The role of the p75 neurotrophin receptor in the morphology of dorsal root ganglion cells in streptozotocin diabetic mice: effects of sciatic nerve crush

Y. Jiang¹ · J. Jakobsen¹

¹ Department of Neurology, Aarhus University Hospital, Aarhus C, Denmark

Abstract

Aims/hypothesis. Neuronal dorsal root ganglion (DRG) cells seem to be vulnerable in diabetes. The aim of this study was to determine whether the p75 neurotrophin receptor stimulates perikaryal shrinkage and neuronal death, and further accelerates neuronal DRG cell loss after axotomy in a mouse model of diabetes.

Methods. Nine non-diabetic BALB/c p75^{+/+} mice, seven diabetic BALB/c p75^{+/+} mice, nine non-diabetic p75^{-/-} mice and nine diabetic p75^{-/-} mice received a unilateral sciatic nerve crush 1 to 2 days after streptozotocin treatment. Tissues were fixed 28 days later by vascular perfusion, and the volume and number of the fifth lumbar DRG neurons were obtained using assumption-free stereological techniques.

Results. In diabetic p75^{+/+} mice there was a 9% reduction in the perikaryal volume of the DRG A cells ($p<0.05$) and a 10% reduction in the perikaryal volume of the DRG B cells ($p<0.05$) on the non-crushed

side compared with in non-diabetic p75^{+/+} mice. However, neuronal cell number was not reduced. Conversely, no perikaryal shrinkage of A cells or B cells occurred on the non-crushed side in diabetic p75^{-/-} mice, and no neuronal cell loss was observed. Following nerve crush, there was a loss of B cells in non-diabetic p75^{+/+} mice (37±6%) and in diabetic p75^{+/+} mice (36±4%). In non-diabetic p75^{-/-} mice, no neuronal cell loss occurred after crush, whereas in diabetic p75^{-/-} mice the loss of B cells (14±4%) was small but significant ($p<0.02$).

Conclusions/interpretation. In experimental diabetes the p75 neurotrophin receptor is involved in neuronal DRG cell body shrinkage without loss of neuronal DRG cells. Following sciatic nerve crush, DRG cell loss is not accelerated in diabetic p75^{+/+} mice.

Keywords Diabetic neuropathy · Dorsal root ganglion cells · Morphology · p75 neurotrophin receptor · Sciatic nerve crush · Stereology · Streptozotocin diabetes

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Y. Jiang (✉)
Department of Neurology, Aarhus University Hospital,
8000 Aarhus C, Denmark
E-mail: jiang@akhphd.au.dk
Tel.: +45-89-493451, Fax: +45-89-493300

Abbreviations: DRG, dorsal root ganglion · JNK, c-Jun-N-terminal kinase · L5, fifth lumbar · NGF, nerve growth factor · STZ, streptozotocin · trk, tyrosine kinase receptor · TUNEL, terminal deoxynucleotidyl transferase-mediated biotin-16-dUTP nick-end-labelling

Introduction

Sensory loss occasionally followed by motor weakness and autonomic failure are characteristic clinical manifestations of long-term diabetic polyneuropathy. The sensory loss involves large, myelinated type A neurons that mediate tactile, vibratory and proprioceptive sensation as well as myelinated and unmyelinated type B neurons that mediate pain and temperature sensation. The most likely explanation for loss of sensation in human diabetes is loss of peripheral nerve fibres and neuronal dorsal root ganglion (DRG) cells [1].

The pathogenesis of diabetic neuropathy is unknown, but studies in the rat streptozotocin (STZ)

model indicate that oxidative stress induced by a variety of mechanisms, including neurotrophin deficiency [2], may be involved. Neurotrophins are involved in the regulation of cell survival [3] and cell death [4], and also play a role in the regulation of neuronal size [5, 6]. In experimental diabetes, target-tissue expression of nerve growth factor (NGF) and neurotrophin-3 is reduced, as is their retrograde transport to the DRG [7, 8, 9, 10, 11]. This reduces the expression of the low-affinity p75 neurotrophin receptor and the high-affinity tyrosine kinase receptor (trk) A and C mRNA in the DRG and the bi-directional axonal transport of the p75 neurotrophin receptor [8, 11, 12, 13].

