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ORIGINAL ARTICLE

# The impact of neurotrophin-3 on the dorsal root transitional zone following injury

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**Study design:** Morphological and Stereological assessment of the dorsal root transitional zone (DRTZ) following complete crush injury, using light microscopy (LM) and transmission electron microscopy (TEM).
**Objectives:** To assess the effect of exogenous neurotrophin-3 (NT-3) on the response of glial cells and axons to dorsal root damage.
**Setting:** Department of Anatomy, University College Cork, Ireland and Department of Physiology, UMDS, University of London, UK.
**Methods:** Cervical roots (C6-8) from rats which had undergone dorsal root crush axotomy 1 week earlier, in the presence ($n = 3$) and absence ($n = 3$) of NT-3, were processed for LM and TEM.
**Results:** Unmyelinated axon number and size was greater in the DRTZ proximal (Central Nervous System; CNS) and distal (Peripheral Nervous System; PNS) compartments of NT-3-treated tissue. NT-3 was associated with a reduced astrocytic response, an increase in the proportion of oligodendrocytic tissue and a possible inhibition or delay of microglial activation. Disrupted-myelin volume in the DRTZ PNS and CNS compartments of treated tissue was lower, than in control tissue. In the PNS compartment, NT-3 treatment increased phagocyte and blood vessel numbers. It decreased myelinating activity, as sheath thickness was significantly lower and may also account for the noted lower Schwann cell and organelle volume in the test group.
**Conclusions:** Our observations suggest that NT-3 interacts with non-neuronal tissue to facilitate the regenerative effort of damaged axons. This may be as a consequence of a direct action or indirectly mediated by modulation of non-neuronal responses to injury.
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**Keywords:** glia; axon; regeneration; NT-3; DRTZ; stereology

## Introduction

Damage to the nervous system causes a structural reorganisation of neuronal and non-neuronal cells and tissue. In the PNS, the Schwann cell response to nerve injury creates an environment which is supportive of regrowth and axons may re-establish synapses with their target tissue, resulting in functional recovery. Together with macrophages, recruited from the blood, Schwann cells phagocytose myelin and degenerating axons and, in addition, secrete trophic factors and adhesion molecules. By contrast, in the (post neo-natal) CNS, glial cell responses to injury contribute to the creation of a *milieu* which is inhibitory to regenerating axons.[1] Many factors account for this poor regenerative capacity including a deficiency of neurotrophic factors, the presence of myelin-associated inhibitory molecules (especially Nogo-A),[2,3] and barrier formation (glial scar) by hypertrophied astrocytes.[1]

Sensory axons transition from the peripheral nervous system to the central nervous system in an anatomically distinct site called the dorsal root transitional zone (DRTZ). Central to this, myelin sheaths are formed by oligodendrocytes and the supporting tissue is astrocytic. Peripheral to it, sheaths are formed by Schwann cells which are enveloped in endoneurium.[4] It was first noted by Ramón y Cajal (1928) that while injured sensory axons regenerate and grow through the PNS compartment of the DRTZ, they fail to enter the DRTZ-CNS compartment.[5] The dorsal root injury model has provided useful information on basic problems of

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regeneration in the CNS as it is possible to assess cell and tissue responses to nerve damage, such as that due to nerve crush axotomy, both distally (PNS compartment) and proximally (CNS compartment) and at the interface.[4] Injury to dorsal roots is also a clinically important entity in itself. Damage to cervical dorsal roots, which sometimes occurs in traffic accidents or during difficult childbirths, results in varying degrees of loss of sensation and motor function in the arm and hand, and often, severe intractable pain.[1]

Neurotrophins comprise a family of endogenous growth factors, which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT4/5. These trophic factors stimulate growth and promote survival of neurons through their interaction with the tropomyosin-related kinase (Trk) receptors; NGF binds to TrkA, BDNF and NT4/5 bind to TrkB, and NT-3 binds to TrkC. In addition, all of the neurotrophins bind to the pan-neurotrophin receptor (NTR) p75. Initially involved in embryogenesis and organogenesis, neurotrophins regulate synaptic activity, neurotransmitter synthesis and neural plasticity in adults.[6] They exercise control over various stages of glial development and in the periphery, impact on Schwann cell physiology.[7]

