Expression of neurotrophic factors in diabetic muscle—relation to neuropathy and muscle strength

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Diabetic polyneuropathy can lead to atrophy and weakness of distally located striated muscles due to denervation. Lack of neurotrophic support is believed to contribute to the development of diabetic neuropathy. In this study, we measured the expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), neurotrophin 4 (NT-4) and ciliary neurotrophic factor (CNTF) in muscle biopsies taken from the gastrocnemius and deltoid muscles in 42 diabetic patients and 20 healthy control subjects. To express the distal neuropathic gradient and to reduce interindividual variation, a distal/proximal ratio between expression levels in the gastrocnemius and deltoid muscles was calculated for all neurotrophic factors. Neuropathic status was determined by clinical examination, electrophysiological studies and quantitative sensory examination in diabetic patients, and muscle strength at both the shoulder and ankle was assessed by isokinetic dynamometry. Distal/proximal ratios for NT-3 were lower in diabetic patients [median (range) 110.7 (39.8–546.8)] than in controls [157.6 (63.3–385.4); (P < 0.05)], and in neuropathic diabetic patients [107.1 (39.8–326.0)] versus patients without neuropathy [134.5 (46.6–546.8); (P < 0.005)]. Further, ratios for NT-3 were related to muscle strength (r_s = 0.41, P < 0.01) and showed a tendency towards a negative relationship to the combined score of all measures of neuropathy [Neuropathy rank-sum score (NRSS)] (r_s = −0.27, P = 0.09). Similar trends were observed for ratios for NT-4. Ratios for NGF (r_s = −0.32, P < 0.05) and BDNF (r_s = −0.32, P < 0.05) were related to NRSS, but not to muscle strength. Ratios for CNTF were higher in diabetic patients [64.6 (23.7–258.7)] compared with controls [50.2 (27.2–186.4); (P < 0.05)], but showed no relationship to neither NRSS nor muscle strength. Our results show that the expression of NT-3 is reduced in striated muscles in diabetic patients and is related to muscle weakness and neuropathy. We suggest that lack of NT-3 contributes to insufficient re-innervation leading to the loss of muscle strength in diabetic neuropathy.

Keywords: diabetic polyneuropathy; neurotrophic factor; striated muscle; muscle strength; human

Abbreviations: BDNF = brain-derived neurotrophic factor; CNTF = ciliary neurotrophic factor; CMAP = compound motor action potential; CV = coefficient of variation; DPN = diabetic polyneuropathy; ISH = in situ hybridization; NGF = nerve growth factor; NIS = neuropathy impairment score; NRSS = neuropathy rank-sum score; NSS = neurological symptom score; NT-3 = neurotrophin 3; NT-4 = neurotrophin 4; PCR = polymerase chain reaction; PNS = peripheral nervous system; UACR = u-albumin/creatinine ratio; VPF = vibratory perception thresholds

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Introduction

Diabetic polyneuropathy (DPN) can result in reduced muscle strength (Andersen et al., 1996b, 2004b; Andreassen et al., 2006) due to progressive muscular atrophy (Andreassen et al., 2009). Incomplete re-innervation plays a role in loss of muscle tissue and strength (Andersen et al., 1998). The aetiology of DPN is uncertain; however, increased polyol pathway activity, accumulation of advanced glycation end-products, impaired vascular function, oxidative stress and altered neurotrophic support are considered as important factors in the pathogenesis (Zochodne, 2007; Tomlinson and Gardiner, 2008).

Neurotrophic factors comprise a heterogeneous group of molecules produced by neurons, Schwann cells and end organs in the central and peripheral nervous system (PNS). Neurotrophic factors contribute during early neuronal development and promote survival and regeneration in adults (Dawborn and Allen, 2003; English, 2003). In humans the neurotrophin family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4) (Pitts et al., 2006). In the adult, PNS neurotrophins are synthesized and released from target tissues and are involved in the ongoing maintenance and regeneration of nervous tissue. Levels of neurotrophins have been shown to change in response to neuronal injury, highlighting the interaction between target tissues and innervating neurons in the PNS (Griesbeck et al., 1995; Fernyhough et al., 1996). Striated muscles are target tissues and produce neurotrophins (Funakoshi et al., 1993; Yamamoto et al., 1996). Denervation causes alterations of muscle neurotrophin levels (Griesbeck et al., 1995; Stuerenburg and Kunze, 1998; Kust et al., 2002), and in several animal studies reduced NGF and NT-3 mRNA as well as NGF protein levels have been observed in diabetic muscle (Fernyhough et al., 1995a, b; Ihara et al., 1996; Fernyhough et al., 1998), whereas an increase in BDNF mRNA level has been reported (Fernyhough et al., 1995a, 1996). NT-4 has not been studied in diabetic muscle, but levels decrease in response to lowered neuromuscular activity and after denervation in non-diabetic muscle (Funakoshi et al., 1993, 1995).

