary antibodies, mouse anti-Elavl3 (formerly HuC/D; Sigma-Aldrich AB1401126), rabbit anti-GFP (ThermoFischer Scientific, Molecular Probes, A6455), mouse anti-GFP (Chemicon, EMD Millipore), and anti-NGFR (ChEX, Weskamp and Reichardt, 1991) were used at a concentration of 1:1000. The TrkB antibodies (Novus NB100-92063 - Chen et al., 2013; Santa Cruz SC-12 - Honjo et al., 2011) were used at concentrations of 1:100, as was the BDNF antibody (Santa Cruz SC-65513; Mo and Nicolson, 2011).

**Western blot**

Tissues were homogenized in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP40, 0.5% Triton X-100, 1 mM EGTA, pH 7.4; Klymkowsky Lab On-line Methods) supplemented with 1 μl protease inhibitor (Halt Protease Inhibitor Cocktail Kit; Pierce Chemicals). Protein extracts were loaded onto sodium dodecyl sulfate-polyacrylamide gels, subjected to electrophoresis, and then transferred to a polyvinylidene fluoride membrane (PVDF, Biorad). Membranes were first blocked in a Tris-buffered saline-Tween solution (TBST: 0.136 M NaCl, 2.68 mM KCl, 24.76 mM Tris, pH 7.4, 0.1% Tween 20) containing 5% nonfat dry milk and then incubated
overnight (4°C) with the anti-NGFR antibody (1:1000; Weskamp and Reichardt, 1991) followed by the species-appropriate secondary antibody (1:2000). The blot was developed using SuperSignal West Dura Extended Duration Substrate (Pierce Chemicals). The positive control, rat DRG protein extract, was kindly provided by Dr. S. Rock Levinson (University of Colorado Anschutz Medical Campus).

**Veratridine, K252a, and Cyclotraxin-B treatments**

Veratridine (Sigma-Aldrich V5754), a blocker of sodium channel inactivation (Catterall, 1975) was prepared as a 10 mM stock solution in ethanol and used at a final concentration of 1 mM in 0.4 M KCl, by injection into the yolk sac of 48 hpf larvae; control injections were performed with vehicle (10% ethanol in 0.4M KCl).

The general Trk kinase inhibitor, K252a, (200 nM; Sigma-Aldrich K1639; Tapley et al., 1992) was added to EM when embryos were 48 hpf. A K252a stock solution (100 µM) was prepared in dimethyl sulfoxide (DMSO). The final concentrations of K252a and DMSO were 200 nM and 0.002%, respectively. At a concentration of 200 nM, K252a inhibits neurotrophin-dependent effects (Borasio, 1990; Wang et al., 1995). Vehicle-control experiments were performed using 0.002% DMSO.

CyclotraxinB (CyTxB; Bio S and T, Inc.), a specific TrkB inhibitor (Cazorla et al., 2010), was prepared as a 10 µM stock solution, and injected into the yolk sac of ~48 hpf embryos, at concentrations ranging between 0-1000 nM. 1% Fast Green was included in the CyTxB and vehicle control solutions to allow visual tracking of the injected material.
Morpholino antisense knockdown

A previously tested zebrafish ntrk2b morpholino (ntrk2bMO; Gasanov et al., 2015), was synthesized by Gene Tools, LLC. The MO target included the predicted start methionine of ntrk2b with the following sequence:

5’ CCATTCCACGAACCCCTGCGGTCAT 3’. A control MO (ctrlMO) with five base mismatches was also synthesized (mismatches in italics):

5’ CCAATGCACCAACCCGTCCGTCAT3’. The MOs (~3 nl, 1-3 ng/nl) were injected into the animal cell of one to two cell stage embryos; Fast Green (1%; Sigma-Aldrich) was included in the MO solutions to allow visual tracking of the injected material. We refer to embryos/larvae that had been injected with MO as morphants.

ntrk2b CRISPR mutant

The online resource ZiFiT (http://zifit.partners.org/ZiFiT/) was used to examine the ntrk2b gene for CRISPR target sites (Sander et al., 2007, 2010). Exon7 was identified as an optimal target. To generate a plasmid for in vitro transcription of a single guide RNA (sgRNA), the following oligonucleotides were annealed and ligated into the sgRNA plasmid pDR274 (Hwang et al., 2013):

5’ TAGGACTTTTCGCCTAATGACAA3’, and 5’ AAACTTGTCCATTAGGCAGAA GT3’. sgRNAs were injected into one cell stage embryos (125 ng/µl) with Cas9 RNA (100 ng/µl) in 0.1M KCl containing 0.2% phenol red to allow visual tracking of the injected solution. RNA purity and concentration were assessed using a nanodrop
spectrophotometer. F0 embryos injected with the sgRNA and Cas9 RNA were raised to adulthood, genotyped (see below) and intercrossed. F2 homozygous mutant and sibling embryos were used for experiments followed by genotyping. Thus data acquisition and analysis were blinded to genotype.

For genotyping, we performed PCR using primers that flanked the ntrk2b CRISPR target site: forward – 5’CTTATGCTGAGC ACTGTATCAG3’, reverse – 5’GAAATATTGAGCATGACTGTAG3’. An endogenous HinfI restriction site adjacent to the CRISPR target site allowed assessment of potential polymorphisms introduced at the target site by CRISPR/Cas9. HinfI digested PCR products were analyzed on a 3% agarose gel (equal parts Metaphor/GeneMate agaroses; Lonza Group, LTD; BioExpress). The PCR products were sequenced by the Barbara Davis Center Molecular Biology Service Center (University of Colorado Anschutz Medical Campus).

**Data analysis**

Data are presented as either box plots or bar graphs. For box plots, the box contains the two inner quartiles, with whiskers showing the 5th and 95th percentiles; outliers appear as circles. The horizontal line within the box indicates the median. Bar graphs plot the mean + SEM. Sample sizes are indicated in the figure legends.

Statistical analyses were performed using either InStat3 (GraphPad Software), Kaleidagraph (Synergy Software) or SAS (SAS Institute, Inc.). Except for Fig. 2E, statistical analysis was performed using either a Mann-Whitney or Kruskal-Wallis nonparametric ANOVA, the latter corrected for multiple comparisons (Dunn).
For Figure 2E, the data were assessed for statistical significance using a repeated measures analysis of variance.

**Results**

*Sensory experience sculpts the size of the emerging sensory system in a BDNF-dependent manner*

In previous work, sensory deprivation, but not enrichment, altered the number of ectopic DRG neurons (Wright and Ribera, 2010), suggesting that sensory experience has a limited ability to regulate DRG neuron position. To test this possibility, we used alternative methods to increase RB cell activity (see Materials and Methods).

First, we used a pharmacological approach and applied veratridine (1 mM, Hines et al., 2015), an inhibitor of sodium channel deactivation (Catterall, 1975), to zebrafish larvae from 48 to 96 hpf. As noted by others (Chopra et al., 2010; Hines et al., 2015) veratridine-treated larvae displayed behaviors consistent with prolonged sodium channel activity, such as increased locomotor activity, a seizure-like state, and eventual transient paralysis. In contrast to the previous sensory enhancement paradigm (Wright and Ribera, 2010), 96 hpf veratridine-treated larvae had fewer ectopic DRG neurons than did controls (Fig. 1A,C,G).

Our second test consisted of transgenic overexpression of the major RB sodium channel isoform, *scn8aa* (Pineda et al., 2005; Novak et al., 2006). In 96 hpf *Tg(CREST3:scn8aa)* larvae, there were fewer ectopic DRG neurons compared to the number present in wildtype (WT) and sibling larvae (Fig. 1D,G).
Our third test entailed exposing embryos/larvae to environmental conditions that provide increased sensory input to RB cells (see Materials and Methods). Consistent with the pharmacological and genetic approaches, the increased stimulation protocol also decreased the number of ectopic DRG neurons (Fig. 1B,G).

In summary, three different methods for increasing RB cell activity all led to a decrease in the number of ectopic DRG neurons. Moreover, a physiologically-based method, environmental stimulation, was effective in regulating DRG neuron position. Thus, sensory experience has the ability to increase as well as decrease the number of DRG neurons that leave the ganglion location.

**BDNF rescues effects of decreased sensory stimulation on the number of ectopic DRG neurons**

Wright and Ribera (2010) implicated BDNF as the factor mediating the effects of decreased RB sodium channel activity on DRG position. If BDNF is also involved in mediating the effects of environmental stimulation of RB cells, it should rescue the effects of decreased sensory stimulation on the number of ectopic DRG neurons. We tested this by raising larvae under conditions of sensory deprivation with or without treatment with BDNF. Consistent with our prediction, the number of ectopic DRG neurons present in sensory-deprived larvae that were treated with BDNF was lower than that of larvae experiencing decreased tactile stimulation alone (Fig. 1E-G,). Moreover, the number of ectopic DRG neurons in larvae that were exposed to both BDNF and sensory deprivation was similar to that of WT embryos raised under control conditions (Fig. 1A,E-G). These results support the view that BDNF mediates effects
of pharmacological or genetic stimulation of RB activity as well as those produced by environmental conditions that modulate RB activity.

*Environmental conditions have no effect on the total number of DRG neurons*

The above results demonstrate that environmental conditions affect the number of ectopic DRG neurons. One possibility is that environmental conditions affect ectopic DRG neuron number independently of the population that remains at the SC:NC boundary (referred to here as normal DRG neurons). That is, environmental conditions could lead to an increase in DRG neurogenesis to provide the population that becomes ectopic. The alternative is that ectopic DRG neurons arise at the expense of normal ones. We distinguished between these possibilities by comparing directly the number of DRG neurons that remain at the SC:NC boundary (Fig. 2A, arrows) versus those that are ectopic (Fig. 2A, arrowhead) under different environmental conditions and treatments.

Interestingly, for all conditions, the total number of DRG neurons (i.e., normal plus ectopic) remained constant (Fig. 2B). However, the number of normally positioned DRG neurons declined when larvae were raised in conditions of sensory deprivation, an effect that was rescued by BDNF (Fig. 2C). Consequently, the ratio of ectopic to normally positioned DRG neurons depended upon the experimental treatment (Fig. 2D). This result supports the view that ectopic neurons arise at the expense of DRG neurons.

To investigate potential functional significance of an increased number of ectopic DRG neurons, we tested the ability of larvae raised under different conditions
to respond to tactile stimulation. Consistent with previous results, larvae raised under conditions of sensory deprivation displayed a reduced touch response compared to control larvae (Fig. 2E; Wright and Ribera, 2010). Interestingly, co-application of BDNF to sensory-deprived embryos partially rescued the touch response (see Figure 2 Legend). The distribution of touch scores more closely resembled that of control. For example, there were a diminished number of larvae that displayed no response (Fig. 2E). These results suggest that the position of a DRG neuron influences its ability to function as a sensory neuron.

Overall, the results support the view that the level of tactile stimulation experienced by zebrafish larvae has the ability to decrease as well as increase the size of the developing somatosensory nervous system. Moreover, for larvae exposed to sensory deprivation, BDNF’s ability to rescue DRG neuron position suggests that the neurotrophin acts to match the size of the developing sensory system to environmental conditions.

Rohon-Beard neurons express bdnf transcripts

Previous work implicated BDNF in mediating the effects of decreased RB sodium channel activity on DRG neuron position (Wright and Ribera, 2010). However, it is not known whether RB cells express BDNF. To address this question, we determined the expression patterns of BDNF mRNA and protein in zebrafish larvae.

At 48-72 hpf, zebrafish larvae expressed bdnf in several regions of the spinal cord (Fig. 3A-D). In particular, RB cells displayed robust expression of bdnf (Fig. 3C, D). Similarly, BDNF protein was also detected in RB cells of 72 hpf larvae (Fig. 3E-
These results indicate that RBs synthesize bdnf/BDNF mRNA and protein during the period when a subset of DRG neurons migrate to new positions.

**NGFR is expressed by DRG neurons, but is not responsible for maintenance of DRG neuron position**

BDNF interacts with two different receptors, NGFR and TrkB (Rodriguez-Tébar et al., 1990; Klein et al., 1991; Reichardt, 2006). Brösamle and Halpern (2009) found that, at 48 hpf, DRG neurons express mRNA for *ngfra*, thus making it a candidate for the relevant BDNF receptor. However, information regarding the protein expression profile for NGFR during embryonic and larval development is lacking. To examine expression of NGFR protein, we used an antibody, ChEX, directed against the extracellular portion of chick NGFR (kindly provided by Dr. Lou Reichardt, UCSF; Weskamp and Reichardt, 1991).

We first used western blot analysis to test whether ChEX recognizes a protein of size corresponding to NGFR. We detected an immunoreactive protein band of expected size (68 kDa) in lysates prepared from 72 hpf larvae, as well as from rat DRG lysate (Fig. 4A, inset). We next performed immunocytochemical experiments to determine the locations of ChEX/NGFR immunoreactivity. In 72 hpf larvae, several central and peripheral nervous system cells, including DRG neurons, were NGFR immunoreactive (Fig. 4A,B). The results support NGFR as a candidate for the BDNF receptor acting to regulate DRG neuron position.

