J. Anat. (2000) **196**, pp. , 279–283, with 2 figures Printed in the United Kingdom

Neurotrophins and other growth factors in the regenerative milieu of proximal nerve stump tips

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(*Accepted 25 October 1999*)

ABSTRACT

Classic ideas on mechanisms for axon sprouting and nerve regeneration from peripheral nerves suggest that there is a prominent role for neurotrophin support. There has been comparatively less attention towards features of the regenerative process that develop from the proximal nerve trunk without the support of target tissues or the denervated trunk of a peripheral nerve. We studied early (2–14 d) expression of local growth factors in proximal nerve stump tips of transected sciatic nerves in rats. Immunohistochemical labelling was used to address specific deposition of BDNF, NGF, NT-3, bFGF, CNTF and IGF-1. We observed a unique localisation of BDNF, and to a much lesser extent, NGF in mast cells of injured nerve trunks but they were also observed in intact uninjured nerves. Macrophages did not express either BDNF or NGF. CNTF and IGF-1 were expressed in Schwann cells of intact nerves and stumps. We did not observe bFGF or NT-3 expression in any of the samples we studied. Mast cells may represent an important reservoir of BDNF in peripheral nerves.

Key words: Peripheral nerve; nerve regeneration; BDNF; NGF; Schwann cells.

INTRODUCTION

To regenerate after transection, peripheral nerves require axons to grow out from the intact proximal portions of the nerve trunk into the denervated distal trunk. If regeneration from the proximal stump of a transected peripheral nerve is prevented by removing the denervated distal nerve trunk, local cellular proliferation and axonal sprouting occurs despite the absence of a distal stump into which to grow. In this scenario, the proximal stump forms a bulbous mass of tissue elements known as a neuroma. Classic concepts of target or distal support for local or retrograde neuronal regenerative changes do not apply. What supports this activity? Local but transformed resident cells, or recruited infiltrating cells could potentially supply retrograde trophic support. The endoneurium of the proximal nerve stump could be exposed to blood borne trophic factors because of local break-down of the blood-nerve barrier. Work to date has concentrated on the possibility that trophic support from the distal stump is critical for regeneration, but similar trophic support at the distal part of the proximal stump, where sprouting first begins has had less attention.

In this work, we studied the distal tips of proximal stumps from transected rat sciatic nerve trunks to search for factors supporting regenerative events: NGF, BDNF, NT-3 and the nonneurotrophin growth factors CNTF, bFGF and IGF-1.

MATERIAL AND METHODS

Animal preparation

The studies used male Sprague-Dawley rats weighing 250–350 *g* housed in sawdust-floored plastic cages with access to rat chow and water ad libitum. Under pentobarbital anaesthesia (65 mg/kg i.p.), the sciatic nerves were exposed and sectioned with a scalpel using aseptic technique at a level approximately half way between the sciatic notch and trifurcation. For the experimental neuroma studies, the rats underwent resection of the portion of the sciatic nerve distal to the section and a further distal 2–3 cm of its branches. After reapposition of the muscles and skin, the rats

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were allowed to recover and were also given Demerol (1.0 mg/kg every 12 h) after awakening for the first 24–48 h after surgery. In an additional experiment (n = 3) the nerve was sectioned only without removing the distal stump or branches. At selected time points after creation of the lesion (48 h, 5 d, 7 d or 14 d; n = 3 for each time point), the rat was killed with high dose pentobarbital and the sciatic nerve stump proximal to the transection site was taken for immunohistochemical studies. All observations were concentrated on transverse sections of this zone of the nerve, just proximal to the 'outgrowth' zone. The whole proximal stump was removed and at its distal end, serial sections progressively proximal, were taken and examined. Studies were carried out on sections just proximal to the 'outgrowth' zone consisting of disorganised connective tissue only ($\sim$ 1 mm deep), at the stump tip proper recognised by the presence of a disorganised perineurial layer and the presence of myelinated fibres. Sections more proximal to this zone (another 1–2 mm deep) resembled intact nerve and were not the specific subject of this study. Control preparations were obtained by using littermates that underwent exposure of the sciatic nerve without injury or manipulation, then wound closure, recovery as above and later nerve removal for immunohistochemistry at identical time points to those described above. The protocol was reviewed and approved by the University of Calgary Animal Care Committee.

