Neurotrophins 3 and 4 differentially regulate NCAM, L1 and N-cadherin expression during peripheral nerve regeneration

Martin R. Thornton*†, Susan G. Shawcross*†, Cristina Mantovani*, Paul J. Kingham*, Martin A. Birchall† and Giorgio Terenghi*

*Blond McIndoe Laboratories, Plastic and Reconstructive Surgery Research, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT, U.K., and †Department of Clinical Veterinary Science, University of Bristol, Langford House, Langford, Bristol BS40 7DU, U.K.

The addition of NT-3 (neurotrophin 3) or NT-4 to injured nerves improves their regeneration potential and may aid axon guidance. It is not well defined whether NTs (neurotrophins) influence other elements, such as the cell-adhesion molecules, which promote nerve guidance and regeneration. Using poly-3-hydroxybutyrate conduits, we applied either NT-3 or NT-4 to axotomized rat sciatic nerves and monitored nerve regeneration and cell-adhesion molecule expression. Regenerating nerves were stained with antibodies against NCAM (neural cell-adhesion molecule) and N-cadherin 2 weeks after injury and staining intensity was quantified. NCAM, N-cadherin and L1 (L1 cell-adhesion molecule) transcription was measured in the proximal and distal stumps and ipsilateral DRG (dorsal root ganglia) (fourth and fifth DRG) using RT (reverse transcriptase)–PCR. Both NT-3 and NT-4 increased NCAM and L1 transcript levels in the DRG of axotomized nerves. This is reflected in the increased NCAM expression at the proximal stump and regeneration front. Increased levels of NCAM were also observed in the distal stump. NT-4 administration increased N-cadherin levels proximal to the injury, but not distally. Following NT-3 administration, N-cadherin expression decreased in proximal and distal stumps compared with the control. In conclusion, NTs differentially alter adhesion molecule expression in regenerating nerves and transcription in the corresponding DRG, although these changes in expression do not alter NT-enhanced regeneration. Thus we propose that retrograde transport of the NTs to the DRG affects adhesion molecule transcription, reflected by protein expression in peripheral nerve axons.

Introduction

In the process of nerve regeneration that follows peripheral-nerve injury, Schwann cells present in the distal stump of the damaged nerves secrete neurotrophic factors [1,2]. Of these neurotrophic factors, NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor) and NT-4 (neurotrophin 4) are up-regulated in response to injury, whereas NT-3 is not [1,3–5]. It has been hypothesized that these factors diffuse across an injury site and exert trophic effects on axons regenerating from the proximal stump [6]. However, this is not sufficient to compensate for the lack of target-derived neurotrophic factor required to maintain cell survival [1,7].

A number of experiments have concentrated on delivering growth factors close to the site of nerve injury in an attempt to improve nerve regeneration. In vitro administration of NT-3 to adult DRG (dorsal root ganglia) has been shown to stimulate neurite outgrowth [8]. In addition, in vivo application of NT-3 to cut sciatic nerve improves the rate and extent of axonal regeneration [9]; it is also capable of restoring the sensory and motor conduction velocity [10]. Local exogenous delivery of NT-4 to axotomized rat sciatic nerve is capable of promoting nerve regrowth [11]. Experiments by our group have demonstrated the selectively beneficial effects of NT-3 and NT-4 upon fast and slow muscle fibre-type reinnervation. Administration of NT-3 to the proximal stumps of cut sciatic nerves results in improved reinnervation of fast motor units by fast fatigable motoneurons [12–14]; NT-4 administration resulted in significant improvement of slow motor-unit reinnervation.

Key words: dorsal root ganglia, L1 cell-adhesion molecule (L1), N-cadherin, neural cell-adhesion molecule (NCAM), neurotrophins 3 and 4, peripheral-nerve regeneration.

Abbreviations used: BDNF, brain-derived neurotrophic factor; DRG, dorsal root ganglia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry; L1, L1 cell-adhesion molecule; NCAM, neural cell-adhesion molecule; NT, neurotrophin; NT-3, neurotrophin 3; NT-4, neurotrophin 4; PC12 cells, pheochromocytoma cells; PHB, poly-3-hydroxybutyrate; PGP 9.5, protein gene product 9.5; RT, reverse transcriptase.

To whom correspondence should be addressed (email sue.shawcross@manchester.ac.uk).
reinnervation [15]. NT-4 has been shown to enhance the survival of injured rat retinal ganglion cells [16] and acts as a potent survival factor for motoneurons [17].