Using unbiased stereological methods,[8] this study seeks to investigate the effect of the endogenous neurotrophin NT-3 on the response of Schwann cells, astrocytes and oligodendrocytes to injury. In addition, effects of the neurotrophin on DRTZ axonal parameters are assessed. The findings focus on ultrastructural features, from morphological and morphometric perspectives. The study complements previous studies, involving immunohistochemistry, immuno-electron microscopy, and physiological and behavioural approaches to analysing the influence of neurotrophins on the regenerative capacity of dorsal root sensory axons, following damage.[9–11]

## Methods

Under sodium pentobarbital anaesthesia (45 mg/kg i.p.), quadruple dorsal root crush rhizotomies central to the dorsal root ganglion (DRG), were performed in 6 Sprague-Dawley (SD) rats at cervical levels C6, C7 and C8, as previously described.[9,11] At the time of surgery, in three animals (test group),osmotic minipumps (Alzet, Charles River, Margate, UK) were implanted into a subcutaneous pocket just below the scapula and the growth factor neurotrophin-3 (NT-3; 12 μg/d) was delivered into the intrathecal space at the cervical enlargement (approximately C6; verified post mortem). Penetration of spinal parenchyma by intrathecally-delivered proteins,using this technique,has been confirmed previously.[12]

The six rats (test/NT-3-treated: $n=3$; control/untreated: $n=3$) were allowed to survive for one week. In the cervical dorsal root rhizotomy model, in which dorsal roots are injured approximately 2 mm from the cord, one week is sufficient for regeneration up to (but in untreated animals, not beyond) the DRTZ; one week's delivery of NT-3 into the intrathecal space has been shown previously to promote regeneration of primary afferent axons into the cord.[9,11,13]

Following transcardiac perfusion with 2.5% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, spinal cord cervical sections were removed and further processed for microscopical analysis. Alternating sequential series of thin (90 nm) and semithin (0.5 μm) transverse sections of rootlets at cervical levels C6, C7 and C8, were prepared. The mid-level of the central tissue projection (CTP), at which the rootlet cross section showed approximately equal proportions of the central (CNS compartment) and peripheral (PNS compartment) tissues of the rootlets, was chosen for the LM and TEM study.

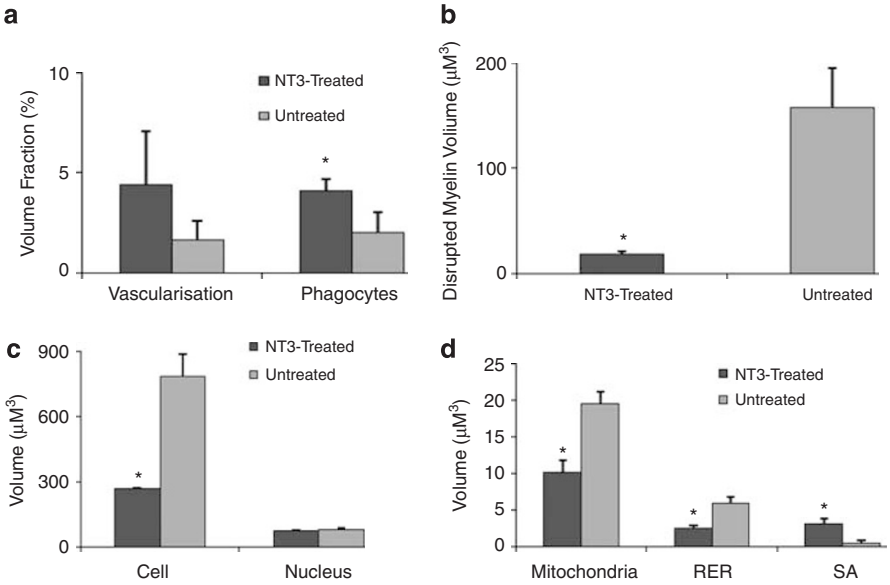


**Figure 1** (**a**) Bar chart showing the proportion of the cross sectional area occupied by blood vessels and phagocytes in the PNS compartment of the DRTZ in untreated and NT-3-treated tissue. (**b**) Mean volume estimates of disrupted myelin in the DRTZ PNS compartment of both groups. (**c**) Mean Schwann cell nuclear and cell volume estimates in NT-3-treated and untreated tissue. (**d**) Mean Schwann cell organelle volume estimates for mitochondria, RER and SA in both groups. *Difference statistically significant.