A study on neurotrophins in amyotrophic lateral sclerosis showed elevated levels of NGF, BDNF, NT-3 and NT-4 in post-mortem muscle tissue, interpreted as a compensatory reaction in muscle tissue due to denervation (Kust et al., 2002). In DPN, the lower motoneuron is affected in a length-dependent manner and to a lesser degree than in amyotrophic lateral sclerosis. Therefore, the most severe nerve damage occurs distally in the lower extremities where denervation is most prominent (Andersen et al., 1996b; Andreassen et al., 2006, 2009). Ciliary neurotrophic factor (CNTF) promotes survival of axotomized motoneurons (Sendtner et al., 1990) and induces sprouting (Siegel et al., 2000). It is mainly expressed in Schwann cells in the PNS and can be measured in muscle biopsies, but its relationship to DPN and muscle strength is unknown. In previous studies, we have documented impairment of muscular function in patients with severe DPN (Andersen et al., 1996b, 1997, 1998, 2004a, b; Andreassen et al., 2006, 2009), emphasizing the interaction between the diseased motor nerve and its target tissue, i.e. striated muscle.

We hypothesized that a compensatory upregulation of BDNF, NT-3, NT-4 and CNTF expression in striated muscle and terminal Schwann cells would occur in DPN, whereas NGF would remain unchanged as it mainly exerts trophic stimuli on sympathetic and sensory neurons.

Research Design and Methods

Patients and control subjects

Forty-two (21 type 1 and 21 type 2) diabetic patients were recruited from the out-patient clinic at the Department of Endocrinology, Aarhus University Hospital. Patients were classified as either neuropathic or non-neuropathic according to the minimal criteria for DPN (Dyck et al., 1987). Twenty healthy age-matched control subjects (10 male and 10 female) were recruited through advertisements in local newspapers and at the local blood bank. Patients were excluded if they suffered from other conditions that could affect the neuro-muscular system, or had signs or symptoms of peripheral artery disease, Charcot neuroarthropathy, painful diabetic neuropathy or abused alcohol. All patients received a written preliminary study invitation and were re-invited once if no response was obtained within a week. All subjects gave informed consent to the study, which was approved by the local ethics committee.

Muscle biopsies

Under sterile conditions biopsy samples were taken from the deltoid muscle of the dominant arm and medial gastrocnemius muscle of the non-dominant leg using conchotome biopsy forceps under local anaesthesia (5% lidocaine with adrenaline). The muscle biopsies were immediately placed in screw-topped cryovials and stored in a freezer at −80°C until analysed.

Biopsies were mixed randomly prior to the laboratory analyses and their identities were kept anonymous to the laboratory personnel.

Quantitative real-time polymerase chain reaction

Homogenization of muscle tissue was performed with an MM301 Mixer Mill (Retsch, Haan, Germany), and total cellular RNA was extracted using a 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). The quality of rRNA was determined by agarose gel electrophoresis and the presence of intact rRNA was verified by the appearance of two distinct bands visible by fluorescence of ethidium bromide. Extracted RNA was quantified by optical density reading at 260 nm. Reverse transcription was performed with a Multiscrypt Reverse Transcriptase kit (Applied Biosystems) under the following conditions: 25°C for 10 min, 48°C for 30 min and 94°C for 29 s. Each sample underwent polymerase chain reaction (PCR) in triplicates using 25 µl wells containing RNA, TaqMan Universal PCR Mastermix and a primer of the target, i.e. NGF (Hs01113193_m1), BDNF (Hs00380947_m1), NT-3 (Hs00267375_s1), NT-4 (Hs01596132) or CNTF (Hs00173456_m1), and a primer of the housekeeping gene 18S (Hs03914313, all purchased from Applied
Biosystems. Liver RNA was used as negative control. The real time PCR reactions ran at 50°C for 2 min, at 95°C for 10 min and in 40 cycles changing between 95°C for 15 s and 60°C for 1.5 min.

**PCR data analysis**

Data were analysed with ABI prism 7000 sequence Detector Software (Applied Biosystems). The output of amplification was measured in the exponential phase of the reaction at the threshold cycle/C\textsubscript{t}-value defined as the cycle number, at which amplification products are detected. This corresponds to the point where fluorescent intensity exceeds the background fluorescent intensity, which is 10 times the standard deviation of the baseline. The average of triplicates from each sample was used. The relative quantification of target gene was calculated using the formula Ct-target gene/Ct-housekeeping gene described in the Users Bulletin 2, 1997 from Perkin-Elmer (Perkin-Elmer Cetus, Norwalk, CT, USA) (Lihn et al., 2004).

