

Ciliary Neurotrophic Factor Improves Nerve Conduction and Ameliorates Regeneration Deficits in Diabetic Rats

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Ciliary neurotrophic factor (CNTF) protein and bioactivity are reduced in the peripheral nerve of hyperglycemic rats with a cause related to metabolism of hexose sugars by aldose reductase. Here the efficacy of CNTF treatment against disorders of nerve function in hyperglycemic rats was investigated. CNTF treatment from the onset of 8 weeks of galactose feeding prevented nerve conduction slowing in a dose-dependent manner. Streptozotocin-induced diabetic rats were maintained for 4 weeks before CNTF treatment was initiated. Four weeks of CNTF treatment significantly improved nerve conduction compared with untreated diabetic rats and also normalized the recovery of toe spread after sciatic nerve crush. One week of CNTF treatment significantly improved the distance of sensory nerve regeneration achieved after nerve crush injury compared with untreated diabetic rats. CNTF was without effects on any parameter in nondiabetic rats. Eight weeks of diabetes did not impair macrophage recruitment 1 and 7 days after nerve crush; neither did intraneural injections of CNTF and CNTFR α enhance recruitment in diabetic or control rats. These observations point to the potential utility of CNTF in treating nerve dysfunction in experimental diabetes. *Diabetes* 53:1807–1812, 2004

Ciliary neurotrophic factor (CNTF) supports the differentiation and/or survival of a variety of neurons during development (1 and references therein). The receptor for CNTF includes a glycosylphosphatidylinositol-linked α component, CNTFR α , present in nerve and muscle (2,3), and a transmembrane β component, the LIFR β /gp130 heterodimer, localized to nerve and macrophages (4,5). CNTF-like activity has been demonstrated by bioassay in adult hypoglossal, sural, and sciatic nerves (6) and is predominately immunolocalized to Schwann cells of larger myelinated fibers (7,8). A role for CNTF as a lesion factor is suggested by trauma-dependent release of the protein from Schwann cells (9), immunolocalization of bioactive peptide to myelin ovoids (8,9), increased retrograde axonal transport of CNTF after

nerve injury (10), and the injury-induced upregulation of CNTFR α in nerve (2) and its release by muscle (3). There is also accumulating evidence that CNTF may contribute to maintenance of uninjured adult neurons, including faint axonal immunostaining for CNTF (7), retrograde axonal transport of CNTF (10), and the ability of CNTF to induce neurofilament synthesis (11). The recent description of abnormal axonal structure in Cntf null ($^{-/-}$) mice (12) is further support for CNTF's role in establishing or maintaining the neuronal phenotype.

CNTF expression is reduced after 1–2 months of hyperglycemia (13) as a result of hyperglycemia-induced increased flux through aldose reductase, a polyol-forming enzyme present in Schwann cells of larger myelinated fibers (14). Blocking exaggerated flux through aldose reductase normalizes nerve CNTF protein and also ameliorates a range of structural and functional defects associated with experimental diabetic neuropathy (reviewed in 15). Given that Schwann cells can influence axonal properties (16), it is plausible that deficits in CNTF resulting from exaggerated polyol flux in Schwann cells may contribute to neuronal disorders characteristic of experimental diabetes. Therefore, the first objective of this study was to determine the efficacy of exogenous administration of CNTF in ameliorating functional and structural disorders of peripheral nerve found in experimental models of diabetic neuropathy. Our finding that CNTF treatment improved nerve regeneration in diabetic rats and previous studies implicating CNTF-mediated macrophage invasion of injured nerve as being essential for normal nerve regeneration (17–20) prompted subsequent studies that investigated the ability of CNTF to modulate macrophage invasion in injured nerves of control and diabetic rats.

