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The Neurotrophic Hypothesis: Where does it Stand?

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The neurotrophic hypothesis: where does it stand?

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SUMMARY

In the developing peripheral nervous system many neurons die shortly after their axons reach their target fields. This loss is thought to match the number of neurons to the size and requirements of their target fields because altering target field size before innervation affects the number of neurons that survive. The neurotrophic hypothesis provides an explanation for how target fields influence the size of the neuronal populations that innervate them. This hypothesis arose from work on nerve growth factor (NGF), the founder member of the neurotrophin family of secreted proteins. Its principal tenet is that the survival of developing neurons depends on the supply of a neurotrophic factor that is synthesized in limiting amounts in their target fields. The neurotrophic hypothesis has, however, been broadened by the demonstration that multiple neurotrophic factors regulate the survival of certain populations of neurons. For example, some neurons depend on several different neurotrophic factors which may act concurrently or sequentially during target field innervation. In addition, there are aspects of neurotrophin action that do not conform with the classic neurotrophic hypothesis. For example, the dependence of some populations of sensory neurons on particular neurotrophins before significant neuronal death takes place raises the possibility that the supply of these neurotrophins is not limiting for survival at this stage of development. There is also evidence that at stages before and after sensory neurons depend on target-derived neurotrophins for survival, neurotrophins act on at least some sensory neurons by an autocrine route. Yet despite the growing wealth of information on the multiple roles and modes of action of neurotrophic factors, the neurotrophic hypothesis has remained the best explanation for how neuronal target fields in the developing peripheral nervous system regulate their innervation density.

1. EVIDENCE FOR THE NEUROTROPHIC HYPOTHESIS

The neurotrophic hypothesis (Levi-Montalcini & Angeletti 1968; Thoenen & Barde 1980; Purves 1988) gained substantial support from work on NGF and has been further substantiated by studies of more recently identified neurotrophic factors. The most important direct evidence for the neurotrophic hypothesis is the demonstration that populations of developing neurons that are supported by NGF *in vitro*, namely sympathetic neurons and certain kinds of sensory neurons, also depend on NGF *in vivo*. Anti-NGF antibodies administered during the phase of target field innervation eliminate these neurons whereas exogenous NGF rescues neurons that would otherwise die (Levi-Montalcini & Angeletti 1968; Johnson *et al.* 1980; Hamburger & Yip 1984). Likewise, these same neurons are lost in mice that have targeted null mutations in the NGF gene (Crowley *et al.* 1994) or the NGF receptor tyrosine kinase (*trk*A) gene (Smeyne *et al.* 1994).

NGF synthesis commences in the peripheral target fields of sensory and sympathetic neurons with the arrival of the earliest axons (Davies *et al.* 1987; Korsching & Thoenen 1988). At the onset of neuronal death in sensory ganglia, the level of NGF mRNA different cutaneous territories is proportional to their final innervation density; high levels in future densely innervated territories and low levels in future sparsely innervated territories (Harper & Davies 1990). After uptake by sensory and sympathetic fibres in their target fields, NGF is conveyed by fast axonal transport to the cell bodies of the innervating neurons where it exerts its survival-promoting effects (Hendry *et al.* 1974; Korsching & Thoenen 1983).

The purification of brain-derived neurotrophic factor (BDNF) and studies of the physiological significance of this factor extended the generality of the neurotrophic theory to a second neurotrophin (Barde *et al.* 1982). BDNF promotes the survival of subsets of embryonic sensory neurons *in vitro* and prevents loss of these neurons *in vivo* when administered to embryos during the phase of naturally occurring neuronal death (Hofer & Barde 1988). Accordingly, mice with targeted null mutations in the BDNF gene (Ernfors *et al.* 1994*a*; Jones *et al.* 1994) or BDNF receptor tyrosine kinase (*trk*B) gene (Klein *et al.* 1993) have specific deficiencies in BDNF-dependent neurons.