Cell-adhesion molecules are believed to play an important role during nerve regeneration. NCAM (neural cell-adhesion molecule) mediates interactions between axons [18] and, along with L1 (L1 cell-adhesion molecule), between Schwann cells and axons [19–22]. In vitro, NCAM and L1 have been shown to promote neurite outgrowth from chick ciliary ganglion on the surface of cultured Schwann and L1 have been shown to promote neurite outgrowth from chick ciliary ganglion on the surface of cultured Schwann cells [23–26]. N-cadherin, a Ca\(^{2+}\)-dependent intercellular adhesion molecule, has been identified on the surfaces of nerve growth cone in vitro [27–29]. N-cadherin is also present on the plasma-membrane contact surface where axon–axon and axon–Schwann cell interactions occur in vivo, but not where axons or Schwann cells were in contact with basal lamina [30]. In addition, both NCAM [31–33] and N-cadherin [30,33] appear to be up-regulated in proximal stumps after nerve injury. Also, in transected adult nerve, L1 and NCAM were detectable in the distal nerve end. Interestingly, the reduction of L1-antigen expression to its normal adult level took more than a year [32].

Some in vitro studies have shown that NTs (neurotrophins) cause an increase in L1, but not NCAM, expression in cultured PC12 cells (pheochromocytoma cells) [34,35], cultured mouse Schwann cells [36] and astrocytes [37], whereas other studies have shown that NGF up-regulated NCAM expression in PC12 cells [38] and down-regulated NCAM and cadherin expression in a mouse fibroblast cell line [39] by exerting its action through the TrkA (tropomyosin receptor kinase A) tyrosine kinase receptor [40]. The in vivo administration of BDNF to TrkB receptor-expressing retinal ganglion cells leads to a 3-fold increase in the number of L1 mRNA-expressing retinal ganglion cells 14 days after injury [41].

In the present study, we have investigated the effects of NT-3 and NT-4 on the expression of the adhesion molecules during nerve regeneration as at present little is known about the specific outcome. Following targeted administration of the growth factors via PHB (poly-3-hydroxybutyrate) nerve conduits, the presence of NCAM and N-cadherin was detected by IHC (immunohistochemistry) and their level was assessed by computerized image analysis 2 weeks after injury. Immunohistochemical detection of L1 proved to be problematic; thus quantification of this molecule was abandoned. In addition to the IHC studies, the NCAM, N-cadherin and L1 transcription levels were assessed by an end-point RT (reverse transcriptase)–PCR. In summary, we found that NTs differentially alter adhesion-molecule expression in regenerating nerves and transcription in the corresponding DRG, but these changes in expression do not change NT-enhanced regeneration.

**Materials and methods**

**Animals and surgical procedure**

All procedures were carried out in compliance with the U.K. Animals (Scientific Procedures) Act 1986. Sprague–Dawley rats (6–8 weeks old), weighing approx. 250 g, were anaesthetized with a Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) and oxygen mixture. The left sciatic nerve was exposed and divided 5 mm distal to the sciatic notch using sharp microscissors. A 5 mm segment of sciatic nerve was removed to produce a 10 mm gap after retraction of nerve ends. Gaps were bridged with a PHB conduit [42] filled with either alginate alone (control) or with the addition of either NT-3 (500 ng/ml; Autogen Bioclear) or NT-4 (1000 ng/ml; Autogen Bioclear). For immunohistochemical analysis, five or six animals from each group were killed by raising the concentration of inhaled carbon dioxide and subsequent cervical dislocation at 2 weeks post surgery. Previous studies showed that this was sufficient time for maximal up-regulation of cell-adhesion molecules after injury [33]. Proximal nerve, grafted conduit and distal nerve segments were harvested en bloc, fixed in 4 % (w/v) paraformaldehyde (Sigma), rinsed in 0.01 M PBS containing 15 % (w/v) sucrose (Sigma) and 0.1 % (w/v) sodium azide (Sigma) and processed for morphological analysis.

For the assessment of adhesion molecule transcript levels, the surgical procedures were performed as outlined above with five or six animals per treatment regime. On the operated side of each animal the fourth and fifth DRGs were harvested and pooled. The proximal stump (50 mm) plus conduit and 50 mm of sciatic nerve immediately distal to the conduit were harvested and immediately snap-frozen in liquid nitrogen then transferred to – 80 °C for storage until all samples had been collected.