**Isokinetic dynamometry**

Maximal isokinetic strength of shoulder abductors and adductors of the dominant arm and of ankle dorsal and planter flexors of the non-dominant leg was determined using an isokinetic dynamometer (Biodex System 3 PRO dynamometer\textsuperscript{a}, Biodex Medical Systems Inc. NY, USA). A standardized validated protocol developed at our laboratory, based on the instructions from Biodex Medical Systems Inc. and similar to the one applied in previous strength measurement studies at our laboratory (Andersen, 1996a), was followed for all participants. For isokinetic measurements, range of motion was 70° at the shoulder and 48° at the ankle, velocities being 60°/s. During the tests, standardized auditory instructions were given. Each test involved eight maximal repetitions. Data were accepted if the coefficient of variation (CV) did not exceed 10% for ankle and 15% for the shoulder measurements. If the CV exceeded these values, participants were re-tested once, and the data were discarded if the CV still exceeded the limit and no outlier torque curve could be identified.

**Clinical examination**

The patients were examined by a neurologist and evaluated according to the neuropathy impairment score (NIS) (Dyck, 1993a) and the neurological symptom score (NSS). NIS is a combined score obtained from the neurological examination of muscle weakness, activity of tendon reflexes, and sensation at the great toe and index finger. NSS includes scores for motor, sensory and autonomic symptoms.

**Biochemical examination, quantitative sensory examination, retinal examination and nerve conduction studies**

In all patients, a blood sample was analysed for blood glucose, HbA\textsubscript{1c}, creatinine and carbamide levels using standard laboratory methods. To evaluate renal function, a sample of first-void urine was analysed for albumin and creatinine, and the u-albumin/creatinine ratio (UACR) was calculated.

Vibratory perception thresholds (VPTs) were determined at the dominant index finger and the non-dominant great toe using a computer-assisted sensory evaluation system (CASE IV \textsuperscript{b}, WR Medical Electronics, Stillwater, MN, USA) (Dyck et al., 1993b).

Nerve conduction studies were performed by applying standardized transcutaneous stimulation and recording techniques using an electromyograph (Keypoint\textsuperscript{b}, Medtronic, Copenhagen, Denmark) with standard filter settings (Stalberg and Falck, 1993; Falck et al., 1994).

Motor nerve conduction velocity and response amplitudes of the compound motor action potential (CMAP) were measured in the dominant forearm segment of the median (elbow to wrist) nerve and in the non-dominant leg segment of the peroneal (below capitulum to ankle) nerve. Sensory nerve conduction velocity of the dominant median nerve (wrist to index finger) and the non-dominant sural nerve was measured with anti-dromic activation. Z-scores were calculated for all the motor nerve conduction velocities.

Retinopathy status was determined within 6 months of participation at the Department of Ophthalmology, Aarhus University Hospital and rated according to the International Clinical Diabetic Retinopathy Disease Severity Scale (The Diabetic Retinopathy Study Research Group, 1981).

**Definitions, calculations and statistical analyses**

According to our hypothesis, altered expression of neurotrophic factors would be expected in distal muscle. Consequently, a ratio between mRNA expression level in the gastrocnemic and deltoid muscles (neurotrophic factor mRNA in the gastrocnemric muscle divided by neurotrophic factor mRNA in the deltoide muscle expressed as a distal/proximal ratio) was calculated for each neurotrophic factor. Furthermore, by calculating this ratio the influence of interindividual differences in mRNA expression was reduced.

To rank patients according to peripheral nerve function, a neuropathy rank-sum score (NRSS) was calculated for each patient adding the rank scores for the NSS, NIS, VPT and nerve conduction velocities. The VPTs were ranked based on the sum of the two percentiles obtained.

Values for neurotrophin expression, neurotrophin ratios, electrophysiological data, VPT and clinical scores were not normally distributed and, therefore, the Wilcoxon–Mann–Whitney test was applied for all comparisons. The Kruskal–Wallis test was applied for comparisons of non-neuropathic diabetic patients, neuropathic diabetic patients and control subjects. To estimate associations between various parameters, Spearman’s rank correlation was applied. The Wilcoxon signed-rank sum test was used for comparisons of distal and proximal mRNA expression. Muscle strength was normally distributed and the t-test applied for comparisons between the groups. Demographic data were compared using the independent samples t-test. For all statistical analyses a 5% limit of significance was applied. STATA software (version 9.2, Statacorp, College Station, TX, USA) was used for all analyses.