Studies of the timing of the neurotrophin survival response have provided additional evidence that supports the neurotrophic hypothesis. Sensory neurons initially survive independently of neurotrophins when their axons are growing to their targets (Davies & Lumsden 1984; Ernsberger & Rohrer 1988; Davies 1989) and become dependent on neurotrophins for survival shortly after their axons reach their peripheral targets (Buchman & Davies 1993; Vogel & Davies 1991) which is associated with a marked increase in the expression of the respective neurotrophin receptors

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(Wyatt *et al.* 1990; Wyatt & Davies 1993; N. Ninkina & A. M. Davies, unpublished results). *In vitro* studies of different populations of cranial sensory neurons have shown that the duration of neurotrophin independence and the timing of the neurotrophin survival response is controlled by an intrinsic timing mechanism in the neurons (Vogel & Davies 1991). The neurons of the vestibulocochlear, geniculate, petrosal and nodose ganglia are derived from neurogenic placodes, are born during the same period of development, but differ in the distances their axons have to grow to reach their peripheral and central targets. Vestibulocochlear neurons, have the closest targets and survive without neurotrophins for only a short time before becoming dependent on BDNF for survival. Nodose neurons have the most distant targets and survive for the longest time without neurotrophins before becoming BDNF dependent. Geniculate and petrosal neurons have intermediate target distances and survive for intermediate times before becoming BDNF dependent. Studies of the survival characteristics of neurons that differentiate *in vitro* from neurogenic placodal cells suggests that the duration of neurotrophin independence is programmed in sensory neuron progenitor cells (Vogel & Davies 1991). Furthermore, heterotopic grafting experiments have shown that the presumptive placodal ectoderm is not yet specified to differentiate into neurons but that signals acting in the vicinity of this ectoderm commits to the cells to a particular neuronal fate (Vogel & Davies 1993).

The molecular cloning of BDNF and the recognition of the homology between NGF and BDNF (Leibrock *et al.* 1989) paved the way for the molecular cloning of additional members of the neurotrophin family, which include neurotrophin-3 (NT3) (Ernfors *et al.* 1990; Hohn *et al.* 1990; Jones & Reichardt 1990; Rosenthal *et al.* 1990) and neurotrophin-4 (NT4) (Berkemeier *et al.* 1991; Hallbook *et al.* 1991; Ip *et al.* 1992). Much of the work on the specific neurotrophin survival requirements of different kinds of sensory neurons during the phase of naturally occurring neuronal death has been carried out on dorsal root ganglion (DRG) neurons. Because DRG contain a variety of functionally distinct classes of sensory neurons for which few *in vitro* markers were available until relatively recently, early work on these neurons was hampered by the inability to determine which kinds of sensory neurons respond to a particular neurotrophin in culture. To circumvent the shortcomings of using cultured DRG neurons and determine if the neurotrophin survival requirements of sensory neurons are correlated with their specific sensory function, *in vitro* studies were carried using cranial sensory neurons which are segregated into groups that serve different sensory modalities. These studies provided the first clear evidence that the neurotrophin requirements of sensory neurons during the phase of naturally occurring neuronal death are broadly related to sensory modality (for reviews, see Davies 1987, 1994). Recent analysis of the kinds of DRG neurons that express different *trk* genes (Mu *et al.* 1993; McMahon *et al.* 1994) and analysis of sensory neuron loss in mice with mutated neurotrophin genes (Crowley *et al.* 1994; Ernfors *et al.* 1994*a*,*b*; Jones *et al.* 1994; Conover *et al.* 1995; Liu *et al.* 1995) and neurotrophin receptor genes (Klein *et al.* 1993 1994; Smeyne *et al.* 1994) has reinforced this conclusion.

2. REFINEMENT OF THE NEUROTROPHIC HYPOTHESIS

In its original form, the neurotrophic hypothesis proposed that for survival, each population of neurons depends on the supply of a single neurotrophic factor from its target field. That multiple neurotrophic factors cooperate in regulating the survival of certain populations of neurons became recognized with the discovery of additional neurotrophins. The first evidence that two different neurotrophins regulate the survival of the same population of neurons came from *in vitro* studies of the proprioceptive neurons of the trigeminal mesencephalic nucleus (TMN). These neurons are supported during the phase of neuronal death by BDNF (Davies *et al.* 1986*b*) and a muscle-derived factor (Davies 1986) that has the same neuronal specificity as NT3 (Hohn *et al.* 1990). Saturating levels of either factor is capable of supporting the survival of almost all of these neurons in culture and low levels of these factors have a synergistic effect on survival (Davies *et al.* 1986*a*). The physiological relevance of these *in vitro* observations has recently been substantiated by analysis of the phenotype of mice with null mutations in the BDNF and NT3 genes. Neuron counts in the TMN of neonatal mutant mice have shown that, compared with wild type mice, there is an approximate 50% reduction in both $BDNF^{-/-}$ mice (Ernfors *et al.* 1994*a*; Jones *et al.* 1994) and $NT3^{-/-}$ mice (Ernfors *et al.* 1994*b*).

