

Selective Loss of Calcitonin Gene–Related Peptide–Expressing Primary Sensory Neurons of the A-Cell Phenotype in Early Experimental Diabetes

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To evaluate the possible role of neuropeptide immunoreactive primary sensory neurons on the development of nociceptive dysfunction in diabetes, the absolute numbers of immunoreactive substance P and calcitonin gene-related peptide (CGRP) dorsal root ganglion (DRG) cell bodies were estimated in diabetic and nondiabetic BALB/C (p75^{+/+}) and p75 receptor knockout (p75^{-/-}) mice with unilateral sciatic nerve crush. The total numbers of immunoreactive substance P A-cells, substance P B-cells, CGRP A-cells, and CGRP B-cells in L5DRG were estimated using semithick consecutive sections and the optical fractionator. After 4 weeks of streptozotocin-induced diabetes, the number of immunoreactive CGRP A-cells was reduced from 692 ± 122 to 489 ± 125 (*P* = 0.004) in p75^{+/+} mice on the noncrushed side. In p75^{-/-} mice, there was no such effect of diabetes on the immunoreactive CGRP A-cell number. In p75^{+/+} and p75^{-/-} mice, there was no effect of diabetes on the immunoreactive CGRP B-cell number, nor was there any effect of diabetes on the immunoreactive substance P B-cell number. Sciatic nerve crush was associated with a substantial loss of L5DRG B-cells in diabetic and nondiabetic p75^{+/+} mice and with substantial loss of immunoreactive substance P cells in diabetic p75^{+/+} mice. In diabetic and nondiabetic p75^{-/-} mice, there was no crush effect on neuropeptide expression. It is concluded that experimental diabetes in the mouse is associated with loss of immunoreactive CGRP primary sensory neurons of the A-cell phenotype, that this loss could play a role for the touch-evoked nociception in the model, and that the neuronal immunoreactive CGRP abnormality possibly is mediated by activation of the p75 neurotrophin receptor. *Diabetes* 53:2669–2675, 2004

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CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglion; NGF, nerve growth factor; p75^{NTR}, p75 neurotrophin receptor; STZ, streptozotocin. © 2004 by the American Diabetes Association.

Sensory polyneuropathy develops insidiously in diabetes and involves all types of nerve fibers. Abnormal sensory perception in diabetic patients includes loss of pain and temperature sensations as well as burning and cutaneous hyperesthesia, typically in the feet and lower legs (1). The mechanisms underlying hypo- or hyperalgesia in diabetes are uncertain, but studies of diabetic rats indicate that unmyelinated afferents (2), myelinated afferents (3), and spinal and superspinal sensory neurons (4) are all involved in the process.

Calcitonin gene-related peptide (CGRP) and substance P are nerve growth factor (NGF)-dependent neuropeptides (5,6). Increased expressions of CGRP and substance P in the peripheral nervous system relate to hyperalgesia or allodynia (7–9). Moreover, the CGRP-related hyperalgesia is abolished with a CGRP receptor antagonist (9), with an antiserum to CGRP (10), or by knockout of the CGRP gene (11).

In dorsal root ganglion (DRG), substance P and CGRP are synthesized predominantly in small B-cells, receiving signals through the peripheral C- and A δ -afferents (12,13). Because of the deficits of NGF support in experimental diabetes (14), it is expected that substance P and CGRP expressions are decreased. Studies of substance P and CGRP content in rat sciatic nerve after 4 weeks of streptozotocin (STZ)-induced diabetes has confirmed this suggestion (15,16), whereas immunohistochemical studies indicate that the relative proportion of immunoreactive CGRP and substance P cells is unchanged in 4-week diabetic BB rats (17) and 12-month STZ-induced diabetic rats (18), despite dramatic reduction of CGRP and substance P mRNA in the DRG (5,14,18).

The mechanical and nociceptive afferents could interact with each other through the interneurons at the spinal cord level. Touch-induced pain seems to involve incoming activity from low-threshold mechanoreceptors to large neuronal A-cells with subsequent presynaptic interaction with central nociceptive afferents in the dorsal horn of the spinal cord (19).

We hypothesize that experimental diabetes is associated with an altered balance between the absolute numbers of large A-cells and small B-cells of CGRP- and substance P-expressing DRG neurons. Recently, we have shown that the low-affinity p75 neurotrophin receptor (p75^{NTR}) influences the morphology of DRG cell bodies in STZ-induced diabetes, and, consequently, we hypothesize that the al-

tered balance between immunoreactive CGRP and substance P A- and B-cells in experimental diabetes is mediated by p75^{NTR}.

