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Neurotrophin-3 as an Essential Signal for the Developing Nervous System

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Abstract

Rapid advances in characterization of the biological actions mediated by the third member of the neurotrophin family, neurotrophin-3 (NT-3), have been made recently in vitro as well as *in situ*. These have been made possible by the cloning of the genes for NT-3 and for its transducing receptor tyrosine kinase TrkC. This article will focus on the roles of NT-3 in the nervous system. *In situ* localization of NT-3 consistent with that of its receptor is manifested at all developmental stages studied and into adulthood. Through TrkC, NT-3 signals a number of trophic effects, ranging from mitogenesis, promotion of survival, or differentiation, depending on the developmental stage of the target cells. The sites of action of NT-3 reside primarily in the peripheral nervous system (PNS), various areas of the central nervous system (CNS), and in the enteric nervous system (ENS). Analyses of the phenotypes of transgenic mice lacking NT-3 or injection of embryos with a blocking antibody have so far revealed the essential role of NT-3 in development of specific populations of the PNS, and in particular of proprioceptive, nodose, and auditory sensory neurons and of sympathetic neurons. The actions of NT-3 also extend to modulation of transmitter release at several types of synapses in the periphery as well as in the adult CNS. In addition, NT-3 may play a role in the development of tissues other than the nervous system, such as the cardiovascular system. Future investigations will widen the understanding of the many roles of NT-3 on both neuronal and nonneuronal cells.

Index Entries: Neurotrophic factors; NT-3; differentiation; neurons; glia; peripheral; enteric; central nervous systems; TrkC; receptor isoforms; NT-3 antibody; transgenic mice.

Introduction

Five years ago five research groups independently cloned neurotrophin-3 (NT-3), the third member of the nerve growth factor (NGF) family of growth factors *(1–5)*. This article focuses on NT-3 because of its many roles both during development and maturity of the nervous system. In the next section the roles of NT-3 will be considered and discussed in a chronological

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sequence from the earliest embryonic stages of neuronal and glial precursor cells, to the subsequent fetal stage at which neuroblasts and glioblasts become postmitotic, to the intermediate perinatal stage, to the mature (postnatal) period, and finally into the aging nervous system. In the following section, the properties and expression of the specific transducing tyrosine kinase receptor for NT-3, TrkC, and its isoforms will be considered. Developmental expression of TrkC will be presented in the same chronological sequence. Data collected from both in vitro and in vivo approaches analyzing effects of exogenous NT-3, as well as data obtained from the phenotypes of transgenic mice lacking the genes either for NT-3 or TrkC, will be described and correlated.

Neurotrophin-3

Brief Description of the NT-3 Molecule

When the sequence of NT-3 was analyzed, common characteristics were revealed with those of its relatives NGF and brain-derived neurotrophic factor (BDNF). This includes two forms of precursors, a short and a long one (termed I and II), that are sequentially cleaved. The mature form of NT-3 has a canonical protease cleavage sequence (Arg-Arg-Lys-Arg) that is followed by the NH_2-terminus and, in contrast to NGF but similar to BDNF, the sequence shows no proteolytic modification at the COOH terminus. There is also a glycosylation acceptor site just upstream of the presumptive cleavage site. The mature form of NT-3 comprises a span of 119 amino acids (aa) with the seven NH_2-terminal aa completely different from either NGF or BDNF and the six cysteine residues found in NGF and BDNF completely conserved *(4)*. There are 54 aa identities with NGF and BDNF, including the cysteines *(2)*. Rat NT-3 shares 57% aa identity with rat NGF and 58% aa identity with rat BDNF *(4)*. Determination of the three-dimensional structure of NT-3 can be anticipated to provide further information about its interactions with the pan-neurotrophin binding receptor p75 and with TrkC. Because of the profound similarities found with the sequences of NGF and BDNF, it was deduced at the time of its cloning that NT-3 would exert neurotrophic activities.

Role of NT-3 on Primary Multipotent Neural Crest Cells, Progenitor Cells of the CNS, and Neural Crest-Derived Cells of the ENS