In addition to the contemporaneous cooperation of different target-derived neurotrophins in regulating the sensory neuron survival, recent work suggests that several different target-derived neurotrophins can act sequentially to promote the survival of developing sensory neurons. Evidence for this came from *in vitro* studies of embryonic mouse trigeminal ganglion neurons (Buchman & Davies 1993; Davies *et al.* 1993). When these neurons are grown at very low density in defined medium at the stage when their axons normally reach their peripheral targets *in vivo*, all of the neurons die unless BDNF, NT3 or NT4, but not NGF, are present in the culture medium. Over the next few days of development, the neurons acquire a survival response to NGF while, at the same time, losing responsiveness to BDNF, NT3 and NT4. The loss of the BDNF survival response is associated with a marked increase in the expression of *trk*B transcripts encoding non-catalytic receptors (N. Ninkina & A. M. Davies, unpublished observations) and a marked shift in the BDNF dose response to higher concentrations (Buj-Bello *et al.* 1994).

The physiological relevance of the early *in vitro* responses of trigeminal neurons to BDNF and NT3 has been strengthened by analysis of mice with null mutations in genes encoding neurotrophins and their receptors. The discovery that $BDNF^{-/-}$ mice (Ernfors *et al.* 1994*a*; Jones *et al.* 1994), but not $NT4^{-/-}$ mice

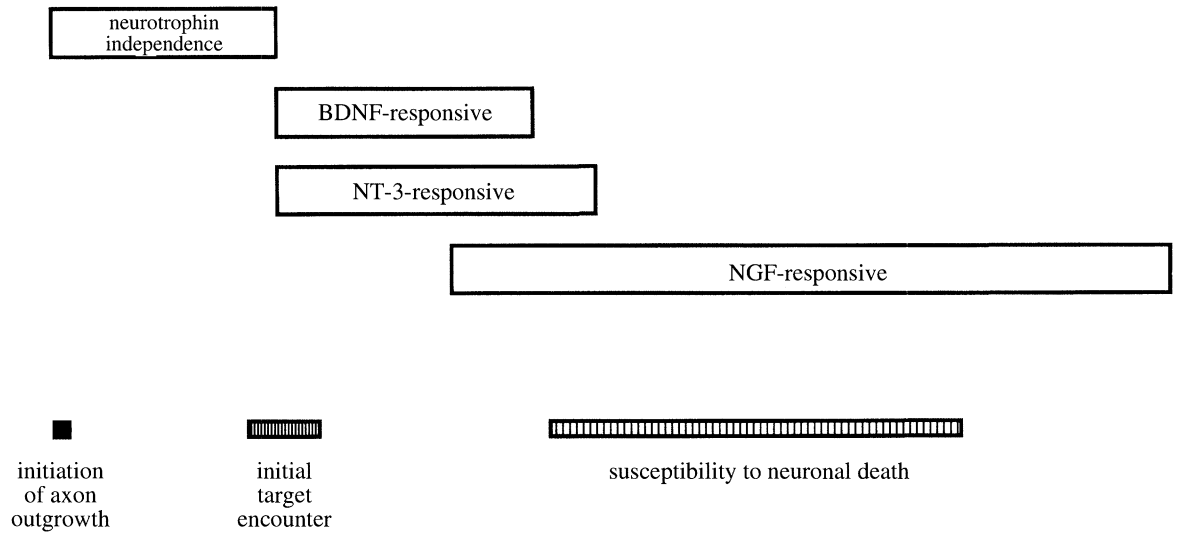

Figure 1. Schematic illustration of the developmental changes in the survival requirements of embryonic mouse trigeminal neurons. The life history of an early trigeminal ganglion neuron is represented from left to right (not necessarily drawn to scale). The onset of axon outgrowth, the approximate time when the peripheral axon comes into proximity with its peripheral target and the period of development during which the neuron is most susceptible to being eliminated by cell death are shown along the lower part of the diagram. From the onset of axon outgrowth to the time the axon comes into proximity with its peripheral target, the neuron survives independently of neurotrophins (upper box). The neuron then becomes responsive to both BDNF and NT3. After a transitional period of responsiveness to BDNF, NT3 and NGF, the BDNF and NT3 responses are then lost, leaving the neuron dependent on NGF for survival during the time when it is competing with other neurons for a limiting supply of NGF.