Immunohistochemistry

Tissue samples were fixed in modified Zamboni's fixative (2% paraformaldehyde, 0.5% picric acid, and 0.2 M phosphate buffer) overnight at 5 °C. Tissues were then washed in phosphate buffered saline (PBS), followed dimethyl sulphoxide, and again with PBS. Tissues were covered with PBS and 20% sucrose, left at 5 °C overnight, and were then embedded in optimum cutting temperature (OCT) compound (Miles), frozen, and sectioned at 16 μm. Sections were placed onto poly-D-lysine coated slides permeabilised with 0.3% Triton X100 in PBS for 2 h and then incubated for 48 h at 4 °C with the following primary

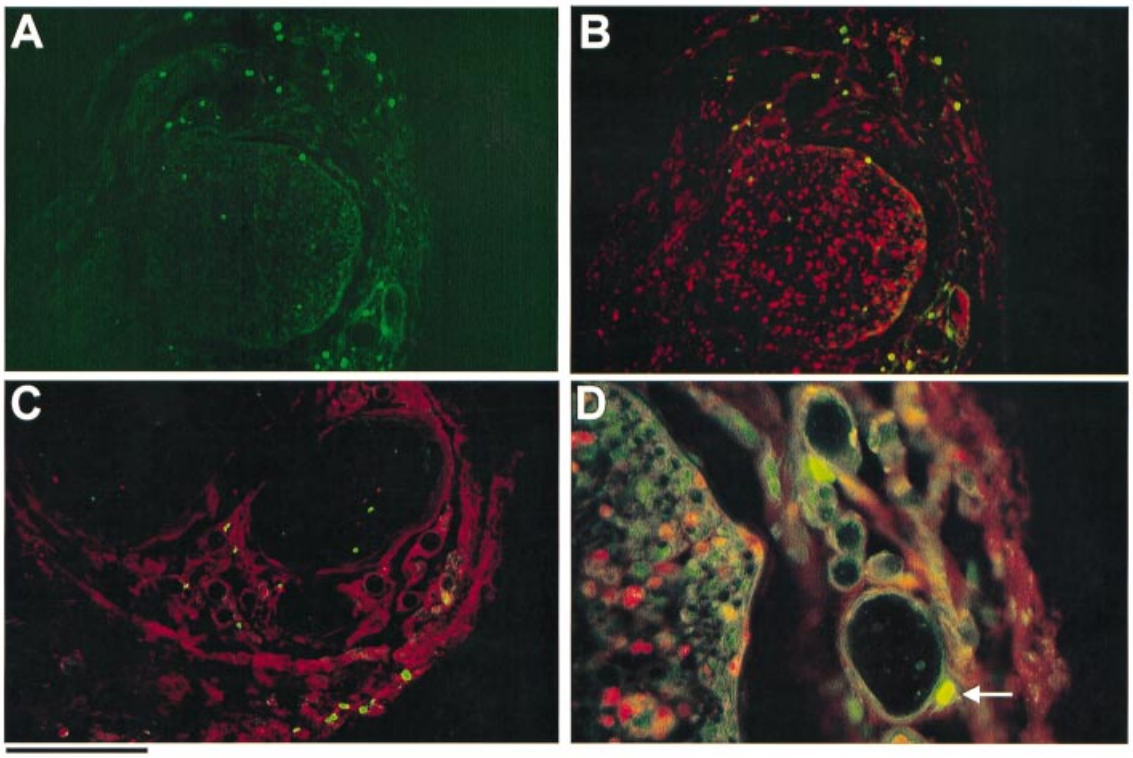

Fig. 1. Immunohistochemical preparations of 14 d proximal nerve stump transverse sections. (*A*) Labelling of cells with an antibody to BDNF and secondary labelling with FITC (green immunofluorescence). Discrete BDNF positive profiles are noted both in the endoneurium and particularly in the epineural connective tissue. The distribution is as expected for mast cells, and the cells colabelled with an antibody to serotonin (see Fig. 2). (*B*) Identical section as (*C*) double labelled with (1) BDNF as above and (2) ED-1 to label macrophages with secondary labelling with CY3 (red immunofluorescence). The red profiles are macrophages that are distinct from, and do not colabel with the green BDNF profiles. (*C*) Sham noninjured nerve at 14 d double labelled as in (*B*) that has BDNF positive profiles (green), as in *B*, but does not contain ED-1 labelled macrophages. (*D*) Higher power magnification as in (*B*), illustrating separate immunoreactivity for BDNF (green immunofluorescence; arrow), probably in mast cells, and red ED-1 labelled macrophage profiles in the endoneurium. Bars, 400 μm (*A–C*), 100 μm (*D*).