RESEARCH DESIGN AND METHODS

In this study, we used 12-week-old male p75^{NTR} knockout mice (p75^{-/-}) and age-matched male BALB/C mice (p75^{+/+}; Taconic M&B, Ry, Denmark), the half-gene background strain for p75^{-/-} mice. The p75^{-/-} offspring of homozygous breeders from The Jackson Laboratories (Bar Harbor, ME) were kindly provided by Martin Koltzenburg, Würzburg, Germany. The mice were housed in top filter-barrier mouse cages, with food and water available at 23°C with 50% relative humidity and a 12-h light/dark cycle. By the end of the experiment, four groups of 16-week-old mice were harvested: p75^{+/+} mice without diabetes (n = 8), p75^{+/+} mice with diabetes (n = 9), p75^{-/-} mice without diabetes (n = 9), and p75^{-/-} mice with diabetes (n = 11).

Diabetes was induced with a single intraperitoneal injection of 190–200 mg/kg STZ (Bie & Berntsen, Roedovre, Denmark) freshly prepared in 10 mmol/l citrate buffer at pH 5.0. At 72 h later and at the end of the experiment, the tail vein blood glucose concentration was determined using a glucose touch meter (Johnson & Johnson, Milpitas, CA). STZ-induced diabetic mice with blood glucose concentrations <15 mmol/l at 72 h or at 4 weeks were excluded.

On the day of STZ treatment, the mice were anesthetized with 240 mg/kg avertin (Sigma-Aldrich, Vallsenbaek Strand, Denmark), and one sciatic nerve in each mouse was crushed at the “sciatic notch” with 0.5-mm forceps (model Tübingen Amann Medizintechnik; S.W. Inox Castroviejo, Emmingen-Liptingen, Germany) Before the operation and for the following 2 days, 3 µg buprenorphin (Schering-Plough, Munich, Germany) was injected subcutaneously twice a day. The crushed side was selected randomly, and the contralateral side served as control. At 4 weeks later, the mice were anesthetized and had a vascular perfusion through the heart with 0.01 mol/l PBS, pH 7.4, for 30 s followed by 4% paraformaldehyde in 0.15 mol/l PBS, pH 7.4, for 10 min.

The L5DRG on both sides were removed and postfixed in 4% paraformaldehyde at 4°C for 2 h, immersed in a cryoprotective solution containing 5% sucrose in 0.15 mol/l PBS, pH 7.4, for 24 h at 4°C, embedded in Tissue-Tek OCT compound, and stored at -80°C. The protocol was in accordance with the guidelines specified in the recommendation by the Federation of European Laboratory Animal Science Associations.

Immunohistochemistry. To minimize the variance induced by technical procedures, the two DRGs from the intact and the crushed sides of the same mouse were sectioned and stained together. The DRGs were cut into 40-µm thick consecutive sections on a cryostat and thawed on gelatin-coated glass slides. All of the sections were separated into two sets, using systematic, uniformly random sampling. One part was selected for the stain with CGRP antibody, and the other set was stained with substance P antibody. The immunohistochemical staining was carried out with the ABC method. The sections were incubated for 1 h with 0.05 mol/l Tris buffer solution (pH 7.6) containing 10% normal goat serum and 0.3% Triton-X, and then they were incubated with polyclonal rabbit anti-rat CGRP antibody (1:1,000 T-4032; Peninsula Lab, San Carlos, CA) or polyclonal rabbit anti-rat substance P antibody (1:800 T-4107; Peninsula Lab) containing 5% normal goat serum and 0.3% Triton-X for 24 h at 4°C. Afterward, sections were incubated with biotinylated goat anti-rabbit antibody (1:200 BA-1000; Vector Lab, Burlingame, CA) for 3 h followed by incubation with StreptABC complex/horseradish peroxidase (K0377; Dako, Glostrup, Denmark) for 2 h, according to the manufacturers’ instructions. The immunoreactive products were visualized with 0.05% diaminobenzidine containing 0.03% H₂O₂ for 10 min. To obtain total numbers of A- and B-cells, nuclei and nucleoli of all sections were counterstained with hematoxylin for 1 min. Finally, tissues were mounted in Aquatex (Merck), coverslipped, and evaluated using light microscopy. Sections were washed thoroughly with Tris buffer solution, containing 0.3% Triton-X, between each step. Control staining was performed without the primary antibodies.

Stereology. The total L5DRG A- and B-cell numbers and the immunoreactive substance P or CGRP A- or B-cell numbers were estimated using the optical fractionator (20,21). A modified Olympus BX50 microscope was connected to an electronic microcator (MT2; Heidenhain, Traunreut, Germany) to record the plane movement in the z direction. On the top of the microscope, a color video camera (3-CCD video camera; Olympus, Copenhagen, Denmark) projected the tissue images to a computer screen. Using interactive graphic software (CAST-grid; Olympus, Albertslund, Denmark) and a 4× lens, tissues were sampled systemically after a random start. The number of sampled neuronal cell bodies depends on the step lengths in the x and y directions and on the density of counting units. Because 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. For total L5DRG neuronal cell counting, the counting frames were 4,720 µm²

TABLE 1

Body weights during the 4-week experimental period in p75^{+/+} and p75^{-/-} mice with and without diabetes