Neuronal Precursors

NT-3 can act as a mitogen on migrating neural crest cells isolated from quail embryos (at the 25 somite stage) and grown in homogeneous cultures. This proliferative effect is enhanced by the presence of somites, suggesting that other signals of somitic origin can modulate the response of these cells to NT-3 *(6)*. In contrast, when neural tubes (NT) of an earlier stage (15–20 somite stage), are explanted and 45 h later clusters of neural crest cells that had migrated away from the NT are excised and grown on other cells transfected with the NT-3 gene, a majority of the clusters are stimulated to grow neurites. Furthermore, NT-3 promotes in a dose-dependent fashion an increase in survival of dissociated neural crest cells grown on mesodermal cells. These results indicate several roles of NT-3 on proliferation, survival, and differentiation of distinct subpopulations of neural crest cells *(7)* depending, for example, on whether they are committed to become nonneuronal vs neuronal cells. Consistent with these effects is the expression of NT-3 mRNA from the earliest stage, E1–2, in quail embryo *(8)* and in E11–12 rat embryos *(9)* and the presence of NT-3 protein in neuroepithelial cells of the NT in E2 and E3 quail embryos *(7)*. Of all the neurotrophins, NT-3 is expressed at the highest level in E12–13 rat spinal cord *(9)*. In combination with basic fibroblast growth factor (bFGF), NT-3 can increase the proliferation of pluripotent crest cells as well as their adrenergic, but not melanogenic potential *(10)*. NT-3 can also promote the differentiation (but not survival) of avian NT progenitor cells from

the 15–18 somite stage into motor neurons. This NT-3 effect is observed in cultures enriched with precursor cells of motoneurons expressing the islet-1 gene. The proliferative rate of these precursor cells remains unchanged by NT-3 *(11)*.

The enteric nervous system (ENS), like the peripheral nervous system (PNS), originates from precursor cells derived from the neural crest that, after following specific pathways of migration, invade the entire gut. These enteric crest-derived cells respond to diffusible factors as well as extracellular matrix proteins (i.e., laminin) generated by the microenvironment of the gut by differentiating into the enteric submucosal and myenteric plexuses. Enteric crest-derived cells that have completed their migration to the gut can be isolated from within the gut wall by magnetic immunoselection utilizing the primary antibody NC-1 (identical to HNK-1), which recognizes a neural crest epitope, and magnetic beads coated with the appropriate species-specific secondary antibodies. NT-3 can induce cultured NC-1-immunoselected cells from E14 fetal rat gut to differentiate as neurons or glia. Of all the neurotrophins tested, NT-3 is the only one to elicit this effect. NT-3 does not change the proliferative rate of these cultured enteric precursors, but promotes neuritic outgrowth of the postmitotic neuronal cells *(12)*. Interestingly, a striking stimulation of fiber outgrowth occurs with NT-3 in the ganglion of Remak *(1)*, an autonomic ganglion in birds that is formed from crest-derived cells of similar origin to those that form the ENS. Consistent with this differentiating role for NT-3 in the developing ENS is the presence of transcripts for NT-3 localized to the enteric mesenchyme of avian fetal gut (C. Kalcheim, private communication) and to the submucosa of the developing stomach in fetal rat *(13)*. In vitro studies utilizing a blocking antibody to NT-3 *(14)* should elucidate whether the mesenchymal cells of cultures derived from fetal bowel actually release endogenous NT-3 and whether this NT-3 is also a survival factor for the differentiated enteric neurons.

Glial Precursors

NT-3 promotes the survival of pure rat postnatal optic nerve 02A progenitor cells of oligodendrocytes *(15)*. In combination with platelet-derived growth factor (PDGF), however, NT-3 increases the proliferative capacity as well as the survival of differentiated oligodendrocytes *(16)*. In contrast, NT-3 was shown not to affect the proliferative capacity of precursors of enteric glia, but promoted their differentiation/survival *(12)*. Thus, NT-3 can also affect the development of those crest-derived cells that evolve into nonmyelinating glia.

Role of NT-3 on Committed Neuroblasts

Sympathetic Neuroblasts

NT-3 controls the number of precursors of sympathetic neurons whether they are still proliferating or whether they have undergone their final mitosis. In vitro analyses have demonstrated that sympathetic precursors from E14–15 rat superior cervical ganglion (SCG) are dependent on NT-3 for survival, particularly those that have ceased to proliferate. This dependency is concomitant with a high mRNA expression of the tyrosine kinase receptor for NT-3, TrkC, in the same age ganglia. TrkC mRNA expression diminishes as development proceeds to be eventually replaced by a dependency on NGF and the reciprocal appearance of TrkA mRNA expression *(17,18)*. Furthermore, these data are consistent with the expression of NT-3 mRNA in a subpopulation of neurons in the embryonic SCG at comparable developmental stages *(19)*. These findings support an autocrine or paracrine role of NT-3 on sympathetic neuroblasts.