We next used ChEX as a function-blocking agent and tested whether its application affected the number of ectopic DRG neurons. ChEX-injected larvae had
a similar number of ectopic DRG neurons versus vehicle control (Fig. 4D,E,H), suggesting that NGFR is not the relevant BDNF receptor.

A potential caveat, however, is that ChEX does not block zebrafish NGFR. To test this possibility, we examined effects of ChEX on a known NGFR-dependent process, myelination of peripheral motor neuron axons (Tep et al., 2012). In contrast to the lack of effect on DRG neuron position, ChEX treatment disrupted the normal association of Schwann cells and motor neuron axons (Fig 4 F, G). Taken together, the results indicate that NGFR is unlikely to mediate effects of BDNF on DRG neuron position.

TrkB is expressed by DRG neurons

The mRNA for the other candidate BDNF receptor, TrkB, has been detected in embryos in the rostral portion of the trunk, the region where ectopic DRG neurons are most often observed (Thisse et al., 2008). In addition, a subset of DRG neurons in juvenile (10 week post fertilization) zebrafish are TrkB immunoreactive (Honjo et al., 2011). Whether TrkB is also present during the larval stages relevant to this study has not been examined. We used immunocytochemical methods to test this possibility.

In 72 hpf Tg(neurogenin:GFP) larvae, we detected TrkB immunoreactivity in several tissues, including the cell bodies and peripherally projecting axons of DRG neurons (Fig. 5A,B,E-J). Interestingly, not all normally-positioned DRG neurons were TrkB immunoreactive, consistent with results obtained from juvenile zebrafish and mammalian DRG neurons (Klein et al., 1990; Mu, et al., 1993; Josephson et al., 2001; Kramer et al., 2006; Honjo et al., 2011). Importantly, ectopic DRG neurons expressed
the receptor (Fig. 5A-G). These results raise the possibility that the TrkB+ DRG subpopulation requires BDNF in order to maintain a position within the ganglion.

Inhibition of the TrkB neurotrophin receptor increases the number of migratory DRG neurons

As an initial approach to test whether TrkB mediates the effects of BDNF on DRG neuron position, we employed pharmacologic methods. First, we used the general trk inhibitor, K252a (Tapley et al., 1992). Larvae treated with K252a had significantly more ectopic DRG neurons than did vehicle control larvae (Fig. 6A,B,D). This result suggests that a trk receptor mediates the effects of BDNF.

To test whether TrkB is the relevant trk, we used the small molecule TrkB-selective inhibitor, CyclotraxinB (CyTxB) (Cazorla et al., 2010). Application of CyTxB led to an increase in the number of ectopic DRG neurons compared to control (Fig. 6A,C,D). Moreover, CyTxB affected the number of ectopic DRG neurons in a dose-dependent manner (Fig. 6E). These results support TrkB as the receptor mediating the BDNF’s effects on DRG neuron fate.

Maintenance of DRG identity requires ntrk2b function

Based on the pharmacologic data implicating TrkB as the relevant BDNF receptor (Fig. 5), we used genetic approaches to inactivate ntrk2b, the gene encoding TrkB. First, we used a morpholino (MO) directed against the ntrk2b translation start site (Fig. 7A, Gasanov et al., 2015). Compared to 96 hpf control
morphants, there was an increased number of ectopic DRG neurons in *ntrk2b* morphants (Fig. 7D,E,H).

We next used the CRISPR-Cas9 system to target the *ntrk2b* gene for inactivation. The sgRNA targeted the *ntrk2b* gene at exon 7, a region encoding the ligand binding domain (Figure 7A; Martin et al., 1995; Reichardt, 2006). Similar to *ntrk2b* morphants, F0 larvae that had been co-injected with *ntrk2b* exon 7 guide RNA and Cas9 RNA displayed an increase in ectopic DRG neurons compared to control (Fig 7D,F,H).

F2 homozygous CRISPR mutant embryos carried a seven base pair deletion within exon 7 of *ntrk2b*, resulting in a frameshift and a stop codon 47 amino acids downstream from the mutation (Fig. 7A-C). Contrary to expectations, homozygous exon 7 CRISPR F2 mutants did not have an increased number of ectopic DRG neurons compared to control (Fig. 7D,G,H). However, the F2 mutants did have an increased ratio of ectopic:normal DRG neurons (Fig. 7I), similar to larvae raised under conditions of decreased stimulation (Fig. 2D). That the ratio of ectopic:normal DRG neurons was increased reflects a reduced number of normal DRG neurons (Fig. 7J). On this basis, the CRISPR F2 mutants did indeed have an increased number of ectopic DRG neurons.

In summary, the data indicate that *ntrk2b* morphants, and transient and stable CRISPR mutants all had an increased fraction of DRG neurons that assumed ectopic locations. In addition, the stable CRISPR mutants showed a reduced number of normally positioned DRG neurons, suggesting that *ntrk2b* loss-of-function in the F2 generation resulted in reduced specification of DRG neurons (Fig. 7J).
Together, these data indicate an essential requirement for \textit{ntrk2b} in specification and maintenance of DRG neuron identity.

**Discussion**

Here we report roles for sensory experience, mediated by BDNF-TrkB signaling, in regulating the fate of DRG neurons. We find that a subset of differentiated DRG neurons respond to increases as well as decreases in environmental stimulation by remaining in or leaving the ganglion location. Further, via TrkB, BDNF mediates the effects of environmental stimulation, consistent with an essential role for this signaling pathway in mediating experience-dependent plasticity.

We previously found that decreases in tactile stimulation led to an increase in the number of DRG neurons that leave the ganglia (Wright and Ribera, 2010). At that time, we were not successful in identifying environmental conditions that would reduce the number of DRG neurons leaving the ganglion. Consequently, questions arose about the extent to which experience could effect plastic changes at the level of the primary sensory neuron. The previous protocol for enhanced tactile stimulation consisted of poking larvae eight times per day and, in retrospect, may have provided insufficient stimulation with respect to that required to influence DRG neuron fate. Here, we used a more robust approach and increased the density of larvae per dish, that was then rocked continually. This protocol induced a decrease in the number of DRG neurons that left the ganglion location (Fig. 1). Thus, sensory experience can either increase or decrease the number that remain within the
ganglion and thereby regulates the somatosensory system at the level of the first neuron.

The effects of sensory experience on the somatosensory system have been studied extensively (for review, Foeller and Feldman, 2004; Margolis et al., 2014). However, prior work has focused on experience-dependent plasticity that occurs at the level of the cortex, far removed from the primary sensory neuron. In rodents, when vibrissae experience decreased or no tactile stimulation, barrel cortex neurons become less responsive to sensory-deprived whiskers but more responsive to whiskers that continued to receive stimulation (Fox, 1992; Diamond et al., 1993; Glazewski and Fox, 1996). Further, available evidence suggests that altered somatosensory experience induces plasticity in the cortex but not the thalamus (Li et al., 1995; Glazewski et al., 1998; Wallace and Fox, 1999).

Experience-dependent plasticity also occurs in cortical and/or subcortical regions of the auditory, visual and olfactory systems (for review, Priebe and McGee, 2014; Skoe and Chandrasekaran, 2014). In the visual system, in addition to cortical effects, experience leads to changes in retinal function, at the level of the first synapse involving a subset of ganglion cells (Tian and Copenhagen, 2003; Dunn et al., 2013). Similarly, in the olfactory system, sensory-deprivation increases the strength of the connection between sensory neurons and their post-synaptic partners in olfactory bulb glomeruli, the site of the first synapse in this pathway (Tyler et al., 2007). Our work contrasts with these findings in that we observe plasticity at the level of the primary sensory neuron itself.
Experience-dependent mechanisms often rely upon the neurotrophin BDNF and/or its receptor TrkB (Cabelli et al., 1995; Galuske et al., 1996; Singh et al., 1997; Lush et al., 2005; Liu et al., 2007; Kaneko et al., 2008; Xu et al., 2010; Anomal et al., 2013; Shimojo et al., 2015). For example, rat pups that experience maternal isolation have altered expression of several neuronal markers; however, subsequent stimulation of whiskers increases the synthesis of BDNF and rescues marker expression (Chatterjee et al., 2007). Similarly, we find that BDNF has the ability to rescue effects produced by sensory deprivation on DRG position (Figs. 1, 2). By matching the number of primary somatosensory neurons to the level of sensory experience, BDNF’s actions on DRG fate are reminiscent of the original role discovered for neurotrophins in coordinating the sizes of input and target tissues (Cohen, 1960; Levi-Montalcini and Cohen, 1960; Levi-Montalcini and Angeletti, 1963; Barde et al., 1982).

The effects of BDNF-TrkB signaling on DRG neuron fate appear to affect a subset of DRG neurons in a restricted region, the rostral trunk of the larva. Consistent with this, ntrk2b is expressed in the rostral portion of zebrafish embryos (CSK and ABR, unpublished observations; Thisse, et al. 2008), where ectopic DRG neurons are most frequently observed. However, ectopic DRG neurons are occasionally found in more caudal regions (Wright et al., 2010). In addition to spatially restricted ntrk2b expression, another potential factor is that the sympathetic ganglia, an alternate fate of ectopic DRG neurons, have a rostral to caudal gradient of differentiation (Stewart et al., 2004).
Nonetheless, even within rostral trunk regions, there is heterogeneity of TrkB expression within the dorsal root ganglia (Fig. 5). Juvenile zebrafish and mammals also display \textit{ntrk2}/TrkB expression in only a subset of DRG neurons. (Klein et al., 1990; Mu, et al., 1993; Josephson et al., 2001; Kramer et al., 2006; Honjo et al., 2011). Given the consequences of tactile stimulation on maintenance of DRG fate that we report, it is interesting that mammalian TrkB-expressing DRG neurons comprise a mechanosensitive subtype (Marmigère and Ernfors, 2007). Further, BDNF plays a role in maintenance of mechanosensitivity in sensory neurons (Carroll et al., 1998).

What is the fate of the DRG neurons that leave the ganglion? Previous work showed that ~25% of ectopic DRG neurons adopt morphological and molecular characteristics indicative of a sympathetic neuron fate (Wright et al., 2010). It is not known what happens to the additional 75% of ectopic DRG neurons. Possible outcomes include death or adoption of yet another fate. In addition, for the neurons that adopt a sympathetic phenotype, it is not known if they functionally integrate into circuits. The possibility that they retain a sensory identity is unlikely given the extensive remodeling of peripheral arbors observed for these neurons (Wright et al., 2010). Further, we find that ectopic DRG neurons arise at the expense of normally positioned DRG neurons (Fig. 2). Moreover, previous work (Wright and Ribera, 2010) and this study demonstrate that sensory deprivation results in reduced touch sensitivity, consistent with loss of sensory identity upon exit from the ganglion.

Stable CRISPR \textit{ntrk2b} mutants, but not transient CRISPR larvae or morphants, had an additional phenotype consisting of a reduced total number of
DRG neurons. Consistent with this observation, genetically-engineered mice that lack either the BDNF or TrkB gene show substantial death of neurons, suggestive of an early effect of TrkB knock-out on sensory neuron differentiation (Klein et al., 1993; Jones et al., 1994). Interestingly, mouse TrkB knock-outs that are complete nulls have less severe phenotypes than do those that express a truncated receptor lacking the kinase domain, presumably because the latter acts as a dominant-negative (Klein et al., 1993; Perez-Pinera et al., 2008). For CRISPR-Cas9 mutagenesis, we targeted exon 7, containing the BDNF binding site that is upstream of the kinase domain (Martin et al., 1995; Reichardt, 2006). The resultant truncated receptor would lack both the ligand binding as well as the kinase domains. This might account for the difference observed between morphants and stable CRISPR ntrk2b mutants, with the latter also showing an early effect of TrkB loss on sensory neuron differentiation. However, this does not explain the different phenotypes of transient versus stable CRISPR mutants. Potential explanations include mosaicism of CRISPR mutagenesis in the transients and potential compensatory mechanisms (Rossi et al., 2015).

Several neural developmental diseases, such as Fragile X, Rett Syndrome, and the autism spectrum disorders present with abnormal levels of BDNF (Nelson et al., 2001; Katoh-Semba et al., 2007; Theoharides et al., 2015). These diseases share a common phenotype of altered sensory function. For example, Rett syndrome individuals display altered sensitivity to pain (Devarakonda et al., 2009). Similarly, individuals diagnosed with an autism spectrum disorder have severe disruptions in multiple sensory modalities, including touch (Biersdorff, 1994; Kern et
al., 2006). While prior work suggests that these symptoms may result from cortical effects, our results raise the possibility that the relevant primary sensory neurons might also be affected.