(Conover *et al.* 1995; Liu *et al.* 1995), have marked reductions of the numbers of neurons in the trigeminal ganglion (between 56–73 % less than normal), suggests that BDNF, but not NT4, is important in maintaining the survival of trigeminal neurons. Because only a small minority of trigeminal neurons depend on BDNF (5–10 %) for survival during the period of development when the number of neurons in the ganglion is decreasing as a result of naturally occurring neuronal death (Buchman & Davies 1993), it is likely that the markedly decreased number of trigeminal neurons in the BDNF$^{-/-}$ mice results from premature neuronal death during the stage of BDNF responsiveness. The physiological relevance of the early BDNF survival response is further strengthened by the substantially reduced neuronal complement of the trigeminal ganglion in *trk*B$^{-/-}$ neonates (60 % less than normal) (Klein *et al.* 1993). The response of early trigeminal neurons to NT3 observed *in vitro* may also be physiologically relevant because the neuronal complement of the trigeminal ganglion in NT3$^{-/-}$ mice is also reduced by 64 % compared with wild type mice (Ernfors *et al.* 1994*b*). Moreover, neuronal death in the trigeminal ganglion of *trk*B$^{-/-}$ mice occurs earlier than in wild type embryos, suggesting that BDNE is required for survival in the early stages of trigeminal development (L. Pinon, L. Minchiello, R. Klein & A. M. Davies, unpublished observations). Figure 1 illustrates the changing survival requirements of developing trigeminal neurons to neurotrophins.

3. ASPECTS OF NEUROTROPHIN ACTION THAT DO NOT CONFORM WITH THE NEUROTROPHIC HYPOTHESIS

Although several novel roles have been discovered for neurotrophic factors that do not involve the regulation of neuronal survival, such as affecting the proliferation and differentiation of neuron progenitor cells and regulating the expression of several differentiated traits of neurons throughout life (reviewed by Davies 1994), these functions of neurotrophins do not come within the bounds of the neurotrophic hypothesis. These studies demonstrate that neurotrophins are multi-functional proteins, but do not invalidate the neurotrophic hypothesis. Recent work has, however, shown that neurotrophins may act on neurons in ways that seem to contradict aspects of the neurotrophic hypothesis.

(*a*) *Non-limiting supplies of neurotrophins in the target field*

The neurotrophic hypothesis, which has traditionally focused on the neurotrophic factor requirements of neurons undergoing naturally occurring neuronal death, proposes that neurotrophic factors are synthesized in limiting amounts so that only the required number of neurons are able to procure enough factor to survive (Thoenen & Barde 1980; Davies 1987). However, the demonstration that embryonic mouse trigeminal neurons are dependent on BDNF and NT3 during the early stages of target field innervation (Buchman & Davies 1993) before significant neuronal death takes place in the ganglion (Davies & Lumsden 1984) implies that BDNF and NT3 are produced in

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sufficient quantities to ensure that the existing compliment of neurons is maintained. That is, the target-derived supply of BDNF and NT3 neurotrophins during this stage of development is probably not limiting. Indeed, it is possible that the reason why early trigeminal neurons exhibit a transitory survival response to BDNF and NT3 may be to sustain the survival of the neurons whose axons reach the target field during the early stages of its innervation. This may delay the onset of neuronal death in the trigeminal ganglion until most of the neurons have started to innervate the target field, thereby ensuring that the majority of neurons compete for a limiting supply of NGF during the same period of development. It may be advantageous for most of the neurons that innervate a given target field to compete for survival at the same time because this would maximize the choice for selectively maintaining neurons on the basis of the appropriateness of their axon terminations in the target field.

