Temporal Analysis of Sensory Neuron Diversification

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Abstract

The vertebrate somatosensory system consists of a multitude of neurons transducing an array of environmental stimuli, ranging from proprioceptive neurons that detect muscle stretch to thermoreceptive neurons that detect temperature. Such a diverse population is required to accurately interpret environmental cues so that the organism can appropriately respond to rewarding or dangerous stimuli. Interestingly, diverse neurons that transduce modality-specific stimuli originate from similar neural progenitor populations, but quickly acquire unique protein expression profiles and mature through temporally independent paths. Naturally occurring cell death and axon degeneration during development permits progressive refinement of sensory circuits. In Chapter II, we interrogated how/if neuronal subtypes differentially die, and we investigated whether the neurotrophin receptor p75NTR is required for developmental cell death across neuronal subtypes. We find that neurons derived from the earlier progenitor population (TrkB+, TrkC+, and eRet neurons) complete developmental cell death prior to neurons derived from the later progenitor population (TrkA+ neurons) (Cheng et al., 2018). We also find that p75NTR restricts the magnitude but not temporal window of death for each subpopulation (Cheng et al., 2018). Within Chapter III, we utilized single cell mass cytometry, CyTOF, to create a high-dimensional developmental map of all DRG cells throughout embryonic and early

postnatal development. This next generation single cell analysis recapitulates observations made via classic approaches (*i.e.* immunohistochemistry). Furthermore, graphical representation of the change in this high dimensional data set (FLOW-MAP) recapitulate lineage progression consistent with previous experiments, where TrkB+ and/or TrkC+ sensory neuron mature prior to TrkA+ sensory neuron populations. This foundational work provides the basis for future studies investigating how candidate proteins influence population dynamics. Collectively, these findings provide a conceptualization of how populations as diverse as sensory neurons with unique temporal and expression characteristics mature throughout development. Additional studies building upon these findings will elucidate how critical and multifunctional proteins, such as neurotrophins, differentially mediate development.

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Chapter 1. Introduction

1. Nervous System Refinement via Multiple Processes

Nervous system development in complex organisms employs a strategy of “trial and error”, which relies on a dynamic, tightly controlled balance between progression and regression of neuronal circuit components. This theme is seen during developmental processes such as neuron survival or death, synapse formation or restriction, and axon growth or degeneration. Dysregulation or imbalance between the cues for construction and destruction may have catastrophic consequences, which manifest as neurological disorders including Alzheimer’s disease and various pain maladies.

The central question of developmental neuroscience is how the nervous system is sculpted despite increasing complexity of body plans throughout evolution. The mature mammalian nervous system consists of billions of neurons communicating through trillions of synapses. The majority of this network forms during development and this network is strictly maintained throughout adulthood. Neurotrophins are a key mediator of neuronal growth during development and network maintenance during adulthood.

While self activation and lateral inhibition have been posited to be key regulators for virtually all natural pattern formation, the identity of these cues directing each opposing function must be defined (Meinhardt and Gierer, 1972). In the development of sensory neurons, stabilization and

destruction is largely mediated by extrinsic cues, such as neurotrophin signaling. However, the molecules governing the modulation and temporal control of such cues mediating patterning between peripheral nervous system (PNS) neuron subtypes are largely unknown. Herein we seek to determine the molecular and temporal rules governing the development of the somatosensory nervous system.

2. Peripheral Nervous System - A Model for Development

The maturation pathways of sensory neuron subsets are interesting avenues for further study to identify how neuronal precursors progressively differentiate specifically within the dorsal root ganglion (DRG). As such, a broader understanding of sensory neuron development will provide insights not only into basic mechanisms driving neuronal differentiation, maturation, and survival, but also into the etiologies and possible therapeutics for both somatosensory disorders, including peripheral neuropathy and hyperalgesia, and neuronal development beyond the PNS (Scholz and Woolf, 2007).

3. Sensory Neuron Subtypes and the Molecular Players Governing their Development

Somatosensory neurons of the vertebrate PNS convey body sensation information to the central nervous system and are therefore critical for the process of responding to the environment cues. Somatosensory neurons are psuedounipolar with cell bodies residing in DRG, tucked between the intervertebral formina of the mature spinal column (Frank and Sanes, 1991). These sensory neurons extend a (1) peripheral process that project to targets like skin and muscle, and (2) a central process the innervates the spinal cord to communicate environmental information to the brain (Frank and Sane, 1991) (Figure 1). DRG neurons represent diverse neuron subtypes, which transmit a variety of stimuli, including proprioceptive, mechanosensitive, and nociceptive neurons for limb position, touch, and pain information, respectively. Early characterization of sensory neuron populations were defined by the general stimuli they detect and gross electrophysiological properties.

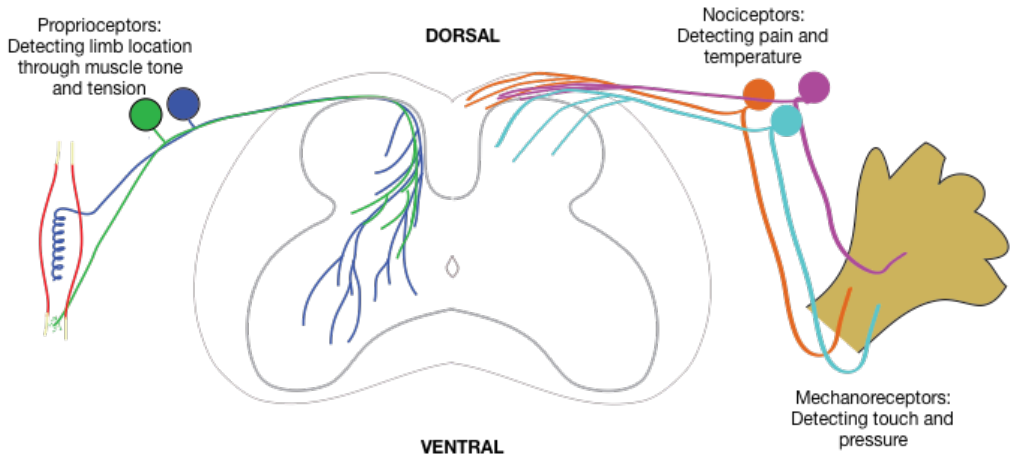

Figure 1. Schematic of neuronal innervation into peripheral and central targets by different sensory neuron subtypes.

Mechanosensitive and nociceptive neurons have cutaneous innervations peripherally and innervate superficial layers of the spinal cord. Proprioceptive neurons detect muscle tone and tension and transduce that information to motor neurons in the ventral horn of the spinal cord.

3.1. Proprioceptors

Proprioceptive neurons, a subset of somatosensory neurons, provide information for the position and movement of body parts, allowing for proper balance and coordination. Proprioceptors (proprioceptive neurons) are involved in the transmission of sensory information encoded by changes in their targeted end-organ, the muscles. Changes in muscle length and tension are measured by proprioceptors innervating the muscle spindles (MS) and Golgi tendon organ (GTO), respectively (Figure 1). These neurons are myelinated large diameter (Aα or Aβ) fibers and sense changes in muscle tone and tension through elegant peripheral innervation structure and the mechanosensitive channel Piezo (Woo et al., 2015). Neuropathies targeting proprioceptive neurons can come about from old age (presbypropria), cytotoxic agents, such as the pyridoxine and the chemotherapeutic drug cisplatin, or from spinal cord injury, resulting in ataxia (Dietz, 2002; Gao et al., 1995; Helgren et al., 1997).

3.2. Mechanoreceptors

Mechanoreceptive neurons provide information about touch and pressure through stretch-sensitive channels at axon terminals (reviewed in Woo et al., 2015) (Figure 1). Glaborous skin (non-hairy skin), are innervated by Aβ-LTMRS that reach specific exteroreceptive target organs, such as Merkel cells, detecting sustained touch, edges, and curvatures (Goodwin et al., 1995), Meissner Corpuscles, detecting low frequency vibrations

(Talbot et al., 1968), Pacinian Corpuscles, detecting high frequency vibrations (Talbot et al., 1968), and Ruffini endings, detecting skin stretch (Johansson and Vallbo, 1979; Johnson et al., 2000). In contrast, hairy skin is innervated by C-, Aβ-, and Aδ-LTMRS (reviewed in Abraira and Ginty, 2013). These specific axon terminals innervate specific terminal organs, such as touch tomes and guard hairs (reviewed in Abraira and Ginty, 2013). Neurons sensing different modalities of touch can vary by adaptation rate (rapid or slow), axon diameter, myelination, and protein expression (Abraira and Ginty, 2013). Improper mechanosensation can manifest as allodynia and is often comorbid with hypersensitivity to touch disorders, such as those found in the autism spectrum disorders (reviewed in Jenkins and Lumpkins, 2017).

3.3. Nociceptor/Thermoreceptors/Pruritoceptive

Nociceptive, thermoreceptive, and pruritoceptive neurons provides information about pain, temperature, and itch, respectively (Figure 1). While these populations transmit relatively disparate stimuli, these populations are derived from a similar lineage and may signal for one modality or several (i.e.multimodel) (Wang and Zylka, 2009). These neurons are predominantly lightly or non-myelinated (C fibers and Aδ fibers) transmitting information about temperature and/or pain (Abraira and Ginty, 2013). These pain, temperature, and/or itch-sensing neurons often transduce stimuli through receptors and channels responsive a wide array

of stimuli, such as chemical irritants, toxins, or mechanical stimuli. Thermoreceptive sensory neurons transduce stimuli *via* temperature-sensitive channels or chemical cues, such as menthol and capsacin (Abraira and Ginty, 2013). Improper development of these neurons manifests in hyper- or hyposensitivity to pain or temperature. Intriguingly, patients with Chronic insensitivity to pain and anhydrosis (CIPA) are insensitive to pain due to an absence of nociceptors (Indo, 1996).

4. Sequential Development from Progenitor to Fully Differentiated Neurons

In early development, the sensory lineage of neural crest cells migrates ventrally to form nascent DRGs. These neural progenitors proliferate into sensory-biased cells and subsequently begin early differentiation to sensory-committed cells. In mid development, sensory-committed cells begin to respond to extrinsic cues, such as neurotrophins, and begin to engage transcriptional programming for terminal differentiation. In late development, terminally differentiated sensory cells continue to refine axonal connections peripherally to targets and centrally to interneurons, corticospinal neurons, and cortical neurons (Figure 2) (de Nooij, Doobar, & Jessell, 2013; Kramer et al., 2006; Lallemend & Ernfors, 2012; Marmigère & Ernfors, 2007; Oliveira Fernandes & Tourtellotte, 2015).

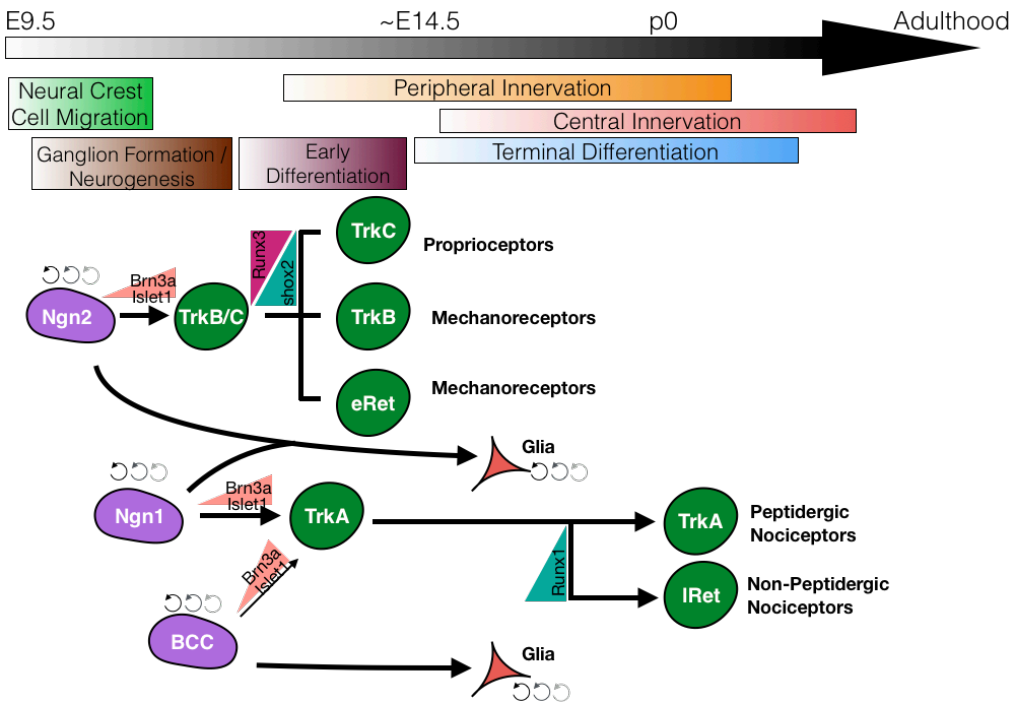

Figure 2. Schematic of development of dorsal root ganglion. Timeline denotes time points within mouse embryonic (E) or postnatal (P) timeline. Sensory neurons and glia arise from three consecutive waves of migrating progenitors, derived from neural crest cells. First from Ngn2+ progenitors, then Ngn1+ progenitors, and ending with boundary cap cell (BCCs) progenitors. Progenitors upregulate Brn3a and Islet1 as they differentiate into immature neurons. Ngn2+ progenitors migrate to the ganglia and differentiate into TrkB+ mechanoreceptors, TrkC+ proprioceptors, and eRet neurons mechanoreceptors. This differentiation is mediated by the transcription factor Runx3 and Shox2. Ngn1+ and BCC progenitors migrate to the ganglia and differentiate into immature TrkA+ neurons, which terminally differentiate into TrkA+ peptidergic

nociceptors and Ret+ non-peptidergic nociceptors (lRet). This differentiation is mediated by the transcription factor Runx1.

4.1. Waves of Migration and Proliferation

Neural crest cells migrate laterally and coalesce to form the DRG. Progenitors continue to migrate and colonize the DRG in mid-embryonic development (reviewed in Marmigere and Ernfors, 2007). During delamination and migration, external cues such as Wnt and β-catenin are produced in the dorsal neural tube which induce expression of the transcription factors Sox10 in sensory-destined neurons, and repression of Mash1 for cells destined for the autonomic lineage (Lo et al., 1998; Carney et al., 2006).

Interestingly, there are multiple waves of migration, and each one contains progenitors fated to become different neuronal subtypes (Lawson and Biscoe, 1979) (Figure 2). The first wave of multipotent progenitors, expressing the neurogenic transcription factor Neurogenin-2 (Ngn2), migrates at E9.5-11.5 in mouse development (Ma et al., 1999). Ngn2+ progenitors preferentially generate large diameter proprioceptors and mechanoreceptors (Ma et al., 1999). The second wave of progenitors, expressing Neurogenin-1 (Ngn1), occurs between E10.5-E13.5 and preferentially generate small diameter TrkA+ sensory neurons (Marmigere and Carroll, 2014). A third smaller wave of migrating progenitors comes from boundary cap cells, a population of progenitors that sits at the intersection of the spinal cord and the peripheral (Dorsal Root Entry Zone (DREZ) and Motor Entry Point (MEP) (Maro et al,. 2004). This population

migrates starting at E11.5 and generates a small population of small diameter nociceptors, as well as the majority of satellite glial cells and Schwann cells (Aquino et al., 2006; Hjerling-Leffler et al., 2005; Maro et al., 2004). The relative proportions of neurons contributed by each population are reviewed in Marmigere and Ernfors, 2007.

4.2. Proneural Gene Expression is Induced Following Migration

As cells mature into immature neurons, the proneural homeobox class transcription factor Islet1 and Brn3a are induced upon intermediate differentiation from neural crest cells to post-mitotic sensory neurons (Sun et al., 2008; Lanier et al., 2009) (Figure 2). Upon differentiation from neuroprogenitor to immature neurons, sensory neurons begin to express distinct transcriptional profiles, including a unique profiles of neurotrophin receptors (Farinas et al., 1998). However, it is unknown how much of this differentiation is dependent on intrinsic transcriptional programming versus extrinsic cues, such as morphogens, to trigger induction of transcription programming.

Progressive differentiation of immature post-mitotic neurons into terminally distinct subsets occurs between E11 and p28 in mouse development. As alluded to, a defining characteristic of sensory neuron subsets is the neurotrophin receptor they express; the expression of these receptors reflect intrinsic differences between sensory neurons and provide a

platform for extrinsic cues, such as neurotrophins, to dictate differentiation. Furthermore, regulations of neurotrophin receptors is a both a regulator and byproduct of transcription programming (Deppmann et al., 2008). As neurons continue to mature, the transcriptional profile of neurotrophin expression becomes more restricted (Kramer et al., 2006; Farinas et al., 1998). For example, at E11.5 TrkB and TrkC colocalization decreases from ~30% to ~18% between E11 and E12 (Kramer et al., 2006). The progressive restriction of neurotrophin receptor expression may result from positive feedback from abundant cognate neurotrophin signaling and transcriptional repression of the less abundant neurotrophin receptors (Kuruvilla et al., 2004; Deppmann et al., 2008; Abdo et al., 2011).

4.3. TrkB/C Lineage

The first wave of migrating neurogenic progenitors are marked by the transcription factor Neurogenin-2 (Ngn2) (Ma et al., 1999). Between E8.5-E10.5 in mouse development, Ngn2+ neural progenitors primarily differentiate into neuronal subsets destined to become TrkB+ mechanoreceptors, TrkC+ proprioceptors, and Ret+ mechanoreceptors in the mature animal (Ma et al., 1999). Neurogenin-2 progenitors integrate extrinsic and intrinsic cues to mediate progressive segregation of sensory neuron subtypes. The critical transcriptional regulator Runx3 represses TrkB expression and maintains TrkC expression (Kramer et al., 2006). In addition, Runx3 is required for further differentiation of TrkC+ cells to

upregulate other proprioceptive markers, such as parvalbumin (Inoue et al., 2002; Levanon et al., 2002; Kramer et al., 2006). In contrast, the transcription factor Shox2 repressed TrkC expression and maintains TrkB expression (Abdo et al., 2011).

The Ret+ population derived from neurogenin-2 progenitor pools are a minor subset of Ret-expressing neurons in the mature animal (Luo et al., 2009). These early-arising Ret+ populations, termed early (e)Ret, are transcriptionally distinct from the later-arising Ret+ populations, termed late (l)Ret, primarily through the transcription factors MafA and cMaf (Bourance et al., 2008; Luo et al., 2009; Hu et al., 2012; Wende et al., 2012).

4.4. TrkA lineage

Multiple waves of TrkA are generated during development, creating a multitude of terminally differentiated neurons. A minor population of TrkA+ neurons are derived from the first migrating progenitor population (marked by Ngn2) at E10.5 (Bachy et al., 2011). This first TrkA+ population arising in the DRG is TrkA+;Cux2+ and develop into high threshold and lightly myelinated A-delta nociceptors (Bachy et al., 2011). Within a day in mouse embryonic development, the second progenitor population (marked by Ngn1) generates the majority of the unmyelinated or lightly myelinated TrkA+ lineages (Ma et al., 1999). This progressively starts at E9.5 in

mouse development (Ma et al., 1999). A majority (~90%) of immature neurons derived from this progenitor population express TrkA (Marmiegere and Ernfors, 2007). A third progenitor neural crest-derived population known as boundary cap cells also generate a small pool of TrkA+ neurons (Maro et al., 2004).

Extensive transcription programming diversifies the TrkA lineage of somatosensory neurons (Kramer et al., 2006; Huang et al., 2015). Single cell RNA sequencing advances paved the way to transcriptionally detect a number of small TrkA+ populations in the mature DRG (Usoskin et al., 2015; Li et al., 2016; Chiu et al., 2015; reviewed in Li et al., 2018). Embryonic TrkA+ neurons differentiate into populations of heat-, cold-, itch-, and mechanical pain-sensing neuronal subsets (reviewed in Lallemend and Ernfors, 2012). Further work has identified the transcriptional programming that differentiates these population (Hadjab et al., 2013; reviewed in Marimiegere and Ernfors, 2007).

Embryonic TrkA+ neurons segregate into those that maintain TrkA-expression and those that downregulate TrkA to express the neurotrophin receptor Ret, termed late (l) Ret (Molliver et al., 1997). Those neurons that maintain TrkA+ expression develop into peptidergic nociceptors, expressing peptidergic proteins such as CGRP or Substance P. In contrast, TrkA+ neurons that downregulate TrkA and coincidentally

upregulate Ret are non-peptidergic nociceptors (Luo et al., 2007; Luo et al., 2009; Golden et al., 2010). The transcription factor Runx1 is a master regulator of transcriptional programming in differentiating immature TrkA+ neurons (Huang et al., 2015; Yang et al., 2013). Runx1 expression is nearly ubiquitous in TrkA+ neurons initially, but is progressively restricted starting at E15.5 and into postnatal development (Abdel Samad et al., 2010; Molliver et al., 1997). Animals lacking Runx1-/- have an impaired segregation between TrkA+ peptidgeric nociceptors and Ret+ nonpeptidergic nociceptors, resulting in an increase in TrkA+ neurons with a coincident decrease in Ret+ neurons (Chen et al., 2006; Kramer et al., 2006, Abdel Samad et al., 2010). In peptidergic nociceptors, FGF-Met receptor activation extinguishes Runx1 expression and permits upregulation of CGRP and Substance P (Gascon et al., 2010). In contrast, NGF-TrkA activation in non-peptidergic nociceptors mediates upregulation of components of the transcription factor complex Runx1 and CBFβ, and induction of non-peptidergic markers, such as Mas-related G protein-coupled receptors, MrgprB4 and MrgprD (Luo et al., 2009). MrgprB4 and MrgprD expressing non-peptidergic neurons are implicated in transducing pleasant and light touch (Luo et al., 2009; Abraira and Ginty, 2013).

4.5. Further Differentiation after Final Target Innervation

While the trajectory of neural progenitors appears stereotyped, there remains a significant amount of compensatory mechanisms and plasticity

in developmental differentiation. For example, each migratory wave preferentially generates functionally disparate sensory neuron populations, but these progenitor populations can compensate for one another. In the absence of the neurogenic transcription factor Ngn2, there is an initial loss of the large diameter TrkC+ proprioceptors and TrkB+ mechanoreceptors, but development appeared normal by E15 (Ma et al., 1999). However, all populations are lost in the absence of Ngn2 and Ngn1, suggesting that Ngn1 may compensate for loss of Ngn2 (Ma et al., 1999). Additionally, extrinsic cues, such as neurotrophins can instruct neuronal fate. Patapoutian and colleagues demonstrated that in mice when TrkC is expressed from the TrkA locus cells fated to become nociceptive shift to proprioceptive-like neurons, characterized by an increase in the proprioceptive marker parvalbumin and innervation into proprioceptive terminal fields, such as muscle and ventral spinal cord (Moqrich et al., 2004).

4.6. Glial development

The glia associated with the somatosensory system include (1) satellite glia cells, (2) myelinating Schwann cells, and (3) non-myelinating Schwann cells. Glia populations in the DRG arise from neural crest-derived and boundary cap cell progenitors and continue to proliferate throughout adulthood (White and Anderson, 1999; Hjerling-Leffler et al., 2005; Maro et al., 2004). Satellite glial cells (SGCs) are the predominant

glial type in peripheral sensory and sympathetic ganglia (Hanani, 2005). Satellite glial cells wrap around the neuronal cell body within the ganglia (Wu et al., 2009; Hanani, 2005; Reviewed in Jessen and Mirsky, 2005). Functionally, they are understudied but they have been shown to scavenge and engulf debris from neighboring dying cells, such as neurons, during development (Wu et al., 2009; reviewed in Hanani, 2010). Additionally, SGCs couple adjacent somatosensory neurons to unify local activation (Kim et al., 2016).

In contrast, both myelinating and non-myelinating Schwann cells are associated with sensory neuron axons. Myelinating Schwann cells myelinate a subset of sensory axons contributing to increased conductance for those axons (Waxman, 1980). Non-myelinating Schwann cells (NMSC) form Remak bundles consisting of multiple small-diameter axons and contribute to the microenvironment by providing structural protection and act as first-responders to injury (reviewed in Griffin and Thompson, 2008).

5. Extracellular Cues Mediate Cell Death in Development

In parallel with the aforementioned developmental processes, massive cell death occurs during early and mid sensory neuron development (Figure 3). In order to establish a more precise neural circuit, cells--and even axons and synapses of sensory neurons--are overproduced and then

culled due to a lack of a constructive trophic signal paired with an increased destructive signal (Bamji et al., 1998; Deppmann et al., 2008; Hamburger and Levi-Montalcini, 1949; Majdan, 2001; Majdan et al., 1997; Oppenheim, 1991; Sharma et al., 2010; Singh et al., 2008).

Victor Hamburger was able to enhance or dampen the total number of peripheral neurons innervating the limbs of chick embryos via limb bud transplantation or ablation, respectively. Chicks with supernumerary wing buds developed an increased number of neurons, while chicks with ablated wing buds showed increased death of brachial spinal motor neurons (Hamburger, 1934). This work provided the first hints that neurons are overproduced during development and target tissues, such as the chick limb bud, can control the innervation it receives (target-matching). Furthermore, loss of central and peripheral connections *via* dorsal rhizotomy (nerve cutting) in newborn rats resulted in a 50% loss of DRG sensory neurons (Yip and Johnson, 1984).

Since peripheral targets are able to drive the magnitude of neuronal innervation, Hamburger and Rita Levi-Montalcini postulated that target tissues secrete a limited amount of an unknown trophic factor. The overproduced neurons would then compete for this limited supply of trophic factor for survival (Neurotrophic Factor Hypothesis) (Levi-Montalcini, 1987) (Figure 3). While it may seem counterintuitive for the

nervous system to eliminate such a substantial subset of newly developed neurons, naturally occurring cell death is critical since it is unlikely that the complex mammalian nervous system relies on a predestined fate for each neuron in the body. Instead, neuronal development is likely governed by a series of rules to tune the nervous system. Hamburger and Montalcini's neurotrophic factor hypothesis was particularly prescient because the notion of final targets mediating nervous system development is now emerging as a common theme that applies to processes well beyond survival/death decisions (i.e. synapse formation/restriction and axon branching/elimination) (Sharma et al., 2010; Singh et al., 2008).

Rita Levi-Montalcini and biochemist Stanley Cohen identified and purified the nerve growth factor (NGF), the first identified member of the neurotrophin family (Cohen & Levi-Montalcini, 1956). Nearly three decades after NGF was discovered, brain-derived neurotrophic factor (BDNF) was identified and found to trophically support several populations of neurons within the peripheral nervous system that were not dependent on NGF for survival (Barde et al., 1982). Additionally, following analysis of nucleotide sequence homology between NGF and BDNF, neurotrophin-3 (NT-3) (Hohn et al., 1990) and neurotrophin-4/5 (NT-4/5) (Berkemeier et al., 1991) were discovered to be structurally related. These four growth factors define the neurotrophin family. However, it is important to note that outside of the classical family of neurotrophins, there are additional factors

that provide target-derived trophic support to neurons, including the glial-derived neurotrophic factors- family ligands (GFL), such as glial-derived neurotrophic factor (GDNF), Neurturin (NTN), Artemin (ARTN), and Persephin (PSPN) (reviewed in (Airaksinen & Saarma, 2002) (Figure 4). Since their initial discovery, neurotrophins have been a persistent field of study to provide insight into nervous system development and adult neuroplasticity.

Nervous system development in complex organisms employs a strategy of “trial and error”, whereby components (e.g. synapses, axons, neurons) are overproduced and a competition ensues, which sculpts these components into a functional circuit (Figure 3). This competition relies on a dynamic and tightly controlled balance between stabilization and destruction of neuronal circuit components. Signals governing stabilization or destruction tune several developmental processes starting with neural progenitor proliferation and spanning neuronal differentiation, survival, death, and axon growth (Oppenheim, 1991). An imbalance between these processes may lead to aberrant tuning (Liu et al., 2014).