Overall, our findings indicate that sensory experience, as a continuum, acts to reinforce or inhibit maintenance of a sensory identity by DRG neurons. Moreover, BDNF-TrkB signaling mediates the effects of sensory experience on the plasticity of DRG neuron identity.
Figure 2.1. Regulation of DRG neuron position. For A-F, all panels but D used Tg(-3.4neurogenin:GFP) larvae to identify DRG neurons on the basis of reporter expression. In D, DRG neurons were identified on the basis of positive Elavl3 immunoreactivity. A-F, Environmental conditions can either increase or decrease the number of ectopic DRG neurons. DRG neurons normally reside at the SC:NC boundary. However, a subset of DRG neurons occasionally acquire ectopic locations. Under control conditions (A), the majority of GFP+ DRG neurons reside at the SC:NC boundary. Increased tactile stimulation (see Materials and Methods) reduces the number of ectopic DRG neurons (B). Similarly, conditions that increase RB cell activity (C, veratidine; D, Tg[Crest3:scn8aa]) reduce the numbers of ectopic DRG neurons. Conversely, and consistent with previous results (Wright and Ribera, 2010), sensory deprivation increases the number of ectopic DRG neurons (E). BDNF rescues the effects of sensory deprivation on ectopic DRG neuron number (F), implicating the neurotrophin in the endogenous mechanism regulating DRG neuron fate. G, The graph summarizes the effects of different sensory, pharmacological and genetic manipulations on the number of ectopic GFP+ neurons. Data are presented as a box plot (see Materials and Methods), *p=0.05, **p=0.001 versus control; ^^p=0.01, ^^^p=0.001 versus decreased stimulation. Scale Bar in A for panels A-F: 100 µm.
**Figure 2.2.** Ectopic DRG neurons arise at the expense of normally-positioned ones. *A,* The dashed line indicates the SC:NC boundary, the typical position of DRG neurons in 3-5 dpf larvae. Arrows and the arrowhead highlight normally positioned and ectopic DRG neurons, respectively. Scale Bar in *A:* 100 µm. For *B-E,* sample size ranged from 12-114 larvae per condition. Data in *B, C* and *D, E* are presented as box plots or a bar graph (mean + SEM), respectively. *B,* Decreased sensory stimulation, with or without co-injection of BDNF, does not alter the total number (normally plus ectopically positioned) of DRG neurons. *C,* Decreased sensory stimulation leads to a reduction in the number of normally positioned DRG neurons, an effect that is rescued by injection of BDNF. *, *p=0.05 versus control, ^^, *p=0.01 versus decreased stim. *D,* The ratio of ectopic to normally positioned DRG neurons increases under conditions of decreased sensory stimulation. Co-application of BDNF with decreased sensory stimulation prevents the increase in the ratio. ***, *p=0.001 versus control; ^^^, *p=0.001 versus decreased stimulation. *E,* Decreased sensory stimulation reduces touch sensitivity compared to control treated embryos (0= no response; 0.5 = abnormal response; 1 = normal response). The effect is partially rescued by co-injection of BDNF. **, *p=0.01 versus decreased stim.
Figure 2.3. RB cells express BDNF mRNA and protein. All images present a lateral view, with rostral to the left and dorsal up. A, B, RNA in situ hybridization with an antisense probe demonstrates the presence of bdnf transcripts in the dorsal spinal cord of a 48 hpf embryo (A, red). In contrast, the sense probe (B) does not detect transcripts. C, D, In 72 hpf larvae, RB cells express bdnf (C, red). The sense probe does not detect transcripts in RB cells (D). RB cells are identified by their positive Elavl3 immunoreactivity (C and D, green). The red channel of the regions outlined by dotted white lines in panels C and D is shown at higher magnification in the insets. E-G, In 72 hpf Tg(-3.4neurogenin:GFP) larvae, dorsal spinal cord cells are positive for BDNF immunoreactivity (E, red). These cells express GFP (F, green) in the Tg(-3.4neurogenin:GFP) line. The majority of RB cells have characteristic large cell bodies and a superficial location (arrowheads) within the dorsal spinal cord. The merged image (G) shows the presence of BDNF immunoreactivity in GFP+ cells that have RB characteristics (arrowheads). The neurons that are GFP+ but with soma at less superficial locations (G, asterisks), adjacent to the nerve tract, are likely to be DoLA interneurons (Moreno and Ribera, 2014). Scale bars: A-D, in B, 10 µm; E-G, in G, 20 µm.
Figure 2.4. DRG neurons express the NGFR, but it is not required for maintenance of DRG neuron identity. A, Both central and peripheral neurons of 72 hpf zebrafish larvae show NGFR immunoreactivity. Inset, The anti-NGFR antibody (ChEX, kindly provided by Lou Reichardt, UCSF; Weskamp and Reichardt, 1991) detects NGFR protein in lysates prepared from 72 hpf zebrafish larvae and rat DRG. Numbers on the left indicate the apparent molecular weight of co-run standards. B, Peripheral NGFR+ neurons also express GFP (arrowheads) in the Tg(3.4neurogenin:GFP) line, identifying them as DRG neurons. C-G, The ChEX antibody does not affect DRG neuron position even thought it disrupts NGFR-dependent motor neuron axon myelination. C, D, ChEX (D) does not affect the number of ectopic DRG versus control treated (C) 96 hpf larvae, suggesting that BDNF interacts with a different receptor to regulate DRG neuron position. E, F, Myelination of peripheral motor neuron axons, an event that depends upon NGFR (Tep et al., 2012), was used as a bioassay to confirm ChEX’s function-blocking actions. In a control 96 hpf larva (E), peripheral Schwann cells (Tg[sox10:mRFP], red) tightly associate (arrowhead) with motor neuron peripheral axons (Tg[folig2:GFP], green). However, injection of the anti-NGFR antibody (F) disrupts motor axon myelination (arrow), indicating that the antibody is function blocking. G, The average number of ectopic DRG neurons does not differ between control and ChEX-treated larvae, indicating that NGFR does not regulate DRG neuron fate. Sample size ranged from 9-11 larvae per condition. Scale bars: A-B, in B, 20 µm; C-D, in D, 100 µm.
Figure 2.5. A subset of DRG neurons express the TrkB neurotrophin receptor. **A-J**, In a transverse sections of a 72 hpf *Tg(-3.4neurogenin:GFP)* larva, a subset of DRG neurons are positive for TrkB immunoreactivity (red). The spinal cord is outlined by a dotted circle in **A-H**, **A,B**, DRG neurons (asterisks) in 72 hpf *Tg(-3.4neurogeninGFP)* larvae display TrkB immunoreactivity (red). **C,D**, In another axial section of the same larva, DRG neurons (open arrowheads) are negative for TrkB immunoreactivity. **E-G**, Normally positioned DRG neurons (open arrowheads) are negative for TrkB immunoreactivity, while an ectopic DRG (white arrowhead) neuron displays TrkB immunoreactivity. **H-J**, The boxed area of **H** is shown at higher magnification in **I** and **J**. In *Tg(-3.4neurogenin:GFP)* larvae, GFP expression reveals a peripheral DRG neuron cell body (I, asterisk) and axon (I, arrowhead). TrkB immunoreactivity is also detected in the axon of the DRG neuron (J, arrow). Scale bars: **A-D**, in **D**, 20 µm; **E-G**, in **G**, 20 µm; **I-J**, in **J**, 10 µm.
Figure 2.6. Maintenance of DRG identity requires TrkB function. 

A-E, Between 48-96 hpf, Tg(-3.4neurogeninGFP) larvae were treated with vehicle (A), K252a, a general trk inhibitor (B), or CyTXB, a small molecule TrkB-selective inhibitor (C). At 96 hpf, compared to control (A), K252a (B) and CyTXB (C) treated larvae have an increased number of ectopic DRG neurons. 

D, Compared to vehicle control, both K252a and CyTXB treatments lead to a significant increase in the number of ectopic DRG neurons. 

E, The median number of ectopic DRG neurons increase in a dose-dependent manner with CyTXB concentration; sample size ranged between 33-59 larvae per condition; *p = 0.05, **p=0.01, ***p=0.001. Scale bars: A-C, in D, 100 µm.
Figure 2.7. Maintenance of DRG identity requires ntrk2b function. A, The ntrk2b gene, encoding TrkB, was targeted for genetic disruption using either an antisense morpholino (MO, exon 1, Gasanov et al., 2015) or CRISPR-Cas9 induced mutation (CRISPR, exon 7). The MO targets the ATG start site, while the exon 7 CRISPR targets the ligand binding domain (Reichardt, 2006). Grey bars indicate the position of primers used for PCR and genotyping. B, Genotyping of individual F2 embryos indicates that CRISPR-Cas9 successfully targeted exon 7 of the ntrk2b gene. HinfI digestion distinguishes between control (white arrowhead) and homozygous CRISPR mutant (open arrowhead) exon 7 PCR products. C, DNA sequencing indicates that WT exon 7 matches the reference sequence while the mutant genome has a seven base-pair deletion, resulting in a frame shift. D-G, At 96 hpf, control Tg(-3.4neurogeninGFP) larvae (D) have few or no ectopic DRG neurons. In contrast, ntrk2b morphants (E) and F0 injected CRISPR-exon 7 transients (F) have several ectopic DRG neurons. F2 homozygous mutant larvae (G) have a decreased number of total DRG neurons. H, Compared to vehicle controls, both nrk2b morpholino injection and CRISPR mutation lead to an increase in the number of ectopic DRG neurons. The number of ectopic DRG neurons in F2 CRISPR mutants is similar to that of control. I, The ratio of ectopic to normal DRG neurons is increased for CRISPR mutants versus control, similar to the effect of decreased sensory stimulation (see Fig. 2D). J, The total number of normal DRG neurons is decreased in ntrk2b homozygous mutant larvae compared to control. Sample size ranged from 11-128 larvae per condition; *p=0.05, **p=0.01, ***p=0.001. Scale bars: D-G, in D, 100 μm
CHAPTER III
MECHANISMS OF PLASTICITY IN
DIFFERENTIATED SENSORY NEURONS

Introduction

Nervous system disease and injury often result in neuronal death. The addition of new neurons after developmental stages occurs only in restricted subpopulations. Therefore, methods to replace injured neurons must incorporate additional strategies, such as directed differentiation of stem cells or transdifferentiation of existing cells (for review, see: Jopling et al., 2011). Identification of an autologous cell type capable of assuming varying neuronal identities would circumvent issues associated with transplantation of stem cells. Further, use of an endogenous neural cell type may retain unknown molecular characteristics that define a cell as a neuron, which may be lost in current stem cell therapies.

Transdifferentiation, whereby cells regress to a state where they can convert to alternative lineages, has been observed in many tissue types, including the lens and retina, endocrine pancreas, adipose tissue, the auditory system and the peripheral nervous system (Roberson et al., 2004; Tsonis et al., 2004; Jee et al., 2010; Kanazawa et al., 2010; Lu et al., 2010; Maki et al., 2010; Wright et al., 2010). Experimentally, transdifferentiation can be accomplished through directed expression of particular genes, and can be achieved in some cases by activation of single genes (Heins et al., 2002; Berninger et al., 2007; Jarriault et al., 2008; Sul et al., 2009; Heinrich et al., 2010; Riddle et al., 2013). Dissecting the mechanisms by
which transdifferentiation occurs in vivo is a critical step towards therapeutic applications.

Derivatives of multipotent precursors such as the neural crest represent an intriguing population that may retain plasticity in the differentiated state. Neural crest cells (NCCs) give rise to a diverse range of cells (for review, see Dupin and Le Douarin, 2014). Though often fate restricted, NCCs can give rise to additional cell types when introduced to new environments (Raible et al., 1992; Raible and Eisen, 1994; Baker et al., 1997). Further, differentiated cells derived from the neural crest also maintain a degree of plasticity, including in vivo (Dupin et al., 2000; Le Douarin and Dupin, 2003; Wright et al., 2010). For example, dorsal root ganglion (DRG) sensory neurons in zebrafish exhibit plasticity in the differentiated state (Wright et al., 2010).

Previous work demonstrated that loss of environmental tactile input and perturbed BDNF/TrkB signaling allow a subset of post-mitotic DRG neurons to migrate away from their stereotypical position (Chapter 2; Wright and Ribera, 2010). Many of these former DRG neurons reside at the location of another neural crest derivative, sympathetic ganglion (SG) neurons, and begin to express markers characteristic of SG neurons, e.g. tyrosine hydroxylase (TH; Wright et al., 2010). While factors that are required to maintain the position and identity of these DRG neurons have been elucidated, less is known about the signals that allow for migratory DRG neurons to assume alternative fates.

We sought to investigate whether mechanisms that direct proper sympathetic ganglion neuron migration and differentiation allow ectopic DRG neurons to assume
SG neuron characteristics. We show that signaling by the chemokine receptor CXCR4 is required for acquisition of an ectopic position by DRG neurons. Further, ectopic DRG neurons display a unique pattern of reporter expression in several transgenic lines, compared to normally located DRG neurons. Specifically, cells that escape from the ganglia maintain expression of a fluorescent reporter driven by the gene encoding the bHLH transcription factor Neurogenin1. Once DRG neurons have left their ganglia and traveled ventrally towards the SG, many begin to express a reporter driven by the gene encoding the SG-associated transcription factor phox2bb, sprout lateral axons reminiscent of SG neurons, and incorporate within the SG.