antibodies: mouse monoclonal antineurofilament 200 (1:800; Sigma, St Louis, Mo) to label axons, rabbit polyclonal anticow glial fibrillary acidic protein (1:1000, Dako Diagnostics, Canada), to label non-myelinating and proliferating Schwann cells, monoclonal mouse antiserotonin (1:5; Dako Diagnostics, Canada) to label mast cells, rabbit antiserotonin polyclonal antibody (1:200; Incstar Corporation, Stillwater, Mn) to label mast cells, rabbit antinerve growth factor (NGF) beta polyclonal antibody (1:500; Chemicon International), rabbit anti-brain derived neurotrophic factor (BDNF) polyclonal antibody (1:500; Chemicon International), rabbit anti-neurotrophin 3 (NT-3) polyclonal antibody (1:1000 and 1:500; Chemicon International), mouse monoclonal anti-ciliary neurotrophic factor (CNTF) monoclonal antibody (1:20; Chemicon International), mouse monoclonal anti-basic FGF antibody (1:50, 1:100; Transduction Laboratories), mouse anti-human insulin-like growth factor 1 [IGF-1] (1:400; Research Diagnostics); mouse monoclonal anti-S-100 (1:100; Chemicon International); mouse monoclonal anti-ED-1 (1:1000; Chemicon International). Slides were then washed with PBS and incubated with fluorescein isothiocyanate-conjugated goat and rabbit immunoglobulin G (Incstar, Stillwater, Mn) diluted 1:50 or sheep antimouse IgG CY3 conjugate (1:100; Sigma, St Louis, Mo) (or both secondary antibodies for double labelling) for 1 h at room temperature. After further PBS washing, coverslips were mounted with bicarbonate-buffered glycerol (pH 8.6) and viewed with a fluorescent microscope (Zeiss Axioplan). The following control procedures were applied to all staining: (1) tissue sections of proximal nerve stumps or neuromas underwent the immunohistochemical protocol but with omission of the primary antibody (to exclude possible nonspecific immunofluorescent staining); (2) sections from proximal stumps were routinely lined up on the same slide as sham-exposed intact nerves so that antibody labelling of experimental and control sections was carried out under identical conditions. Each slide contained 4–5 stump and 4–5 control sections (n = 3 rats for each primary antibody); (3) dilutions of primary antibodies were

