The structural effect of systemic NGF treatment on permanently axotomised dorsal root ganglion cells in adult rats

T. TANDRUP1,2, S. VESTERGAARD1, D. R. TOLMINSON3, L. T. DIEMEL3 AND J. JAKOBSEN4

1 Department of Neurology, University Hospital of Aarhus, and 2 Stereological Research Laboratory, University of Aarhus, Aarhus, Denmark and 3 Department of Pharmacology, Queen Mary and Westfield College, University of London, UK

(Accepted 1 December 1998)

ABSTRACT

The effect of systemic NGF treatment on loss and shrinkage of dorsal root ganglion cells was studied in adult male rats after permanent axotomy. Nineteen 16 to 18-wk-old rats had their right 5th lumbar spinal nerve ligated and cut approximately 7 mm peripheral to the ganglion. Two days before the operation, treatment with subcutaneous injections of human recombinant NGF (1.0–0.5 mg/kg/day) was started in 9 test rats; 10 controls were given saline injections. After 1 mo the levels of substance P (SP) and calcitonin gene related peptide (CGRP) were significantly increased in intact sciatic nerve. The number and mean volume of perikarya were estimated using assumption-free stereological techniques including vertical sections, the Cavalieri principle, optical disectors, the planar rotator and systematic sampling techniques. Systemic NGF administration had no influence on survival of primary sensory neurons after axotomy. The number of perikarya was 14300 (± 1800) in axotomised ganglia in control rats versus 14700 (± 2100) in axotomised ganglia of NGF treated rats. The reduction of perikaryal volume after axotomy was significantly less after NGF treatment (11600 µm³ in the control group versus 8000 µm³ in the NGF treated group). However, the apparent protection of NGF-treatment on perikaryal volume is explained by a hitherto unrecognised size effect on nonaxotomised dorsal root ganglion cells. The untreated rats had a mean volume of 24700 µm³ (± 2700 µm³) whereas rats treated with NGF had a volume of 20400 µm³ (± 1700 µm³) on the nonaxotomised side. In conclusion, systemic NGF treatment in adult rats has no effect on dorsal root ganglion cell loss in permanent axotomy whereas perikaryal size of intact nonaxotomised cells is reduced.

Key words: Axotomy; nerve transection; neuronal degeneration; neurotrophic factors.

INTRODUCTION

During the embryonic and early postnatal period, nerve growth factor (NGF) influences the survival of dorsal root ganglion cells (Johnson et al. 1980; Miyata et al. 1986; M. D. Gjerstad et al. unpublished observations). Also, it has been suggested that the NGF responsiveness is preserved in adult rats and that NGF plays a role in the maintenance of mature neurons (Longo et al. 1993). Changes of expression pattern and presentation of the NGF receptor occur after neuronal damage in adult rats (Verge et al. 1989a). Local application of NGF at the proximal stump of axotomised primary sensory neurons in adult rats prevents reduction in number and volume of the cell bodies (Otto et al. 1987; Rich et al. 1987). NGF therefore seems to have therapeutic potential in peripheral nerve disorders. In a previous study we have shown that there is a significant loss of dorsal root ganglion cells 2 wk after permanent axotomy in adult rats (Vestergaard et al. 1997). The purpose of the present study was to examine the effect of systemic NGF treatment in adult animals on cell loss and reduction of perikaryal volume of dorsal root gang-
lion cells after permanent axotomy with assumption-free stereological techniques.