Sensory Neuroblasts

Early during development, cultured neurons from the dorsal root ganglia (DRG) of E4.5 chick can undergo accelerated maturation when exposed to NT-3 before they have developed a dependency for survival for exogenous NT-3 or other neurotrophins *(20)*. Since the transcripts for NT-3 are already present in DRGs

of the E2.5 chick *(21)*, one can invoke an autocrine effect of NT-3 at this early stage in these neurons, which, like the sympathetic neurons, are derived from the neural crest. At a later stage (E8), chick spinal sensory neurons continue to respond to NT-3 with extended neuritic outgrowth. This neuritogenesis also occurs in mouse and rat DRGs and also in E8 chick nodose ganglionic neurons, which are derived from the placode *(1,2,4,5)*. It is to be noted that mouse E12–13 trigeminal sensory neurons (both neural crest- and placode-derived) can be supported by NT-3 prior to developing their dependency on NGF and the period of naturally occurring cell death *(22)*. This transient state of responsiveness to NT-3 is analogous to that observed for sympathetic neuroblasts. Furthermore, concomitant expression of NT-3 mRNA peaks at E12–13 in the maxillary peripheral target field of innervation of the trigeminal ganglionic neurons *(22)*. Interestingly, sensory DRG from cervical and lumbar levels of chick embryos that comprise neurons innervating limb muscles specifically respond to NT-3 *(23)*, and NT-3 mRNA transcripts are also present in limb buds *(24)*. The expression of NT-3 mRNA has been reported to occur in DRGs of various species: E7 quail *(7)*, E13.5 rat DRGs where it is found expressed at high level *(25,26)*, and E15.5 mouse *(19)*. In addition, at a later stage, systemic administration of recombinant NT-3 to E14–19 rat embryos has a specific trophic effect in increasing the size of the large DRG sensory neuron perikarya (presumably of the proprioceptive type) *(27)*.

In the early chick retina (E7), NT-3 stops proliferation and induces differentiation of the neuroepithelial cells via a high affinity mechanism. By E14, NT-3 was found to exert trophic support of postmitotic differentiated amacrine and ganglion cells. Two distinct peaks of expression of NT-3 receptors occur, one at E6–7 and another at E14 *(28)*. Furthermore, during development of the mammalian visual system the use of a crosslinking agent has demonstrated that NT-3 and TrkC are coexpressed at all stages in the retina.

In the auditory system, NT-3 mRNA recently has been localized to the developing inner ear, particularly in the hair cells of the organ of Corti *(25,29–32)*. The hair cells constitute the peripheral target for the neurons of the spiral ganglion. Concomitantly, in the developing ear these spiral ganglion neurons express TrkC (i.e., ref. *25* and *see* Expression of *trk*C During Development, Loss vs Maintenance of Expression in the Adult). Consistent with these *in situ* analyses, culture studies of explanted cochleovestibular ganglia (CVG) from early chick embryos (E3) show powerful mitogenic effect of NT-3 *(33)* on these ganglion cells. At a later stage of development (chick E7 and rat E13) NT-3 was shown to exert survival and neuritic outgrowth effects on these CVG neurons *(32,34)*. Overall these data gathered from developing sensory neurons of various modalities point to numerous trophic roles of NT-3 as an autocrine- or paracrine-derived factor at the early stages, then as a target-derived factor at later stages when the neurons are in the process of establishing connections with their peripheral fields of innervation.

Role of NT-3 in Developing Neurons of the CNS

Cultured embryonic motor neurons isolated from E15 rat spinal cord and identified with such markers as L14, p75, and islet-1 respond in a dose-dependent fashion to NT-3 with increased survival *(24)*. In addition, NT-3 upregulates the cholinergic phenotype of cultured rat E14 motor neurons *(35)*. By Northern blot analyses as well as by *in situ* hybridization NT-3 has been localized not only within the E11.5 mouse *(19)*, E12–13 rat *(4)*, and E15 rat ventral spinal cord, but also in the limb bud *(24)*.

As was the case for the retina, analysis using a crosslinking agent has shown in the ferret that at all developmental stages NT-3 and TrkC are also coexpressed in the higher structures, such as the lateral geniculate (LGN), superior colliculus, and cortex. These crosslinking studies further revealed that in the adult LGN and superior

colliculus there was an increase in NT-3 binding to truncated forms of TrkB and to p75 compared to binding to TrkC because of the large amount of truncated TrkB and the significant level of p75 at this stage *(36)*. These data suggest a functional role for NT-3 in the visual system both during development and at the adult stage.