RESEARCH DESIGN AND METHODS

These studies were conducted with the approval of the San Diego Veterans Administration Animal Studies Subcommittee. Adult female Sprague-Dawley rats (Harlan, San Diego, CA) were allowed free access to food and water and maintained under a 12-h light/dark cycle. For galactose intoxication, rats were provided with standard diet supplemented with D-galactose (40% wt/wt) and the control rats with standard diet containing 40% solka flock, a nonnutritive fiber. For insulin-deficient diabetes, rats were fasted overnight before receiving a single intraperitoneal injection (50 mg/kg in 0.9% sterile saline) of streptozotocin (STZ). Hyperglycemia (15 mmol/l or greater) was confirmed 2 days later by measurement of tail-vein blood glucose concentration (Ames Glucostix; Myles, Elkhart, IN).

CNTF was supplied by Regeneron Pharmaceuticals (Tarrytown, NY). An initial dose-efficacy study was performed in the galactose intoxication model of hyperglycemia. CNTF (0.25 or 1.0 mg/kg) or saline vehicle was delivered thrice weekly for 8 weeks by subcutaneous injection to control and galactose-fed rats from the onset of intoxication. In subsequent efficacy studies using the insulin-deficient model of hyperglycemia, STZ-induced diabetic rats were untreated for an initial 4 weeks before onset of treatment with saline or CNTF (1 mg/kg by subcutaneous injection thrice weekly). Treatment was continued

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CNTF, ciliary neurotrophic factor; MNCV, motor nerve conduction velocity; NCV, nerve conduction velocity; SNCV, sensory nerve conduction velocity; STZ, streptozotocin.

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for either 1 (sensory nerve regeneration studies) or 4 (nerve conduction and toe spread studies) weeks.

Nerve conduction velocity. Nerve conduction velocity (NCV) was measured under halothane anesthesia (4% for induction and 2% for maintenance) using evoked early (M) and late (H) waves, respectively (21). Late responses were routinely verified as H waves by their presence at stimulus intensities too low to elicit M or F waves. The distance between stimulation sites was divided by the median latency difference determined from three pairs of notch- and ankle-evoked M or H waves to calculate sciatic motor (MNCV) or sensory NCV (SNCV). **Axonal morphometry.** At the conclusion of the study, animals were anesthetized with an intraperitoneal injection (2 ml/kg) of pentobarbital (12.5 mg/ml) and diazepam (1.25 mg/ml) in 0.9% NaCl. Sciatic nerves were removed and fixed overnight by immersion in cold (4°C) 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer before postfixing in 1% aqueous osmium and subsequent processing to alradite resin blocks. Thick sections (1 μ m) were cut with glass knives and stained with *p*-phenylenediamine before computer-assisted analysis of axonal size-frequency distributions of myelinated fibers as described earlier (21). Nonoverlapping fields were sampled by systematic serpentine progression across the entire nerve fascicle, and axonal diameters were calculated by assuming that axonal areas were derived from circles of equivalent perimeter.

Nerve regeneration after crush injury. Nerve crushes were performed on halothane-anesthetized control and diabetic animals 4 weeks after induction of experimental diabetes as described in an earlier report (22). Nerve regeneration was assessed in two ways. Sensory nerve regeneration distance was measured 7 days after bilateral sciatic nerve crush in rats that were anesthetized with an intraperitoneal injection of the previously described pentobarbital cocktail. After exposure of both sciatic nerves, each nerve was stimulated (10 V, 0.05 ms pulse width; 58019 Square Wave Stimulator; Stoelting, Chicago, IL) with a bipolar electrode at a point 30 mm distal to the crush site and then proximally in 0.5-mm increments. The most distal point of sensory nerve regeneration was indicated when the stimulus resulted in contractions in the upper leg and lower back musculature via spinal reflexes (23). For each animal, regeneration distance was calculated as the average of the distance measured in the right and left nerves. Recovery of hind-foot toe spread after unilateral nerve crush, a functional index of nerve regeneration (24), was measured from prints made on paper after coating the plantar surface of both hind feet liberally with Betadine. The median distance between the first and fifth digits from three different prints was recorded from each animal just before nerve crush and at assorted time points up to 26 days after crush. Toe spread measurements for each animal were expressed as a ratio of precrush values.