The STZ model of diabetic neuropathy is characterised by shrinkage of neuronal DRG cell bodies, swelling of nerve root axons and dwindling of peripheral nerve axons [14, 15, 16, 17, 18]. It is uncertain whether axons are lost in experimental diabetes as they are in the human condition. However, recent studies from two laboratories indicate that neuronal DRG cells in STZ-diabetic rats are lost and show increased apoptotic activity [19, 20, 21].

The p75 neurotrophin receptor mediates cell apoptosis by activation of c-jun-N-terminal kinase (JNK) or ceramide and their downstream signalling pathways [22, 23]. The p75 neurotrophin receptor may be involved in the neuronal DRG cell abnormalities observed in experimental diabetes and it is possible that the condition could give rise to accelerated neuronal cell loss after nerve crush. We hypothesised that in experimental diabetes the p75 neurotrophin receptor is involved in: (i) an excessive loss of neuronal DRG cells after crush; and (ii) in neuronal DRG cell body shrinkage and cell loss in the non-crushed condition. To test the hypothesis, the sciatic nerve was crushed unilaterally in diabetic and non-diabetic p75-receptor homozygous knockout mice and in their wild-type BALB/c strain. Neuronal number and cell body volume of the fifth lumbar (L5) DRG were quantified with assumption-free stereological methods using the optical dissector and the vertical planar rotator.

Materials and methods

Animals. Eight-week-old, male p75-receptor knockout mice (p75^{-/-}) and age-matched male BALB/c mice (p75^{+/+}), the genetic background strain for p75^{-/-} mice, were used in this study. The p75^{-/-} offspring of homozygous breeders from Jackson Laboratories (Bar Harbor, Me., USA) were kindly provided by M. Koltzenburg (University of Würzburg, Germany). BALB/c mice were obtained from Taconic M&B (Ry, Denmark). The mice were housed in top filter-barrier mouse cages at 23 °C with 50% relative humidity and a 12-h light–dark cycle. All mice had free access to food and water. At the end of the experiment, four groups of 12-week-old mice were harvested: (i) p75^{+/+} mice without diabetes ($n=9$); (ii) p75^{+/+} mice with diabetes ($n=7$); (iii) p75^{-/-} mice without diabetes ($n=9$); and (iv) p75^{-/-} mice with diabetes ($n=9$).

Diabetes was induced by a single i.p. injection of 190–200 mg/kg of STZ (Bie & Berntsen, Roedovre, Denmark) dissolved

in 10 mmol/l citrate buffer, pH 5.0 immediately before application. Tail-vein blood glucose concentration was determined 48 h after injection and at the end of the study using a glucose touch meter (Johnson & Johnson Company, Milpitas, Calif., USA). STZ-treated mice with blood glucose concentrations <15 mmol/l at 48 h or at 4 weeks were excluded from the study.

Within 48 h after STZ injection, mice were anaesthetised with 240 mg/kg of avertin (Sigma-Aldrich Denmark, Copenhagen, Denmark) and one sciatic nerve in each mouse was crushed at the “sciatic notch” with a 0.5-mm S. W. Inox. Castroviejo forceps (model Tübingen Amann Medizintechnik, Emmingen-Liptingen, Germany). Before the operation and for 2 days after, 3 µg of buprenorphin (Meda, Solna, Sweden) was injected subcutaneously twice daily. The crushed side of the sciatic nerve was selected at random, and the contralateral side served as the control. Four weeks later, the mice were anaesthetised and were fixed by vascular perfusion transcardially with Tyrode buffer for 30 s followed by 4% glutaraldehyde dissolved in a 0.08 mol/l phosphate buffer (pH 7.4) for 10 min. The L5 DRGs on both sides were removed and post-fixed in 4% glutaraldehyde at 4 °C until embedded in agar. The protocol was in accordance with the guidelines specified by the Federation of European Laboratory Animal Science Association.

Tissue preparation. The dissected ganglia were embedded in 3.5% agar (Difco Laboratories, Detroit, Mich., USA) on an aluminium block with semicircular grooves, and rotated along their vertical axis to reduce sampling variation due to anisotropy of DRG cells. The agar-embedded tissues were dehydrated in ascending concentrations of alcohol (70%, 96%, 99%) for 1 h in each solution before being embedded in glycolmethacrylate (Technovit 7100, Heraeus Kulzer, Wehrheim, Germany). To obtain the ‘vertical sections’ required for estimation of cell volume [24], the embedded ganglia were cut with a glass knife into consecutive 30-µm thick sections, parallel to the vertical axis of the ganglia. After selecting a random start point, every third section was systematically sampled and stained with 0.25% cresyl violet acetate, pH 3.45 (Sigma-Aldrich, Steinheim, Germany) for 3 h and then differentiated in 1% acetate acid. Samples were subsequently dehydrated in ascending concentrations of alcohol, unfolded in water and dried at 60 °C.