(*b*) *Autocrine supply of neurotrophins in developing sensory neurons*

In accordance with the neurotrophic hypothesis, numerous studies have shown that neurotrophins are expressed in the peripheral and central targets of developing sensory neurons. However, the detection of BDNF mRNA and NT3 mRNA in subsets of DRG neurons (Ernfors *et al.* 1988; Schecterson & Bothwell 1992) has raised the possibility that sensory neurons could obtain BDNF or NT3 by paracrine or autocrine routes operating in the ganglion. The first direct experimental evidence for the operation of a neurotrophin autocrine loop came from *in vitro* studies of DRG neurons isolated from chicken embryos at the stage when their axons are growing to their targets (Wright *et al.* 1992). These early sensory neurons undergo a clearly recognizable morphological change during the first 24 h in culture before they become dependent on added neurotrophins for survival. Initially the neurons have small, spindle-shaped, phase-dark cell bodies and short neurites. Subsequently they develop spherical, phase-bright cell bodies and extend long neurites. Although the rate at which this maturational change takes place is accelerated by exposure to either BDNF or NT3, the neurons need not obtain these factors from other cells to mature because they still mature when cultured as single cells in separate culture wells containing chemically defined medium without added neurotrophins. Because RT/PCR revealed that early DRG cells express BDNF mRNA, antisense BDNF oligonucleotides were used to investigate if BDNF might act by an autocrine route to promote maturation. Each of three different antisense BDNF oligonucleotides, but none of the corresponding sense control oligonucleotides, reduced by 40–50 % the number of neurons that underwent the maturational change. The inhibition of neuronal maturation by antisense BDNF oligonucleotides was not due to a non-specific reduction of protein synthesis because the effect of the oligonucleotides could be specifically reversed by adding very low concentrations of BDNF to the culture medium. The antisense BDNF oligonucleotides did not affect the survival of early DRG neurons, indicating that the BDNF autocrine loop is not required for survival at this stage.

The expression of BDNF mRNA in a subset of embryonic DRG neurons when naturally occurring neuronal death is taking place (Schecterson & Bothwell 1994), has raised the possibility that BDNF may act by an autocrine route in some neurons during this period of development. However, measurement of BDNF mRNA in different populations of cranial sensory neurons that respond to either NGF or BDNF has shown that during the phase of naturally occurring neuronal death BDNF mRNA is expressed predominantly in neurons that respond to NGF, not BDNF (M. Robinson & A. M. Davies, unpublished observations). Thus during the period of naturally occurring neuronal death BDNF is unlikely to act by an autocrine route in at least most cranial sensory neurons. This is consistent with the neurotrophic hypothesis because an autocrine loop at this stage in development would interfere with the selection of neurons by target-derived neurotrophins. It is possible that the BDNF made by NGF-dependent sensory neurons during this stage acts on other cells such as the neurons in the CNS with which they synapse.

Recent work using antisense-BDNF oligonucleotides in cultures of adult DRG neurons has demonstrated the operation of a BDNF autocrine loop in a subset of these neurons (Acheson *et al.* 1995). In contrast to early sensory neurons, the BDNF autocrine loop at this stage appears to be required for survival. Although this loop operates long after limiting levels of target-derived neurotrophins regulate neuron number along the lines of neurotrophic hypothesis, this work provides a clear example of how neurons may depend on neurotrophins for survival yet obtain these from sources other than their targets.

4. CONCLUSIONS

Since its formulation, the neurotrophic hypothesis has remained an attractive scheme for explaining how neuronal target fields in the peripheral nervous system regulate their innervation density. Although recent work has shown that neurotrophins and other neurotrophic factors have functions in addition to regulating neuronal survival and may act on neurons in ways that were not originally anticipated, the neurotrophic hypothesis has gained increasing experimental support. Although there may be particular circumstances in which not all of the tenets of this hypothesis are satisfied in the regulation of neuronal survival during development, there is no experimental evidence to cast serious doubt on the validity of this hypothesis. There is, however, one important caveat. The experimental validation of the neurotrophic hypothesis has relied almost exclusively on studies of neurons of the peripheral nervous system. With the exception of motoneurons, which innervate clearly defined muscle groups in the periphery, relatively little is known about the control of neuronal survival in the CNS. The extent to which different classes of CNS neurons depend on

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neurotrophic factors for survival and whether they obtain these by local autocrine or paracrine routes, from post-synaptic or pre-synaptic neurons or from other cells in their projection territories or local environments is largely unknown. This important and challenging work will confirm or refute the generality of the neurotrophic hypothesis.

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