Macrophage recruitment after nerve crush. After 8 weeks of untreated diabetes, control and diabetic rats were anesthetized with halothane and both sciatic nerves were exposed via a small incision below the sciatic notch. Each nerve was crushed as above, and an intraneural injection of 10 μ l of a solution that contained either 0.2 ng of CNTF and 0.2 ng of CNTFR α (R&D Systems, Minneapolis, MN) or 0.5 ng of CNTF and 0.5 ng of CNTFR α in PBS was made at the crush site of the left nerve using a 50- μ l glass syringe and 33-gauge needle (N733; Hamilton, Reno, NV). These amounts are within the range that elicited macrophage recruitment in a microchemotactic assay (25). In the right sciatic nerve, a 10- μ l intraneural injection of PBS served as a vehicle control.

For macrophage counts, sciatic nerves were removed from noninjured or from crush-injured animals 1 and 7 days after injury. After overnight fixation in 4% phosphate-buffered paraformaldehyde, segments that contained the crush site were processed to paraffin blocks and immunostained as described earlier (26) with ED-1 (0591; Serotec, Raleigh, NC), a monoclonal mouse anti-rat antibody marker for macrophages and monocytes. Immunostaining specificity was verified by omitting the ED-1 antibody. Sections were lightly counterstained using Vector Gill's Formula hematoxylin before macrophage recruitment was assessed by counting only immunostained profiles with a visible nucleus. Counts were normalized to either the epineurial or the endoneurial area, determined using point-counting techniques and a grid with a magnified distance of 0.08 mm between intersection points, to obtain macrophage density (macrophage number per mm²).

Statistical analysis. All experiments and data collection were conducted with animals and tissues coded to avoid the possibility of bias. Differences between groups were tested using two-way unpaired or paired *t* tests and one-way or two-way ANOVA as appropriate. When statistically significant differences were indicated ($P < 0.05$), multiple comparisons were made using Student-Newman-Keuls test (one-way ANOVA) or Bonferroni's test (two-way ANOVA). When SDs were significantly different, comparisons were made with the Mann-Whitney *U* test or Kruskal-Wallis nonparametric ANOVA followed by the method of Dunn.

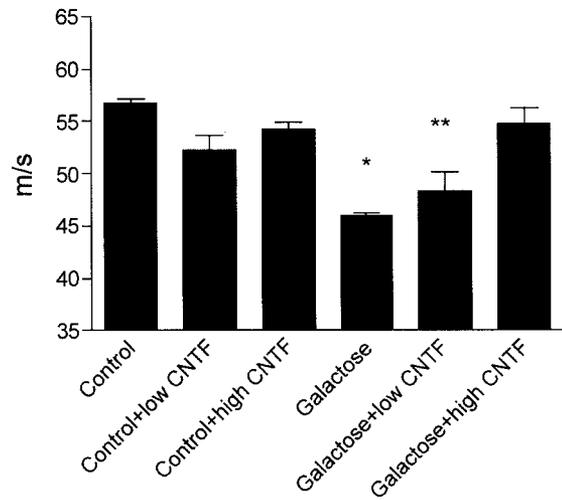


FIG. 1. Effects of CNTF on SNCV in control and galactose-fed rats. Data are presented as means \pm SE ($n = 7-8$ per group) and were analyzed with a one-way ANOVA, after which multiple comparisons were made with the Student-Newman-Keuls test. *Galactose versus control and both CNTF-treated galactose groups (at least $P < 0.01$); **galactose + low CNTF versus control and galactose + high CNTF groups ($P < 0.001$).

