Research Article

Neurotrophin-3: A Neurotrophic Factor Related to NGF and BDNF

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The development and maintenance of the nervous system depends on proteins known as neurotrophic factors. Although the prototypical neurotrophic factor, nerve growth factor (NGF), has been intensively studied for decades, the discovery and characterization of additional such factors has been impeded by their low abundance. Sequence homologies between NGF and the recently cloned brain-derived neurotrophic factor (BDNF) were used to design a strategy that has now resulted in the cloning of a gene encoding a novel neurotrophic factor, termed neurotrophin-3 (NT-3). The distribution of NT-3 messenger RNA and its biological activity on a variety of neuronal populations clearly distinguish NT-3 from NGF and BDNF, and provide compelling evidence that NT-3 is an authentic neurotrophic factor that has its own characteristic role in vivo.

WIDESPREAD NEURONAL CELL DEATH ACCOMPANIES normal development of the central and peripheral nervous systems, and apparently plays a crucial role in regulating the number of neurons that project to a given target field (*1, 2*). Ablation and transplantation studies of peripheral target tissues during development have shown that neuronal cell death results from the competition among neurons for limiting amounts of survival factors (neurotrophic factors) produced in their projection fields. These observations led to the identification of nerve growth factor (NGF), which remains, by far, the best characterized neurotrophic molecule (*3, 4*). Understanding the role and mechanism of action of NGF has been aided by the discovery of a rich source of this protein in male mouse submaxillary glands, which allowed for the purification and cloning (*5, 6*) of NGF, as well as the generation of neutralizing antibodies.

In that NGF only supports a limited set of neuronal populations, the existence of additional neurotrophic factors has long been postulated (*7–9*). While it is now clear that such factors do exist, their extremely low abundance has impeded their molecular characterization. Nevertheless, purification of small amounts of two such proteins, namely, brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF), has recently permitted their partial sequence analysis and molecular cloning (*10–12*). Despite specificities for distinct neuronal populations, BDNF and NGF (but not CNTF) display sufficient structural homology to be regarded as members of a gene family (*10*). We used this homology to design a cloning strategy to search for additional members of this gene family. We now describe the cloning of a gene encoding a third member of this family, which we have designated neurotrophin-3 (NT-3). This protein displays distinct biological activity and different spatio-temporal characteristics from those of NGF and BDNF.

Molecular cloning of NT-3. NGF and BDNF are basic proteins of approximately 120 amino acids that share about 50 percent amino acid sequence identity, including absolute conservation of six cysteine residues that, in active NGF, have been shown to form three disulfide bridges (*10, 13*). Comparison of the sequences of NGF from evolutionarily divergent species has revealed that the amino acids flanking these cysteine residues represent the most highly conserved regions of the molecule (*14, 15*). These are also the regions that show the most similarity between BDNF and NGF (*10*). Degenerate oligonucleotides corresponding to four of these regions were used in polymerase chain reactions (PCR) (*16, 17*) to

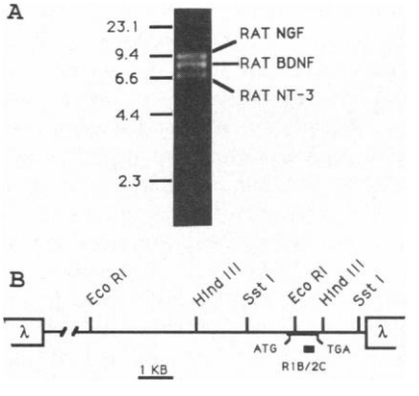

Fig. 1. (**A**) PCR product derived with degenerate 1B and 2C primers, designated R1B/2C (*18*), detects a novel gene, NT-3, as well as the NGF and BDNF genes in rat genomic DNA. DNA was prepared from the livers of Fischer rats (*21*) and digested with Eco R1; 10-μg sample was then fractionated on a 1 percent agarose gel. The DNA was transferred to nitrocellulose with 10× SSC (standard saline citrate) (*21*), hybridized (*47*) to the ^{32}P-labeled R1B/2C PCR product (*48*) at 60°C, and washed in 2× SSC containing 0.1 percent SDS at 65°C. The NT-3, NGF, and BDNF bands are indicated; position of NGF and BDNF bands was as previously determined with specific probes (*19*). (**B**) Restriction map of a rat NT-3 genomic clone. Two independent bacteriophage clones specifically hybridizing to the R1B/2C probe were isolated from a genomic library (Clontech), prepared from Sprague-Dawley rat DNA (partially digested with Sau 3A restriction endonuclease and cloned in the EMBL3/SP6/T7 bacteriophage vector), as described in the text. A schematic representation of the restriction map of one of these clones, containing a 19.5-kb insert, is depicted. The thickened line indicates the open reading frame (ORF) of NT-3 (Fig. 2A). The position of the R1B/2C probe is indicated.

