Neurotrophins and their receptors in chicken neuronal development

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ABSTRACT A review on current studies of chicken neurotrophins and their receptors is given. Chicken NGF, BDNF and NT-3 have been cloned and sequences have been used to synthesize oligonucleotides for specific localization of expression during development. Also, chicken TrkA, TrkB and TrkC have been cloned, sequenced and studied by *in situ* hybridization. Recombinant NT-3 was applied to chicken ganglia at different developmental stages to examine acquirement of responsiveness to NT-3 compared to NGF. Phylogenetic analyses of the chicken neurotrophins and Trk receptors were carried out based on parsimony. Finally, some data on apoptosis in chicken embryo sympathetic ganglia are presented.

KEY WORDS: *avian, BDNF, evolution, NGF, NT-3, sensory, target-derived factor, Trk, tyrosine kinase receptors*

Introduction

Neuronal death has been identified as a widely occurring phenomenon in the development of the vertebrate nervous system. Hamburger and Levi-Montalcini (1949) demonstrated the extent of neuronal death during normal development in the nervous system. They found that marked degeneration of neurons occurred in chick dorsal root ganglia (DRG) around embryonic days 5 to 7. Subsequent work has shown that naturally occurring neuronal death is present in many neuronal cell populations both in the peripheral and central nervous systems. Examples include motoneurons (Oppenheim *et al.*, 1982), ciliary neurons (Landmesser and Pilar, 1974) and sensory neurons of the chick (Hamburger *et al.*, 1981). Naturally occurring neuronal death usually takes place during restricted time periods, as in the case of the chick ciliary ganglion where half of the neurons die between embryonic days E8 and E14 (Landmesser and Pilar, 1974). The extent of the naturally occurring neuronal death can be up to 60% of the original number of neurons (Berg, 1982).

Numerous studies have shown that removal of the target tissue in the embryo causes massive cell death in the neuronal populations that project to the target. These regressive events caused by the ablation experiments follow the same time course as the naturally occurring neuronal death (reviewed in Cowan *et al.*, 1984). The results indicated that the neurons acquire a dependence on the target tissue (Hamburger, 1977). Together, the data have led to a model with neurons that depend on target tissue or on factors secreted from the target. These factors mediating the survival effects were named neurotrophic factors. This model was also supported by studies of the effect on neuronal survival after supplying additional target tissue. Transplantation of extra limbs to the embryonic tadpole before periods of naturally occurring neuronal death increased the number of surviving motoneurons in frog (Hollyday and Hamburger, 1976). The neuronal death has been shown to be an active process involving the mechanisms of apoptosis (Martin *et al.*, 1988; Oppenheim *et al.*, 1990; Garcia *et al.*, 1992; Allsopp *et al.*, 1993).

Nerve growth factor (NGF) has served, since it was discovered (Bueker, 1948; Levi-Montalcini and Hamburger, 1951; Levi-Montalcini and Hamburger, 1953), as a prototype for the neurotrophic factors. NGF belongs to a family of four structurally related proteins known as neurotrophins, which support neuronal survival both in the developing and adult nervous system. In addition to NGF, the neurotrophin family includes brain-derived neurotrophic factor (BDNF; Barde *et al.*, 1982; Leibrock *et al.*, 1989), neurotrophin-3 (NT-3; Ernfors *et al.*, 1990; Hohn *et al.*, 1990; Jones and Reichardt, 1990; Kaisho *et al.*, 1990; Maisonpierre *et al.*, 1990a; Rosenthal *et al.*, 1990) and neurotrophin-4 (NT-4; Hallböök *et al.*, 1991; Ip *et al.*, 1992) also known as neurotrophin-5 (Berkemeier *et al.*, 1991). All four factors have similar binding characteristics to a low-affinity receptor (Ernfors *et al.*, 1990; Rodriguez-Tébar *et al.*, 1990; Hallböök *et al.*, 1991; Squinto *et al.*, 1991), which is represented by a transmembrane glycoprotein of about 75 kDa (p75LNGFR) (Johnson *et al.*, 1986; Radeke *et al.*, 1987). The neurotrophins also bind and activate a second class of receptors, which are tyrosine kinase receptors, known as the Trk receptors. NGF binds and activates the *trk* proto-oncogene product, which is a glycoprotein

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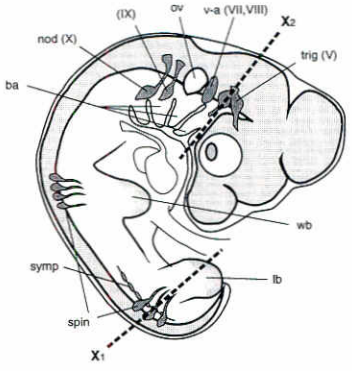


Fig. 1. Schematic representation of a chicken E4.5 embryo. *The shaded areas are parts of the avian developing nervous system. Indicated are cranial, sensory and future sympathetic ganglia. The plan of sectioning showed in Figs. 2 and 5 are indicated as X1 and X2, respectively. ba: branchial arches, lb: leg bud, ov: otic vesicle, spin: spinal sensory ganglia, symp: sympathetic ganglia (future position), trig: trigeminal ganglion, v-a: vestibulo-acoustic ganglion,*

of about 140 kDa, TrkA (Cordon *et al.*, 1991; Hempstead *et al.*, 1991; Kaplan *et al.*, 1991a,b; Nebrada *et al.*, 1991). There are two more TrkA-related tyrosine protein kinases, namely the 145 kDa glycoprotein gp145TrkB (Glass *et al.*, 1991; Klein *et al.*, 1991b; Soppet *et al.*, 1991; Squinto *et al.*, 1991) and the 145 kDa glycoprotein gp145TrkC (Lamballe *et al.*, 1991). These have been shown to constitute functional receptors for BDNF and NT-3, respectively. The TrkB receptor has also been shown to be activated by NT-4 (Ip *et al.*, 1992). The Trk tyrosine kinase receptors mediate biological responses of the neurotrophins by activating several signal transduction pathways which include regulators of the phosphatidyl inositol metabolism and the activity of the $p21^{ras}$ protein and Raf-1 proteins (Vetter *et al.*, 1991; Loeb *et al.*, 1992; Soltoff *et al.*, 1992; Stephens *et al.*, 1994). The function of the p75LNGFR is not fully clear for BDNF, NT-3 and NT-4 but in the case of NGF, evidence is presented that it contributes to the high-affinity NGF binding including the TrkA receptor (Mahadeo *et al.*, 1994; Verdi *et al.*, 1994).

In this report we review our current research and approaches to understand and illustrate the developmental mechanisms that include the actions of neurotrophins. We are using the chicken embryo as a model (Fig. 1), and molecular cloning of genes for the avian neurotrophins and their Trk receptors has allowed us to perform detailed comparative studies of the sites and patterns of synthesis of the corresponding mRNA.

Results and Discussion

Neurotrophins are expressed in the target fields of developing neurons

NGF, BDNF and NT-3 have been cloned from the chicken (Ebendal *et al.*, 1986; Hallböök *et al.*, 1991) and are likely to mediate a host of neurotrophic interactions in chicken development both in CNS and PNS. In particular we focus our attention here on peripheral ganglia in the developing chicken embryo. Neurons in the peripheral nervous system of the chicken embryo include sensory neurons located to cranial and spinal ganglia. The sensory innervation already starts soon after formation of the ganglia and neurites are extended towards their future terminal fields. Neurons in the lumbar sensory spinal ganglia innervate the developing limb buds. Both exteroceptive and proprioceptive sensory neurons in these ganglia have their future target areas in the developing buds, and in the E4 chicken embryo the ganglia are still close to the limb buds, and growth factors produced in the target can reach the neurites (Fig. 1). Using *in situ* hybridization analysis with probes for the neurotrophins, expres-