**Tissue processing and immunohistochemical analysis**

The specimens were blocked in Tissue-Tek\textsuperscript® O.C.T. Compound (BDH Laboratory Supplies, Poole, Dorset, U.K.) with a piece of rat liver marking the proximal end of the nerve to identify the orientation of each sample. Cryostat sections (15 \(\mu\)m) were cut longitudinally, and collected on Vectabond-coated slides (Vector Laboratories) and then dried overnight at 37 °C. The sections were permeabilized with 0.1 % (w/v) Triton X-100, washed in PBS (5 min) and blocked with 1 % (w/v) normal goat serum for 1 h. Sections were incubated overnight with primary antibodies against NCAM (rabbit polyclonal to all NCAM isoforms, dilution 1:500; Chemicon), N-cadherin (rabbit polyclonal, dilution 1:20; Santa Cruz Biotechnology), S-100 (protein gene product 9.5) (mouse monoclonal, 1:600; Affiniti) or PGP 9.5 (protein gene product 9.5) (mouse monoclonal, 1:2000; Biogenesis). Sections
were washed with PBS (2 × 5 min) and incubated with either polyclonal goat anti-rabbit–FITC (1:100; Vector Laboratories) for adhesion molecules or incubated with monoclonal goat anti-mouse–Cy3™ (1:100; Amersham Biosciences) for double staining with either S-100 or PGP 9.5 for 1 h. Following a final rinse in PBS (2 × 5 min), the sections were mounted in Vectashield (Vector Laboratories) to minimize decay of fluorescence.

**Imaging, quantification and morphometric analysis**

Specimens were examined with a BX60 microscope (Olympus) and high-definition monochrome images were captured with an Evolution™ QEI digital camera (Media Cybernetics). Quantification of staining intensity requires mandatory precautions to minimize artefactual variation in staining intensity and these were carried out as follows. All images for quantification were taken in monochrome to ensure that there was no overlap or duplication of the sections, adjacent fields for each image were captured allowing that there was no overlap or duplication of the measured area. For each captured image, the areas of positive staining were edited above the threshold levels to exclude background noise. Image-Pro Plus® software (Media Cybernetics) was used to measure the overall intensity of positively stained areas. Intensity per unit area (arbitrary units) was then calculated from these values and used as a relative indicator of adhesion molecule expression.

**Assessment of L1, NCAM and N-cadherin transcript levels in axotomized sciatic nerve and their associated DRGs**

**Total RNA preparation**

Total RNA was extracted from the axotomized, conduit-wrapped sciatic nerve proximal and distal segments (Figure 1) and the ipsilateral DRG (fourth and fifth) using a Qiagen RNaseasy Protect mini-column system (Qiagen). All extracts were treated with RNase-free DNase I (Qiagen). RNA concentrations were determined spectrophotometrically at 260 nm by using RNase-free cuvettes (Eppendorf UVette®) and a WPA 1101 photometer (Biotech). The integrity of the RNA was assessed by agarose-gel electrophoresis. An aliquot of each RNA was diluted to 1 ng/µl using RNase-free water (Sigma) and stored at −80 °C; the concentrated stock RNAs were also stored at −80 °C.

RT–PCR Four pairs of oligonucleotides (Invitrogen Life Technologies, Paisley, Renfrewshire, Scotland, U.K.) were used for the one-step RT–PCRs: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) [43], NCAM [44,45], L1 [46] and N-cadherin [47] (see Table 1). To exclude the possibility of amplification from genomic DNA, all primers, with the exception of GAPDH, amplified across exon–exon boundaries. The RT–PCRs were performed using the Qiagen One-Step RT–PCR kit (Qiagen) in a total volume of 10 µl and using a preheated (50 °C) MJ Research PTC-200 thermal cycler with hot bonnet. BioPlastics® (BioPlastics, Landgraaf, The Netherlands) 0.2 ml thin-walled PCR tubes were used throughout. The optimum annealing temperature (59.2 °C) for the primer pairs was determined using rat Schwann cell total RNA as a target. A standard curve of band intensity versus amplification cycle number was constructed using the GAPDH primer pair and 1 ng of rat sciatic nerve total RNA per reaction. From this standard curve, it was determined that, for all primer pairs, a total of 35 cycles of PCR would be used, which ensured that all reactions were captured and the data were collected in the exponential phase of amplification. These amplicons were sequenced using a BigDye® Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) and its associated protocol, followed by electrophoresis on an Applied Biosystems ABI 3100 capillary sequencer.