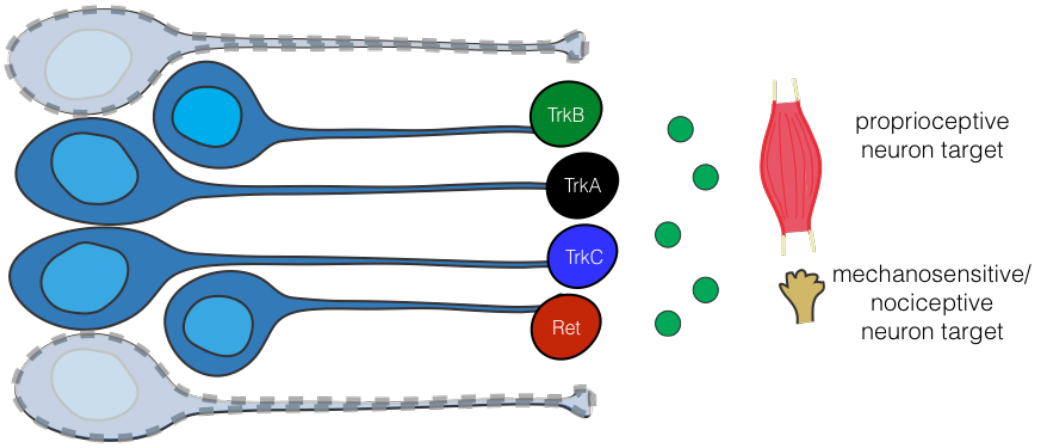

Figure 3. Schematic of neurotrophin dependence and competition for survival. Sensory neurons express a variety of receptor tyrosine kinases (TrkA, TrkB, TrkC, and Ret), which compete for a limiting amount of target-derived neurotrophin for survival.

6. Neurotrophin and Neurotrophin Receptor Families

The neurotrophins are a well-characterized phylogenetically conserved family of polypeptide growth factors, with members expressed in zebrafish and lampreys (Hallböök, 1999). The early chordate Amphioxus expresses a protoTrk (AmphiTrk) gene that likely emerged prior to the cephalochordate/vertebrate split. Interestingly, AmphiTrk interacts with mammalian neurotrophins to promote survival and differentiation (Benito-Gutierrez, 2005).

During development, DRG neuron subtypes are differentially responsive to target derived neurotrophic ligands, which can mediate long-distance survival signaling (Ernfors et al, 1994; Luo et al., 2007; Moqrich et al., 2004). Each neurotrophin selectively with binds with highest affinity to one member of the Trk family - NGF binds with highest affinity to TrkA, BDNF and NT-4/5 to TrkB, and NT-3 to TrkC (Reviewed in Skaper, 2012) (Figure 4). Similarly, the Ret receptor tyrosine kinase forms a coreceptor with a glycosyl phosphatidyl inositol (GPI)-linked binding subunit, including GFRα1, GFRα2, GFRα3, and GFRα4, to bind a number of neurotrophic factors within the GDNF family of ligands, GDNF, neurturin, artemin and persephin, respectively (reviewed in Lallemend and Ernfors, 2012).

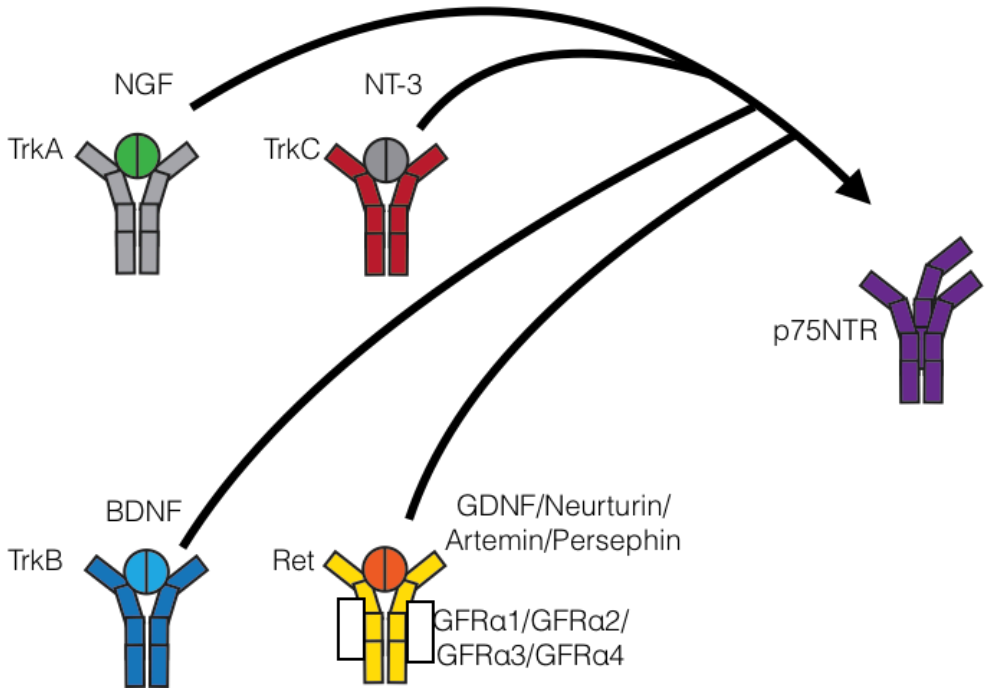

Figure 4. Neurotrophin receptors and corresponding potential ligands. Neurotrophins (NGF, NT-3, BDNF, GDNF, Neurturin, Artemin, and Persephin) binding to the receptor tyrosine kinases (TrkA, TrkB, TrkC, and Ret - with the co-receptors GFRα1/GFRα2/GFRα3/GFRα4) or the TNFRsf member p75NTR.

7. Source of Neurotrophins

Neurotrophic factors have been shown to influence development, maintenance, and disease in both autocrine and paracrine manners (Huang and Reichardt, 2001). Throughout development, neurotrophins are available en route to targets (Kuruvilla et al., 2004), at the final target (Kuruvilla et al., 2004), and are secreted by DRG SGCs and neurons (Mannion et al., 1999; reviewed in Huang and Reichardt, 2001). In adulthood and disease contexts, neurotrophins are released during injury and inflammation (Huang and Reichardt, 2001). The temporal control and localization of neurotrophin release dictates downstream function (Huang and Reichardt, 2001).

8. Target-derived neurotrophins mediate survival

Neurotrophins bind with high affinity to members of the tropomyosin receptor kinase (Trk) family to promote survival and other developmental processes. Studies injecting antibody against neurotrophins into peripheral targets and limb bud ablation studies result in drastic loss of neurons, providing the first insight into the critical role of target derived trophic factors in survival (Huang and Reichardt, 2001). Neurotrophin dependence varies between neuronal populations, an effect that is largely determined by RTK receptor expression within the population (Farinas et al., 1998).

The portion of neurons typically expressing a given neurotrophic receptor are lost in corresponding genetic mutants (NGF - Crowley et al., 1994 TrkA - Smeyne et al., 1994 NT-3 - Ernfors et al., 1994 TrkC - Tessarollo et al., 1994 BDNF - Klein et al., 1993 TrkB - Jones et al., 1994 GFL and GFRalpha - Ernsberger, 2008, Lindfors et al., 2006 CKO Ret - Franck et al., 2011; Golden et al., 2010; Luo et al., 2007). Neurotrophin signaling through neurotrophic receptors are critical in the development of both sensory progenitor and terminally differentiated populations. For example, mice harboring null alleles of *NGF* displayed a loss of TrkA+ neurons (Patel et al., 2000), whereas mice harboring null alleles for *NT-3* revealed (1) a loss of TrkC+ neurons and (2) premature differentiation to sensory neurons and a subsequent loss of proliferating progenitors (Farinas et al., 1996). This suggests that NGF is solely required in terminally differentiated TrkA+ neurons, whereas NT-3 is critical at multiple stages in sensory system development (Farinas et al., 1996).

Naturally occurring cell death in sensory neurons occurs predominantly between E12.5 and E14.5 in mouse development, and repression of naturally occurring apoptosis results in excessive neuronal number postnatally (Cheng et al., 2018; Suzuki et al., 2010). Apoptosis occurs in all neuronal subsets but the relative amount of death and the temporal pattern of apoptosis reflects the temporal pattern of migration and

differentiation, where TrkB+ and TrkC+ neurons complete developmental cell death prior to TrkA+ neurons (Cheng et al., 2018; Suzuki et al., 2010).

Neurotrophin-RTK signaling is most often studied in the sympathetic and sensory nervous system, where neuronal cell bodies reside in ganglion near the spinal column with axons projecting distances ranging from millimeters to meters depending on the target. In these peripheral neurons, neurotrophin-RTK interactions primarily occur on the axon terminals, where they receive neurotrophins secreted by target tissues. Following RTK activation on the axon terminals, this growth signal can either phosphorylate effector proteins locally to promote a spatially precise axon growth process or travel back to the cell bodies via retrogradely-traveling endosomes to induce transcription associated with growth (Harrington & Ginty, 2013).

Neuronal survival is critically dependent on the three primary signaling pathways (PI3K-Akt, PLCγ, and Ras-MAPK - described below) emanating from neurotrophin activation. The majority of the cell biological pathway in neurotrophin signaling stems from the sympathetic nervous system model, where these neurons are also dependent on NGF for survival. Minimally, for sympathetic neuron survival, these signals must direct the retrograde transport and prevent degradation of the signaling endosome, inactivate

apoptotic proteins, and increase expression of pro-survival proteins (Bodmer et al., 2011; Datta et al., 1997; Riccio, 1997; Barford et al., 2017).

9. Local and long distance cues cues mediate axon innervation

The neurotrophic factor hypothesis may represent a generalizable mechanism for how a circuit is tuned to the needs of the target, with the final target regulating several other neurodevelopmental events beyond survival (e.g. synapse formation, branching, axon growth). Neurotrophins can induce multiple growth processes through a number of downstream signals and through diverse trafficking patterns (Barford et al., 2018).

9.1. Axon Growth is mediated by neurotrophins and other extrinsic cues

Sensory neurons of different modalities develop unique innervation patterns with terminal structures specialized to sense different environmental stimuli, and and convey that information to different regions of the central nervous system.

How does this innervation occur in development? And how do different neurons define where to terminate (1) at different lamina within the spinal cord to construct appropriate post-synaptic connections, and (2) with vastly different peripheral targets with morphologically distinct target organs? Correct innervation patterning is mediated by an ensemble of

cues, including contact mediated inhibition, neurotrophins, and other trophic cues (Lallemend and Ernfors, 2012).

Neurotrophins have pleiotropic functions beyond survival/death decisions include axon growth. Axon growth in DRG neurons is enhanced in a neurotrophin dose-dependent manner *in vivo* (NGF - Marckus et al., 2002; Newbern et al., 2011; BDNF - Hory-Lee et al., 1993; NT-3 - Hory-Lee et al., 1993; GDNF - Paveliev et al., 2004). Mice lacking the function gene for the pro-apoptotic protein *Bax* do not undergo apoptosis; researchers use these transgenics to investigate the roles of neurotrophins in growth beyond survival (Deckwerth et al., 1996; Suzuki et al., 2010). In these instances, peripheral sensory innervation is halted at the border between intermediate (i.e. blood vessels) and final target (i.e. skin) and central projections do not penetrate into the appropriate lamina (TrkC-/-; Bax-/- - Patel et al., 2003; TrkA-/-; Bax-/- - Patel et al., 2000; Ret-/-; Bax-/- -Honma et al., 2010). These studies demonstrate the critical role of neurotrophin signaling *in vivo* in axon growth and final target-innervation (reviewed in Lallemend and Ernfors, 2012).

9.2. Central Innervation

Sensory neuron subtypes terminate to unique locations in the spinal cord. The most superficial lamina (laminae I and II) of the spinal cord are

innervated by nociceptors and thermoreceptors, when deeper lamina are innervation by proprioceptive neurons which can directly synapse onto motor neurons in the ventral horn (Lallemend and Enfors, 2012) (Figure 1).

What allows for different populations to terminate in restricted lamina in the spinal cord? Neurotrophin signaling is critical for mediating central innervation. For example, *NT-3*$^{-/-}$; *Bax*$^{-/-}$ results in lack of innervation in the spinal cord beyond the intermediate zone, resulting in a lack of synaptic connections between sensory and motor neurons in the ventral horn (Patel et al., 2003). However, this innervation deficit can be rescued with by overexpressing NT-3 in muscle (Wright et al., 1997). In contrast, motor neuron-derived neurotrophins are not sufficient to drive central innervation, suggesting long-distance peripheral target-derived, but not locally motor neuron-derived cues mediate proper central spinal cord innervation (Patel et al., 2000; Patel et al., 2003).

Chemorepellent and chemoattractant cues differ in expression over time and this allows for progressive innervation with each sensory neuron subset (reviewed in Marmigere and Ernfors, 2007). Sema3A is a chemorepellent and causes growth cone collapse in the Nrp1-high expressing TrkA neurons and not in Nrp1-low expressing TrkC proprioceptive neurons (Fu et al., 2000). Sema3A is selectively expressed

in the ventral spinal cord (Fu et al., 2000). This region that is enriched for motor neurons repels innervation by TrkA+ nociceptors and thermoreceptors, but do not repel TrkC+ proprioceptors destined to terminate onto these motor neurons (Fu et al., 2000).

9.3. Peripheral Projections

Peripheral branch axons innervate the forelimb by E10.5, and reach targets like skin at E13.5 (Berg and Farel, 2000). Sensory neuron axons terminal endings possess function-fitting morphology, where proprioceptive neurons develop coil-like structures around intrafusal muscle fibers to detect changes in muscle tone and mechanoreceptors develop tight connections with hair cells to detect mild deflections due to hair movement (Abraira and Ginty, 2012). How does this innervation pattern form during development? Initial sensory system differentiation precedes final target innervation, suggesting that sensory neuron differentiation is not strictly defined by target-derived trophic cues (Lallemend and Ernfors, 2012).

Neurons and peripheral targets communicate during development to create a mature functional circuit. For example, neurotrophin signaling is required to develop peripheral structures; muscle spindle formation is contingent on proper proprioceptive neuron innervation (Patel et al., 2003). Whereas mechanosensitive Merkel cell secrete BDNF by E16.5

and this target-derived BDNF in hair follicles may regulate polarity in hair cells (Rutlin et al., 2014). Transcription factors downstream of target-derived cues can also mediate innervation patterns (Yang et al., 2013). In nociceptive neurons, Runx1 is required for nociceptive superficial epidermal innervation, but not deep tissue peripheral innervation, such as dermis, muscle, bone, and visceral organs (Yang et al., 2013). Therefore, these target-derived neurotrophins and transcriptional cascades carefully interact during peripheral target innervation.

10. Neurotrophin-Trk Signaling

10.1. Long distance signaling

Target-derived neurotrophins are retrogradely trafficked from distal tips of axon to the cell body to induce transcriptional changes (reviewed in Harrington and Ginty, 2013). Target derived neurotrophin signaling mediates long distance effects through a signaling endosome that consists of an endocytosed ligand/receptor complex (Kuruvilla et al., 2004). The neurotrophin retrograde signaling pathway is most extensively elucidated in the sympathetic nervous system, where postganglionic sympathetic neurons are uniformly dependent on target-derived NGF-TrkA signaling for survival (reviewed in Harrington and Ginty, 2013 and Ascano et al., 2012). In the sympathetic nervous system, the high affinity NGF-TrkA complex must be retrogradely transported back to the cell body to promote neuronal survival (Kuruvilla et al., 2004). Coronin-1 is induced upon final

target innervation and stabilizes the NGF-TrkA signaling endosome to prevent lysosomal degradation (Suo et al., 2014). Interestingly, experiments using radiolabelled neurotrophic factors reveal that neurons are selectively in which neurons they take up and traffic retrogradely; this lays the foundation for differential neurotrophin responsiveness for each sensory neuron subtype (DiStefano et al., 1992).

10.2 Signaling Cascades

There are **three major signaling cascades** that are highly enhanced following neurotrophin exposure: (1) PI3K-Akt, (2) PLCγ, and (3) Ras-MAPK. These pathways are generally associated with receptor tyrosine kinase activation and contain multiple points of crosstalk (Reichardt, 2006). These pathways represent branch points where functional outputs are partitioned. In the following sections, I will describe how functions diverge between these signaling pathways.

10.2.1. PI3K - Akt

PI3K signaling is necessary for initiation of retrograde transport of the NGF-TrkA signaling endosome and is critical for NGF-dependent survival of sympathetic neurons (Kuruvilla et al., 2000). Additionally, within the sensory system, NGF locally activates PI3K pathway to inhibit GSK3b, which allows APC, a microtubule plus-end binding proteins, to promote axon growth (Zhou et al., 2004). Interestingly, PI3K inhibitor injections into

the eye, a target of the sympathetic nervous system, attenuate retrograde transport of radiolabelled ^{125}NGF in sympathetic neurons, but not sensory neurons (Bartlett et al., 1997).

10.2.2. PLCγ

In the sympathetic nervous system, NGF-TrkA endocytosis leads to PLCγ–dependent activation of calcineurin, which in turn, dephosphorylates the endocytic GTPase dynamin1 (Bodmer et al., 2011). Activation of dynamin1 further upregulates NGF-TrkA endocytosis to promote survival (Bodmer et al., 2011). Additionally, PLCγ-dependent signaling promotes synaptic plasticity and alters transcription following BDNF-TrkB signaling in the hippocampus (Minichiello et al., 2002).

10.2.3. Ras-MAPK

Ras-MAPK activation promotes growth and differentiation by inducing the phosphorylation of the transcription factor cAMP response element binding protein (CREB) and the anti-apoptotic family of proteins Bcl-2 (Kaplan & Miller, 2000; Riccio, 1997). The mode of Ras-MAPK pathway activation following NGF exposure can vary depending on the locale of activation on neurons. For example, NGF exposure to the cell body compartment will activate protein serine/threonine kinases Erk 1, 2, and Erk 5. However, local exposure of NGF to only the distal axon compartment selectively activates Erk5, suggesting that the locale of

NGF-stimulation can dictate the combination of MAPK pathway activation (Watson et al., 2001). Interestingly, mice lacking *Erk5* did not have notable embryonic defects (Newbern et al., 2011). In contrast, mice lacking *Erk1* and *Erk2* have defects in sensory neuron survival, axon fasciculation, and cutaneous innervation *in vivo* (Newbern et al., 2011). The variability of unique MAPK pathway is critical because each MAP kinases can phosphorylate unique substrates (Heerssen & Segal, 2002).

11. Death Signaling

When peripheral neurons are deprived of neurotrophins, the cells initiate a process of cell death dependent on protein synthesis and an activation cascade of cysteine-dependent aspartyl-specific proteases (caspases) (Deckwerth et al., 1996; Martin et al., 1988; McCarthy et al., 1997). More specifically, in the absence of neurotrophins, neurons activate c-Jun N-terminal kinase (JNK) and c-Jun to upregulate expression of pro-apoptotic BH3 members. BH3 family members activate the Bcl-2 family member Bax, which permeabilizes the mitochondrial outer membrane to cytochrome-C. Cytosolic cytochrome-C assembles with Apaf-1 and caspase-9 to form the apoptosome complex, which in turn cleaves and activates caspase-3 to initiate an effector caspase cascade (Wu, 2013). Interestingly, one study found that forced premature expression of neurotrophin receptors in a period preceding neurotrophin expression resulted in increased cell death (Nikoletopoulou et al., 2010). This suggest

that the Trk receptors may function as dependence receptors, where in the presence of their respective cognate ligand, they will signal survival (Nikoletopoulou et al., 2010). However, in the absence of such ligands, death cues are transduced instead (Nikoletopoulou et al., 2010; Tauszig-Delamasure et al., 2007). This theory is still controversial and has also not yet been shown to be relevant in development *in vivo*.

12. P75 Neurotrophin Receptor

P75 neurotrophin receptor (p75NTR) is a member of the TNFR superfamily and is expressed throughout the nervous system, namely the peripheral nervous system, in sympathetic, motor, and sensory neurons (Kaplan & Miller, 2000). p75NTR has a been implicated in mediating neurotrophin signaling activity and survival in both the sympathetic and sensory nervous system (Lee et al., 1992; Lee et al., 1994).

P75NTR can bind both immature pro-neurotrophins and mature neurotrophins (Barker, 2007). The binding affinity of p75NTR toward specific ligands is defined by the availability of other higher-affinity receptors for the ligand in question (Barker, 1998).

Animals lacking p75NTR have significant loss of sensory neurons, increased developmental sensory neuron death, and cutaneous hypoinnervation (Lee et al., 1992; Lee et al., 1994; Yamashita et al., 1999;

Bergmann et al., 1997; Vaegter et al., 2011). As a result of decreased sensory neurons, mice lacking *p75NTR* also exhibit behavioral deficits, such as hyposensitivity to noxious temperatures and locomotor deficits (Lee et al., 1992; Vaeger et al., 2010). The contribution of p75NTR in sensory system development could occur at multiple points in development. *In vitro* studies also identified that NGF-dependent survival and TrkA activation is perturbed in *p75NTR*$^{-/-}$ (Barrett and Barlett, 1994; Davies et al., 1993; Lee et al., 1994). Animals lacking p75NTR also demonstrate increased apoptosis in post-mitotic DRG neurons during normal periods of developmental cell death, suggesting the role of p75NTR is to temper the magnitude but not temporal window of developmental cell death (Cheng et al., 2018). However, conditional excision of p75NTR in post-mitotic (Islet1+) neurons results in selective loss of lRet neurons, which suggests non-cell autonomous roles of p75NTR in maintaining neuronal survival (Chen et al., 2017).

The mechanism underlying p75NTR-dependent cell survival has been elusive. However, three prevailing theories exist (1) p75NTR and Trk family members form a high affinity pocket for neurotrophins (Hempstead et al., 1991), (2) p75NTR sequesters neurotrophin ligand for neighboring neurotrophin receptors, thereby enhancing neurotrophin signaling (Barker 2007), and (3) p75NTR promotes cell surface localization of cognate neurotrophin receptors (Chen et al., 2017). However, the p75NTR crystal

structures argues against a high affinity pocket formed by a TrkA-p75NTR tetramer and instead suggest a ternary heterocomplex, where a NGF dimer drives a head:head heterocomplex with TrkA and p75NTR (Covaceuszach et al., 2015; Wehrman et al., 2007). Extensive work has been done to elucidate the role of p75NTR in sensory neurons by examining the biochemical pathways influenced by loss or activation of p75NTR. In sensory neurons, p75NTR prolongs Trk receptor activation and is required for NF-kB activation by neurotrophins (Hempstead et al., 1991; Barker and Shooter, 1994, Hantzopoulos et al., 1994; Makkerh et al., 2005; Hamanoue et al., 1999).

p75NTR has also been implicated in contributing to vastly different processes in different cell types, include survival in Schwann cells (Gentry et al., 2004; von Schack et al., 2001), death in sympathetic neurons (Deppmann et al., 2008; Bamji et al., 1998; Bertrand et al., 2008), myelination in Schwann cells (Cosgaya et al., 2002), neurogenesis in subventricular zone (Young et al., 2007), avoiding myelinated areas in neurons (Park et al., 2010). and in restriction to regions within the central nervous system, such as the cortex, cerebellum, hippocampus, basal forebrain, and caudate putamen (Kaplan & Miller, 2000). This question is further complicated by the potential for the multi-faceted nature of a single protein. Could these dual-responding receptors be a source of diversity within the sensory nervous system?

Herein, my work seeks to identify key regulators of sensory neuron diversification. I seek to identify the role of p75NTR in developmental cell death (Chapter II), and to create a comprehensive map of development using high-dimensional single cell analysis (Chapter III).

Chapter II. Temporally Restricted Death and the Role of p75NTR as a Survival Receptor in the Developing Sensory Nervous System

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ABSTRACT

The peripheral somatosensory system overproduces neurons early in development followed by a period of cell death during final target innervation. The decision to survive or die in somatosensory neurons of the dorsal root ganglion (DRG) is mediated by target derived neurotrophic factors and their cognate receptors. Subsets of peripheral somatosensory neurons can be crudely defined by the neurotrophic receptors that they express: peptidergic nociceptors (TrkA+), non-peptidergic nociceptors (Ret+), mechanoreceptors (Ret+ or TrkB+), and proprioceptors (TrkC+). A direct comparison of early developmental timing between these subsets has not been performed. Here we characterized the accumulation and death of TrkA, B, C, and Ret+ neurons in the DRG as a function of developmental time. We find that TrkB, TrkC, and Ret-expressing neurons in the DRG complete developmental cell death prior to TrkA-expressing neurons. Given the broadly defined roles of the neurotrophin receptor p75NTR in augmenting neurotrophic signaling in sensory neurons, we investigated its role in supporting the survival of these distinct subpopulations. We find that TrkA+, TrkB+, and TrkC+ sensory neuron subpopulations require p75NTR for survival, but proliferating progenitors do not. These data demonstrate how diverging sensory neurons undergo successive waves of cell death and how p75NTR represses the magnitude, but not developmental window of this culling.

MAIN TEXT

INTRODUCTION

Somatosensory neurons of the peripheral nervous system (PNS) convey environmental information to the central nervous system. These neurons reside in the dorsal root ganglia (DRG), and encode distinct modalities of sensory information, such as pain, temperature, touch, and limb position. Terminally differentiated sensory neurons can be distinguished by their distinct axon terminal morphologies, receptive modalities, and proteomics profile (reviewed in Lallemend and Ernfors, 2012).

In mice, neural crest-derived progenitors migrate ventrally and coalesce into DRGs by embryonic day (E) 9.5. Neural progenitors migrate into the nascent ganglia in three waves and proliferate both en route to and within the DRG (reviewed in Marmigère and Carroll, 2014; Marmigère and Ernfors, 2007). The first migratory wave (E9.5) selectively generates myelinated neurons including proprioceptors and mechanoreceptors, whereas the second (E10) primarily generates unmyelinated neurons including nociceptive neurons and c-fiber mechanoreceptors, and the third (E11) generates additional nociceptive neurons (Ma et al., 1999; Maro et al., 2004; Marmigère and Carroll, 2014; Marmigère and Ernfors, 2007; George et al., 2010). By E10.5-E13.5 of embryonic mouse development, sensory-committed cells begin to respond to extrinsic cues (i.e. target derived neurotrophic factors) and initiate transcriptional programs that

specify growth, terminal differentiation, and synapse formation (Marmigère and Ernfors, 2007; Ladle, Pecho-Vriesling, and Arber, 2007). These progenitors continue to differentiate into at least 13 molecularly distinct subpopulations of neurons, ranging from thermosensitive to proprioceptive (Marmigère and Ernfors, 2007; Lallemend and Ernfors, 2012). Sensory neuron axons arrive at target tissues starting at E11.5 (Berg and Farel, 2000). Upon innervation of peripheral targets, including skin and muscle, neurons encounter a limiting amount of target-derived trophic factor, which supports neuronal survival and promotes further differentiation (Luo et al., 2007). Importantly target-derived trophic factors are limited to quantities sufficient to support survival of only a fraction of the neurons innervating a target (Hamburger and Montalcini, 1949; Lee et al., 1994; Ernsberger, 2009; Deppmann et al., 2008). This initial overproduction and subsequent competition for survival leads to a functional postmitotic circuit. Very little is known about how these distinct subpopulations develop relative to one another.

Each mature sensory neuron subpopulation has unique neurotrophin receptor expression profiles. These neurotrophin receptors bind with high affinity to a cognate target-derived neurotrophic factor to enable survival and axon growth: (i) TrkA binds NGF, (ii) TrkB binds BDNF and NT-4, (iii) TrkC binds NT-3, and (iv) in concert with co-receptors, Ret binds GDNF, Neurturin, Artemin or Persephin (reviewed in Lallemond and Ernfors,

2012). Although there are exceptions, generally neurons that express TrkA transmit nociceptive pain, Ret and TrkB transmit mechanical touch and pressure, and TrkC transmit proprioceptive information for determining limb position. Throughout development, these four populations further diverge to adopt other molecular and functional profiles. For example, subsets of neurons that transduce temperature or mechanical information lose TrkA expression and gain expression of Ret (Molliver et al., 1997).