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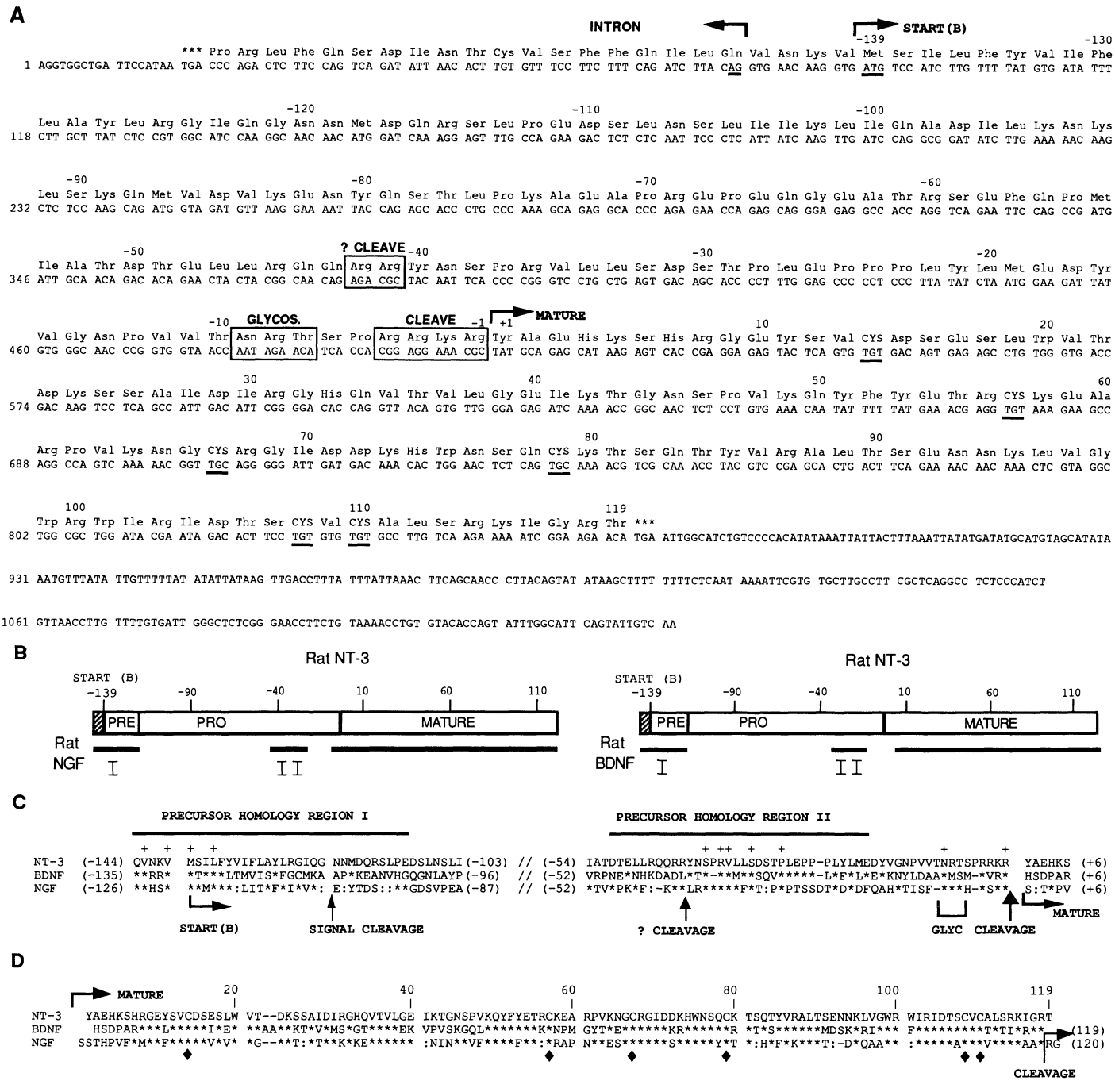