**Agarose-gel electrophoresis and amplicon quantification**

Following RT–PCR, the amplicons were electrophoresed through 2% (w/v) agarose (Melford Laboratories, Chelsworth, Ipswich, Suffolk, U.K.) gels in 1 × TAE (Tris/acetate/EDTA) buffer [48] and stained by incubation in a
Figure 1  Schematic diagram showing which areas of the transected nerve and conduit were harvested and analysed using immunostaining methods.

The proximal and distal stumps were harvested and analysed by both immunostaining and RT–PCR procedures. The nerve regeneration fronts were analysed by immunostaining only and the ipsilateral DRG are not shown, but were harvested and analysed in the RT–PCR experiments. The PHB conduit was 14 mm long with an internal diameter of 1.6 mm. A 2 mm length of the proximal stump and of the distal stump of the transected nerve were inserted into the conduit, which created a 10 mm gap between the cut ends.

Table 1 Oligonucleotide primer pairs used for the RT–PCRs

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Primer sequence (5′–3′)a</th>
<th>Amplicon size (bp)</th>
<th>GenBank® accession number</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH (rodent)</td>
<td>ACCACAGTCCATGCCATCAC</td>
<td>433</td>
<td>M17701.1</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td>TCCACCCACCTGGCTGTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCAM (rodent)</td>
<td>GCAGGTAGATATTGGTTCCCA</td>
<td>137</td>
<td>NM_031521</td>
<td>[44,45]</td>
</tr>
<tr>
<td></td>
<td>GGTTCCTGCTACGCTTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1 (rat)</td>
<td>ATGGTCATGATGCTGTGGTACGT</td>
<td>545</td>
<td>X59149</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td>GCCCATGGACACCCGCTCATC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-cadherin (rat)</td>
<td>ACTGAGGAGCCGATGGAAGGAACCAC</td>
<td>234</td>
<td>NM_031333</td>
<td>[47]</td>
</tr>
<tr>
<td></td>
<td>GTTGATGATGAGATGCCCGCGTGGGA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aLower case indicates mismatched base in the rat sequence.

solution of 0.5 μg/ml ethidium bromide for 30 min with gentle agitation, and then washed for 20 min in water with gentle agitation. The gels images were captured under UV transillumination using standardized conditions with an Alpha Imager 2200 gel documentation system (Alpha Innotech, San Leandro, CA, U.S.A.). The intensity of the bands was determined using the AlphaEaseFC (Alpha Innotech) software using non-saturated images. To account for slight sample-to-sample and gel-to-gel variations, the adhesion-molecule transcript levels (band intensity) were expressed as a proportion of the GAPDH transcript (band intensity) for each sample.

Statistical analysis  Analysis of the immunostaining results was carried out using SigmaStat® software program (SPSS Science). Data were significantly skewed. Therefore Kruskal–Wallis one-way ANOVA on ranks was used to analyse the median values (25th–75th centiles) of intensity of adhesion-molecule staining per unit area of nerve in NT-3- and NT-4-treated nerves and the control. Differences between control and experimental groups were identified by the use of a multiple comparisons procedure (Dunn’s test). A Mann–Whitney rank sum test was used to compare differences between NT-3- and NT-4-treated groups. Statistical analysis of the adhesion-molecule transcript data was carried out using GraphPad Prism4 and GraphPad InStat3 (GraphPad Software, San Diego, CA, U.S.A.). The data (parametric) were analysed by ANOVA, with a post hoc Tukey’s multiple comparison test, to compare differences between the control group and the groups treated with NT-3 or NT-4.
Results

In the present study, we have used the intensity of immunostaining expressed per unit area (arbitrary units; IA) as an indirect measure of adhesion molecule expression. Using this measurement, we have compared the expressions of NCAM and N-cadherin at the regeneration front, proximal nerve stump or distal stump, 2 weeks after the exogenous application of NT-3 and NT-4 to regenerating rat sciatic nerves. In addition to the immunostaining experiments, we used an RT–PCR-based methodology to quantify the level of adhesion molecule transcription in the proximal and distal stumps of the axotomized nerves and their associated DRG.

Expression of NCAM in the proximal stump
NCAM expression was associated with PGP 9.5-labelled regenerating axons in the proximal stump (Figure 2C). NCAM levels in the proximal stump of control nerve (710 IA, 312–1041) were similar to those at the regeneration front (778 IA, 429–1353) (Table 2a). NT-3 and NT-4 administration increased expression in the proximal stump (NT-4: 2880 IA, 1801–5204; NT-3: 4223 IA, 1063–5606) (Table 2a), which was significantly greater than the control ($P < 0.05$). However, the increase was less than that observed at the regeneration front (Table 2a). Once again, the increase in NCAM expression in the presence of NT-3 was greater than for NT-4; however, this did not reach statistical significance.