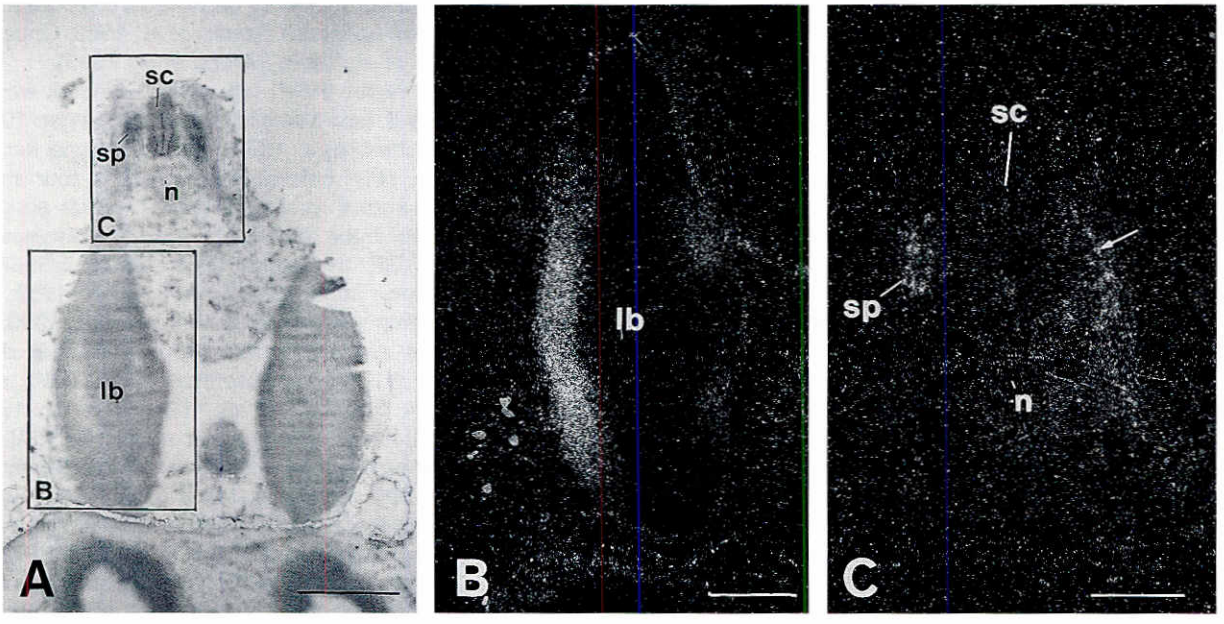


Fig. 2. *In situ* hybridization analysis of BDNF and trkB mRNA in the developing limb bud and spinal sensory ganglia. (A) *Bright-field microphotograph showing in situ analysis of transversal sections through the leg bud and lumbar region of an E4.5 chicken embryo. Boxed regions indicate the magnified areas in panel* **B** *and* **C. (B)** *Darkfield micrograph showing BDNF expression in the developing limb bud.* **(C)** *trkB mRNA expression in the spinal sensory ganglia. The trkB probe recognizes the mRNA encoding the catalytic trkB receptor. lb: limb bud, n: notochord, sc: spinal cord, sp: spinal sensory ganglia. Scale bars: A, 300 μm; B, 50 μm; C, 50 μm.*

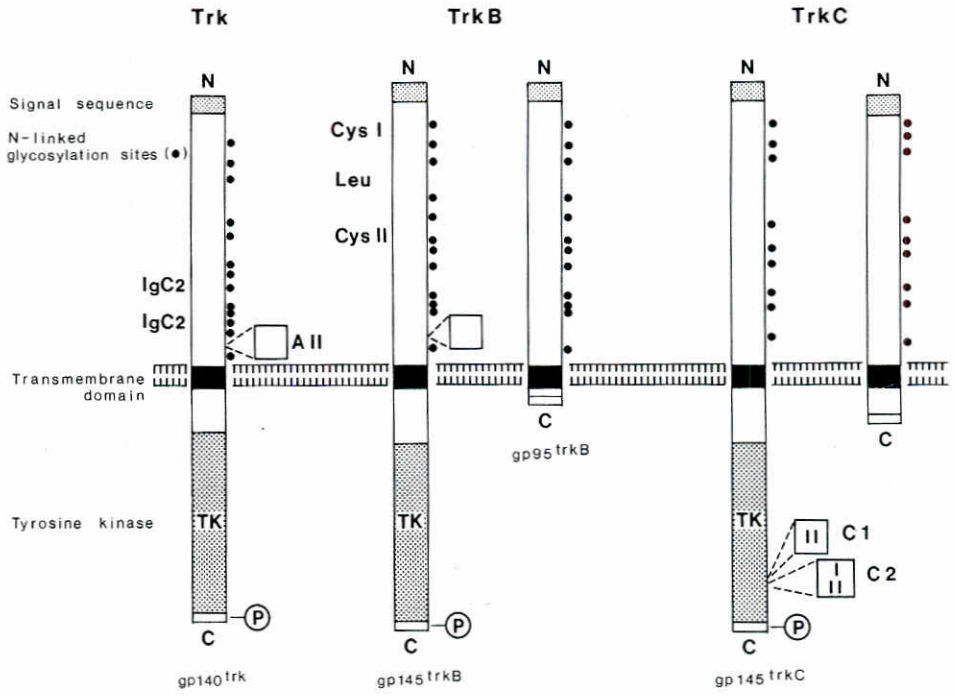


Fig. 3. Schematic presentation of the chicken TrkA, TrkB and TrkC receptor isoforms. *Overview of the chicken Trk isoforms sequenced and used for synthesis of oligonucleotides applied to in situ hybridization analysis. Based on detected insertion sites in the TrkA extracellular region (Barker et al., 1993) and in kinase domain of TrkC (Lamballe et al., 1993; Tsoulfas et al., 1993; Valenzuela et al., 1993) and in the case of trkB in the extracellular portion (unpublished results) we were able to detect different isoforms of the receptors.*

sion of neurotrophin mRNA during the critical periods of innervation and cell death can be studied. Complex, but specific expression patterns for each of the neurotrophin mRNAs in the developing embryo are described and the spatio-temporal patterns are in many cases consistent with a target-derived mode of action where the neurotrophin mRNA is detected in the target fields of neuronal innervation, and mRNA for the corresponding Trk receptors are detected in the innervating neuron body. As shown in Figure 2, using *in situ* hybridization analysis on fresh frozen sections, BDNF mRNA is expressed in the E4.5 limb bud which corresponds to the target field for sensory neurons in the lumbar spinal ganglia. An adjacent section including the lumbar spinal ganglia hybridized with a probe for the mRNA encoding catalytic TrkB receptors shows that *trkB* mRNA expressing neurons are present in the ganglia. Neurons in the spinal ganglia have been shown to be supported by BDNF, and the trkB mRNA expressing neurons are most likely supported by BDNF during the period of naturally occurring neuronal death and will respond with neurite outgrowth when BDNF protein is encountered. It has also been shown that NT-3 and NT-4 are expressed in the developing limb bud (Hallböök *et al.*, 1993; Henderson *et al.*, 1993) and most probably NGF mRNA is expressed in the epithelium of the developing extremities (Wyatt *et al.*, 1990). NT-4 still remains to be cloned and sequenced in the chick.