**Results**

Demographic and clinical data are presented in Table 1. Diabetic patients had higher body weight than controls, but BMI was similar in the two groups. Neuropathic patients had higher HbA\textsubscript{1c} and longer duration of diabetes than non-neuropathic patients. There was a tendency towards a higher proportion of males in the diabetic patient group compared with controls (P = 0.06).
Clinical findings, nerve conduction, vibratory perception and muscle strength

As expected, NIS and NSS were higher in neuropathic patients (Table 2). Clinical signs of muscle weakness were found in the lower extremities in two neuropathic patients, and seven neuropathic patients reported symptomatic weakness of the legs. No response could be obtained for the peroneal nerve in five neuropathic patients, whereas low motor nerve conduction velocities (Z-score < −1.96) were recorded in eight neuropathic and one non-neuropathic patient. For the sural nerve, no response could be obtained in 13 neuropathic and 2 non-neuropathic patients. VPTs were abnormal (≥ 98th percentile) at both the index finger and great toe in 10 neuropathic patients.

The percentage of expected muscle strength at the shoulder and ankle for all patients and control subjects is presented in Table 2. No difference was found between patients and controls for either shoulder adduction (P = 0.2) or abduction (P = 0.07). Ankle plantar flexion was reduced in patients, compared with controls, and there was a tendency towards reduced strength for ankle dorsal flexion in patients (P = 0.05). No difference was found between neuropathic and non-neuropathic patients for shoulder adduction (P = 0.13) or abduction (P = 0.41), whereas ankle dorsal flexion was reduced in neuropathic patients with a tendency for impaired plantar flexion (P = 0.06). A relationship was found between NRSS and shoulder abduction (r = −0.34, P < 0.05). Furthermore, relationships were established between NRSS and strength for ankle dorsal (r = −0.62, P < 0.0001) and plantar flexion (r = −0.44, P < 0.005).

Table 1 Demographic data, duration of diabetes and biochemical findings in diabetic patients with and without neuropathy and in control subjects

<table>
<thead>
<tr>
<th></th>
<th>Diabetic patients</th>
<th>Control subjects</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>Neuropathic</td>
<td>Non-neuropathic</td>
</tr>
<tr>
<td>n</td>
<td>42</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>31/11</td>
<td>18/4</td>
<td>13/7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.0 (28.0–67.0)</td>
<td>54.0 (28.0–67.0)</td>
<td>54.5 (30.1–66.0)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>82.5 (51.0–124.0)*</td>
<td>85.0 (64.0–124.0)</td>
<td>78.5 (51.0–124.0)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>174.5 (153.0–189.0)</td>
<td>177.0 (153.0–189.0)</td>
<td>173.5 (156.0–187.0)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.3 (19.2–43.6)</td>
<td>26.3 (21.1–43.6)</td>
<td>26.2 (19.2–35.6)</td>
</tr>
<tr>
<td>Type 1/type 2 diabetes</td>
<td>21/21</td>
<td>15/7</td>
<td>6/14</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>17.0 (3.5–49.5)</td>
<td>25.0 (8.0–49.5)</td>
<td>11.5 (3.5–36.0)</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>9.8 (2.7–20.9)</td>
<td>10.6 (2.8–20.9)</td>
<td>8.6 (2.7–17.3)</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>8.0 (5.6–10.6)</td>
<td>8.4 (6.7–10.0)</td>
<td>7.4 (5.6–10.6)</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>74.0 (41.0–142.0)</td>
<td>79.0 (41.0–142.0)</td>
<td>69.5 (46.0–134.0)</td>
</tr>
<tr>
<td>UACR (mg/mmol)</td>
<td>0.7 (0.3–106.9)</td>
<td>0.8 (0.3–106.9)</td>
<td>0.7 (0.4–2.8)</td>
</tr>
<tr>
<td>Retinopathy (none/simplex/proliferative)</td>
<td>17/18/7</td>
<td>3/12/7</td>
<td>14/6/0</td>
</tr>
</tbody>
</table>

Data are mean ± SD.
*P < 0.05 compared with the control subjects.
†P < 0.01, ‡P < 0.001 compared with the non-neuropathic diabetic patients.

Table 2 NSS, NIS and strength measurements in diabetic patients with and without neuropathy and in control subjects

<table>
<thead>
<tr>
<th></th>
<th>Diabetic patients</th>
<th>Control subjects</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>Neuropathic</td>
<td>Non-neuropathic</td>
</tr>
<tr>
<td>n</td>
<td>42</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>NIS</td>
<td>6 (0–56)</td>
<td>15 (2–56)§</td>
<td>3 (0–12)</td>
</tr>
<tr>
<td>NSS</td>
<td>0 (0–7)</td>
<td>1 (0–7)‡</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>Shoulder strength (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abduction</td>
<td>96.8 ± 17.7</td>
<td>94.6 ± 20.1</td>
<td>99.2 ± 14.8</td>
</tr>
<tr>
<td>Adduction</td>
<td>100.5 ± 20.7</td>
<td>95.9 ± 25.2</td>
<td>105.6 ± 13.1</td>
</tr>
<tr>
<td>Ankle strength (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsal flexion</td>
<td>85.7 ± 22.3</td>
<td>77.7 ± 24.5†</td>
<td>94.4 ± 16.1</td>
</tr>
<tr>
<td>Plantar flexion</td>
<td>75.3 ± 21.7*</td>
<td>69.3 ± 23.2</td>
<td>81.9 ± 18.3</td>
</tr>
</tbody>
</table>