Although NT-3 does not produce survival of cultured hippocampal neurons from rat E18 embryos, it increases the number of neurons expressing calbindin immunoreactivity, thereby indicating its role in the maintenance of a subpopulation of hippocampal neurons *(37,38,75)*. Cultured rat hippocampal astrocytes express transcripts for NT-3 *(39)*, suggesting a local paracrine role for NT-3 on the neurons of this structure. In addition to neurons and hippocampal astroglia, astrocytes derived from other brain regions of postnatal rats recently have been shown to express mRNAs for NT-3 and TrkC after growth in culture *(40)*. This indicates a potential role of this neurotrophin in neuron-glial interaction in the CNS.

In embryonic rat cerebellum *(25)* as well as in newborn cerebellum, diencephalon, hippocampus, and cortex, transcripts for NT-3 are expressed at a strikingly high level *(9)* when proliferation, migration, and differentiation of neuronal precursors are taking place. By inducing the production of NT-3 in cerebellar granule cells, tri-iodothyronine can indirectly promote the differentiation of Purkinje cells in culture *(41)*. Neuronal colocalization of NT-3 mRNA and p75 NGFR mRNA has been shown to occur in some of the neurons in the developing cortex, hippocampus, and the anterior and lateral hypothalamus of P9–10 mouse and rat, suggesting a possible autocrine action of NT-3 in these brain regions *(42)*. In contrast to an earlier report *(43)*, NT-3 can increase the cholinergic phenotype and survival of E16 basal forebrain neurons and the survival of tyrosine hydroxylase (TH)-containing neurons cultured from the locus ceruleus *(44)*. NT-3 can also increase survival of TH-expressing neurons isolated from the E14 ventral mesencephalon, as well as their dopaminergic uptake. Additionally, NT-3 promotes survival and differentiation of cultured neurons of the developing substantia nigra by increasing their GAD activity and GABA content *(45)*.

NT-3 in the Mature and Aging Nervous System; Its Role in Maintenance and Regeneration

NT-3 has been shown to selectively stimulate sprouting of the descending corticospinal tract in the postnatal rat. In the adult rat (4–7 wk old) local injection of NT-3 can increase regenerative sprouting of the lesioned spinal tract and long distance NT-3-induced regeneration occurs in the presence of an antibody neutralizing the myelin-associated neurite growth inhibitory proteins *(46)*. Elsewhere, NT-3 partially rescues motor neurons of the facial motor nucleus after lesion of the facial nerve in newborn rats *(47)*. It could also rescue motor neurons in the neonate following sciatic nerve lesion *(48)*. Additionally, NT-3 has been shown to rescue a majority of the DRG neurons that are destined to die after sciatic nerve lesion. This rescue effect is maintained up to 3 wk after axotomy *(49)*. In the CNS 6-hydroxydopamine-lesion model that results in degeneration of locus ceruleus neurons, implantation of transfected fibroblasts that synthesize and release NT-3 prevents loss of 80% of the noradrenergic neurons within 1 wk after lesion *(50)*. In another pharmacological lesion model, hippocampi that have been injected with quinolinic acid exhibit degeneration of the CA1 and CA4 pyramidal cell layers. NT-3 mRNA expression has been shown to increase in these regions following seizure and onset of neurodegeneration *(51)*. In aged (22–24 mo old) rats that display impairment of spatial learning and memory, intracerebroventricular injection of NT-3 (for 4 wk) reduces the ongoing atrophy of the cholinergic neurons, which occurs in the septum, nucleus basalis, and striatum *(52)*. NT3 has been found to be the predominant neurotrophin in the adult organ of Corti *(53)*. In cultured neurons of adult spiral ganglia, NT-3 dramatically increases survival and neuritogenesis *(54)*.

In the adult rat, localization of the NT-3 protein using a specific antibody indicates its presence in various peripheral organs, including the epithelial cells of the intestinal villi and the longitudinal muscle layer of the gut *(55)*. This observation suggests that beyond the developmental period, NT-3 continues to play a role as a target-derived factor within the environment of the gut to maintain subpopulations of ENS ganglion neurons. In summary, all of these studies point to a role of NT-3 in rescuing mature neurons that have been deprived of the trophic influence of their targets.