The dynamic expression patterns for each neurotrophin receptor has also lent insight into their possible role in proliferation, differentiation, and competition for survival (Ernsberger, 2009). Immature sensory neurons transiently express multiple neurotrophin receptors simultaneously, and terminally differentiated neurons often express a single neurotrophin receptor (Lallemend and Ernfors, 2012). At E11.5, TrkC is the mostly widely expressed neurotrophin receptor in the ganglia, with expression in neural progenitors and immature sensory neurons, which is consistent with the role of NT-3 and TrkC in migration and proliferation (Fariñas et al., 1998; Lefcort et al., 1996). However, nearly universal TrkC expression in DRG cells is transient because by E13, TrkA becomes the most prevalent receptor on sensory neurons (Fariñas et al., 1998).

Interestingly, TrkA is critical for the development of a subset of Ret+ neurons. Two populations of sensory neurons express Ret: (1) rapidly adapting- (RA-) mechanoreceptors and (2) non-peptidergic nociceptors (Luo et al., 2007; Luo et al., 2009). Ret expression in sensory neuron subsets arises in two waves (1) an early subpopulation emerging from the first neurogenic wave termed “early (e)Ret” with detectable Ret expression starting at E10.5 or (2) a later subpopulation that is born from the second neurogenic wave but only expresses Ret perinatally (starting at E15.5) termed “late (l)Ret” (Luo et al., 2007; Molliver et al., 1997; Silos-Santiago et al., 1995; Lallemend and Ernfors, 2012; Luo et al., 2009). The (l)Ret population is derived from TrkA+ neurons with high levels of NGF-TrkA signaling, suggesting that altered neurotrophin signaling can promote or suppress differentiation (Luo et al., 2007; Wheeler et al., 2014). Intriguingly, neurotrophin signaling via the GDNF family of ligands in Ret-expressing neurons is required for developmental processes such as differentiation or axon branching, but not survival (Luo et al., 2007; Luo et al., 2009). Mice null for *Ret*, *NRTN*, or the Ret co-receptor *GFRa2*, do not display loss of RA-mechanoreceptors or non-peptidergic nociceptors compared to WT (Luo et al., 2007; Luo et al., 2009).

Beyond classic trophic factors and their cognate receptors, other receptors have been shown to dampen or augment trophic signaling. Members of the tumor necrosis factor receptor superfamily (TNFRsf) synergize or

antagonize neurotrophic signaling in a receptor- and cell type-specific manner. Interestingly, the TNFRsf member, p75 neurotrophin receptor (NTR), works in concert with TrkA to promote survival in the sensory system, yet antagonizes TrkA to promote death in the sympathetic nervous system (Lee et al., 1992; Fan et al., 1999; Lee, Davies, & Jaenisch, 1994). p75NTR can bind all mature and immature neurotrophins as a homodimer or as a heterodimer with the receptor tyrosine kinase neurotrophin receptors TrkA, TrkB, TrkC, and Ret (Hempstead, 2002; Vilar et al., 2009). Sensory neurons lacking *p75NTR* require a higher concentration of neurotrophins to promote survival *in vitro* compared to wild type controls (Buchman and Davies et al. 1993; Lee et al. 1992; Hantzopoulos et al., 1994; Makkerh et al., 2005; Hamanoue et al., 1999; Chen et al., 2017). Consistent with this, mice lacking *p75NTR* have a decreased number of all analyzed sensory neuron subtypes, including peptidergic nociceptors, non-peptidergic nociceptors, TrkB+ mechanoreceptors, and TrkC+ proprioceptors, resulting in a 50% decrease in lumbar sensory neurons by E14.5 (Murray et al., 1999; Bergmann et al., 1997; Barrett and Bartlett, 1994; Fan et al., 1999; Lee et al., 1994; Vaegter et al., 2010).

Here we sought to examine the development of these subpopulations relative to each other by asking the following: (1) Does refinement through cell death occur simultaneously between subpopulations? (2) Does

p75NTR-Trk/Ret synergism apply to all neurotrophin-responsive neurons or just a subset? (3) Does p75NTR mediate survival during normal periods of developmental death? The progressive migration and differentiation of sensory neurons subtypes prompts the question of whether developmental cell death coincides with the progression of birthdate. Staggered windows of neuronal birth and death may reflect cell intrinsic differences that permit neuronal diversity.

Our results indicate that developmental cell death of sensory neuron subsets occur at different rates mirroring the temporally successive and overlapping birth dates of each subset. We find that TrkB+, TrkC+, and Ret+ neurons derived from the first neurogenic wave complete developmental cell death prior to TrkA+ neurons derived from the second and third neurogenic waves. Additionally, p75NTR is critical for restricting the magnitude of developmental death in TrkA+, TrkB+, and TrkC+ populations, but is dispensable for proliferating progenitors. However, loss of *p75NTR* does not prolong the window of developmental death for any sensory neuron subpopulation, suggesting that it does not mediate the restricted period of competition for survival as it does for sympathetic neurons (Deppmann et al 2008; Bertrand et al., 2008). Interestingly, loss of *p75NTR* did not increase the number of dying Ret-expressing neurons at the timepoints we investigated, but total Ret-expressing neurons were drastically decreased by birth. Our findings support a model whereby

terminally differentiated sensory neurons undergo sequentially staggered birth and death in a subset-dependent manner, and the magnitude, but not windows of developmental cell death in TrkA+, TrkB+, and TrkC+ neurons are dependent on p75NTR.

MATERIAL AND METHODS

Animals

All experiments were carried out in compliance with the Association for Assessment of Laboratory Animal Care policies and approved by the University of Virginia Animal Care and Use Committee. *p75NTR*$^{-/-}$ mice were purchased from Jackson Labs (#002213, RRID:IMSR_JAX:002213), first developed by Lee et al. (1994), and were maintained on a B6;129s mixed background and genotyped with primers against intron II in *p75NTR* - Intron II (p75-IntII, 5′-CGATGCTCCTATGGCTACTA), Intron III (p75IntIII, 5′-CCTCGCATTCGGCGTCAGCC), and the pGK-Neo cassette (pGK, 5′-GGGAACTTCCTGACTAGGGG). For timed pregnancies, animals were mated overnight and removed the next day once per week. Animals were housed on a 12-hour light/dark cycle with food and water *ad libitum*.

Tissue Processing

P0 and embryonic mice were euthanized by decapitation; spinal columns (lower lumbar or L3-L6) were dissected and fixed in 4% PFA (in 1x PBS; pH=7.4) overnight at 4°C. Adult L4 DRG tissue was dissected and fixed in

4% PFA for 2 overnight at 4°C. Fixed tissues were cryoprotected in 30% sucrose (in 1x PBS) for at least 2 days at 4°C, embedded in OCT (VWR Catalog Number: 25608-930), and then cryosectioned at 10μm sequentially between 5 slides. The DRG from lower lumbar segments were analyzed at E11.5 and E12.5, immediately above the lower limb buds. The L4 DRG was analyzed at E13.5, E14.5, and p0, using the last rib as a landmark for T13.

Immunostaining

Staining was performed as described previously (Barford et al. 2017). Mounted sections were warmed to room temperature and washed with 1 X PBS three times for 5 min each. Antigen retrieval was performed for all antibodies by microwave boiling slides/sections in sodium citrate buffer (10 mM sodium citrate, pH 6.0). Sections were cooled to room temperature, sodium citrate buffer was replaced, and sections were microwaved until boiling again. Sections were then rinsed three times with 1 X PBS and incubated with blocking solution (0.2% Triton X-100, 3% normal donkey serum) for 1 hour at room temperature. Sections were incubated with primary antibodies diluted as detailed below in blocking solution overnight at 4°C. Sections were washed with 1 X PBS three times for 5 min each, incubated with secondary antibodies for 1 hr at room temperature protected from light, and then washed with 1 X PBS three times for 5 min each. Sections were mounted in Fluoromount-G with DAPI

(SouthernBiotech). Primary antibodies used in this study: Goat Anti-TrkA (R&D, AF1056, 1:200 or 0.2ug/mL, RRID:AB_2283049), Goat Anti-TrkB (R&D, AF1494, 1:100 or 0.2ug/mL, RRID:AB_2155264), Goat Anti-TrkC (R&D, AF1404, 1:500, RRID:AB_2155412), Rabbit Anti-Cleaved Caspase-3 (Cell Signaling, 9661S, 1:200, RRID:AB_2341188), Mouse Anti-Islet1/2 (DSHB, 39.4D5, 1:100, RRID:AB_528173), Goat Anti-Ret (Neuromics, GT15002, 1:1000, RRID:AB_1622006), Rabbit Anti-Ki67 (Abcam, AB15580, 1:300, RRID:AB_805388), Rabbit Anti-Sox10 (Gift from S. Kucenas, 1:5,000), Rabbit Anti-p75NTR (Promega, G3231, 1:250, RRID:AB_430853), Rabbit Anti-BFABP (Gift from C. Birchmeier and T. Müller, 1:10,000, Kurtz et al., 1994), Cleaved Caspase-3 (Asp175) (D3E9) Rabbit mAb (Alexa Fluor® 488 Conjugate, 9603, 1:50, RRID:AB_11179205). Secondary antibodies used in this study: Alexafluor 488 Donkey anti-mouse (ThermoFisher, A-21202, 1:500, RRID:AB_141607), Alexafluor 548 Donkey anti-mouse (ThermoFisher, A10037, 1:500, RRID:AB_2534013), Alexafluor 647 Donkey anti-mouse (ThermoFisher, A-31571, 1:500, RRID:AB_162542), Alexafluor 488 Donkey anti-goat (ThermoFisher, A-11055, 1:500, RRID:AB_142672), Alexafluor 633 Donkey anti-goat (ThermoFisher, A-21082, 1:500, RRID:AB_141493), Alexafluor 568 Donkey anti-rabbit (ThermoFisher, A10042, 1:500, RRID:AB_2534017).

Cell Count Quantification

All tissue was sectioned into fifths (five representative sets) and each section was collected and stained with the indicated marker. All tissue was imaged on the laser scanning confocal Zeiss 780 NLO at 20X resolution in z-stacks at 3 μm intervals for manual or automated quantification in Fiji (Schindelin et al., 2012, RRID:SCR_002285). Counts for L4 DRG in E13.5, E14.5, and p0 were the total number of cells summed within one set and multiplied by five (i.e., the number of sets) to yield the total number of cells in a given DRG, modified from Wheeler et al. (2014). Counts of the number of cells per section were averaged between at least five, but up to ten, sections for each animal (N). Islet1/2 counts in E12.5, E13.5, E14.5, and p0 animals were performed using Cell Profiler (Carpenter et al., 2006, RRID:SCR_007358) with manual quantification corrections. Islet1/2 counts in E11.5 animals were performed manually.

Statistical Analyses

Statistical analysis was performed in GraphPad Prism software as indicated in L legends. Data are presented at mean ± standard error of the mean. Sample size (n) was defined as the number of animals from which DRGs were dissected.

RESULTS

Mechanosensitive and Proprioceptive Neurons Complete Developmental Cell Death Prior to Nociceptive Neurons

We first examined accumulation of each sensory neuron subset in lower lumbar DRGs or specifically L4 DRGs as a function of development. DRGs were collected, sectioned, and immunostained from E11.5, E12.5, E13.5, E14.5, and p0 mice; these timepoints represent a dynamic period of proliferation, neuronal differentiation, and death in sensory neuron development. Multipotent neural precursors migrate into the dorsal root ganglion in three waves, and each wave preferentially generates different sensory neuron subsets (Figure 1). Sensory neuron subsets were identified by expression of neurotrophin receptors (NTR), including TrkA, TrkB, TrkC, and Ret, and the pan-sensory neuron transcription factor Islet1/2. The ratios of TrkA+, TrkB+, TrkC+, and Ret+ neurons across development are consistent with the ratios reported by others (Fariñas et al., 1998) (Figure 2A-D). These relative ratios give clues as to the sequence of differentiation. For example, the second and third wave of precursor migration begins at E10 and E11, respectively; these precursors proliferate and preferentially terminate into TrkA+ neurons (nociceptors). At E11.5, the sensory neurons in the DRG are predominantly TrkC+ (88.21 ± 4.75% (TrkC+; Islet1/2+/Total Islet1/2)*100), which is similar to the previously reported molecular identity of immature neurons in the DRG, suggesting that while the ganglia has started to coalesce, the majority of neurons have yet to terminally differentiate (Figure 2A, 2C) (Fariñas et al., 1998). A loss of TrkC expression in immature neurons throughout development is reflected in the decrease in TrkC+ neurons

from 88.21 ± 4.75 % of TrkC+ neurons at E11.5 to 28.83 ± 7.93 % at E13.5 (Figure 2C). We also carefully quantified the total number of neurons per Lumbar 4 (L4) ganglia because ganglionic volume varies along the anterior-posterior axis and increases throughout development (Fariñas et al., 1996; Fariñas et al., 1998). The lack of anatomical landmarks at E11.5 and E12.5 prevented analysis of total cell counts per DRG. As such, we presented this data as the relative precentage of each neuronal subtype at E11.5 and 12.5 (Figure 2A, 2C). When we investigate total numbers of each sensory neuron subtype in the L4 ganglia, we observe an increase of Ret+ neurons between E14.5 and p0; this can be attributed to late differentiation of TrkA+ neurons to late (l) Ret+ neurons and is consistent with other studies (Figure 2D) (Luo et al., 2009). Intriguingly, we also a relatively consistent population of TrkB+ and TrkC+ (mechanoreceptors and proprioceptors) within the L4 ganglia between E13.5 and p0 (Figure 2D). At first blush, this might be surprising given the known role of neuronal cell death during development. If developmental death contributes to loss of neurons, what could account for consistent cell counts in these neuronal subsets? Previous birthdating pulse chase studies suggest DRG neurogenesis is complete by E14.5 (Lawson and Biscoe, 1974). Rather than late proliferation, the consistency of TrkC+ and TrkB+ neuronal counts can be associated with (1) an increase in neurotrophin receptor expression after final target innervation (Kuruvilla et al., 2004; Deppmann et al., 2008), (2) an increase in cell size particularly

in TrkC+ and TrkB+ cells (large-diameter neurons) between E14.5 and p0 which results in an increased ease of identification (Figure 1A, 2B, 2D) (Luo et al., 2009), and/or (3) late terminal differentiation where neurons adopt new neurotrophin receptor expression (Lallemend and Ernfors, 2012).

This led us to ask if this differential accumulation of subpopulations also reflect differential periods of naturally occurring death. In order to identify the temporal waves of death in each population, we co-immunostained DRG sections obtained at several timepoints across early development for neurotrophin receptors, TrkA, TrkB, TrkC, or Ret and cleaved caspase-3 (Figure 3A-E). Cleaved caspase-3 is the active form of caspase-3, the predominant effector caspase critical in the late stages of apoptosis (Walsh et al., 2008). Consistent with previous findings, we find that sensory neurons undergo cell death at time points reported to correspond to final target innervation and late stage differentiation (Berg and Farel, 2000; Pinon et al., 1996). TrkB+, TrkC+, and Ret+ neurons exhibit peak levels of cell death at E12.5 and a progressive decrease in dying cells by E13.5 (Figure 3B-D). In contrast to the peaks of death in the TrkB+, TrkC+, and Ret+ neurons at E12.5, we found that TrkA+ nociceptors display consistent levels of death at E12.5, E13.5, and E14.5, but is virtually absent by p0 (Figure 3E). Interestingly, the larger window of birth for TrkA+ nociceptors, may result in a longer window of maturation and

target innervation, resulting in persistent levels of death between E12.5 and E14.5. Overall, these results indicate that developmental cell death in TrkB+, TrkC+, and Ret+ sensory neuron precedes TrkA+ sensory neurons.

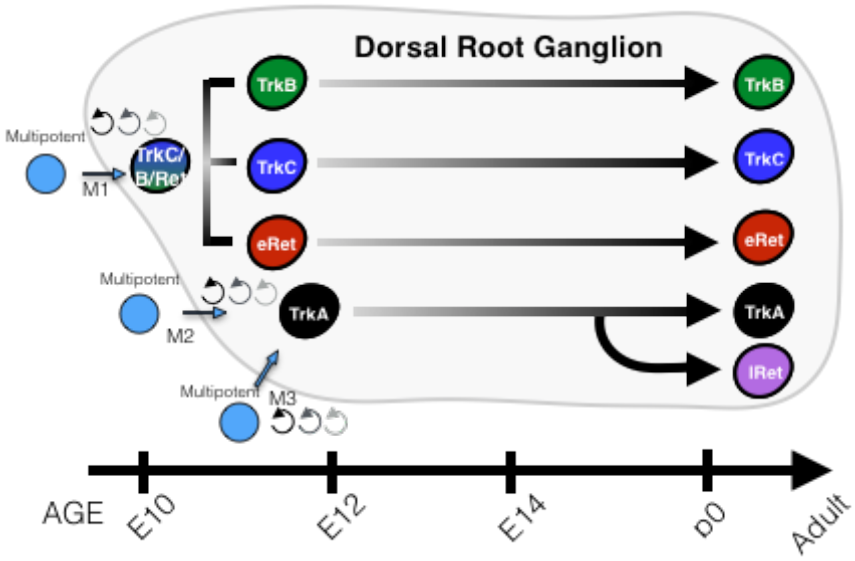

Figure 1. Schematic Representation of Neurogenic Waves in Dorsal Root Ganglia Development

Sensory neuron migration, proliferation, and differentiation mouse sensory neuron development. M(1/2/3): Migration of progenitors (Wave 1/2/3), cyclic arrow (↻) denotes proliferation.

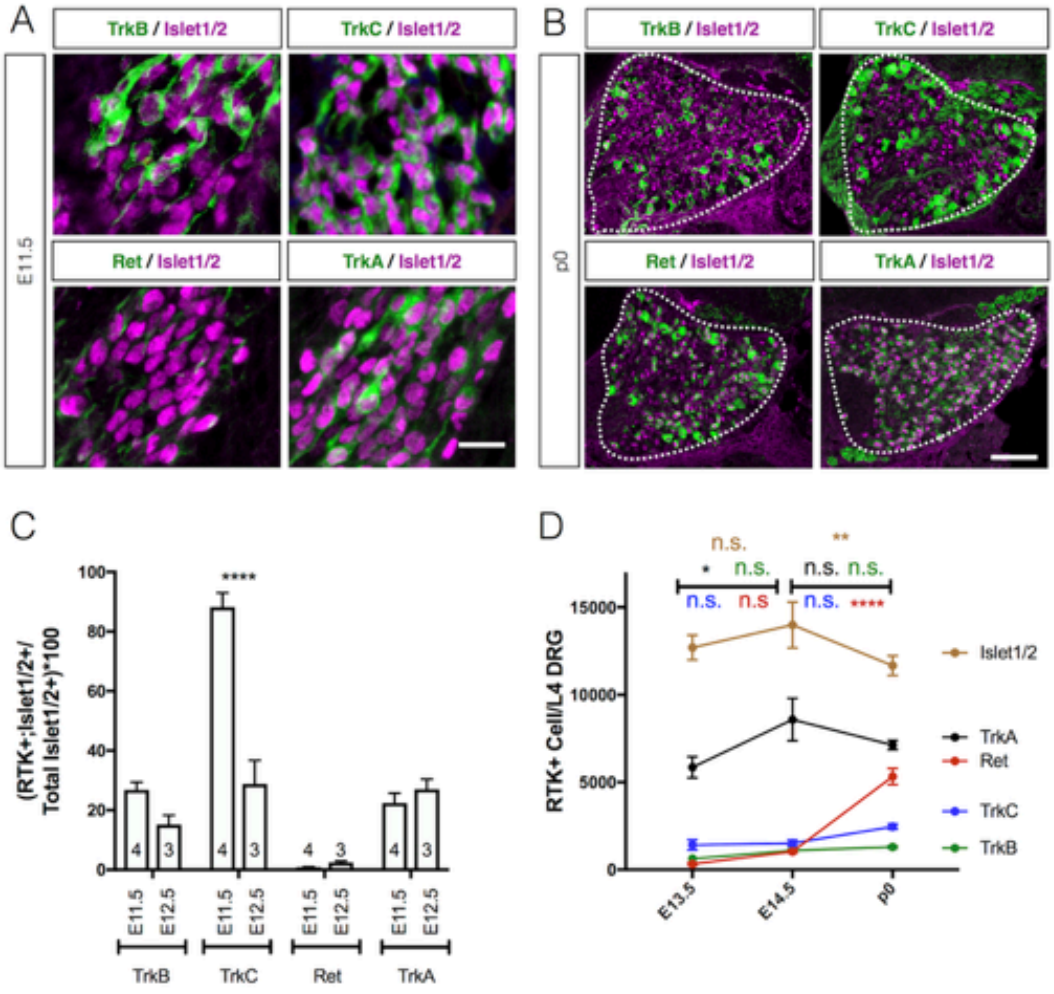

Figure 2. TrkB+, TrkC+, Ret+, and TrkA+ Sensory Neuron Developmental Accumulation

(A) Representative co-immunofluorescence of E11.5 lower lumbar DRGs with anti-neurotrophin receptors (NTR) (TrkA/B/C/ or Ret) or anti-Ret and sensory neuron marker anti-Islet1/2. Scale bar, 20μm.

(B) Representative co-immunofluorescence p0 L4 DRGs with anti-TrkB/C/A or anti-Ret and sensory neuron marker anti-Islet1/2. Scale bar, 100μm.

(C) Quantification of percent of neurons expressing TrkB/C/A or Ret (Islet1/2+; NTR+/Total Islet1/2*100) from E11.5 or E12.5 lower lumbar DRGs. Values are expressed as means ± SEM with N (animals) labelled on the graph.

(D) Quantification of total RTK+ expressing cells in L4 DRG at E13.5, E14.5, and p0. N=3-8 animals for each timepoint.

* denotes $p<0.05$, ** $p<0.01$, **** $p<0.0001$ with two-way Anova with Sidak post hoc (B) or Tukey (D).

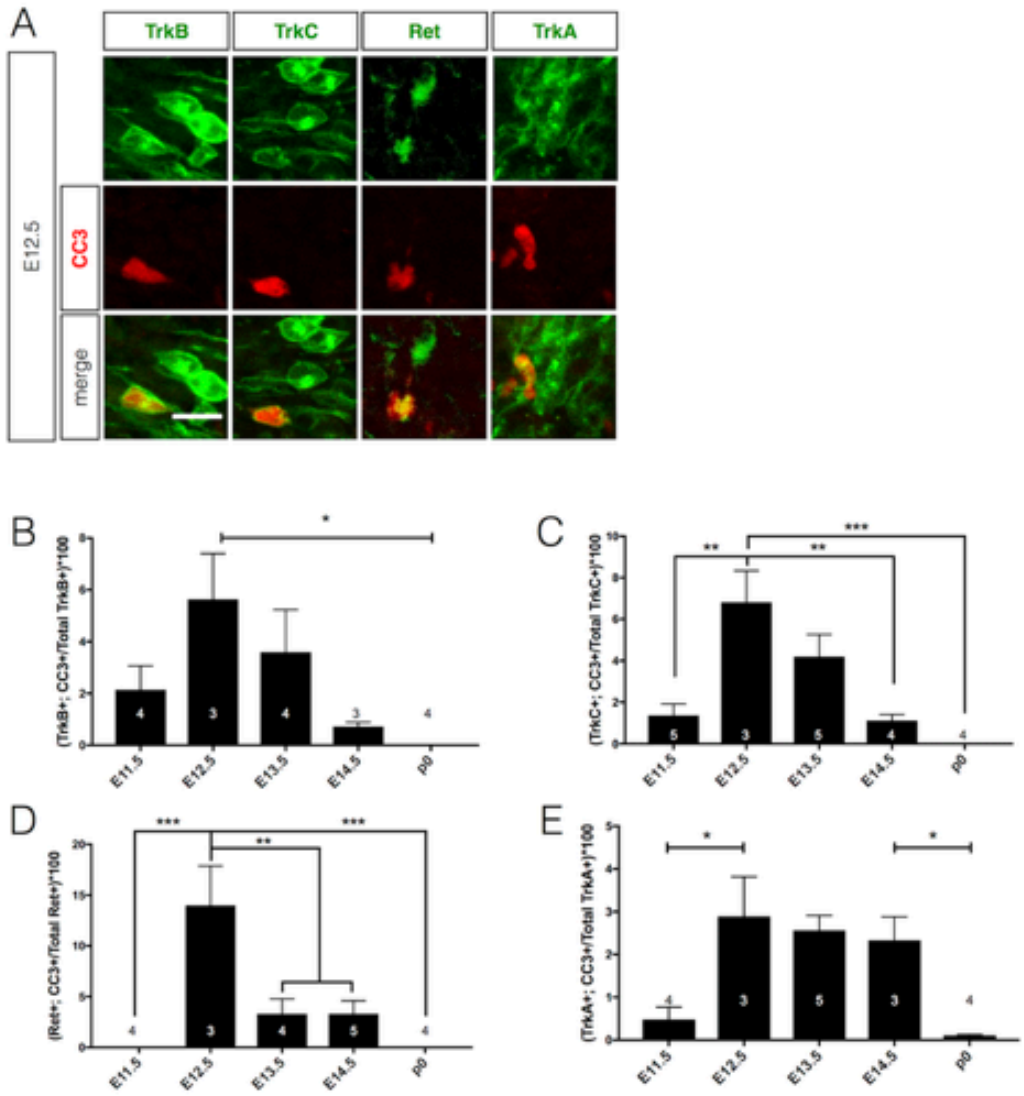

Figure 3. TrkB+, TrkC+, and Ret+ Neurons Complete Developmental Cell Death Prior to TrkA+ Neurons

(A) Representative co-immunofluorescence of E12.5 lumbar DRG with anti-TrkB/C/A or anti-Ret and anti-cleaved caspase-3. Scale bar, 20μm. (B-E) Quantification of dying TrkB/C/A or Ret (% cells double positive for CC3 and TrkB/C/A or Ret divided total TrkB/C/A or Ret*100).

* denotes $p<0.05$, ** denotes $p<0.01$, and *** denotes $p<0.001$, with one-way Anova with Tukey post hoc (B-E). Values are expressed as means ± SEM with N (animals) labelled on the graph.

p75NTR Expression in Sensory Neurons Coincides with Periods of Elevated Cell Death

p75NTR is thought to be universally required for neurotrophic signaling in sensory neurons, however, a direct analysis of the precise timing of p75NTR-dependent survival across sensory neuron subpopulations has not been performed. We first examined the expression pattern of p75NTR over development. Consistent with previous reports (Chen et al., 2017), we find that p75NTR is expressed in nearly all sensory neurons at E12.5 (Figure 4A). In contrast, by p0, p75NTR is only expressed in a subset of TrkA+ and Ret+ sensory neurons, but is expressed in all TrkB+ and TrkC+ sensory neurons (Figure 4A-D). Interestingly, Chen et al. (2017) identified expression of p75NTR in satellite glial cells in the DRG and the potential role of p75NTR non-cell autonomous neuronal survival. To determine the developmental window where p75NTR-mediates neuronal survival, we examined mice lacking *p75NTR* during periods of known developmental cell death (*i.e.* E11.5-E14.5). Compared to wild type, animals lacking *p75NTR* had similar numbers of Islet1/2+ sensory neurons at E13.5, but significantly lower numbers by E14.5 and p0 (Figure 4E-F). Furthermore, compared to wild type, $p75NTR^{-/-}$ sensory neurons undergo excessive apoptosis in embryonic development (Figure 4E, 4G). The highest percentage of sensory neurons dying occurs at E13.5 in both WT and $p75NTR^{-/-}$, with 1.67 ± 0.23% to 5.19 ± 0.90% sensory neurons dying, respectively (Figure 4G). A similar peak in sensory neuron death between

WT and *p75NTR*$^{-/-}$ suggests that p75NTR influences the magnitude of death but does not effect the developmental window in which death occurs for sensory neurons.