Stereology. The fractionator, the optical dissector and nucleator theories were used to evaluate neuronal cell number and cell volume of L5 DRGs [25]. A modified Olympus BX50 microscope (Olympus Denmark, Albertslund, Denmark) was connected to an electronic microcator (MT2; Heidenhain, Traunreut, Germany) to record the plane movement in the Z direction. A colour video camera (3-CCD video camera; Olympus Denmark) on top of the microscope projected the tissue images onto a computer screen. Using interactive graphic software (CAST-Grid, Olympus Denmark) and a 4× lens, tissues were sampled systemically after selecting a random start point. The number of sampled neuronal cell bodies is dependent on the step lengths in the X and Y directions and on the density of counting units. As the density of B cells is higher than that of A cells, the counting frame for B cells was smaller than that for A cells. The counting frames were 4806 µm² for A cells and 2067 µm² for B cells using a 60× oil-immersion lens. The thickness of every fourth vision was measured using a 100× oil lens. The optical dissector started at 3 µm under the top plane where the tissue came into focus, and continued through a depth of 15 µm (Z direction).

A cells and B cells were distinguished under the light microscope based on their morphology. A cells have a large, centrally located nucleolus with one or two smaller nucleoli and have granular Nissl substance distributed evenly throughout

Table 1. Body weight of p75^{+/+} and p75^{-/-} mice with or without diabetes during the 4-week experimental period

Group	Weight (g)		
	Day 0	Week 4	Difference
p75 ^{+/+} without diabetes	27.3±1.9	29.3±1.6	2.0±0.9
p75 ^{+/+} with diabetes	27.3±1.3	25.3±1.6 ^a	-2.0±0.7
p75 ^{-/-} without diabetes	21.4±2.0	24.4±2.2	3.0±1.2
p75 ^{-/-} with diabetes	23.2±1.9	18.8±3.3 ^a	-4.5±2.6

Values are means ± SD. Start body weight was comparable in diabetic and non-diabetic mice in the p75^{+/+} group and in the p75^{-/-} group. ^a*p*<0.001 vs non-diabetic control group

the bright cytoplasm. B cells usually have several peripherally located small nucleoli and dark, homogeneous cytoplasm [26]. The biggest nucleolus was used as the counting unit. If two nucleoli were of the same size, one was selected at random. Cells were counted if the largest nucleolus was inside the optical dissector without touching one of the two forbidden lines of the counting frame. Nucleoli coming into focus at the top plane were not included, whereas those at the bottom plane were counted. The following formula was used to calculate the cell number:

$$N = \frac{\text{step length}(X \cdot Y)}{a} \cdot \frac{t}{h} \cdot 3 \cdot \sum Q$$

where *a* is the area of the counting frame, *t* is the mean thickness of the sections, *h* is the predetermined height of the optical dissector (15 μm) and *Q* is the total counted number of A cells or B cells.

In this study, the step length was 90–100 μm for p75^{-/-} mice and 120–140 μm for p75^{+/+} mice. In each of the 68 ganglia studied, a mean of 162±30 A cells and 142±27 B cells were counted on the intact side, compared with 156±29 A cells and 108±23 B cells on the crushed side. The mean section thickness was 24.88±2.24 μm.

Perikaryal volume was estimated by the vertical planar rotator principle using the biggest nucleolus as the cell centre [27].

Statistical analysis. The p75^{+/+} mice and p75^{-/-} mice have different numbers of neuronal DRG cells. Therefore, all comparisons between non-diabetic and diabetic groups were exclusively performed within the wild-type p75^{+/+} group or within the genetically modified p75^{-/-} group using the unpaired Student's *t* test. For comparison of the intact and crushed sides in the same group of animals, paired Student's *t* test was applied.

Cell volume distributions are right-skewed and natural-log-transformed data were applied to compare the differences between groups. Values are presented as means ± SD, and data on the relative loss of neurons are presented as means ± SEM. A *p* value of less than 0.05 was considered statistically significant.