In addition to the target field-derived mode of action of the neurotrophins, evidence suggesting a local, either autocrine or paracrine mode of action, is emerging. Thus, BDNF and NT-3 mRNA are expressed in embryonic sensory ganglia and that some of these ganglia contain cells that may express both the neurotrophin and its corresponding Trk receptor (Ernfors and Persson, 1991; Schecterson and Bothwell, 1992). These results suggest a local action of the neurotrophins within both neural crest and placode-derived sensory neurons in the peripheral ner-

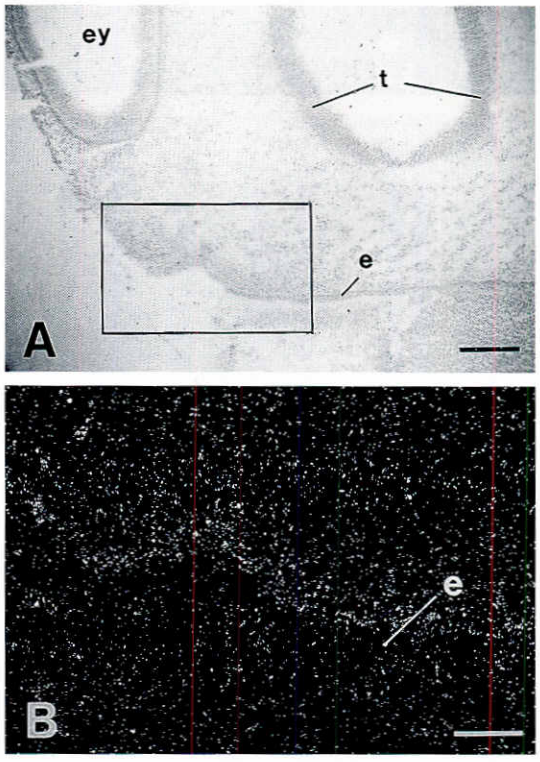
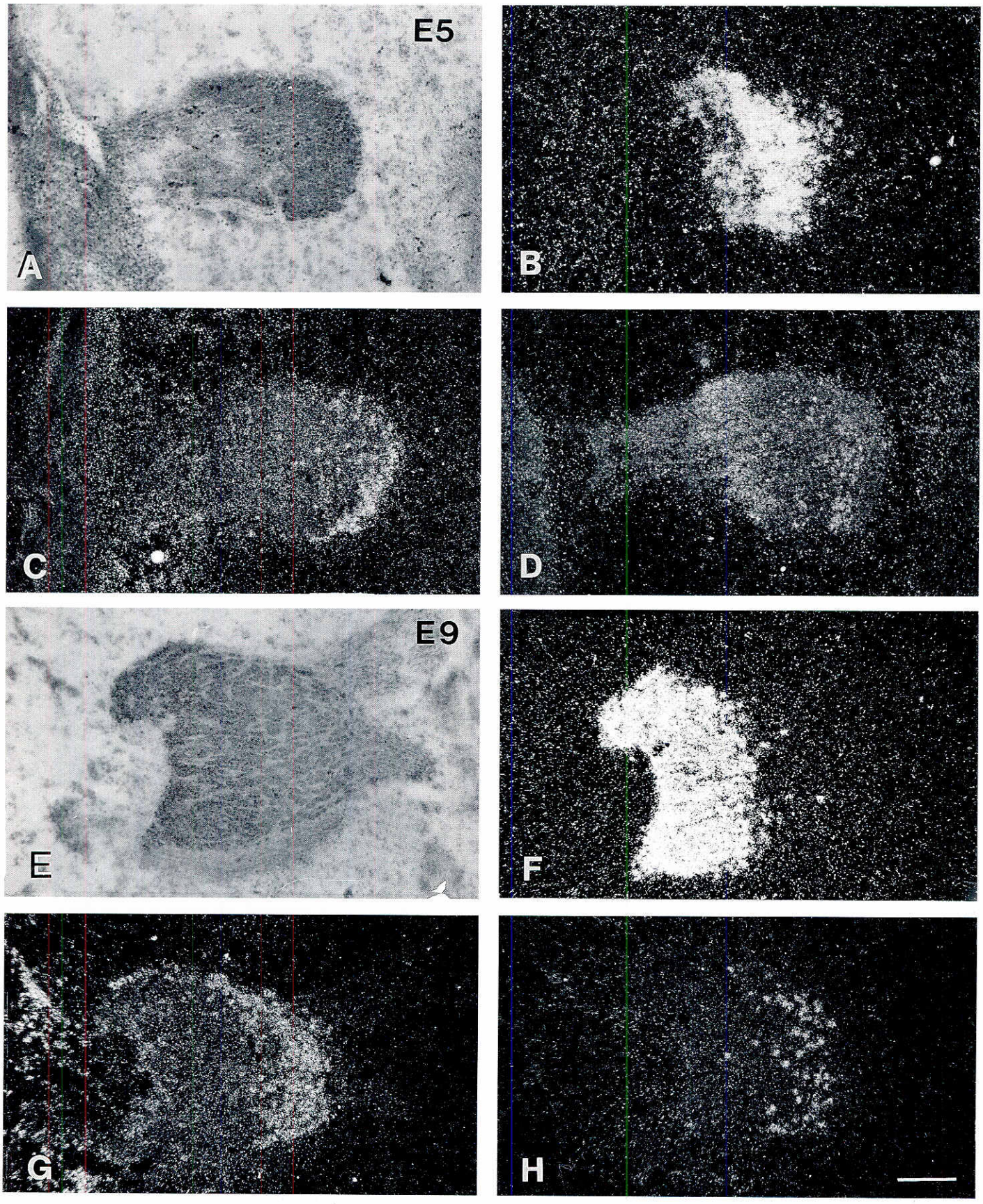


Fig. 4. Detection of NT-3 mRNA in developing skin of the E4 chicken embryo head. (A) *Section through the head of an E4 chicken embryo through the telencephalon, eyes and the nasal pits shown by bright-field microscopy after hybridization with the NT-3 probe.* **(B)** *Dark-field illumination and higher magnification of the boxed region in panel A, showing labeling for NT-3 mRNA in the developing skin. Epithelium (e), retina of the eye (r), telencephalon (t). Bars: A, 250 μm; B, 100 μm.*


E5
A
B
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E9
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vous system. It remains to be determined how the local action of neurotrophins function together with a target-derived mode of action.

Neurotrophin receptors

A strategy based on PCR and degenerate primers was used to isolate the chicken TrkA, TrkB and TrkC. An oligonucleotide probe for the *trkB* mRNA (used in the analysis shown in Fig. 2C) is directed to the part of the *trkB* mRNA encoding the intracellular tyrosine kinase domain of the receptor. This domain is essential for signal transduction pathway activation. There are several isoforms of the Trk receptors with insertions and deletions in the extra- and intracellular domains (Fig. 3). Isoforms lacking the intracellular tyrosine-kinase domain have been characterized for TrkB and TrkC and these truncated receptors can bind neurotrophins but are believed to be unable to activate the signal transduction pathway (Klein *et al.*, 1989; Middlemas *et al.*, 1991). The system is complex and TrkC isoforms have been found with amino-acid insertions in the tyrosine-kinase domain. These TrkC isoforms have different biological properties (Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993) and substrate specificity (Lamballe *et al.*, 1993). Using probes of 45 to 55 nucleotides, particular mRNA sequences for isoforms can be specifically detected. We have probes that are directed to the region of insertions in the tyrosine kinase domain spanning the insertion position and will therefore be specific for mRNAs that encode catalytic TrkC receptors without insertions in the tyrosine kinase domain. It is not clear whether there are isoforms of the TrkA or TrkB receptors that have insertions in the tyrosine kinase domain but we have oligonucleotide probes for *trkA* and *trkB* mRNA that are directed to the same region of the tyrosine kinase domain as the *trkC* probe. These probes will also be specific for catalytic TrkA and TrkB receptors (Fig. 3).

Another example of a neuronal population that has been extensively examined is the sensory neurons in the trigeminal ganglion which innervate the skin and epithelium of the face and oral cavity (Fig. 1). Starting around E3, neurons in the newly formed trigeminal ganglion send out neurites into the upper branchial arches. *In situ* hybridization analysis with a probe for chicken NT-3 shows that NT-3 mRNA is expressed at E4.5 in the epithelium which will be the future endothelium in the oral cavity and the facial skin (Fig. 4). As shown in several studies, the target fields of neurons in the trigeminal ganglion express NGF, BDNF and NT-4 mRNA in addition to NT-3 mRNA (Davies *et al.*, 1986a, 1987; Ernfors *et al.*, 1992; Buchmann and Davies, 1993; Hallböök *et al.*, 1993; Ibáñez *et al.*, 1993). In agreement with expression of all neurotrophins in the target fields for the neurons in the trigeminal ganglion, mRNA encoding all three neurotrophin Trk receptors have been detected in the ganglion (Fig. 5; Ernfors *et al.*, 1993; Ibáñez *et al.*, 1993; Williams *et al.*, 1993, 1994).