Materials and Methods

Animal care

The University of Colorado Committees on Use and Care of Animals approved all animal protocols. Adult zebrafish (Danio rerio) were maintained at 28.5°C on a 10/14h dark/light cycle in the Center for Comparative Medicine at the University of Colorado Anschutz Medical Campus and bred according to established protocols (Westerfield, 1995). Fish were raised in embryo media (EM: 130 mM NaCl, 0.5 mM KCl, 0.02 mM Na₂HPO₄, 0.04 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, 0.4 mM NaH₂CO₃) and staged according to external morphology (Kimmel et al., 1995).
Transgenic lines

Dr. Uwe Strähle (University of Heidelberg; Blader et al., 1997) provided the Tg(-3.4neurogenin:GFP)sb4 transgenic zebrafish line. The transgenic line Tg(islet2b:EGFP) was obtained from Dr. Chi-Bin Chien (University of Utah; Pittman et al., 2008). The transgenic lines TgBAC(neurod:EGFP) and Tg(neurogenin:dsRed) were obtained from Dr. David Raible (University of Washington; Obholzer et al., 2008; Drerup and Nechiporuk, 2013). The Tg(phox2bb:EGFP) line was provided by Dr. Bruce Appel (University of Colorado Anschutz Medical Campus; Nechiporuk et al., 2007).

Analysis of DRG position

Analysis of DRG neuron position was performed blinded to experimental condition. Larvae were mounted laterally in 1.0% low melting point agarose containing 0.01% tricaine (Sigma-Aldrich, E10521). DRG position was analyzed in the 10 most rostral trunk segments of each embryo. DRG neurons were identified on the basis of either GFP expression in Tg(-3.4neurogenin:GFP)sb4 larvae or Elavl3 immunoreactivity. DRG neurons were considered to be ectopic if they were located ventrally more than one cell body diameter from the spinal cord – notochord boundary (Wright et al., 2010).

Confocal microscopy

All images were acquired using a Zeiss LSM5 Pascal Confocal Upright Microscope, equipped with a 10x, 40x, or 63x water-immersion objective. Slice
thickness of confocal images ranged from 2-4 µm, depending on the objective used. The pinhole for each image was set to one Airy unit. Data are presented as projections of a subset of a z-stack and typically include those slices that captured DRG neurons on the left side of the larva for the ten most rostral trunk segments (10-30 slices, depending upon objective used and mounting).

**Immunocytochemistry**

Whole mount immunocytochemistry was performed as described by Svoboda et al. (2001) with minor modifications. Embryos were fixed overnight at 4°C in 4% paraformaldehyde in phosphate buffered saline (PBS: 137mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.8mM KH2PO4, pH 7.4) containing 0.1% Tween-20 (PBSTw). Fixed embryos were permeabilized, blocked and incubated with primary antibodies. The primary antibodies, mouse anti-Elavl3 (formerly HuC/D; Sigma-Aldrich AB1401126) and rabbit anti-GFP (ThermoFischer Scientific, Molecular Probes, A6455), were used at a concentration of 1:1000. Following primary antibody incubation, embryos were exposed to species-specific secondary antibodies (1:1000 goat anti-mouse Alexa 488 or 1:1000 goat anti-rabbit Alexa 568; Molecular Probes-Invitrogen).

**AMD3100 treatment**

AMD3100 (Sigma-Aldrich V5754), a blocker of CXCR4 (Donzella et al., 1998), was dissolved in dH20, and added to the embryo media at 10-200 ng/µl beginning at the one-cell stage (Novoa et al., 2009; Packham et al., 2009; Shayegi
et al., 2014). Formation of cranial ganglia IX and X was assessed at 52 hours post fertilization (hpf) using Elav3 immunoreactivity as described previously (Olesnicky-Killian et al., 2009). For fish in which DRG neuron position was analyzed, embryos were treated until 96 hpf.

Data analysis

Data are presented as a box plot; the box contains the two inner quartiles, with whiskers showing the 5th and 95th percentiles; outliers appear as circles. The horizontal line within the box indicates the median. Statistical analyses were performed using InStat3 (GraphPad Software). Data were analyzed using a Kruskal-Wallis nonparametric ANOVA, corrected for multiple comparisons (Dunn).

Results

Former dorsal root ganglion neurons reside at the sympathetic ganglion

In live larvae, we monitor the position of DRG neurons using transgenic lines that express fluorescent reporter proteins (e.g., Fig. 1). For example, promoter elements of the gene encoding the basic helix loop helix transcription factor Neurogenin1 drive expression of reporter proteins in DRG as well as other neurons (McGraw et al., 2008). At 72 hpf, the typical position of DRG neurons is lateral to the SC:NC boundary (Fig. 1A). In addition, a small subset of differentiated DRG neurons migrate away from their ganglia in the ventral direction (Fig1B,C). Interestingly, some of these ectopic DRG neurons travel as far ventrally as the
location of another neural crest derivative, sympathetic ganglion (SG) neurons (Fig. 1C,C’).

The mechanisms that direct differentiation of SG neurons have been well-studied, and include activity of the forkhead transcription factor Phox2bb (Stanke et al., 1999; Howard, 2005). In the transgenic line Tg(phox2bb:EGFP), SG neuron somas reside next to the dorsal aorta and extend characteristic short axons that project either rostrally or caudally (Fig 1C,D).

The chemokine receptor CXCR4 enables the ectopic position of DRG neurons

The expression of the SG marker TH by DRG neurons that migrate towards the SG (Wright et al., 2010) raises the possibility that signals directing the normal development of SG neurons could mediate acquisition of SG-like characteristics by ectopic DRG neurons. Further, as migratory DRG neurons expressing TH were present both at the location of the SG and also between the DRG and SG, it is likely that the signal participating in this process acts over long distances. On this basis, candidates governing this migration and subsequent acquisition of SG fate are likely secreted molecules, present at or near location of the SG at the correct developmental time, and play a role in the differentiation of SG neurons.

The chemokine CXCL12 and its receptor CXCR4 are required for proper migration of many neuronal subtypes (for review, see Tiveron and Cremer, 2008). CXCL12/CXCR4 signaling is required for accumulation of sympathetic neuron precursor cells at the dorsal aorta, prior to differentiation of SG-neuron phenotype (Kasemeier-Kulesa et al., 2010; Saito et al., 2012). When signaling by either SDF-1
or CXCR4 is inhibited, SG neuron precursors do not congregate at the dorsal aorta, and subsequent differentiation (e.g. TH hydroxylase expression) does not occur (Kasemeier-Kulesa et al., 2010). In zebrafish, SDF-1 is expressed during embryonic and larval stages by cells comprising the dorsal aorta, where SG neurons differentiate and ectopic DRG neurons often reside (Li et al., 2004; Siekmann et al., 2009). Accordingly, we examined whether inhibition of CXCR4 signaling altered the number of ectopic DRG neurons.

Olesnicky-Killian et al. (2009) demonstrated that perturbed CXCR4 signaling impairs formation of cranial ganglia IX and X due to improper cranial neural crest migration. Consistent with this finding, when the CXCR4 inhibitor AMD3100 (Donzella et al., 1998) is applied to developing zebrafish embryos beginning at the one cell stage, these ganglia are absent at 52 hpf (Fig 2A-C). Unlike cranial neural crest cells, migration of trunk neural crest DRG precursors and their initial differentiation as DRG neurons was unaffected with AMD3100 treatment (Fig 2C). Consistent with this, neural crest cell migration and DRG differentiation occur normally when CXCL12 is overexpressed ectopically (Svetic et al., 2007). However, when larvae were examined at later stages (96 hpf), it was evident that treatment with AMD3100 led to a significant decrease in the number of ectopic DRG neurons compared to vehicle treated controls, in a dose-dependent manner (Fig2E-G). Together, these data indicate that the chemokine receptor CXCR4 underlies the ability of DRG neurons to acquire an ectopic position near the sympathetic ganglion.
Ectopic DRG neurons have a unique molecular signature

DRG neurons comprise an impressively heterogeneous population (Usoskin et al., 2015). For example, in zebrafish, the TrkB neurotrophin receptor is expressed in only a subset of DRG neurons in both larval and juvenile zebrafish, a property that likely underlies the ability of some DRG neurons to migrate from their ganglia under proper conditions (Chapter 2, Honjo et al., 2011). To gain further insights into the degree of heterogeneity in zebrafish DRG neurons in early larval stages, and the ability of a subset to leave their normal position, we took advantage of several transgenic lines that express reporter proteins in DRG neurons.

The basic helix-loop-helix transcription factor neurod directs precursor cells towards a neuronal fate (Lee et al., 1995, for review: Chae et al., 2004). In another sensory system of the zebrafish, e.g. the lateral line, and in the endocrine pancreas, specification of cellular subtypes is directed by differential expression of neurod (Sarrazin et al., 2006; Sato and Takeda, 2013; Dalgin and Prince, 2015). In the zebrafish, neurod is expressed by DRG precursors and a subset of differentiated DRG neurons (Fig 3.; Ungos et al., 2003; Honjo et al., 2008; McGraw et al., 2012). To examine whether differential expression of neurod may underlie the ability of a subset of DRG neurons to migrate from their ganglia and assume SG characteristics, we compared expression in the transgenic lines TgBAC(neurod:EGFP) and Tg(neurogenin:dsRed) in normally versus ectopically positioned cells (Obholzer et al., 2008). At 7 dpf, normally positioned DRG neurons display variable expression of both reporters -- dsRed+/EGFP--; dsRed-/EGFP+; dsRed+/EGFP+ (Fig. 3A). Interestingly, ectopic DRG neurons at 7 dpf continue to
express dsRed in the transgenic line \textit{Tg(neurogenin:dsRed)}, but had low or undetectable levels of expression of EGFP in the \textit{TgBAC(neurod:EGFP)} line (Fig. 3B).

Another transcription factor expressed in DRG neurons in zebrafish from embryonic to adult stages is the LIM homeobox transcription factor Islet2b (Fig. 4, Won et al., 2012). We used the transgenic line \textit{Tg(islet2b:EGFP)} to examine reporter expression driven by promoter elements of the \textit{islet2b} gene in normally versus ectopically positioned DRG neurons (Pittman et al., 2008). At 48 hpf, all DRG neurons, positioned at the SC:NC boundary, were labeled in the \textit{Tg(neurogenin:dsRed)} and \textit{Tg(islet2b:EGFP)} lines (Fig. 4A-C). By 60 hpf, there were additional EGFP+ cells present, though only a subset expressed dsRed (Fig 4D-F). At 10 dpf, all DRG neurons were EGFP+ (Fig 4G,H). Interestingly, the majority of normally positioned DRG neurons at this time did not express dsRed, while dsRed expression was maintained in ectopic DRG neurons (Fig. 4G-I). When larvae were examined at later stages (15 dpf), ectopic DRG neurons continued to express both dsRed and EGFP, while the majority of normally positioned DRG neurons were dsRed-/EGFP+ (Fig. 4J-K).

Taken together, these data suggest that ectopic DRG neurons may have a unique molecular makeup, including sustained expression of the basic helix-loop-helix factor Neurogenin1.
Ectopic DRG neurons are labeled in the transgenic line Tg(phox2bb:EGFP)

A remarkable quality of ectopic DRG neurons is their ability to express markers of an alternative neuronal identity, e.g. SG neurons (Wright et al., 2010). We next investigated whether genetic factors that control SG neuron differentiation could be involved in transdifferentiation of ectopic DRG neurons to a sympathetic identity.

The forkhead transcription factor Phox2bb is required for the differentiation of SG neurons (Pattyn et al., 1999; for review, Howard, 2005). SG neurons fail to differentiate and do not express sympathetic markers such as tyrosine hydroxylase when Phox2bb is absent. Because ectopic DRG neurons begin to express the sympathetic marker tyrosine hydroxylase, we investigated if they were labeled in the transgenic line Tg(phox2bb:EGFP), as this could shed light on the ability of this differentiated cell type to assume an alternative identity. A subset of ectopic DRG neurons indeed were EGFP+ in Tg(phox2bb:EGFP) larvae (Fig. 4A). These EGFP+ cells had pseudounipolar processes and soma shapes reminiscent of DRG neurons (Fig 4-7). However, cells co-expressing dsRed and EGFP were absent in Tg(neurogenin:dsRed x Tg(phox2bb:EGFP)) fish, suggesting that ectopic DRG neurons that are reprogrammed to an SG identity may do so by turning off expression of the sensory neuron related transcription factor Neurogenin1 (Fig 5B,C).