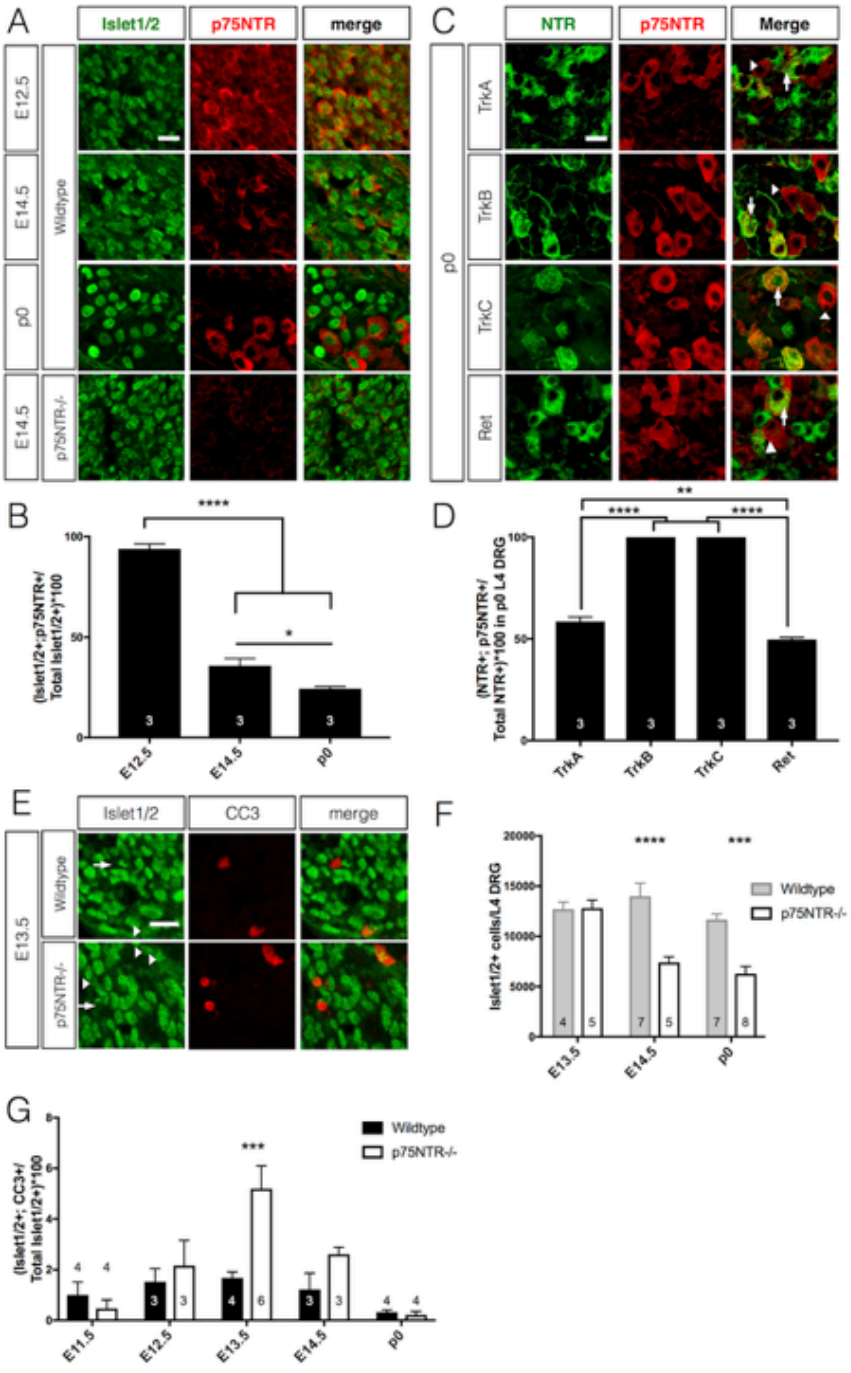

Figure 4. p75NTR is Required for Developmental Sensory Neuron Survival

(A) Co-immunofluorescence staining of WT and p75NTR$^{-/-}$ DRG from lower lumbar DRGs (E12.5) or L4 DRG (E14.5 and p0) with anti-p75NTR and anti-Islet1/2.

(B) Quantification of percent of neurons expressing p75NTR (Islet1/2+; p75NTR+/Total p75NTR*100). N=3 animals per time point with average over 3 sections/animal.

(C) Representative co-immunofluorescence of TrkA/B/C or Ret and p75NTR in p0 L4 DRG. Arrow denotes p75NTR+; NTR+ cells and arrowhead denotes p75NTR+; NTR- cells.

(D) Quantification of percentage of each neuronal subset expressing p75NTR in p0 L4 DRG.

(E) Co-immunofluorescence of Islet1/2 and cleaved caspase-3 in E13.5 DRGs. Arrow denotes CC3+; Islet1/2^{low} cells. Arrowhead denotes CC3+; Islet1/2^{high} cells.

(F) Quantification of Islet1/2+ cells per section from DRGs from WT or p75NTR$^{-/-}$. WT numbers (grey) are repeated from Fig2D.

(G) Quantification of percent dying neurons from DRGs from WT or p75NTR$^{-/-}$.

* denotes $p<0.05$, ** denotes $p<0.01$, *** $p<0.001$, **** $p<0.0001$ analyzed through one-way Anova with Tukey post hoc (B, D) or two-way Anova with Sidak post hoc (F-G). Values are expressed as means ± SEM with N (animals) labelled on the graph. Scale bar in denotes 20 μm.

p75NTR is not Required for Early Proliferation

p75NTR has a clear role in the survival of sensory neurons, but it is unknown whether p75NTR also influences proliferating progenitors. Early neurotrophin signaling by NT-3 and p75NTR has been implicated in maintenance of a proliferative state in sensory neuron progenitors (Fariñas et al., 1996; Hapner et al., 1998). Therefore if p75NTR augments neurotrophin-dependent survival in neurons, it is critical to also investigate whether p75NTR also augments neurotrophin-dependent proliferation in progenitors. To this end, we immunostained proliferating cells (Ki67+) in DRGs at E12.5, E13.5, and E14.5 in WT and *p75NTR*$^{-/-}$ mice. The number of proliferating cells in the DRG decreased drastically between E13.5 and E14.5 in both WT and *p75NTR*$^{-/-}$ animals, but this analysis revealed no difference in the number of proliferating cells between WT and *p75NTR*$^{-/-}$ DRGs at any time analyzed (Figure 5A , 5B). Additionally, following the majority of migration of neural crest cells (E14.5), we found no difference in the total number or percentage of multipotent cells and Schwann cells (Sox10+) dying in WT and *p75NTR*$^{-/-}$ (Figure 5C-F) (Kim et al., 2003; Sonnenberg-Riethmachera et al., 2001). Taken together with the observation that there are equivalent numbers of Islet1/2+ cells at E13.5 between WT and *p75NTR*$^{-/-}$ (Figure 4F), these data suggest that p75NTR is not required for maintenance or establishment of progenitor populations.

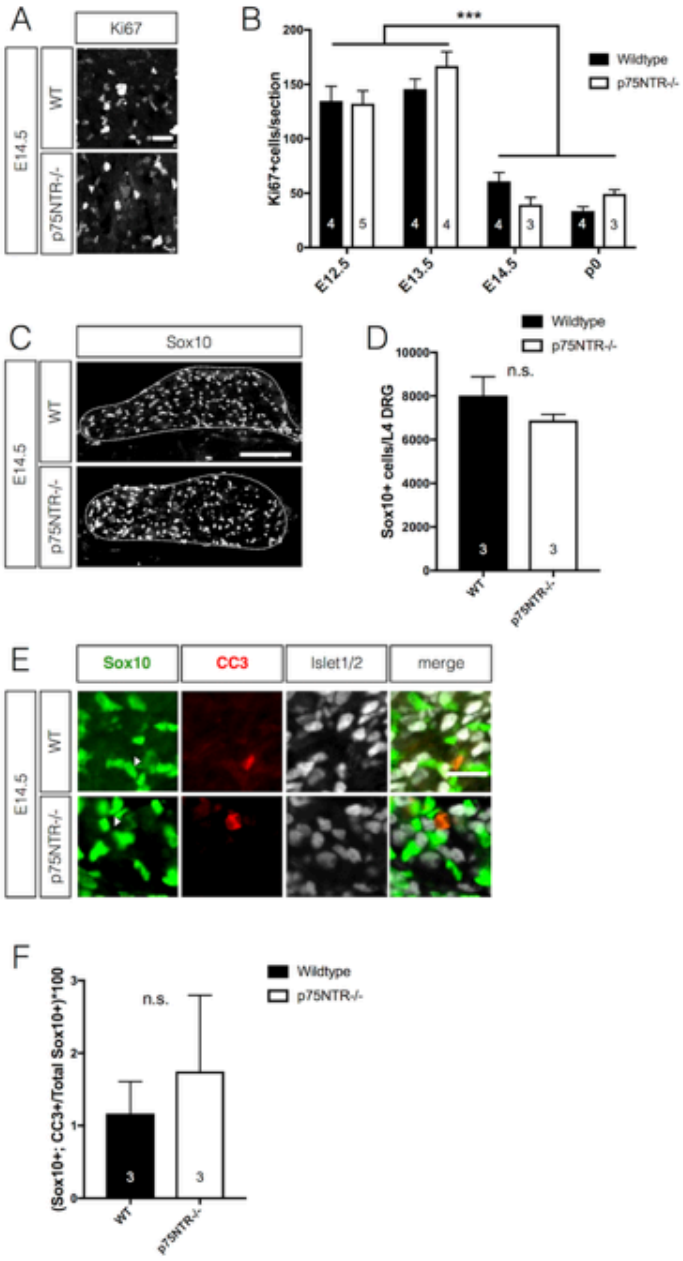

Figure 5. p75NTR is Dispensable for Developmental Proliferation in the DRG

(A-B) Representative immunofluorescence against Ki67 in E14.5 DRG with quantification of Ki67+ cells in DRGs from WT or p75NTR$^{-/-}$. *** denotes $p<0.001$ analyzed through two-way Anova with Sidak post hoc

and no significance (p>0.05 - n.s.) was found between the number of ki67+ cells in WT and p75NTR-/- within any timepoint.

(C-D) Immunofluorescence of Sox10+ cells, multipotent and Schwann cells, in E14.5 DRG with quantification of total number of Sox10+ cells. No significance (n.s.) was found though unpaired T-test.

(E-F) Co-immunofluorescence of Sox10+ and CC3+ cells in E14.5 DRG with quantification of dying Sox10+ cells (Sox10+; CC3+/Total Sox10+*100). Arrow denotes Sox10+;CC3+ cells.

No significance (n.s.) was found though unpaired T-test. Scale bar in denotes 20 μm (A, C) or 100 μm (B).

p75NTR is Required for Satellite Glia Cell Accumulation

Evidence from p75NTR conditional knockouts in post-mitotic sensory neurons suggests that p75NTR may exert survival cues in neurons through neighboring cells, such as p75NTR-expressing satellite glial cells (SGC) (Chen et al., 2017). Consistent with this, we find that DRGs from *p75NTR*$^{-/-}$ mice have a decreased number of satellite glial cells (B-FABP+) by p0 (Figure 6A, 6B), suggesting that p75NTR is required for establishment or maintenance of SGCs. However, it remains unclear if the decrease in satellite glial cell number is due to a cell-autonomous role of p75NTR.

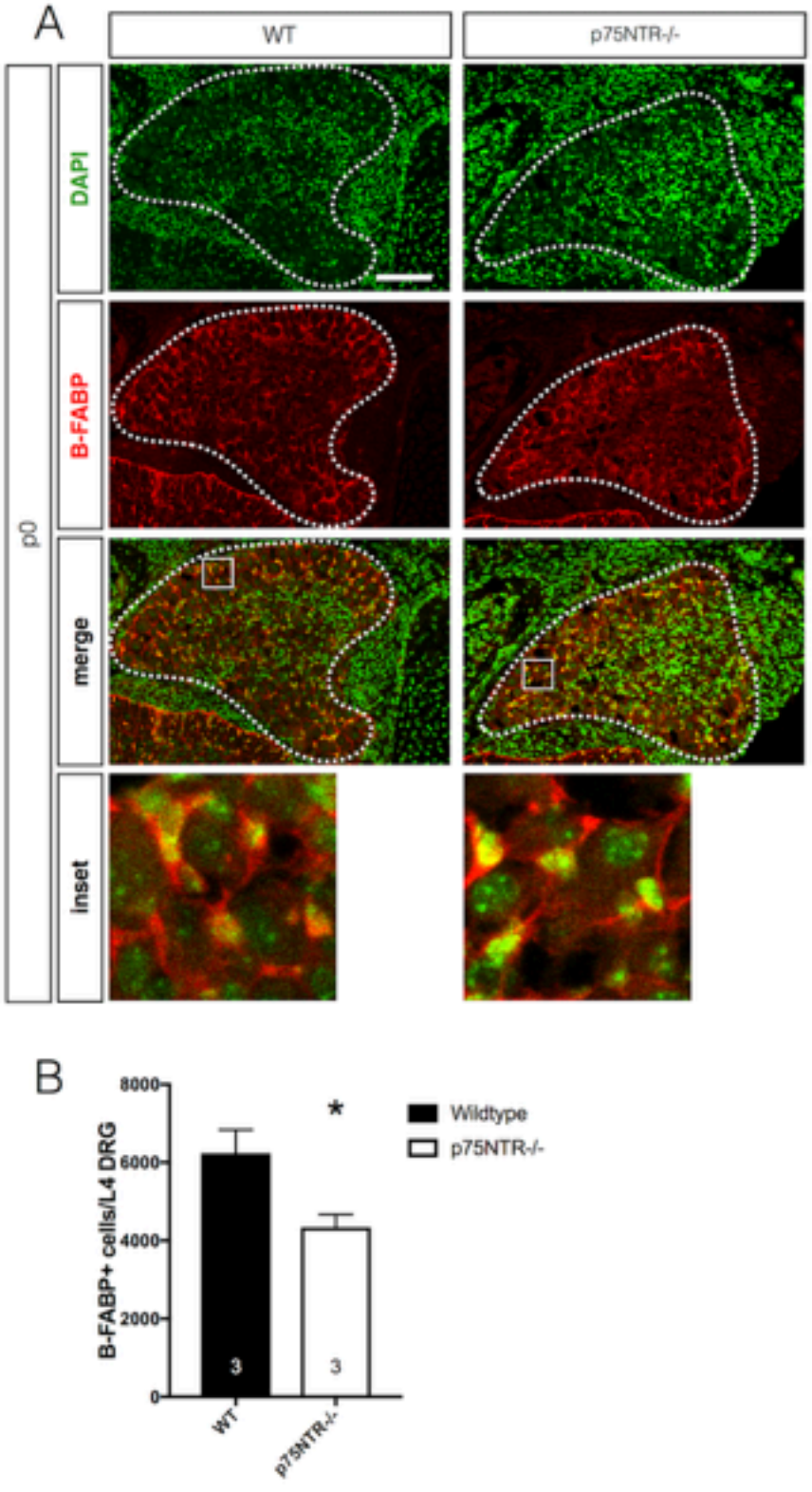

Figure 6. p75NTR is Required for Satellite Glia Cell Accumulation

(A) Immunofluorescence of B-FABP+ and DAPI+ cells in p0 DRG with quantification (B).

* denotes $p<0.05$ analyzed through unpaired T-test. Values are expressed as means ± SEM with N (animals) labelled on the graph. Scale bar in denotes 100 μm.

p75NTR Represses Developmental Cell Death During Restricted Temporal Windows

Is the role of p75NTR simply to sensitize neurons to their corresponding neurotrophic factors or does it play a more subtle role in governing developmental windows of cell death for each of these populations? Previous studies found *p75NTR*$^{-/-}$ mice have increased cell death in sensory neurons mid-embryogenesis resulting in loss of all analyzed cell types, including proprioceptors and nociceptors (Lee et al., 1994; Vaegter et al., 2010; Bogenmann et al., 2011). We quantified the percentage of dying cells in each major sensory neuron subset throughout development in WT and *p75NTR*$^{-/-}$ animals. In the absence of *p75NTR*, animals display higher levels of cleaved caspase-3 positive TrkA+, TrkB+, and TrkC+ sensory neurons at times coinciding with their normal peaks of apoptosis (Figure 7A,B,C). The increase in apoptosis in animals lacking *p75NTR* corresponds with a decrease in overall cell number in TrkA+, TrkB+, and TrkC+ populations by E14.5 (Figure 7E,F,G).

Ret+ populations of sensory neurons can be further subdivided into those arising early (eRet+) and late (lRet+) (Molliver et al., 1997). The eRet population, rapidly adapting- (RA-) mechanoreceptors, arises from the earlier progenitor population at E10.5, whereas the lRet+ population, non-peptidergic nociceptors, arises at E15.5 and are derived from a TrkA+ population (Luo et al., 2007; Molliver et al., 1997; Silos-Santiago et al.,

1995; Lallemend and Ernfors, 2011). Similar to the Trk receptors, p75NTR also interacts with Ret receptor to augment neurotrophin signaling (Chen et al., 2017). In contrast to Trk+ neurons, the number of Ret+ cells dying at E11.5, E12.5, E13.5, E14.5, or p0 does not differ between WT and *p75NTR*$^{-/-}$ mice (Figure 7D). Animals lacking *p75NTR* display the same number of Ret+ neurons at E14.5 as wild type but have a substantial decrease in Ret+ cells at p0 (Figure 7H). This discrepancy in Ret+ loss in *p75NTR*$^{-/-}$ suggests a more complicated path in development of Ret+ sensory neurons. For example, a loss in Ret+ DRG neurons at p0, but not E14.5, can be attributed to (1) a late wave of apoptosis in eRet neurons in late embryonic development (E15.5-E18.5) that is exacerbated in *p75NTR*$^{-/-}$, (2) a loss of fated lRet progenitors due to a diminished pool of early post-mitotic TrkA+ by E14.5 in *p75NTR*$^{-/-}$, and/or (3) a defect in terminal differentiation into lRet neurons due to aberrant neurotrophin signalling in *p75NTR*$^{-/-}$. However, late-expressing Ret neurons appear between E14.5 and p0 in both WT and *p75NTR*$^{-/-}$ animals, suggesting conversion of TrkA neurons to lRet neurons in *p75NTR*$^{-/-}$ animals remains intact (Figure 7H). It is likely that the dramatic decrease in Ret+ neurons in *p75NTR*$^{-/-}$ animals by p0 is due to the combined effects of late apoptosis and a diminished pool of TrkA neurons prior to terminal differentiation.

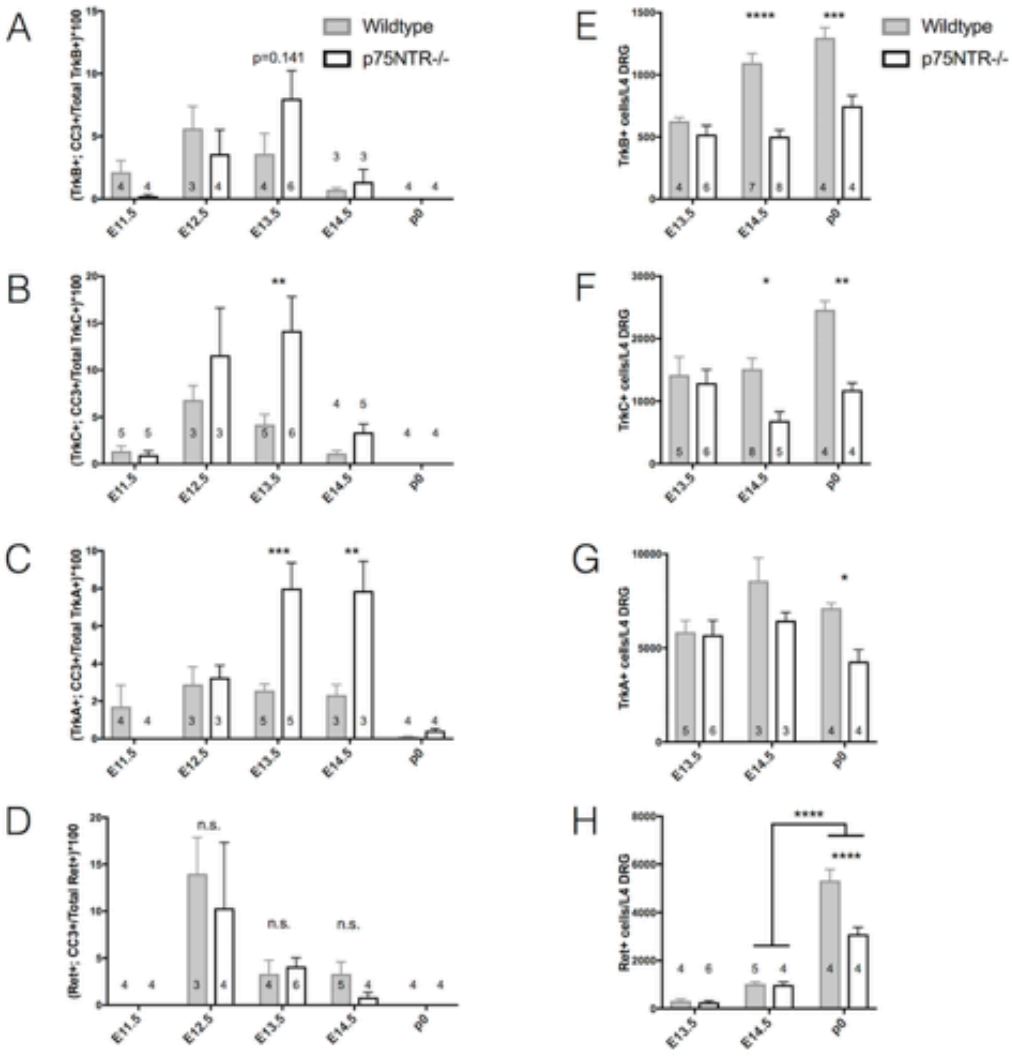

Figure 7. p75NTR Promotes Survival of TrkA+. TrkB+, and TrkC+ Sensory Neurons During Normal Periods of Cell Death

(A-D) Quantification of dying TrkB/C/A or Ret (% cells double positive for CC3 and TrkB/C/A or Ret in DRGs from WT and p75NTR$^{-/-}$ mice between E11.5 and p0. WT numbers (grey) are repeated from Figure 3B-E.

(E-H) Quantification of total TrkB/C/A or Ret+ cells in DRGs from E13.5, E14.5, and p0 WT and p75NTR$^{-/-}$ mice. WT numbers (grey) are repeated from Figure 2D.

Values are expressed as means ± SEM with N (animals) labelled on the graph. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$ analyzed through two-way Anova with Sidak post hoc.

DISCUSSION

This study sheds light onto the sequential nature of sensory neuron subset development. We find that death of TrkB, TrkC, and Ret-expressing sensory neurons peaks at E12.5, whereas TrkA-expressing neurons undergo cell death for an extended period of developmental time (from E12.5 to E14.5). Additionally, we found that *p75NTR*$^{-/-}$ mice display elevated cell death during typical windows of naturally occurring cell death in TrkA, TrkB, and TrkC expressing sensory neurons, resulting in substantially lower numbers of sensory neurons postnatally. p75NTR-dependent survival within the typical period of developmental cell death further supports a role for p75NTR in neurotrophic signaling sensitization. Furthermore, the series of staggered developmental birth and death suggests distinct intrinsic cues between sensory neuron subsets, permitting neural progenitors to differentiate into molecularly and functionally distinct sensory neuron subtypes. These data provide insight into the temporal windows and mechanism of neurotrophin signaling in development of sensory neuron subtypes.

Sensory Neuron Subtypes Die in the Order that They are Born

What are key determinants for timing of developmental death between sensory populations? Our data illustrate that neurons undergo developmental cell death in an order that correlates with developmental birth (reviewed in Marmigere and Ernfors, 2007). One may intuit that

earlier born neurons reach final targets and experience neurotrophic signaling earlier than later born neurons. Our results revealed that TrkB, TrkC, and Ret expressing neurons derived from the first neurogenic wave undergo rapid cell death at E12.5. In contrast, the multi-day window of TrkA+ neuron death over at least three embryonic days likely reflects the larger window of birth for TrkA+ neurons from the second and third neurogenic wave (Ma et al., 1999; Maro et al., 2004; George et al., 2010). Our findings of disparate tempos of death between sensory neuron subsets are in accord with previous findings that mice null for *TrkC* or *NT-3* display excessive death at E11, whereas mice null for *TrkA* or *NGF* show later excessive death at E13.5 (Pinon et al., 1996).

While our work carefully chronicles developmental death of major neuronal subsets as defined by co-localization of cleaved caspase-3 and the neurotrophic receptors they express, there are caveats of this study. Firstly, cleaved caspase-3 is the activated form of the primary executioner caspase responsible for proteolytic degradation of during apoptosis. A portion of apoptotic cells showcasing cleaved caspase-3 may have already degraded neurotrophin receptors, thereby resulting in an underestimation of dying sensory neurons (Slee et al., 2001). Secondly, neurotrophin receptor expression during development is dynamic and final sensory neuron identity cannot always be defined by a single neurotrophin marker, particularly in early differentiation (Rifkin et al., 2000). Neural-

committed progenitors express TrkC and progressively restrict their neurotrophin receptor expression; early TrkC signaling in progenitors has been shown to repress neurogenic development and promote proliferation of sensory neuron precursors (Kirstein and Fariñas, 2002; Gaese et al., 1994; Lefcort et al., 1996; Hapner et al., 1998). Early TrkC-expressing sensory-committed cells have been shown to terminally differentiate into Ret+ RA-mechanoreceptors, TrkB+ mechanoreceptors, and TrkC+ proprioceptors (Lallemend and Ernfors, 2012). Therefore, while our data analyzes the concurrent expression of apoptotic and neurotrophic markers, lineage-tracing studies may further reveal survival/death decisions of each fated neuronal subset.

The regulation of neurotrophin receptor expression levels and type is dependent on concurrent positive- and negative-feedback loops. Target-derived trophic signaling is a robust positive regulator of neurotrophin receptors; in sympathetic neurons, retrogradely trafficked NGF-TrkA signaling endosomes autonomously upregulates TrkA mRNA expression (Deppmann et al., 2008). Therefore, neurons in a mature functional circuit often express high levels of a defining neurotrophin receptor. Additionally, neurotrophin signaling at targets can also provide transcriptional feedback to alter expression and refine their neurotrophic identity, and therefore change their sensory neuron identity. For example, subsets of TrkA-expressing neurons in embryonic development upregulate Ret and other

non-peptidergic nociceptive related genes upon final target innervation and NGF-exposure (Luo et al., 2007). These findings highlight the critical importance of target-derived extrinsics cues in shaping neuronal identity.

p75NTR Promotes Survival of Trk+ Sensory Subtypes

We further characterized the expansive role of p75NTR in sensory neuron development by determining the role of p75NTR on sensory subtype survival as a function of developmental time.

Consistent with previous results, we find that mice lacking *p75NTR* displayed a larger magnitude of sensory neuron death in TrkA TrkB, and TrkC populations compared to WT mice, but we find that this death remained restricted to the same temporal window that each of these populations would normally undergo apoptosis (Davies et al. 1993; Lee et al. 1992; Hantzopoulos et al., 1994; Makkerh et al., 2005; Hamanoue et al., 1999; Chen et al., 2017). However, the comparable synchrony of death suggests that p75NTR does not change the timing of birth, target innervation, or competition for survival of these subpopulations. Instead, temporal control of birth, axon growth, and survival is tightly regulated by a number of other intersecting pathways, including cell autonomous intrinsic cues (e.g. transcription factor networks) and external cues (e.g. neurotrophins and other morphogens).