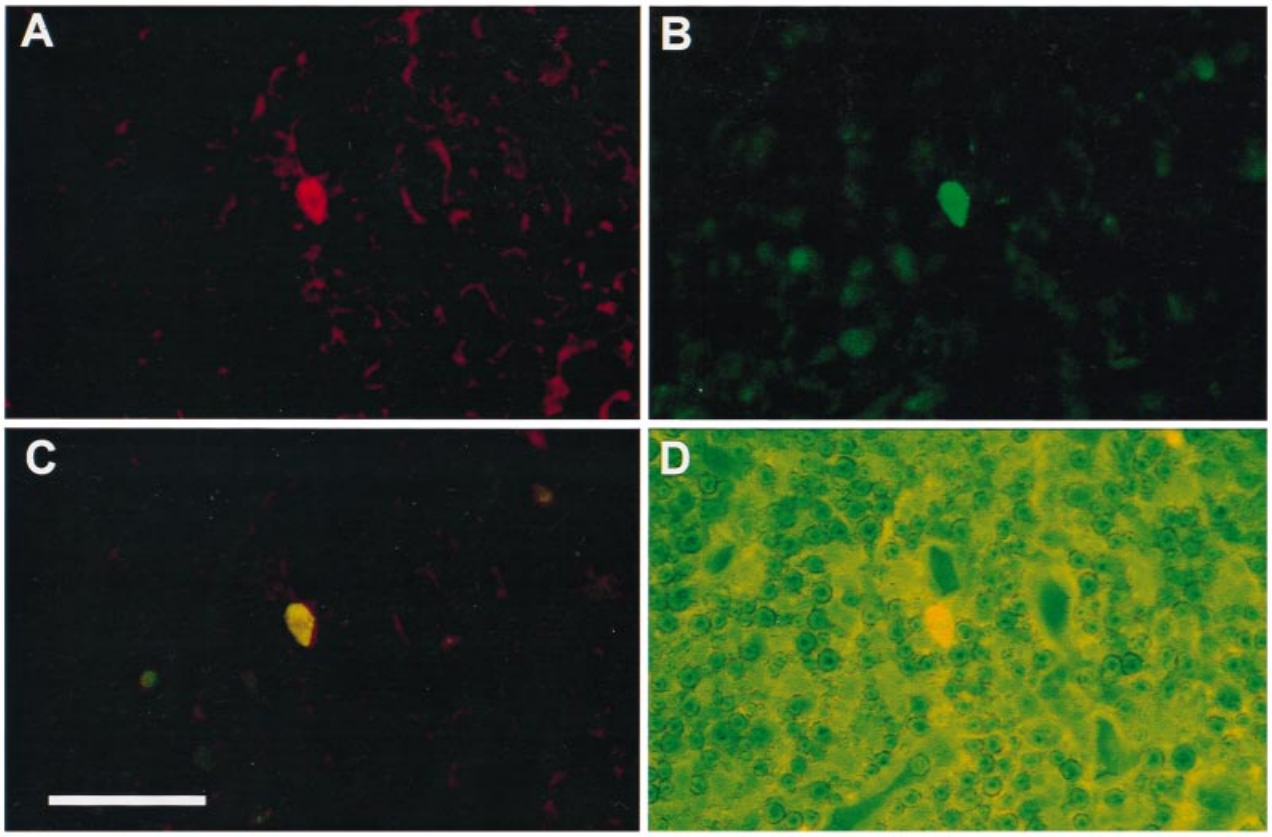

Fig. 2. Immunohistochemical preparation of a 48 h proximal nerve stump transverse section. A perivascular cell is double labelled with: (1) a primary antibody to serotonin to identify it as a mast cell secondarily labelled with CY3 conjugated antibody (red immunofluorescence); (2) a primary antibody to BDNF secondarily labelled with FITC conjugated antibody (green immunofluorescence). The upper left photomicrograph (*A*) identifies the cell as containing serotonin. On the upper right (*B*), green immunofluorescence indicates the presence of BDNF. The lower photomicrographs (*C*, *D*) indicate the double labelling. The cell is seen in relationship to a vessel and myelinated fibres (*D*). Bar, 100 μm.

varied to provide optimum staining to visualise areas of interest; (4) an additional control replaced the primary antibody with normal rabbit serum. Extra shortpass filtering (Zeiss SP560) was used to eliminate 'bleed through' of CY3 fluorescence when FITC labelled structures were studied. The specificity of the NGF antibody was confirmed by its staining of NGF in mouse submaxillary salivary glands. Similar BDNF staining was not observed, rendering crossreactivity between NGF and BDNF unlikely. Finally, to further evaluate the specificity of staining for BDNF, we added 0.10 g/l of a blocking peptide corresponding to amino acids 128–147 at the carboxy terminus of mature human (identical to mouse sequences) BDNF (Santa Cruz Biotechnology, cat. no. sc-546 P; Santa Cruz, Ca) to the primary antibody. Staining for BDNF was eliminated. The peptide had no effect on staining with other primary antibodies.

RESULTS

The milieu of the proximal stump tip we examined was disorganised and had features suggestive of local proliferative activity and axonal sprouting by 7 d. While not quantitated in this work, the evidence for this was the presence of disorganised axons (Nf 200 labelled), frequently in the perineurium, and prominent GFAP labelling of Schwann cells, indicating an apparent proliferating phenotype (Jessen et al. 1990; Dubovy & Svizenska 1992; Thomson et al. 1993) again involving perineurium. While occasional resident macrophages (ED-1 labelled) were present in the endoneurium of intact nerves or earlier stump tips, prominent invasion was present by 5 d, then particularly 7 d. We next examined whether trophic factor labelling coincided with any of the above events.