RESULTS

Galactose model of experimental diabetes. There was no effect of treatment with either 0.25 mg/kg (285 \pm 23 g; mean \pm SD) or 1.0 mg/kg (285 \pm 21 g) CNTF on body weight of control rats when compared with vehicle-treated control rats (299 \pm 18 g). Rats had a significantly lower body weight after 8 weeks of galactose feeding (272 \pm 13 g), and this was not altered by treatment with either 0.25 mg/kg (266 \pm 10 g) or 1.0 mg/kg (273 \pm 17 g) CNTF (all $P < 0.05$ vs. control groups). NCV of control rats was not altered by either dose of CNTF (Fig. 1). Galactose-fed rats showed a significantly (at least $P < 0.01$) reduced NCV compared with control and CNTF-treated galactose groups. CNTF prevented the nerve conduction deficit in a dose-dependent manner, with greater efficacy at the higher dose. **STZ model of experimental diabetes.** After 8 weeks of STZ-induced diabetes, rats had significantly lower body weights ($P < 0.0001$) and elevated plasma glucose levels ($P < 0.0001$) compared with control rats (Table 1). Treatment with 1 mg/kg CNTF for the last 4 weeks of diabetes did not alter body weight or plasma glucose levels in either control or diabetic rats. To determine the efficacy of CNTF treatment at reversing diabetes-induced functional deficits, we measured the conduction velocity of myelinated fibers. After 8 weeks of diabetes, both MNCV and SNCV were significantly (at least $P < 0.01$) lower than in controls (Table 1). Initiation of CNTF treatment after 4 weeks of untreated diabetes and continuation for 4 additional weeks partially protected both MNCV and SNCV such that values were significantly (at least $P < 0.05$) greater than untreated diabetic animals but significantly (at least $P < 0.01$) less than values of treated control groups. As with body weight and plasma glucose, CNTF treatment had no effect on nerve conduction in control animals.

Mean axonal diameter and the size-frequency distribution of myelinated fibers in the sciatic nerve were measured as structural correlates to nerve conduction. After 8 weeks of untreated diabetes, mean axonal diameter was

TABLE 1

Effects of CNTF on body weight, plasma glucose, NCV, axonal structure of myelinated fibers and sensory nerve regeneration distance in control and diabetic rats

	<i>n</i>	Control	Control + CNTF	Diabetic	Diabetic + CNTF	<i>P</i>
Body weight (g)	14–21	244 ± 5 ^a	238 ± 4 ^a	165 ± 4 ^b	166 ± 7 ^b	<0.0001 (a vs. b)
Plasma glucose (mmol/l)	14–21	3.2 ± 0.3 ^a	3.6 ± 0.7 ^a	28.2 ± 1.1 ^b	26.2 ± 1.0 ^b	<0.0001 (a vs. b)
MNCV (m/s)	8	58.2 ± 0.8 ^a	58.1 ± 0.9 ^a	45.2 ± 0.9 ^b	52.2 ± 1.3 ^c	<0.001 (a, c vs. b)
SNCV (m/s)	8	57.3 ± 1.8 ^a	58.4 ± 1.0 ^a	43.1 ± 1.3 ^b	49.3 ± 1.9 ^c	< 0.01 (a vs. b, c); < 0.05 (b vs. c)
Mean axonal diameter (μm)	6–8	5.80 ± 0.16 ^a	5.94 ± 0.18 ^a	5.11 ± 0.19 ^b	5.41 ± 0.17 ^c	< 0.05 (a vs. b); NS (a, b vs. c)
Fibers ≤4 μm (%)	6–8	11.9 ± 1.9 ^a	11.6 ± 2.3 ^a	27.4 ± 3.8 ^b	21.9 ± 3.2 ^b	< 0.05 (a vs. b)
Fibers ≥8 μm (%)	6–8	25.0 ± 3.7	25.5 ± 3.7	15.0 ± 3.5	19.3 ± 2.7	NS
Regeneration distance (mm)	5–8	20.7 ± 0.6 ^a	21.0 ± 0.6 ^a	18.7 ± 0.8 ^b	21.9 ± 0.7 ^a	< 0.05 (a vs. b)

Data are means ± SE and were analyzed by one-way ANOVA, after which multiple comparisons were made with the Student-Newman-Keuls test. Axonal size-frequency distributions were derived from 4,032 (control), 3,040 (control + CNTF), 3,094 (diabetic), and 5,082 (diabetic + CNTF) myelinated fibers. NS, not significant.

significantly ($P < 0.05$) lower than in control animals (Table 1). CNTF treatment partially ameliorated the decrease in diabetic animals such that mean axonal diameter in this group was not significantly different from that in either the control or diabetic animals. Diabetes was associated with a significant ($P < 0.05$) increase in the relative number of small ($\leq 4 \mu\text{m}$) fibers and a trend toward a decrease in the proportion of larger ($\geq 8 \mu\text{m}$) fibers (Table 1). CNTF treatment did not have a significant impact on the axonal size-frequency distribution of either the control or diabetic animals.