Results

Blood glucose and body weight. Blood glucose values at 2 days and 4 weeks were 20.5±3.1 mmol/l and 26.8±1.5 mmol/l in the diabetic p75^{+/+} mice and 19.7±2.3 mmol/l and 26.1±2.5 mmol/l in the diabetic p75^{-/-} mice respectively. Foot turnover was observed

Fig. 1. Chromatolysis with eccentricity of the nucleus and condensation of Nissl substance at the nuclear membrane in neuronal L5 DRG cells 28 days after sciatic nerve crush in p75^{+/+} mice (a) and p75^{-/-} mice (b). Arrows indicate chromatolytic A cells; arrow heads indicate chromatolytic B cells. Bar = 20 μm

on the ipsilateral side after sciatic nerve crush in all diabetic and non-diabetic mice.

Table 1 shows the mean body weight in all groups. At the start of the study, the body weights of the diabetic and non-diabetic p75^{+/+} mice were comparable, as were those of the two groups of p75^{-/-} mice. The p75^{-/-} mice weighed 6 g less than the p75^{+/+} mice. The non-diabetic mice achieved moderate weight gain during the course of the study, whereas the diabetic p75^{+/+} group lost 14% of their body weight and the diabetic p75^{-/-} group lost 23% of their body weight.

Histological appearance of DRG cell bodies. The DRG cell bodies were of similar appearance in both p75^{+/+} and p75^{-/-} mice. Twenty-eight days after sciatic nerve crush, chromatolysis with eccentricity of the nucleus and condensation of Nissl substance at the nuclear membrane was observed in a few ipsilateral DRG A cells and B cells in both groups of mice (Fig. 1).

Table 2. Number of non-crushed L5 DRG neurons in 12-week-old p75^{+/+} and p75^{-/-} mice with or without diabetes

Group	Total cells	A cells	B cells	Unidentified cells
p75 ^{+/+} without diabetes	9962±1143	2920±414 (29.3±2.5%)	6941±853 (69.7±2.7%)	101±43 (1.0±0.4%)
p75 ^{+/+} with diabetes	9355±728	2710±222 (29.0±1.9%)	6533±597 (69.8±2.0%)	112±25 (1.2±0.3%)
p75 ^{-/-} without diabetes	4178±788	1528±202 (37.2±4.8%)	2610±647 (61.9±4.9%)	40±19 (1.0±0.4%)
p75 ^{-/-} with diabetes	4484±581	1594±185 (35.8±4.2%)	2843±488 (63.2±4.4%)	47±13 (1.1±0.3%)

Values are means ± SD

Table 3. Geometric mean volume of crushed and non-crushed neurons in 12-week-old p75^{+/+} and p75^{-/-} mice with or without diabetes

Group	Non-crushed neurons		Crushed neurons	
	A cell volume (μm ³)	B cell volume (μm ³)	A cell volume (μm ³)	B cell volume (μm ³)
p75 ^{+/+} without diabetes	29,315±1527	5272±374	32,698±2200 ^{bb}	5820±414 ^b
p75 ^{+/+} with diabetes	26,642±2029 ^a	4751±513 ^a	29,104±2023 ^{b, cc}	5299±426 ^{b, c}
p75 ^{-/-} without diabetes	28,105±1776	4911±292	29,044±1483	5413±284 ^{bbb}
p75 ^{-/-} with diabetes	27,680±2568	4997±509	28,406±3361	5316±467

Values are geometric means ± SD. ^a*p*<0.05 vs the non-diabetic group on the non-crushed side; ^b*p*<0.05, ^{bb}*p*<0.01 and ^{bbb}*p*<0.001 vs the intact side in the same mice; ^c*p*<0.05 and ^{cc}*p*<0.01 vs the non-diabetic group on the crushed side

Effect of diabetes on L5 DRG structure on the non-crushed side of p75^{-/-} and p75^{+/+} mice. In non-diabetic p75^{+/+} mice there were 9962±1143 neuronal cell bodies in the L5 DRG on the non-crushed side. The distributions of A cells, B cells and unidentified neuronal DRG cell bodies were 29%, 70% and 1% respectively (Table 2). The geometric mean A cell body volume was 29 315±1527 μm³ and the geometric mean B cell body volume was 5272±374 μm³ (Table 3).