	Weight (g)		
	Day 0	4 weeks	Difference
p75 ^{+/+} without diabetes	30.0 ± 1.8	30.6 ± 1.3	0.6 ± 1.1
p75 ^{+/+} with diabetes	30.8 ± 2.3	23.2 ± 3.7*	-7.5 ± 3.3
p75 ^{-/-} without diabetes	24.0 ± 1.9	25.8 ± 1.9	1.8 ± 1.9
p75 ^{-/-} with diabetes	25.6 ± 2.7	20.7 ± 2.6*	-4.9 ± 2.6

Data are means ± SD. *P < 0.001 in comparison with nondiabetic controls.

for A-cells and 2,029 µm² for B-cells using a 60× oil immersion lens. The tissue thickness of every fourth sampling frame was measured.

Using light microscopy A- and B-cells were distinguished, based on their morphology. A-cells have a large centrally placed nucleolus with one to two smaller nucleoli, and the granular Nissl substance is distributed evenly throughout the bright cytoplasm. B-cells usually have several peripherally located small nucleoli and a dark, homogeneous cytoplasm (22). The biggest nucleolus was the counting unit. If two nucleoli were of the same size, one of them 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:

$$N = \frac{1}{SSF} \times \frac{1}{ASF} \times \frac{1}{HSF} \times \Sigma Q^- = 2 \times \frac{dx \times dy}{A(frame)} \times \frac{t_q^-}{h} \times \Sigma Q^-$$

where SSF is the section sampling fraction; ASF is the area sampling fraction; HSF is the height sampling fraction; dx = dy is the step length in the x and y directions; A(frame) is the area of counting frame; h is the dissector height; Q⁻ is the number of counted cells; t_q⁻ is the q⁻ weighted section thickness;

$$t_q^- = \frac{\Sigma(t_i q_i)}{\Sigma(q_i)}$$

and q_i is the number of sampled neurons for that particular section thickness.

The 4,720-µm² counting frame was applied for estimation of the total numbers of immunoreactive cells. To count the maximum number of immunoreactive cells and to avoid double counting, a 90-µm step length was applied in the x and y directions for immunoreactive substance P A- and B-cells, whereas the step length for immunoreactive CGRP A- and B-cell counting was 120–140 µm in p75^{+/+} mice and 90–100 µm in p75^{-/-} mice. According to the penetration of antibodies and Z calibration of the stained neurons (20) (in the present study, the curves of position distribution of immunoreactive CGRP and substance P neurons in tissue sections showed stable density in the depth from 5 µm to 16 µm), the optical dissector started 5–7 µm under the top plane and continued for 9 µm in the depth (z direction). Tissue thickness of every second sampling frame was measured. The average of the final section thickness was 22.3 ± 3.5 µm.

Statistical analysis. p75^{+/+} mice and p75^{-/-} mice have different numbers of neuronal DRG cells. Therefore, all comparisons between nondiabetic and diabetic groups were exclusively performed within the wild-type p75^{+/+} group or within the genetically modulated p75^{-/-} group. The primary study parameters in the p75^{+/+} group as well as in the p75^{-/-} group were the absolute numbers of immunoreactive CGRP A- and B-cells and substance P B-cells on the noncrushed side in diabetic mice as compared with their nondiabetic controls. For statistical analysis of the three primary end points in the p75^{+/+} group and in the p75^{-/-} group, an unpaired Student’s t test with a 2% level of significance was applied (Bonferroni correction). All other comparisons were considered secondary end points and were analyzed with unpaired (between groups) or paired (within groups) Student’s t test using a 5% level of significance. Values were shown as the means ± SD.

RESULTS

At 3 days after treatment with STZ, blood glucose levels were 21.7 ± 4.7 mmol/l in p75^{+/+} mice and 20.1 ± 1.9 mmol/l in p75^{-/-} mice. At the end of the study, blood glucose values were >22.2 mmol/l in all diabetic mice.

Table 1 shows the mean body weight in all groups. The body weights at start were comparable in diabetic and