**Materials and Methods**

During anaesthesia performed with a mixture of pentobarbital 50 mg/ml and diazepam 5 mg/ml 19 male Wistar rats aged 16–18 wk had their right 5th lumbar spinal nerve carefully exposed from the dorsal side, removing the crest of ilium and some of the transverse process. After localisation of the lumbar spinal nerves the L5 spinal nerve was ligated and cut approximately 7 mm distal to the ganglion. Two days before the operation, 9 of the rats had begun daily treatment with subcutaneous injection of 1 mg recombinant human NGF (Genentech Inc.) per kg. The dose was lowered to 0.5 mg NGF/kg/day on the 1st postoperative day and this treatment dose was continued throughout the study. The 10 remaining rats served as controls and received saline injections. One month after axotomy 2 1 cm pieces of the non-axotomised sciatic nerve were removed distal to the sciatic notch under deep anaesthesia (a mixture of pentobarbital 50 mg/ml and diazepam 5 mg/ml). Nerve segments were flash-frozen in liquid nitrogen and rats were killed by perfusion. The perfusion started with a prerinse with Tyrode’s buffer for 10 s followed by 10 min perfusion with 4% glutaraldehyde dissolved in a 0.08 M phosphate buffer at a pH of 7.40. Then the right and left 5th lumbar dorsal root ganglia were cut off from nerve and roots at a distance of 2–3 mm from the ganglion. Tissues were stored in the fixative.

The frozen nerve segments were transferred to boiling extraction buffer (2 M acetic acid, 10 mM HCl, 1 mM EDTA, 1 mM benzamidine, 2 µg/µl pepstatin A) for 10 min. Subsequently, the samples were homogenised and vortex mixed. An aliquot was removed from each sample by the Folin–Lowry method. The remainder of the homogenates were centrifuged (9000 g) for 10 min at 4 °C and the supernatants were freeze-dried overnight. Standard radioimmunoassays were used for determination of amount of substance P (SP) (Amersham) and calcitonin gene-related peptide (CGRP) (Peninsula Laboratories) (see Diemel et al. 1992).

To obtain vertical sections (Baddeley et al. 1986) with the vertical axis along the fibre direction (Tandrup, 1993) each ganglion was rotated randomly around its long axis and cut in half. Again the 2 ganglion parts were randomly rotated around their long axis and embedded in 7% agar. Tissues were dehydrated in ascending graded series of alcohol (2 h in 70%, 3 h in 96%, 2 h in 99%), embedded in glycolmethacrylate (Technovit, Kulzer, GmbH) and cut in 30 µm serial sections. For further study every 3rd section was sampled using a random starting point. Sections were stained with cresyl violet acetate.

The total number of neurons was the product of neuron density and ganglion volume (Tandrup, 1993). Neuron density was estimated using optical disectors (Gundersen, 1986; Braendgaard et al. 1989) obtained from systematic randomly sampled fields of vision. Counting of neurons with disectors and measurements of perikaryal volume was performed on a microscope computer apparatus (Olympus, Denmark). A video camera projected the image from the microscope to a computer screen with superimposed counting frames (CAST Grid, Olympus Denmark, Albertslund, Denmark). A microcator controlled movements of the focal plane in the vertical direction and a stepping motor moved the sections in the transverse direction for systematic random sampling of counting fields. As the plane of focus was moved 15 µm down through the section the numbers of neurons appearing within the counting frame (Gundersen, 1977) were counted, the counting unit being the nucleus. The disector was started 3–4 µm under the section surface. Cells present in the initial plan of the optical disector were not counted, whereas all cells in the final optical plan were included. A × 60 oil immersion lens (NA = 1.40, depth of focus ~ 0.5 µm) was used which gave a total magnification of ×1708. The counting frame for A cells was 3489 µm² and the for B cells 1745 µm².

The Cavalieri principle (Gundersen & Jensen, 1987) was applied for estimation of the ganglion volume, defined as the whole tissue block, using parallel sections separated by a known distance. The volume is the sum of the areas of all cross sections multiplied by 3 times the section thickness. The average section thickness was achieved from 4 measurements of every section focusing through the section using a 100 × oil lens (NA = 1.40, depth of focus ~ 0.5 µm) and the microcator. The tissue area of the sections was estimated by point-counting using a projection microscope. The total magnification was × 34 and the area associated with each point was 0.19 mm².