To investigate whether ectopic DRG neurons labeled by the Tg(phox2bb:EGFP) line had reverted to a dedifferentiated state, we examined whether they expressed the post-mitotic marker Elavl3 (Kim et al., 1996). Consistent
with previous studies that ectopic DRG neurons maintain their expression of Elavl3 once they migrate away from their ganglia, we found co-expression of the post-mitotic marker Elavl3 in EGFP+ ectopic DRG neurons in Tg(phox2bb:EGFP) larva (Wright et al., 2010; Fig 5A-E).

Taken together, these data suggest that the plasticity of DRG neurons in the differentiated state allow them to acquire characteristics of sympathetic ganglion neurons, including expression of a phox2bb-driven reporter protein.

**Tg(phox2bb:EGFP)+ DRG neurons begin to morphologically resemble SG neurons**

Wright and Ribera (2010) demonstrated the ability of ectopic DRG neurons to sprout lateral processes that resemble those of SG neurons. Accordingly, we examined whether EGFP+ DRG neurons of Tg(phox2bb:EGFP) larva begin to resemble sympathetic ganglion neurons. Interestingly, some EGFP+ ectopic DRG neurons seemed to retract their centrally projecting processes as they approached the location of the sympathetic ganglia (Fig 7 A-C). Further, these former sensory neurons begin to sprout lateral processes that resemble those of the sympathetic ganglion neurons (Fig. 7D-F). While arrival at the SG was not required for sprouting of these lateral axons, lateral axons appeared to intermingle with the projections of normal sympathetic ganglion neurons, and occasional EGFP+ ectopic DRG soma were seen projecting axons into the SG (Fig 7B,F).

In summary, the SG-related transcription factor gene phox2bb drives reporter expression in a subset of ectopic DRG neurons, which begin to morphologically resemble sympathetic neurons and physically incorporate into the SG.
Discussion

It is increasingly clear that maintenance of a neuronal identity is an active process (Deneris and Hobert, 2014). As our understanding of these maintenance processes improves, another question arises. What happens to cells that lose these maintenance signals? While the loss of maintenance signals sometimes results in cell death (Zhao et al., 2006; Tsarovina et al., 2010), it is also possible that this will result in adoption of different identities. Frequently, the loss of maintenance signals results in loss of a particular neuronal characteristic, such as neurotransmitter expression (Saucedo-Cardenas et al., 1998; Coppola et al., 2010). Here we report mechanisms by which former differentiated sensory neurons are able assume multiple characteristics of another neuronal subtype. Our data suggest that cues that direct the normal development of sympathetic ganglion neurons allow for a subset of DRG neurons to transdifferentiate to a sympathetic phenotype (Fig. 8A-D).

The assumption of SG-like tyrosine hydroxylase immunoreactivity by ectopic DRGs suggested that signals directing normal SG neuron development may be involved in the acquisition of a new identity by these cells (Wright et al., 2010). The later development of SG neurons compared to DRG neurons affords the possibility that signals directing development and differentiation of SG neurons can influence already differentiated DRG neurons (Stewart et al., 2004). The chemokine receptor CXCR4 is required for proper migration of sympathetic neuron precursors; when it is functionally inhibited, SG neurons do not arrive at the proper location, and do not differentiate (Kasemeier-Kulesa et al., 2010; Saito et al., 2012). As in other systems, the CXCR4 ligand CXCL12 is expressed during early zebrafish development at the
location of the developing SG neurons and ectopic DRG neurons, the dorsal aorta (Siekmann et al., 2009). We found that inhibition of CXCR4 dramatically reduced the number of DRG neurons that migrate ventrally from their ganglia without affecting DRG differentiation (Fig. 2). This is consistent with the idea that signals directing SG neuron differentiation are involved in DRG neuron transdifferentiation. While CXCR4 is expressed by DRG neurons in other systems, it has not been detected in zebrafish DRG neurons, suggesting that this signaling may be unique to the cells that escape the ganglion environment (McGrath et al., 1999; Chong et al., 2001; Belmadani et al., 2005). The requirement for CXCR4 signaling in ectopic DRG neurons further highlights these cells as a novel population.

Previous studies implicated BDNF/TrkB signaling as required for maintenance of position and sensory neuron identity of DRG neurons. Interestingly, mice heterozygous for bdnf show increased levels of CXCR4, while overexpression of BDNF reduces CXCR4 expression (Bachis et al., 2003; Nosheny et al., 2007; Ahmed et al., 2008). These are consistent with the idea that without signals that maintain a sensory identity (e.g. BDNF/TrkB), CXCR4 signaling allows ectopic DRG neurons to migrate ventrally to the SG. Another possibility is that SG neurons themselves are directing the migration of ectopic DRG neurons, and that inhibited CXCR4 signaling indirectly alters DRG neuron position through an effect exerted by SG neurons. Finally, as several diffusible signals are involved in SG neuron development, it will be interesting if other factors, such as the bone morphogenetic protein (BMP) or neuregulin family of ligands, influence ectopic DRG neuron migration (Saito et al., 2012). Intriguingly, BMP4, a key regulator of sympathetic
neuron development, is expressed during embryonic and larval zebrafish development in the heart and dorsal aorta (Reissmann et al., 1996; Chin et al., 1997; Schneider et al., 1999; Wilkinson et al., 2009).

What makes the subset of migratory DRG neurons unique from their normally positioned counterparts? Zebrafish DRG neurons, as in other systems, comprise a heterogeneous group. As a population, they express several transcription factors, including Neurogenin1, Neurod and Islet2b. Examining the expression of reporter lines in ectopic versus normally positioned DRG neurons revealed interesting clues about the identity of the ectopic cells. While normally positioned DRG neurons expressed reporters driven by neurogenin1 and neurod in combination and alone, we did not detect expression of neurod-driven EGFP in ectopic DRG neurons (Fig 2). Neurod expression typically follows expression of Neurogenin and directs cells towards a neuronal fate. It is possible that the absence of neurod-driven reporter expression in ectopic DRG neurons indicates a less differentiated state. Further, levels of Neurod expression dictate cell subtype specificity in another zebrafish sensory cell type, the lateral line, and are also expressed in only a subset of cranial sensory cells, suggesting that differing levels of Neurod expression may yield a subtype that allows DRG neurons to migrate from their ganglia to assume alternative identities (Holzschuh et al., 2005; Sato and Takeda, 2013).

In contrast to their lack of expression in the TgBAC(neurod:EGFP) line, ectopic DRG neurons continue to express reporters driven by both islet2b and neurogenin1. While both normally and ectopically positioned cells express islet2b-driven EGFP, dsRed in the Tg(neurogenin:dsRed) line is downregulated in most
normally positioned DRG neurons (Fig 3,4). Interestingly, our examination of ectopic DRG neurons through 15 dpf revealed sustained expression in ectopic cells in the Tg(neurogenin:dsRed) line.

The Neurogenin transcription factors help define DRG subtypes by directing expression of the Trk family of neurotrophin receptors (Ma et al., 1999). Though neurogenin1 is expressed by the majority of DRG neurons in the developing zebrafish, its actions are known to be dependent on environmental cues (Blader et al., 1997). The TrkB neurotrophin receptor is expressed in ectopic DRG neurons, and the level of TrkB signaling determines whether cells can exit the ganglia, so it is tempting to speculate that Neurogenin1 in ectopic DRG neurons specifies TrkB expression in a subset of neurons (Chapter 2, Fig. 4,5). Whether the activity of Neurogenin1 determines TrkB expression in the rostral DRG neurons where ectopic cells are typically observed is an intriguing avenue for further study. The presence of neurogenin-driven dsRed in cells at the normal DRG position may represent TrkB+ cells that received sufficient levels of BDNF signaling to maintain their position and identity; they may also represent a TrkB- population that do not respond to BDNF/TrkB signaling. Finally, the diminished touch sensitivity in larvae with an increased number of ectopic DRG neurons (Chapter 1; Wright and Ribera, 2010) suggests a loss of sensory neuron identity; interestingly, Neurogenin is able to specify mechanosensitive markers in DRG neuron subtypes (Bourane et al., 2009). Thus, although markers for a mechanosensitive DRG neuron in zebrafish are not known, expression of a neurogenin-driven reporter in ectopic neurons could be indicative of a previous identity as a mechanosensitive neuron that has been lost
with exit from the ganglion. Whether the transcription factor RUNX3 is also expressed by ectopic DRG neurons is an interesting question, as RUNX3 expression has been shown to direct potential mechanosensitive DRG neurons towards another subtype during development (Kramer et al., 2006).

The expression of the sympathetic ganglion neuron marker TH by a subset of ectopic DRG neurons suggested that mechanisms that direct normal SG differentiation may allow them to acquire this alternative fate. Expression of a reporter driven by phox2bb, a gene encoding the forkhead transcription factor Phox2bb, which is required for normal differentiation of SG neurons, was found in a subset of ectopic DRG neurons (Fig 6-8). Further, and consistent with findings by Wright et al. (2010) that ectopic DRG neurons maintained markers of differentiation, EGFP+ DRG neurons in the Tg(phox2bb:EGFP) line maintained expression of the post-mitotic marker Elavl3 (Fig. 6). Only a subset of ectopic DRG neurons express EGFP in the time frame we that examined. While others may turn on phox2bb-driven EGFP expression at a later time, it is also possible that the subset expressing EGFP are the same subset that will express TH (Wright et al., 2010). Further, whether additional genes involved in SG neuron differentiation, such as phox2aa or paralogues of the aschaete-scute family (ascl1a, ascl1b in zebrafish), are expressed by ectopic SG-like DRG neurons remains unknown. Finally, it is noteworthy that these reporter lines suggest loss of Neurogenin1 expression may precede expression of Phox2bb in these ectopic neurons, as Neurogenin activity is thought to fate restrict neural crest cells to a sensory, rather than sympathetic, phenotype (Fig. 5; Zirlinger et al., 2002). Interestingly, Neurogenin1 knockdown in zebrafish results in
touch-insensitive fish, consistent with a loss of sensory neuron function and identity in these ectopic cells (Cornell and Eisen, 2000). The finding that the centrally projecting axons begin to retract in some ectopic cells may also explain the diminished touch sensitivity of larvae with an increased number of ectopic DRG neurons (Chapter 2).

Do SG neuron-like ectopic cells begin to function in an autonomic manner? The reduction of touch sensitivity in fish with increased numbers of ectopic DRG neurons suggests that mechanosensory function and sensory identity is lost when these cells exit the ganglia (Chapter 1; Wright and Ribera, 2010). However, it is unknown if expression of \textit{phox2bb}-driven EGFP and TH in a subset of these cells correlates with an acquired sympathetic function. Interestingly, we found that EGFP-expressing ectopic DRG neurons begin to sprout SG-like lateral axons, and some appear to incorporate within the ganglia (Fig. 7). Future studies that investigate activity patterns of these ectopic cells could indicate the degree to which these cells function as the SG neurons they begin to resemble.

Here we provide insight into the mechanisms by which a differentiated peripheral neuron is able to acquire characteristics of another neuronal subtype, \textit{in vivo}. Current clinical efforts aimed at facilitating repair or regenerative processes following nervous system insult often involve the transplantation and directed differentiation of stem cells, a process that is complex and with limitations (Lehmann and Ho, 2015). Peripheral nervous system diseases, such as autonomic peripheral neuropathy encountered in the settings of diabetes or malignancy, lead to significant morbidity (Watkins et al., 1995; Koike and Sobue, 2013; Vinik and Erbas, 2013;
Freeman, 2014). Autonomic dysfunction in general causes a variety of insults, including exercise intolerance and postural hypotension, and leads to increased mortality (Maser et al., 2003; Benarroch, 2014). Interestingly, treatment of autonomic neuropathy with sympathomimetic drugs mitigates high peripheral blood flow, reducing the incidence of arteriovenous shunting and neuropathic edema (Boulton et al., 1982; Edmonds et al., 1983). Our data showing the acquisition of SG neuron traits by another neuronal population provide an intriguing method by which sympathetic tone may be increased endogenously.

Additionally, interactions between sympathetic and sensory fibers (Fig. 1C’) have been proposed as a mechanism for neuropathic pain (McLachlan et al., 1993; Ramer and Bisby, 1998; Kim et al., 1999; Ali et al., 2000; Chung and Chung, 2001; Chien et al., 2005; for review, see Nickel et al., 2012). Our data show that an ectopic DRG neuron location, dependent on CXCR4 signaling, may lead to increased interactions between sympathetic and sensory fibers. Interestingly, activity of the chemokine receptor CXCR4, shown here to be responsible for the ectopic position of DRG neurons and interaction with sympathetic fibers, has been implicated in the development and maintenance of neuropathic pain (Bhangoo et al., 2007, 2009; Menichella et al., 2014; Luo et al., 2016). This effect is ameliorated with treatment of AMD3100 (Menichella et al., 2014). Further, signaling by the neurotrophin nerve growth factor (NGF) and its receptor TrkA causes an increase in TH immunoreactivity and sympathetic sprouting in rat DRG (Jones et al., 1999; Deng et al., 2000); whether NGF/TrkA signaling is involved in the transdifferentiation of ectopic DRG neurons is unknown. Finally, increased
excitability of DRG neurons has been suggested to underlie some components of neuropathic pain (Hoeijmakers et al., 2014; Huang et al., 2014; Xie et al., 2010; Zhang and Strong, 2008). Whether the excitability of ectopic DRG neurons or the ion channel makeup of these cells differs from their normal positioned counterparts is an avenue for future study.