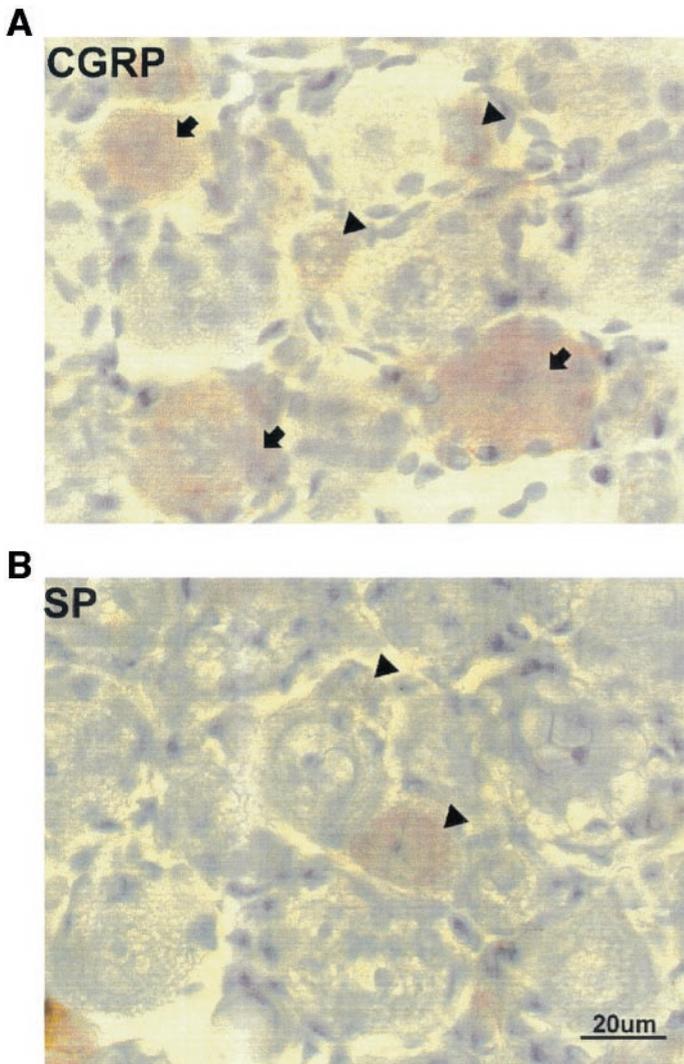


FIG. 1. CGRP (A) and substance P (B) immunostained DRG cell bodies of a $p75^{+/+}$ nondiabetic mouse. The cytoplasm of B-cells is stained homogeneously, whereas the CGRP staining of A-cells is more granular. Arrows show A-cells, arrowheads show B-cells. SP, substance P.

nondiabetic $p75^{+/+}$ mice as well as in diabetic and nondiabetic $p75^{-/-}$ mice. The mean body weight of $p75^{-/-}$ mice was 20% less than that of $p75^{+/+}$ mice. Nondiabetic $p75^{-/-}$ mice had a mild weight gain during the study, whereas no weight change occurred in nondiabetic $p75^{+/+}$ mice. Diabetic mice in the $p75^{+/+}$ group lost 24% of their body weight, and in the $p75^{-/-}$ group, the loss was 19%.

Morphology. Immunostaining for CGRP and substance P was located in the cytoplasm, with diffuse granular staining of large immunoreactive CGRP A-cells and with homo-

geneous staining of immunoreactive substance P B-cells and CGRP B-cells (Fig. 1). There was no apparent difference of stain intensity among the different groups. Cells with less staining intensity displayed sufficient contrast difference to the background.

Effects of diabetes and nerve crush on the number of L5DRG neuronal cells in $p75^{+/+}$ and $p75^{-/-}$ mice. Table 2 shows the number of total neuronal DRG cells of the A and B subtype in diabetic and nondiabetic $p75^{+/+}$ and $p75^{-/-}$ mice with and without sciatic nerve crush. There was no effect of diabetes on neuronal cell number in either $p75^{+/+}$ or $p75^{-/-}$ mice with intact nerves. Sciatic nerve crush was associated with a substantial loss of the B subtype of neuronal L5DRG cells in $p75^{+/+}$ mice, whereas the loss in $p75^{-/-}$ mice was modest, only.

Effect of diabetes on L5DRG CGRP expression on the noncrushed side of $p75^{+/+}$ and $p75^{-/-}$ mice. In nondiabetic $p75^{+/+}$ mice, there was a total of $3,682 \pm 756$ or $40.1 \pm 8.0\%$ neuronal immunoreactive CGRP cells. CGRP immunoreactivity was present in both A- and B-cells. There was 692 ± 122 or $25.2 \pm 5.9\%$ immunoreactive CGRP A-cells and $2,990 \pm 660$ or $47.2 \pm 11.8\%$ immunoreactive CGRP B-cells (Table 3), the A-cell-to-B-cell ratio being 0.24 ± 0.04 (Fig. 2).

Diabetes did not induce any changes in the number of total immunoreactive CGRP cells. However, in $p75^{+/+}$ diabetic mice, the number of immunoreactive CGRP A-cells was reduced by 29% ($P = 0.004$), and the relative number of immunoreactive CGRP A-cells was reduced from 25.2 to 16.9% ($P = 0.002$) (Table 3). Likewise, the A-cell-to-B-cell ratio of immunoreactive CGRP cells declined to 0.15 ± 0.03 ($P < 0.001$) (Fig. 2). There was no effect of diabetes on immunoreactive CGRP B-cells.

The nondiabetic $p75^{-/-}$ mice had approximately half the number of neuronal L5DRG cells and total immunoreactive CGRP cells. The relative number of immunoreactive CGRP A-cells in nondiabetic $p75^{-/-}$ mice was 8% lower than that in nondiabetic $p75^{+/+}$ mice ($P = 0.012$). However, in $p75^{-/-}$ mice, diabetes did not cause any alteration in the total number of immunoreactive CGRP cells, the number of absolute or relative immunoreactive CGRP A-cells or B-cells (Table 3), or the A-cell-to-B-cell ratio of immunoreactive CGRP cells (Fig. 2).