BDNF immunostaining was observed in discrete endoneurial and epineural sites in all preparations studied, whether intact nerve or stump. The location of the profiles, the presence of identified granules within them, and their colabelling with serotonin identified them as mast cells (Figs 1, 2). BDNF did not colabel with the macrophage marker ED-1 (Fig. 1). A similar pattern of staining to BDNF was observed with NGF, but the labelled profiles were less intense and far fewer in number. NGF labelling was also seen in both stumps and intact nerves. The NGF positive profiles did not colocalise with the ED-1 marker for macrophages. Unlike BDNF or NGF, there was no staining in any of the preparations studied for NT-3. CNTF antibody labelled Schwann cells in intact sham-exposed uninjured nerves, and proximal nerve stumps from all timepoints. Staining was similar in the stump tip preparations, irrespective of the time point, compared to sham-exposed control nerves. In stumps distal to the nerve section, CNTF labelling of Schwann cells disappeared. IGF-1 labelling was present on Schwann cells but was far less intense than that of CNTF. The staining intensity did not appear to differ among the different time points tested with intact or sham exposed nerves. No labelling with anti bFGF was found in any of the sections studied. In summary, labelling of the growth factors studied did not appear to coincide with other cellular changes at the stump tip or with macrophage invasion.

DISCUSSION

In this work we (1) showed that proximal nerve stump tips following nerve transection expressed BDNF, and to a much lesser extent NGF, in mast cells, but not macrophages or Schwann cells. This labelling was also seen in intact uninjured nerves and did not coincide with cellular changes of the stump tip; (2) showed that CNTF and IGF-1 were expressed in Schwann cells of proximal nerve stumps and in control intact nerves; CNTF expression was absent in distal denervated stumps; (3) did not observe expression of NT-3 or bFGF.

Our studies used immunohistochemistry to provide a reflection of protein expression. In our sections there were only very low levels of NGF protein in mast cells, in agreement with the studies of Heumann et al. (1987*b*) despite findings that similar stumps express relatively high levels of NGF mRNA. Rapid capture and retrograde transport of NGF by axonal stumps has been postulated to explain this interesting discrepancy. The potential pitfalls of our approach were that very low level expression of NT-3 and bFGF may not have been detected, although it might be questioned how relevant such limited expression of growth factors might be. We did not identify an overlap in the staining of NGF, BDNF and NT-3 despite their structural similarities, an important negative control. Our NGF antibody, but not the BDNF antibody appropriately stained mouse submaxillary glands, and the IGF-1 antibody and CNTF antibodies had expected staining of Schwann cells, as described in previous studies. Finally, BDNF staining of mast cells was appropriately blocked by inclusion of a BDNF antigenic peptide.

Mast cell localisation of NGF has been previously described by Leon et al. (1994). While not counted in this study, mast cell numbers do increase in proximal stumps similar to those studied here (Zochodne et al. 1994). NGF synthesis rises in the distal stump of

transected peripheral nerves, but probably from Schwann cells and not mast cells and apparently stimulated by interleukin-1 (Heumann et al. 1987*a*; Lindholm et al. 1987; Anand et al. 1997). Rises in NGF in neuromas were reported in some 'pockets' of human neuromas, but these were at much later time points than we observed (Anand et al. 1997).