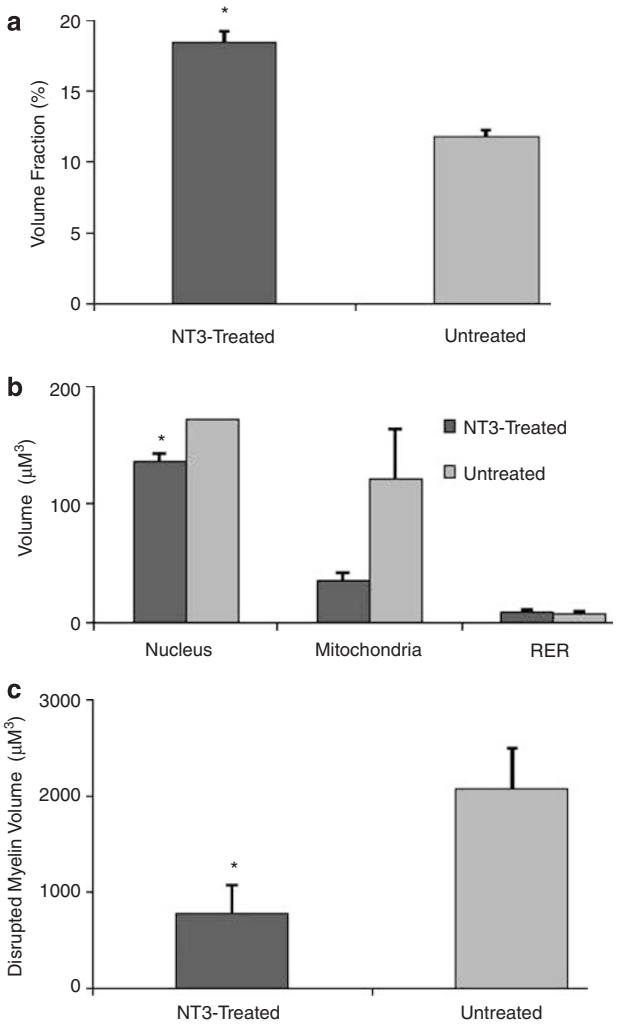


**Figure 2** (**a**) Mean oligodendrocytic tissue volume fraction (%) in NT-3-treated and untreated tissue. (**b**) Mean oligodendrocyte nuclear, mitochondrial and RER volume estimates in untreated and NT-3-treated tissue. (**c**) Mean volume estimates of disrupted myelin in the DRTZ CNS compartments of both groups. *Difference statistically significant.