The level of NCAM transcript in the proximal stump ($0.707 \pm 0.052$) of the NT-4-treated nerves was significantly greater ($P < 0.01$) than that in the same region of the control, alginate-only-filled conduits ($0.433 \pm 0.024$). Whereas the NCAM transcript level was almost the same in the NT-3-treated ($0.434 \pm 0.056$) and the control nerves (Table 3a).

Expression of NCAM in the distal stump
Expression of NCAM in the distal stump of control nerves (32 IA, 8–165) was significantly lower than that seen at either the regenerating front or the proximal stump ($P < 0.05$; Table 2a). However, in the presence of NT-4 and NT-3, an increase in NCAM expression was observed (NT-4: 420, 210–956; NT-3: 925, 525–2814) (Table 2a). These increases were significantly greater than in the distal stump of control nerves ($P < 0.05$), but were an order of magnitude less than those seen at any site proximal to the injury. Here again, the increase in expression in the presence of NT-3 was twice as high as that seen in the main proximal stump (cf. C). (B) After NT-4 administration, NCAM (green) staining and S-100 (red)-positive Schwann cells were seen at the regeneration front. The inset shows in detail the co-localization of the two fluorescent stains (yellow) in a single Schwann cell. The cell body is indicated by an arrow. (C) After NT-4 administration, axons stained with PGP 9.5 (red) in the proximal stump also co-localized (yellow) with NCAM (green). Scale bar, 50 µm.

Expression of NCAM at the regeneration front
NCAM expression co-localized well with both PGP 9.5 in regenerating axons (Figure 2A) and S-100 in Schwann cells (Figure 2B) at the regeneration front. In control PHB conduits, which were filled with alginate alone as vehicle, a low level of NCAM expression was measured at the regeneration front (778 IA, 429–1353). In the presence of both NT-4 and NT-3, a significant increase in the expression of NCAM in this area was observed (NT4: 3946 IA, 2092–6458;
M. R. Thornton and others

NT-3 administration (14873 IA, 6197–21625) (∗∗P < 0.01 using ANOVA/Tukey's multiple comparison test) was lower than that seen with axons regenerating after regenerating through alginate alone (7248 IA, 3704–11568). N-cadherin expression in the main proximal stump of axons N-cadherin expression co-localized with some Schwann cells (Figure 3A) and partially with PGP 9.5-positive Schwann cells (Figure 3A) and partially with PGP 9.5-positive axons regenerating after NT-4 administration (14873 IA, 6197–21652) (∗∗P < 0.01). Administration of NT-3 had little effect on N-cadherin expression, which showed values similar to those seen in control (6799 IA, 4675–9154). This contrasts with the decreased expression seen at the regeneration front (Table 2b).

The level of N-cadherin transcript in the proximal stump (0.156 ± 0.019) (Table 2b) of the axotomized nerves was almost 6-fold lower compared with the ipsilateral DRG (0.893 ± 0.051). At the proximal stump, the N-cadherin level was increased slightly, but not significantly, in the presence of NT-4 (0.199 ± 0.033), whereas in the presence of NT-3 there was a slight, but not significant, decrease in the level (0.121 ± 0.020) (Table 2b).

**N-cadherin expression in the distal stump**

N-cadherin expression in the distal stump of controls (alginate alone) (10094 IA, 6927–19405) was comparable with that observed in controls at the regeneration front, but greater than that measured in the controls at the proximal stump (∗P < 0.05) (Table 2a). An increase in N-cadherin expression in distal stumps was seen following NT-4 administration (14555 IA, 9837–19735), but this was not statistically significant. After NT-3 delivery, reduced expression of N-cadherin was seen in the distal stump in comparison with the control (8528 IA, 4758–11558, P < 0.05), similar to the effect of NT-3 upon N-cadherin expression at the regeneration front (Table 2b). N-cadherin expression in the distal stump also co-localized with Schwann cells, which displayed the typical morphology seen during Wallerian degeneration (Figure 3D).

The N-cadherin transcript levels at the distal stump of the NT-4-treated (0.362 ± 0.054) and NT-3-treated (0.307 ± 0.036) nerves were not significantly different from untreated (alginate only), control transcript levels (0.355 ± 0.023) in the distal stump (Table 3b).