In diabetic p75^{+/+} mice there was no loss of neuronal DRG cells on the non-crushed side and the distribution of A cells, B cells and unidentified cells was similar to that observed in the non-diabetic p75^{+/+} mice (Table 2). Compared with non-diabetic p75^{+/+} mice, the geometric mean cell body volume of the A cells and B cells was reduced by 9% (*p*<0.05) and 10% (*p*<0.05) respectively (Table 3). Figure 2 shows the size–frequency distribution curves of non-crushed A cells and B cells in non-diabetic and diabetic p75^{+/+} mice. In diabetic p75^{+/+} mice the curves for both A cells and B cells are shifted towards smaller values.

In non-diabetic p75^{-/-} mice there were 4178±788 neuronal cell bodies in the L5 DRG on the non-crushed side. The distributions of A cells, B cells and unidentified cells were 37%, 62% and 1% respectively (Table 2). The geometric mean A cell body volume was 28 105±1776 μm³ and the geometric mean B cell body volume was 4911±292 μm³ (Table 3). In diabetic p75^{-/-} mice the number and distribution of the neuronal DRG cell bodies were similar to those observed in non-diabetic p75^{-/-} mice (Table 2). The neuronal perikaryal volumes in the two groups were also comparable (Table 3).

Fig. 2. Non-crushed DRG cell volume distribution curves of A cells and of B cells in diabetic p75^{+/+} mice and non-diabetic p75^{+/+} mice. The diabetic distribution curves are slightly shifted to the left. Data are means ± SEM. The x axes indicate cell volume using a log scale. filled triangles, A cells; open triangles, B cells; dotted lines, non-diabetic p75^{+/+} mice; solid lines, diabetic p75^{+/+} mice

Effect of diabetes on L5 DRG structure after sciatic nerve crush in p75^{-/-} and p75^{+/+} mice. The effect of nerve crush was assessed by comparing values obtained on the crushed side with those measured on the non-crushed side. In non-diabetic p75^{+/+} mice the loss of neuronal L5 DRG cell bodies was 27±6% (2832±1959, *p*=0.002) 4 weeks after crush. The loss was restricted to the B cells (37±6%, 2676±1495, *p*<0.001) (Fig. 3, Table 4).

In diabetic p75^{+/+} mice, nerve crush reduced the number of neuronal L5 DRG cells by 27±4%

Fig. 3. Number of neuronal cells (all) (a), A cells (b) and B cells (c) in the L5 DRG of diabetic (D) and non-diabetic (ND) p75^{+/+} and p75^{-/-} mice after nerve crush. Filled circles, crushed side; open circles, non-crushed side

(2529±1080, $p=0.001$). As in the non-diabetic p75^{+/+} mice, the loss was limited to the B cells (36±4%, 2369±850, $p<0.001$). The B cell loss in diabetic and non-diabetic p75^{+/+} mice was similar (36% and 37% respectively) (Fig. 3, Table 4).

Sciatic nerve crush significantly increased the geometric mean A cell perikaryal volume by 4059±3043 μm^3 in non-diabetic p75^{+/+} mice ($p<0.01$) and 2462±2837 μm^3 in diabetic p75^{+/+} mice ($p<0.05$). The geometric mean B cell perikaryal volume was in-

Table 4. Relative loss of A cells, B cells and total cells after sciatic nerve crush in 12-week-old p75^{+/+} and p75^{-/-} mice with or without diabetes

Group	Relative loss of total cells(%)	Relative loss of A cells (%)	Relative loss of B cells (%)
p75 ^{+/+} without diabetes	27±6 ^b	4±6	37±6 ^c
p75 ^{+/+} with diabetes	27±4 ^b	5±6	36±4 ^c
p75 ^{-/-} without diabetes	1±7	-0.3±5	2±9
p75 ^{-/-} with diabetes	12±4 ^a	8±4	14±4 ^a

Values are means ± SEM. ^a $p<0.05$ vs the non-crushed side; ^b $p<0.01$ vs the non-crushed side; ^c $p<0.001$ vs the non-crushed side

creased by 655±585 μm^3 ($p<0.05$) and 548±674 μm^3 ($p<0.05$) in non-diabetic and diabetic p75^{+/+} mice respectively (Table 3). On the crushed side, the geometric mean A cell perikaryal volume was 11% lower ($p<0.01$) in diabetic p75^{+/+} mice than in non-diabetic p75^{+/+} mice, and the geometric mean B cell perikaryal volume was 9% lower ($p<0.05$) (Table 3).