NIS and NSS are median (range), strength values are mean ± SD.
Strength values are expressed as percent of expected strength.
*P < 0.005 compared with control subjects.
§P < 0.05, ‡P < 0.01, †P < 0.001 compared with non-neuropathic diabetic patients.
Neurotrophin mRNA expression
mRNA levels for all neurotrophic factors measured in the deltoid and gastrocnemic muscles, and ratios expressing the relative amount of neurotrophic factor mRNA in the medial gastrocnemic muscle (distal muscle) as a percentage of the level in the deltoid muscle (proximal muscle) are shown in Table 3.

Neurotrophin mRNA expression in healthy controls
Females [0.035 (0.003–0.061)] had lower levels of BDNF mRNA in the medial gastrocnemic muscle compared with males [0.052 (0.016–0.067); (P<0.05)]. No other differences in neurotrophic factor expression were found between the genders.

For all controls, a relationship was established between height and mRNA levels of BDNF in the gastrocnemic muscle (r=0.49, P<0.05) and CNTF mRNA in the deltoid muscle (r=-0.47, P<0.05). Age and BMI showed no such relationship to neurotrophic factor mRNA levels.

BDNF ratio was related to height (r_s=0.64, P<0.005), but not BMI or age. No other neurotrophic factor distal/proximal ratios were related to these variables.

NGF (P<0.0005), BDNF (P<0.005) and CNTF (P<0.0005) mRNA levels were lower in the gastrocnemic muscle compared with the deltoid muscle, whereas no difference was found for NT-4 mRNA levels (P=0.79). NT-3 mRNA levels were higher in the gastrocnemic than in the deltoid muscle (P<0.005).

For NGF, there was a close relationship between mRNA expression levels in the gastrocnemic and deltoid muscles. Tendencies towards related neurotrophin expression levels in the two muscles were found for BDNF, NT-3 and NT-4, but not for neurotrophic factor (Table 4).

Relationships were found between distal/proximal ratios for NT-4 and NGF, and between NT-4 and NT-3, indicating inverse proportionality of the relative expression of NT-4 and NGF and proportionality of the relative expression of NT-4 and NT-3 (Table 4).

There were no relationships between neurotrophin levels in either the deltoid or the gastrocnemic muscle and strength for shoulder abduction or plantar flexion, respectively, or between neurotrophic factor distal/proximal ratios and strength for plantar flexion.

Neurotrophin mRNA expression in diabetic patients
Neurotrophic factor mRNA expression levels in the medial gastrocnemic and deltoid muscles were similar in diabetic males and females.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Deltoid muscle</th>
<th>Gastrocnemius muscle</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>2.0 (0.7–15.4)</td>
<td>1.3 (0.2–10.2)</td>
<td>58.7 (15.7–217.5)</td>
</tr>
<tr>
<td>Males</td>
<td>2.1 (0.7–15.4)</td>
<td>1.5 (0.2–10.2)</td>
<td>56.5 (15.7–101.1)</td>
</tr>
</tbody>
</table>

Table 3 Neurotrophic factor mRNA levels (×10^2) relative to housekeeping gene (18S) in the deltoid and gastrocnemic muscles, and ratios expressing the amount of neurotrophic mRNA synthesized in the gastrocnemic muscle relative to the deltoid muscle in diabetic patients with and without neuropathy and in control subjects

<table>
<thead>
<tr>
<th>Factor</th>
<th>Diabetic patients</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All patients</td>
<td>Neuropathic patients</td>
</tr>
<tr>
<td>NGF</td>
<td>2.0 (0.7–15.4)</td>
<td>2.1 (0.7–15.4)</td>
</tr>
<tr>
<td></td>
<td>1.3 (0.2–10.2)</td>
<td>1.5 (0.2–10.2)</td>
</tr>
<tr>
<td></td>
<td>55.7 (15.7–217.5)</td>
<td>56.5 (15.7–101.1)</td>
</tr>
<tr>
<td>BDNF</td>
<td>5.5 (1.0–11.0)</td>
<td>6.2 (1.9–9.6)</td>
</tr>
<tr>
<td></td>
<td>2.9 (0.03–11.8)</td>
<td>2.9 (0.1–11.8)</td>
</tr>
<tr>
<td></td>
<td>54.1 (2.7–193.4)</td>
<td>52.7 (4.9–124.6)</td>
</tr>
<tr>
<td>NT-3</td>
<td>15.0 (8.7–54.0)</td>
<td>15.0 (10.0–54.0)</td>
</tr>
<tr>
<td></td>
<td>16.9 (10.3–87.2)</td>
<td>15.0 (10.3–87.2)</td>
</tr>
<tr>
<td></td>
<td>110.7 (39.8–546.8)</td>
<td>107.1 (39.8–326.0)</td>
</tr>
<tr>
<td></td>
<td>157.6 (33.7–165.1)</td>
<td>157.6 (33.7–165.1)</td>
</tr>
</tbody>
</table>

Data are median (range).

n=21 for measurements of neurotrophic factors from the gastrocnemic muscle in neuropathic patients, and n=19 for measurements of neurotrophic factors from the gastrocnemic muscle in non-neuropathic patients.