Understanding the process by which differentiated neurons acquire characteristics of alternative neuronal fates in vivo is an exciting step towards reprogramming of endogenous cells for nervous system repair, and provides further evidence for the impressive degree of plasticity retained by neurons in a differentiated state.
**Figure 3.1.** Ectopic DRG neurons migrate to the sympathetic ganglion. **A,B.** In wild type 72 hpf *Tg(-3.4neurogenin:GFP)* larvae, DRG neurons express GFP (**A**, asterisks). At 72 hpf, the majority of DRG neurons have cell bodies that reside lateral to the SC:NC boundary (**A**, dotted line). In addition, a subset of DRG neurons that migrated properly as neural crest DRG precursors and initiated neuronal differentiation have cell bodies that reside outside of the ganglion (**B**, arrowhead; Wright and Ribera 2010; Chapter 2). **C,C’,** In a 7 dpf *Tg(neurogenin:dsRed)* larva, the soma of an ectopic DRG neuron (arrowhead), has migrated to the location of SG neurons (arrow, *Tg(phox2bb:EGFP)*). **C’,** The processes of a SG neuron (arrow) envelop the soma and axon of an ectopic DRG neuron (arrowhead). **D,** At 7dpf, sympathetic ganglion neurons typically have axons that emanate from the cell body and run parallel to the rostral-caudal axis (empty arrowheads). Scale bar, in **B**, for **A-C; C’**, 20 µm; **D,** 10 µm.
Figure 3.2: CXCR4 blockade reduces the number of ectopic DRG neurons. **A-C**, AMD3100 inhibits a known CXCR4-dependent event, development of cranial ganglia IX and X. In vehicle-treated 52 hpf control larvae (**A**), or larvae treated with 10 µg/ml AMD3100 beginning at the one cell stage (**B**), cranial ganglia IX and X (solid arrowheads) develop normally, as assessed by Elav3 immunoreactivity (red). However, when treated with 100 µg/ml AMD3100, cranial ganglia IX and X are not present at 52 hpf (**C**, open arrowhead). **D**, In contrast, after AMD3100 treatment (100 µg/ml; 0 – 60 hpf), 60 hpf Tg(-3.4neurogenin:GFP) larvae have DRG neurons (asterisks) that reside lateral to SC:NC boundary (dotted line), indicating that migration of neural crest DRG precursors occurs normally, despite effective blockade of CXCR4. Further, the presence of axons indicates that DRG neurons are undergoing normal morphological differentiation (arrows). **E-G**, Compared to controls (**E**), 96 hpf larvae treated with AMD3100 (100 µg/ml) have fewer ectopic DRG neurons (**F**). **G**, The median number of ectopic DRG neurons (**F**, arrows) decreases in a dose-dependent manner with increasing AMD3100 concentration. Sample size ranged between 18-76 larvae per condition; ^p=0.05, **p=0.01. Scale bars: **A-C**, in **C**, 100 µm; **D**, 20 µm; **E** and **F**, in **F**, 100 µm.
Figure 3.3. As early as initial larval stages, DRG neurons comprise a heterogeneous population. In all images, the SC:NC boundary, the characteristic location of DRG neurons, is indicated by a dotted line. **A-C**, In 7 dpf *Tg(neurogenin:dsRed) x TgBAC(neurod:EGFP)* larvae, normally positioned DRG neurons include three subtypes: dsRed⁺ (asterisk), GFP⁺ (arrowheads), and dsRed⁺/GFP⁺ (arrow). **D-F**, In a double *Tg(neurogenin:dsRed ; neurod:EGFP)* larva, an ectopic DRG neuron (arrowhead) expresses dsRed, but not EGFP. Scale bar: **A-F**, in **F**, 10 µm.
Figure 3.4: In the Tg(neurogenin:dsRed) line, ectopic DRG neurons continue to express dsRed as late as 15 dpf. In all images, a dotted line indicates the SC:NC boundary; the transgenic line Tg(neurogenin:dsRed) is the red signal in all panels, and the transgenic line Tg(islet2b:EGFP) is the green signal in all panels. A-C, At 48 hpf, normally positioned DRG neurons (asterisks) express both dsRed and GFP. D-F, By 60 hpf, individual dorsal root ganglia comprise several DRG neurons that collectively express different repertoires of reporter proteins, either EGFP alone or both EGFP and dsRed (F, empty arrowheads). G-I, The boxed area in G is shown at higher magnification in H, I. DRG neurons continue to express EGFP (H; asterisk), while only a subset express dsRed (G; empty arrowheads). An ectopic DRG neuron (H, I; arrowhead) expresses both GFP and dsRed. J-K, At 15 dpf, an ectopic DRG neuron (L, arrowhead) continues to express dsRed. Scale bars: C, F, I, and L: 20 µm.
**Figure 3.5**: Ectopic DRG neurons begin to express EGFP in the transgenic line Tg(phox2bb:EGFP). In all images, a dotted line demarcates the SC:NC boundary. **A-C**, Ectopic DRG neurons in a double [Tg(neurogenin:dsRed);Tg(phox2bb:EGFP)] larva; one ectopic DRG neuron is dsRed\(^+\) (A-C, arrowheads), while a dsRed\(^-\) ectopic DRG neuron expresses EGFP (asterisk); this ectopic DRG neuron is adjacent to an EGFP\(^+\) SG neuron (arrow). **D-F**, At 7 dpf, a [Tg(neurogenin:dsRed);Tg(islet2b:EGFP)] larva contains ectopic DRG neurons that are EGFP+/dsRed+ (F, arrowheads). **G-I**, At 7dpf, a [Tg(neurogenin:dsRed);Tg(islet2b:EGFP)] larva has one double EGFP\(^+\)/dsRed\(^+\) ectopic DRG neuron (H, arrowhead), and one EGFP\(^+\) only ectopic DRG neuron (G-H, empty arrowhead). Scale bars: C, F, and I; 20 µm.
Figure 3.6: Ectopic DRG neurons that are EGFP+ in the transgenic line Tg(phox2bb:EGFP) express the post-mitotic marker Elavl3. In all images the SC:NC boundary is marked by a dotted line. **A**, The boxed area in **A** is enlarged in **B-E**. Elavl3 expressing ectopic DRG neurons are present in a 7 dpf Tg(phox2bb:EGFP) larva. One ectopic DRG neuron (arrowhead) is Elavl3+/EGFP+, while others (asterisks) are Elavl3−/EGFP+. EGFP+ SG neurons (empty arrowheads) also express Elavl3+. **B-E**, Normally positioned DRG neurons adjacent to the SC:NC boundary are Elavl3+/EGFP−, while ectopic DRG neurons (asterisks) that have migrated towards SG neurons (arrowheads) are Elavl3+/EGFP+. Scale bars: **A** 100 μm; **B-E**, in **E**, 20 μm.
Figure 3.7: EGFP+ ectopic DRG neurons in the Tg(phox2bb:EGFP) transgenic line retract axons and incorporate into the SG. A-A”, An ectopic DRG neuron (arrowhead) in a live 7 dpf [Tg(neurogenin:dsRed);Tg(phox2bb:EGFP)] larva expresses GFP, but not dsRed, and resides ventral to a normally positioned DRG neuron that does express dsRed but not EGFP (asterisk). A’, A” Time lapse examination of the EGFP+ ectopic DRG neuron shows that it has retracted its centrally projecting process (empty arrowhead) between 7 (A’) and 9 dpf (A’’). B-F, An ectopic DRG neuron resides at the location of the SG in a 7 dpf [Tg(neurogenin:dsRed);Tg(phox2bb:EGFP)] larvae. The solid box in B is enlarged in C-E; the dotted box in B is enlarged in F. C-E, Normally positioned dsRed+/EGFP- DRG neurons (C, D, asterisks) reside at the SC:NC boundary. An ectopic dsRed-/EGFP+ DRG neuron (C, arrowhead) has travelled ventrally and sprouted a lateral axon (E, arrow) similar to those of SG neurons. F, SG neurons and an ectopic EGFP+ DRG neuron from (C-E) showing incorporation of the ectopic DRG soma (arrowhead) and its axons (arrows) within the SG. Scale bars: A-A”, in A”, 20 µm; B, 20 µm; C-F, in E, 20 µm.
Figure 3.8. Model for mechanism of transdifferentiation by DRG neurons. In all panels the SC:NC boundary is indicated by a dotted line. Axons of other DRG neurons have been omitted for simplicity. **A**, Neural crest cell migration and DRG neuron differentiation occur normally. A subset of differentiated neurons that reside within the ganglia express the bHLH transcription factor Neurogenin1 (red). **B**, Upon blockade of BDNF/TrkB signaling (Wright and Ribera, 2010; Chapter 2), Neurogenin1+ DRG neurons can migrate ventrally towards sympathetic ganglion neurons (black), via DA-derived CXCL12 signaling to the chemokine receptor CXCR4. **C**, While continuing to express the post-mitotic marker Elavl3 (not shown), a subset of ectopic DRG neurons turn off Neurogenin1 expression, and begin to express the transcription factor Phox2bb (green). Phox2bb+ DRG neurons begin to retract their stereotypic bipolar axons. **D**, Phox2bb+ neurons begin to incorporate into the ganglia to and sprout SG-like lateral axons. DA: dorsal aorta, SDF-1: stromal derived factor-1.
CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

Summary of Findings

My work has increased understanding of the role that the environment plays in maintenance of neuronal identity, and has provided insights into molecular components of this maintenance signal. Further, my studies have begun to delineate factors that govern plasticity of differentiated neurons that do not receive proper identity maintenance signals.

The level of activity in the transient, mechanosensitive Rohon-Beard cells determines the initial size of the permanent DRG neuron sensory pool. Increased activity in RB cells, including in a physiologic paradigm, results in maintenance of position and sensory identity in DRG neurons. Further, the neurotrophin BDNF represents the signal communicated from RBs to DRGs in an activity-dependent manner, as exogenous BDNF rescues DRG neuron position and touch sensitivity in sensory-deprived fish. Although two BDNF receptors, NGFR and TrkB, are expressed by embryonic and early larval zebrafish, pharmacologic and gene targeting methods implicate TrkB as the relevant receptor to maintain a sensory DRG neuron identity.

When a sensory identity is lost in these cells, differentiated DRG neurons respond to signals normally associated with development of another neuronal subtype and neural crest derivative, the sympathetic ganglion neurons. Specifically, signaling by the chemokine receptor CXCR4 underlies the ability of former sensory neurons to travel ventrally to the the location of SG neurons. Further, a subset of
ectopic neurons lose markers indicative of a sensory identity, and begin to express a reporter driven by the gene encoding the transcription factor Phox2bb, which governs differentiation (e.g. TH hydroxylase expression) of sympathetic ganglion neurons. The morphology of former sensory neurons begins to resemble that of sympathetic ganglion neurons, and some projections appear to infiltrate within the SG.

**Maintenance of a Sensory Neuronal Identity**

The understanding that many populations require sustained regulation of cellular identity is continuing to emerge (Holmberg and Perlmann, 2012). Particularly in the study of neurons, which have generally been regarded as an immutable population, the notion of identity maintenance raises interesting questions (Deneris and Hobert, 2014). Is this maintenance typically governed in a cell-autonomous manner? Indeed, several studies have identified individual genes that seem to act as gatekeepers, maintaining a particular identity in differentiated neurons (Carney et al., 2013; Price et al., 2014; Southall et al., 2014). Also becoming clear is the notion that many factors that direct aspects of development (e.g. survival, differentiation) continue to function as maintenance signals in post-mitotic neurons (e.g. Sun et al., 2008; Deneris and Hobert, 2014). In addition to cell-autonomous mechanisms, both extracellular signals and sensory inputs shape many aspects of development. The maintenance of DRG neuron identity in zebrafish is reminiscent of several of these paradigms, including the iterative use of signaling partners (BDNF/TrkB) and the role of external, sensory inputs in maintaining this neuronal identity. Continued investigation of these maintenance programs not only bolsters our understanding of
what constitutes a neuronal identity, but may one day be harnessed to liberate previously unappreciated plasticity of post-mitotic cells for the treatment of disease.

Rohon-Beard cells are classically considered a mechanosensitive primary neuron that mediate the zebrafish touch response. My work and other work from our lab (Wright and Ribera, 2010) have revealed another role for early detection of tactile inputs in sculpting the size of the permanent sensory neuron pool. Other studies have revealed a role for sensory inputs in governing neuronal traits. For instance, depriving mature mice of odor inputs via nare closure results in downregulation of the nuclear orphan receptor COUP-TFI and diminished tyrosine hydroxylase immunoreactivity in the olfactory bulb glomerular layer (Bovetti et al., 2013). While transducing mechanosensitive inputs is a well-known role for the transient RB cell population, their maintenance of DRG neuron identity suggests they have more diverse roles than previously appreciated.

