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M. Ockel, D. Von Schack, A. Schropel, G. Dechant, G. R. Lewin and Y.-A. Barde

Phil. Trans. R. Soc. Lond. B 1996 **351**, 383-387
doi: 10.1098/rstb.1996.0032

Roles of neurotrophin-3 during early development of the peripheral nervous system

M. OCKEL, D. VON SCHACK, A. SCHRÖPEL, G. DECHANT, G. R. LEWIN AND Y.-A. BARDE

Max-Planck Institute for Psychiatry, Department of Neurobiochemistry, 82152 Planegg-Martinsried, Germany

SUMMARY

The neurotrophins are structurally related proteins regulating cell numbers in the developing vertebrate nervous system. They are necessary survival factors preventing the death of specific neuronal populations. Previous experiments have indicated that the administration of nerve growth factor or of brain-derived neurotrophic factor during the formation of sensory ganglia and of target innervation increases the number of neurons by preventing normally occurring neuronal death. These results support the view that during development, neuronal numbers are adjusted to the size of the target tissue by the release of limiting amounts of neurotrophins. However, increasing the levels of neurotrophin-3 during the formation of sensory ganglia results in a marked decrease in neuronal numbers, possibly as a consequence of premature cessation of sensory neuroblast proliferation. In sympathetic ganglia, the application of neurotrophin-3 during the formation of the sympathetic chain causes cell numbers to increase, a result also observed following the application of nerve growth factor. It thus appears that neurotrophin-3 and nerve growth factor can regulate cell numbers well before the period of target-derived control, and that neurotrophin-3 affects neuronal numbers in sensory and sympathetic ganglia in opposite ways.

1. INTRODUCTION

Neurotrophins are best known for their ability to prevent the death of a variety of embryonic neurons. Their remarkable specificity has been most clearly documented with neurons of the peripheral nervous system (PNS). Thus in the dorsal root ganglia (DRG), neuronal sub-populations could be distinguished on the basis of their survival requirements for either nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) (Barde *et al*. 1982). In addition, the death of cultured sympathetic neurons could be prevented by the addition of NGF (Levi-Montalcini & Angeletti 1963), but not of BDNF (Barde *et al*. 1982).

Whereas *in vitro* studies also played an essential role in the identification and characterization of both NGF and BDNF (Levi-Montalcini *et al*. 1954; Cohen 1960; Barde *et al*. 1982), experiments with cultured neurons can be misleading, and it has not always been possible to demonstrate that a factor-induced survival of PNS neurons *in vitro* accurately predicts prevention of neuronal death *in vivo*. For example, when applied *in vivo*, neither fibroblast growth factor 1 and 2, nor ciliary neurotrophic factor prevent normally occurring neuronal death in the variety of peripheral ganglia that have been examined (Oppenheim *et al*. 1991,1992; see also below a further example with neurotrophin-3). This has not been the case with NGF and BDNF. For reasons that might be related to the Spartan tissue culture conditions used for the characterization of both NGF (fibre outgrowth in a plasma clot) and BDNF (neuronal survival on a polyornithine, laminin-free substrate), it seems that only robust activities survived the purification procedure from the complex tissue sources that were used. Following their purification, both NGF and BDNF could be subsequently shown *in vivo* to selectively prevent neuronal death in peripheral sensory (Hamburger *et al*. 1981; Hofer & Barde 1988) or sympathetic ganglia (Oppenheim *et al*. 1982). The specificity predicted from *in vitro* experiments (Lindsay *et al*. 1985) could be confirmed *in vivo* in that placode-derived neurons of the nodose ganglion are rescued by BDNF, but not by NGF (Hofer & Barde 1988). NGF, but not BDNF, prevents cell death in the sympathetic chain (Oppenheim *et al*. 1982). These results have also been useful in supporting the idea that neurons are eliminated during normal development because of limiting amounts of neurotrophins.

Two additional neurotrophins have been identified in mammals, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Unlike NGF or BDNF, their identification did not make use of their biological activity. But interestingly, both were also found to be active on sensory neurons. Whereas unique targets have been difficult to identify for NT-4/5 by using cultured peripheral neurons, *in vivo* studies revealed that neuronal losses in some visceral ganglia appear to be unique to animals lacking the NT-4/5 gene, compared with BDNF -/- animals (Conover *et al*. 1995; Liu *et al*. 1995). In the DRG, NT-3 has been shown to have a striking specificity for muscle afferents (Ernfors *et al*. 1994; Fariñas *et al*. 1994).