*P<0.05 compared with control subjects.

†P<0.005 compared with non-neuropathic patients.
females. mRNA levels did not differ between diabetic patients and controls or between neuropathic and non-neuropathic patients (Table 3).

There was a relationship between NT-4 mRNA levels in the gastrocnemous muscle and HbA1c ($r_s = 0.35, P < 0.05$), while age, BMI, height, UACR and diabetes duration did not relate to neurotrophic factor levels.

Neurotrophic factor distal/proximal ratios for diabetic patients were not related to height, age, UACR, HbA1c or duration of diabetes, though a tendency was found for NT-3-ratio and BMI ($r_s = -0.28, P = 0.08$).

mRNA levels were lower in the gastrocnemous muscle than in the deltoid muscle for NGF ($P < 0.0001$), BDNF ($P < 0.0001$), CNTF ($P < 0.0001$) and NT-4 ($P < 0.05$), whereas NT-3 mRNA levels were higher in the gastrocnemous than in the deltoid ($P < 0.05$), similar to the observation made in controls. Neurotrophic factor mRNA expression levels in the gastrocnemous and deltoid muscles were related for NGF, BDNF, NT-4 and CNTF, and a trend was found for NT-3 (Table 4).

Analysis of distal/proximal ratios for neurotrophic factors showed that the ratio for NT-3 was related to strength for plantar flexion (Fig. 1F), and for the ratio for NT-4, a tendency towards a positive correlation was also seen (Fig. 1H). This pattern was not found for ratios for NGF (Fig. 1B), BDNF (Fig. 1D) or CNTF (Fig. 1J).

NT-3 distal/proximal ratios were reduced in diabetic patients compared with controls, indicating lower relative levels of expression of NT-3 mRNA in the gastrocnemous muscle in patients (Table 3). CNTF distal/proximal ratios, on the other hand, were increased in patients compared with controls, indicating higher relative levels of CNTF mRNA expression in the gastrocnemous muscle in diabetic patients (Table 3). This was not observed for either NT-4 ($P = 0.25$), NGF ($P = 0.37$) or BDNF ($P = 0.53$). Furthermore, the distal/proximal ratio for NT-3 was lower for neuropathic patients compared with non-neuropathic patients (Fig. 2).

Correlations were found between distal/proximal ratios for the expression of NT-4 versus BDNF, NT-4 versus NGF, NT-4 versus NT-3, NT-4 versus CNTF and NGF versus CNTF, demonstrating the presence of proportionality in the relative expression of NT-4 and all other measured factors, as well as in the relative expression of NGF and CNTF. Tendencies towards relationships were seen for distal/proximal ratios for NGF versus BDNF, NGF versus NT-3 and BDNF versus NT-3 (Table 4).

Neurotrophin mRNA levels in the deltoid and gastrocnemous muscles were not related to strength for shoulder abduction or plantar flexion, respectively.

### Table 4 Relationship between distal and proximal mRNA expression levels for each individual neurotrophic factor (upper section), and between neurotrophic factor distal/proximal mRNA ratios in diabetic patients and control subjects (lower section)

<table>
<thead>
<tr>
<th>Individual neurotrophic factors:</th>
<th>All diabetic patients (n = 40)</th>
<th>Control subjects (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td>$r_s = 0.63^*$</td>
<td>$r_s = 0.73^*$</td>
</tr>
<tr>
<td>BDNF</td>
<td>$r_s = 0.75^*$</td>
<td>$r_s = 0.39$</td>
</tr>
<tr>
<td>NT-3</td>
<td>$r_s = 0.31$</td>
<td>$r_s = 0.44$</td>
</tr>
<tr>
<td>NT-4</td>
<td>$r_s = 0.67^*$</td>
<td>$r_s = 0.35$</td>
</tr>
<tr>
<td>CNTF</td>
<td>$r_s = 0.52^1$</td>
<td>$r_s = 0.26$</td>
</tr>
</tbody>
</table>

Distal/proximal ratios:

| NT-3 versus NGF                  | $r_s = 0.28$                  | $r_s = -0.10$            |
| NT-3 versus BDNF                 | $r_s = 0.29$                  | $r_s = 0.13$             |
| NT-3 versus NT-4                 | $r_s = 0.36^3$                | $r_s = 0.48^5$           |
| NT-4 versus NGF                  | $r_s = 0.42^8$                | $r_s = -0.5^5$           |
| NT-4 versus BDNF                 | $r_s = 0.57^7$                | $r_s = 0.21$             |
| NT-4 versus CNTF                 | $r_s = 0.31^5$                | $r_s = -0.27$            |
| NGF versus BDNF                  | $r_s = 0.30$                  | $r_s = 0.13$             |
| NGF versus CNTF                  | $r_s = 0.32^5$                | $r_s = 0.41$             |

All correlations are presented as Spearman’s rho ($r_s$).