For estimation of mean perikaryal volume a representative sample of cells was sampled with the optical disectors. To avoid assumptions about shape, size and orientation, a combination of vertical sections (Baddeley et al. 1986) and the vertical planar rotator principle (Jensen & Gundersen, 1993) was applied. The planar rotator technique requires that each cell has a unique reference point and that cell perikarya
are clearly defined. The centre of the largest nucleolus was the unique reference point. In case 2 or more nucleoli appeared to be of equal size one was chosen at random. The vertical axis of the tissue was defined as the long axis of the halved ganglion. Measurements with the vertical planar rotator were performed in the focal plane of the unique reference. The computer was able to place a line grid random but perpendicular to the vertical axis over the profile. In order to hit cells by 3 lines the dimensions of the line grid were varied with the extension of the cell in the direction of the vertical axis. The distance from the intersection between a line and the cell border to the vertical axis running through the unique point was measured on both halves of the profile. For each line the computer calculated the average squared distance for each line in the grid. Perikaryal volume was then obtained by multiplying the sum of average squared intercepts with the distance between the lines in the line grid multiplied by $\pi$ (equation 4.1 in Jensen & Gundersen, 1993).

In normal dorsal root ganglia the cell population can be divided into 2 subtypes on light microscopy (Andres, 1961; Lieberman, 1976; Duce & Keen, 1977; Rambourg et al. 1983). In this study neurons were characterised as A cells if the nucleus of the cells was large and light, with a single large centrally placed nucleolus only. Cells characterised as B cells typically had a light nucleus with multiple smaller nucleoli often located at the periphery of the nucleus. The cytoplasm of the B cells generally appeared darker than the A cells often with an uneven distribution within the perikarya. The 3-D information obtained from the thick sections applied in this study was important for correct validation of cell type. Less than 2% of the neurons could not be classified as either A or B cells. Since several A and B cells of the axotomised ganglia appeared chromatolytic and cytoplasmic characteristics of the cells could not be used for separation. In chromatolytic and normal A cells the nucleus was large and light with only 1 large centrally placed nucleolus whereas in the chromatolytic and normal B cells the nucleus was light with multiple smaller nucleoli located at the periphery of the nucleus (Fig. 1). The cell population was therefore divided in subtypes using the characteristics of the nucleus.

Cut ends of all ganglia were observed for com-
pleteness of ganglion cells. All counting and volume measurements were carried out blindly. For statistical evaluation of the 2 groups of 9 and 10 animals, respectively, the 2-tailed paired t test was applied, using a 5% limit of significance.

**Results**

Animals given the NGF injections reacted aggressively to the injections compared with those receiving saline. Otherwise they did not show any behavioural changes. Three days after the axotomy there was a minor initial lack of increment of body weight of 10 g in the control group and 30 g (2P < 0.001) in the NGF treated group. Both groups gained weight during the study period, but a significant difference of body weight remained throughout the experiment (Table 1).

SP levels in intact sciatic nerve were significantly increased by 75% and CGRP by 266% in the NGF treated animals (Table 2).

In the ganglia chromatolysis occurred to a similar extent in treated and untreated animals. A as well as B cells showed signs of chromatolysis with reorganisation of the Nissl substance, and displacement of the nucleus to the periphery of the cytoplasm. No other qualitative morphological differences between NGF treated and untreated rats were apparent (Fig. 1).

There was a significant loss (2P < 0.05) of dorsal root ganglion cells of 12% (1890 neurons, s.E.M. = 690) on the axotomised side compared with the nonaxotomised side during the experimental period in control animals. In the NGF treated group the significant loss (2P < 0.05) of cells was 16% (2700 neurons, s.E.M. = 1090). Both A and B cells were lost (Table 3). Despite NGF treatment the axotomised ganglia in treated and untreated animals had an almost identical number of cells. The results for the A and B cell groups were also similar.