Indeed, new transgenic lines and an expanding repertoire of molecular markers hint at a heterogeneous RB cell population, and introduce additional questions in the context of DRG neuron sensory identity maintenance by RB cells. Is a subset of RB cells responsible for synthesis and release of BDNF, and how can we identity that subset? The runt-related transcription factor encoding gene runx1 is expressed in some RB cells in both *Xenopus laevis* and zebrafish (Kataoka et al., 2000; Burns et al., 2002). Knockdown of runx1 causes both decreased touch sensitivity and abnormal RB cell axon morphology (Kalev-Zylinska et al., 2002; Park et al., 2012). Most intriguing is the presence of a RUNX1 binding site in a promoter region of the zebrafish *bdnf* gene (Heinrich and Pagtakhan, 2004). Whether
disruption of RUNX1 activity leads to decreased bdnf transcription or loss of DRG sensory neuron identity are attractive avenues for future studies.

Do some DRG axons purposefully seek out RB processes in the periphery during their initial innervation? While RB and DRG processes are known to overlap during zebrafish development (Wright and Ribera, 2010), neither the peripheral branching patterns of DRG neurons nor their interactions with RB cell projections have been investigated in settings where BDNF or TrkB signaling is abrogated. Altered outgrowth of DRG axons in these conditions would not be unexpected, considering even the historical roles of neurotrophins in neurite outgrowth (Levi-Montalcini, 1964; Davies et al., 1986). Further, as neurotrophin/trk interactions are capable of guiding axon growth and direction (Gundersen and Barrett, 1979; Tucker et al., 2001; Horch and Katz, 2002; Hellard et al., 2004; Park and Poo, 2013), one potential mechanism is that RB-derived BDNF signals to TrkB on approaching DRG peripheral axons to facilitate their interaction; this would be a logical avenue for the finite number of TrkB expressing DRG neurons to connect with BDNF-producing RB cells.

Also unknown are regulatory factors that govern synthesis and release of BDNF in response to tactile inputs. Whether there are transcripts present that are translated locally as a result of stimuli detected at the periphery, or if these inputs are communicated back to the RB cell body where new bdnf transcripts are produced, is unknown. CamKII-mediated phosphorylation of MeCP2 underlies the ability of physiologic inputs to elicit activity-dependent bdnf transcription in some contexts (Zhou et al., 2006). Interestingly, sustained activation of CamKII by sensory
inputs to Aβ-LTMRs is also required for maintenance of mechanosensitive signal transduction; without this LTMR-mediated CamKII activity, these inputs can shift to nociceptive pathways (Yu et al., 2015). Further, signaling by the cAMP response element-binding (CREB) protein is involved in sensory-induced activity-dependent bdnf transcription (Hong et al., 2008), and represents another avenue for future study, particularly with the presence of a predicted CREB binding site within the zebrafish bdnf gene (Huynh and Heinrich, 2001).

Whether sensory inputs continue to govern plasticity of DRG neurons in juvenile and adult animals, or if the stage during the initial differentiation of DRG neurons represents a true critical period for this identity maintenance, is unknown. The sustained expression of TrkB by a subset of juvenile DRG neurons hints at a role beyond embryonic and early larval stages (Honjo et al., 2011). DRG neurons also maintain expression of trk receptors in adult mammalian systems, including in humans (Yamamoto et al., 1993; McMahon et al., 1994; Muragaki et al., 1995; Phillips and Armanini, 1996). While RB cells are not thought to be present at later stages, at least in their canonical embryonic form, the idea of autocrine / paracrine maintenance of identity by sensory neurons is attractive (Schecterson and Bothwell, 1992). Consistent with this idea, DRG neurons also synthesize neurotrophins, including in adult humans (Yamamoto et al., 1996). Other than TrkB, expression of neurotrophins and trk receptors has not been examined in juvenile and adult zebrafish DRG neurons. Whether neurotrophin/trk signaling continues to govern zebrafish DRG identity in later stages is unknown, though continued trk expression suggests this as a possibility.
The maintenance of sensory neuron identity by BDNF/TrkB signaling is reminiscent of several classically described roles for neurotrophins. The requirement of environmentally-stipulated BDNF by DRG neurons for maintenance of sensory identity is similar to original descriptions of NTs are target-derived factors. This is also similar to a role described for target-derived cytokine signaling in the maturation of cholinergic neurons in mouse sweat glands (Stanke et al., 2006). Further, while BDNF/TrkB signaling is required for maintenance of a sensory neuronal identity in DRG neurons, it also may be required for survival of nascent DRG neurons or neural crest cell precursors (Chapter II). Interestingly, other insights into maintenance of neuronal identity have benefited from analysis of hypomorphic alleles (Doitsidou et al., 2013). Future examination of NCC and early DRG neuron survival and differentiation in ntrk2 mutants may clarify an iterative role for BDNF/TrkB signaling in DRG neurons, and dependence of these cells on neurotrophin signaling for survival, similar to mammalian models (Jones et al., 1994; Snider, 1994). Further, the downstream signaling partners of this BDNF/TrkB signaling are unknown; their investigation will increase our understanding of the maintenance of neuronal identity (see Future Directions, below).

**The Identities of Ectopic Cells**

The ability to characterize and perturb signals directing maintenance of neuronal identity has the exciting potential to yield new tools for the treatment of diseased or damaged nervous systems. While identification of plasticity retained by differentiated cells represents an important step in the process, understanding the
mechanisms that allow for plasticity when maintenance signals are lost, and the consequences of this plasticity, are critical if they are to ultimately be harnessed in a therapeutic setting.

Cells that migrate from the dorsal root ganglia in zebrafish appear to lose their sensory identity, as evidenced by diminished touch sensitivity in larvae with an increased number of ectopic cells, and the adoption of characteristics of sympathetic neurons by a subset of these cells. However, it seems that these ectopic cells comprise a heterogeneous population, and that multiple cell fates may arise with absence of a BDNF identity maintenance signal. Some ectopic cells continue to express a *neurogenin*-driven reporter while others appear to downregulate this expression in favor a reporter driven by sympathetic neuron associated transcription factor gene, *phox2bb*. Some but not all ectopic DRG neurons begin to express TH, or morphologically resemble SG neurons, further highlighting the apparent heterogeneity of this group (Wright et al., 2010). While these differences could be ascribed merely to a temporal heterogeneity, other possibilities exist.

The diminished touch sensitivity in fish with an increased number of ectopic cells suggests that an ectopic position correlates with a loss of mechanosensitivity. It is possible, however, that rather than losing a sensory neuronal identity, some of these ectopic DRG neurons simply lose the ability to transduce mechanosensitive inputs. The ability of BDNF/TrkB signaling to modulate the mechanosensitive properties of LTMRs leaves open the possibility that BDNF/TrkB signaling is required for not only positioning of DRG neurons, but also the mechanosensitive properties of these cells (Carroll et al., 1998; Rutlin et al., 2014). Further, it is
possible that the BDNF/TrkB identity maintenance mechanism, rather than representing a binary choice (i.e. loss of sensory identity if no signaling is present, and vice versa), exists on a spectrum, and that a more dramatic reduction in signaling enables a greater level of plasticity in these cells, away from any semblance of a somatosensory identity. A continuum of BDNF/TrkB signaling in maintenance of sensory identity could potentially explain some of the variability in phenotype encountered in \textit{ntrk2b}MO vs \textit{ntrk2b}CRISPR (transient F0 and F2 \textit{-/-}) larvae. An attractive hypothesis is that continued expression of Neurogenin1 by some cells in an ectopic position indicates that they have managed to retain some degree of somatosensory identity, but that rather than being reprogrammed to a sympathetic fate, they are simply released from their mechanosensitive subtype identity in the absence of tactile stimulation-derived maintenance signals.

Interestingly, the level of signaling by the neurotrophin NT-3, in a target-derived manner, influences proprioceptive neuron subtype identity (de Nooij et al., 2013). While currently there are few markers to investigate stimulus-specific heterogeneity in zebrafish DRG neurons, their future identification could allow investigation of either a) loss of mechanosensitive markers (e.g. short stature homeobox2 - Scott et al., 2011; \textit{piezo2b} - Faucherre et al., 2013;) or b) gain of non-mechanosensitive DRG markers, which would indicate a subtype switch rather than a complete loss of sensory neuron identity. For example, initiation of expression of the transcription factor RUNX1 or the neurotrophin receptor TrkA in ectopic neurons could indicate an adoption of a nociceptive sensory identity (Kramer et al., 2006; Lallemand and Ernfors, 2012). This subtype switching would be reminiscent of the rod-to-cone
transdifferentiation that occurs with conditional knockout of the basic leucine zipper transcription factor gene *nrl* in adult retina (Montana et al., 2013). Initial clues to DRG heterogeneity in zebrafish may also be found by investigation of soma size distribution in larval or juvenile ganglia, as stimulus specificity is thought to segregate with varying soma sizes.

Based on the role of *phox2b* in SG neuron development (Stanke et al., 1999), it is possible that expression of *phox2bb* underlies the ability of a subset of ectopic DRG neurons to express TH. Further, the morphological changes that occur in ectopic DRG neurons (e.g. retraction of central process, lateral axon sprouting) are indicative of a loss of sensory identity and adoption of SG-like characteristics. Another possibility, consistent with the notion posited above of a continuum of BDNF/TrkB signaling, is that some TH expressing cells represent a particular DRG neuron subtype, rather than a newly transdifferentiated SG neuron. While TH expression has not been detected in normally positioned DRG neurons in zebrafish, it is found in DRG neurons of other systems (Lallemend and Ernfors, 2012; Usoskin et al., 2015); specifically, TH+ DRG neurons have been found in a Ret+/VGLUT3+ C-fiber LTMR population (Li et al., 2011). Co-expression of the Ret receptor or VGLUT3 in *Tg(phox2bb:EGFP)*-expressing ectopic cells has not been examined, but these findings would be consistent with the idea that these ventrally positioned cells perhaps represent the spatial segregation of a DRG subtype. However, the diminished touch sensitivity in larvae with increased ectopic neurons would argue against adoption of another mechanosensitive fate. As *phox2b* is not known to be expressed in DRG neurons of other systems, including TH-expressing DRG
neurons, this would seem to represent a novel gene directing subtype specificity within the DRG lineage. Conversely, sustained expression of phox2b is required for maintenance of a sympathetic neuronal identity (Coppola et al., 2010), and thus it would be intriguing if this expression was maintained in former sensory neurons.

Though proximity to the SG and SG-like morphology in ectopic DRG neurons are suggestive of an alternative neuronal fate in some ectopic DRG neurons, future studies will clarify whether these phox2bb-EGFP and TH expressing cells represent fully transdifferentiated neurons. For example, expression of dopamine beta-hydroxylase, which converts dopamine to norepinephrine, would be further indicative of a sympathetic, catecholamine-producing cell type (Stanke et al., 1999; Howard, 2005; Lucas et al., 2006). Like Phox2b, the transcription factor Hand2 is also required for maintenance of a sympathetic identity, and its expression in ectopic cells would also suggest a true sympathetic transdifferentiation (Schmidt et al., 2009).

**Human Disease Relevance**

The work presented here has the potential to shed light on the pathophysiology of several disease states. In addition, beginning to understand the mechanisms that direct in vivo plasticity of differentiated neurons is an important step towards reprogramming a patient’s own cells to combat disease.

Several human diseases include both deficits in sensory function or processing, and dysregulation of BDNF levels. For example, patients with Rett syndrome, caused by a mutation in the gene encoding the transcriptional regulator MeCP2, have diminished somatosensory function, particularly in the ability to sense
high threshold mechanical stimuli (Devarakonda et al., 2009; Downs et al., 2010; Battaglia, 2011). Intriguingly, mice with mutated or diminished levels of MeCP2 also display reductions in both thermal and mechanical sensitivity (Manners et al., 2015; Samaco et al., 2008, 2013). Further, mutation of mecp2 results in decreased levels of BDNF and altered BDNF secretion (Chen et al., 2003; Wang et al., 2006). With increasing support for a role of BDNF in the pathophysiology of Rett Syndrome (Chang et al., 2016; Kasarpalkar et al., 2014) and the recovery of some neuronal deficits (e.g. synaptic dysfunction) in mecp2/− mice when treated with exogenous BDNF (Kline et al., 2010), it will be interesting if BDNF is tested in clinical trials for treatment of human Rett Syndrome patients, and to determine whether BDNF treatment ameliorates the sensory deficits experienced by some patients. While BDNF treatment has been proposed for treatment of diseases of the central nervous system, and even tested in clinical trials in patients with Amyotrophic Lateral Sclerosis (Ochs et al., 2000; Ankeny et al., 2001), technical challenges arise when attempting to deliver the relatively large BDNF protein past the CNS blood brain barrier (Nagahara and Tuszynski, 2011); these challenges may be circumvented in BDNF treatment of peripheral nervous system disease components.