**N-cadherin expression at the regeneration front**

N-cadherin expression co-localized with S-100-positive Schwann cells (Figure 3A) and partially with PGP 9.5-positive axons (Figure 3B) at the regeneration front: this was

### Table 2 Quantification of immunofluorescence staining of NCAM and N-cadherin

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NT-3</th>
<th>NT-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute intensity/area of the adhesion molecule staining after nerve injury and repair with PHB conduit containing either alginate alone as a control or with the addition of NT-3 or NT-4 (median values; 25th–75th percentile ranges).</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) NCAM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal stump</td>
<td>0.328±0.024</td>
<td>0.344±0.025</td>
<td>0.362±0.026</td>
</tr>
<tr>
<td>Distal stump</td>
<td>0.372±0.026</td>
<td>0.394±0.027</td>
<td>0.407±0.028</td>
</tr>
<tr>
<td>DRG</td>
<td>0.390±0.028</td>
<td>0.412±0.029</td>
<td>0.425±0.030</td>
</tr>
<tr>
<td>(b) N-cadherin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal stump</td>
<td>0.156±0.019</td>
<td>0.178±0.021</td>
<td>0.199±0.033</td>
</tr>
<tr>
<td>Distal stump</td>
<td>0.355±0.023</td>
<td>0.370±0.025</td>
<td>0.385±0.027</td>
</tr>
<tr>
<td>DRG</td>
<td>0.893±0.051</td>
<td>0.947±0.024</td>
<td>0.950±0.018</td>
</tr>
<tr>
<td>(c) L1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal stump</td>
<td>0.156±0.019</td>
<td>0.178±0.021</td>
<td>0.199±0.033</td>
</tr>
<tr>
<td>Distal stump</td>
<td>0.355±0.023</td>
<td>0.370±0.025</td>
<td>0.385±0.027</td>
</tr>
<tr>
<td>DRG</td>
<td>0.893±0.051</td>
<td>0.947±0.024</td>
<td>0.950±0.018</td>
</tr>
</tbody>
</table>

NT-3: 5950 IA, 4050–8788, P < 0.05 (Table 2a). The increase in expression was greater for NT-3 than for NT-4 (P < 0.001).

### Table 3 Quantification of the NCAM, N-cadherin and L1 adhesion molecule transcript levels

The transcript levels were measured after nerve injury and repair with PHB conduit containing either alginate alone as a control or with the addition of NT-3 or NT-4. The adhesion molecule amplicon band intensities were normalized to the GAPDH band intensity for each sample. The values are means ± S.E.M. ∗P < 0.05 and ∗∗P < 0.01 using ANOVA/Tukey’s multiple comparison test.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NT-3</th>
<th>NT-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute intensity/area of the adhesion molecule staining after nerve injury and repair with PHB conduit containing either alginate alone as a control or with the addition of NT-3 or NT-4 (median values; 25th–75th percentile ranges).</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) NCAM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal stump</td>
<td>0.156±0.019</td>
<td>0.178±0.021</td>
<td>0.199±0.033</td>
</tr>
<tr>
<td>Distal stump</td>
<td>0.355±0.023</td>
<td>0.370±0.025</td>
<td>0.385±0.027</td>
</tr>
<tr>
<td>DRG</td>
<td>0.893±0.051</td>
<td>0.947±0.024</td>
<td>0.950±0.018</td>
</tr>
<tr>
<td>(b) N-cadherin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal stump</td>
<td>0.156±0.019</td>
<td>0.178±0.021</td>
<td>0.199±0.033</td>
</tr>
<tr>
<td>Distal stump</td>
<td>0.355±0.023</td>
<td>0.370±0.025</td>
<td>0.385±0.027</td>
</tr>
<tr>
<td>DRG</td>
<td>0.893±0.051</td>
<td>0.947±0.024</td>
<td>0.950±0.018</td>
</tr>
<tr>
<td>(c) L1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal stump</td>
<td>0.156±0.019</td>
<td>0.178±0.021</td>
<td>0.199±0.033</td>
</tr>
<tr>
<td>Distal stump</td>
<td>0.355±0.023</td>
<td>0.370±0.025</td>
<td>0.385±0.027</td>
</tr>
<tr>
<td>DRG</td>
<td>0.893±0.051</td>
<td>0.947±0.024</td>
<td>0.950±0.018</td>
</tr>
</tbody>
</table>

**NCAM transcription in the DRG**

There was significantly (∗P < 0.05) more NCAM transcript present in the DRG (L4 + L5) of the axotomized nerves that were exposed to NT-4 (0.700 ± 0.024) and NT-3 (0.694 ± 0.019) compared with the alginate alone (0.543 ± 0.049) (Table 3a).