In non-diabetic p75^{-/-} mice, nerve crush did not reduce the total number of DRG cells (1±7% reduction, $p=0.85$) and did not affect the distribution of the neuronal subtypes (Fig. 3, Table 4). In diabetic p75^{-/-} mice the total number of neuronal DRG cell bodies was 4484±581 on the non-crushed side versus 3904±536 on the crushed side (Fig. 3, Table 4), which translates into a difference of 12±4% ($p=0.014$) between the two sides (Fig. 3, Table 4). The reduction in neuronal DRG cell bodies was mainly due to B cell loss (14±4%, $p=0.011$), and A cell loss was not significant (8±4%, $p=0.09$) (Fig. 3, Table 4). There was no significant difference in the extent of cell loss after crush between the diabetic and non-diabetic p75^{-/-} mice ($p=0.21$).

There were no differences in A cell perikaryal volume between the crushed and non-crushed sides in either non-diabetic or diabetic p75^{-/-} mice. Nerve crush significantly increased B cell perikaryal volume in non-diabetic p75^{-/-} mice (502±197 μm^3 increase, $p<0.001$) but not in diabetic p75^{-/-} mice (Table 3). The difference between the two groups was not significant. The geometric mean values for A cell and B cell perikaryal volume after nerve crush were similar in diabetic and non-diabetic p75^{-/-} mice (Table 3).

Comparison of L5 DRG neuronal cell body loss in diabetic and non-diabetic p75^{+/+} and p75^{-/-} mice. Table 4 shows the relative loss of A cells and B cells 4 weeks after crush for all groups. This table shows that following sciatic nerve crush: (i) B cells are lost in diabetic and non-diabetic p75^{+/+} mice; (ii) there is a small loss of B cells in diabetic p75^{-/-} mice; and (iii) no B cell loss occurs in non-diabetic p75^{-/-} mice.

Discussion

The present study shows that the reduction in the perikaryal volume of neuronal DRG cells in early experimental diabetes can be prevented by genetic knockout of the low-affinity p75 neurotrophin receptor. There was no perikaryal volume reduction in diabetic p75^{-/-} mice as compared with non-diabetic p75^{-/-} control mice, indicating that the volume reduction observed in p75^{+/+} mice was not induced by hyperosmolarity associated with hyperglycaemia.

An overall 18% reduction in perikaryal area has previously been reported in rats with a diabetic duration of 4 weeks [18]. In a recent study, stereological methods were applied to show that A cell and B cell perikaryal volume is reduced by 33% and 62% respectively in rats after 6 weeks of diabetes [28]. Perikaryal volume has not previously been studied in diabetic mice. In the present study, using optical dissector and vertical rotator techniques, we found a 9 to 10% reduction in the geometric mean volume of A cells and B cells of the L5 DRG in p75^{+/+} mice after 4 weeks of diabetes. Compared with that in non-diabetic mice, cell-volume distribution was uniformly shifted towards smaller sizes, suggesting overall shrinkage of both A cells and B cells in diabetic p75^{+/+} mice.

The pathogenesis of perikaryal volume reduction in diabetes is uncertain. Early on in experimental diabetes, reduced production of NGF and neurotrophin-3 in the target tissues leads to the reduced retrograde axonal transport of these proteins to the DRGs [7, 9, 11]. This, in turn, leads to impairment of p75 neurotrophin receptor and trkA and trkB mRNA synthesis in DRGs [8, 11, 12], reducing neurotrophin receptor occupancy [29]. It was recently observed that experimental diabetes causes changes in primary sensory neuronal cytoskeleton mRNA levels that mimic those caused by axotomy, including deprivation of target-derived neurotrophic factors as well as perikaryal shrinkage and axonal atrophy [30].