To analyze the distribution of neurons within the trigeminal sensory system, we have thus performed *in situ* hybridization analysis on serial sections through the trigeminal ganglion at various ages of development using the oligonucleotide probes for *trkA*, *trkB* and *trkC* mRNA (Fig. 5). We found a very early expression of *trkC* mRNA prior to the formation of the ganglion with subsequent expression of *trkA* and *trkB* mRNA at later stages (Williams *et al.*, 1994). Neurons that express mRNA for each of the Trk receptors occupy discrete regions of the ganglion which shows that the ganglion is comprised of distinct neuronal subpopulations, each of which has a different capacity to

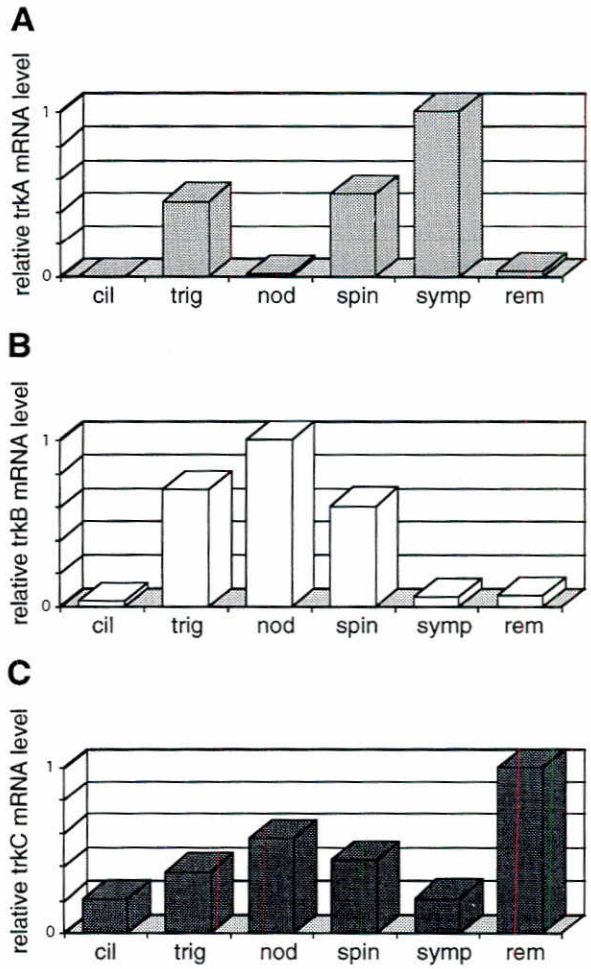

Fig. 6. TrkA, TrkB and TrkC mRNA expression in E9 chicken embryo peripheral ganglia. *The diagrams show the relative TrkA* **(A),** *TrkB* **(B)** *and TrkC* **(C)** *mRNA levels in total RNA prepared from E9 ciliary, trigeminal, nodose, spinal sensory, sympathetic and Remak ganglia. The mRNA levels were measured using RNase protection assay with probes to the region of mRNA encoding the extracellular region of the Trk receptors. Bars represent the total level of receptor mRNA, including mRNA encoding catalytic and truncated isoforms.*

Fig. 5. Neurotrophin receptor expression in the E9 chicken trigeminal ganglion. *Photomicrographs of the developing trigeminal ganglion at E5* **(A-D)** *and E9* **(E-H)** *showing cresyl violet stained sections* **(A and E)** *as well as the sections with labeling for mRNA encoding each of TrkA* **(B and F),** *TrkB* **(C and G)** *and TrkC* **(D and H)** *in dark field. TrkA mRNA is localized only within the dorso-medial aspect of the ganglion, whereas both TrkB and TrkC mRNAs are within the ventrolateral regions. The mRNA for TrkB seems to be more expressed in neurons which are more distally positioned than those expressing TrkC mRNA, both at E5 and E9). Scale bar, 100 mm.*

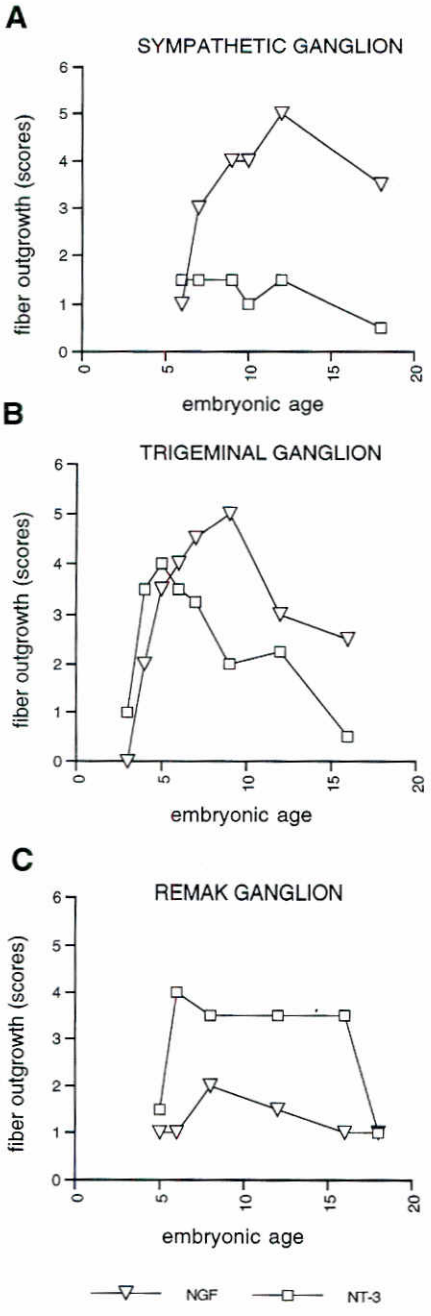

Fig. 7. Temporal profile of the neurite outgrowth response from peripheral ganglia on stimulation with NGF and NT-3. **(A)** *Sympathetic ganglia were grown as explants in a collagen gel and stimulated with NGF or NT-3 and the neurite outgrowth was scored on a scale between 1 and 6 as shown in the diagram.* **(B)** *Trigeminal ganglion, and* **(C)** *Remak's ganglion grown and stimulated with NGF or NT-3.*

respond to the neurotrophins (Williams *et al.*, 1994). From this study it is also clear that the expression of the Trk receptors changes during development and generally the expression decreases during development (Williams *et al.*, 1994) suggesting that neurons depend to a less degree on neurotrophins at later stages of development (Lindsay and Harmar, 1989).

Thus, both the trigeminal and spinal ganglia contain subpopulations of neurons that express each of the Trk receptors (Fig. 5; Mu *et al.*, 1993; Williams *et al.*, 1994). Neurons in the sympathetic ganglia express mainly *trkA* mRNA (Ernfors *et al.*, 1992) with lower levels of *trkC* mRNA expression in development (Williams *et al.*, 1993). The different levels of Trk receptor mRNAs in E9 chicken embryo ciliary, trigeminal, nodose, spinal, sympathetic and Remak ganglia were analyzed using RNase protection assay (Fig. 6). These results are in agreement with the *in situ* hybridization data showing mainly *trkA* mRNA with lower levels of *trkC* mRNA in sympathetic ganglia (Fig. 6A,C) and all *trkA*, *trkB* and *trkC* mRNAs in RNA prepared from trigeminal and spinal ganglia. *trkB* mRNA is also found in the nodose ganglion (Fig. 6B) whereas *trkC* mRNA is found in both the nodose and Remak ganglia and at low levels in sympathetic and ciliary ganglia (Fig. 6C).