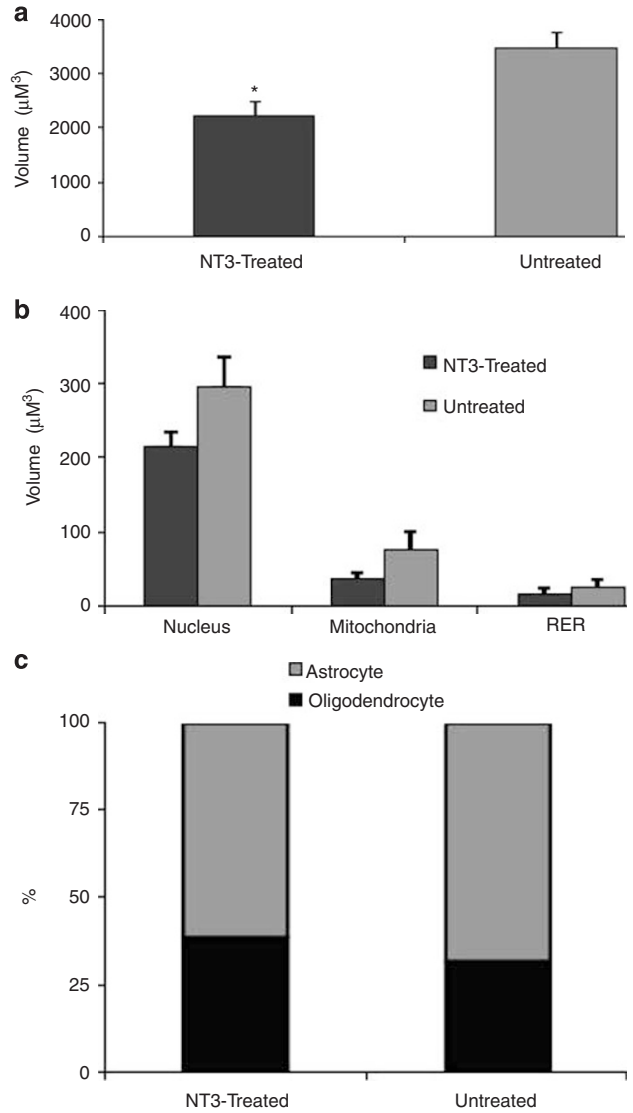


**Figure 3** (**a**) Mean astrocytic process volume in both groups. (**b**) Mean astrocyte nuclear, mitochondrial and RER volume estimates in untreated and NT-3-treated tissue. (**c**) Relative proportions of oligodendrocyte and astrocyte tissue in NT-3-treated and untreated tissue. *Difference statistically significant.

*Stereology*

Stereological analyses of the composition of the transversely sectioned root face were carried out, using unbiased techniques.[8]

*Light microscopy.* Mean volume-weighted nuclear volumes of Schwann cells (Figure 1c), oligodendrocytes (Figure 2b) and astrocytes (Figure 3b) were estimated, using the point-sampled intercept (PSI) method applied to toluidine blue-stained semithin sections.

*Transmission electron microscopy*

(1) Glial cell parameters

- Simple point-counting was applied to estimate mean volume density (Vv) nucleus:cell, which in combination with nuclear volume estimates, enabled an estimation of the mean cell volume for the three cell types: Schwann cells, oligodendrocytes and astrocytes (Table 1). Mean volume density (Vv) organelle:cell was estimated for glial cell mitochondria and rough endoplasmic reticulum *(RER)*. Additionally, Schwann

**Table 1** Glial cell volume (Mean ± s.e.m.; $\mu m^3$) in untreated and NT3-treated tissue

| | *Untreated* | *NT3-Treated* | *t-Test* |
|---|---|---|---|
| Schwann Cell | 783 ± 104 | 270 ± 6 | *$P<0.04$ |
| Oligodendrocyte | 3050 ± 566 | 1391 ± 376 | *$P<0.005$ |
| Astrocyte | 4444 ± 401 | 2811 ± 232 | *$P<0.04$ |

*Difference statistically significant.