Effect of diabetes on L5DRG substance P expression on the noncrushed side of $p75^{+/+}$ and $p75^{-/-}$ mice. In nondiabetic $p75^{+/+}$ mice, the total number of immunoreactive substance P cells was $2,245 \pm 610$ or, expressed relatively, $24.1 \pm 4.5\%$. Substance P was almost exclusively observed in small B-cells, but a few A-cells displayed immunoreactivity (41 ± 41 [$1.9 \pm 1.8\%$]). Diabetes did not

TABLE 2

Total numbers of total L5DRG neuronal cells and of the A- and B-cell subtypes in $p75^{+/+}$ and $p75^{-/-}$ mice with intact and crushed nerves and in those with and without diabetes

Groups	Total neurons		A-cells		B-cells	
	Intact	Crushed	Intact	Crushed	Intact	Crushed
$p75^{+/+}$ without diabetes	$9,233 \pm 973$	$6,973 \pm 1,368^*$	$2,791 \pm 439$	$2,510 \pm 428$	$6,442 \pm 892$	$4,463 \pm 1,097^*$
$p75^{+/+}$ with diabetes	$9,951 \pm 1,667$	$7,986 \pm 1,462^*$	$2,890 \pm 494$	$2,825 \pm 624$	$7,061 \pm 1,261$	$5,161 \pm 971^\dagger$
$p75^{-/-}$ without diabetes	$4,408 \pm 773$	$4,176 \pm 899$	$1,443 \pm 219$	$1,466 \pm 342$	$2,965 \pm 636$	$2,709 \pm 650$
$p75^{-/-}$ with diabetes	$4,396 \pm 1,082$	$4,066 \pm 868^*$	$1,461 \pm 356$	$1,451 \pm 348$	$2,935 \pm 811$	$2,614 \pm 645^\dagger$

Data are means \pm SD. $*P < 0.05$ and $^\dagger P < 0.01$ in comparison with the intact nerve side in the same group.

TABLE 3

Absolute and relative numbers of total immunoreactive CGRP L5DRG neuronal cells, A-cells, and B-cells in p75^{+/+} and p75^{-/-} mice with intact and crushed nerves and in those with and without diabetes

	All immunoreactive CGRP neurons (%)		Immunoreactive CGRP A-cells (%)		Immunoreactive CGRP B-cells (%)	
	Intact	Crushed	Intact	Crushed	Intact	Crushed
p75 ^{+/+} without diabetes						
Absolute	3,682 ± 756	2,901 ± 803	692 ± 122	629 ± 252	2,990 ± 660	2,273 ± 582
Relative (%)	40.1 ± 8.0	41.2 ± 5.4	25.2 ± 5.9	24.6 ± 7.7	47.2 ± 11.8	51.4 ± 9.6
p75 ^{+/+} with diabetes						
Absolute	3,721 ± 666	3,269 ± 1,000	489 ± 125*	539 ± 229	3,232 ± 586	2,730 ± 812
Relative (%)	37.5 ± 4.4	40.3 ± 6.4	16.9 ± 3.2*	18.5 ± 5.6	46.0 ± 5.5	52.1 ± 7.5†
p75 ^{-/-} without diabetes						
Absolute	1,686 ± 273	1,510 ± 323	258 ± 101	216 ± 74	1,429 ± 206	1,293 ± 269
Relative (%)	38.4 ± 2.6	36.7 ± 6.7	17.6 ± 5.1‡	14.6 ± 3.4	49.0 ± 5.6	49.2 ± 10.8
p75 ^{-/-} with diabetes						
Absolute	1,710 ± 547	1,550 ± 319	254 ± 118	224 ± 121	1,457 ± 470	1,325 ± 247
Relative (%)	38.7 ± 6.5	38.3 ± 4.4	16.9 ± 6.0	15.1 ± 6.4	49.4 ± 7.4	51.9 ± 10.0

Data are means ± SD. **P* < 0.01 and ‡*P* < 0.05 in comparison with nondiabetic p75^{+/+} mice with intact nerves; †*P* < 0.05 in comparison with diabetic p75^{+/+} mice with intact nerves.

induce any changes in total or in A or B subtype numbers of immunoreactive substance P cells in p75^{+/+} mice. In nondiabetic p75^{-/-} mice, the number of immunoreactive substance P cells was approximately half that of p75^{+/+} mice. Again, diabetes did not induce any changes in substance P immunoreactivity in p75^{-/-} mice (Table 4).

