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Partial deletion of p75^{NTR} in large-diameter DRG neurons exerts no influence upon the survival of peripheral sensory neurons *in vivo*

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Significance Statement:

The p75 neurotrophin receptor (p75^{NTR}) is required for peripheral sensory neuron survival, however the cellular mechanism underpinning this effect is unclear. Adopting a neuronal-specific conditional knockout strategy, we identify that neuronal p75^{NTR} expression is not essential for maintaining peripheral sensory neuron survival in health and after demyelinating neuropathy, suggesting a potential role for glial expression of p75^{NTR} in mediating neuronal survival. Thus, our findings provide new insights into the mechanism of p75^{NTR} mediated neuronal survival in the peripheral nervous system.

Abstract

The p75 neurotrophin receptor (p75^{NTR}) is required for maintaining peripheral sensory neuron survival and function, however the underlying cellular mechanism remains unclear. The general view is that expression of p75^{NTR} by the neuron itself is required for maintaining sensory neuron survival and myelination in the peripheral nervous system (PNS). Adopting a neuronal-specific conditional knockout strategy, we demonstrate the partial depletion of p75^{NTR} in neurons exerts little influence upon maintaining sensory neuron survival and peripheral nerve myelination in health and after demyelinating neuropathy. Our data show that the density and total number of dorsal root ganglion (DRG) neurons in 2months old mice is not affected following the deletion of p75^{NTR} in large diameter myelinating neurons, as assessed by stereology. Adopting experimental autoimmune neuritis induced in adult male mice, an animal model of demyelinating peripheral neuropathy, we identify that deleting p75^{NTR} in myelinating neurons exerts no influence upon the disease progression, the total number of

DRG neurons, and the extent of myelin damage in the sciatic nerve, indicating that the expression of neuronal p75^{NTR} is not essential for maintaining peripheral neuron survival and myelination after a demyelinating insult *in vivo*. Together, results of this study suggest that the survival and myelination of peripheral sensory neurons is independent of p75^{NTR} expressed by a subtype of neurons *in vivo*. Thus, our findings provide new insights into the mechanism underpinning p75^{NTR} mediated neuronal survival in the PNS.

Key words: p75^{NTR}, DRG, myelination, BDNF, peripheral nervous system, EAN, remyelination, AB_11205760, AB_2801575, AB_2267254, AB_2340380, AB_2732073, AB_2534102, AB_11205760, AB_2340375, AB_304560, AB_1658411.

Introduction

Genetic analyses of neurotrophins and their receptors have established that they are required for peripheral nervous system (PNS) function including neuronal survival and myelination during normal development and after injury (Carter and Lewin 1997, Chao 2003, Xiao, Kilpatrick et al. 2009). The survival of peripheral sensory neurons, in particular dorsal root ganglion neurons (DRGs), is particularly sensitive to the neurotrophins, with approximately 80% dependent upon Nerve Growth Factor (NGF) for survival, ~30% dependent on Brain-derived neurotrophic factor (BDNF) and ~20% on Neurotrophin-3 (NT-3) (Wright and Snider 1995, Conover and Yancopoulos 1997). The receptors of neurotrophins can be co-expressed in the same cell type and their expression in neurons changes dynamically during development (Chao 1994, Chao 2003). The biological effects of the neurotrophins are mediated via two distinct classes of receptors: the tropomyosin-related kinase (Trk) receptors and the p75 neurotrophin receptor (p75^{NTR}) (Chao 1994, Huang and Reichardt 2001, Chao, Rajagopal et al. 2006). The analysis of germline p75^{NTR} knockout (KO) mice shows an approximately 50% reduction in the number of postnatal sensory neurons but not motor neurons (Lee, Li et al. 1992, Lee, Davies et al. 1994, Stucky and Koltzenburg 1997, Murray, Bartlett et al. 1999), accompanied by hypomyelination of surviving neurons (Cosgaya, Chan et al. 2002) and sensory function deficits (Lee, Li et al. 1992), indicating that the

expression of p75^{NTR} is required for the survival of peripheral sensory neurons during normal development.

In addition to neuronal survival, neurotrophin signalling plays critical roles in regulating Schwann cell migration and myelination in the PNS (Chan, Cosgaya et al. 2001, Cosgaya, Chan et al. 2002, Chan, Watkins et al. 2004, Xiao, Kilpatrick et al. 2009, Xiao, Wong et al. 2009, Gonsalvez, Tran et al. 2017). During development, different neurotrophins exert distinct influences upon peripheral nerve myelination depending upon the cognate receptor expressed by the neuron (Xiao, Kilpatrick et al. 2009, Xiao, Wong et al. 2009). NGF enhances Schwann cell myelination of NGF-dependent neurons via activation of neuronal TrkA receptors, but has no effect on BDNF-dependent neurons which do not express TrkA (Chan, Watkins et al. 2004). NT-3 inhibits peripheral myelination *in vitro* (Chan, Cosgaya et al. 2001, Cosgaya, Chan et al. 2002), whereas analysis of NT3^{-/-} mice suggests it exerts a pro-myelinating influence (Meier, Parmantier et al. 1999, Woolley, Tait et al. 2008), suggesting that the role of NT3 in peripheral myelination is currently inconclusive. BDNF enhances peripheral myelination during development via p75^{NTR} (Xiao, Wong et al. 2009) and remyelination after injury (Zhang, Luo et al. 2000, Song, Zhou et al. 2006). Amongst the neurotrophin receptors, the expression of p75^{NTR} coincides with the onset of peripheral nerve myelination and has been identified as a positive regulator of PNS myelination (Cosgaya, Chan et al. 2002).

Despite the important roles that p75^{NTR} signalling plays in the PNS, the underpinning mechanism is unclear. This is partially due to the broad cellular expression profile of p75^{NTR}. In the PNS, p75^{NTR} is present in subpopulations of DRG neurons, unmyelinating Schwann cells (Jessen and Mirsky 2005, Jessen, Mirsky et al. 2015, Jessen and Mirsky 2019), and possibly other subtypes of glial cells (Lee, Li et al. 1992, Koike, Tanaka et al. 2019). Indeed, it is unclear whether the expression of p75^{NTR} by subpopulations of DRG neurons itself is required for maintaining sensory neuron survival in the PNS *in vivo*, and whether its function on neuronal survival is similar in the healthy PNS and after injury. While previous analysis of full p75^{NTR} KO mice demonstrated an essential function of this receptor in aspects of PNS development, later studies suggested that these full KO models are different mouse strains suffered from alternative splicing and gain-of-function products (Lee, Li et al. 1992, Paul, Vereker et al. 2004, Nykjaer, Willnow et al. 2005). The mutant models mostly used so far are the p75^{NTR}^{ExIII} and