To investigate the impact of CNTF treatment on impaired nerve regeneration in experimental diabetes, we assessed sensory nerve regeneration distance and toe spread recovery after sciatic nerve crush. Sensory nerve regeneration distance 7 days after a crush injury was significantly ($P < 0.05$) less in untreated diabetic rats than in control rats (Table 1). The deficit in regeneration distance was completely prevented by treating diabetic animals with CNTF, whereas no impact of CNTF on sensory nerve regeneration distance in control animals was noted.

As a functional measure of regeneration after nerve crush, the spread of the first and fifth digits of the hind feet was assessed in untreated and CNTF-treated control and diabetic animals. Nerve crush in control rats induced a marked reduction in toe spread that recovered by day 24 (Fig. 2). A similar pattern was noted in control rats that were treated with CNTF for the period after nerve crush. Diabetic rats exhibited a delayed recovery from nerve crush, and toe spread was significantly ($P < 0.01$) lower at day 26 after crush than in control rats, whereas those that were treated with CNTF for the period after nerve crush injury had a toe spread that was not different from controls at this time point.

Macrophage recruitment after nerve crush in STZ diabetes. In the cohort of diabetic and control animals used to examine the impact of intraneural injections of CNTF and CNTFR α on macrophage recruitment after nerve crush, similar effects of diabetes on final body weight (214 ± 4 vs. 273 ± 3 g; $n = 17$ per group; $P < 0.0001$) and plasma glucose (44.1 ± 1.6 vs. 7.6 ± 0.6 mmol/l; $n = 12$ per group; $P < 0.0001$) were observed.

To explore a possible cause of the diabetes-induced reduction in sensory nerve regeneration, we assessed macrophage recruitment 1 and 7 days after crush injury with and without intraneural injections of this Schwann cell-derived neurotrophic factor and its α receptor component. Diabetes had no significant effects on macrophage recruitment into either the epineurium or the endoneurium (Fig. 3), although there was a nonsignificant trend for a reduction in endoneurial macrophages in diabetic animals 7 days after nerve crush. In the epineurium, there was a significant increase ($P < 0.01$) in macrophages 1 day after nerve injury compared with counts in uninjured nerve or 7 days after injury (Fig. 3). In contrast, endoneurial macrophage numbers were significantly increased ($P < 0.001$) by 7 days after crush compared with uninjured and 1 day postcrush counts.

Intraneural injections of CNTF/CNTFR α did not alter the overall pattern of epineurial or endoneurial macrophage recruitment after nerve crush, compared with similar injections of the vehicle PBS (Fig. 3; 0.5 ng of intraneural injection, data not shown). In the epineurium,

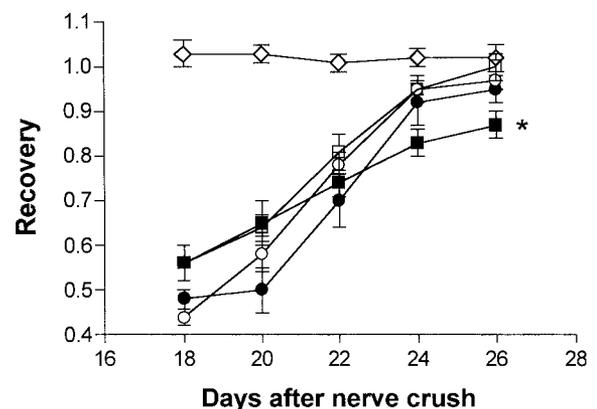


FIG. 2. Recovery of toe spread after crush injury in control (□), control+CNTF (○), diabetic (■), and diabetic+CNTF (●) rats. Toe spread recovery is expressed as a ratio of precrush values. Toe spread in the limb of control rats that did not undergo sciatic nerve crush is shown for comparison (◇). Data are presented as means ± SE ($n = 6-8$ per group) and were analyzed with a one-way ANOVA, after which multiple comparisons were made with the Student-Newman-Keuls test. *Untreated diabetic versus control groups ($P < 0.01$).