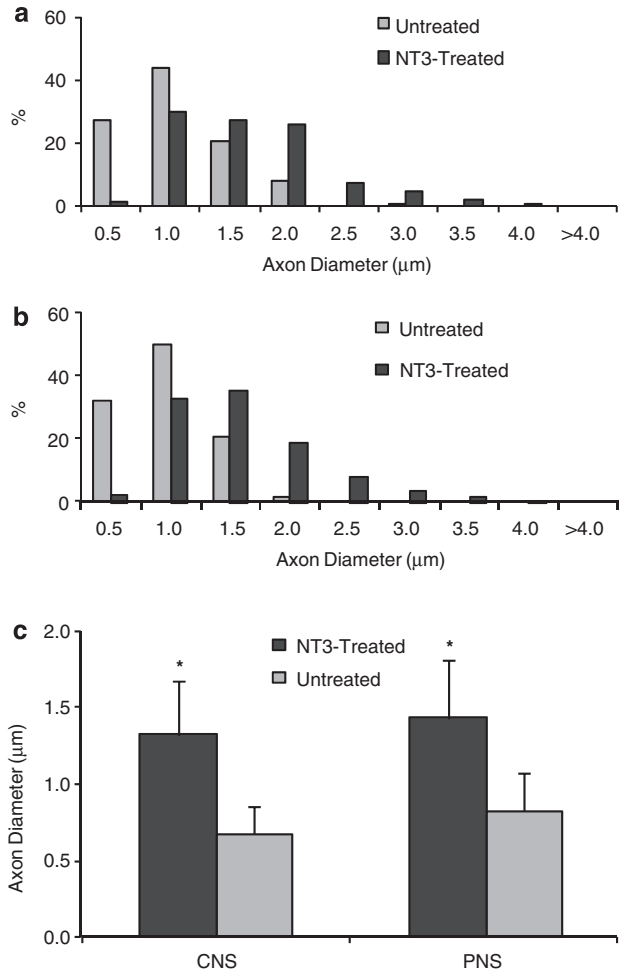


**Figure 4** DRTZ diameter distributions of unmyelinated axons in NT-3-treated and untreated tissue in the (**a**) PNS compartment, and (**b**) CNS compartment. (**c**) Mean axon diameter of unmyelinated axons in the PNS and CNS DRTZ compartments of both groups. *Difference statistically significant.

cell Vv (volume density) secretory apparatus (SA):cell was assessed.

$$\mathrm{Vv} = \frac{\text{Volume of feature in reference space}}{\text{Volume of reference space}}$$

The corresponding organelle volumes were then estimated based on the cellular-volume for each cell type (Figures 1d, 2b and 3b). In the present study, secretory apparatus includes secretory vesicles, smooth endoplasmic reticulum and the Golgi complex. Mean volume of disrupted myelin was estimated per compartment: PNS (Figure 1b) and CNS (Figure 2c).

(2) Axonal parameters

- Using measurements of transversely-sectioned axon perimeters, the equivalent diameters were estimated on the basis that the true cross sectional profile was circular. Axon diameter distribution is shown as percentage frequency histograms (Figures 4a and b); mean values are shown in Figure 4c.

**Table 2a** Mean total axon count per root (Mean ± s.e.m.; $n = 3$) in untreated and NT-3-treated tissue

| | *Compartment* | |
|---|---|---|
| | *CNS* | *PNS* |
| Untreated | 29.7 ± 10.3 | 62.7 ± 18.3 |
| NT-3-Treated | 92.7 ± 8.9 | 99.3 ± 4.6 |
| *t*-Test | *$P<0.01$ | $P>0.05$ |

*Difference statistically significant.

**Table 2b** Mean myelinated axon count (Mean ± s.e.m.) and myelin sheath thickness (Mean ± s.e.m.; μm), the PNS compartments of NT-3-treated and untreated tissue

| | *PNS compartment* | | |
|---|---|---|---|
| | *Untreated* | *NT-3-Treated* | *t-Test* |
| Myelinated axon | 7.0 ± 4.0 | 11.0 ± 6.0 | $P>0.05$ |
| Myelin Sheath (μm) | 0.2 ± 0.06 | 0.1 ± 0.03 | *$P<0.01$ |

- Axons were counted in both compartments (Tables 2a and b). Myelin sheath thickness was measured (Table 2b) in the small number of myelinated which occurred in the PNS compartment of both groups.
- Microtubules were counted in unmyelinated axons in each of the compartments and plotted against axon cross sectional area (Figures 5a–d); the strength of the relationship between the two parameters was also calculated (Table 3).

*Data analysis*

Data were analysed using unpaired Student's *t*-test; the conventional probability of $P<0.05$ was taken as the limit of statistical significance. All the tests were performed using SPSS software.

## Results

*Morphological assessment*

Disrupted myelin was evident in the CNS compartment of both groups. NT-3-treated tissue (Figure 6a) contrasted strongly with untreated tissue in having a much larger incidence of regenerating axons. In the PNS compartment, features of degeneration were also prominent in both groups. These comprised typical myelin breakdown products, including sheath fragmentation and disruption, lamellar separation and the formation of lipid inclusions, as well as axon degeneration and phagocytosis.

