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 acquisition 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; Maisonnier 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 (p75NGFR) (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...
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 p21ras 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 p75NGFR 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. 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.

Expression 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 (Hällböök 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.
vorous 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; Ibañez 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; Ibañez 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. 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.
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 responses to NT-3 were 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-elicted 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
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 (Emfors et al., 1990). In contrast, NGF did not affect the ciliary ganglion at any time between E5-E18 (Ebenholtz et al., 1979; Ebenholtz et al., 1990).

In contrast to the three autonomic ganglia examined, the sensory trigeminal ganglion (Hedlund and Ebenholtz, 1978) responded to both NGF and NT-3 during development (Figs. 7-9). As described before (Ebenholtz 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 Ebenholtz, 1980; Davies and Lindsay, 1985; Ebenholtz 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 (Ebenholtz, 1979; Ebenholtz 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.

Fig. 10A. The topology of the phylograms with respect to each neurotrophin is in agreement with the consensus evolutionary rela-
relationship 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 (Smyame 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 (Emfors 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 thermosensation, 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 depleting 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-

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**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 TGFβ (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 TGFβ 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.
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controlof the entry into the cell cycle (Shi et al., 1994) as well as
the function of these genes and their mammalian homologs are not clear but results suggest that the
products are involved in inhibition of lipid peroxidation by
reactive oxygen intermediates (Hockenbery 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 apop-
tosis 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 neu-
rotrophic factors and their receptors are well conserved.
Especially in the peripheral neurons it is easy to see shared com-
mon features in Trk expression in different neuronal populations
during development. This indicates a selective advantage during
evolution for maintaining basic neurotrophic mechanisms regu-
lating development and apoptotic cell death in peripheral neu-
rons.

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 per-
formed 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 pro-
tocols (Pharmacia, Uppsala, Sweden). The corresponding amino acid
sequences for the 5' and 3' PCR primers were NNGDYT and IENPQYF,
respectively. The sequence(s) for the PCR fragments are spanning the
membrane 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 α[32P]-dATP (Amersham,
Buckinghamshire, UK). Full length sequences were screened for in
JZAP1cDNA libraries as described by Williams (1993) using PCR frag-
ments 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 β-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[32P]-dATP (Amersham,
Buckinghamshire, UK) by in vitro transcription. The cRNA probes were
hybridized to 5 μg of total RNA. After hybridization, the samples were
untreated 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 5.

In situ hybridization analysis

For in situ hybridization, synthetic oligonucleotide probes comple-
mentary 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 73 of the NT-3 mature pro-
tein (Hallböök et al., 1993). The oligonucleotides complementary to
chicken trk receptor mRNAs correspond to the tyrosine kinase domain
including amino acids 669 to 688 of the human TrkA receptor (Martin-
Zanca et al., 1989), amino acids 696 to 713 of the chicken TrkB recep-
tor (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 unlabelled oligonucleotides at 100 times excess together with
the labeled probes.

The oligonucleotide probes (50 ng) were labeled at the 3'-end with α-
[32P]-dATP using terminal deoxynucleotidyl transferase (Promega,
Madison, WI) to a specific activity of approximately 1x10⁶ cpm/μg. The
probes were purified on Norsorb 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 elec
troporation 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 con-
structs 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 cen-
tration 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 relations-
ships of the neurotrophin sequences to calculate and visualize the evol-
utionary relationship. A heuristic search for the shortest trees composed
of the neurotrophin sequences using the chicken TGFß 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 by Klein et al., 1990a and Rosenthal
Maisonpierre (1990), Leibrock et al. (1989) and
Maisonpierre et al. (1990b). NT-3 sequences are taken from
D-Iysine and laminin. Neurons were first kept for 36h with NGF and then
antibodies capable to inhibit

Neurons were grown in HAM’s F12 medium (GIBCOBRL) supplemen-
ted with 20 ng/ml NGF, BDNF and NT3proteins were prepared by elec-
troporation of cells with transient expression vector constructs contain-
ing DNA fragments encoding the human neurotrophins. 1x10^6 COS cells
were electroporated with 30 µg of neurotrophin expression vector con-
structs 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 cen-
tration of which was measured using an enzyme immunoassay
(Söderström et al., 1990).

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