p75^{NTRExIV} knockout mice. The p75^{NTRExIII} KO mice that have been created by deleting Exon III (Lee, Li et al. 1992, Murray, Bartlett et al. 1999) are hypomorphic because they still express a short variant of p75^{NTR} (Nykjaer, Willnow et al. 2005, Busch, Baldus et al. 2017). While the p75^{NTRExIV} KO mice were created by deleting Exon IV that results in a loss of both the full-length and the short isoform of p75^{NTR} (von Schack, Casademunt et al. 2001), this mouse mutant results in the production of a truncated protein encoding the intracellular domain, which may account for some of the discrepant phenotypes (Paul, Vereker et al. 2004), indicating that a part of p75^{NTRExIV} KO mice phenotype may reflect a gain-of-function rather than loss of p75^{NTR} function. Thus, results obtained from the different strains of p75^{NTR} KO mice further complicate our understanding of the roles of p75^{NTR}. Therefore, selectively deleting p75^{NTR} receptor in neurons free of functional splice products is important in order to precisely determine its function in the PNS. To address this, we generated a neuronal-specific p75^{NTR} mutant mouse via crossing a neuronal specific cre mouse with a p75^{NTR} flox colony targeting the Exon I. Our data show that deleting p75^{NTR} specifically in myelinating neurons does not influence DRG neuron density or number in the normal PNS, or following demyelinating peripheral neuropathy induced in male adult mice, indicative of no effect on survival. The extent of myelin damage in the sciatic nerve including axonal calibre, the number of dysmyelinated axons and the thickness of myelin sheaths following demyelinating peripheral neuropathy remains similar between the p75^{NTR} mutant and control mice. Together, the findings of this study reveal that the survival and myelination of peripheral neurons are independent of neuronal p75^{NTR} *in vivo*.

Materials and methods

Animals

All animal procedures are approved by the Animal Experimentation Ethics Committees at the University of Melbourne. The neuronal-specific p75^{NTR} mutant mice were generated by crossing p75^{NTR} floxed (p75^{NTR fl/fl}) mice (Boskovic, Alfonsi et al. 2014) against the Neurofilament-Light chain Cre (NFL-Cre) mice in which the expression of cyclization recombinase (Cre) gene was driven by NFL promoter restricted to neurons (Schweizer, Gunnensen et al. 2002). In p75^{NTR fl/fl} mice, an inverted mCherry gene was inserted behind the exon 1 of the p75^{NTR} gene, and together they were flanked by a mutated loxP pair (Boskovic, Alfonsi et al. 2014) The mCherry and the STOP signal sequences were integrated within the first intron so that, when Cre recombinase was absent (in a wild-type context), p75^{NTR} expression

remained unchanged and there will be no expression of mCherry. However, when Cre recombinase was present, the coding sequence for p75^{NTR} exon 1 was replaced with the mCherry gene sequence and the cells in which p75^{NTR} was deleted turned red (Figure 1A). p75^{NTR} in/in NFL^{Cre +/-} (p75^{NTR} cKO) and Cre negative littermate control (p75^{NTR} in/in NFL^{Cre -/-}, control) mice at the age between 8-16 weeks were used for this study. Unless otherwise specified, mice of either gender were used for experiments.

EAN induction in mice

Male p75^{NTR} in/in NFL^{Cre +/-} (p75^{NTR} cKO) and Cre negative littermate control mice (all on a C57/B6 background) were induced with EAN disease as previously described (Gonsalvez, De Silva et al. 2017, Gonsalvez, Fletcher et al. 2017, Gonsalvez, Tran et al. 2017). Briefly, eight-weeks old male mice (p75^{NTR} cKO and control) were immunized twice (Day 0 and Day 8 post induction) by subcutaneous injection of PO peptide 180-199 (PO₁₈₀₋₁₉₉, sequence S-S-K-R-G-R-Q-T-P-V-L-Y-A-M-L-D-H-S-R-S), and 0.5mg Mycobacterium (M.) tuberculosis (strain H 37 Ra; Difco #231141, Detroit, MI) emulsified in 25µl saline and 25µl of complete Freund's adjuvant (Difco #263910 comprising; 3.75µl of mannide monoolate + 21.25µl of paraffin oil and 12.5µg of desiccated killed and dried Myobacterium butyrcum). Mice received pertussis toxin (Ptx, Sigma, St, Louis, MO) on Day -1 (400 ng/mouse), and Days 1 and 3 (300ng/mouse) by intraperitoneal injection. All Ptx and inoculation injections were carried out on mice anesthetized by aerosol isoflurane 2% in normal air. Animals were monitored daily for weight loss, and clinical disease was scored as: 0 for normal, 1 for less lively, 2 for mild tail and hind limb paresis, 3 for mild ataxia and limb paresis, 4 for severe ataxia and limb paresis and 5 for limb paralysis. L4/L5 DRGs and sciatic nerves were isolated at the peak of ENS disease (Day 26) for histological analyses.

Tissue collection and processing

Mice were transcardially perfused with 4% paraformaldehyde (PFA). L4/L5 DRG neurons and sciatic nerves were dissected for immunohistochemical, stereological or histological analyses. For immunohistochemistry and stereological analyses, tissues were cryoprotected with 30% sucrose in 0.01M MT-PBS for 48h at 4°C followed by Tissue-Tek O.C.T. embedding. For histology analysis, tissues were post-fixed in Karnovsky's buffer for 24h at 4°C, followed by Osmium tetroxide processing and subsequent resin embedding, as previously described (Xiao, Wong et al. 2010, Wong, Xiao et al. 2013, Gonsalvez, Tran et al. 2017, Fletcher, Wood et al. 2018).

Immunohistochemistry and Imaging

10µm thick sections were prepared from DRGs (n=3 mice per genotype, n= 6 technical replicates per mouse). Cryostat sections were blocked, incubated in primary antibodies overnight, followed by incubation with the appropriate secondary antibodies. Primary antibodies used were directed against chicken anti-NeuN (1:400, Millipore, #ABN91, RRID:AB_11205760), mouse anti-DSRed (1:500, Santa Cruz Biotech, #sc-390909, RRID:AB_2801575), goat anti-p75NTR (1:200, Santa Cruz Biotech, #sc-6188, RRID:AB_2267254), chicken anti-NF200 (1:1000, Abcam #ab4680, RRID:AB_304560) and mouse anti-CGRP (1:1000, Abcam #ab81887, RRID:AB_1658411). Secondary antibodies were used as follows: Alexa Fluor® 647 donkey anti chicken IgY (1:200, J.I.L. Lab, #703-606-155, RRID:AB_2340380), Alexa Fluor® 594 donkey anti mouse IgG (1:400, Abcam, #Ab150108, RRID:AB_2732073), and Alexa Fluor® 488 donkey anti goat IgG (1:400, Molecular Probes, A-11055, RRID:AB_2534102), Alexa Fluor® 488 Donkey anti-Chicken (J.I.L. Lab, #703-545-155, RRID:AB_2340375), Alexa Fluor® 405 Donkey anti mouse (Abcam ab175658, RRID:AB_2687445). Images of adjacent sections were captured using confocal microscope and cellular analysis were performed using Fiji ImageJ (ImageJ 1.50i NIH, USA), using our previously published method (Wong, Xiao et al. 2013, Peckham, Giuffrida et al. 2016, Fletcher, Wood et al. 2018).