Neurite outgrowth in response to neurotrophin stimulation of explanted peripheral ganglia

Since the Trk tyrosine kinase receptors (Fig. 3) are mediating the biological capacities of the neurotrophins and are recruiting a signal transduction cascade, the presence of receptors is a prerequisite for a neuron to be able to respond to a neurotrophin. This was confirmed by the correlation of Trk receptor mRNA expression and neuronal response to neurotrophin stimulation. Various E9 chicken ganglia were dissected and placed in collagen gels in culture and stimulated with neurotrophins. Fibre outgrowth from the explants was estimated after two days in culture. The fibre outgrowth responses to NGF and NT-3 (Figs. 7-9) during development were thus studied in explanted chicken ganglia (Hedlund and Ebendal, 1978). NGF has previously been examined in this assay (Ebendal, 1989; Ebendal *et al.*, 1978) using sympathetic and Remak's ganglia (Ebendal, 1979) as well as the trigeminal ganglion (Ebendal *et al.*, 1990; Williams *et al.*, 1994) of different ages. These studies show that background fibre outgrowth in control medium is very low or absent from E6 until E16 or E18. Both mouse or chicken NGF began stimulating fibre outgrowth at E7 (stage 30) in sympathetic lumbosacral paravertebral ganglia (Ebendal, 1979) and robust responses were obtained at E8 reaching peak outgrowth densities at E9 to E14 followed by a slight decline at E16 and E18. These findings were repeated and confirmed here (Fig. 7A). In contrast, the new data obtained in this study show that NT-3 stimulates fibre outgrowth only marginally throughout development of the sympathetic ganglia. Thus only weak fibre outgrowth stimulation, earlier described from the E9 sympathetic ganglion in this assay (Ernfors *et al.*, 1990), is seen from E6 (stage 29) throughout E12 and then further being reduced at E18 (Fig. 7A). Thus no indications for a switch in neurotrophic dependencies between NT-3 and NGF were obtained by studying the fibre outgrowth responses in E6 to E18 sympathetic ganglia.

For Remak's ganglion the situation was the reverse (Fig. 7C). A weak response to NT-3 was seen in E5 ganglia (stage 27-29, Fig. 9) and vigorous outgrowth responses (Ernfors *et al.*, 1990; Kullander and Ebendal, 1994) were found in E6 to E16. No response to NT-3 was seen at E4 (stage 24-25). Only weak responses to NGF were seen during development of the Remak ganglion. However, the ganglion does show fibre outgrowth above the control levels in response to NGF (Ebendal, 1979; Ebendal *et al.*, 1990) from E5 until E18, with a peak at E8-E12. The receptor mechanism for the NGF-elicited outgrowth is not known but the NT-3 stimulation is most likely to be mediated by the TrkC receptor abundantly expressed in Remak's ganglion (Williams *et al.*, 1993). Ebendal and Jacobson (1977) observed a slight selectivity in the attraction of fibre outgrowth from

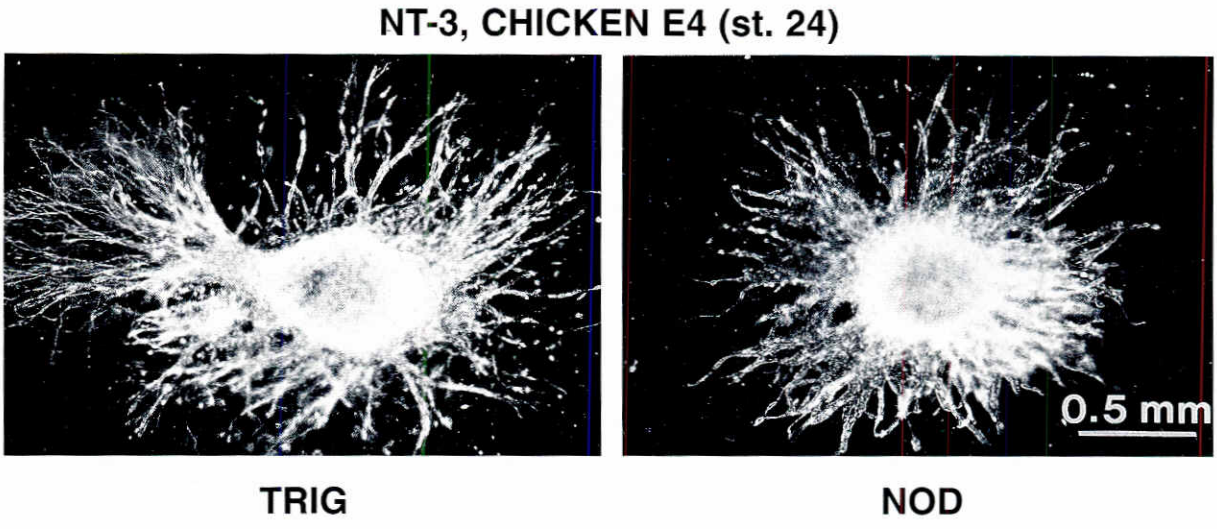

Fig. 8. Neurite outgrowth from peripheral ganglia. *An early response to neurotrophin-3 (NT-3) in the trigeminal (TRIG) and nodose (NOD) ganglia of the chicken embryo. Ganglia were dissected from stage 24 (E4) embryos and explanted to a collagen gel. NT-3 was present at a concentration of 5 ng/ml during the two days of culture resulting in a distinct outgrowth of nerve fibres. In the absence of NT-3 no outgrowth occurred. Dark-field micrographs.*

Remak's ganglion to intestinal tissue. In retrospect the effect may well have been elicited by NT-3 being released from the explanted colo-rectal tissue.

The ciliary ganglion responds to NT-3 at E5 by a limited formation of some few, thick fibre fascicles (Fig. 9), also formed at E6-E10 (Ernfors *et al.*, 1990). In contrast, NGF did not affect the ciliary ganglion at any time between E5-E18 (Ebendal, 1979; Ebendal *et al.*, 1990)

In contrast to the three autonomic ganglia examined, the sensory trigeminal ganglion (Hedlund and Ebendal, 1978) responded to both NGF and NT-3 during development (Figs. 7-9). As described before (Ebendal *et al.*, 1990) the response to NGF develops early, already at E4 (Fig. 7B). Peak responses to NGF were obtained at E8 and E9 followed by declined responses at E12 and E16. The outgrowth effect by NT-3 develops even earlier in the trigeminal ganglion, the first NT-3 evoked effects seen at E3 (stage 18-19). At E4 (stage 24-25) the trigeminal ganglion explants responded well to NT-3 (Figs. 7B and 8, Williams *et al.*, 1994), thus before the time when NT-3 mRNA is detectable in the target cells for trigeminal innervation (Fig. 4). The nodose ganglion was the only other ganglion found to be stimulated also by NT-3 at E4 (Fig. 8), that is one day before the nodose ganglion responds to NGF by modest fiber outgrowth (Hedlund and Ebendal, 1980; Davies and Lindsay, 1985; Ebendal *et al.*, 1990). The trigeminal ganglion showed an early peak in responsiveness to NT-3, already at E5 followed by a marked decline at E9 until E16. The differences in maturation of TrkC and TrkA expressing neurons may account for these different time courses in trigeminal fibre outgrowth evoked by NT-3 and NGF, respectively (Williams *et al.*, 1994).

The sensory spinal ganglia (dorsal root ganglia) from the lumbosacral region became distinctly responsive to NT-3 (Fig. 9) at E5 (stage 26-27). The spinal ganglia at this stage were only slightly affected by NGF (Ebendal, 1979; Ebendal *et al.*, 1990). At E6 (stage 29) both NT-3 and NGF gave dense fibre outgrowth in these ganglia. The time schedule of early fibre outgrowth responses to NT-3 resembles that found at E4 for BDNF in early ventrolateral trigeminal and nodose ganglion explanted into collagen gels (Davies *et al.*, 1986b). The present data for NT-3, beginning to stimulate spinal ganglia at E5, also coincide with BDNF distinctly affecting spinal ganglion fibre outgrowth at E5 (Davies and Lindsay, 1985). Our data also fit with the finding that spinal sensory neurons are responsive to NT-3 and BDNF already at E4.5 (stage 25/26) as measured in a maturation assay with dissociated neurons, although at this stage they do not depend yet these neurotrophins for their survival (Wright *et al.*, 1992). Moreover, the maximum neurite responses to BDNF occurred in all responding sensory neurons at E10-12 according to the same authors. The general pattern fits well with the developmental expression pattern of TrkB and TrkC in different populations of the chicken trigeminal ganglion (Williams *et al.*, 1994).