Curiously, we find that apoptosis in Ret-expressing sensory neurons is not increased in *p75NTR*$^{-/-}$ E12.5-E14.5 mice. Ret is expressed in sensory neuron subsets that convey mechanical and nociceptive stimuli, where the individual modality-sensing subset arises from molecularly distinct lineages. In wild type neurons, we observe a peak of apoptosis in Ret+ neurons at E12.5 (14.0± 3.9%) which rapidly drops to below 4% for all subsequent ages examined. Importantly, loss of *p75NTR* does not significantly influence the amount of death that we observe in this population at any time point that we examined. Our findings that the loss of *p75NTR* does not influence Ret+ neuron numbers or magnitude of death at early time points in development suggest that eRet neurons do not require *p75NTR* for survival. What could be unique about this eRet population? Perhaps this population does not require target-derived Ret ligands for survival in embryonic development. Indeed, mice lacking *Ret* have impaired axon growth *in vitro* and *in vivo*, but not impaired survival of eRet and lRet populations by P14 in mice (Luo et al., 2007; Luo et al., 2009). However, conditional gene excision of *Ret* in non-peptidergic neurons results in lower numbers of Ret+ neurons in adult mice, suggesting that Ret critically mediates survival/maintenance after P14 (Golden et al., 2010). Interestingly, Chen et al. (2017) also demonstrated that loss of *p75NTR* in sensory neurons results in a decreased number of nonpeptidergic Ret+ nociceptors, suggesting a cell autonomous role of p75NTR in postnatal development. These findings showcase the unique

resilience of Ret-expressing neurons against developmental death in early embryogenesis; however future studies must be done in order to investigate if (1) Ret+ neurons undergo later waves of developmental death in embryogenesis, and/or (2) lRet-fated neurons complete developmental cell death before or after their TrkA+ precursor state. Perinatal developmental cell death in lRet neurons is supported by *in vitro* evidence identifying the neurotrophin- and p75NTR-dependent survival in lRet p0 neurons (Chen et al., 2017).

p75NTR is Required for Sensory Neuron Development in Post-Mitotic Neurons

The overall role of p75NTR in sensory neuron survival is well established, however the cell autonomous role of p75NTR within neurons has been debated. Prior studies suggest a role of p75NTR and TrkC in the regulation of proliferation in sensory neuron progenitors (Kirstein and Fariñas, 2002; Gaese et al., 1994; Lefcort et al., 1996; Hapner et al., 1998). Additionally, Chen et al. (2017) finds that Islet-driven excision of *p75NTR* by E12.5 results in no loss of peptidergic nociceptors, proprioceptors, and mechanoreceptors populations, but does result in loss of the late Ret population in the DRG. These results prompt the question of whether p75NTR-dependent survival originates from (1) maintenance of neural crest cell progenitors prior to differentiation, (2) non-neuronal and glial support of neurons, or (3) a cell survival priming mechanism prior to

terminal differentiation or Islet-expression. We find that proliferation of progenitor populations and the number of neurons at E13.5 were similar between WT and *p75NTR*$^{-/-}$ animals, suggesting that neuronal loss in *p75NTR*$^{-/-}$ is due to increased death of neurons. Curiously, we find that compared to WT animals, *p75NTR*$^{-/-}$ animals have less satellite glial cells (SGC) in their DRG. Interplay between SGCs and sensory neurons is well established; SGCs are critical for debris scavenging of apoptotic neurons and proliferate in response to injury, inflammation, and pain (Wu et al., 2009; reviewed in Hanani, 2005). Interestingly, SGCs express p75NTR, but they are largely absent during periods of p75NTR-dependent survival (*i.e.*E12.5) (Chen et al., 2017). Further work must be done to determine whether p75NTR promotes SGC survival in a cell autonomous fashion.

Disparate Roles of p75NTR in Development of Neuronal Populations

Our observations suggest that p75NTR mediates survival in a sensory neuron-specific manner but it does not influence the developmental window in which survival/death decisions are made. Interestingly, p75NTR also plays critical roles in development by regulating axon growth, synapse formation, protein trafficking, and many more processes (Lee et al., 1994; Bamji et al., 1998; Singh et al., 2008; Sharma et al., 2010; Chen et al., 2017). Whether p75NTR uniformly contributes to these processes in all sensory neuron subsets is still unknown. Perhaps, additional intrinsic differences between sensory neuron subsets result in subset-specific

p75NTR-signaling pathways. We have previously described subset-specific signaling of TNFRsf members in the development of the sensory system; TNFR1 and its canonical ligand TNFα promote naturally occurring pruning and differentiation only in nociceptors (Wheeler et al., 2014). The distinguishing signaling capabilities between neuron cell subtypes may explain how extrinsic cues allow for progressive molecular segregation of differentiating neurons.

Chapter III. Mass Cytometry Provides High Dimensional Analysis of Somatosensory System Development

Introduction

Sensory neurons residing in the dorsal root ganglion (DRG) transmit diverse sensory stimuli, such as mechanical pressure, changes in limb position, temperature, pain, and itch. Neural crest-derived neurogenic progenitors rapidly distinguish themselves during development to expressing unique proteomics profiles that endows several populations of neurons and glia with unique functions. This proteomic diversity underlies differential responsiveness to stimuli modality, cell size diameter, timing of maturation, and divergence in developmental lineage.

There is no standardized classification scheme for neural cell types, but current approaches use some combination of cell morphology, localization, connectivity, molecular profiling, electrophysiological behavior, and modality sensitivity (Zeng and Sanes, 2017). For higher-throughput molecular profiling of neural cell types, the current state-of-the-art is single-cell RNA sequencing (scRNA-seq), which measures thousands of mRNA transcripts per cell (Usoskin et al., 2015; Li et al., 2018). However, cell throughput is still a limiting factor for scRNA-seq, as the reported number of analyzed cells in these studies is limited to tens of thousands, which is insufficient for comprehensive analysis, given the size and complexity of the nervous system. There is good support for the notion that relative abundance of mRNA transcripts can be used to phenotype and classify cell types, but it is also clear that mRNA levels do not precisely correspond with protein abundance, particularly in dynamic

transitioning cell populations in the developing nervous system (Mrdjen et al., 2018). Furthermore, transcriptomics can define cellular identify but proteomics analyses are required to more accurately define cellular activity. In this study, we extend the state-of-the-art for neural cell profiling to proteomic-level measurements with an order-of-magnitude greater throughput via single-cell mass cytometry.

Historically, it has been challenging to map the development of a discrete neuronal nuclei or ganglia. High-throughput analysis of the nervous system has been stymied because the tissue is highly diverse, relatively limited, and difficult to dissociate (Lacar et al., 2016). However, the high-dimensional analysis of protein expression within a single cell has been advanced by the development of mass cytometry, also known by its commercial name, CyTOF. CyTOF is a flow cytometry variant that uses isotopically pure rare earth metals conjugated to antibodies or other types of affinity reagents, permitting measurement of over 40 markers simultaneously (reviewed in Spitzer and Nolan, 2016). These markers include cell surface receptors (Ornatsky et al., 2010), intracellular signaling molecules (Bendall et al., 2011), transcription factors (Zunder et al., 2015), proliferation (Behbehani et al., 2012), and viability (Fienberg et al., 2012), as well as mRNA transcripts (Frei et al., 2016). Labeled cells subjected to mass spectrometry revealing the relative abundance of an epitopes examined at a single cell level at a rate of 1 million cell per hour. Interrogating so many markers simultaneously at the single-cell level

provides extraordinary intersectional power to identify both known and unknown cell subtypes, and this approach has proven success in the immune cells in the nervous system (Spitzer and Nolan et al., 2016; Mjrden et al., 2018; Ajami et al., 2018). However, to date, no one has applied this technology to examine neural cell types (Spitzer and Nolan et al., 2016; Mjrden et al., 2018; Ajami et al., 2018). This chapter represents the first study to profile neural cells using mass cytometry.

Both conventional immunohistochemistry and novel scRNAseq studies identified up to 13 subpopulations of mature sensory neurons by the first month of development in mice (reviewed in Lallemend and Ernfors, 2012; Usoskin et al., 2015; reviewed in Li et al., 2018). These diverse somatosensory neurons subpopulations originate from multipotent neural crest-derived progenitors that diverge upon exposure to extrinsic soluble cues triggering intrinsic transcriptional programming.

Herein, we validate and apply cell mass cytometry to neural tissues from DRGs. We collected DRGs at developmental timepoints, from shortly after gangliogenesis (E11.5) to postnatal periods where neurons have primarily terminally differentiated (P4) (reviewed in Lallemend and Ernfors, 2012). This timeframe reflects a known window when subpopulations progressively segregate over time to acquire mature proteomic profiles. This timeframe also reflects a window where there is much to learn about the precise molecular transitions that occur during development. We developed a curated antibody panel of previously defined critical

transcription factors, neurotrophic receptors, and other key protein markers, and our results provide a high-throughput atlas that chronicles the progressive segregation of cells within the somatosensory system. We find that our CyTOF results are consistent with conventional immunohistochemistry-based approaches (Farinas et al., 1998; Cheng et al., 2018). For example, that neuronal populations are the dominant population in early development, but other non-identifiable cell types and glial populations progressively represent a larger proportions of cells in later development. Using sequential hierarchical gating and unbiased FLOW-MAP analysis, we also observe maturation-based population shifts in neuronal subsets in a fashion that reflect previous literature. Collectively, these findings provide a foundation for high-throughput analysis of development and differentiation in the somatosensory system and beyond.

Methods

Animals

All experiments were carried out in compliance with the Association for Assessment of Laboratory Animal Care policies and approved by the University of Virginia Animal Care and Use Committee. C57Bl6 mice were purchased from Jackson Labs and were bred in house. For timed pregnancies, animals were mated overnight and removed the next day

once per week. Animals were housed on a 12-hour light/dark cycle with food and water *ad libitum*.

Tissue Collection, Cell Dissociation, and Fixation

Dorsal root ganglia collected from embryonic and postnatal mice were dissociated to single cell suspension through enzymatic and mechanical processing. DRG from E11.5–E14.5 and E15.5–P4 animals were processed as described in Wheeler et al., 2014, Suo et al., 2014, respectively. The single cell suspension was then treated with 10 μM cisplatin (Sigma Aldrich, P4394) for 30 seconds, then immediately quenched with 0.5% BSA in 1X PBS, centrifuged for 500 × g for 10 minutes, and then re-quenched with 0.5% BSA in 1x PBS. Cisplatin-stained cells were then fixed in 1.6% PFA for 10 minutes and stored in cell staining media (CSM; 0.5% BSA and 0.02% NaN3 in 1X PBS) at -80°C. Trypan Blue (Sigma) exclusion assay was performed prior to fixation with 0.2% solution of trypan blue solution. Trypan Blue-positive cells were identified on a hemocytometer and imaged on a Zeiss Axio Observer inverted widefield fluorescence microscope.

Flow cytometry

Cells were thawed on ice, pelleted by centrifugation at 600 × g for 3 minutes at 4ºC, and the supernatant was discarded. After washing once with CSM, cells were resuspended in 0.5 mL of cold saponin solution

(0.02% in PBS) containing specific concentrations of Alexa Fluor 647 and/or Alexa Fluor 750 NHS Esters (Thermo Fisher Scientific, A20006 and A20111) to barcode up to eight samples (data not shown). For surface staining, cells were blocked in PBS containing 10% (v/v) normal donkey serum (Millipore, S30-100ML) for 30 minutes at room temperature. Primary antibodies listed in Table 1 were diluted in CSM and added to cells (100 uL staining volume per 1 x 10^6 cells), which were incubated on a shaker at 1300 rpm for 30 min at room temperature. After incubation, tubes were centrifuged at 600 × g for 3 minutes at 4ºC, the supernatant was discarded, and cells were washed three times with CSM. For intracellular staining, cells were permeabilized by adding enough ice-cold 100% methanol to fill the tube and vortexing every 2 minutes. After incubation on ice for 10 min, tubes were centrifuged at 600 × g for 3 minutes at 4ºC, and the supernatant was discarded. Cells were washed once with CSM, and primary antibodies listed in Table 1 were diluted in CSM and added to cells on a shaker at 1300 rpm for 1 hour at room temperature. After incubation, tubes were centrifuged at 600 × g for 3 minutes at 4ºC, the supernatant was discarded, and cells were washed three times with CSM. For secondary antibody staining, an appropriate volume of 1 ug/mL Alexa Fluor 488-conjugated anti-species-specific IgG

(listed in Table 1) was added to cells, which were incubated for 30 min at room temperature. After incubation, cells were washed three times with CSM before resuspension, filtering through 40-um nylon mesh, and analysis on an Attune NxT Flow Cytometer (Applied Biosystems). Data were analyzed using CytoBank software (https://cytobank.org). All staining was done by Amy Van Deusen.

Mass-Tag Cell Barcoding (MCB)

Prior to antibody staining, individual samples were thawed on ice, pelleted by centrifugation at 600 × g for 3 minutes at 4ºC, and the supernatant was discarded. Pellets are washed once with CSM, then resuspended in 500µL of cold saponin solution (0.02% in PBS) containing specific combinations of different concentrations of palladium metal isotopes to barcode samples, as described in Fread et al. (2018) and Zunder et al. (2015). Primary antibody staining, (antibodies and vendors summarized in Table 1), were processed as described above for flow cytometry. Cells were also stained with DNA intercalator (1:5000, Cell-ID™ Intercalator-Ir, Fluidigm Corporation) in 1.6% PFA for 15 minutes at room temperature on a shaker at 1300 rpm, or overnight at 4ºC. Following intercalator staining, samples were washed sequentially with CSM, water, 0.05% Tween-20 (in water), and water. Cells were pelleted and resuspended in water with 1:20

EQ Four Element Calibration Beads (Fluidigm Corporation), strained through a 40-um nylon mesh, and finally ran on a Helios CyTOF System (Fluidigm Corporation) as described below. All staining was done by Amy Van Deusen and cells were processed by Eli Zunder.

Mass Cytometry Antibodies

Antibodies used this this study (summarized in Table 1) were conjugated using the MaxPAR antibody conjugation kits (DVS Sciences), according to the manufacturer's recommended protocol. Antibodies were stored prior to conjugation according to manufacturer's instructions and in Antibody Stabilizer (Candor Bioscience, Cat # 131 050) at 4°C after metal-conjugation. All conjugations were done by Amy Van Deusen.

Mass Cytometry Measurements and Data Processing

Stained DRG cell samples were processed on a CyTOF mass cytometer (Helios) at > 500 cells/second, as described in Zunder et al., 2015. Signal sensitivity variability across runtime was normalized through normalization beads, as described in Finck et al. 2013. Collective data from a single run is de-barcoded to identify individual samples, then stored as FCS files, and processed in Community Cytobank or FLOW-MAP, as previously described or described below (Zunder et al., 2015; Ko et al., unpublished).

FLOW-MAP Analysis

The FLOW-MAP graphs were built using R language script (http://www.R-project.org/) and the igraph package (http://igraph.sf.net), adapted from Zunder et al., 2015 and Ko et al., unpublished. FLOW-MAP graphs were built with the following parameters: subsamples - 500, number clusters - 250 , distance metric - euclidean, clustering variables - CD45, CD31, Islet1, PGP9.5, GFAP, TrkA, TrkB, and TrkC, minimum edge # - 2, maximum edge # -5, seed # - 1. The resultant FLOW-MAP graphs (graphml files) were analyzed in the Gephi software package (http://www.gephi.org) (Bastian et al., 2009). Force-directed layouts were built using the ForceAtlas2 algorithm (Jacomy et al., 2014).

Results

Neuro-CyTOF probes multiple markers simultaneously on a single-cell level

Until now, mass cytometry has not been used to examine cells of the nervous system, primarily because neural tissue is difficult to dissociate into a single-cell suspension. In this study, we dissociated DRGs from entire mouse litters from E11.5 to P4, paving the way to construct a proteomic cell atlas for the developing somatosensory system (Figure 1A). Single-cell suspensions from these cells yield an average of 138476.60 ± 11612 cells/animal, with varying yield through development (Figure 1B). These cells are largely viable, as exemplified by a majority of cells excluding Trypan Blue (Figure 1A). The single-cell suspension was also

treated with an acute dose of the viability stain cisplatin, a cell impermeant stain detectable *via* mass cytometry. Once all developmental timepoints were collected, individual cell samples were labelled using metal-tag barcoding and then simultaneously stained with an antibody cocktail (Fread et al., 2018) (Figure 1A). This barcoding strategy stains each sample with a unique combination of three metal isotopes (Fread et al., 2017). By barcoding samples prior to staining, a single cocktail of antibodies can be applied to all samples, which reduces staining variability (Fread et al., 2018). Pooled antibody-stained cell samples are then subjected to mass cytometry and processed through multiple computational analyses (Figure 1A, 1D). Recorded events are normalized to standard polysterene beads uniformly coated with five different metals to combat signal decay (Finck et al., 2013).

Pre-processing is performed to deconvolute all recorded events and isolate viable cellular events. Such hierarchical pre-processing gates include (1) equilibration beads^{low}, (2) background^{low}, (3) barcode separation distance^{high}, (4) mahalanobis distance^{low}, (5) high intercalator^{high}, and (6) cisplatin^{low}. First, the polysterene beads standards are removed (Figure 1C). Samples exhibiting excessive signal in an unutilized channel, 190BCKG, are also eliminated (Figure 1C). Cells that do not fall cleanly within a barcode gate are eliminated based on barcode separation distance (Fread et al., 2017) (Figure 1C). Extreme events with

high antibody binding, likely represent promiscuous binding to debris and are also excluded based on a low Mahalanobis distance (Figure 1C). Lastly, live cells are gated based on DNA intercalator positivity and cisplatin negativity (Feinberg et al., 2012) (Figure 1C). En masse analysis of all viable cells *via* heatmaps demonstrates how markers of more differentiated cell types (i.e. sensory neuron subsets)increase as animals age (Figure 1D). We also visualized multidimensional data sets of protein expression over time as a heatmap across sequentially gated populations. In neuron (Islet1+) gated populations, we were able to identify higher collective expression of neuronal markers in comparison to all cells (Figure 1D).

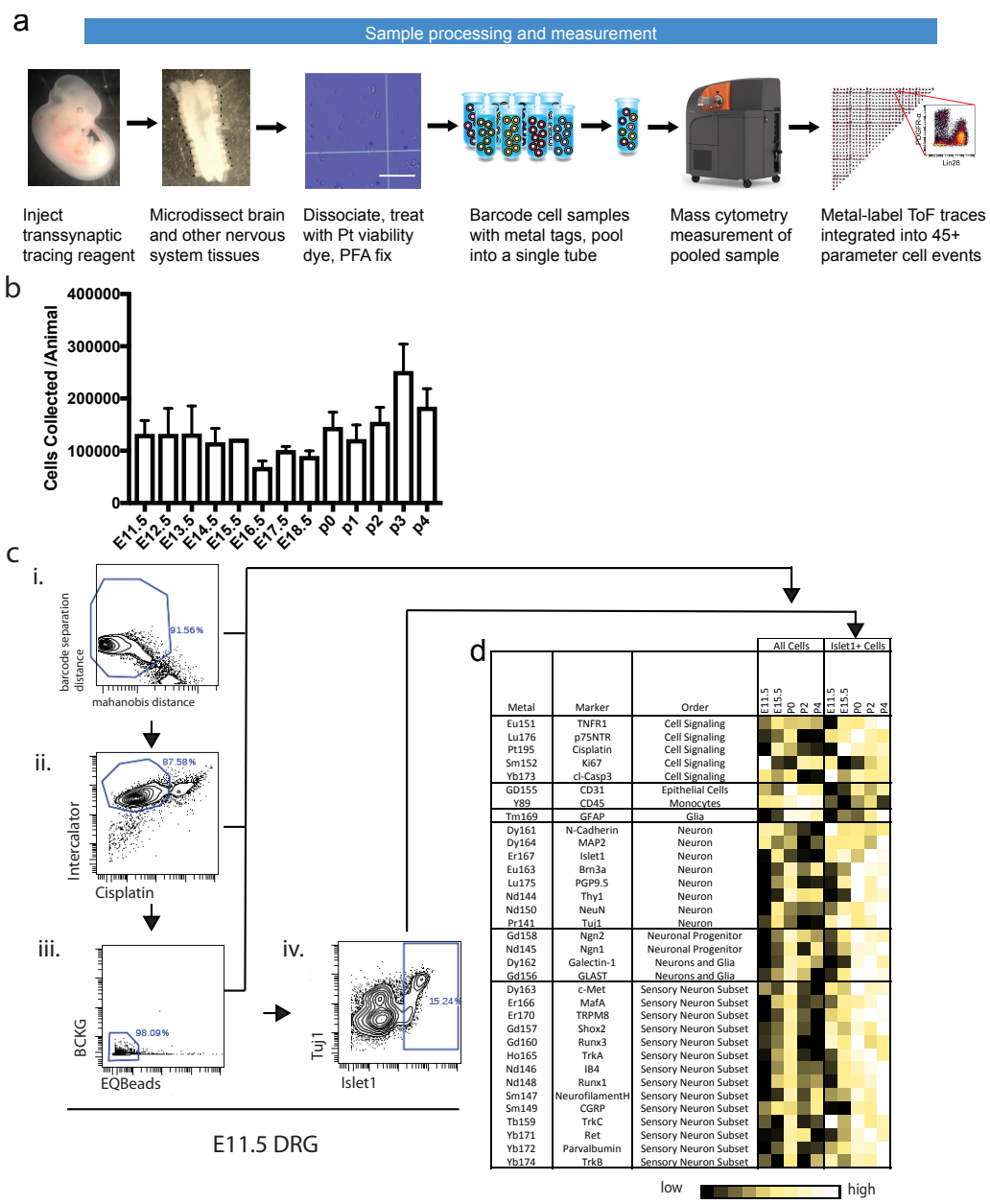

Figure 1. (A) Schematic of sample collection and processing for mass cytometric analysis. E11.5 to P4 mice are dissected and all DRGs are pooled (Pictured is E12.5 pup and spinal cord with DRGs denoted by arrowheads.) Then ganglia are dissociated through enzymatic and

mechanical dissociation. Pictured is a representative image of trypan blue staining following E11.5 dissociation DRG cells. The single cell suspension is stained with cisplatin and fixed for long term storage. Upon staining, individual cell suspension samples are barcoded with heavy metal tag combination, stained with a cocktail of antibodies, and put through CyTOF machine. Mass cytometry data is processed for analysis. (B) Quantification of cells collected from DRG enzymatic dissociations at all ages. N≤ 2. (C) Hierarchical gating strategy to identify viable cell events (barcode separation distance^{high}; mahalanobis distance^{low}; background^{low}; equilibration beads^{low}; high intercalator^{high}; cisplatin^{low}) and neurons (Tuj1+, Islet+). (D) Antibody panel and heatmap of percent of cells positive across development in gated cells or neuronal populations.

Antigen	Cassette	AKA	Vendor
TNFR1	Cell Signaling	TNFR1	BD
Ki67	Cell Signaling	MKI67	BD
Cleaved-Caspase-3	Cell Signaling	Caspase 3 (Cleaved Form)	BD
P75NTR	Cell Signaling	P75 neurotrophic receptor, TNF receptor 16	R&D Systems
CD31	Endothelial Cells	PECAM-1	Biolegend
GFAP	Glia	Glial fibrillary acidic protein	BD Bioscience
CD45	Monoctyes	Tyrosine phosphatase receptor type C	Fluidigm
Tuj1	Neuron	Beta3-tubulin	Gift (A. Spano)
Thy1	Neuron	CD90.2	Biolegend
NeuN	Neuron	Neuronal Nuclei	Novus
N-cadherin	Neuron	N-cadherin	Biolegend
MAP2	Neuron	Mmicrotubule-associated protein 2	Novus
Islet1	Neuron	ISL LIM homeobox 1	Novus
PGP9.5	Neuron	Protein gene product 9.5	Millipore
GLAST	Neuron and Glia	Glutamate asparate transporter	Novus
Galectin-1	Neuron and Glia	Galactose-binding lectin, Gal-1	R&D Systems
Neurofilament-H	Sensory Neuron Subset	Neurofilament-H	Novus
Runx1	Sensory Neuron Subset	Runt-related transcription factor 1	Biolegend
CGRP	Sensory Neuron Subset	Calcitonin gene-related peptide	Bio-Rad
Shox2	Sensory Neuron Subset	Short stature homeobox 2	Abcam
TrkC	Sensory Neuron Subset	Neurotrophic tyrosine kinase receptor type 3	Thermo Fisher
Runx3	Sensory Neuron Subset	Runt-related transcription factor 3	R&D Systems
TrkA	Sensory Neuron Subset	Neurotrophic tyrosine kinase receptor type 1	Millipore
Ret	Sensory Neuron Subset	Ret proto-oncogene	R&D Systems
Parvalbumin	Sensory Neuron Subset	Parvalbumin	Novus
TrkB	Sensory Neuron Subset	Neurotrophic tyrosine kinase receptor type 2	Thermo Fisher

Table 1. Antibody Panel and Vendor.

Mass Cytometry identifies previously defined and novel population changes throughout development

However, the power of single cell analysis via CyTOF is masked in grouped heatmap analysis, where many expression patterns between many different cells types are averaged together. DRGs contains multiple neuronal and glial subtypes with diverse functionality. A serial enzymatic and mechanical-based dissociation may selectively eliminate populations, resulting in a non-representative single-cell suspension. However, our mass cytometry data identify a spectrum of cell types, including epithelial cells, monocytes, glial cells, and neurons in our dissociated samples (Figure 2). We identify near reciprocal expression of most markers between epithelial (CD31), monocyte (CD45), and glial (GFAP) markers and neuronal markers (Islet1+) (Figure 2). Whereas, multiple neuron markers, such as NeuN, PGP9.5, and Islet1, are found within the same cells, suggesting that we are not selectiving losing cells (Figure 2). Overall, this high-dimensional analysis simultaneously probing for canonical cell type markers demonstrates that we can identify classic macro cell populations; however, this informative analysis may identify novel and intermediate cell types.

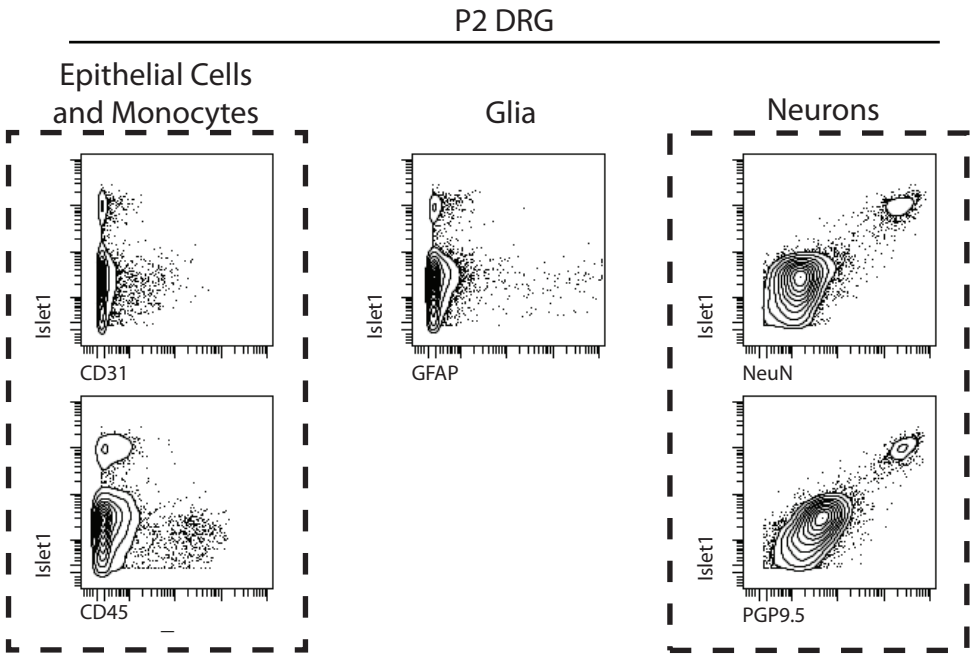

Figure 2. Neuro-CyTOF identies distinct cell populations from mouse DRGs. Biaxials from cells from P2 DRGs detecting monocytes, epithelial cells, glial subsets, and neurons.