**N-cadherin expression in the proximal stump**

N-cadherin expression co-localized with some Schwann cells stained with S-100 in the proximal stump (Figure 3C) and partially co-localized with axons (results not shown). N-cadherin expression in the main proximal stump of axons regenerating through alginate alone (7248 IA, 3704–11568) was lower than that seen with axons regenerating after NT-4 administration (14873 IA, 6197–21652) (∗∗P < 0.05) (Table 2b).
Neurotrophins regulate adhesion molecule expression during nerve regeneration

Similar to the pattern of staining seen with NCAM, N-cadherin expression in nerves regenerating thorough alginate alone was also measured as a baseline (12086 IA, 7450–16990) (Table 2b). Following the administration of NT-4, N-cadherin expression was increased at the regeneration front (15309 IA, 12094–18231) (P < 0.05). However, administration of NT-3 resulted in significantly reduced (P < 0.05) levels of expression when compared with alginate alone (9660 IA, 6845–13096) (Table 2b).

**N-cadherin transcript levels in the DRG**

There was a high level of N-cadherin transcript present in the DRG (L4+L5) of the axotomized nerves both in the presence of NT-4 and NT-3 and in the control (alginate alone). The transcript levels in the DRG of the NT-4 treated (0.950 ± 0.018) and NT-3 treated (0.947 ± 0.024) were little different from the control DRG (0.893 ± 0.051) (Table 2b).

**L1 transcript levels in the proximal stump**

The level of L1 transcript in the proximal stump (0.256 ± 0.005) of the untreated, axotomized nerves was approximately half of that in the ipsilateral DRG (0.513 ± 0.053). In the presence of NT-4 or NT-3, the level of L1 in the proximal stump did not change significantly (0.183 ± 0.038 and 0.207 ± 0.034 respectively) (Table 3c).

**L1 transcript levels in the distal stump**

The L1 transcript level at the distal stump of the control nerves (0.647 ± 0.071) was slightly higher than in the ipsilateral DRG (0.513 ± 0.053). In the presence of NT-4 or NT-3, the level of L1 in the distal stump did not change significantly (0.628 ± 0.037 and 0.587 ± 0.049 respectively) (Table 3c).

**L1 transcript levels in the DRG**

There was a highly significant (P < 0.01) increase in the level of L1 in the DRG (L4+L5) of those nerves treated with NT-4 (0.779 ± 0.013) or NT-3 (0.743 ± 0.025) compared with the untreated, alginate-only controls (0.513 ± 0.053) (Table 3c).

**Discussion**

NTs are important mediators of nerve regeneration, yet their ability to influence the expression of cell-adhesion molecules has been studied previously only in vitro [34–37,40] or in retinal ganglion cells [41]. The recent findings that NT-3 and NT-4 may preferentially stimulate re-innervation
of either fast- or slow-type muscle fibres [12,13,15] not only increased our understanding of their actions, but also highlights their potential effects on the molecular composition of regenerating nerves. In the present study, we have chosen to examine the effect of NT-3 and NT-4 on the levels of protein and mRNA expression of NCAM, N-cadherin and L1. Our results show that administration of NT-3 and NT-4 appears to be associated with increased NCAM immunostaining and NCAM transcript levels at the regeneration front and proximal stump during nerve regeneration. However, the effect of NT-3 and NT-4 on N-cadherin expression immunostaining levels is selective. In the presence of NT-4, increased N-cadherin immunostaining was seen at the regeneration front and in the proximal stump. In contrast, the administration of NT-3 was associated with decreased expression of N-cadherin at the regeneration front and in the distal stump. Interestingly, in the presence of either NT-3 or NT-4, the levels of N-cadherin transcript were not significantly different from the control (alginate alone) in either the DRG or regenerating nerve. However, there was a higher level of N-cadherin transcript present in the DRG than in other regions.

The decision to examine expression of adhesion molecules at 2 weeks post-injury was based on the findings of previous studies by this group [33] and others [31]. These studies showed that 2 weeks post-injury represents the time point at which maximal adhesion molecule expression is to be expected. At this time point, the regeneration front of the axons is still within the conduit and this allowed us to analyse separately the different areas of the re-grown nerve. The concentrations of NT-3 and NT-4 we have used have been shown to be effective in enhancing nerve regeneration and restoring muscle phenotype [9,12,14,15]. However, because we have not yet conducted a detailed dose–response analysis, we acknowledge that these concentrations may be insufficient to elicit maximal change in expression of adhesion molecules.