Previous studies of axon calibre in experimental diabetes have demonstrated proximal swelling with accumulation of the structural proteins neurotubulin, triple neurofilament and actin, in addition to dwindling of the remaining part of the axon with impairment of the transport of structural proteins [14, 31, 32]. In experimental diabetes, administration of neurotrophin-3 has been shown to ameliorate the neurofilament defects and the perikaryal and axonal atrophy [33], and to prevent the accumulation of neurofilaments in the proximal axon of DRG [34]. Also, the activation of stress-activated JNK correlates with hyperphosphorylation of neurofilaments, which leads to slow neurofilament transport [35, 36, 37] in experimental diabetes [38]. Based on our finding that a lack of p75 neurotrophin receptors prevents perikaryal volume reduction in 4 weeks' experimental diabetes, it is possible that reduced support of NGF and neurotrophin-3 in

DRGs could lead to a lack of occupancy of the p75 neurotrophin receptor, which could in turn activate the JNK and modulate perikaryal volume via changes in neurofilament phosphorylation. In addition, lack of occupancy of the p75 neurotrophin receptor could induce perikaryal volume and axonal calibre reduction by activating GTPase RhoA which regulates actin dynamics [39] and, therefore, cell dimensions.

It remains controversial whether neuronal DRG cell loss occurs in experimental diabetes. A previous study showed enzymatic apoptotic activity of DRG neurons after exposure to hyperglycaemia in vitro with increased expression of caspase 3 and TUNEL staining [20] and in vivo in rats after 1 month of diabetes with TUNEL staining [20]. TUNEL staining of DRG neurons in vivo and in vitro indicates DNA fragmentation [20]. However, the interpretation of positive TUNEL staining is uncertain, because the time interval between the signal and cell death could be long or the equilibrium between DNA fragmentation and DNA repair could permit a condition of neuronal dysfunction without leading to final dissolution and phagocytosis. A recent study confirmed the presence of an increased proportion of TUNEL-positive and caspase 3-positive DRG neuronal cells after 1, 3 and 12 months of STZ-induced diabetes in rats [21]. A study from the same laboratory reported no change in the total neuronal DRG cell number after 12 months of STZ-induced diabetes in rats. Further analysis, however, indicated that a subpopulation of the largest DRG cells was lost [19]. If perikaryal volume was reduced in this study, as in the present study, the apparent loss of the largest neurons could instead be explained by overall cell body shrinkage. Rat DRG neurons with increased caspase-3 levels have recently been shown to survive experimental diabetes without positive TUNEL staining reaction for 12 months [40]. Our finding of a lack of neuronal DRG cell loss in early experimental diabetes is consistent with previous observations [13].

The present study applied assumption-free stereological techniques to estimate neuronal DRG cell size and number. Using the same technique, other authors found similar numbers and sizes of A cells and B cells in the L5 DRG of non-diabetic p75^{+/+} mice and non-diabetic p75^{-/-} mice to those reported here [26, 41].

Non-diabetic and diabetic p75^{+/+} mice in the present study exhibited an increase in the geometric mean perikaryal volume of A cells and B cells 26 to 28 days after nerve crush. The increase in B cell perikaryal volume might be due to the loss of smaller B cells, whereas the increase in A cell perikaryal volume occurred without cell loss. In non-diabetic p75^{-/-} mice the geometric mean volume of A cells tended to increase after crush, whereas B cell volume increased significantly without cell loss. In a previous study in rats, mean A cell volume diminished as early as 4 days after spinal nerve crush, but was normalised after

120 days. In contrast, B cell volume was only mildly affected [42]. The present study differs from the previous report with respect to the species of animal used and the location of crush in relation to the DRG. The closer the crush is located to the DRG, the more neurons are lost. A sciatic nerve crush was applied in the present study, which probably produces smaller volume changes than a crush closer to the DRG. In addition, the life span of mice is shorter than that of rats, indicating that the insult and the repair process are faster in mice. Therefore, early nerve regeneration may well allow compensatory hypertrophy in DRG cells, whereas the lack of an increase in neuronal perikaryal volume in diabetic $p75^{-/-}$ mice after crush might be due to more severe neurotrophin deficiency.

In line with previous findings [41], nerve crush did not reduce the number of neuronal DRG cells in non-diabetic $p75^{-/-}$ mice. It could be argued that the prevention of neuronal DRG cell loss in $p75^{-/-}$ mice after crush is caused by the loss of a neuronal subpopulation during development. However, in comparison with in $p75^{+/+}$ mice, the neuronal loss in $p75^{-/-}$ mice was not confined to a specific subtype of cells or to specific functions [43, 44], making it less likely that the absence of a reduction in the number of cells in $p75^{-/-}$ mice after crush is due to the loss of a particular vulnerable subpopulation during development.