Still very little is known about *in vivo* effects of elevated levels of NT-3 during early development, and we report here on the results of such experiments. They were prompted by an unexpected observation resulting

Phil. Trans. R. Soc. Lond. B (1996) **351**, 383–387
Printed in Great Britain

from the neutralization of endogenous NT-3 by using a monoclonal antibody (Gaese *et al.* 1994). Cell counts in the nodose and dorsal root ganglia revealed that over 30% of the neurons were lost in these ganglia already at the end of gangliogenesis. This early decrease was not seen with NGF-antibodies, and when examined several days later after the period of cell death, the DRG of embryos treated with a combination of antibodies to both NGF and NT-3 showed effects that were less than additive, suggesting that some NGF-requiring neurons first depend on NT-3 (Gaese *et al.* 1994).

2. NT-3 AND SENSORY NEURONS

In previous experiments with NGF or BDNF, the daily application of 1 µg or 5 µg (with quail or chick embryos, respectively) was found to be necessary to prevent neuronal death in peripheral ganglia (Oppenheim *et al.* 1982; Hofer & Barde 1988). When neuronal numbers were assessed after the period of normally occurring cell death, substantial increases in cell numbers were observed in the corresponding peripheral ganglia. Treatment of chick embryos with NT-3 (5 µg daily between E3 and E6) led to marked decreases in neuronal numbers in both nodose and dorsal root ganglia when analysed after the period of normally occurring cell death (figure 1, see also Ockel *et al.* 1996). These effects were only observed when NT-3 was applied during the period of cell proliferation in the sensory ganglia (D'Amico-Martel 1982), and not when the same treatment was applied during E6 and E9 (figure 1). Also, the reduction in cell numbers seems to be specific for NT-3 in that identical, early treatments with BDNF did not decrease neuronal numbers (Ockel *et al.* 1996). Whereas in these experiments, cell counts were done after the period of normally occurring cell death, the nodose ganglia were already reduced in size at E7 when compared with ganglia of control animals. It thus appears that NT-3 exerts its effects when cell division is still observed in sensory ganglia, before the target-related cell death phase. The possibility that NT-3 affects cell proliferation during gangliogenesis was tested. Using immunostaining for PCNA (proliferating cell nuclear antigen, a DNA polymerase δ-associated protein), the percentage of dividing cells was determined on sections of nodose ganglia at E4.5, and was found to be reduced by about half.

The reduction in cell numbers did not seem to selectively affect any particular sub-population of sensory neurons. Size-frequency analysis done at E11 showed a normal cell size distribution of the remaining neuronal populations, and tracing experiments using the dye DiI revealed no changes in the projection patterns in the spinal cord of the surviving neurons (Ockel *et al.* 1996). This implies that the neurons remaining after the short initial treatment with 5 µg NT-3 have the potential to innervate the normal range of spinal cord laminae. Either the treatment has equal effects on different sensory precursor populations or it is affecting a pool of uncommitted sensory precursors. In contrast, recent experiments with lower doses, but longer term NT-3-treatment resulted in changed projection patterns, especially affecting projections to laminae II (Eide *et al.* 1994; G. Lewin, unpublished data). In the rat, it has also been shown that the application of NT-3 starting at E14 does affect the projections of IA afferents in the spinal cord (Zhang *et al.* 1994*b*).

When NT-3 was applied between E6 and E9, a time interval during which the net number of neurons decreases in the avian nodose and dorsal root ganglia (Hofer & Barde 1988), an increase of about 30% in cell survival was observed in the DRG. This presumably results from the prevention of normally occurring cell death, like previously observed with NGF and BDNF (see §1). However, no change in neuronal numbers could be observed in the number of nodose neurons. This result is surprising in view of *in vitro* studies demonstrating that NT-3 prevents the death of a proportion of nodose neurons isolated during the period of normally occurring cell death (Hohn *et al.* 1990), and that neuronal death can be prevented *in vivo* by BDNF (Hofer & Barde 1988). This seems to be a further, and surprising, example of a situation where