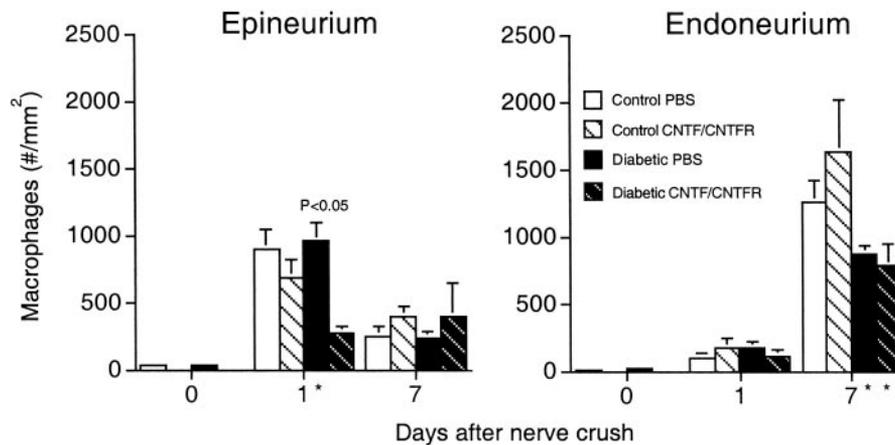


FIG. 3. Macrophage recruitment after nerve crush in control and diabetic rats and the effect of intraneural injections of CNTF (0.2 ng) and CNTFR α (0.2 ng). Data are presented as means \pm SE ($n = 3$ per group). The impact of time and diabetes on macrophage recruitment in PBS-injected nerves were analyzed with a two-way ANOVA, after which multiple comparisons were made with Bonferroni's test. In both the epineurium and the endoneurium, diabetes had no significant effect on macrophage recruitment. Compared with the other time points, macrophage density was significantly increased at 1 day (*) in the epineurium ($P < 0.01$) and 7 days (**) in the endoneurium ($P < 0.001$). The impact of intraneural injections of CNTF and CNTFR α at 1 and 7 days in control and diabetic animals was analyzed with a two-tailed, paired t test. No significant differences were apparent except in the epineurium, where macrophage density in diabetic animals was significantly decreased ($P < 0.05$) in nerves that received CNTF and CNTFR α 1 day after crush.

CNTF/CNTFR α injections were associated with a significant reduction ($P < 0.05$) in macrophage counts 1 day after nerve crush in diabetic but not control animals. In the endoneurium, there was no significant difference in macrophage recruitment when counts in CNTF/CNTFR α - and vehicle-injected nerves were compared.

DISCUSSION

CNTF treatment dose-dependently prevented NCV slowing in galactose-fed rats, a model of elevated hexose sugar metabolism by aldose reductase, and was without effect in control rats. We have previously shown that galactose intoxication markedly reduces nerve CNTF and that this depletion can be prevented by aldose reductase inhibition (13,14). As aldose reductase inhibitors also prevent NCV slowing in galactose-fed rats (14), it seems reasonable to suggest that nerve CNTF depletion is an intermediary in the causative sequence that leads from increased hexose sugar metabolism by aldose reductase in Schwann cells to NCV slowing. Providing exogenous CNTF may replace the deficient production of this factor by metabolically stressed Schwann cells, although the site of action of exogenous CNTF is not yet known and further studies are required to establish how CNTF maintains NCV.