It is possible that neuronal DRG cell loss after crush is mediated by activation of the $p75$ neurotrophin receptor. In the present study, the total number of L5 DRG cells in $p75^{+/+}$ mice was reduced by 27% 4 weeks after sciatic nerve crush, with the reduction predominantly affecting B cells. This observation is in accordance with axotomy studies in rats in which the extent of neuronal loss was 15–22% and 30–35%, 15 and 45 days after L5 spinal nerve crush or transection respectively [42, 45]. In a different study, a 54% neuronal loss was observed 28 days after mid-thigh sciatic nerve transection in C57BL/J6 mice [46]. A recent study investigating the time course of cell death reported that neuronal death is modulated by peripheral nerve repair and timing after axotomy [47].

The sensitivity of B cells to crush in $p75^{+/+}$ mice could be due to their specific *trk* phenotype. The $p75$ neurotrophin receptor is expressed throughout the neurons in lumbar DRG and is usually coexpressed with *trks*. The type of *trk* expressed is dependent upon the cell type: *trkA* is expressed in small-to-medium-sized cells, *trkB* is expressed in medium-to-large-sized cells and *trkC* is expressed in large A cells [48]. Although the neurotrophic receptor expression has not been reported in A and B cells, it is most likely that part of the small B cells coexpress *trkA* and the $p75$ receptor. Several lines of evidence indicate that the $p75$ neurotrophin receptor induces apoptosis when *trkA* is either absent or down-regulated. High levels of expression of the $p75$ receptor have been shown to cause the death of postnatal sensory neurons after

withdrawal of NGF [49], and antisense oligonucleotides directed against the $p75$ neurotrophin receptor markedly reduce the loss of axotomised sensory neurons [50]. Seven days after sciatic nerve transection, $p75$ receptor expression and *trk* mRNA synthesis were dramatically down-regulated [51] due to reduced neurotrophin retrograde transport [8, 29]. Unsaturated *trkA* and reduced $p75$ levels will reduce NGF signalling to *trkA*. The down-regulation of *trkA* signalling could trigger $p75$ -receptor-mediated B cell loss after nerve crush in our study. The survival of large A cells after axotomy may be explained by different interactions between *trkB* and the $p75$ receptor, and *trkC* and the $p75$ receptor.

We hypothesised that experimental diabetes accelerates the loss of DRG cells after crush. However, neuronal DRG cell loss was not accelerated in the diabetic $p75^{+/+}$ mice compared with that in non-diabetic $p75^{+/+}$ mice. Surprisingly, a small loss of neuronal DRG cells was observed in diabetic $p75^{-/-}$ mice. This finding could indicate that the $p75$ neurotrophin receptor partly protects the DRG neurons in the diabetic condition. Since *trkA* homodimerisation and phosphorylation initiated by NGF binding promote neuronal survival [52, 53], the protective effect of the $p75$ neurotrophin receptor in diabetes after crush could be due to increased affinity of *trkA* for NGF. Interaction between the $p75$ neurotrophin receptor and *trkA* has been shown in several studies. Embryonic DRG [54] and trigeminal DRG neurons [55], which are $p75$ deficient, are less sensitive to sub-saturating concentrations of NGF than wild-type neurons during the period of naturally occurring cell death. Conversely, the response of $p75$ -deficient sensory neurons to lower doses of brain-derived neurotrophic factor and neurotrophin-4/5 is normal. Moreover, the disruption of NGF binding to the $p75$ neurotrophin receptor attenuates NGF binding to *trkA* and *trkA* phosphorylation [56]. The ability of the $p75$ receptor to increase the sensitivity of sensory neurons to NGF [55, 54] and to increase *trkA* phosphorylation [56] is limited to lower NGF concentrations and disappears at high concentrations. Fast binding and dissociation of NGF to the $p75$ neurotrophin receptor could increase the local concentration of NGF and thereby promote NGF binding to *trkA* when NGF is limited. Since *trkA* is mainly expressed in small, unmyelinated DRG neuronal cells [48], we suggest that selectively reduced sensitivity of *trkA* to NGF after sciatic nerve crush in diabetic $p75^{-/-}$ mice could account for the small loss of NGF-dependent DRG B cells.

In conclusion, our observations indicate that the $p75$ neurotrophin receptor is the major, but not the only, molecule involved in induction of DRG neuronal apoptosis after sciatic nerve crush.

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