Myelinated axons were almost non-existent in the CNS compartment of both groups. In the PNS compartments, a small number of axons were myelinated and or undergoing incipient myelination (Figure 6b).

*Stereological assessment*

*Non-neuronal tissue.* Our morphometric and stereological studies at one week after operation showed changes in the proportions of the non-neuronal tissue components. Statistical comparison was by unpaired Student's *t*-test, unless stated otherwise.

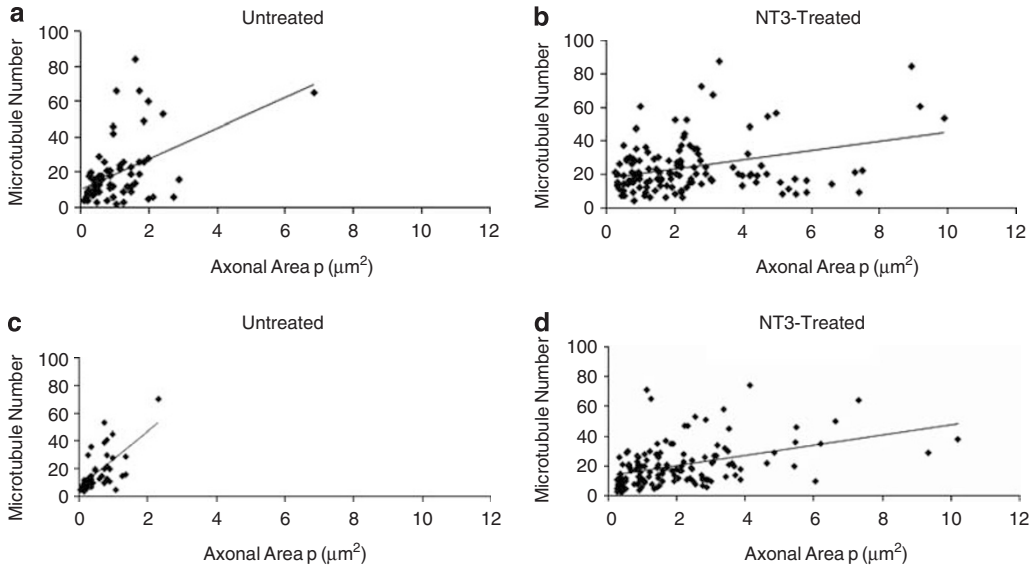


**Figure 5** Scattergrams showing relationships between microtubule number and axonal area, of untreated and NT-3-treated unmyelinated axons in: (**a**–**b**) DRTZ PNS compartment; linear regression lines: (a): $y = 8.6x + 10.5$; (b): $y = 2.7x + 17.8$; (**c**–**d**) DRTZ CNS compartment; linear regression lines: (c): $y = 19.4x + 7.32$; (d): $y = 3.4x + 13.5$.

**Table 3** Coefficients of correlation [$r$] and of determination [$r^2$] for microtubule numbers vs axonal cross-sectional area, in PNS and CNS unmyelinated axons of NT3-treated and untreated tissue

| | *CNS* | | *PNS* | |
|---|---|---|---|---|
| | $r$ | $r^2$ | $r$ | $r^2$ |
| Untreated | 0.6 | 0.4 | 0.5 | 0.2 |
| NT3-Treated | 0.4 | 0.2 | 0.3 | 0.1 |

*PNS*: The mean volume fraction of phagocytes was higher ($P<0.04$), vascularistion more prominent (albeit not significant; $P>0.05$) and disrupted myelin volume estimates lower ($P<0.02$) in NT-3-treated tissue compared with control tissue (Figures 1a and b). Mean Schwann cell ($P<0.04$; Table 1), mitochondrial and RER volumes were lower ($P<0.04$ and $P<0.02$, respectively), and SA volume higher ($P<0.01$) in NT-3-treated tissue (Figure 1d). However, there was no significant difference ($P>0.05$) between the mean nuclear volume estimates for the two groups (Figure 1c).