Stereological analysis

L4 and L5 DRG (n=3 mice per genotype, n= 2 technical replicates per mouse) were identified by tracing sciatic nerve trunks back to their nerve root entry points on the spinal cord, also by counting subsequent nerve roots using the T13 nerve as a landmark. L4 and L5 ganglia were serially sectioned (25µm thick) on a freezing microtome. All sections were processed for immunofluorescence using a primary antibody directed against chicken anti-NeuN (Millipore, #ABN91, RRID:AB_11205760) and followed by secondary antibody Alexa Fluor® 488 Donkey anti-Chicken (J.I.L. Lab, #703-545-155, RRID:AB_2340375) and the nuclear stain dye Hoechst 33342 (1:10000, Abcam, #ab228551). To determine the total volume of each DRG and the number of neurons within each ganglion we utilised the Optical Fractionator Probe within Stereo Investigator version 11.01.02; (MBF Bioscience). In brief, sections were imaged, traced and then overlaid with a grid. The counting sites were exhaustively imaged in the Z-plane using a Zeiss Axio M2 with Apotome (63 X 1.4NA lens). The total section thickness was measured at every single counting site to maximise the accuracy in volume estimates. The grid frame and counting window

sampling protocol used ensured a Gundersen M0 Coefficient of Error (M0 CE) of <0.05, effort was taken to ensure this was significantly less than the MBF recommended M0 CE value of 0.1.

Histological analysis

Transverse semi-thin sections (0.5 μm) of sciatic nerve resin blocks were cut and stained with toluidine blue. Bright field images were acquired at 100x magnification using Zeiss AxioVision[®] microscope (Oberkochen, Germany). The quantification of dysmyelinated axons, axonal diameter and G-ratio (the ratio of axonal diameter to fibre diameter [axon and myelin]) were performed using Fiji/ImageJ (ImageJ 1.50i NIH, USA) software. For quantification, minimum 3 images per animal per group (n=3 mice per genotype) were taken randomly and analysed covering the majority of the tissue sections. 300-500 axons per animal per group were used for the analysis of G-ratio and axonal diameter.

Statistical analysis

Assessors were blind to animal groups or genotypes during all data acquisition and analyses. Graph preparation and statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA)). All data are presented as mean \pm standard error of the mean (sem). To compare the cellular parameters between genotypes, unpaired t-tests were performed for a minimum of three mice per genotype per group. For G-ratios, linear regressions were performed, and axon diameter frequency distribution were assessed using χ^2 distribution tests. Clinical scoring of EAN disease progression between genotypes was analysed using Mann-Whitney's U-test. Differences between the mean responses were considered significant if $p < 0.05$.

Results

Selectively deleting p75^{NTR} in neurons in vivo

To determine the influence that neuronal p75^{NTR} exerts upon peripheral neurons and myelination *in vivo*, we generated conditional knockout mice (p75^{NTR} cKO) in which p75^{NTR} was specifically deleted from neurons by crossing p75^{NTR} ^{fl/fl} mice with neuronal-specific NFL^{Cre} mice (Schweizer, Gunnarsen et al. 2002). This Cre -induced recombination resulted in the expression of mCherry in cells of the NFL^{Cre+/-} mice where p75^{NTR} is deleted (Boskovic, Alfonsi et al. 2014). The fidelity of p75^{NTR} deletion was verified via assessing both DsRed and p75^{NTR} expression in DRG neurons using triple immunostaining of for the

neuronal marker NeuN together with DsRed and p75^{NTR}. We first assessed the specificity of DsRed expression in L4-L5 DRGs and found that DsRed was only expressed in neurons from the p75^{NTR} cKO mice, with approximately 50% of DRG neurons being DsRed+ (Figure 1B, quantitated in Figure 1C). No DsRed+ neurons were detected in control mice (Figure 1B, quantitated in Figure 1C), confirming the specificity of the NFLere. Quantitative analysis of p75^{NTR} immunoreactivity demonstrated that approximately 50% of neurons are p75^{NTR}+ in control mice (Figure 1D), and that p75^{NTR} cKO mice displayed a significant reduction in the proportion (approximately 40%) of p75^{NTR} + neurons at 8 weeks of age (Figure 1B, quantitated in Figure 1D). Together, our results demonstrate that p75^{NTR} expression is selectively deleted in the neurons of p75^{NTR} cKO mice but that recombination was not complete.

Partial deletion of p75^{NTR} in large-diameter neurons exerts no influence upon neuronal number within DRGs in vivo

p75^{NTR} is widely expressed in DRG neurons and its germline deletion leads to approximately 50% loss of these neurons during normal postnatal development (Murray, Bartlett et al. 1999). To determine whether the expression of neuronal p75^{NTR} is required for maintaining DRG neuron survival, we first analysed neuronal density in the L4-L5 DRGs of 8-week-old p75^{NTR} cKO and littermate control mice. No significant difference in the density of neurons within DRGs was observed between genotypes (Figure 2A), suggesting no loss of neurons. However, neuronal number is a function of both neuronal density and ganglion volume, which could be altered as a result of neuronal p75^{NTR} deletion and confound the result seen in Figure 2A. Therefore, to definitively determine if neuronal p75^{NTR} is required for DRG neuron survival, we undertook a stereological approach and assessed the absolute number of neurons within the L4-L5 DRGs. We found there are approximately 11,000 neurons in the L4/L5 DRGs of littermate control mice (10949.08333 ± 1466.97, data = mean ± sem), consistent with the published literatures (Shi, Tandrup et al. 2001, Lyu, Lyu et al. 2017). Similarly, no significant difference in neuronal number was observed between genotypes (Figure 2B, cKO= 10947.6 ± 1457.69, data = mean ± sem). Due to the incomplete recombination of this p75^{NTR} cKO mouse, it is possible that p75^{NTR} expression is only deleted from a sub-population of DRG neurons, ultimately resulting in the negative phenotype. To address this, we investigated the expression of p75^{NTR} in distinct subpopulations of DRG neurons including the large-diameter A neurons that give rise to myelinated axons and small-diameter C neurons that give rise to unmyelinated axons, the latter of which can be further classified as peptidergic or non-

peptidergic C neurons, via triple-immunostaining of p75^{NTR}, NF200 (marker for myelinated A neurons) and CGRP (marker for peptidergic neurons) in L4/L5 DRG sections (Figure 2C). Interestingly, the immunoreactivity for p75^{NTR} is detected in NF200+ myelinating neurons and NF200-/CGRP+ peptidergic neurons but absent in the NF200-/CGRP- non-peptidergic C-fibre (Figure 2C). There is a significant reduction in the proportion of NF200+ neurons that express p75^{NTR} in cKO mice compared to the littermate control mice (Figure 2D). In contrast, the proportion of peptidergic neurons that express p75^{NTR} remains similar between genotypes (Figure 2E). These results suggest that, in the cKO mice, p75^{NTR} expression is specifically deleted from a substantial fraction of myelinating neurons however the recombination is again incomplete. We have also analysed the density of NF200+ myelinating A neurons (Figure 2F), NF200-/CGRP+ peptidergic (Figure 2G) and NF200-/CGRP- non-peptidergic C-fibre (Figure 2H) neurons and found no significant difference in any of the neuronal subpopulations between genotypes. Thus, our data demonstrate that only the large-diameter myelinating neurons are depleted of p75^{NTR} in the cKO mice, and that deleting p75^{NTR} expression in these neurons exerts no influence upon either the density or number of DRG neurons.