In the control group the perikaryal volume of the dorsal root ganglion cells was significantly diminished by 47% (11600 µm³, s.E.M. = 800 µm³) on the axotomised side compared with the nonaxotomised side. However, the reduction of perikaryal volume was significantly less (2P < 0.01) in the NGF treated group (Table 4). The reduction amounted to 8000 µm³ (s.E.M. = 600 µm³) or to 39% only. This effect of NGF on the perikaryal volume, however, can be explained by a significantly smaller (2P < 0.001) dorsal root ganglion cell size on the nonaxotomised side.
side in the NGF treated rats (Fig. 2). In NGF treated rats perikaryal size was 20 400 µm³ (s.d. = 1700 µm³) on the nonaxotomised side as compared with 24 700 µm³ (s.d. = 2700 µm³) in untreated rats. In axotomised ganglia the mean perikaryal volume of the various cell populations were similar in treated and untreated rats.

**DISCUSSION**

There are several reports on the therapeutic effect of NGF on rescue of damaged or degenerating neurons in adult animals (Kromer, 1987; Koliatsos et al. 1990). Two earlier studies have demonstrated that NGF treatment of primary sensory neurons after sciatic nerve axotomy prevents the reduction in number of the cell bodies of adult rats. Otto et al. (1987) and Rich et al. (1987) found no loss of neurons 4 and 6 wk after NGF treatment in axotomy, whereas cell body loss in the control groups was 34% and 16%, respectively. However, in a study on dorsal root ganglion cells in adult rats using stereological methods no cell loss could be detected within the first 8 wk after permanent axotomy of the sciatic nerve in the mid thigh region (Tandrup et al. unpublished results). The quantitative method used in the 2 earlier NGF studies estimates corrected numbers of neurons from counts of nucleolar profiles. Swelling or shrinkage of cell bodies due to the experiment can lead to changes in the estimated neuronal number. In fact, cell bodies exposed to axotomy in the present study had reduced size. In our study we have used techniques based on stereological principles where all cells have the same probability of being included in the estimate (Coggeshall, 1992; Tandrup, 1993). Furthermore, the results are independent of changes in shape, size and orientation of the cells. Different counting methods therefore can explain why our results are in conflict with the results of the previous studies.

The absence of effect of NGF in our study might be explained by the experimental conditions. We have used an axotomy model where the axons are cut closer to the ganglion. The reason was that a pilot study showed that loss of neurons in the ganglion could not be detected stereologically 1 mo after the axotomy at midthigh level. When the nerve is cut closer to the ganglion the cell loss is significant within this period (Vestergaard et al. 1997). Production of NGF in nonneuronal cells of nerve trunks increases proximal to an axotomy. The NGF made at this site is presumed to be captured by neurons and transported retrogradely to the cell bodies, where it stimulates events normally associated with regeneration (Heumann et al. 1987; Taniuchi et al. 1988). Although there probably still is a considerable number of NGF producing cells left in the 7 mm proximal nerve stump it is possible that the shorter proximal axon stump of the present axotomy model could have an influence on the capability of the neurons to respond to NGF treatment.

It is possible that the lack of effect of NGF on neuron numbers in our study is due to nonoptimal concentration in relevant cell compartments. The dose used is not very different from that applied in other studies (Yip et al. 1984) but still the concentration could be too low to pass the blood-nerve barrier, the exposure to NGF too short or the rats might have developed antibodies against NGF. However, the observed rise in SP and CGRP after NGF treatment is due to stimulation of transcription of SP and CGRP genes (Fernyhough et al. 1995). The administered NGF therefore has gained access to the dorsal root ganglion cells. Also, the half-life for NGF is 4.5 h in blood after subcutaneous administration.
(Tria et al. 1994) and further 3 h for the retrogradely transported NGF (Ure & Campenot, 1997).