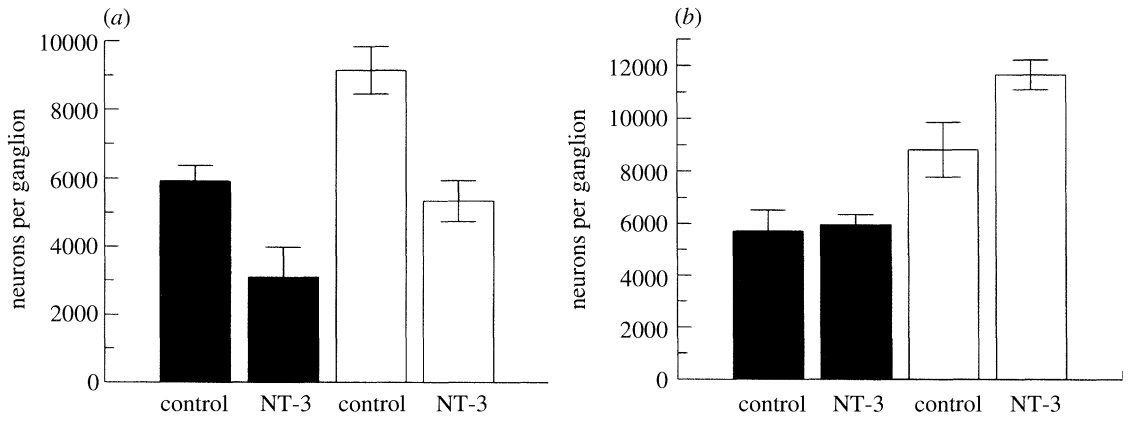

Figure 1. Cell counts in chick nodose (black columns) and dorsal root (white columns) ganglia after daily treatments with 5 µg NT-3 between E3-E6 (*a*) or between E6-E9 (*b*). Black columns neuronal numbers (±s.d.) were determined at E10 or E11 either on histological sections of the ganglia or following their dissociation by trypsin (for further details, see Ockel *et al.* 1996).

predictions made on the basis of *in vitro* experiments cannot be verified *in vivo*, and it would be interesting to see if NT-3 also rescues nodose neurons cultured in the absence of laminin (see §1).

Taken together, these findings indicate that NT-3 has multiple effects on sensory neurons during different phases of development. The time window for these different actions is narrow and represents a critical parameter in the NT-3-mediated regulation of neuronal numbers. Early in gangliogenesis, NT-3 might take some dividing neuroblasts out of the cell cycle, and at the same time, it might also be a survival factor for these cells. Thus, the net results of deprivation paradigms like the application of antibodies would be in any case fewer neurons when the ganglia are analysed (Gaese *et al.* 1994). Whereas it is novel for the action of neurotrophin *in vivo*, this effect of NT-3 during sensory gangliogenesis is reminiscent of what has long been observed with PC12 cells treated with NGF. NGF blocks proliferation of these cells, and in the absence of serum, NGF is also a survival factor for post-mitotic PC12 cells (Greene & Tischler 1976; Greene 1978).

The effects of late applications of NT-3 on DRG cell numbers can be rationalized according to the classical NGF model. NT-3 is made in the target of some proprioceptive neurons, for which it is an essential survival factor (Oakley *et al.* 1995). In the DRG, some of these neurons would die because of the lack of sufficient quantities of target-derived NT-3. Such does not seem to be the case for visceral neurons in the nodose ganglion between E5 and E11.

3. NEUROTROPHIN-3 AND AUTONOMIC NEURONS

As early applications of NT-3 markedly decrease the number of neurons in sensory ganglia, the question arises as to how widespread these effects are in the PNS. Parasympathetic neurons of the ciliary ganglion have been previously reported not to respond to NT-3 (Hohn *et al.* 1990; Maisonpierre *et al.* 1990). In NT-3-treated embryos with markedly smaller sensory ganglia, neuronal numbers in the ciliary ganglia were not different from control embryos (Ockel *et al.* 1996). The lack of receptors able to transduce an NT-3 response in these neurons might explain this result. In this context, it is interesting to note that whereas the death of ciliary neurons resembles in many ways that observed in other peripheral ganglia, these neurons are curiously unresponsive to the neurotrophin-trk-system: even when they artificially express trkA following the intracellular injection of the corresponding plasmids, they remain NGF-unresponsive (Allsopp *et al.* 1993).

With regards to the effects of early applications of NT-3, the sympathetic chain is an especially interesting structure to study. Indeed, previous *in vitro* work has indicated that the precursors of sympathetic neurons can be cultured, and that they even continue to divide *in vitro* (Rohrer & Thoenen 1987). This is in contrast with what has been observed with sensory neuroblasts which do not survive and divide in culture after their isolation from early sensory ganglia (Rohrer & Thoenen 1987; Ernsberger *et al.* 1989). Early survival effects of NT-3 have been reported with cultured sympathetic neuroblasts of chick (at E7) and rats (Birren *et al.* 1993; Dechant *et al.* 1993; DiCicco-Bloom *et al.* 1993), and high concentrations of NT-3 block proliferation of these cells *in vitro* (Verdi & Anderson 1994). These studies also indicated that, *in vitro*, sympathetic neurons first dependent on NT-3 during early stages of development, later switch their factor requirement for NGF (Birren *et al.* 1993; Dechant *et al.* 1993; DiCicco-Bloom *et al.* 1993). This switch might result from the gradual substitution of trkC for trkA, and evidence for cell non-autonomous signals inducing trkA expression in culture sympathoblasts has been provided. With E6.5 chick neurons, the addition of retinoic acid causes a marked inrease of trkA expression (von Holtz *et al.* 1995), whereas with immuno-isolated E14.5 rat sympathoblats, NT-3 and other agents producing mitotic arrest, but not retinoic acid, increase trkA mRNA levels (Verdi & Anderson 1994). However, recent data indicate that in the chick *in vivo*, trkA mRNA can already be detected at E4.5 in the primary sympathetic chain, and that during the period that corresponds to the switch from NT-3 to NGF-dependency of cultured neurons, trkA mRNA levels remain constant in freshly isolated sympathetic chains (Schröpel *et al.* 1995). Maximal expression levels are already reached at E7 in sympathetic ganglia, an age at which trkC is also expressed.