Partial deletion of p75^{NTR} in large-diameter neurons exerts no influence on neuronal number or myelin damage after demyelinating peripheral neuropathy in vivo

It has been well established that the expression of p75^{NTR} is highly responsive to injury with its upregulation found in different models of peripheral nerve injuries such as demyelinating peripheral neuropathy (Yamamoto, Ito et al. 2002, Gonsalvez, Tran et al. 2017). Thus, having identified that p75^{NTR} ablation in myelinating neurons exerts little influence on DRG neuron survival in the normal PNS *in vivo* (Figure 2), we sought to determine if it is required for maintaining the survival of myelinating DRG neurons and myelination after a demyelinating insult *in vivo*. To address this question, we used a murine EAN model, an established animal model of demyelinating peripheral neuropathy (Gonsalvez, De Silva et al. 2017, Gonsalvez, Fletcher et al. 2017, Gonsalvez, Tran et al. 2017). EAN course mirrors many of the morphological and functional aspects of Guillain-Barré syndrome (GBS) and Chronic Inflammatory Demyelinating Polyneuropathy (CIDP) (Maurer and Gold 2002, Hughes and Cornblath 2005, Hughes, Swan et al. 2007, Hughes 2008, Yuki and Hartung 2012). In this study, EAN disease was induced in both p75^{NTR} cKO and littermate control mice. Analysis of murine EAN disease progression

using the published scoring method (Gonsalvez, De Silva et al. 2017, Gonsalvez, Fletcher et al. 2017, Gonsalvez, Tran et al. 2017) demonstrates that there is no significant difference in the progression and severity of EAN disease between genotypes (Figure 3), indicating that p75^{NTR} expression in myelinating neurons exerts no influence on the clinical phenotype of EAN.

To ascertain the influence that neuronal p75^{NTR} exerts on the neuronal and myelin pathology following EAN, we first performed the stereological analysis of DRGs collected at the peak of EAN disease (Day 26) from both p75^{NTR} cKO and littermate control mice. We found the total number of neurons within L4/L5 DRGs remained unchanged between genotypes after EAN (Figure 4A), indicating that neuronal p75^{NTR} exerts no influence on sensory neuronal survival following demyelinating peripheral neuropathy. We then went to analyse the extent of myelin pathology through assessing the percentage of dysmyelinated axons (Figure 4B, quantitated in C) and the thickness of myelin sheaths of these axons (Figure 4D). For all mice inducted with EAN, using the published method, we analysed the presence of dysmyelinated axons that had myelin debris in close proximity, indicating ongoing pathology or a failure of remyelination to occur (Gonsalvez, De Silva et al. 2017, Gonsalvez, Fletcher et al. 2017, Gonsalvez, Tran et al. 2017). However, again we found no significant difference in these measurements between genotypes. The analysis of axonal diameter (Figure 4E) and frequency distribution of demyelinated axons relative to axonal diameter (Figure 4F) also remained unchanged between genotypes after EAN. Overall, our data revealed that deleting the expression of p75^{NTR} in myelinating neurons exerts no influence on neuronal number, axonal number, axonal diameter and the extent of myelin pathology following EAN, suggesting that p75^{NTR} in a subtype of neurons (e.g. myelinating neurons in this study) is not required for maintaining peripheral neurons and myelination after demyelinating neuropathy.

Discussion

While p75^{NTR} plays multi-facet roles in the PNS, little is known about the cellular source of p75^{NTR} in maintaining peripheral neurons and their myelination in normal PNS or after injury. Results of this study provide evidence that the expression of p75^{NTR} in myelinating neurons is not essential to maintain the survival of peripheral sensory neurons in health and after peripheral demyelinating neuropathy. Furthermore, the expression of P75^{NTR} in myelinating neurons is not essential to protect against myelin damage after a peripheral demyelinating insult *in vivo*. Thus, the overall results suggest that the

maintenance of peripheral neurons and their myelination is independent of p75^{NTR} expression in a subtype of neurons, suggesting other cellular source of p75^{NTR} in mediating these effects.

Previous studies analysing p75^{NTR} global knockout mice have demonstrated a 50% loss of large and small diameter DRG neurons, and these losses occur across sensory modalities during postnatal development (Lee, Li et al. 1992, Lee, Davies et al. 1994, Stucky and Koltzenburg 1997, Murray, Bartlett et al. 1999). In line with these findings, a more recent analysis of p75^{NTR} full knockout mice further show that subsets of peripheral somatosensory neurons require p75^{NTR} expression for survival during late embryonic development but not after birth (Cheng, Jin et al. 2018), suggesting a temporal regulation of p75^{NTR}-induced neuronal survival. However, the interpretation of these studies are complicated by different strains of full KO models with alternative splicing and gain-of-function products being used (Lee, Li et al. 1992, von Schack, Casademunt et al. 2001, Paul, Vereker et al. 2004, Nykjaer, Willnow et al. 2005). Thus, the roles that neuronal p75^{NTR} plays in supporting PNS neuron survival still remain unclear. Recently, Chen et al., (Chen, Donnelly et al. 2017) has shown that deleting p75^{NTR} selectively in spinal motor and sensory neurons (*Isl1-Cre^{+/-};p75^{NTR}^{fl/fl}*) exerts no influence upon DRG neuron survival during embryonic and postnatal development. However, this neuronal p75^{NTR} deletion results in ~20% loss of adult DRGs neurons compared to the control mice, further suggesting that neuronal p75^{NTR} expression is not essential for promoting peripheral neuron survival during postnatal development but is important for maintaining a subset of DRG neurons in the adult PNS, indicative of a temporally specific and contained role. Interestingly, our study reveals that a partial deletion of p75^{NTR} in a subpopulation of DRG neurons (large-diameter A myelinating neurons) exerts no influence on either the density or the total number of DRG neurons in healthy adult PNS, suggesting that the expression of p75^{NTR} in this specific population of neurons is not absolutely required for maintaining their survival during development and into adulthood.