In the previous studies NGF has been applied locally around the transected sciatic nerve in a chamber at midthigh level. This form of administration could be biologically meaningful but not feasible if NGF were to be used in the treatment of generalised neurological disorders. The systemic administration of NGF applied in this study could result in insufficient levels of NGF to maintain cell number after axotomy. However, systemic NGF treatment has been used in neonatal rats with remarkable effect on neuron survival (Yip et al. 1984; Miyata et al. 1986). Also, in the present study a remarkably large increase in SP and CGRP in intact NGF treated ganglion cells was observed, indicating sufficient access, at least via terminals and retrograde transport, to boost phenotype. However, axotomy causes ipsilateral down-regulation of NGF receptors, which may limit the capacity of affected neurons to respond. Even so, this study used a large dose of NGF.

In cultured adult dorsal root ganglion cells increased cell size during NGF treatment has been reported (Yasuda et al. 1990). Also, in vivo studies found enlargement of the perikarya (Yip et al. 1984; Rich et al. 1987). In the present study, however, NGF treatment resulted in shrinkage of perikarya of the nonaxotomised dorsal root ganglia. Again, these conflicting results could be explained by the difference in size estimators used. We have applied stereological methods where no assumptions are needed. To obtain reliable estimates of cell size it is important both to be able to make a representative sample of the cell population and to be able to measure cell volume of the sampled cells without making assumptions about the orientation and shape of the cells.

The mean perikaryal volume is reduced in axotomised dorsal root ganglion cells and the phenomenon is present before cell loss can be observed (Vestergaard et al. 1997; Tandrup et al. unpublished observations). In ganglia with cell loss it is difficult to evaluate whether cell dimensions are changed or selective cell loss has occurred.

High affinity NGF receptors have been found on approximately half of the cells in the dorsal root ganglion and it has been shown that these cells contains SP and CGRP (Verge et al. 1989b). Therefore, it is expected that the NGF responsive cells are mainly contained in the group of cells in the ganglia morphologically defined as B cells. However, the results of this study show no signs of differential effect of NGF between A and B cells.

The most surprising observation in this study is the lower mean perikaryal volume in ganglia from the nonaxotomised side in NGF treated animals. The phenomenon is present both in the A and the B cell population. From the number estimates it is known that no neurons are lost and, therefore, the change cannot be explained by a predominant loss of large neurons. The underlying mechanism might either be lack of increment in size or shrinkage of the perikarya or a combination of both. However, it is less likely that the magnitude of change can be explained by lack of increment alone. The influence of NGF on the peripheral sensory neurons is complex and unexplained. The reduction in cell size might represent a toxic effect of a large NGF dose but might also present a physiological effect of NGF. Thus NGF has been reported to induce apoptosis (Bredesen & Rabizadeh, 1997). The effect observed in normal dorsal root ganglion cells may be related to the apoptotic process including cell shrinkage and cell loss.

The rats receiving NGF in this study were more aggressive. It is well known that NGF results in hyperalgesia (Lewin et al. 1993) and the rats therefore probably use more energy than the controls which could explain the lower body weight in the NGF treated group. Even though hyperalgesia has been a not negligible side effect in human trials it has been proposed that neurotrophic factors have a therapeutic potential in treatment of neurodegenerative diseases. It has been suggested that NGF can prevent or delay the loss of neurons in Parkinson’s disease, Alzheimer’s disease and amyotrophic lateral sclerosis (Hefti & Weiner, 1986; Stewart & Appel, 1988; Phelps et al. 1989). Nevertheless, in the present study on adult animals NGF did not prevent perikaryal loss in dorsal root ganglia during permanent axotomy.

ACKNOWLEDGEMENT

Genentech Inc., California, is acknowledged for providing human recombinant NGF. Kirsten Kandborg and Nicola Havenhand provided excellent technical assistance.

REFERENCES


BRAENDGAARD H, EVANS CH, GUNDERSEN HJG (1989) The total number of neurons in the human neocortex unbiasedly