The present understanding of Trk expression in the chicken PNS can be compared with findings from null mutations of members of the neurotrophin gene family. Data obtained in mice (see below) suggest important functions for the neurotrophins and their receptors during peripheral nervous system development.

Evolution of the neurotrophin and trk gene families

Comparison of twenty amino-acid sequences encoding the prepro neurotrophin proteins shows that neurotrophin primary amino acid structure is highly conserved from fish to man. The formation of the neurotrophin gene family has involved at least two gene duplications producing the currently known four mem-

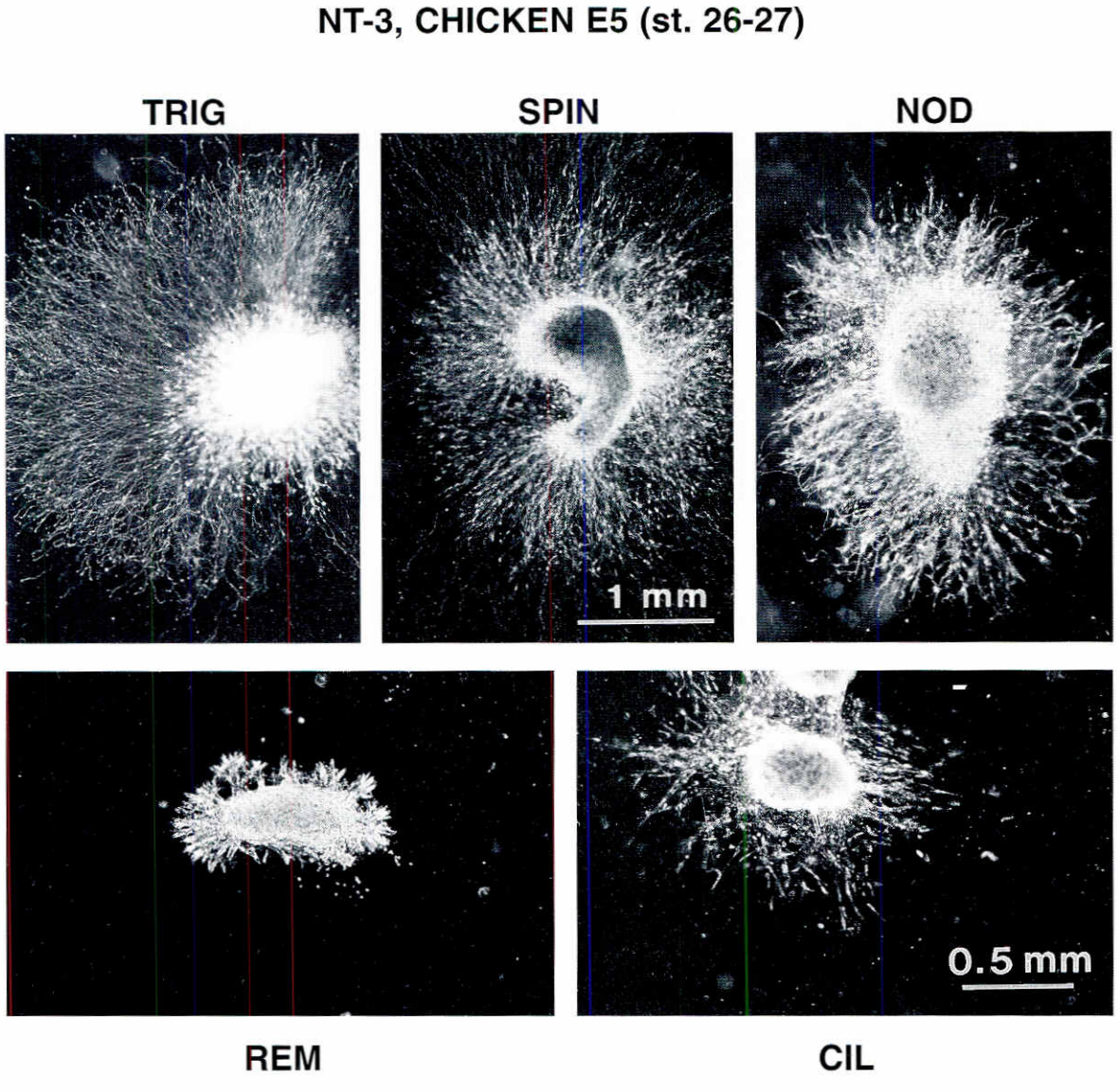

Fig. 9. Neurite outgrowth from peripheral ganglia. *Neurotrophin-3 stimulation on a range of peripheral ganglia from stage 26-27 (E5) chicken embryo. Fibre outgrowth is prominent at this stage in the sensory ganglia (upper microphotographs), i.e. the trigeminal (TRIG), spinal dorsal root (SPIN) and nodose (NOD) ganglia. Remak's ganglion (REM) at this stage is also responsive to NT-3 but the response is less prominent than at E9. Single fibre fascicles are formed by the ciliary ganglion in response to NT-3 at this stage as is the case also at E9. It is not possible to dissect the sympathetic paravertebral ganglia for explantation at this stage of chicken development. Dark-field micrographs of 2 day-old cultures.*

bers of the family. Using the software PAUP, we performed heuristic searches on the computer for the shortest trees, representing the relationship between the different genes, composed of the neurotrophin and Trk amino acid sequences. Analysis of the prepro neurotrophin sequences gave 21 equally short and probable trees. In all 21 trees, BDNF and NT-4 are grouped on the same branch node (Fig. 10A) indicating that BDNF and NT-4 are more closely related to NT-3 or NGF than BDNF, and it seems possible that NT-4 and BDNF were formed by a duplication of an ancestral BDNF/NT-4 gene. 11 of the 21 trees have NT-3 on the same branch node as NGF and one of these trees is shown in Figure 10A. Ten of the 21 trees have BDNF/NT-4 on the same branch node as NGF. The difference in number of trees and branch node topologies is not significant, indicating that NT-3 and BDNF sequences are equally related to the NGF sequences. Neurotrophins are found in vertebrates from fish to mammals, which indicates that neurotrophins existed 400 million years ago and probably supported neuronal development in primitive fish, which were the ancestors of today's vertebrates (Hallböök *et al.*, 1991; Ebendal, 1992). It is not clear whether neurotrophins exist in invertebrates. NGF can stimulate neurite outgrowth from ganglionic formations in the snail *Lymnaea stagnalis* (Ridgway *et al.*, 1991) indicating the possibility that invertebrates may also have neurotrophic factors. As shown in Figure 10A the topology of the phylograms with respect to each neurotrophin is in agreement with the consensus evolutionary rela-

tionship among species. This is also valid for the comparison of the Trk receptor sequences shown in Figure 10B.

Neuronal cell death induced by neurotrophic deprivation is similar to apoptosis

NGF is essential for survival of developing sympathetic neurons (Thoenen and Barde, 1980; Levi-Montalcini, 1987; Oppenheim, 1991). This has been demonstrated *in vitro* and confirmed *in vivo* with depletion of NGF using antibodies (Pearson *et al.*, 1983) or NGF gene knockout (Crowly *et al.*, 1994). NGF promotes survival by activation of TrkA receptors which are expressed by sympathetic neurons (Fig. 6) and the subsequent activation by signal transduction pathways. However, it is not clear what exact mechanisms are necessary for survival, nor is it clear by what mechanism the neurons die. Non-neuronal cells that undergo apoptosis exhibit several characteristics including increased membrane budding and degradation of genomic DNA into oligo-nucleosomal fragments (Duke *et al.*, 1983). These degradation products can be seen as a DNA ladder when analyzed on an agarose gel.