Modulatory Role of NT-3 on Synaptic Activity

Recent and exciting findings have demonstrated an acute role for NT-3 in modulating the development and function of synaptic interactions between various types of neurons. In most cases NT-3 has been shown to enhance synaptic efficacy. The first evidence was obtained at developing neuromuscular junctions that showed enhancement of the amount of transmitter released in cultures of *Xenopus (56)*. Enhancement of excitatory activity for several minutes is observed in rat somatosensory cortex cultures on perfusion of NT-3, whereas a decrease in GABA-mediated synaptic transmission occurs in these cultures under the same conditions *(57)*. NT-3 has been recently shown to increase dopamine metabolism in the striatum and to interact with amphetamine effects *(58)*. One underlying mechanism for synaptic plasticity can be the adjustment of the intracellular Ca^{2+} concentration to critical levels so that activation or inhibition of second messenger pathways can occur. Acute application of neurotrophins, such as NT-3, elevates intracellular Ca^{2+} in hippocampal neurons *(59)*. A long-lasting enhancement in synaptic strength can be induced and maintained for 2–3 h by a 20-min bath application of NT-3 at the Schaeffer collateral-CA1 synapses in hippocampal slices of adult rat *(60)*.

Experimental Models in Which NT-3 Is Lacking or Prevented to Act Demonstrate Its Crucial Role in the Development of Specific Sensory Structures of Avians and Mammals In Vivo

The wealth of previously described experiments argues strongly for a role of NT-3 in the development, maintenance, and repair of many neuronal structures of the PNS, CNS, and ENS. However, most of this work has been carried out by the exogenous application of NT-3 either in vitro or in vivo. Within the past year, the crucial role of this neurotrophin has been unambiguously demonstrated by experiments performed in vivo by treatment with specific antibodies that block NT-3 function during the period of gangliogenesis, or by analyses of homozygous mice lacking the NT-3 gene. Injection of a blocking anti-NT-3 into the chorioallantoic membrane of E3 quail embryos produces (when analyzed at stage E6) a loss of about 30% of neurons in the nodose (placode-derived) ganglion and a 30% loss of neurons in DRG no. 25 (neural-crest-derived), which innervates the limb *(14)*. It is notable that during this period when neuronal precursors are still dividing, and prior to the period of naturally occurring cell death, NT-3 exerts an early supporting role on a subpopulation of sensory neurons. These data are also consistent with the expression of mRNA for NT-3 at comparable stages in quail DRGs, both in neurons and support cells *(21)*, and emphasizes the local paracrine/autocrine action of NT-3 within these avian ganglia.

Homozygous transgenic mice deficient for the NT-3 gene display at birth a loss in portions of the peripheral sensory and sympathetic system and in particular, an absence of the muscle spindles and Golgi tendon of the proprioceptive sensory modality *(61–63)*. In addition, in the auditory sensory systems a major neuronal loss (up to 85%) occurs in the cochlear (spiral) ganglion and a minor loss (25%) in the vestibular ganglion *(64)*. This loss was found to be insignificant in the study of Fariñas et al. *(62)*.

In contrast to the defects found in the development of specific populations of sensory neurons, the lack of NT-3 did not induce any obvious deficit either in the development of motor neurons in the brain stem, nor of neurons in the cortex, hippocampus, cerebellum, or locus ceruleus, or in the development of neurons in the ganglionic plexuses of the ENS *(62)*. At first glance these data would suggest that NT-3 is not essential to the development of neuronal structures in the CNS or the ENS.

Receptors for Neurotrophin-3

The Tyrosine Protein Kinase TrkC Is the Transducing Receptor for NT-3 and Several Isoforms Encoded by the trkC Locus Have Been Characterized

The isolation of the third major member of the Trk (tropomyosin receptor kinase) family, the glycoprotein gp145^{trkC}, and its molecular and biological characterization as the transducing receptor for NT-3 were achieved within 9 mo after the cloning of NT-3 itself *(65)*. The molecular structure of gp145^{trkC} comprises 825 amino acids (aa). On the extracellular portion (NH2 terminal) there is a 31-aa signal peptide sequence followed by an extracellular binding domain with leucine-rich and cysteine-rich motifs, and C2 Ig-like motifs. There is a single transmembrane domain and the intracellular portion of the receptor comprises a 90-aa juxtamembrane region, the tyrosine kinase (TK) catalytic domain, and a 15-aa carboxyl terminal cytoplasmic tail with a single tyrosine residue *(66)*.