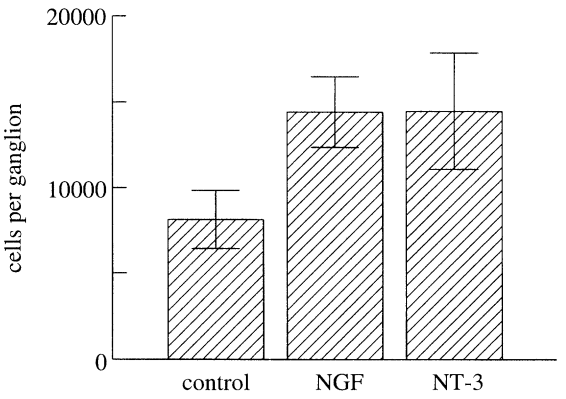

Figure 2. Cell counts in the E7 lumbar sympathetic chain after daily treatments with 5 μg NT-3 or NGF. Cell numbers (±s.d.) were obtained either by DNA measurements or following dissociation of the ganglia by trypsin (D. von Schack, unpublished results).

In view of these results, it was necessary to examine the effects of NT-3 and of NGF administration during the earliest stages of formation of the sympathetic chain, and to compare them with the results obtained with the sensory ganglia. Chick embryos were treated between E3 and E5 with 5 μg NT-3 or NGF and their lumbar sympathetic chains examined at E7, which is before the onset of target innervation. Both factors were found to induce a marked increase in cell numbers compared with untreated embryos (figure 2; see also Oppenheim *et al.* 1982). Whether or not NGF and NT-3 affect the same population of sympathoblasts is not known. In the same embryos, the nodose ganglia were markedly reduced in size.

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Currently, it is unclear if these increases in cell numbers result from increased proliferation, or from a reduction in early programmed cell death before target innervation. In any case, these results indicate that *in vivo*, cells in the sympathetic chain have functional receptors for both NGF and NT-3 at these early stages, and that in marked contrast with the effects of NT-3 on sensory ganglia, the size of sympathetic ganglia does not decrease, but increases after early NT-3 treatments.

4. CONCLUSIONS

The main conclusion from the experiments involving the application of exogenous NT-3 during gangliogenesis is that the development of the sensory ganglia, but not of the sympathetic ganglia, is affected in a way that is opposite to that observed following identical experiments done previously with either NGF or BDNF.

At present, it is difficult to know if the reduction in cell numbers seen in these ganglia reflects a physiological role of NT-3 during development. Indeed, manipulations such as the application of NT-3-antibodies or the deletion of the NT-3 gene can only show the net result of the manipulation, namely fewer neurons, as NT-3 seems to be also a survival factor for neurons during gangliogenesis (Gaese *et al.* 1994). The prediction that larger sensory ganglia should be formed in the absence of NT-3 can thus not be tested with the deprivation paradigm. Nonetheless, it appears possible that the anti-proliferative effects of NT-3 revealed by the administration of NT-3 during gangliogenesis represent an integral part of NT-3's physiology. The mRNAs coding for NT-3 and for trkC, the probable NT-3 receptor involved in these early effects, can be detected in the neural tube and the DRGs of the avian embryos at E3 (Hallböök *et al.* 1993; Pinco *et al.* 1993; Kahne & Kalcheim 1994; Zhang *et al.* 1994*a*).

In view of the results obtained with sensory ganglia, the observations made with the sympathetic ganglia are surprising because more cells, and not fewer, are seen with increased levels of neurotrophin-3. Similar results are obtained with NGF, indicating the presence of functional receptors for NGF at early stages on these neurons.

Why sensory and sympathetic gangliogenesis is affected in opposite ways by increased levels of NT-3 will also be an interesting question to address in future experiments. Finally, it will be useful to do similar experiments in the central nervous system, including the developing retina, where the presence of high affinity NT-3 receptors has been observed during neurogenesis (Rodrírguez-Tébar *et al.* 1993). Such experiments would shed light on the generality and diversity of the early roles of NT-3 during the formation of the nervous system.

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