Progressive differentiation, accumulation, and diminishment of sensory neuron subtypes

Migrating neural crest cells coalesce to form the DRG in mice between E9.5 and E11.5. Following this, the majority of developmental transitions and terminal differentiation events in neurons are thought to occur between E11.5 and P7 (Marmieger and Ernfors, 2007; Lallemend and Ernfors, 2012). As with many other neuronal systems, DRG cell types infiltrate, proliferate, and terminally differentiate at different rates. We used a development-focused antibody panel, designed and validated through flow cytometry (data not shown), to track these neural precursors as they terminally differentiate.

We examined DRG cells from E11.5, E15.5, P0, P2, and P4 animals, and investigated the relative proportions of cellular subtypes through hierarchical gating and biaxial plots (Figure 3A-B). Neurogenic differentiation predominantly precedes gliogenic differentiation in the central and peripheral nervous system (Marmigere and Ernfors, 2007; Jessen and Mirsky, 2005). Through mass cytometry in the somatosensory system, Islet+ neuronal populations were the predominant macro-population at E11.5, E15.5, and P0, but decreased to 3.48% at P1 (Figure 3A-B). However, we also observed a slight drop at E17.5, which can likely be attributed to dissociation variability and could be rectified with an increased sample size. Whereas Islet+ is solely expressed in post-mitotic

neurons, Sox10 is expressed in multiple cell types at different developmental stages (Jessen and Mirsky, 2005). For example, in early development, all neural crest-derived cells are Sox10+ (Lallemend and Ernfors, 2007). However, immature neurons downregulate Sox10, while Schwann cell precursors and mature Schwann cells maintain Sox10 expression (Jessen and Mirsky, 2005). CyTOF analysis identifies a peak Sox10+ population at E17.5, a period that likely represents a stage where Sox10+ Schwann cell precursors and immature Schwann cells are proximal to the DRG, but have yet to distally migrate towards nerves (Jessen and Mirsky, 2005). Similarly, GFAP+ Schwann cell precursors increase from 0.44% of the cellular population at E11.5 to a peak of 4.25% at P0 (Figure 3A-B). These data demonstrate that Neuro-CyTOF can faithfully recapitulate the dynamism in multiple cellular populations over development.

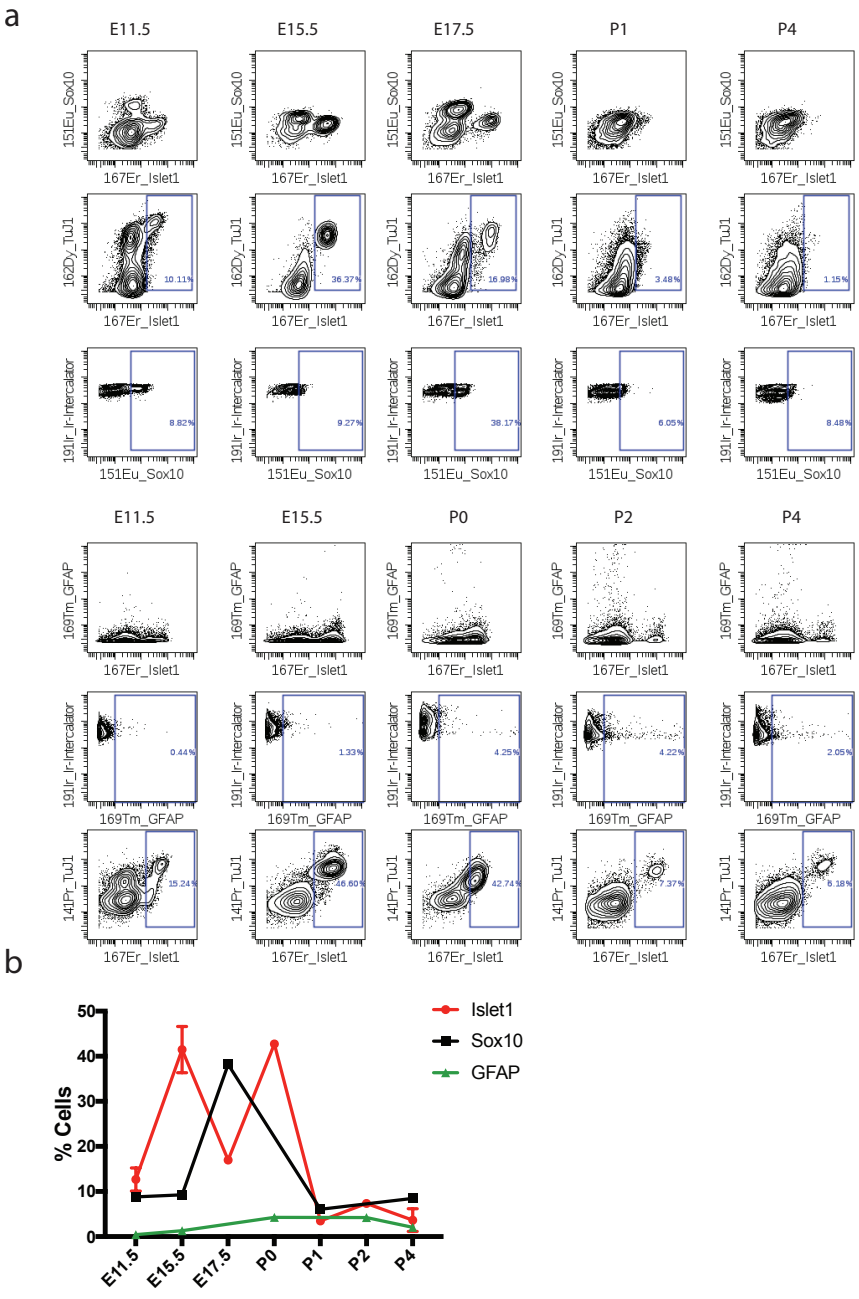

Figure 3. Cell type representation shifts throughout development. (A) Biaxials of Sox10+, Islet+, and GFAP+ population across development. (B) Quantification of percent in each gated populations following pre-processing. N = 1-2 litters.

Mass Cytometry analysis identifies waves sensory neuron subsets differentiation

Sensory neurons subsets differentially rely on different neurotrophins for trophic-based development, and neurotrophin receptor expression can be a proxy of sensory neuron subset identification (reviewed in Lallemend and Ernfors, 2012). Therefore, we further validated CyTOF using a Flag-TrkA knock-in mouse, whereby the Flag epitope is inserted in frame into the locus for the neurotrophin receptor TrkA (Sharma et al., 2010). We found that Flag antibody correlated with TrkA antibody in Flag-TrkA P1 knock-in mice (94.3% of Flag+ cells) (Figure 4A-C). In early postnatal mice, TrkA is predominantly expressed in two populations of somatosensory neurons: (1) peptidergic neurons that maintain TrkA expression, and (2) non-peptidergic neurons that progressive downregulate TrkA and upregulate expression of the neurotrophin receptor Ret (Molliver and Snider, 1997; Molliver et al., 1997). CyTOF-processed P1 FLAG-TrkA DRGs reveal that a large portion of Ret+ cells (47.67%) are also Flag+ at P1, which reflects the transient TrkA+;Ret+ population that occur prior to late ret+ terminal differentiation of RA LTMRs (Figure 4A-C) (Molliver et al., 1997). We also observed minor populations of TrkB+; Flag+ and TrkC+; Flag+ neurons (2.59% and 24.35% colocalization, respectively) (Figure 4B-C). This is consistent with previous studies identifying a small population of TrkA+;TrkC+ neurons at P0 (Genc¸ et al., 2004). The functional significance of these populations

remains unclear. These data not only further validate several of the antibodies used in this study but also validate that data collected from mass cytometry is comparable with previously published data sets (Farinas et al., 1999; Molliver et al., Genc et al., 2004) .

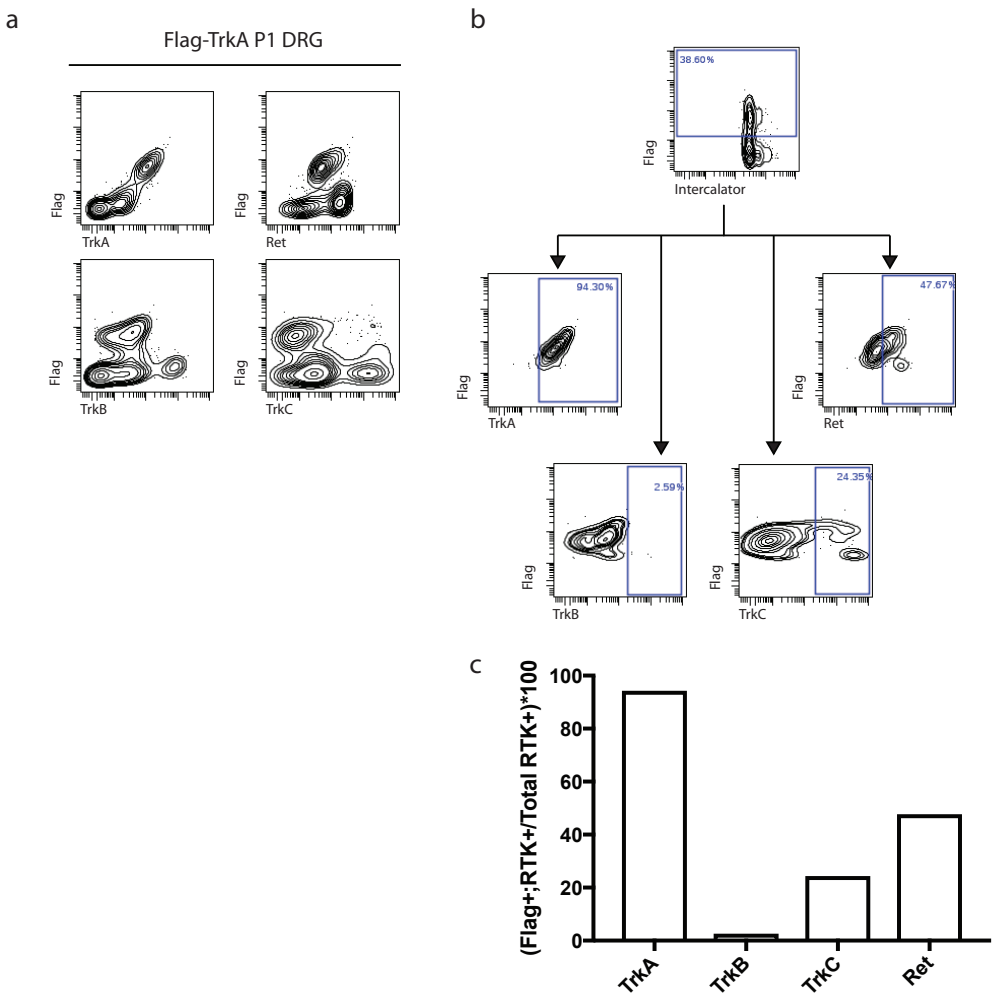

Figure 4. Flag Reporter mice validates CyTOF staining. (A) Biaxials from P1 DRG from P1 Flag-TrkA mice against indicated RTKs. (B) Biaxials with hierarchical gating strategy to identify Flag+;RTK+ events. (C) Quantification of cells (RTK+;- Flag+/Total Flag+)*100. N=1 litter.

CyTOF identifies shifts in neuronal populations that are consistent with previously performed immunohistochemistry studies (Farinas et al., 1998; Cheng et al., 2018). Neurogenesis in the DRG is stereotyped and is generated from three sequential waves of migration of neural crest-derived progenitors (Ma et al., 1999; Maro et al., 2004; reviewed in Marmigere and Ernfors, 2007). The first wave of migration delivers progenitors that primarily generate terminally differentiated TrkB+, TrkC+, and a subset of Ret+ sensory neuron populations starting at E9.5 in mice (Ma et al., 1999). A second neural crest-derived progenitor population begins to migrates ventrally to the DRG at E10 and predominantly generates TrkA+ sensory neuron populations (Ma et al., 1999). The last neural crest-derived progenitor population temporarily resides as boundary cap cells and migrates at E11; these progenitors predominantly differentiate into TrkA+ neurons and glial cells (Maro et al., 2004). As expected, during nascent gangliogeneis (E11.5), the predominant population of sensory neurons are TrkC+ (Figure 5A-B). However, by E15.5, TrkA+ neurons become the predominant population (Figure 5A-B). Interestingly, a Ret+ population arises from a transiently immature population of TrkA+ neurons that downregulate TrkA expression and upregulate Ret, termed late (l) Ret, starting at E15.5 (Luo et al., 2007) (Figure 5C-D). Our CyTOF analysis reflects this differentiation nexus, whereby Ret+ expression increases from 13.34% of Islet1+ at E17.5 to 44.77% of Islet1+ in P0 DRGs (Figure 5A-D). Upon further maturation,

TrkA and Ret+ colocalization decreases, demonstrating progressive terminal differentiation postnatally(Figure 5C-D).

FLOW-MAP visualization subsamples individual cells into 500 nodes, each of which is located relative to the molecular similarities between nodes within a set of manually preset parameters (Zunder et al., 2015). FLOW-MAP analysis of cells from early gangliogenesis built around the quantal measurements of CD45, CD31, Isl1, PGP9.5, GFAP, TrkA, TrkB, and TrkC to identify distinct cell types (Figure 6A). At E11.5, Islet+ neurons segregate into three distinct population that are (1) TrkC+; TrkB-; TrkA-, (2) TrkC+; TrkB+; TrkA-, (3) TrkA+;TrkC-; TrkB-, which represent the largest populations found in previous literature (Figure 6A) (Cheng et al., 2018; Farinas et al., 1998).

FLOW-MAP compiled from E11.5, E15.5, P0, P2, and P4 DRG cells illustrate how these temporally defined progenitor populations change over developmental time. The progressive protein expression changes that occur during differentiation can be recapitulated through FLOW-MAP analysis. We were able to create a network of subsampled nodes and identified nodal populations as they shift as a function of time and expression (Figure 6B). Connections between nodes represent potential stages of differentiation. FLOW-MAP unsupervised analysis identifies two presumptive progenitor populations that differentiate into (1) an earlier

population that is TrkC+ and/or TrkB+, and (2) a later TrkA+ population. The originating population that generates TrkC+ and/or TrkB+ neurons is presumably from the first migratory progenitor wave (Ngn2+). In contrast, the TrkA+-fated progenitor populations are likely from the second and third wave of migrating progenitors. Further antibody panel refinement and design will allow for increasingly distinguishable subpopulations.

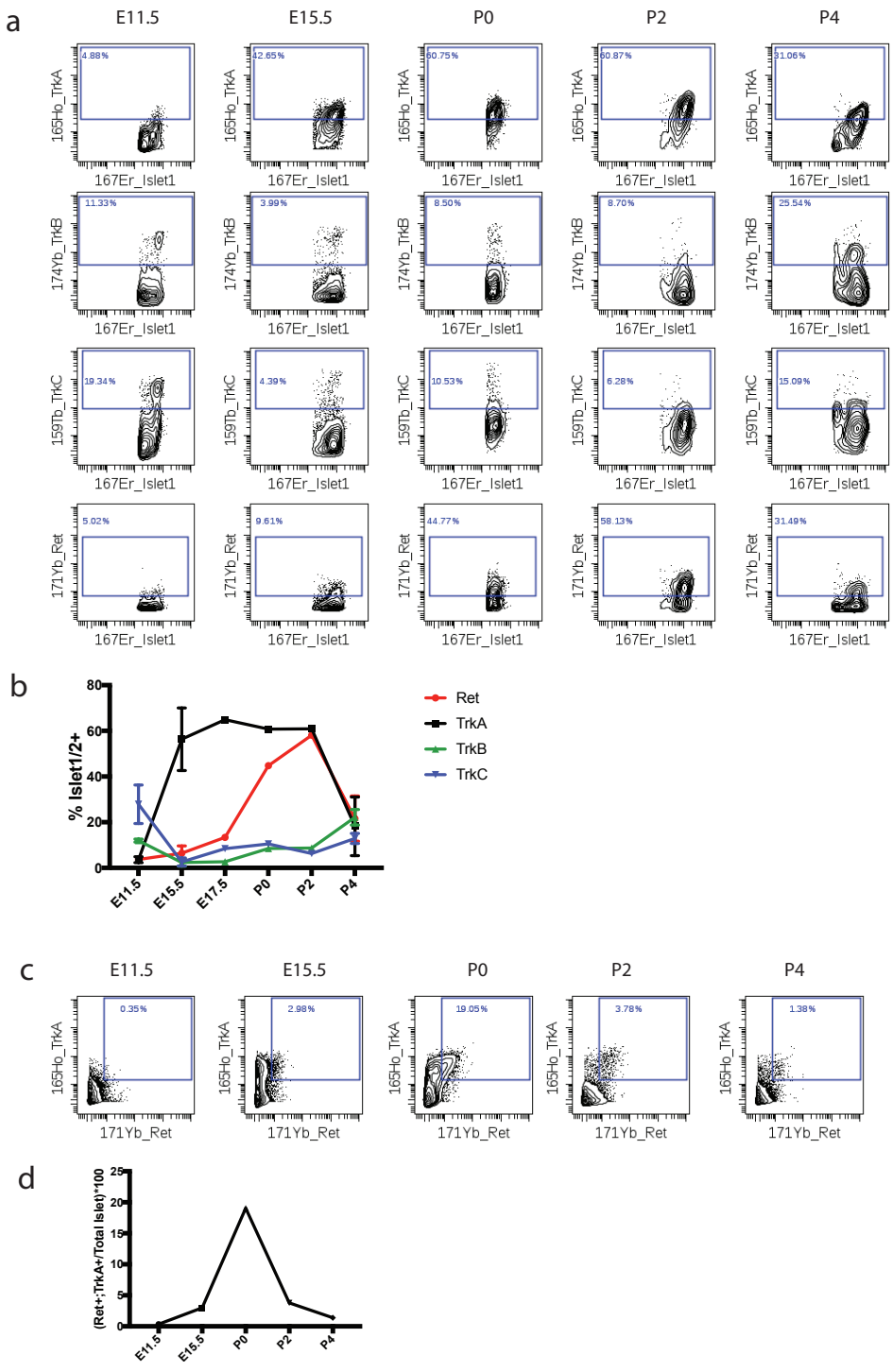

Figure 5. Neuronal populations populate the DRG in asynchronous waves in development. (A) Biaxials of TrkA+, TrkB+, TrkC+, and Ret+ and Islet+ population across development. (B) Quantification of percent in

each gated populations in Islet1+ gated populations. (C) Biaxials identifying the Ret+; TrkA+ population across development. (D) Quantification of percent Ret+; TrkA+ in Islet1+ gated populations. N= 1-2 litters.

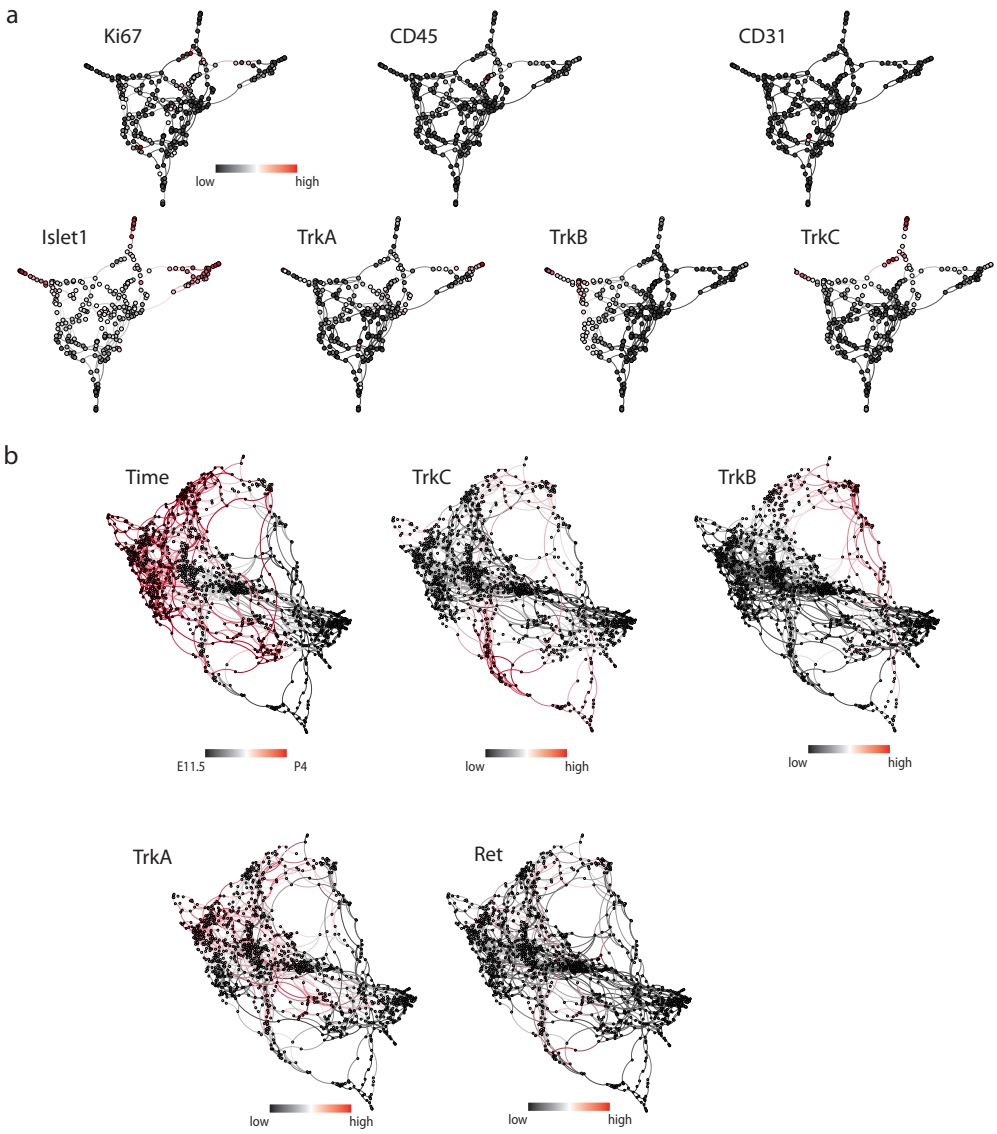

Figure 6. FLOW-MAP identifies the progressive differentiation, accumulation, and diminishment of sensory neuron subtypes. (A) FLOW-MAP from E11.5 DRG. Scale is by expression. (B). FLOW-MAP compiled from E11.5, E15.5, P0, P2, and P4 DRG samples. Color scale indicates time/age or expression levels. N=1 litter.

Discussion

Comprehensive cell profiling (i.e. scRNA seq) has been nothing short of miraculous for our understanding of the composition of several organ systems including the nervous system. While these approaches give us a good clue about the molecular identity of many different cell types, they don't inform cellular function. For that we need comprehensive single cell analysis at the protein level, permitted by mass cytometry. Mass cytometry is a flow cytometry alternative that uses antibodies conjugated to metals instead of fluorophores to detect the relative abundance of various epitopes. Because we are no longer limited by spectral overlap of fluorophores we can detect the relative abundance of roughly 50 epitopes on a single cell at a rate of 1 million cells per hour via mass spec. If we choose the right antibodies and epitopes, this should be sufficient for complete coverage of an organ system (50 to the 50th marker combinations). The speed and depth of coverage allows us to finish a project that would take 5 years using conventional approaches in a matter of months with tissue collection being the limiting factor. Mass cytometry can define new cell types, allow for rapid classification of ambiguous cell types, and distinguish small variances between cell types in the nervous system, a tissue known for asynchronous development (Reemst et al., 2016). Additionally, mass cytometry allows for assessment of multiple signaling pathways at discrete developmental transition, which cannot be accomplished with other single cell profiling approaches (Bendall et al., 2014).

During embryogenesis, neural crest-derived progenitors migrate ventrally to form DRGs along the spinal cord, and these progenitors progressively differentiate into a diverse population of neurons and glial cells (Marmigere and Ernfors, 2007). In our study, we perform mass cytometry on DRGs during this dynamic temporal window. We collected samples at daily timepoints to identify progressive changes in molecular profile over the course of development. Our analysis utilizes canonical markers, such as cell-type specific transcription factors and neurotrophin receptors, to identify different cell populations. We find that the constitution of our samples reflect accurate proportions of each cell type seen throughout development (Lallemend and Ernfors, 2012; Marmigere and Ernfors, 2007). For example, we identify glia and neuron subsets at populations that reflect those seen in previous literature.

Future studies building on this work will interrogate fundamental questions in development, including how extrinsic signals intersect with intrinsic programming to define distinct cell types with unique proteomic profiles and functions. For example, NGF-TrkA signaling regulates the transcription factor Runx1 to allow for differential intrinsic programming cascades and divergent pathways into two distinct nociceptive neuron populations (Luo et al., 2007). However, a systematic investigation into the developmental role of extrinsic cues, such as the neurotrophins NGF, NT-

3, BDNF, and GDNF in differentially biasing or supporting the survival of each sensory neuron subpopulation has not been performed. Such studies could provide insight into how these structurally and functionally similar extracellular cues can compensate for one another in development.

CyTOF can distinguish between membrane-bound protein and vesicular protein through sequential staining of differently metal-labeled antibodies against the same neurotrophin receptor. Neurotrophin induced signaling cascades occur (1) locally at neurotrophin receptors on the plasma membrane and (2) across long-distances through trafficked signaling endosomes containing neurotrophin receptors (Harrington and Ginty, 2013; Kuruvilla et al., 2004). Intracellularly trafficked TrkA endosomes actively signal *en route* and at the cell body, where they initiate transcriptional (survival) and non-transcriptional (i.e. synapse formation) dependent developmental events . Mass cytometry promises the deepest conceptualization of neurotrophic signaling to date.

There are a few drawback to this approach that we will work to mitigate in the future. Neuronal tissue exists as a dense interwoven mesh of cell bodies and axons. In order to achieve a single cell suspension for CyTOF analysis, we must dissociate target tissues with proteases and mechanical force. Our results are able to distinguish distinct cell types, including neuronal and glial subtypes. However, these aforementioned dissociation

steps contribute to limitations, including (1) loss of physiological signaling associated with a multi-hour protocol, and (2) loss of spatial information.

Importantly, CyTOF permits quantification of antibody-based post-translational modifications, allowing us to probe activation of multiple cell signaling cascades simultaneously on a single-cell level (Bendall et al., 2014). The current dissociation protocol requires an extended dissociation process, such that we cannot yet investigate short-lived signaling. However, rapid fixation following *in vitro* studies coupled with mass cytometry show great potential. Canonical signaling cascades intersect with each other through positive and negative-feedback loops. The ability to quantify several signaling events simultaneously at temporal snapshots would provide new insights into the biochemical pathways required during development. Neurotrophin signaling mediates a host of growth processes in development via activation of multiple pathways, include MAPK, PI3K, and PLCγ (Harrington and Ginty, 2013). The differential activation of these pathways can further delineate how neurotrophins promote divergent processes such as survival axon growth and synapse formation (Harrington and Ginty, 2013).

While single cell analysis via mass cytometry has several advantages (enumerated above), the act of dissociation loses valuable anatomical information (i.e. protein localization and cell morphology). Nervous system

function relies not only on a molecularly diverse pool of neural cells, but also on connectivity between these populations. Future studies using mass cytometry-compatible tracing reagents could rapidly define the connections made by specific neuronal subpopulations. Mapping synaptic connectivity is a promising strategy to understand the function and behavior of neural circuits. The reagents used for this method could include proteins and viral particles that are transported in the cell along neuronal processes, and are able to cross the synapse (Lerner et al., 2016).

Metal-conjugated antibodies designed for CyTOF can also be utilized for emergent technologies that do not lose anatomical information. Multiplexed ion beam imaging (MIBI) uses the same principles of CyTOF, however, MIBI utilizes ion beams on tissue sections stained with metal-conjugated antibodies (Angelo et al., 2014). MIBI technology has nanometer resolution while simultaneous analyzing over 100 parameters (Angelo et al., 2014; Rost et al., 2017). Both directed synaptic tracing and imaging CyTOF-based studies can supplant current visualization techniques and provide immense insight into neuronal and subcellular architecture.