The reason that underpins the different neuronal phenotypes of the above studies remains unclear. It is noticed that p75^{NTR} expression is deleted from ~95% of DRG neurons in the study by Chen et al., (Chen, Donnelly et al. 2017) whereas ~40% reduction of p75^{NTR} positive neurons is achieved in our study, demonstrating different recombination efficiencies in the two p75^{NTR} mouse mutants and suggesting that partial deletion of neuronal p75^{NTR} is not sufficient to alter their survival. It is important

to note that the p75^{flox} colony used in this study achieved a high recombination efficiency (>90%) when crossed against the ChAT-Cre mice in the previous study (Boskovic, Alfonsi et al. 2014), whilst the NFLCre line has previously been shown to generate <50% recombination efficiency in neurons when intercrossed against the Stat3 flox mouse line (Schweizer, Gunnensen et al. 2002). These findings collectively suggest that the low rate of recombination observed in our study is likely due to the NFLCre line. In addition, analysis of p75^{NTR} full KO demonstrates, whilst it regulates sensory function, it is not required for the survival of specific subpopulations of sensory neurons (Stucky and Koltzenburg 1997). This is in line with our finding that depletion of p75^{NTR} in a fraction of the large diameter neurons is not sufficient to influence their survival. Collectively, our results together with the published work indicate that neuronal p75^{NTR} is not essential for the survival of specific subpopulations of peripheral neurons during normal development, and that there is also a dose-dependent effect of p75^{NTR} -mediated neuronal survival in the adult PNS.

It has been well established that there is a dramatic increase of p75^{NTR} protein in DRG neurons in response to peripheral nerve injury (Zhou, Rush et al. 1996, Obata, Katsura et al. 2006), implicating a role in neuroprotection. Concordant with this, we have previously shown that p75^{NTR} expression is significantly upregulated in peripheral nerves after a demyelinating insult *in vivo* (Gonsalvez, Tran et al. 2017). Here we identify that deleting p75^{NTR} in neurons exerts no influence on the total number of DRG neurons after peripheral demyelinating neuropathy *in vivo*, indicating that the expression of p75^{NTR} in neurons is not sufficient to maintain their survival after peripheral demyelinating neuropathy. Analysis of p75^{NTR} full knockout mice also revealed its role in promoting remyelination after peripheral nerve injury (Song, Zhou et al. 2006), as evident by significantly fewer myelinated axons and thinner myelin sheaths in the injured sciatic nerves of p75^{NTR} knockout mice compared with the wild-type controls (Song, Zhou et al. 2006). Interestingly, whilst p75^{NTR} expression is required for BDNF -induced neuroprotective effects in EAN, we identify that p75^{NTR} haploinsufficiency exerts no influence upon the EAN disease progression and the extent of myelin damage at the basal level (Gonsalvez, Tran et al. 2017). In line with this finding, in this study, we identify that deleting p75^{NTR} expression in myelinating neurons exerts no influence upon the total number of neurons and the extent of myelin damage after peripheral nerve demyelination *in vivo*. Together, our data collectively suggest that the expression of p75^{NTR} in myelinating neurons is not essential for maintaining peripheral sensory neurons and for

protecting against myelin damage after injury in adult PNS.

How could p75^{NTR} regulate peripheral neuron survival and myelination? Although the role of p75^{NTR} in modulating sensory neuronal survival (Lee, Li et al. 1992, Lee, Davies et al. 1994, Murray, Bartlett et al. 1999) and peripheral nerve myelination (Cosgaya, Chan et al. 2002, Xiao, Wong et al. 2009) is well established, the precise role that p75^{NTR} signalling plays in the PNS remains controversial. One prevailing hypothesis is that DRG neuronal loss occurs as a result of impaired neurotrophin signalling (Lee, Li et al. 1992, Lee, Davies et al. 1994, Murray, Bartlett et al. 1999). A few studies suggest that p75^{NTR} can serve as a modulator of neuronal survival and myelination in response to neurotrophin signalling. For example, p75^{NTR} null sensory neurons are approximately two-fold less sensitive to NGF-induced pro-survival effect *in vitro*, but not to other neurotrophins (Davies, Lee et al. 1993, Lee, Davies et al. 1994). It is possible that, in the absence of p75^{NTR} expression on a proportion of peripheral neurons, the level of neurotrophin ligands remain intact and is still sufficient to maintain neurotrophin signalling required for neuronal survival and/or myelination. Thus, a signalling redundancy may exist to compensate the attenuated signalling of p75^{NTR}. Moreover, p75^{NTR} interacts with Schwann cell-expressing proteins involved in myelination, at least *in vitro* (Chan, Jolicoeur et al. 2006). Chan et al., (Chan, Jolicoeur et al. 2006) found that a polarity protein Par-3 localized asymmetrically in Schwann cells can form a protein complex with p75^{NTR}, the interaction of which subsequently recruits p75^{NTR} to the axon/glia junction, a cellular event critical to myelin formation by Schwann cells. However, as Par-3 can interact with multiple binding partners via its PDZ domain, whether the Par-3 -induced myelinating phenotype *in vitro* is due to its direct interaction with p75^{NTR} is still unclear. The role of p75^{NTR} signalling in regulating PNS function is further complicated by its broad cellular expression profile in both neurons and Schwann cells as well as satellite glial cells (Lee, Li et al. 1992, Gai, Zhou et al. 1996, Koike, Tanaka et al. 2019). Indeed, the expression of p75^{NTR} protein in DRG neurons and glial cells displays differing change in response to peripheral nerve transection, with the sciatic nerve lesions resulting in a reduction of neuronal p75^{NTR} expression while an increase of glial p75^{NTR} in ipsilateral DRG (Zhou, Rush et al. 1996). Adopting a Schwann cell transplantation approach in p75^{NTR} null mice, it has been shown that mice transplanted with p75^{NTR} - expressing Schwann cells had significantly more remyelinating axons and bigger axon calibre accompanied by thicker myelin sheaths after sciatic nerve injury, accompanied by improved motor function (Tomita, Kubo et al. 2007). This finding implicates a

neuroprotective role of Schwann cell p75^{NTR} in promoting both neuron survival and myelination after peripheral demyelinating neuropathy. During normal development, p75^{NTR} signalling in Schwann cells, as assessed by its downstream transcription factor NF-kappaB, is required for peripheral myelin formation (Nickols, Valentine et al. 2003). Interestingly, we have recently found that deleting p75^{NTR} expression in Schwann cells *in vivo* (MPZ-Cre^{+/-}:p75^{NTR} fl/fl) exerts no significant influence on the extent of remyelination following sciatic nerve crush injury (Goncalves, Mohseni et al. 2019), however it is unclear whether the number of peripheral neurons remains intact in these Schwann cell-specific p75^{NTR} mutant mice. Our data indicates that partial deletion of p75^{NTR} in specific population of neurons exerts no influence upon the neuronal survival. However, due to the incomplete recombination efficiency, the role of neuronal p75^{NTR} in maintaining DRG neuron survival still remains inconclusive and a neuronal-specific p75^{NTR} mutant with a more complete recombination efficiency is required. In addition, these published findings together with our results suggest that another cellular source of p75^{NTR} such as glial cells and/or glial-axonal interactions may be responsible for p75^{NTR} signalling-induced peripheral neuronal survival.