In the original report of the cloning of gp145^{trkC} *(65)*, *in situ* hybridization already indicated expression of mRNA for TrkC in the same structures where NT-3 had been shown to have biological effects, including the pyramidal cell layer of the hippocampus, the dentate gyrus, the cerebral cortex, and the granular cell layer of the cerebellum as well as the intestine wherein is contained the ENS. NT-3 was shown to mediate mitogenic responses by NIH3T3 cells expressing gp145 ^{trkC}, concomitant with a phosphorylation of the receptor on tyrosine residues. In addition, binding of ^{125}I-labeled NT-3 to NIH3T3 cells expressing gp145^{trkC} was shown to be efficiently competed by unlabeled NT-3, but not by other neurotrophins, such as NGF or BDNF *(65,66)*. Binding of NT-3 to the p75 NGFR as well as to a high affinity receptor was subsequently characterized on avian embryonic sensory neurons *(67)*. However, the presence of p75 NGFR appeared to disfavor the interaction of NT-3 with nonpreferred Trks, such as TrkB *(68)* and recent experiments also suggest that the presence of p75 NGFR may also prevent interaction of NT-3 with TrkA *(69)*. Thus, for neurons that may concomitantly express p75 NGFR and several of the Trks, NT-3 would preferentially bind to gp145^{trkC}. Several isoforms of TrkC can be encoded by the *trk*C locus. These include three full-length receptor tyrosine kinases that differ by novel amino-acid insertions in the kinase domain (TrkC14, TrkC25, and TrkC39) and a fourth truncated form that lacks the catalytic domain *(70,71)*. Northern blot analyses of TrkC transcripts also indicated the presence of both the full-length and truncated forms in 1-wk-old mouse brain and intestine. The functional significance of the truncated forms of TrkC are not known but their transcripts have been localized to astrocytes, peripheral nerve, and nonneural tissue *(72)*.

The Signal Transduction Pathways for TrkC Are Beginning to be Elucidated

On binding of a neurotrophin to its preferred receptor tyrosine kinase a number of events occur that have been already established for NGF binding to its preferred receptor, TrkA. A ligand-induced dimerization of the receptor followed by a transphosphorylation on specific tyrosine residues occurs and peaks within 5–10 min after onset of NGF binding. Formation of molecular complexes occurs with binding of secondary signaling elements, such as SHC, PI-3 kinase, and PLC-γ1, to specific tyrosine phosphate residues of the receptor *(73)*. This in turn,

leads to activation of downstream signaling pathways, including the ras, MEK, ERK pathway, and one that is characterized by tyrosine phosphorylation of the nuclear-localized protein SNT. Interestingly, SNT becomes tyrosine phosphorylated when neurotrophins exert differentiating effects, but not under conditions leading to mitogenesis *(73)*.

Because of the close homology of intracellular sequences between the various Trks, it is highly likely that the signaling pathways will be analogous among the various neurotrophins. The intracellular targets of Trk activation by NT-3 appear to be qualitatively the same as those activated by NGF (D. Kaplan, personal communication). However, the signaling molecules involved in the various biological actions of NT-3 are not yet completely characterized in primary neurons or their precursors. Full-length TrkC phosphorylates PLC-γ1 and PI-3 kinase in NIH3T3 cells expressing this receptor *(71)*. A recent report indicates that Trk tyrosine phosphorylation can be induced by NT-3 in various brain areas at embryonic and early postnatal stages *(74)*. NT-3 can also induce Trk phosphorylation as detected by immunoprecipitation using a pan-Trk specific antibody in E18 hippocampal cultures *(38)*. Interestingly, tyrosine phosphorylation of PLCγ1, Erk-1, and SNT are induced by NT-3 only in the embryonic brain, but not in the adult *(74)*. These signal transduction cascades eventually produce activation of immediate early genes, such as c-*fos* and c-*jun*. In fact, NT-3 can induce transient expression of c-Fos only in nuclei of process-bearing cells in cultures enriched with the crest-derived precursor cells of the developing ENS *(12)*. NT-3 has also been shown to induce c-Fos expression, in contrast to NGF, in cultured hippocampal neurons *(75,38)*. All of the full-length forms of TrkC autophosphorylate rapidly after binding to NT-3, but they do not always transduce the same type of biological activity *(70,72)*. For example, the PC12 cell line expressing $gp145^{trkC}$ undergoes survival in serum-free medium in response to NT-3, whereas a PC12 cell line expressing the isoform with a 14-aa insert (TrkC14/TrkCK2) does not. In contrast to the full-length TrkC, isoforms with amino acid insertions do not phosphorylate PLCγ1 and PI-3 kinase *(71)*. Thus, different isoforms of TrkC may mediate significantly different biological responses.

Expression of trk*C During Development; Loss vs Maintenance of Expression in the Adult*

The localization of *trk*C expression during ontogeny is consistent with the developmental role characterized for NT-3 early in development that was described earlier. TrkC mRNA is already present in the dividing neuroepithelium and it increases in dorsal areas of the NT concomitant with migration of neural crest cells *(76)*. *trk*C is present at E2–3 on the precursor cells of avian DRG *(21,76)* and in all avian cranial sensory ganglia *(76)* and persists later in E11.5 mouse DRG *(77)*. *trk*C is also present on the precursor neurons of early avian sympathetic ganglia *(21)*, in E14.5 *(18)* and E15.5 *(17)* rat sympathetic ganglia, and in E13.5 mouse trigeminal and otic ganglia *(77)*. In the developing ear, *trk*C recently has been localized more specifically to the spiral and vestibular ganglia *(25,32,78,79)*.