*CNS*: In NT-3-treated tissue, a trend for greater vascularisation was noted but to a much smaller degree than in the PNS compartment (data not shown). In addition, there was pronouncedly less disrupted myelin present, compared with untreated tissue ($P<0.005$; Figure 2c). The mean oligodendrocytic tissue volume fraction was greater ($P<0.02$; Figure 2a) and the nuclear volume estimate smaller ($P<0.04$; Figure 2b) in the presence of the neurotrophin. Oligodendrocyte RER and mitochondrial volumes were not different ($P>0.05$) between the two groups, however mitochondrial volume estimates exhibited a larger trend in untreated tissue (Figure 2b).

Mean astrocyte cell volume (Table 1) and specifically that of the processes (Figure 3a), was less in NT-3-treated tissue ($P<0.04$ and $P<0.03$ respectively). While not statistically significant ($P>0.05$; Figure 3b), the trend was one of lower mean estimates for astrocyte nuclear, mitochondrial and RER volumes in NT-3-treated tissue. Astrocytic and oligodendrocytic tissue volume fractions showed opposite trends in the presence and absence of the neurotrophin (Figure 3c).

Volume fraction estimates for microglia were notably lower, although not significantly, in NT-3-treated tissue (0.1 vs 0.6% respectively; $P>0.05$; data not shown).

*Axons*. As myelinated axons were present at very low numbers in the PNS compartments and almost non-existent in the CNS compartments of both groups, the data presented are from morphometric analyses of unmyelinated axonal parameters.

Unmyelinated axon calibre distributions were shifted to the right (Figure 4a and b) and mean axonal diameters (Figure 4c) were larger ($P<0.005$) in both compartments of NT-3-treated tissue, compared with the control group.

Mean axon number per root, was higher in the CNS compartment of the NT-3-treated group compared untreated animals ($P<0.1$); a similar trend (although not significant), was noted in the respective PNS compartments (Table 2a). Correlation coefficients (Table 3) and regression line slopes for linear relationships between microtubule number and axonal area were lower in NT-3-treated tissue (Figures 5a–d).

While the mean number of myelinated axons counted was similar ($P>0.05$), myelin sheath thickness was significantly lower ($P<0.01$) in NT-3 treated PNS tissue (Table 2b).

## Discussion

The overall conclusion from this study is that exogenous NT-3 had clearly defined effects on neuronal and non-neuronal tissue in both the peripheral (PNS) and central (CNS) DRTZ