Effect of sciatic nerve crush on L5DRG CGRP expression in p75^{+/+} and p75^{-/-} mice. In nondiabetic p75^{+/+} mice, there was no effect of sciatic nerve crush on numbers of immunoreactive CGRP A-cells or B-cells after 4 weeks. In diabetic p75^{+/+} mice, the proportion of immunoreactive CGRP B-cells increased from 46.0 ± 5.5% on the noncrushed side to 52.1 ± 7.5% on the crushed side (*P* = 0.04), and the A-cell-to-B-cell ratio of immunoreactive CGRP cells rose from 0.15 ± 0.03 to 0.20 ± 0.06 (*P* = 0.04) (Fig. 2). Compared with nondiabetic p75^{+/+} mice, the A-cell-to-B-cell ratio of immunoreactive CGRP cells after crush fell from 0.27 ± 0.05 to 0.20 ± 0.06 (*P* = 0.02) in those with diabetes (Fig. 2). In p75^{-/-} mice there was no

effect of sciatic nerve crush on the number of immunoreactive CGRP cells in either diabetic or nondiabetic mice after 4 weeks (Table 3).

Effect of sciatic nerve crush on L5DRG substance P expression in p75^{+/+} and p75^{-/-} mice. There was no difference of substance P immunoreactivity after nerve crush between diabetic and nondiabetic p75^{+/+} mice. In diabetic p75^{+/+} mice, total immunoreactive substance P cell numbers were reduced by 32% (*P* = 0.008) and immunoreactive substance P B-cell numbers by 32% (*P* = 0.007) as compared with the nonaxotomized side in the same mice. In neither diabetic nor nondiabetic p75^{-/-} mice was there any influence of sciatic nerve crush on substance P immunoreactivity after 4 weeks (Table 4).

DISCUSSION

Intracellular electrophysiological recordings have shown that large DRG cells of the A subtype project myelinated

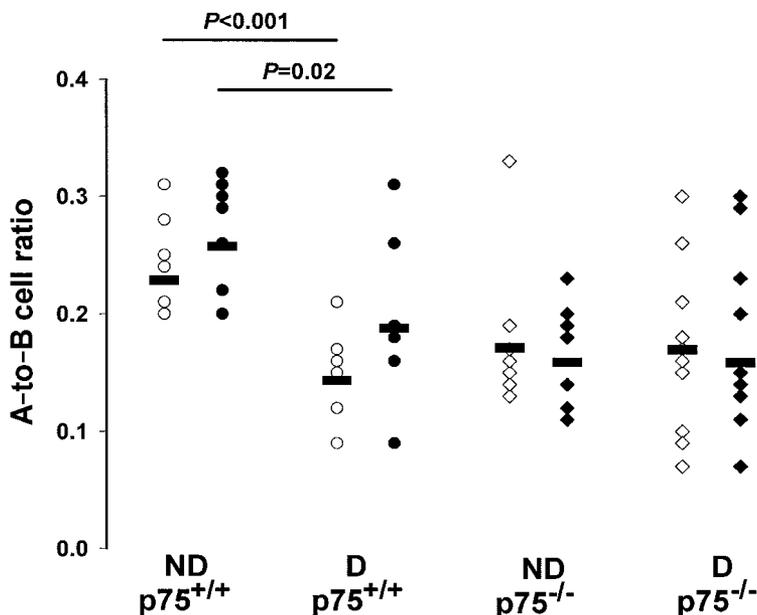


FIG. 2. A-cell-to-B-cell ratio of immunoreactive CGRP L5DRG cell bodies on the noncrushed side (open symbols) and on the crushed side (filled symbols) in diabetic (D) and nondiabetic (ND) p75^{+/+} (circles) and p75^{-/-} (diamonds) mice.

TABLE 4

Absolute and relative number of total immunoreactive substance P L5DRG neuronal cells and B-cells in p75^{+/+} and p75^{-/-} mice with intact and crushed nerves and in those with and without diabetes

	All immunoreactive substance P neurons (%)		Immunoreactive substance P B-cells (%)	
	Intact	Crushed	Intact	Crushed
p75 ^{+/+} without diabetes				
Absolute	2,245 ± 610	1,688 ± 481	2,204 ± 615	1,651 ± 472
Relative (%)	24.1 ± 4.5	23.9 ± 3.8	34.2 ± 7.6	36.9 ± 5.5
p75 ^{+/+} with diabetes				
Absolute	2,761 ± 836	1,888 ± 547*	2,734 ± 831	1,871 ± 539*
Relative (%)	27.4 ± 4.8	23.6 ± 4.4†	38.4 ± 7.1	36.2 ± 7.1
p75 ^{-/-} without diabetes				
Absolute	996 ± 210	922 ± 223	972 ± 208	918 ± 227
Relative (%)	22.5 ± 3.2	22.1 ± 4.2	33.4 ± 6.4	34.1 ± 6.8
p75 ^{-/-} with diabetes				
Absolute	1,015 ± 374	877 ± 278	994 ± 367	861 ± 265
Relative (%)	23.1 ± 7.0	21.9 ± 6.5	34.0 ± 9.9	34.1 ± 11.7

Data are means ± SD. **P* < 0.01; †*P* < 0.05 in comparison with the intact nerve side in the same group.