* $P < 0.0001$, † $P < 0.0005$, ‡ $P < 0.001$, § $P < 0.01$, ¶ $P < 0.05$. 

### Discussion

To our knowledge, there are no human studies on muscular expression and synthesis of neurotrophic factors in diabetes, or on the association to neuropathy for which neurotrophic factors are appraised as a potential treatment.

The main findings of our study are altered expression of NT-3, CNTF and possibly NT-4 in the distal gastrocnemous muscle, compared with the proximal deltoid muscle. NT-3 expression in the gastrocnemous muscle was downregulated in diabetic patients and a similar trend was observed for NT-4. Subgroup analysis revealed further downregulation of NT-3 expression in neuropathic subjects compared with non-neuropathic patients. Also, the extent of downregulation corresponded to the degree of muscle weakness. NT-4 expression levels did not reach statistical significance, but paralleled NT-3 in all analyses indicating a similar, but weaker response. CNTF expression in the gastrocnemous was upregulated compared with the deltoid muscle in diabetic patients. This finding can be interpreted as a distal upregulation of CNTF expression in terminal Schwann cells, present in small quantities...
Figure 1  Ratios for mRNA expression in distal muscle (medial gastrocnemius) relative to proximal muscle (deltoid) expressed as a percentage for all neurotrophins in relation to NRSS (A, C, E, G and I) and percentage of expected strength of plantar flexion (B, D, F, H and J) for all diabetic patients. White dots = neuropathic patients; black dots = non-neuropathic patients; \( r_s \) = Spearman correlation coefficient.
ameliorating the detrimental effects of hyperglycaemia. Have been treated with anti-diabetic medication for many years, term hyperglycaemia and nerve lesion. Furthermore, our patients died, whereas animal studies evaluate the acute reactions to short-term hyperglycaemia and nerve lesion. The interaction occurring at the neuromuscular junction as well as in the presence of BDNF in rat muscle (Copray et al., 1995). Expression of NGF is found in Schwann cells, including those surrounding large myelinated fibres (Lee et al., 1996). Our results, therefore, reflect not only altered muscular expression of neurotrophic factors, but also the intricate interaction occurring at the neuromuscular junction as well as in other cell types within striated muscle.

BDNF, NT-3, NT-4 and CNTF exert action on motoneurons with regard to excitability, survival and conduction velocity (Apfel et al., 1993; Gonzalez and Collins, III 1997; de Carrizosa et al., 2009), whereas NGF stimulates sensory fibres and sympathetic neurons (Dawbarn and Allen, 2003). Therefore, according to our hypothesis that neurotrophic expression is altered in muscle tissue due to DPN, we would expect changes in neurotrophin expression of BDNF, NT-3, NT-4 and CNTF to correlate to measures of motor dysfunction. We observed that reduced expression

The proportions found between expression levels of mRNA for the various neurotrophic factors in both the medial gastrocnemius and deltoid muscle in controls and patients correspond with findings reported in other studies (Griesbeck et al., 1995; Kust et al., 2002; Pitts et al., 2006). Even in healthy subjects greater amounts of NGF, BDNF and CNTF are expressed in proximal muscle, whereas the opposite is seen for NT-3. One explanation could be that neurotrophin expression depends on muscle fibre type, although conflicting findings have been reported (Funakoshi et al., 1995; Sakuma et al., 2001). The deltoid is a mixed muscle, whereas slow-twitch fibres predominate in the gastrocnemius muscle. If neurotrophic factors are expressed differently in fibre types 1 and 2, change in fibre type distribution following denervation could account for the observed differences. Another explanation for the observed proximo-distal variations in mRNA expression could be differences in the intensity of neuromuscular activity in the lower and upper extremities (Funakoshi et al., 1995; Schinder and Poo, 2000). Motor units are smaller in arm muscles than in leg muscles, leading to increased neuron to muscle fibre ratio.

In neuropathic patients, the differences in distal/proximal ratios seem to be due to a combination of reduced mRNA expression in distal muscles and an increased expression in proximal muscles. It could be speculated that denervation and thus impaired neuromuscular signalling contributes to insufficient feedback between neuron and muscle fibre distally, resulting in downregulation of mRNA expression, whereas proximally, nerve damage is less severe allowing communication between cells leading to upregulation of mRNA expression to stimulate re-innervation.