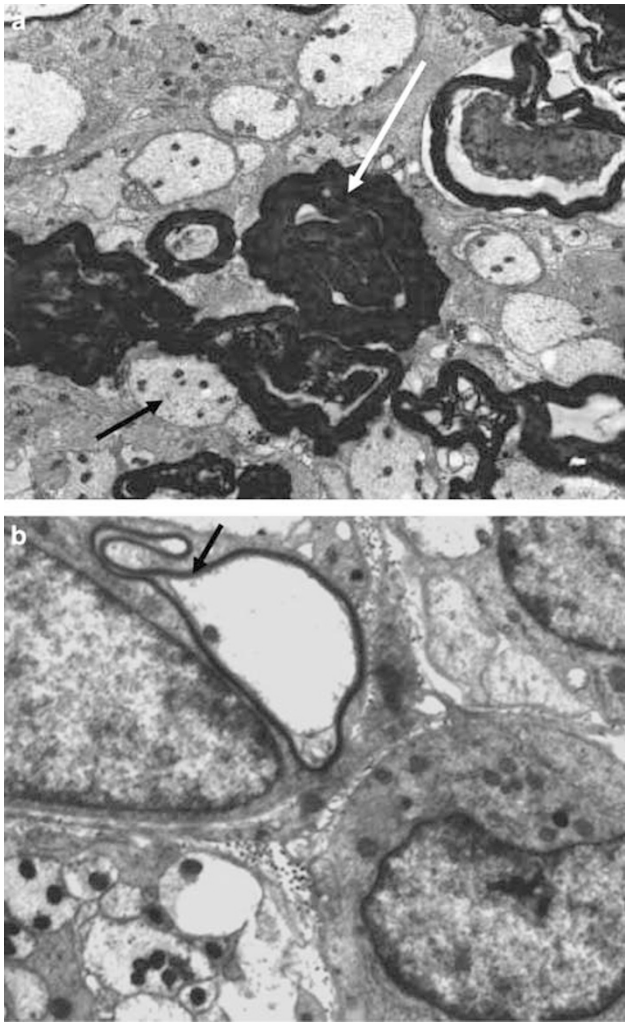


**Figure 6** Electron micrographs of transverse DRTZ sections of NT-3-treated tissue (**a**) Axons (black arrow) are abundant in the CNS compartment, surrounded by degenerating myelin profiles (white arrow). (**b**) Early remyelination of a regenerating axon (black arrow) in the PNS compartment.

compartments. Our findings point to positive actions of NT-3 on the response of glial cells to injury, as the astrocytic response was lower and there was evidence of greater oligodendrocytic survival, in its presence. It is possible that these effects may in part be an indirect consequence of the action of the neurotrophin on microglia. Activated microglia are thought to be involved in evoking apoptosis in oligodendrocytes following injury[14] and while the trigger(s) involved in astrocytic activation are unknown, it has been suggested that they occur secondary to microglial activation.[15] Our finding of a trend of lower microglial volume fraction in NT-3-treated tissue may indicate a delay or even an inhibition of microglial activation, by the neurotrophin.[16]

The trend of increased vascularisation in the PNS (and CNS, to a smaller extent) compartment of NT-3-treated tissue noted in this study, would support the report of angiogenic properties of the neurotrophin.[17] This could, in turn, facilitate recruitment of macrophages to the site of injury and intensify removal of disrupted myelin by phagocytosis. This is further supported by our finding that phagocytic and disrupted myelin measurements are inversely linked, in the PNS compartment of NT-3- treated tissue.

Correlation coefficients for linear relationships between microtubule number and axonal area were lower in unmyelinated axons from treated animals in both compartments of the DRTZ. This is consistent with findings in elongating axons due to a dynamic instability of microtubules in the growth cone region.[18]

In the DRTZ PNS and CNS compartments of un-operated (normal) adult SD rats, unmyelinated axonal diameters range from 0.5–1.5 μm, with the highest frequency occurring in the 0.5–1.0 μm region (93.0 ± 1.8 and 94.8 ± 5.2%, respectively). At diameters of 2.0 μm and greater, the profile is one of myelinated axons (data not shown). In this study, axon diameter distribution in untreated damaged tissue mirrors that seen in undamaged (normal) tissue.

In NT-3-treated roots, the profile is quite different as the mean axonal diameter of unmyelinated axons in both compartments is increased and the diameter distributions are shifted to the right, compared with untreated tissue. Axons of 2.0 μm and greater remain unmyelinated and in the small number of myelinated axons within the PNS compartment of both experimental groups, sheath thickness is significantly lower in NT-3-treated tissue. This strongly suggests that NT-3 may be inhibiting myelinating activity in Schwann cells. Both exogenous and endogenous NT-3 is reported to have an inhibitory effect on myelination.[19,20]

A delay in myelination would potentially prolong access to axolemmal receptors by trophic agents and may be correlated to the overall increase in axon size noted in this study. It may also have been significant in enabling a larger number of axons to successively extend within the 'hostile' *milieu* of the damaged CNS compartment, evidenced by our finding of a greater axon count in NT-3-treated tissue.

In the nervous system, neurons and glia share a mutual dependence in both establishing and maintaining a functional relationship; injury causes a sudden disruption of the status quo. The outcome of non-neuronal tissue response to injury can be favourable in the PNS, with the potential for nerve regeneration and reestablishment of function.[1] In the CNS, injury causes the relationship between neuronal and non-neuronal tissue to go awry, leaving nerves hindered in their regenerative effort. Agents such as NT-3, offer the potential of effective therapeutic responses following CNS damage, which may result in axonal regenerative success.

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