A α / β -fibers and thinly myelinated A δ -fibers associated with signal transmission from mechanoreceptors, whereas the small DRG cells of the B subtype project thinly myelinated A δ -fibers and unmyelinated C fibers, which transmit nociceptive signals, mainly (23). The neuropeptides CGRP and substance P are predominantly located in the small neuronal B-cells (12,13).

In the present study, the relative numbers of all neuronal immunoreactive CGRP and substance P cells in L5DRG in p75^{+/+} as well as p75^{-/-} mice are in accordance with those previously obtained in mice and rats (17,24). Our estimation of the absolute numbers of immunoreactive substance P and CGRP A- and B-cells has been obtained with stereological techniques not previously applied.

In diabetic p75^{+/+} and p75^{-/-} mice, we observed no change in the absolute or relative number of total immunoreactive CGRP and substance P neurons. Similar results have been obtained in BB rats with 4 weeks of diabetes (17) and in STZ-induced diabetic rats with 12 months of diabetes (18). Nonetheless, it is well established that the expression of CGRP and substance P is reduced in diabetes. The mRNA of CGRP and substance P in DRG declines (5,14,18), and the immunoreactivity and the content of CGRP and substance P are both reduced in peripheral nerves (15,16). In diabetic BB rats, the immunoreactive CGRP neurons are smaller, whereas immunoreactive substance P neurons have an unchanged size (17). An *in vitro* study showed that the proportion of large immunoreactive CGRP DRG neurons was dramatically increased in diabetic mice in an NGF-free medium and that NGF supplementation normalized neuronal size (6). In the two former studies, small and large immunoreactive CGRP neurons were not separated, and A- and B-cells were not distinguished. The new finding of the present study is that the absolute and relative numbers of immunoreactive CGRP A-cells are markedly reduced in diabetic p75^{+/+} mice without any change of the number of the immunoreactive CGRP B-cell subtype, leading to reduction of the A-cell-to-B-cell ratio of immunoreactive CGRP neurons. Because there was no DRG A-cell loss in diabetic p75^{+/+} mice without nerve crush, the reduced number of immunoreactive CGRP A-cells is mostly likely caused by a loss of the

immunoreactive CGRP phenotype. Because the DRG A- and B-cells were classified based on other cell morphology characteristics besides cell volume, the selective loss of immunoreactive CGRP A-cells can hardly be explained by misclassification leading to a shift from A- to B-cells. Furthermore, the number of total DRG A-cells and the ratio of DRG A-cells to B-cells in diabetes are unchanged. The pronounced reduction of the immunoreactive CGRP A-cell number in diabetic p75^{+/+} mice did not influence the total immunoreactive CGRP cell number because the fraction of immunoreactive CGRP A-cells is small. In the present study, a 29% reduction of immunoreactive CGRP A-cells will give rise to a 6% reduction in total immunoreactive CGRP DRG cells, only.

In experimental diabetes nociception has been studied with behavioral escape responses, using stimulus intensity or withdrawal latencies as effect parameters. In STZ-induced diabetic rats, the findings in studies of the nociceptive response to thermal stimulation are equivocal (25–27), whereas mechanically induced nociception is reported to be increased (28,29). However, in STZ-induced diabetic mice, a severe hypoalgesic response to mechanical stimulation occurs, and this can be improved after treatment with NGF (30).

Small C fibers are considered to play a significant role for abnormal nociception in diabetes. A study of the saphenous nerve showed that a subpopulation of C-fibers is hyperexcitable to sustained suprathreshold stimuli in diabetic rats (2). However, Khan et al. (3) reported that tactile allodynia appeared earlier in diabetic rats than thermal hyperalgesia, and this was associated with ectopic discharges and a higher spontaneous activity in A δ - and A β -fiber afferents with an augmented response to mechanical stimuli. These findings indicate that myelinated as well as unmyelinated immunoreactive CGRP and substance P fibers could be involved in pain transmission in diabetes. The central branches of immunoreactive CGRP DRG neurons end in laminae I-IV of the spinal cord dorsal horn (17), where interactions between A-fibers and C-fibers can occur (19). Under normal conditions, activation of A β -afferents evokes presynaptic inhibition of nociceptive afferents, whereas under pathological conditions, intensive

activation of A β -afferents (31) and C-afferents (19) could sensitize the interneurons in the spinal cord that mediate the presynaptic link between low-threshold mechanoreceptors and nociceptors (19). We therefore suggest that the altered expression of CGRP between DRG A- and B-cells influences the balance between non-nociceptive and nociceptive sensations.