Collectively, our high dimensional analyses demonstrate replicable ground truths associated with sensory development. This technique provides

inroads for future studies to ask fundamental developmental questions at a pace and resolution unmatched in history.

Chapter IV. Conclusion and Future Directions

Abstract

A functional somatosensory circuit is composed of diverse populations of sensory neurons detecting many different environmental signals and relaying those signals to appropriate targets in the CNS. The development of the sensory system is relatively stereotyped and is therefore an accessible model to investigate diversification of cell types in development. We find that the temporal pattern of migration of sensory neurons into the dorsal root ganglia (DRG) is later reflected in the temporal pattern of death in sensory neurons (Chapter II). This insight can be attributed to intrinsic transcriptional differences and responsiveness to different cues. We also iteratively investigated somatosensory system development through mass cytometry, using a panel of antibodies to characterize differentiation of populations of neurons and glia (Chapter III). Through this technique, we are able to precisely track the development of the sensory system from immature progenitors to terminally differentiated neurons. We identify mass cytometry as a viable method to rapidly identify cell populations as they undergo molecular diversification throughout development; this provides the platform for future multi-spanning investigations.

Sequential Waves of Naturally Occurring Death of Sensory Neuron Development

We sought to identify how the nervous system is constructed by investigating windows of cell death. We find that sensory neuron death occurs in concurrent and overlapping waves that reflect the sequential waves of migration of neural derived progenitor to the DRG (Cheng et al., 2018). More specifically, in that study, we find earlier-arriving TrkC+ and TrkB+ cells complete their window of cell death prior to the later-arriving TrkA+ populations (Cheng et al., 2018). The window of cell death in TrkA+ neurons is also longer than that of TrkB+, TrkC+, and eRet neurons (Cheng et al., 2018). The relatively expansive window of TrkA+ death could result from the more expansive window of migrating progenitors fated to be TrkA+ (Ma et al., 1999; Maro et al., 2004). Overall, this suggests that while these neuron subtypes undergo cell death in overlapping but distinct windows.

Multiple Death Receptors Can Mediate Multiple Destructive Processes

The sequential pools of migrating neural crest cells generate a functionally and molecularly diverse array of neurons in a stereotyped fashion. These cells are exposed to a similar milieu of extracellular cues, but each lineage follows a developmental lineage at its own pace (Marmigere and Ernfors, 2007; Cheng et al., 2018). We asked if the pan-neuronal marker p75NTR was a master regulator of competence for survival. We find that the p75NTR is required to temper developmental cell death in TrkA+, TrkB+,

and TrkC+ populations, but not eRet populations (Cheng et al., 2018). Additionally, we find that p75NTR is expressed in neurons beyond the typical window of cell death yet p75NTR does not govern the length of this window (Cheng et al., 2018). Overall these data clarify that p75NTR is required to support cell survival in a cell-type specific manner during periods of naturally occurring cell death, however the mechanism of p75NTR-dependent survival remains unclear.

The mechanism of p75NTR in survival has been difficult to assess because p75NTR is multifunctional. p75NTR is a death receptor in the Tumor Necrosis Factor Receptor Superfamily (TNFRsf), and therefore is capable of promoting apoptosis through the extrinsic pathway (Haase et al., 2008). However, p75NTR functions in a cell-type specific manner. For example, p75NTR is critical to support survival in sensory neurons, whereas p75NTR refines the sympathetic nervous system by promoting cell death, axon pruning, and synapse elimination (Bamji et al., 1998; Lee et al., 1994). The functional disparity in p75NTR between sensory and sympathetic neuron function is likely due to a combination of differences in ligand availabilty, downstream signaling and p75NTR coreceptor partners (Barker et al., 2007).

Additionally, p75NTR can also signal through cell autonomous and non-autonomous mechanisms (Chen et al., 2017). Islet1-driven Cre-excision in

presumptive sensory neurons results in typical survival of sensory neurons in all analyzed cell types except non-peptidergic nociceptors (Chen et al., 2017). These results taken with the results identifying cell loss in mice lacking *p75NTR* in the germline suggests that p75NTR may support neuronal survival through neighboring cells, such as BFABP+ satellite glial cells (Chen et al., 2017).

Interestingly, we recently found that in addition to p75NTR, the highly related TNFR family members Tumor Necrosis Factor Receptor-1 (TNFR1) and Death Receptor-6 (DR6) are also expressed in sensory neurons during development (Wheeler et al., 2014). Simultaneous expression of a trio of similar TNFRsf death receptors could contribute to cell-specific and process-specific developmental refinement in the sensory system. Interestingly, TNFR1 and DR6 participate in destructive processes much like p75NTR, in the sympathetic nervous system. Specifically, TNFR1 has been reported to participate in axon pruning in peptidergic nociceptors sensory neurons and cell death in sympathetic neurons, and DR6 is implicated in sensory and motor neuron degeneration (Wheeler et al., 2014; Barker et al., 2001; Gamage et al., 2017). It is plausible that, similar to neurotrophin receptors dictating differentiation, a variety of death receptors in sensory neuron subsets could underlie how neuronal populations diversify and respond differently to extrinsic cues.

Transcription Factors Dictate Sensory Neuron Diversification

Recent single cell RNAseq data have identified 17 subtypes of putative somatosensory neurons, possessing distinct transcriptomic profiles (Usoskin et al., 2015). Differential expression of these profiles start to mediate progressive segregation of each subtype by the time progenitors migrate to the DRG (Marmigere and Ernfors, 2007). Each wave of migrating neural-crest derived progenitors produces a stereotyped pool of terminally differentiated somatosensory neurons. Interestingly, these progenitors already present intrinsic differences, where the first wave expresses the transcription factor Neurogenin1 (Ngn1), and the second wave expresses Neurogenin2 (Ngn2) (reviewed in Marmigere and Ernfors, 2007). These early diverging progenitors (Ngn2+ and Ngn1+) generate different terminally differentiated populations. Ngn2-expressing cells predominantly generate proprioceptors and mechanoreceptors, whereas Ngn1 expressing cells predominantly generate nociceptors (Ma et al., 1999). Such intrinsic transcriptional programming allows for progressive differentiation of precursors fated to be somatosensory neurons.

High-Dimensional Analysis of Individual Sensory Neurons

Low-dimensional and low-throughput methods has prevented us from expansively investigating the interplay between intrinsic transcriptional cascades and extracellular cues (Lallemend and Ernfors, 2012).

Furthermore, as neuroscience continues to advance, we as a community are finding new markers to identify new cell types; however, oftentimes cell types can only be identified using an intersectional labelling approach. Recent technological advances in the field, such as single cell RNAseq and mass cytometry, have overcome previous limitations by providing multidimensional analysis of a single cell (Spitzer and Nolan, 2016).

Mass cytometry, commercially known as CyTOF, is mass spectrometry on single cell suspensions stained with antibodies coupled to isotopically pure heavy metals (Spitzer and Nolan, 2016). This mass-based analysis of protein expression currently allows for precise quantification of expression of about 40 markers simultaneously (Spitzer and Nolan, 2016). This high-dimensional analysis allows researchers to investigate combinatorial code that may function in a cell-type specific manner. Our CyTOF analysis recapitulates the results with conventional lower throughput approaches, where we see a progressive diversification of cell types within the sensory systems. In Chapter III, we find accurate representation of all analyzed cell types, such as neurons and glia. Furthermore, we are able to identify shifts in relative proportions of sensory neuron subsets, marked by their neurotrophin expression, throughout development.

High throughput single cell analysis allows researchers to investigate progressive differentiation and identify close relationships. In Chapter III,

we utilized FLOW-MAP (force-directed layout of a weighted graph containing multidimensional agglomeratively clustered points) analysis to identify the progressive processes of development by creating a network of randomly sub-sampled Islet1+ neurons. FLOW-MAP constructs a network where individual cells are spatially mapped based on similarities between designated parameters, and cells mapped closer together represent more similarity. Our analysis is able to identify distinct and diverging populations of sensory neurons, such as non-overlapping populations of TrkA+ neurons and TrkC+ neurons. Furthermore, FLOW-MAP analysis across concatenated cell samples from multiple developmental periods correctly showcases the progressive differentiation of immature neurons into mature terminally differentiated neurons (Chapter III). We demonstrate that FLOW-MAP, a non-biased computational approach, can replicate the known developmental trajectory elucidated from traditional lineage tracing studies.

Similar to other forms of high-dimensional analyses, these data will inform and direct future hypotheses. However, all analyses must be reinforced with parallel investigatory techniques. Immediate tangible questions include screening how somatosensory populations shift in transgenic animals to rapidly phenotype population shifts.

High Dimensional Analysis Permits Rapid and Informative Analysis of Morphology and Circuit Formation

Our current usage of CyTOF requires dissociation of intact neural tissue into a single cell suspension, resulting in loss of crucial spatial information. However, in the future, we will capture this critical information using Multiplex Ion Beam Imaging (MIBI), a recent advance in mass-based immunostaining where an ion beam releases tissue stained with metal-conjugated antibodies and spatial information is reconstructed post-hoc (Angelo et al., 2014). MIBI marries the increased parameterization associated with CyTOF with the informative spatial information associated with classic fluorescence immunohistochemistry (Angelo et al., 2014).

MIBI can identify how somatosensory neurons precisely project into the spinal cord lamina and synapse onto correct post-synaptic neurons (Chen et al., 2003). Previous literature suggests that such precise target matching are dictated by attractive and repressive cues, such as PlexA1 and Sema6D which mediates proprioceptive-specific axons but not axons from peptidergic neurons (Yoshida et al., 2006). For example, a rapid MIBI screen of adhesion proteins, including cadherins, protocadherins, and semaphorins, could provide insight into target matching.

MIBI provides robust spatial information on a nanometer scale, allowing us to ask questions concerning cytoarchitecture at subcellular levels. High-dimensional analysis would permit analysis of protein trafficking and subcellular localization endosomes, potentially identifying pockets of critical signaling domains. In neurotrophin-dependent neurons, neurotrophin signal endosomes are diversified in different endosomal compartment; this diversification may underlie how neurotrophins can engage in differential growth processes, such as survival versus synaptogenesis (Barford et al., 2018). MIBI-based high-dimensional analysis simultaneously investigating endosomal populations, localization, and downstream signaling activation would provide foundational knowledge as to how one signal engages in numerous processes .

High Dimensional Analysis Permits Rapid and Informative Analysis of Cell Signaling

Signaling cascades are key executioners which lead to the morphological and transcriptional changes that lead to progressive differentiation into disparate cell types. Mass cytometry can probe simultaneous signaling pathways through immunostaining-based investigation of post-translational modification (Bendell et al., 2014). Historically, it has been difficult, to delineate how neurotrophin-dependent signaling pathways, such as PI3K, MAPK, and PLCγ, differs between neurotrophins and cell types. Conclusions are difficult to dissect because signaling cascades are

intersectional and are involved in concurrent positive and negative feedbacks. For example, the PI3K signaling cascade is required for neurotrophin signaling endosome formation, which continues to activate the MAP kinase cascade intracellularly (Ceni et al., 2014). However, multi-dimensional analysis of signaling pathway activation on a single cell level could provide insight into cell type-specific signaling in a variety of conditions.

It is unclear how neurotrophin signaling differs downstreams of each neurotrophin receptor activation (e.g. TrkA versus TrkB versus TrkC). The majority of studies investigating neurotrophin-dependent signaling has been performed in NGF/TrkA-dependent sympathetic neurons and the findings have largely been assumed to apply to all neurotrophin-dependent neurons. Interestingly, neurotrophins are sufficient to shift population profile; TrkC expressed from the TrkA locus results in sensory neuron population shift towards a primarily proprioceptive population and against a nociceptive population (Moqrich et al., 2004). If neurotrophin receptor replacements are capable of shifting cell fate, this suggests that neurotrophin signaling is not uniformly trophic and actively instructs differentiation (Moqrich et al., 2004). Furthermore, neurotrophin signaling can promote differential growth processes, such as survival, axon growth, and synapse formation, in a localization-specific manner. Multidimensional analysis in *in vitro* studies using microfluidic devices, that spatially and

fluidically isolate cell bodies from axons, could link subcellular differences in signaling directly to function.

Implications in Disease and Injury

Insights into basic sensory system development can be utilized to identify therapeutic targets. In Chapter II, we identify p75NTR as an actively regulator of the magnitude, but not temporal window, of cell death in the sensory. Previous studies implicate p75NTR in injury-induced axon degeneration and Alzheimer's disease (Gamage et al., 2017; Hu et al., 2013). Our insight into the temporally-restricted role of p75NTR in developmental cell death provides insight into the tight regulation of death in the sensory system during development. Furthermore, if we can understand the unique cellular pathway associate with p75NTR in naturally occurring cell death, we can co-opt this pathway for survival and revival of cells.

In Chapter III, we perform a high-dimensional temporal analysis of sensory system development. This expansive analysis of neuronal development provides the critical foundation for future studies. Our fundamental insights can be directly applicable to the stem cell field. At present times, neurons derived from iPSCs can only reliably produce select neuron types limiting therapeutic applications. Furthermore, it is still understudied how exogenous cells incorporate into a circuit (Blanchard et al., 2014). Further

analysis of the basic biology behind the intrinsic and extrinsic cues that mediate construction of the nervous system may lend cues into disease treatments.

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Appendix I. The Role of p75NTR and TNFα in Proprioception

Portions of this appendix were adapted from Lucy Jin's, B.A., University of Virginia Thesis.

Introduction

The proprioceptive nervous system provides information on the position and movement of body parts, namely limbs, in space. The ligand-receptor pair, Neurotrophin 3 (NT3)-TrkC represents the pro-construction signal required for proprioceptor survival and axon growth. In addition to pro-constructive signaling, pro-destructive signaling is required for fine-tuning and refinement of the proprioceptive system. Here, we investigate the potential role of TNFRSF members in destructive signaling through immunohistochemical and behavioral analyses on knockout mice lacking TNFRSF members and ligands.

Methods

Animals

All experiments were carried out in compliance with the Association for Assessment of Laboratory Animal Care policies and approved by the University of Virginia Animal Care and Use Committee.

Tissue Processing

Tissue collection was performed as previously described (Cheng et al., 2018).

Immunostaining

Immunostaining was performed as previously described (Cheng et al., 2018). Primary antibodies: Rabbit anti-Parvalbumin (Swant PV27, 1:5000), Goat anti-TrkC (AF1404, 1:500), and Mouse anti-Islet (Neuromics 39.4D5, 1:100). Following secondary antibody incubation, slides were washed 3 times for 5 minutes with 1x PBS and mounted in Fluromount-G DAPI (SouthernBiotech).

Quantification of Images

Quantitative analyses were performed with ImageJ and Excel. The genotypes of the mice were blinded beforehand. Parvalbumin-positive cells in both the left and right DRG of L4 were individually counted for each section of the spinal cord. For each mouse, the averages of the left and right DRG for each section were summed and multiplied by 3 to produce the total number of Parvalbumin-positive cells per DRG.

Gait Analysis

Gait was scored as either normal or abnormal based on positioning of the hind limbs as the mice walked. Observations were blindly performed on 6-7 week old littermate mice.

Rotarod Test

6-7 week old littermate mice were initially trained on the rotarod for 4 days. At noon each day, mice were placed on the rotarod at a constant speed for a maximum time of 60 seconds. Mice repeated this three more times on the same day with at least 15 minutes between each trial. After training, mice performed the rotarod test for 4 days. The speed of the rotarod was set to increase gradually over time, and the time it took for each mouse to fall was recorded. Mice repeated the test each day after a minimum of 15 minutes following the first trial. The averages of the two trials each day was taken.

Statistical Methods

All statistical analyses were performed with Prism 6 software. Statistics were determined by one-way ANOVA with Bonferroni post-hoc test. Data were assumed to be normal although no formal normalization test was conducted. Data are all presented at mean±SEM in each figure. Statistical significance threshold was set at $p<0.05$ for all analyses.

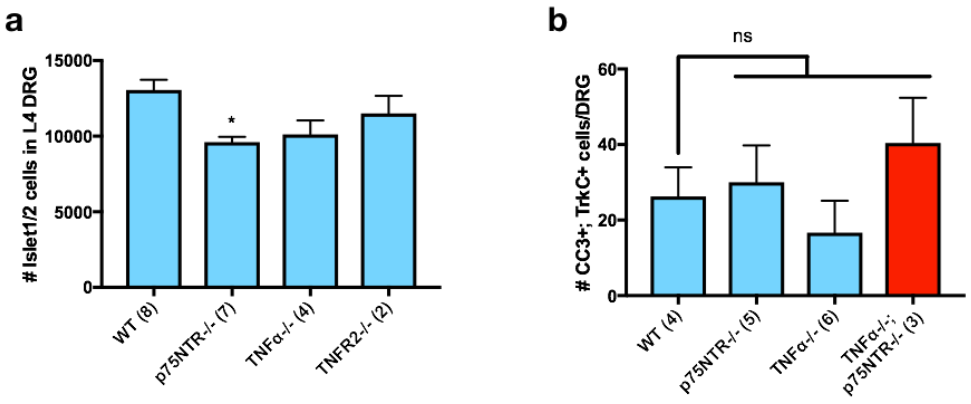


Figure 1. p75NTR is required for normal neuronal cell number. (A) Number of Islet1/2+ neurons in L4 DRG at E14.5. (B) Number of cleaved caspase-3+; TrkC+ neurons in L4 DRG at E14.5. Number of animals tested is included in (). Data represent mean ± SEM. * dictates p<0.05.

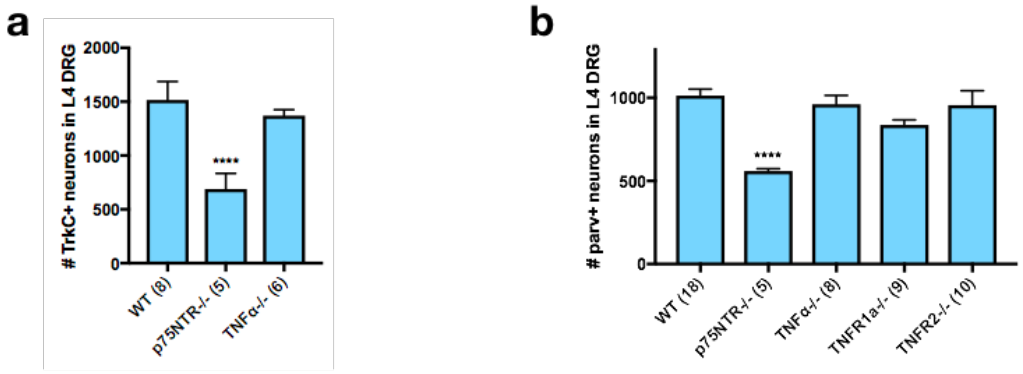


Figure 2. p75NTR is required for normal proprioceptive cell number. (A) Number of TrkC+ cells in L4 DRG at E14.5. (B) Number of parvalbumin+ cells in L4 DRG at p0. Number of animals tested is included in (). Data represent mean ± SEM. **** dictates p<0.0001.

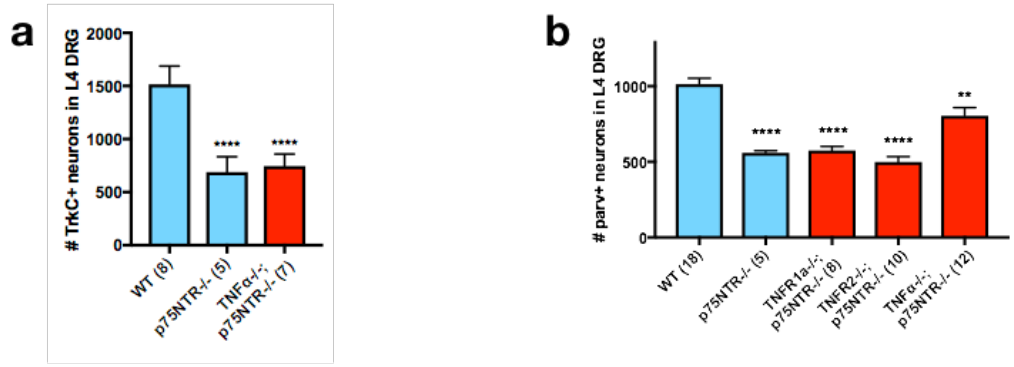


Figure 3. Parv+ proprioceptive cell loss associated with an absence of p75NTR is rescued in animals also lacking TNFα at p0, but TrkC+ proprioceptive cell loss associated with an absence of p75NTR is not rescued in animals also lacking TNFα at E14.5. (A) TrkC+ cells in L4 DRG at E14.5. (B) Parvalbumin+ cells in L4 DRG at p0. Number of animals tested is included in (). Data represent mean ± SEM. **** dictates p<0.0001 and ** dictates p<0.01.

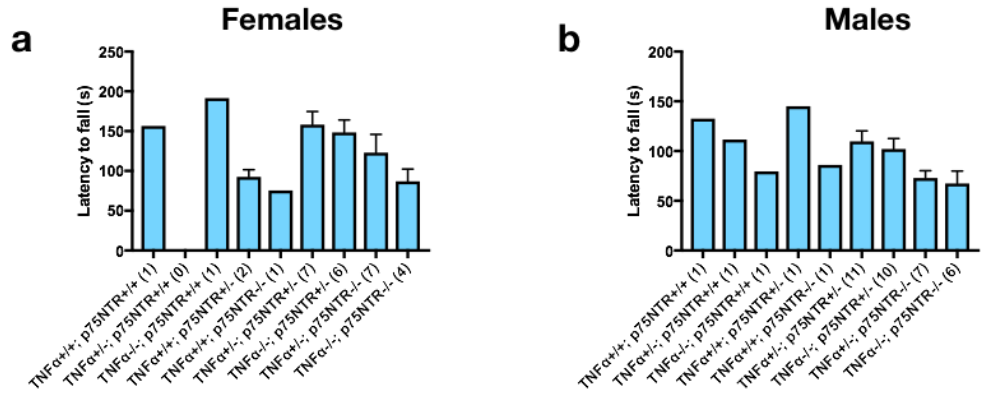


Figure 4. Proprioceptive behavior and locomotion. (A-B) Behavioral sensitivity 7-8 week old littermate mice were tested in an accelerating rotarod test (A – females, B – males). Number of animals tested is

included in (). Data represent mean ± SEM. No statistical test was performed.

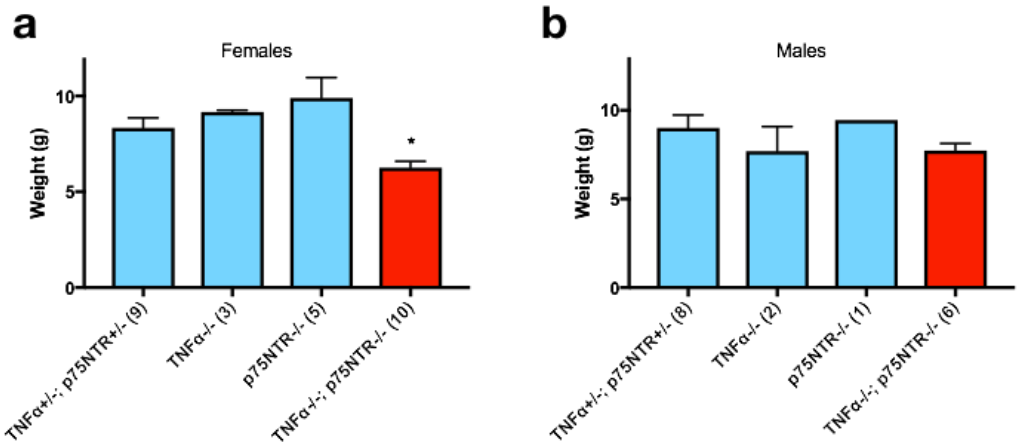


Figure 5. P75NTR and TNFα are required for normal weight. (A-B) Weight of 7-8 weeks old littermate mice (A – females, B – males). Number of animals tested is included in (). Number of animals tested is included in (). Data represent mean ± SEM. * dictates $p<0.05$.

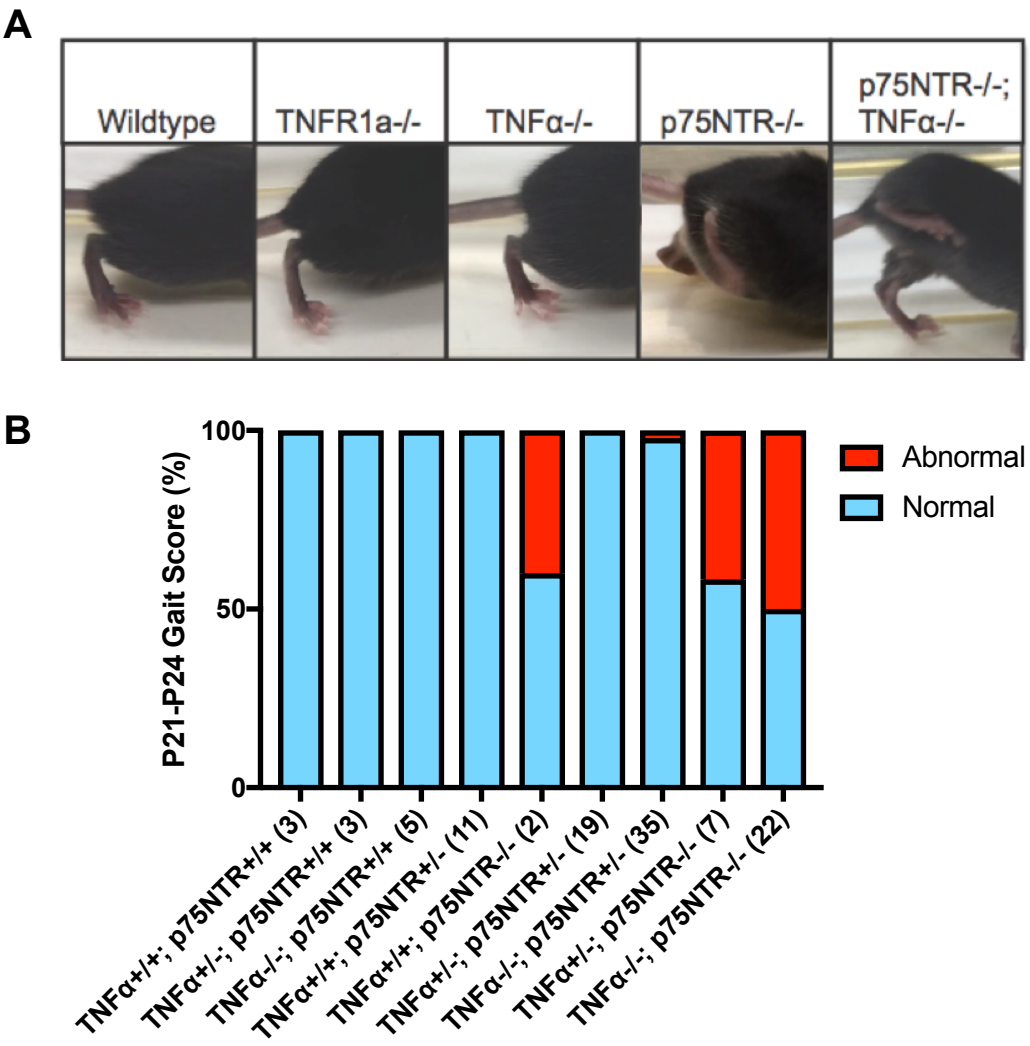


Figure 6. Abnormal hindlimb gait associated with a loss of p75NTR is not corrected through a combined absence with TNFα. (A) Visualization of normal and abnormal (hyperelevated) gaits. (B) Proportion of animals in each genotype with gait corresponding to the categories: Normal versus Abnormal. Littermate animals were tested at 7-8 weeks old. Number of animals tested is included in ().

Conclusion

Consistent with previous literature, our results show that the absence of p75NTR results in reduced proprioceptive cell survival and increased motor impairment (Cheng et al., 2018; Vaegter et al., 2010). The absence of TNFα, though, partially rescues proprioceptor death induced by the absence of p75NTR.