In summary, results of this study identify that neuronal p75^{NTR} does not play an essential role in maintaining peripheral sensory neurons and their myelination *in vivo*. Findings of this study reveal the potential importance of glial cells-expressing p75^{NTR} in supporting peripheral neuron survival, providing new insights into the cellular mechanism underpinning p75^{NTR} signalling-induced PNS function.

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Conflict of interest statement

The authors declare no conflicting financial or other interests.

Authors contribution

ZQ performed experiments and analyzed data; DG and RW performed experiments; RW, SY and FD contributed to data analysis; DG, SM and JI contributed to data analyses and edited the manuscript; EC generated and provided the p75^{ffl} mice and edited the manuscript; JX conceived the study and supervised the project; ZQ and JX wrote the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures and Figure Legend

Figure 1. Deleting p75^{NTR} expression in neurons *in vivo*

(A) Schematic showing the p75^{NTR} ^{fl/fl} mouse and inversion strategy used to generate p75^{NTR} cKO mice (p75^{NTR} ^{in/in} NFL^{Cre}, adapted from (Boskovic, Alfonsi et al. 2014)). In cells expressing cre recombinase, the loxP-flanked region inverts to generate p75^{NTR} cKO that express mCherry rather than p75^{NTR} from the endogenous p75^{NTR} promoter. UTR, Untranslated region.

(B) Representative immunostaining of NeuN, DsRed and p75^{NTR} in the DRGs of eight-weeks old p75^{NTR} cKO and littermate control mice (scale bar 200µm).

(C) Quantification of the proportion of DsRed+/NeuN+ neurons (relative to total NeuN+ cells) in the L4/L5 DRGs. ~50% of NeuN+ neurons are DsRed+ cells in p75^{NTR} cKO mice and no DsRed+ cells were detected in the littermate control mice. (n = 3 mice per genotype, n= 6 replicates per mouse, data = mean ± SEM, unpaired t-test, *p < 0.0001).

(D) Quantification of the proportion of p75^{NTR} +/NeuN+ neurons (relative to total NeuN+ cells) in the

L4/L5 DRGs. There is a significant reduction (~40%) in the proportion of p75^{NTR}+ neurons in p75^{NTR} cKO mice compared to littermate control mice (n = 3 mice per genotype, n= 6 replicates per mouse, data = mean ± SEM, unpaired t-test, *p < 0.0001).

Figure 2. Partial deletion of p75^{NTR} in large-diameter neurons exerts no influence upon neuronal number in L4/L5 DRGs

(A) Quantification of the density of neurons within the L4/L5 DRGs showing no significant difference between genotypes (n = 3 mice per genotype, data = mean ± SEM, unpaired t-test, ns = no significant difference).

(B) Quantification of total neuronal number in L4/L5 DRGs using stereology showing no significant difference between genotypes (n = 3 mice per genotype, data = mean ± SEM, unpaired t-test, ns = no significant difference).

(C) Representative immunostaining of NF200, CGRP and p75^{NTR} in the DRGs of p75^{NTR} cKO and littermate control mice (scale bar 100µm).

(D-E) Quantification of the proportion of NF200 + myelinating A neurons (D) and NF200-/CGRP+ peptidergic neurons (E) that express p75^{NTR} in L4/L5 DRGs. (n = 3 mice per genotype, n= 3 replicates per mouse, data = mean ± SEM, unpaired t-test, *p < 0.01).

(F-H) Quantification of the density of NF200 + myelinating A neurons (F), NF200-/CGRP+ peptidergic neurons (G) and NF200-/CGRP- non-peptidergic C-fibre neurons (H) in L4/L5 DRGs showing no significant difference between genotypes. (n = 3 mice per genotype, , n= 3 replicates per mouse, data = mean ± SEM, unpaired t-test, ns = no significant difference).

Figure 3. Deleting p75^{NTR} in myelinating neurons exerts no influence on EAN disease progression

Time courses of EAN disease induced in p75^{NTR} cKO and littermate control mice showing no significant difference between genotypes. (n=6 mice per genotype per condition, Mann-Whitney's U-test, ns = no significant difference between p75^{NTR} cKO and littermate control mice induced with EAN).

Figure 4. Partial deleting p75^{NTR} in myelinating neurons exerts no influence upon DRG neuron number and myelin damage in EAN mice.

(A) Quantification of total DRG numbers in EAN mice at Day 26 using stereology showing no significant difference between genotypes (n = 3 mice per genotype, data = mean ± SEM, unpaired t-test, ns = no significant difference).

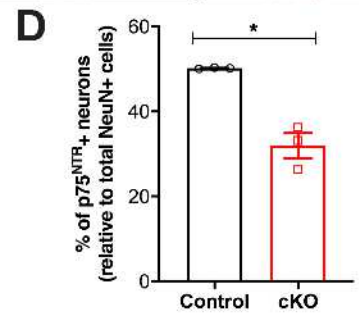
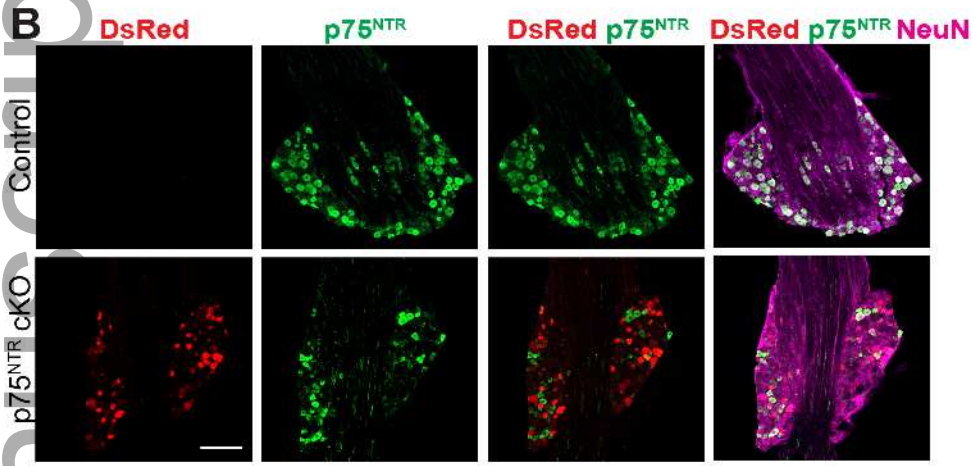
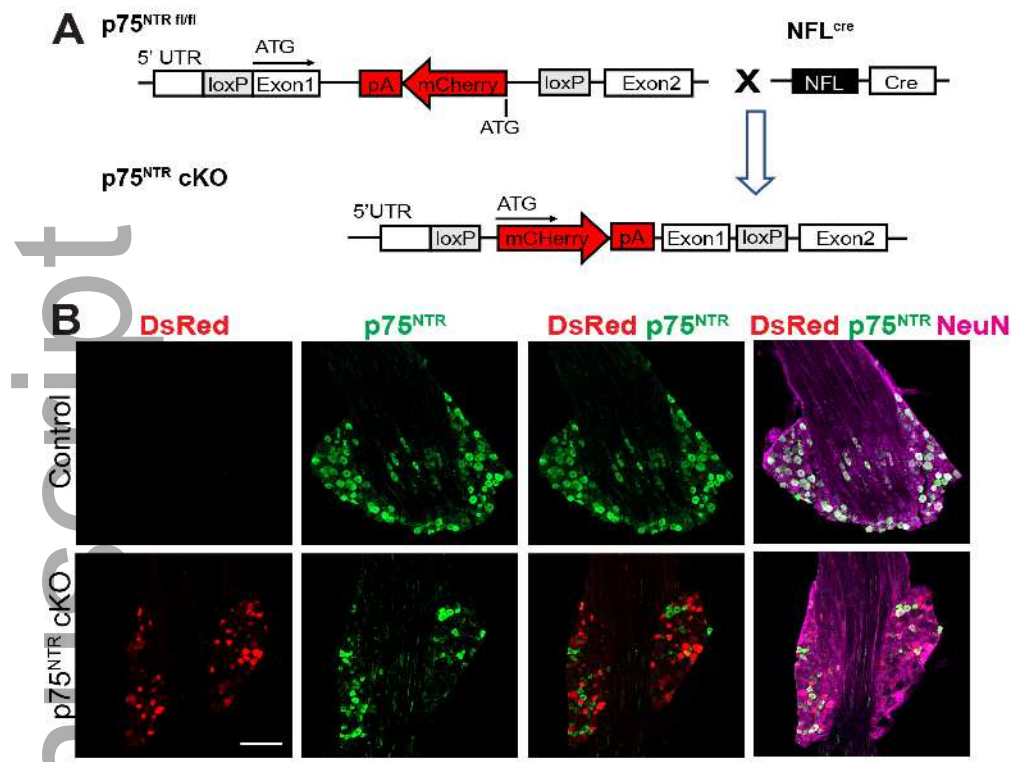
(B) Representative bright field images of sciatic nerves collected from p75^{NTR} cKO and littermate control mice induced with EAN disease at Day 26 (scale bar 20µm). (asterisks indicate dysmyelinated axons in the inset images)