In wild-type mice the selective immunoreactive CGRP A-cell loss in early STZ-induced diabetes observed in the present study can account for the reported impairment of the nociceptive response to mechanical stimuli (30), whereas rats treated with STZ develop mechanical hyperalgesia within 2 weeks (29). Different cutaneous immunoreactive CGRP innervation in diabetic rats and mice could account for this discrepancy of sensation. Cutaneous immunoreactive CGRP nerves are largely lost in diabetic mice (32), similar to findings in diabetic patients (33), whereas in STZ-induced diabetic rats, numbers of cutaneous immunoreactive CGRP fibers are increased (34).

The p75^{-/-} mice have half the number of DRG neuronal cells, increased thermal and mechanical thresholds for noxious stimuli, and depletion of immunoreactive CGRP and substance P fibers in the footpad skin (22,24,35). As reported previously, we observed that the relative number of total immunoreactive CGRP neurons in p75^{-/-} mice is similar to that in p75^{+/+} mice (24). However, we find that the relative number of immunoreactive CGRP A-cells is reduced in nondiabetic p75^{-/-} mice. This new finding may well correspond to the selective reduction of the relative number of A δ -mechano fibers shown electrophysiologically (36). Because A δ -mechano nociceptors are sensitive to NGF during development (37,38), and because >90% of immunoreactive trkA (high-affinity tyrosine kinase receptor for NGF) DRG neurons coexpress CGRP (39), it is reasonable to speculate that A δ -mechano neurons are CGRP immunoreactive. The selective loss of immunoreactive CGRP A-cells in nondiabetic p75^{-/-} mice could be responsible for the A δ -mechano fiber alterations, including the loss of heat sensitivity (36). P75^{NTR} deficiency leads to reduced sensitivity to NGF in sensory neurons during development (40,41) and, subsequently, could be responsible for the loss of A δ -mechano fibers and immunoreactive CGRP A-cells.

Diabetes had no effect on relative or absolute numbers of immunoreactive CGRP A-cells, nor did it have an effect on the A-cell-to-B-cell ratio of immunoreactive CGRP cells in p75^{-/-} mice. This observation might indicate that the p75^{NTR} is involved in the downregulation of the number of immunoreactive CGRP A-cells and in the A-cell-to-B-cell ratio of immunoreactive CGRP cells in diabetes in adult mice. However, the number of DRG cell bodies in the L5DRG is halved in p75^{-/-} mice, and the relative number of immunoreactive CGRP A-cells is also reduced. Therefore, conclusions on the role of the p75^{NTR} should be made with caution.

There was no influence of sciatic nerve crush on CGRP or substance P immunoreaction in either p75^{+/+} or p75^{-/-} mice after 4 weeks. Peripheral nerve axotomy leads to downregulation of substance P and CGRP in the DRG (42,43). At 2 weeks after axotomy, there is an overall decrease of CGRP immunoreaction in the DRG, but the A subpopulation of immunoreactive CGRP neurons and the

A fibers in the spinal cord are dramatically increased (44). Upregulation of large immunoreactive CGRP neurons has also been observed during inflammation (8). The ratio changes in those studies were suggested to play a role in the development of hyperalgesia during axotomy or inflammation. The different regulation of immunoreactive CGRP A-cells in STZ-induced diabetes and in inflammation or axotomy suggests different pain mechanisms between the different conditions. The lack of effect on neuropeptide expression after nerve crush in the present study might well be caused by early recovery of CGRP expression. In C57BL/6 mice, immunoreactive CGRP cells were transiently reduced at 7 days after sciatic nerve transection, but they recovered to normal at 28 days (45). The fast recovery of CGRP and substance P immunoreaction could be induced by the recovery of NGF support in DRG neurons. Axotomy dramatically reduces the retrograde transport of NGF (46), leading to decreased NGF protein content in the ipsilateral DRG as early as 6 h after spinal nerve ligation in rats (47). However, the NGF protein levels recover almost to normal after 1–2 days with an increased mRNA NGF in the DRG (47,48). The fast recovery of NGF levels in DRG may be caused by new synthesis of NGF from non-neuronal satellite cells in the DRG (47) and from Schwann cells in the peripheral nerves (49).

In diabetic p75^{+/+} mice, the immunoreactive substance P B-cell number decreased significantly after nerve crush. There was no significant reduction of the immunoreactive CGRP A- and B-cell number after nerve crush, but the relative number of immunoreactive CGRP B-cell increased, indicating that non-neuropeptide immunoreactive B-cells are preferentially lost. The lack of an additional effect of diabetes on changes in cell number and CGRP phenotype at 1 month after nerve crush is in accordance with previous electrophysiological studies showing that diabetes delays, but does not ultimately impede, peripheral nerve regeneration (50).

The low-affinity p75^{NTR} plays a role for both myelinated and nonmyelinated peripheral nerve fibers, especially for the development of thinly myelinated and unmyelinated fibers. The influence of p75^{NTR} on immunoreactive CGRP A-cells is unknown, but our findings suggest that it could be involved in the regulation of CGRP expression in neuronal DRG cells of the A subtype. The effect of p75^{NTR} on CGRP expression in A-cells could be biologically significant during development and in various pathological disorders such as diabetes in adults.

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