Appendix II. The Role of DR6 in Sensory Perception

Introduction

DR6 has been implicated in injury-induced axon degeneration and developmental axon pruning in sensory neurons (Gamage et al., 2017; Nikolaev et al., 2009). We sought to investigate if DR6 is involved in proprioceptive neuron development and motor and nociceptive function.

Methods

Immunostaining

Refer to Appendix I for immunostaining methods. Antibodies used this study include Rabbit anti-Parvalbumin (Swant, PV27, 1:5000), Guinea Pig anti-CGRP (Bachem, T-5053.0050BA, 1:4000)

Behavior

All behavior was performed in 6-8 week old Bl6 mice unless otherwise noted.

Rotarod

Refer to Appendix I for rotarod experimental design.

Open Field

Open field was performed on Noldus EthoVision XT and data was collected from 15 minute runs.

Hot Plate

Mice were contained on a hot plate heated to a given temperature. The latency to lick the hindpaw was recorded, but the time on the hot plate was capped at 60 seconds. Three trials for each temperature, with a 15 minute break between trials, were performed over the span of three days.

Hargreaves

Mice were manually restrained with each a hindpaw placed on the plantar surface over the infrared heat and the latency to withdraw the hindpaw was recorded. Three trials for each level, with a five minute break between trials, were performed over the span of three days.

Weight

Weight was collected in the morning for all animals across development.

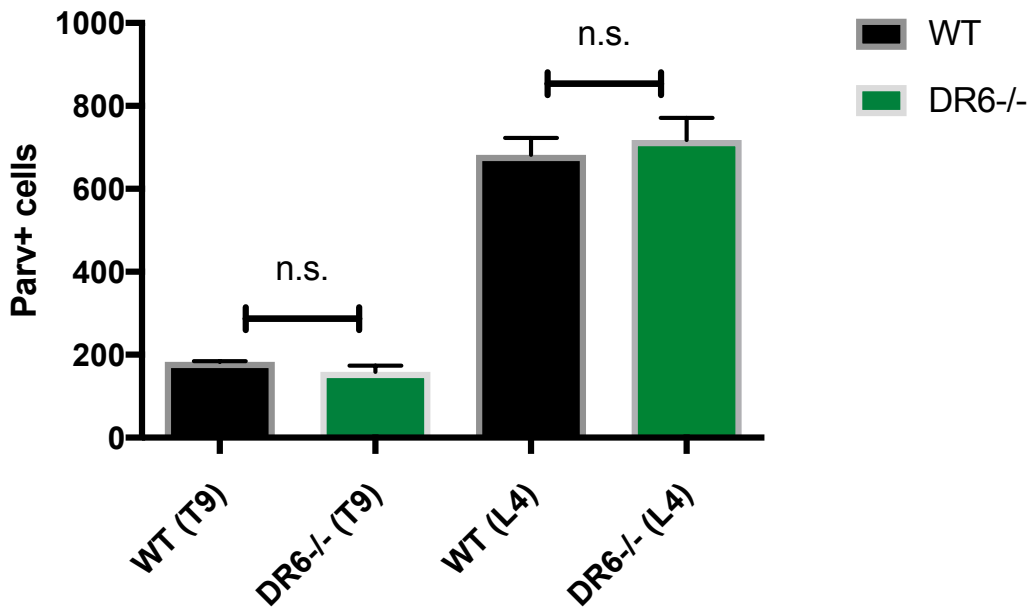


Figure 1. DR6 is not required for propioceptive neuron number. (A) Parv+ cells in L4 DRG at P0 in Thoracic 9 (T9) or Lumbar 4 (L4) DRG. No significance was found with unpaired T-test. N>3 animals.

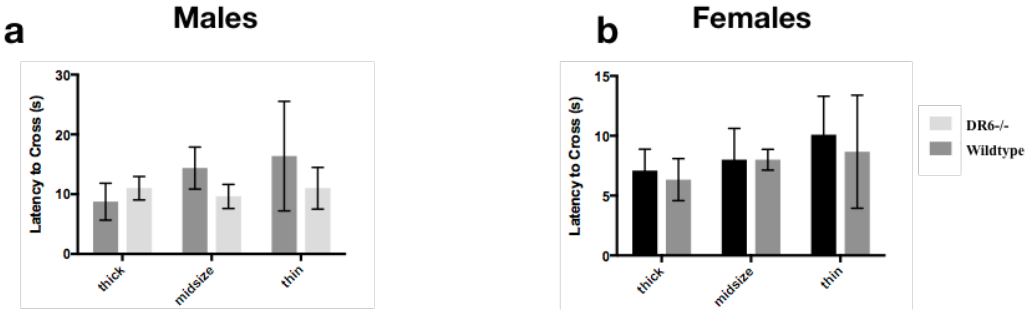


Figure 2. DR6 is not required for propioceptive performance. (A-B) Balance beam in 6-8 week males (A) and females (B). N=3-5 animals. No significance found via unpaired T-test

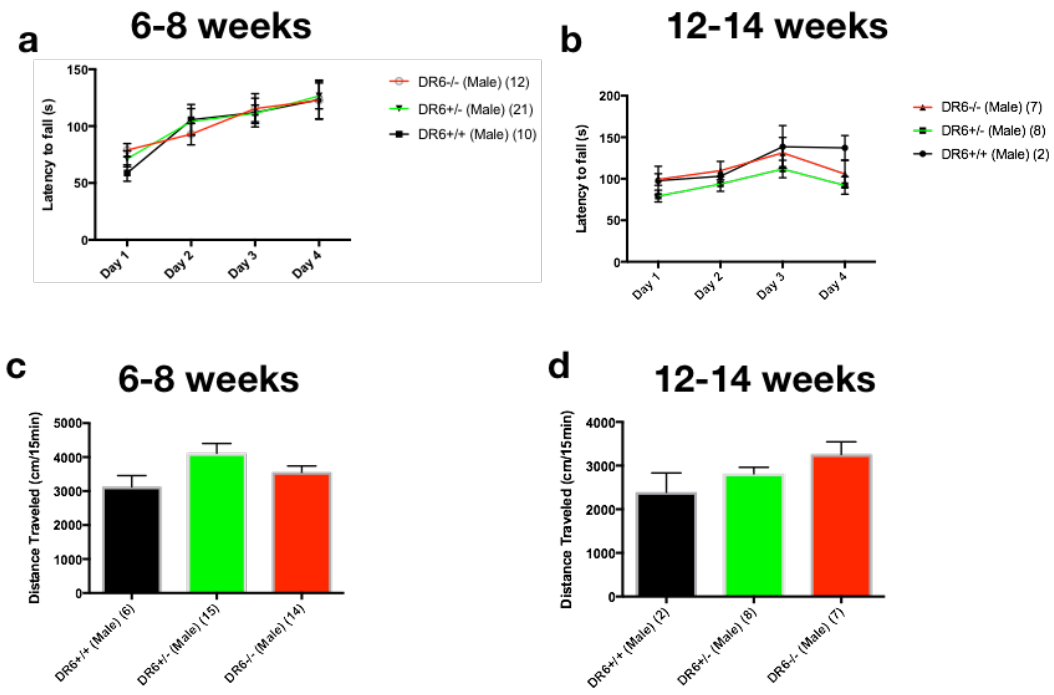


Figure 3. DR6 is not required for proprioceptive function. (A-B) Rotarod in 6-8 week old males (A) and 12-14 week old males (B). No significance with two-way anova with tukey postdoc. (C-D) Distance travelled in open field for 15 minutes in 6-8 week old males (C) and 12-14 week old males (D). No significance with one-way anova with tukey postdoc. Animals analyzed are in ().

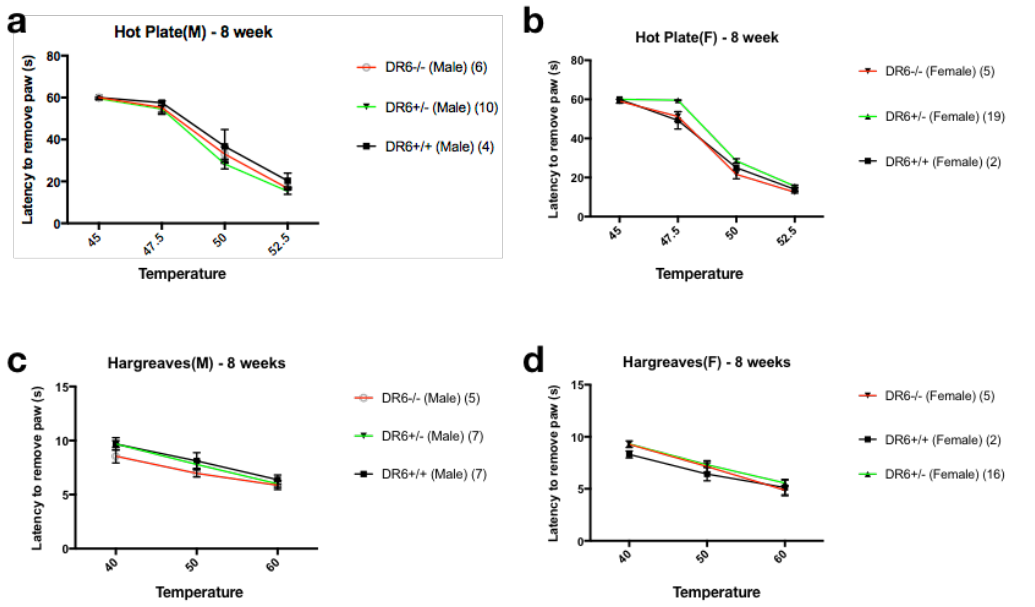


Figure 4. DR6 is not required for thermosensation. (A-B) Hot plate assay in 8 week mice (A – females, B – males). (C-D). Hargreaves assay in 8 week mice (C – females, D – males). No significance with one-way anova with bonferroni postdoc. Animals analyzed are in ().

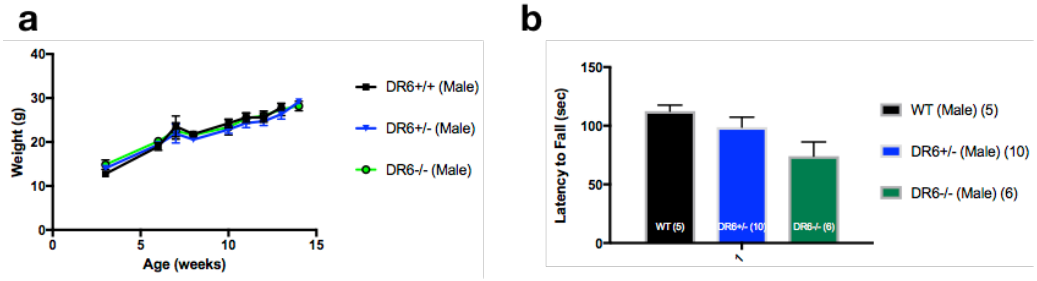


Figure 5. DR6 is not required for weight or strength. (A) Weight across development. N=1-8 animals. No statistic tests were performed. (B) Cage hang assay where latency to fall was recorded. No statistical significance was found with one-way anova with Tukey postdoc. $p>0.05$. Animals analyzed are in ().

Conclusion

These data suggest that the TNFRsf member DR6 is not required for sensory function, as assay by total number of proprioceptive neurons, balance beam, rotarod, open field, hot plate, hargreaves, or cage hand.

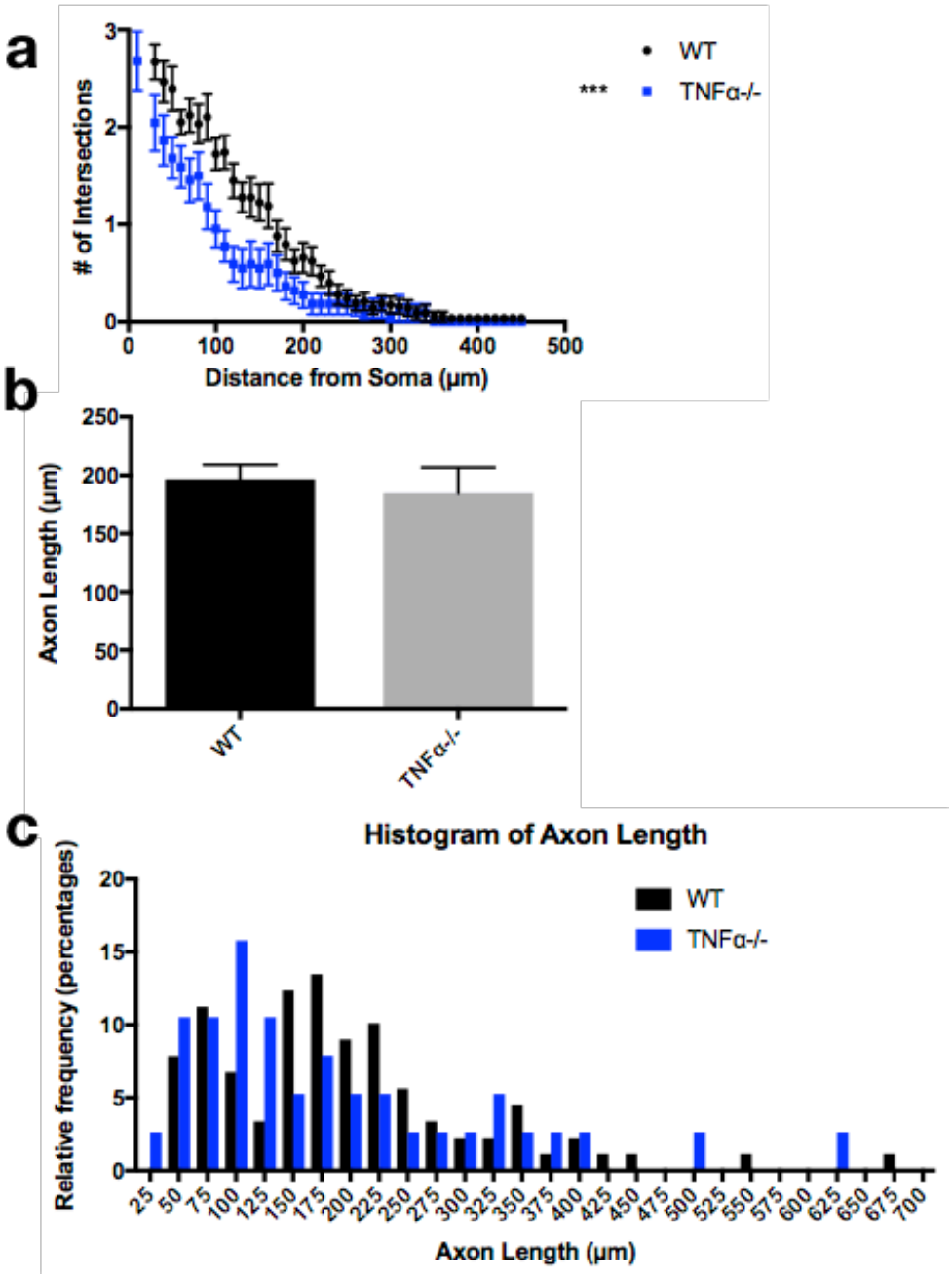
Appendix III: The Role of TNFα in Sensory Neuron Axon Branching

Introduction

TNFα-TNFR1 signaling has been implicated in axon growth and pruning in nociceptive neurons (Wheeler et al., 2014). We investigated the role of TNFα in *in vitro* proprioceptive axon growth and branching.

Methods

Sensory neurons were dissociated as previously described (Wheeler et al., 2014). Neurons cultured and stained as described in Keeler et al., 2017. Neurons were incubated with 20ng/mL of NT-3 (PeProTech, 450-03) and 4 μg/mL aphidicolin (Sigma, A0781) for 24 hours, fixed, and immunostained with Tuj1 and anti-Parvalbumin (Swant, PV27, 1:5000). Images were taken on Zeiss Axiozoom Inverted Widefield Scope and analyzed using FIJI.



TNFα-/- proprioceptive neurons extend shorter neurites and branch less compared to WT. (A) Sholl analysis from neurons were cultured in NT-3 (20ng/mL) for 24 hours and identified with anti-parvalbumin and Tuj1 (*** P<0.0001, comparison with two-tailed paired t-test). (B) Length of longest axon. No significance was found through unpaired T-test (C)

Histogram of longest axon lengths. No significant was found through paired T-test of histogram.

Conclusion

This study demonstrates that TNFα can reduce axon branching in proprioceptive neurons *in vitro*. This provides a potential mechanism for developmental axon pruning in proprioceptive neurons.

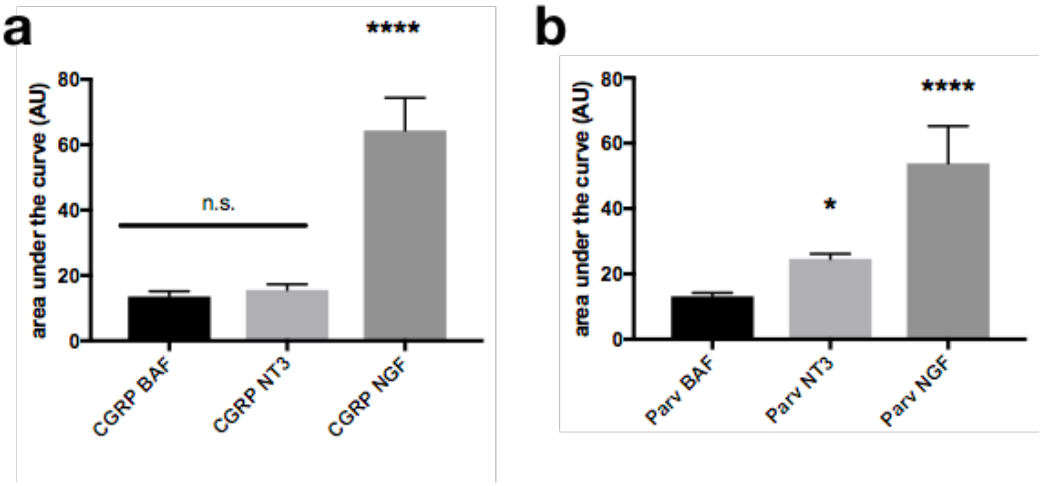
Appendix IV: Non-Cognate Neurotrophins Signaling in Sensory Neuron Axon Branching

Introduction

Sensory neuron subsets can be defined by their neurotrophin receptor expression pattern (Lallemend and Enrfors, 2012). Nociceptors express the neurotrophic receptor TrkA and are thought to be responsive to the neurotrophin NGF, whereas proprioceptors express TrkC and are thought to be responsive to the neurotrophin NT-3 (Lallemend and Enrfors, 2012). We sought to investigate if non-cognate neurotrophin-neurotrophin receptor pairs are capable of eliciting axon growth in sensory neurons. We tested if CGRP+ nociceptive axons grow in the presence of NT-3 and if parvalbumin+ proprioceptive axons grow in the presence of NGF.

Methods

Refer to Appendix III for immunostaining methods and tissue culture. E14.5 were cultured with 20ng/mL of NT-3 (PeProTech, 450-03), 10uM BAF (BOC-Asp(OMe)CH2F, MP Biomedicals, 03FK01105), 20ng/mL NGF (gift from Tony Spano), for 24 hours, fixed and immunostained. Images were taken on Zeiss Axiozoom Inverted Widefield Scope and analyzed using FIJI.



NGF is sufficient to induce axon branching in both peptidergic nociceptors and proprioceptors. Area under the curve calculated from histogram in CGRP+ neurons (A) and Parvalbumin+ neurons (B). One way anova with Dunnett post-hoc. N = 10-17 neurons from 1 experiment.

Conclusion

This experiment suggests that NGF can induce axon growth and branching in Parv+ proprioceptive neurons *in vitro*. This finding that Parv+ neurons, which presumably are TrkC+;TrkA-, may induce NGF-dependent axon branching through TrkC. However, future studies should be done to repeat this study and to validate that TrkA is not expressed in Parv+ neurons.

Appendix V: TNFRsf Members in Developmental Sympathetic Neuron Death

Introduction

The best-characterized destruction receptor in the peripheral nervous system is p75NTR, a tumor necrosis factor receptor (TNFR) family member, which promotes several processes including apoptosis, synapse restriction, neuronal atrophy, and axonal degeneration. P75NTR is unlikely to be the only destruction receptor that antagonizes trophic signals (Deppmann et al., 2008; Bamji et al., 1998; Majdan et al., 2001; Sharma et al., 2010; Singh et al., 2008). For example, the observation that loss of p75NTR only delays and does not prevent naturally occurring sympathetic neuron death lends evidence towards the existence of additional mediators of destruction (Deppmann et al., 2008).

Method

Immunohistochemistry

Refer to Appendix I for immunohistochemistry protocol. Antibodies used this experiment: Rabbit anti-cleaved Caspase-3 (Cell Signaling Technologies, 9661s).

Sympathetic SCG Neuron Count

Sympathetic neuron counts were performed on cryosectioned and Nissl stained superior cervical ganglia (SCGs) as previously described (Deppmann et al., 2008).

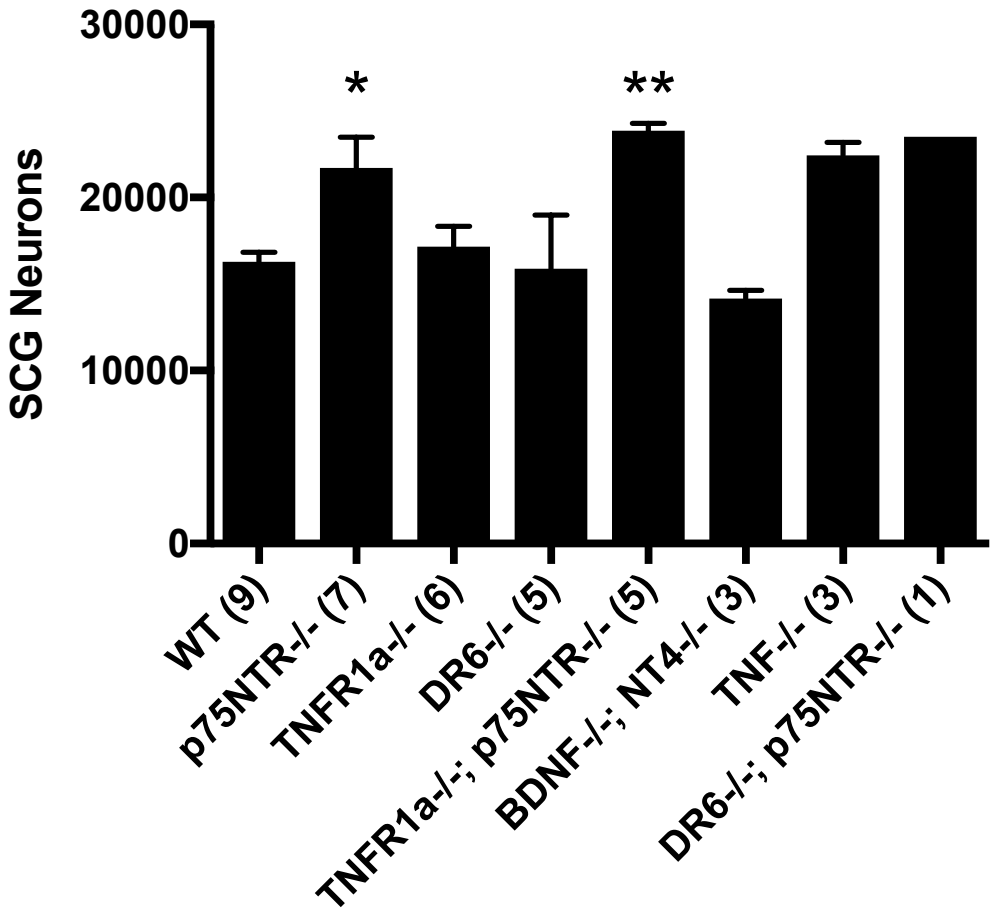


Figure 1. Sympathetic neuron counts in the p0 superior cervical ganglia in Wildtype mice and mice lacking TNFRsf members or ligands. Counts were performed off Nissl stained sections. One-way Anova with Tukey post-hoc. * denotes $p<0.05$. All were compared to WT. Animals analyzed are in ().

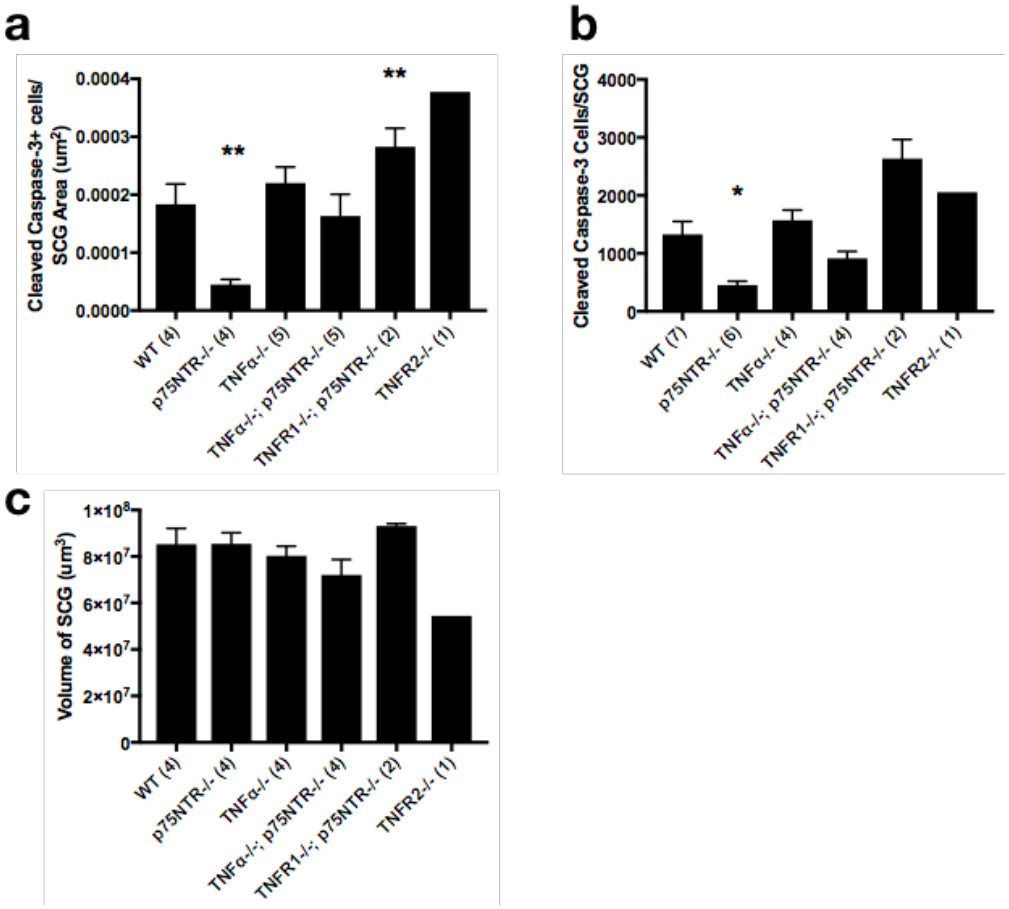


Figure 2. Apoptosis in sympathetic neurons in p0 Wildtype mice and mice lacking TNFRsf members or ligands. Cells reactive for anti-cleaved caspase-3 in P0 SCG per area (A) or per SCG (B). (C) Volume of SCG at p0 in WT and transgenic mice. Animals analyzed are in (). One-way Anova with Tukey post-hoc. * denotes $p<0.05$. All were compared to WT. Animals analyzed are in ().

Conclusion

Our findings are consistent with previous findings that p75NTR is required for naturally-occurring cell death in the sympathetic nervous system (Bamji

et al., 1998; Majden et al., 2001; Deppmann et al., 2008). Interestingly, we see wildtype or higher levels of apoptosis in animals also lacking TNFR1 or TNFα; this suggests that p75NTR-dependent sympathetic neuron death may require TNFR1-TNFα signaling. However, this does not influence total SCG volume.