Detailed analyses of the developmental appearance of *trk*C transcripts in mouse embryos from E9.5 to E17.5 and continuing at P6 and P10, emphasize its very early expression throughout the CNS, and in the NT, the neural crest, and the aortic arches *(77,79)*. Expression of *trk*C was noted in cells of the developing ENS of the E13.5 mouse stomach and the E17.5 mouse intestine *(77,79)* and in the E14 and E16 rat bowel *(12)*. In accordance with these data, NT-3 promotes the differentiation of crest-derived cells immunoselected with NC-1 from E14 rat gut that mature into neurons and glia; these enteric precursor cells express transcripts for full-length *trk*C as well as for one isoform with 25-aa insert (*trk*C K3) *(12)*. A more detailed analysis of expression of various TrkC isoforms in the developing rat ENS shows a predominance of TrkC and

TrkC14 in the fetal gut, whereas the insert form TrkC39 is limited to the proximal gastrointestinal tract and the TrkC25 form is evident in developing stomach and colon *(80)*.

A most striking example of a decrease in *trk*C expression that occurs with age is that of the neurons derived from the neural crest, such as the sensory DRG and sympathetic ganglia *(17,18,21)*. Interestingly, NT-3 can induce Trk phosphorylation and activation of secondary intracellular substrates in certain brain regions only in embryonic, but not at adult stages *(74)*. However, *trk*C expression remains prominent in adult mouse cortex, hippocampus, thalamus, hypothalamus, cerebellum, and spinal cord *(77)*. Adult neurons do tend to express the full-length form of TrkC more abundantly than the truncated forms. This is particularly striking in retina. In contrast, the sciatic nerve, which does not contain neuronal perikarya, only expresses truncated forms of TrkC *(72)*. These observations may reflect a changing biological role for NT-3 in the development/differentiation of specific regions of the PNS and CNS. In adult ENS, TrkC mRNA is expressed in neurons of both the myenteric and submucosal plexus *(81)*. Demonstration of a continued role for NT-3 in the maintenance of adult neurons in the ENS, however, remains to be demonstrated.

One of the hallmarks of a functional neurotrophin receptor is the ability to mediate retrograde transport of the appropriate neurotrophin in adult neural structures. Retrograde transport studies with ^{125}I NT-3 show that large size neurons scattered in the lumbar DRG exhibit dense radiolabeling throughout the ganglion similar to that exhibited by neurons labeled with retrograde-transported iodinated NGF. In contrast, in sympathetic ganglia only 5–7% of the neurons that are labeled with ^{125}I NGF show retrograde labeling by iodinated NT-3 when it is injected in the anterior chamber of the eye *(82)*. Adult spinal motor neurons projecting ipsilateral to an injected crushed sciatic nerve can also retrogradely transport iodinated NT-3. In the CNS, intrahippocampal injection of ^{125}I NT-3 results in the retrograde labeling of neurons of the dorsal hippocampus (Hilus/CA4), the medial septal nucleus, and, in the diagonal band of Broca and the supramammilary nucleus, all nuclei projecting to the hippocampus *(82)*. These data thus argue for continuing local as well as target-derived effects of NT-3 in the adult PNS, ENS, and CNS. Such effects may include long-term potentiation, regeneration, and plasticity of local synaptic circuitry.

Experimental Models with an Inactive trk*C Gene Indicate an Essential Role for NT-3 in the Development and Maintenance of Proprioceptive Sensory Neurons*

Homozygous mutant mice defective in the portion of the *trk*C gene encoding the catalytic domain of the receptor develop until birth. However, postnatal mutant mice can survive for up to 4 wk, at which time most of them die. The subpopulation (19% of DRG neurons) known to express *trk*C transcripts is lost in these mice *(83)*. These sensory neurons have their peripheral endings in muscle spindle and correspond to the Ia and II afferents that project to the dorsal column nuclei and send collaterals to motor neuron pools. Loss of proprioception explains the abnormal locomotion and posture in these mutant mice. There is also loss of the neurons in the mesencephalic trigeminal ganglion (I. Silos-Santiago, personal communication). The homozygous mutant animals do not show gross defects in the CNS, but because of their limited lifespan, abnormalities may occur during the postnatal week in subpopulations of neurons in other structures *(83)*, including the ENS. Such defects remain to be demonstrated. Consistent with the phenotype of *trk*C gene-deficient mice, mutant mice lacking NT-3 also show no Ia afferents. However, a widespread reduction in several types of sensory neuronal populations occurs in the NT-3 gene deletion in contrast to the *trk*C gene deletion paradigm. In addition to the sensory deficit, the NT-3 gene deletion results in a deficit among sympathetic ganglion neurons whose