(C) Quantification of the percentage of dysmyelinated axons in p75^{NTR} cKO and littermate control mice induced with EAN (n = 3 mice per genotype, data = mean ± SEM, unpaired t-test, ns = no significant difference).

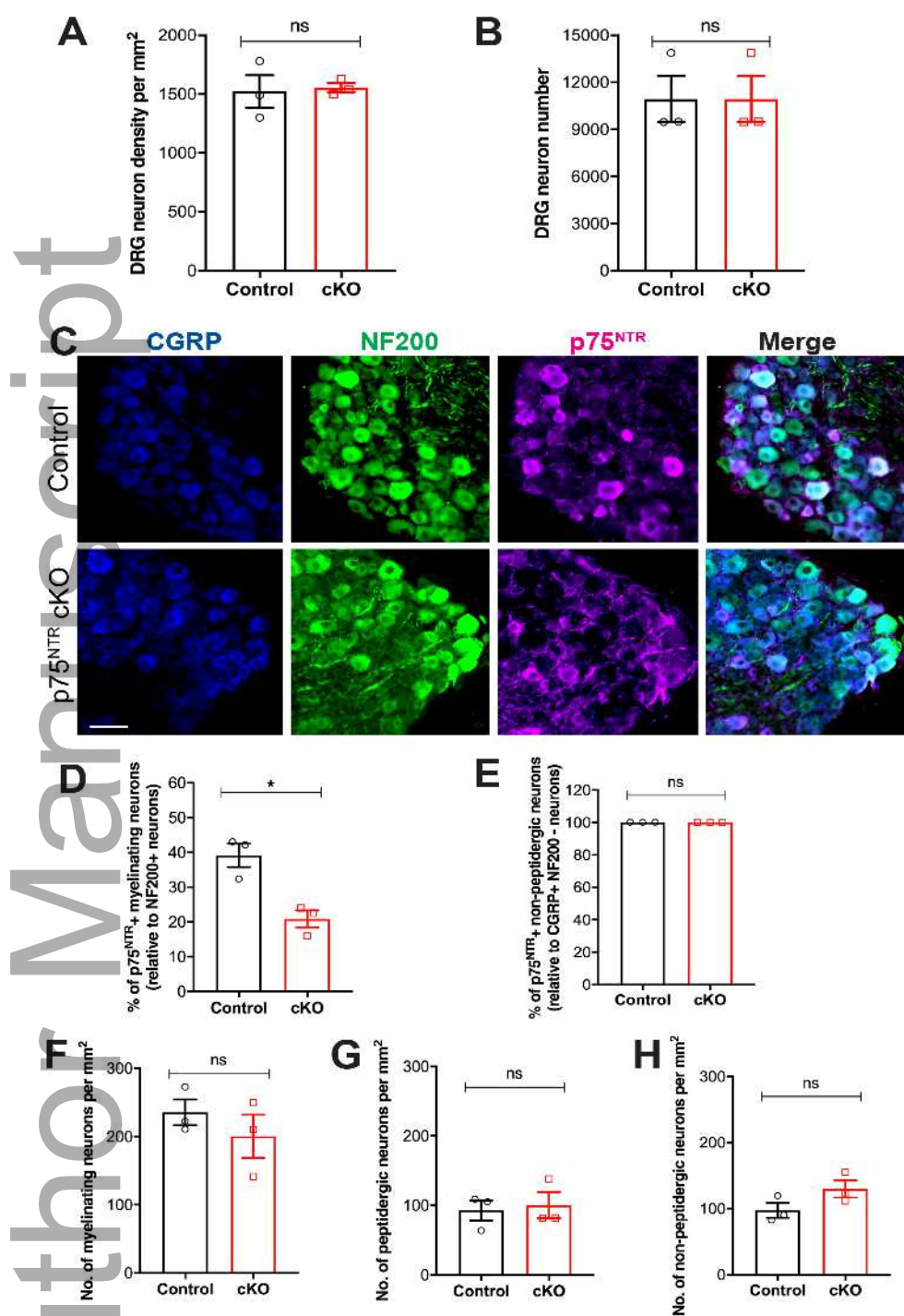
(D) Graphical representation of the g-ratios of dysmyelinated axons in relation to axon diameter (presented as scatterplots) in the sciatic nerves of p75^{NTR} cKO and control mice induced with EAN. There is no significant difference in the distribution of g-ratios in relation to axon diameter between genotypes. Dark lines represent the linear regression line for each dataset (n = 3 mice per genotype, linear regression analysis of the y-intercepts indicating no significant difference, n = 300–400 axons analysed per mouse per genotype).

