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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.

Keywords

Neurotrophic factor; sensory; development; apoptosis; p75NTR; dorsal root ganglion; peripheral nervous system

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Conflict of Interest Statement

The authors declare no conflict of interest.

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 Lallemand 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; Lallemand 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 Lallemand 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., 2011).

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 neuron 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 $\pm$ 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+ ($88.21 \pm 4.75\%$ (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 $88.21 \pm 4.75\%$ of TrkC+ neurons at E11.5 to $28.83 \pm 7.93\%$ 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 percentage 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.

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 $1.67 \pm 0.23\%$ to $5.19 \pm 0.90\%$ 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.

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.

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.

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., 2011; 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; Lallemand 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.

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 extrinsic 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 TNF α 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.

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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, 20 μ m.

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

(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 $\pm$ 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, 20 μ m.

(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 $\pm$ SEM with N (animals) labelled on the graph.

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.

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 μ m (A, C) or 100 μ m (B).

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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 $\pm$ SEM with N (animals) labelled on the graph. Scale bar in denotes 100 μ m.

Figure 7. p75NTR Promotes Survival of TrkB+, 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 $\pm$ 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.