The counteraction of developmental sensory neuronal death by the neurotrophins has been repeatedly confirmed during recent years. Administration of exogenous NGF during chick embryonic development will result in the decrease of the number of pycnotic cells in sensory and sympathetic ganglia (Dimberg *et al.*, 1987) and injection of antibodies to NGF will have the opposite effect. More recently it has been shown that mice homozygous for a NGF null mutation develop severe perinatal sensory and sympathetic deficits as a result of a complete absence of NGF (Crowly *et al.*, 1994). Thus the superior cervical ganglion will lose neurons in the mice homozygous for null mutations of NGF as well as for trkA (Smeyne *et al.*, 1994). Mice homozygous for NGF null mutant (Crowly *et al.*, 1994) show marked reductions in the sympathetic superior cervical ganglion, with up to 80% reduction of the ganglionic volume, losses of neurons and the presence of abundant pycnotic nuclei 3 days after birth. Moreover, mice with null mutations for BDNF and NT-3 (Ernfors *et al.*, 1994; Jones *et al.*, 1994) also show severe sensory deficits. The sensory deficiency induced by the NGF null mutation mainly affects nociception and thermoception, which show the specificity of the action of NGF on particular subsets of sensory neurons which mediate these types of sensory stimuli. The affected neurons are most probably those neurons that express TrkA receptors during development, which is further confirmed by the similarity of the phenotypes of NGF and trkA null mutants. Combining these results with the result of the localization of trkA expressing neurons (Fig. 5), it is possible to locate nociceptive/thermoceptive neurons within sensory ganglia and follow their localization during development.

We have studied the degradation of genomic DNA in dissociated chicken sympathetic neurons in culture resulting from the removal of NGF. Dissociated neurons were plated on laminin and grown in the presence of NGF for 36 h. At time 0, neurons were washed and blocking anti-NGF antibodies were added to the cells. Cultures were harvested after 0, 10, 15, 20, 25 and 35 h and analysis for DNA fragmentation was performed. The results show that no DNA degradation occurs in cultures that have been grown in the presence of NGF and that DNA is degraded after neurons are depleted of NGF (Fig. 11). The results also show that DNA degradation does not occur before 15 h in culture after depletion of NGF and this time scale is in agreement with the induction of DNA degradation in non-neuronal cells (Wyllie *et al.*, 1984). These results taken together with other data show that the mechanisms of neuronal and non-neuronal cell death are shared, at least in part. The time scale of DNA degradation correlates with the time point of no return for when sympathetic neurons can be rescued by re-addition of NGF to cultures (Thoenen and Barde, 1980; Hamburger and Oppenheim, 1982; Oppenheim *et al.*, 1990).

Even though the mechanisms of sympathetic neuronal death are not clear the molecular pathways that regulate apoptosis are beginning to be unraveled. Inhibition of protein and RNA synthesis can prevent sympathetic neurons deprived of NGF from dying (Martin *et al.*, 1988; Oppenheim *et al.*, 1990; Scott and Davies, 1990; Edwards *et al.*, 1991). This is in line with the con-

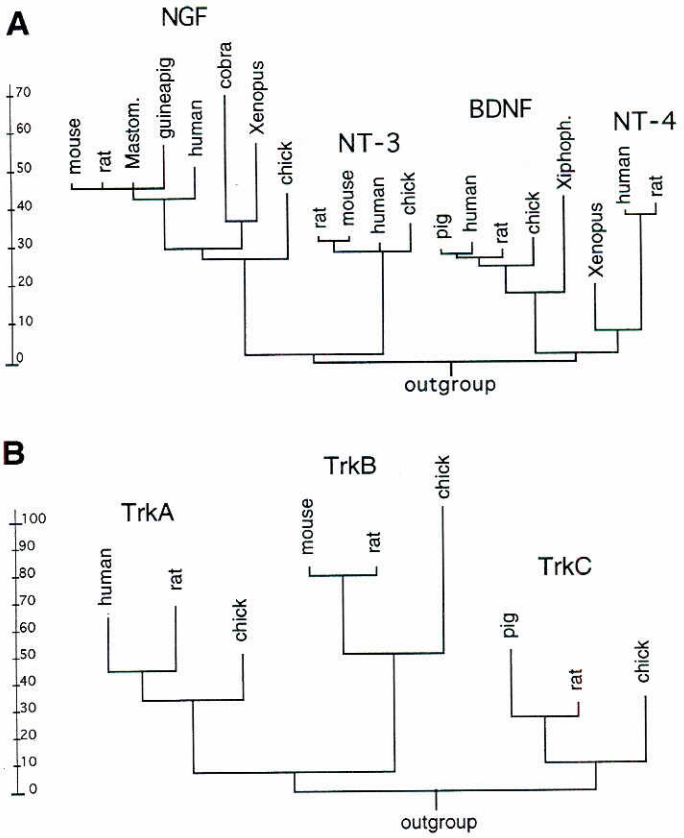

Fig. 10. Evolutionary analysis of the neurotrophin and trk gene families. *Cladistic trees showing a likely evolutionary relationship between members of the neurotrophin gene family and Trk family of tyrosine receptor kinases. The length of each branch represents an average change of amino acid substitutions. The calculations are based on full-length protein amino-acid sequences (as in Ebendal, 1992) regarding the neurotrophins.* **(A)** *Chicken TGFß (Barnett et al., 1994) was used to root the tree as a hypothetical ancestor. The heuristic search gave rise to 21 equally parsimonious phylograms.* **(B)** *The chicken TGFß II receptor (Barnett et al., 1994) was used as an outgroup to root the tree. The heuristic search only gave rise to one parsimonious phylogram.*

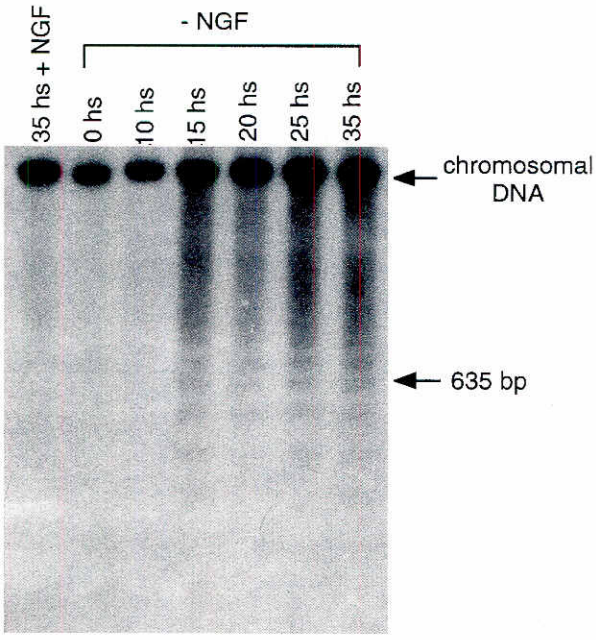

Fig. 11. Fragmentation of genomic DNA from sympathetic neurons as a result of NGF depletion. *Dissociated sympathetic neurons were grown in culture with NGF for 36 h before the NGF support was depleted (time 0 h) and fragmentation of genomic DNA as a result of increased cell death was measured using Southern blot analysis. The Figure shows an autoradiogram of a Southern blot analysis of a 1.2 % agarose gel with DNA samples from cultures harvested at times after NGF depletion indicated in the figure. As a probe, labeled total genomic chicken DNA digested with Mbo1 was used.*

cept of apoptosis in non-neuronal cells (Wyllie *et al.*, 1984). *bcl-2*, a proto-oncogene found in follicular B-cell lymphomas can prevent and delay apoptosis in lymphocytes as well as in neurons induced by trophic factor deprivation (Allsopp *et al.*, 1993; Garcia *et al.*, 1992). Major contributions to the understanding of the molecular pathways that regulate apoptosis have been achieved by studies of the nematode *Caenorhabditis elegans* (Hengartner *et al.*, 1992). The *bcl-2* gene product can substitute the *ced-9* gene product in the control of cell death in *Caenorhabditis* (Vaux *et al.*, 1992) which was identified together with a few other genes by their key roles in the control of apoptosis in *Caenorhabditis*. The functions of these genes and their mammalian homologs are not clear but results suggest that the gene products are involved in inhibition of lipid peroxidation by reactive oxygen intermediates (Hockenbery *et al.*, 1993), inhibition of specific proteases (Gagliardini *et al.*, 1994) as well as control of the entry into the cell cycle (Shi *et al.*, 1994).