Muscle biopsies contain intramuscular nerve endings, Schwann cells, vascular endothelial cells and connective tissue, in addition to muscle fibres. As in situ hybridization (ISH) was not performed, we cannot determine which cells contributed to the altered expression of neurotrophic factors. Küst et al. (2002) localized NGF, BDNF and NT-4 expression to muscle fibres in humans. NT-3 was mainly observed in connective tissue and intramuscular nerve endings, although some expression was also seen in muscle (Kust et al., 2002). Other studies using ISH have confirmed the presence of BDNF in rat muscle (Copray et al., 2000), but Schwann cells surrounding motoneurons were also stained (Griesbeck et al., 1995). Expression of NGF is found in Schwann cells, but has been located to motoneurons and muscle (Amano et al., 1991; Anand et al., 1997). CNTF is expressed in Schwann cells, including those surrounding large myelinated fibres (Lee et al., 1996). Our results, therefore, reflect not only altered muscular expression of neurotrophic factors, but also the intricate interaction occurring at the neuromuscular junction as well as in other cell types within striated muscle.

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of NT-3 was related to lower muscle strength and the results for NT-4 parallel these findings. Furthermore, both factors show trends towards a relationship to NRSS. BDNF may play a less important role in neuromuscular interaction as it was not downregulated in diabetic patients and showed no relationship to strength. NGF was related to NRSS, probably reflecting neuropathy of sensory and sympathetic fibres. As expected, it showed no relation to muscle strength. CNTF ratio was the only factor upregulated in diabetic patients compared with control subjects, but showed no relationship to NRSS or muscle strength. This was an unexpected finding as CNTF supports motor neurons both in vitro and in vivo (Sendtner et al., 1991, 1997). The CNTF receptor is also abundant in muscle (Davis et al., 1991). CNTF, therefore, is not a classic target-derived growth factor and it has been proposed that it exerts its effects indirectly via the release of a muscle-derived sprouting factor (English, 2003). Reports have stated a myotrophic effect of CNTF, and CTNF has been related to loss of motor neurons and reduced muscle strength (Masu et al., 1993). Consequently, upregulation of CNTF expression distally could be the result of insufficient synthesis of this unknown factor by muscle, explaining the lack of correlation to DPN and muscle strength.

Although it did not reach statistical significance, the control group included relatively more females than the patient group. This might have resulted in a lower BDNF ratio for controls as females had lower amounts of BDNF mRNA in the gastrocnemius muscle than men. A substantial difference in fibre distribution between genders seems an unlikely explanation as previous studies have reported varying results (Staron et al., 2000; Toft et al., 2003).

In accordance with previous reports, levels of neurotrophic factor mRNA showed substantial variation in both patients and controls (Table 3) (Fernyhough et al., 1995a; Kust et al., 2002). In diabetic patients, mRNA expression levels in the deltoid and gastrocnemius muscle were related for all neurotrophic factors except NT-3, which just failed to reach significance. These observations suggest high interindividual variation, but considerably less intra-individual variation. Consequently, up- or downregulation of mRNA expression in distal and proximal muscle can be assessed with less variation using distal/proximal ratios.

A multiple comparisons problem might have occurred due to several statistical calculations being performed during analysis. Studies separately evaluating each individual factor are, therefore, needed to confirm the novel findings reported in this study.

Synthesized protein was not measured in our study. Several studies have reported coherence between levels of expressed neurotrophin mRNA and synthesized protein in muscle (Kust et al., 2002; Omura et al., 2005). Accordingly, the differences in mRNA expression observed in our study most likely constitute corresponding alterations in protein synthesis. Complex interactions exist between neurotrophic factors and their receptors in the neuromuscular system as a response to neuropathy and denervation (Sobue et al., 1998; Michalski et al., 2008). In our study, mRNA expression corresponded for several neurotrophic factors suggesting an interdependent regulation. Exogenous administration of NT-3 improves motor and sensory nerve function in diabetic rats (Mizisin et al., 1999; Pradat et al., 2001). However, our study indicates that there is a complex interplay between the various neurotrophic factors, suggesting that treatment with one or even more of these factors may be insufficient to normalize nerve and muscle function.

In this study, we have found that neuropathy induces reduced expression of NGF and BDNF in distal muscle compared with proximal muscle. Expression levels of NT-3 in distal muscle, relative to proximal muscle, were positively related to strength, suggesting that reduced expression of NT-3 is involved in loss of muscle strength in diabetic patients. Studies measuring expression and synthesis of neurotrophic factors and their correspondent receptors combined with ISH to locate the altered expression and synthesis are needed to map the intricate interrelationship between these factors in diabetic muscle in response to neuropathy.

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### References