To complement these findings of BDNF dysregulation in Rett Syndrome, it would be interesting to investigate the regulation of sensory neuron identity in a zebrafish model of Rett Syndrome (i.e. mecp2 mutant; Pietri et al., 2013). Reminiscent of my work showing a requirement for environmentally stipulated, activity-dependent BDNF signaling in maintenance of DRG neuron identity, MeCP2 is required for stimulus-mediated refinement of circuitry in both primary visual cortex
and olfactory neuron projections (Degano et al., 2014; Lee et al., 2014; Krishnan et al., 2015). Whether MeCP2 (or epigenetic mechanisms) influence activity-dependent BDNF transcription, and the role it may play in maintaining sensory neuron identity in zebrafish, is unknown. Interestingly, in zebrafish with a mutation in the histone deacetylase encoding gene hdac1, DRG neurons are often found in ectopic positions (Ignatius et al., 2013).

In addition to their relevance to diseases with sensory deficits, the data presented here are applicable to our understanding of some models of neuropathic pain. Wright and Ribera (2010) demonstrated an SG-like morphology and expression of the sympathetic marker TH in some ectopic DRG neurons. However, evidence suggesting an interaction between the two cell types had yet to be demonstrated. I have shown here the intermingling of fibers from normally positioned sympathetic ganglion neurons and ectopic DRG neurons. While inappropriate sprouting of sympathetic fibers within the DRG has been proposed as a mechanism of neuropathic pain (McLachlan et al., 1993; Ramer and Bisby, 1998; Kim et al., 1999; Ali et al., 2000; Chung and Chung, 2001; Chien et al., 2005; for review, see Nickel et al., 2012), my findings here suggest another, converse mechanism, whereby normally positioned SG neurons are able to interact with DRG neurons as a result of an ectopic position of the sensory neuron. Further, the finding that CXCR4 signaling seems to underlie the ability of these cells to migrate ectopically is intriguing given the clinical data associating CXCR4 activity with neuropathic pain, in patients infected with the human immunodeficiency virus (Bhangoo et al., 2007, 2009; Menichella et al., 2014; Luo et al., 2016). Whether
these fibers make synaptic connections, and whether activation of sympathetic neurons elicit responses in ectopic DRG neurons, would be intriguing avenues for future studies (see below). One particular subtype of neuropathic pain, termed Static Mechanical Allodynia, has components of both atypically-induced pain (allodynia) and an underlying inability to detect normal mechanical stimuli in the same dermatomal distribution (Spicher et al., 2008); a potential intriguing pathophysiologic mechanism for such a condition could be loss of a sensory (e.g. mechanosensitive) identity, and subsequent interaction with sympathetic fibers.

Finally, my studies have begun to unravel the mechanisms of plasticity of these former sensory neurons. The ability to direct transdifferentiation of endogenous cells represents an exciting potential treatment option for repair of nervous system disease and damage, as it does not require transplantation of stem cells, and the endogenous cell type may retain unknown neuronal characteristics absent in exogenous treatment options (Jopling et al., 2011). Further, because ectopic DRG neurons begin to resemble sympathetic ganglion neurons, it is intriguing to speculate about their potential utility in diseases with dysautonomia, such as diabetes or infectious disease (e.g. HIV). While the discovery of expression of a phox2bb-driven reporter by a subset of ectopic DRG neurons may yield insights into the ability of differentiated neurons to assume characteristics of differentiated SG neurons, much remains to be discovered about the function of these cells, the degree of plasticity they retain, and thus the range of their therapeutic potential.
Future Directions

My work provides mechanistic insights into both the maintenance of neuronal identity by environmental sensory input, and plasticity of cells in which these maintenance signals are absent. However, there are questions that remain to be answered, including avenues for investigation suggested by the current studies.

RB cells interpret the amount of environmental sensory input and communicate these levels as maintenance signals to differentiated DRG neurons. Work by Wright and Ribera (2010) suggested that Na\textsubscript{v}1.6 knockdown in individual RB cells was sufficient to free DRG neurons for the ganglion environment. However, the heterogeneity of the RB population hints that not all RB cells may be involved in the maintenance of DRG neurons. The degree to which BDNF is synthesized by only a subset of RB cells is unknown. Further, past work suggests that apoptosis by RB cells is an activity-dependent process (Svoboda et al., 2001). The notion that RB cells that synthesize and release BDNF in an activity-dependent process are those that subsequently show signs of apoptosis is an intriguing possibility, as though their information transfer to the permanent sensory population relinquishes them from their somatosensory duties. Though it seems unlikely given the current data, the possibility remains that an alternative source provides DRG neurons with BDNF. Possibilities include autocrine or paracrine secretion by DRG neurons themselves, or from the hindbrain where centrally projecting DRG axons are thought to terminate. Optogenetic stimulation of RB cells, both groups of cells and individually, and monitoring of DRG positioning could provide more evidence that RB cells are in fact the relevant BDNF source, while mosaic \textit{bdnf} morpholino injection may shed light on
other sources. The recent identification of *piezo2b* transcripts in RB cells (Faucherre et al., 2013) suggests it could be the molecular player mediating mechanosensitive inputs that stipulate levels of BDNF as a maintenance signal – it would be interesting to investigate effects of *piezo2b* knockdown or mutation on the maintenance of DRG neuron sensory identity. Additionally, as a subset of RB cells appear to persist for longer than previously thought, the handoff of somatosensory function from RB to DRG neurons is seemingly less clear. The finding that mechanosensitive inputs are transduced by rodent DRG neurons coincident with peripheral innervation (Lechner et al., 2009) makes it reasonable that zebrafish DRG neurons are competent to transduce inputs in embryonic stages; however, it remains unknown the degree to which RB and DRG cells share responsibility for mechanosensation. Monitoring activity patterns (e.g. Ca\(^{2+}\) activity in an *islet2b* or *neurogenin*-driven GCaMP6 line) with a stereotyped mechanosensitive stimulus could shed light on this transfer of somatosensory duties, though the potential movement of the specimen upon tactile stimulation may make this imaging technically difficult. Finally, expression of several markers by RB cells enable or suggest the potential for detection of non-mechanosensitive inputs by RB cells (e.g. the opsin encoding gene *valopab*, Kojima et al., 2008; *trpA1*, Prober et al., 2008; *trpV1*, Gau et al., 2013;), and the possibility that non-tactile sensory inputs could also govern DRG neuron identity maintenance. Interestingly, an exon within the zebrafish *bdnf* gene that is restricted in expression to RB cells contains a predicted heat shock factor binding site, suggesting the possibility of thermal-mediated *bdnf* transcription, and potential regulation of DRG identity by thermal, in addition to mechanosensitive, inputs (Heinrich and Pagtakhan,
Fish exposed to a brief thermal stimulus indeed show increased production of *bdnf* transcripts; interestingly, this study used 5 dpf zebrafish, and so could also lend support for BDNF production by DRG neurons themselves (Malafoglia et al., 2014). The transient receptor potential channel gene *trpv1*, expressed by zebrafish RB cells and responsible for thermally-induced behaviors, represents an attractive molecular target for investigation of thermal sensory inputs on DRG neuron identity (Gau et al., 2013).

Investigation of *piezo2b* transcripts in DRG neurons, and activity-patterns in DRG neurons themselves in the presence specific stimuli also represent intriguing avenues for future study, and will allow us to begin to probing subtype specificity with DRG neurons. While my studies and others hint at a not-unexpected diversity within the DRG neuron population, in zebrafish a detailed characterization of modality specific markers, and how this diversity arises, has not yet been undertaken. It will be interesting to see if markers of a non-mechanosensitive DRG identity are expressed in ectopic DRG neurons. The sustained expression of a *neurogenin*-driven reporter signal in ectopic DRG neurons is intriguing, as Neurogenin1/2 in mouse has been shown to govern trk expression in developing DRG neurons (Ma et al., 1999). Whether expression of Neurogenin1 in DRG neurons enables TrkB expression, and thus the ability to respond to a BDNF maintenance signal, is an attractive idea. This Neurogenin directed TrkB expression would likely involve yet unidentified local cues, as Neurogenin is detectable in DRG neurons along the entire rostral-caudal axis (Cornell and Eisen, 2002); interestingly, zebrafish Neurogenin action is known to depend on environmental factors (Blader et
al., 1997). Additionally, signaling components downstream of BDNF/TrkB that maintain a sensory identity in DRG neurons are unknown. Expression of the alpha isoform of protein kinase C (PKC) in zebrafish DRG neurons (Slatter et al., 2005; Patten et al., 2007), the conserved PLCγ (upstream of PKC) binding site in the intracellular portion of zebrafish NTRK2 (Heinrich and Lum, 2000), and involvement of PKC signaling in transcriptional regulation make it an intriguing candidate (Reichardt, 2006). Genetic or pharmacologic perturbation of PKC signaling and analysis of DRG neuron position would provide evidence about whether this signaling component present in these cells is responsible for maintenance of their identity.

Finally, at least a subset of ectopic neurons begin to resemble sympathetic ganglion neurons, and evidence presented here suggests that signals governing normal SG development may govern the plasticity of these former DRG neurons. CXCR4 signaling appears to dictate the ability of DRG neurons freed from their sensory identity to migrate ventrally to the SG. However, whether these effects are cell-autonomous within the ectopic neurons is unknown; knockdown of CXCR4 signaling specifically within DRG neurons (e.g. mosaic cxcr4a/b morpholino injection) would inform whether DRG neurons specifically employ this signaling to travel ventrally. Though it would not inform about the cell-autonomy of CXCR4 action, it would also be interesting if dorsally placed SDF1 coated beads could induce atypical migration directions of DRG neurons freed from the ganglion environment.
The exciting finding that ectopic DRG neurons are identifiable in the
*Tg(phox2bb:EGFP)* line provides a potential mechanism of TH-expression by these
former sensory neurons. Time lapse imaging of *Tg(neurogenin:dsRed) x
*Tg(phox2bb:EGFP)* larvae will clarify whether loss of Neurogenin1-driven reporter
expression indeed precedes the expression of EGFP in these cells, or if a resident
progenitor population at the DRG gives rise to newly EGFP-expressing DRG
neurons; however, the expression of the post-mitotic marker Elavl3 by EGFP+
(Chapter III) and TH+ ectopic cells (Chapter III; Wright et al., 2010) hints at a
process of direct transdifferentiation of DRG neurons to an SG-like phenotype. Also,
whether Phox2bb expression is predictive of TH expression in ectopic cells is
unknown, though reasonable to suspect given the requirement of Phox2bb activity in
TH expression in normally developing SG neurons (Stanke et al., 1999). Further, it is
unknown to what degree the DRG to SG transition stems from reversion to an
alternative fate of a common precursor cell, or simply the response to locally
secreted cues once maintenance signals are lost in these cells. A technically
challenging, though potentially rewarding experiment could involve transplantation of
cells recently freed from the ganglion environment to locations where different
molecular environments would be encountered. Are *phox2bb*-driven EGFP and TH
hydroxylase present in cells transplanted dorsally, away from the SG, or more
caudally, where SG development takes place at a later time? The expression of
these markers regardless of location would hint at a plasticity governed by cell-
autonomous factors, while the finding that their expression depends on a ventral
position would be an exciting hint that their non-sensory identity is influenced by
external cues, consistent with the current data. Interestingly, Phox2bb and TH expression could also indicate adoption of an enteric fate (Elworthy et al., 2005; Olsson, 2015); whether transplant of these ectopic cells to a location near gut neurons enabled expression of other enteric markers such as the GDNF receptor GFRα1 would be interesting, and indicative of even greater plasticity of these cells (Shepherd et al., 2004). Finally, though single transcription factors are sufficient to govern some instances of transdifferentiation (Riddle et al., 2013), examining the role of other factors that direct normal SG neuron development in the acquisition of sympathetic traits by ectopic DRG neurons, e.g. BMP4 (Reissmann et al., 1996; Schneider et al., 1999; Ernsberger et al., 2000) or neuregulin (Saito et al., 2012), and could complement the discovery of phox2bb-driven reporter expression in a subset of these cells.

Finally, is it possible that the number of sensory neurons devoted to detection of a specific stimulus could continue to change depending on the environment that an organism encounters? For example, if fish were exposed to chronic noxious stimuli, would there be a transition from other stimulus subtypes and upregulation of noxious nociceptors receptors to match the environment, or would there be a decrease in pain-sensing neurons to decrease the detection of the chronic stimulus? Either result would be exciting, and indicative of a retention of plasticity even at later stages in the life of the zebrafish.

My work has increased our understanding of both maintenance and plasticity of differentiated neuronal identity. It has become clear that every answered question elicits many more – I hope that my efforts here represent stepping stones rather than
conclusions, and that future investigations of plasticity in zebrafish DRG neurons will aid in unraveling the many mysteries of our complex nervous systems.
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