Perspectives

The present paper shows that the neurotrophins and their receptors have preserved many basic characteristics between mammals and the chick. In particular, the same genes are found in birds and mammals, and the expression patterns of these neurotrophic factors and their receptors are well conserved. Especially in the peripheral neurons it is easy to see shared common features in Trk expression in different neuronal populations during development. This indicates a selective advantage during evolution for maintaining basic neurotrophic mechanisms regulating development and apoptotic cell death in peripheral neurons.

Materials and Methods

In order to isolate DNA fragments encoding the different Trks from chick we have designed degenerate oligonucleotides based on *trkA* and *trkB* gene sequences. cDNA was synthesized and RT-PCR was performed at low stringency (annealing temperatures ranging from 40°C to 50°C). Total RNA was prepared from E9 spinal ganglia and was further selected for polyA$^+$ RNA which was used as template for the synthesis of cDNA. Synthesis was performed according to the manufacturer's protocols (Pharmacia, Uppsala, Sweden). The corresponding amino acid sequences for the 5´ and 3´ PCR primers are NNGDYT and IENPQYF, respectively. The sequence(s) for the PCR fragments are spanning the transmembrane region and ends in the juxtamembrane region. PCR fragments were cloned into pBS KS$^+$ (Stratagene, La Jolla, USA) and the plasmids were subjected to DNA sequencing of both strands, using Sequenace (USB, Cleveland, OH, USA) and $\alpha(^{35}S)$-dATP (Amersham, Buckinghamshire, UK). Full length sequences were screened for in λZAPIIcDNA libraries as described by Williams (1993) using PCR fragments as probes.

RNA preparation and RNase protection assay

Total RNA was isolated from E9 peripheral ganglia as indicated in Fig. 6. RNA was prepared as described previously (Williams *et al.*, 1993). Briefly, 0.1-0.3 g tissue was homogenized in extraction buffer containing guanidinium isothiocyanate and ß-mercaptoethanol using a Polytron homogenizer. The samples were layered on cesium trifluoroacetate and spun in an ultracentrifuge.

The RNase protection assay (RPA) was performed using the RPAII Ribonuclease Protection Assay kit (Ambion, Austin, TX, USA) according to the manufacturer's recommendations. Briefly, cRNA probes for the avian Trk receptors were labeled with α-[^{32}P]-UTP (Amersham, Buckinghamshire, UK) by *in vitro* transcription. The cRNA probes were hybridized to 5 µg of total RNA. After hybridization, the samples were treated with ribonuclease and protected cRNA fragments were analyzed on a denaturing polyacrylamide gel (Williams *et al.*, 1993). The mRNA levels were quantified using a PhosphorImager (Molecular Dynamics) and the mean mRNA levels from two series were calculated and plotted in Figure 6.

In situ hybridization analysis

For *in situ* hybridization, synthetic oligonucleotide probes complementary to the chicken NGF, BDNF, NT-3, trkA, trkB and trkC mRNAs were used. The oligonucleotide complementary to chicken neurotrophin mRNAs correspond to amino acids 61 to 77 of the BDNF protein (Isackson *et al.*, 1991) and amino acids 62 to 78 of the NT-3 mature protein (Hallböök *et al.*, 1993). The oligonucleotides complementary to chicken *trk* receptor mRNAs correspond to the tyrosine kinase domain including amino acids 669 to 686 of the human TrkA receptor (Martin-Zanca *et al.*, 1989), amino acids 696 to 713 of the chicken TrkB receptor (Dechant *et al.*, 1993) and amino acids 683 to 700 of the chicken TrkC receptor (Okazawa *et al.*, 1993). Specificity controls were performed by addition of unlabeled oligonucleotides at 100 times excess together with the labeled probes.

The oligonucleotide probes (50 ng) were labeled at the 3'-end with a-[^{35}S]ATP using terminal deoxynucleotidyl transferase (Promega, Madison, WI) to a specific activity of approximately 1x10^9 cpm/µg. The probes were purified on Nensorb 20 column (DuPont, Wilmington, Delaware, USA) prior to use. *In situ* hybridization was performed as pre-

viously described (Hallböök *et al.*, 1993; Williams *et al.*, 1993) at 42°C for 15 h.

Dissections, cell culture and neurotrophin production

Recombinant NGF, BDNF and NT3 proteins were prepared by electroporation of cells with transient expression vector constructs containing DNA fragments encoding the human neurotrophins. $1x10^6$ COS cells were electroporated with 30 µg of neurotrophin expression vector constructs in phosphate buffer at 400 V and 250 µF at 22°C as previously described in detail (Kullander and Ebendal, 1994). Concentration of recombinant neurotrophins as calculated in relation to NGF, the concentration of which was measured using an enzyme immunoassay (Söderström *et al.*, 1990).

Computer analysis of amino acid sequences

We have used the computer softwares PAUP (Swofford and Olsen, 1990) and MacClade for phylogeny analysis of parsimonious relationships of the neurotrophin sequences to calculate and visualize the evolutionary relationship. A heuristic search for the shortest trees composed of the neurotrophin sequences using the chicken TGFb sequence (Barnett *et al.*, 1994) as an outgroup, gave 21 equally short and equally probable trees. The NGF sequences for mouse, rat, the African rat *Mastomys natalensis*, guinea pig, human, cobra, *Xenopus* and chicken have been presented (Scott *et al.*, 1983; Ullrich *et al.*, 1983; Ebendal *et al.*, 1986; Fahnestock and Bell, 1988; Whittemore *et al.*, 1988; Schwarz *et al.*, 1989). The sequences for BDNF from pig, human, rat, chick and *Xiphophorus* are from Hohn *et al.* (1990), Leibrock *et al.* (1989) and Maisonpierre *et al.* (1990b). NT-3 sequences are taken from Ernfors *et al.* (1990), Hohn *et al.* (1990), Kaisho *et al.* (1990), Maisonpierre *et al.* (1990a) and Rosenthal *et al.* (1990). Finally the NT-4 sequences from *Xenopus laevis*, human and rat are taken from Hallböök *et al.* (1991), Berkemeier *et al.* (1992; named NT-5) and Ip *et al.* (1992). The chicken TGFß II receptor (Barnett *et al.*, 1994) was used as an outgroup to root the tree for *trk* receptor sequences . The heuristic search only gave rise to one parsimonious phylogram. The different Trk sequences have been presented by Klein *et al.* (1991a), Lamballe *et al.* (1991), Merliot *et al.* (1992) and Squinto *et al.* (1991). The trees were imported to the computer program MacClade for layout design.

DNA fragmentation analysis

Sympathetic ganglia from E9 chicken embryos were dissected, washed in PBS and treated with 0.25 % trypsin in PBS for 15 min at 37°C. Trypsin was inhibited by serum, the ganglia were trituated with a fine glass pipette and the dissociated sympathetic neurons were washed, counted and plated at a density of 400000 cells in 35 mm dish. Neurons were grown in HAM's F12 medium (GIBCO BRL) supplemented with 10 ng/ml of NGF and 2 mM glutamate in dishes coated with poly-D-lysine and laminin. Neurons were first kept for 36 h with NGF and then washed to remove the NGF, dead cells and debris. Blocking anti-NGF antibodies capable to inhibit 20 ng/ml NGF were added to the medium to deplete NGF. Cultures were harvested after 0, 10, 15, 20, 25 and 35 h and analysis for fragmentation of DNA was performed. Cells were collected by centrifugation, re-suspended in 50 ml of lysis buffer (1 mg/ml proteinase K, 0.5% SDS, 10 mM EDTA, 10 mM Tris-HCl) and incubated for 30 min at 45°C. The lysate was phenolized and run on a 1.2% agarose gel with subsequent Southern blot analysis using a probe consisting of labeled complete chicken genomic DNA digested with Mbo1 restriction enzyme. The fragmented DNA was visualized by autoradiography using X-ray film (Fig. 11).

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