Both NT-3 and NT-4 are subject to retrograde transport to the neuronal soma after nerve injury [49–51]. In addition, both NTs are capable of reducing cell death in motor neurons [52–54]. NT-3 has been shown to rescue sensory neurons [10,55] and can modulate their neuropeptide Y levels [56]. If more neuronal cells expressing NGF and NT-3 to cultured trigeminal mesencephalic nucleus neurons in culture has no effect on expression of L1 and N-cadherin seen in acutely denervated Schwann cells [58]. It is therefore possible that the local administration of NT-3 and NT-4 affects both proximal and distal stumps of axotomized nerves and it may further stimulate expression of NCAM and N-cadherin seen in acutely denervated Schwann cells [58]. This is particularly relevant given recent studies showing that NT-3 is capable of enhancing migration of Schwann cells via TrkC [59], which may in turn enhance NCAM levels, as soluble NCAM chimaeras have been shown to promote Schwann-cell migration [60].

In the untreated axotomized nerves we have observed higher levels of NCAM immunoreactivity expression in both the proximal stump and regenerating front than in the distal stump. This decrease in expression level may be attributable to the lack of NCAM-positive regenerating axons in the distal stump; NCAM present in the distal stump may be contributed by Schwann cells alone. Studies examining the effect of growth factors on adhesion molecule expression in neurons are limited. One study has shown that addition of NGF and NT-3 to cultured trigeminal mesencephalic nucleus neurons in culture has no effect on expression of L1 and N-cadherin, but increased the amount of polysialylated NCAM [61]. Therefore further studies of neuronal cell cultures are required to define the exact role of NTs in these cells.

NTs may also be important in modulating axon guidance [62]. For example, NGF changes the pattern of DRG outgrowth from a preferred central-nervous-system substrate to a peripheral nervous system substrate [63]. NT-3 and BDNF rapidly sensitize cultured DRG neurons to the
effect of the inhibitory axon guidance molecule collapsin-1 [64], and NGF can stimulate β1-integrin accumulation at the tips of filopodia in the growth cones of sympathetic neurons [65]. In our results, we observed a higher level of immunoreactive adhesion molecule expression at the regeneration front, where larger numbers of growth cones are expected, than in the proximal stump; therefore it is possible that NT-3 and NT-4 stimulate adhesion molecule accumulation in this area.

The response of axons to NTs at the regeneration front is likely to be particularly important; indeed, it is plausible that, if NTs are differentially distributed along the regeneration pathway, then they may alter the growth cone responses to guidance cues by changing their adhesion molecule phenotype dynamically. It appears that in developing neurons, an alteration in responsiveness to attractant factors is important so that growth does not stall at intermediate targets, but is able to reach the final target destination [66]. It is also possible that the effect of NT-3 and NT-4 on NCAM and N-cadherin is linked to their involvement in common intracellular signalling pathways. It has been demonstrated that NCAM promotes neurite outgrowth by organizing a very large supramolecular complex consisting of N-cadherin, fibroblast growth factor receptor-4, phospholipase Caγ and adaptor proteins Src, SHC (Src homology and collagen homology) and FRS2 (fibroblast growth factor receptor substrate 2) [67]. In addition, it has been demonstrated that NCAM is capable of acting as a signalling receptor for ligands of the glial-derived neurotrophic factor family [68], which suggests the possibility that soluble factors could act locally with cell-adhesion molecules to modify growth cone advance or migration.

There is also evidence from transfected cell lines that the NT-4 receptor, TrkB, may be involved in cell adhesion with cadherins, although it must be noted that aggregation may be significantly different in vivo [69]. Nevertheless, these studies demonstrate that important interactions occur between NTs and adhesion molecules.

In conclusion, we have shown that application of exogenous NT-3 and NT-4 to an injured nerve can have a dramatic effect on NCAM, N-cadherin and L1 during peripheral-nerve regeneration. Further studies, particularly using in vitro models of regenerating neurons and Schwann cells, should bring greater understanding of the mechanisms by which these effects are brought about.

Acknowledgments

We thank The Wellcome Trust for funding M. R. T. on a Research Training Fellowship. We also thank Astra Tech for donating PHB.

References