Despite its presence, we consider that BDNF is unlikely to act as a primary proliferative signal for axons in neuromas since there was similar expression in mast cells of intact nerve. Increased BDNF signalling following injury through enhanced expression of TrkB or the low affinity neurotrophin receptor, P75, on nonneuronal cell types remains a possible stimulus for axon sprouting (Johnson et al. 1988; Stoll et al. 1993) that we did not examine. Meyer et al. (1992) reported that BDNF levels rose dramatically in the rat nerve trunk distal to a section starting at postlesion d 3 and reaching a maximum by wk 3–4. BDNF elaboration was argued to arise from Schwann cells because they express BDNF in culture (Meyer et al. 1992). Proximal nerve stump expression of BDNF mRNA, however, did not appear prominent (Meyer et al. 1992). CNTF ranks as a possible growth signal from Schwann cells in proximal nerve trunks (Dobrea et al. 1992) but we did not observe alterations in its expression, except distal to a nerve transection, where immunoreactivity disappeared. We did not detect expression of bFGF or NT-3. Macrophages might elaborate alternative proliferative signals we did not examine in proximal stumps (Hall, 1989; Lu & Richardson, 1991). Finally, several other nonneurotrophin growth factors such as GDNF, aFGF cardiotrophin, EGF and others require study in what we believe is a relevant and active 'regenerative milieu'.

ACKNOWLEDGEMENTS

Brenda Boake provided expert secretarial assistance. The work was supported by the Muscular Dystrophy Association of Canada. DWZ is a Medical Scholar of the Alberta Heritage Foundation for Medical Research.

REFERENCES

ANAND P, TERENGHI G, BIRCH R, WELLMER A, CEDARBAUM JM, LINDSAY RM et al. (1997) Endogenous NGF and CNTF levels in human peripheral nerve injury. *NeuroReport* **8**, 1935–1938.

DOBREA GM, UNNERSTALL JR, RAO MS (1992) The expression of CNTF message and immunoreactivity in the central and peripheral nervous system of the rat. *Brain Research* **66**, 209–219.

DUBOVY P, SVIZENSKA I (1992) Migration of Schwann cells from the distal stump of the sciatic nerve 1 week after transection: the effects of insulin and cytosine arabinoside. *Glia* **6**, 281–288.

HALL SM (1989) Regeneration in the peripheral nervous system. *Neuropathology & Applied Neurobiology* **15**, 513–529.

HEUMANN R, LINDHOLM D, BANDTLOW C, MEYER M, RADEKE MJ, MISKO TP et al. (1987*a*) Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: role of macrophages. *Proceedings of the National Academy of Sciences of the USA* **84**, 8735–8739.

HEUMANN R, KORSCHING S, BANDTLOW C, THOENEN H (1987*b*) Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection. *Journal of Cell Biology* **104**, 1623–1631.

JESSEN KR, MORGAN L, STEWART HJ, MIRSKY R (1990) Three markers of adult non-myelin-forming Schwann cells, 217c(Ran-1), A5E3 and GFAP: development and regulation by neuron-Schwann cell interactions. *Development* **109**, 91–103.

JOHNSON EMJR, TANIUCHI M, DISTEFANO PS (1988) Expression and possible function of nerve growth factor receptors on Schwann cells. *Trends in Neurosciences* **11**, 299–304.

LEON A, BURIANI A, DAL TOSO R, FABRIS M, ROMANELLO S, ALOE L et al. (1994) Mast cells synthesize, store, and release nerve growth factor. *Proceedings of the National Academy Sciences of the USA* **91**, 3739–3743.

LINDHOLM D, HEUMANN R, MEYER M, THOENEN H (1987) Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve. *Nature* **330**, 658–659.

LU X, RICHARDSON PM (1991) Inflammation near the nerve cell body enhances axonal regeneration. *Journal of Neuroscience* **11**, 972–978.

MEYER M, MATSUOKA I, WETMORE C, OLSON L, THOENEN H (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. *Journal of Cell Biology* **119**, 45–54.

STOLL G, LI CY, TRAPP BD, GRIFFIN JW (1993) Expression of NGF-receptors during immune-mediated and lysolecithin-induced demyelination of the peripheral nervous system. *Journal of Neurocytology* **22**, 1022–1029.

THOMSON CE, GRIFFITHS IR, McCULLOCH MC, KYRIAKIDES E, BARRIE JA, MONTAGUE P (1993) In vitro studies of axonally-regulated Schwann cell genes during Wallerian degeneration. *Journal of Neurocytology* **22**, 590–602.

ZOCHODNE DW, NGUYEN C, SHARKEY KA (1994) Accumulation and degranulation of mast cells in experimental neuromas. *Neuroscience Letters* **182**, 3–6.