(E) Quantification of the diameters of dysmyelinated axons in the sciatic nerve showing no significant difference in the range of axonal diameters between genotypes. (n=3 mice per genotype, error bars = mean ± SD, Descriptive statistical analysis).

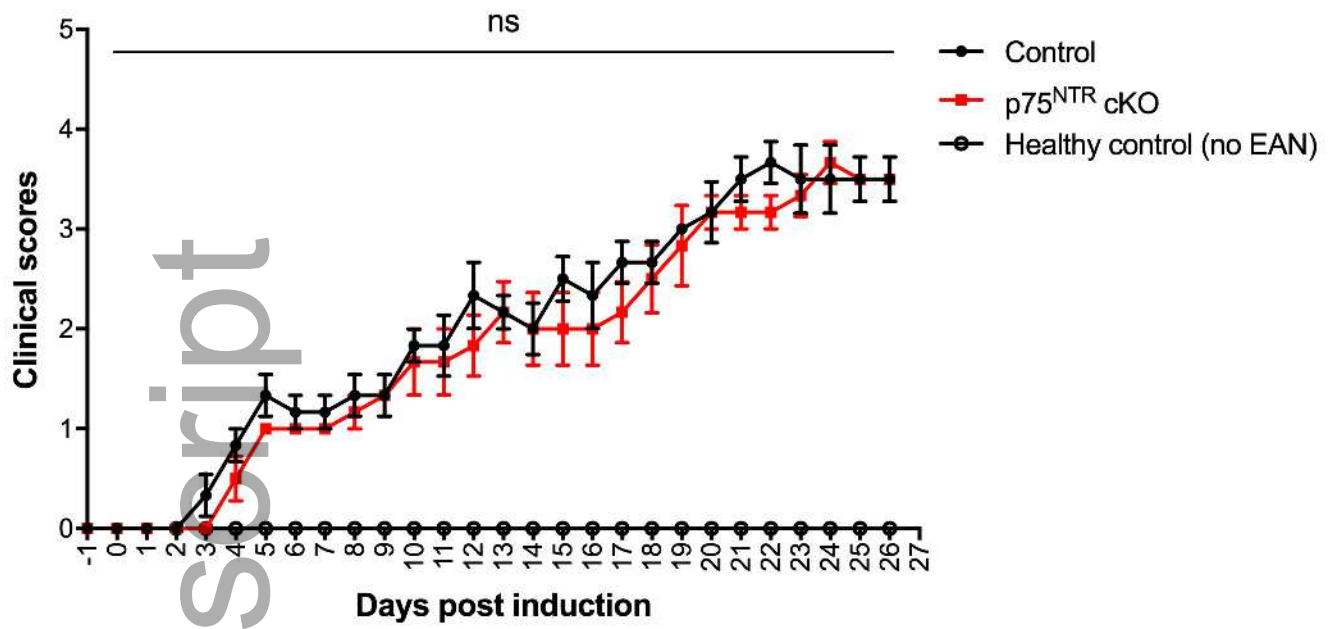
(F) Frequency distribution plot of dysmyelinated axon relative to axonal diameter in the sciatic nerves of p75^{NTR} cKO and control mice induced with EAN indicating no significant difference between genotypes (n = 3 mice per genotype, 300–400 axons analysed per mouse per genotype).



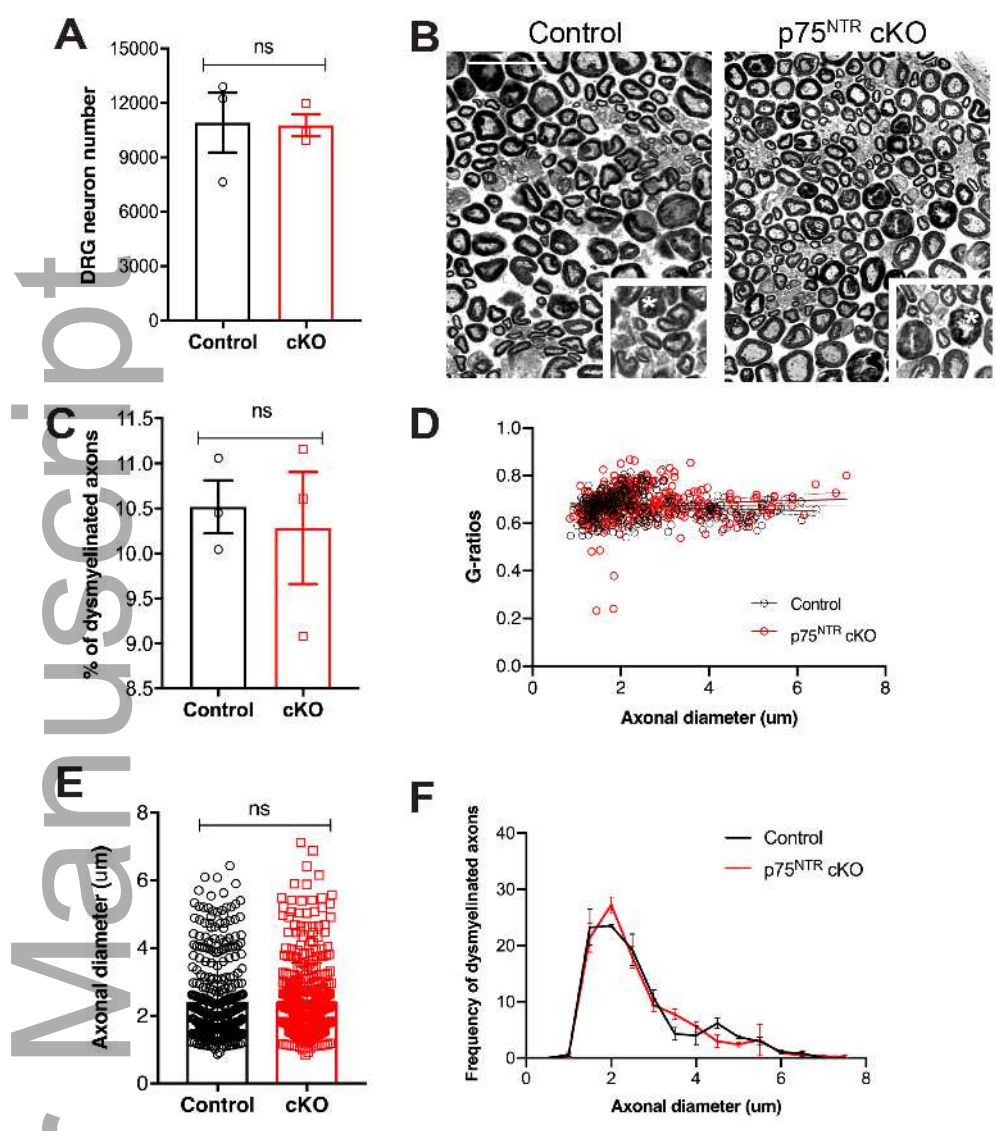
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jnr_24665_f3.jpg



jnr_24665_f4.tif