precursor cells depend on NT-3. Surprisingly, sympathetic ganglia are not affected in the *trk*C knock-out (K.O.) mice so that NT-3 may signal through another Trk (personal communication, I. Silos-Santiago and M. Barbacid). So far, the ligand gene deletion seems to produce a more severe phenotype than the receptor gene deletion, suggesting that NT-3 can use other Trks (i.e., TrkA) to transduce signals in the embryo. It will be of interest to analyze the phenotype of double (*trk*C/*trk*A) deletion mutants in this respect.

Conclusions and Perspectives

The wealth of data that has so far accumulated points to widespread effects of NT-3 early in development of the peripheral (sympathetic and specific sensory) nervous system. Because NT-3 is the most promiscuous of all the neurotrophins in its binding properties to neurotrophin receptors (to p75 as well as to TrkB and even to TrkA under certain conditions, in addition to TrkC), the phenotype of NT-3 K.O. mice is understandably more severe than the phenotype of *trk*C K.O. mice. However, development of many areas of the CNS and ENS are not grossly affected in the NT-3 K.O. mice even though in vitro effects of NT-3 have been revealed in these systems as well as correlated with in vivo expression of NT-3 and its receptors. Further analyses of the phenotypes in the K.O. animals are required, particularly within the CNS (i.e., visual system, hippocampus) and in neuronal subpopulations of the plexuses of the ENS. Perhaps analyses carried out on mice lacking several Trk receptors may detect further disruptions in phenotype. Mitogenic, survival-promoting, and differentiating effects of NT-3 have been described so far, depending on the developmental stage of the target cells. Notably, NT-3 also may have synergistic or cooperative effects with other factors to promote survival or to accelerate the acquisition of differentiated neuronal phenotypes, such as with bFGF (i.e., ref. *10*) or, similar to NGF, with other cytokines, such as ciliary neurotrophic factor (CNTF) (i.e., refs. *84–86*). In the hippocampus, a new role for NT-3 is emerging in modulating the strength of synaptic excitability. Thus, NT-3 should play a vital role in the plasticity and maintenance of the adult nervous system. Potential therapeutic roles for NT-3 can also be envisioned in case of injury (i.e., ref. *46*) or as a candidate for prevention of certain neural degenerative disorders, for example, in sensory proprioception, in the auditory system, or in the CNS (i.e., ref. *50*).

Because a good functional correlation exists between the localization of NT-3 and its TrkC receptor, new avenues of investigation should focus on the earliest appearance of either the ligand or the receptor in development, such as during formation of the neural plate. Will the expressions be concomitant with one another or will the receptor be expressed before the ligand, or vice versa? Concomitant expression of mRNA for NT-3 and TrkC is already present at stage 1 in the quail embryo *(8)*, thus sites of early localization may point to new roles for NT-3 in differentiation of tissues other than the nervous system. For instance, little information is known about roles of NT-3 in neural crest cell migration or in development of the wall of arteries that derive from cardiac neural crest cells *(87)*. Another intriguing question pertains to the roles of the isoforms of TrkC, those with inserts as well as the truncated forms remain to be elucidated, as well as the types of cells where they are expressed (glia vs neurons). For instance, a new insert isoform of the avian TrkC receptor cloned from chick brain mediates neurite outgrowth, but not survival in transfected PC12 cells *(88)*. Since such isoforms are expressed in increasing amount with age *(89)*, they may exert a critical influence in maintenance of the mature nervous system. Finally, the nature of the molecular signals that regulate the expression of the genes for NT-3 and its receptors remains to be elucidated. Does the activation of these genes occur early in development under the control of early "master genes," that is, is it programmed? Can the regulation occur under the influence of other differentiating factors, such as transforming

growth factor β (TGFβ), which has been shown to downregulate NT-3 mRNA and to upregulate NGF mRNA in sensory target fields when sensory neurons may switch their dependency from NT-3 to NGF *(90)*? Can it be under the influence of increasing or decreasing synaptic input to NT-3 responsive neurons? In the adult stage, could this regulation be governed by injury- or stress-released factors? These are some of the challenging questions that remain to be addressed to further understand the functions of NT-3.

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