Exogenous administration of CNTF also had a significant impact on nerve function in rats with already established STZ-induced diabetes, without having an effect in nondiabetic animals. Reports of involvement of CNTF in nerve function are limited to effects in nondiabetic animals and several in vitro experiments. Exogenous administration of CNTF increases NCV in immature rats, an effect not present in older, more mature animals (27). A diabetes-induced CNTF expression deficit (13) coupled with possible age-dependent expression differences in nondiabetic rats may explain why CNTF was effective only in the diabetic and not the mature, treated-control rats studied here. There are a number of possible ways by which CNTF may modulate nerve conduction. In vitro, CNTF supports survival of DRG neurons and maintains Na⁺/K⁺-ATPase

activity (28). A role for CNTF in the development or maintenance of peripheral nerve is also suggested by decreased axonal caliber, myelin sheath disruption, and loss of axon-Schwann cell networks at the node of Ranvier observed in *Cntf* null ($-/-$) mice (12). In the present study, the amelioration of nerve conduction deficits in rats with established diabetes was associated with a partial restoration of mean axonal caliber but not a significant shift in axonal size-frequency distribution toward larger myelinated fibers. The partial effect on diabetes-induced nerve defects may be the result of beginning CNTF treatment after 4 weeks of untreated diabetes and/or the duration of treatment. As seen with galactose-fed rats, a more robust effect on axonal caliber and conduction deficits might be expected with treatment initiated at the onset of diabetes or continued beyond 4 weeks.

As previously reported (29,30), there was a delay in sensory nerve regeneration 7 days after crush injury in untreated diabetic rats. Three subcutaneous CNTF injections over 7 days completely prevented the diabetes-induced decrease in regeneration distance without any effect in nondiabetic animals. In vivo, exogenous CNTF induces sprouting in undamaged adult motor neurons (31) and enhances anterograde transport in axons regenerating after nerve transection (32). Further studies are needed to determine whether the effect of CNTF treatment on sensory nerve regeneration distance in diabetic rats seen here might be related to overcoming diabetes-induced defects in sprouting (33) or anterograde axonal transport (34,35).

Toe spread recovery is a functional assessment of regeneration after nerve crush (24) and is dependent on both the rate of axonal regeneration and reestablishment of motor and sensory innervation of interosseal muscles. CNTF treatment restored toe spread in diabetic rats to normal, consistent with this neurotrophic factor's influence on the development and function of synapses (36) and the previously reported ability to enhance functional recovery after nerve transection (37). The lack of an effect of

CNTF in treated nondiabetic animals suggests that there is sufficient CNTF available to promote recovery in adult normal rats and that this cannot be enhanced further, at least with the dose and treatment regimen used here.

Macrophage recruitment to the site of injury is important for subsequent nerve regeneration (17–20,26). Diabetic rats have impaired nerve regeneration, and previous studies have shown impaired macrophage recruitment, albeit at a number of weeks after injury (38,39). In the present study, there was a trend toward fewer endoneurial macrophages in diabetic nerves 7 days after nerve crush, which might be the precursor to the deficit in macrophage recruitment seen at later time points by others. However, the pattern of macrophage recruitment in the days immediately following nerve crush was not different in control and diabetic rats, despite impaired nerve regeneration at this time, suggesting that deficient early macrophage recruitment is not responsible for delayed nerve regeneration in experimental diabetes.

In vitro, macrophage chemotaxis in response to CNTF and CNTFR α is concentration-dependent and receptor-mediated and involves the phosphoinositide-3 kinase and mitogen-activated protein kinase signaling proteins (25). However, intraneural injections of these peptides into the crush site immediately after nerve injury did not enhance early macrophage recruitment in either nondiabetic or diabetic animals. Unexpectedly, these peptides reduced epineurial macrophage recruitment in diabetic animals. The data obtained using this experimental approach are not consistent with a role for CNTF and CNTFR α in initiating the early recruitment of macrophages after nerve injury and do not provide support for a mechanism linking deficient expression of CNTF to decreased macrophage recruitment in experimental diabetes.

In summary, exogenous administration of CNTF to rats with galactose or STZ-induced hyperglycemia improved nerve conduction deficits that, in STZ-induced diabetes, may be partially dependent on restoration of axonal caliber. After crush injury, CNTF prevented diabetes-induced deficits in sensory nerve regeneration distance and promoted toe spread recovery in diabetic animals. Regeneration defects in diabetic rats were not associated with insufficient early recruitment of macrophages.

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