Fig. 2. Sequence of rat NT-3 and its similarity to rat NGF and rat BDNF. The DNA was sequenced by the dideoxynucleotide chain termination method (*49*), with the Sequenase version 2.0 kit and recommended protocols (U.S. Biochemical). (**A**) Nucleotide and amino acid sequence of NT-3 showing the DNA sequence spanning the open reading frame (ORF) encoded by NT-3 gene, with the amino acid translation indicated above DNA sequence; the asterisks mark the beginning and end of ORF. Amino acids are numbered with position +1 assigned to first residue of mature NT-3 (119 amino acids). The cleavage site that is used to release mature NT-3 is boxed, as is the conserved glycosylation site just upstream to this cleavage site; another potential cleavage site, which is similarly located to a proposed intermediate processing site in NGF (*22*) (but which is not conserved in BDNF), is boxed and marked with a "? cleave". The six cysteines in mature NT-3 are underlined. The methionine initiation codon for the short precursor form of NT-3 (at position −139), which marks start site B, is also underlined. The proposed boundary of the splice acceptor site and intron (*25*) upstream of the B start site is indicated. (**B**) Sequence alignments of rat NT-3 with rat NGF and rat BDNF. The MacVector sequence analysis software (International Biotechnologies) was used to generate a matrix alignment of the rat NT-3 ORF with the ORF's of the NGF and BDNF genes (using a window size of 20 and a minimum match of 20 percent). Significant matches along the diagonal of this matrix are represented underneath NT-3 protein product; two regions of homology upstream to the mature NT-3, which are seen in comparisons to both NGF and BDNF, are designated I and II. Region I extends upstream of the B start site used to generate the short precursor form of NT-3, supporting the contention that a longer precursor exists (*25*). (**C**) Sequence comparisons between NT-3, NGF, and BDNF in homology regions I and II. Sequences are aligned to maximize homology; with gaps inserted for alignment indicated by a dash (-). Identities of either BDNF or NGF with the NT-3 sequence are indicated by an asterisk, while identities of NGF with BDNF are indicated by a colon (:) in the NGF sequence. A plus sign on top of the sequence indicates residues that are completely conserved between rat NT-3 and the NGF and BDNF sequences from all species examined. The following sites defined for NGF, previously predicted for BDNF and proposed here for NT-3 are indicated: the B start site of the methionine initiation codon; the signal sequence cleavage site (*24*); a proposed NGF intermediate cleavage site, which is absent in BDNF but is present in NT-3; a glycosylation acceptor site; the proteolytic cleavage site which releases the mature factors. (**D**) Sequence comparions of the mature forms of NT-3, BDNF, and NGF. Conserved cysteines are indicated by ♦. Asterisk, colon, and dash are as in (C).

amplify novel related sequences (*18*). Without any knowledge of a specific tissue source in which novel neurotrophic factors might be expressed at high levels, we chose genomic DNA, rather than cDNA, from diverse species for use as templates for the PCR reactions; the use of genomic DNA appeared to be appropriate because the coding regions for NGF and BDNF are not interrupted by introns (*5, 19*).

Amplification products of the expected sizes (as predicted from the NGF and BDNF sequences) were obtained with the different pairs of degenerate oligonucleotides. These products were first subjected to restriction enzyme analysis to determine the relative content of NGF, BDNF, or novel DNA sequences. In all cases (*18*), restriction fragments corresponding only to NGF and BDNF sequences were detectable. However, use of the same PCR products as hybridization probes on DNA (Southern) blots of rat genomic DNA revealed that one product, designated R1B/2C (*18*), identified a novel genomic DNA sequence, in addition to NGF and BDNF (Fig. 1A); thus, screening of PCR products by Southern blotting allowed for identification of rare amplified sequences that were undetectable by other means (*20*). The R1B/2C probe also detected novel sequences in the genomic DNA of evolutionarily divergent species, including human, mouse, chicken, and *Xenopus*, supporting the notion that this probe identifies a functional gene.

In order to isolate this gene, the R1B/2C probe and probes specific for NGF and BDNF were used to screen (*21*) a rat genomic DNA library (Fig. 1B). We found two independent bacteriophage clones that hybridized to the R1B/2C probe, but not to the other two probes. Restriction map analyses of the rat genomic inserts in these clones demonstrated that they correspond to the same gene (Fig 1B). Sequence analysis proved that the gene identified by R1B/2C encodes a new member of the NGF/BDNF family (Fig. 2, below), which we have named neurotrophin-3.

Sequence analysis of mature NT-3 and of its precursors. NGF has two distinct precursor forms, termed the long (initiating at start site A) and short (initiating at start site B) precursors, which differ by the length of their amino-terminal sequences (*22–24*). Both the long and short precursors can be proteolytically cleaved to yield the mature form of NGF, which essentially constitutes the carboxyl terminal 120 amino acids of each precursor. BDNF may also have similar long and short precursor forms (*19*). Alignment of the NT-3 gene sequence with the sequences of NGF and BDNF revealed substantial similarities that have allowed us to define the protein sequence of the mature NT-3 product, as well as to predict that there are both long and short precursors for this protein (Fig. 2B). The predicted NH_2-terminus of mature NT-3 follows a canonical protease cleavage sequence (Arg-Arg-Lys-Arg), very similar to those seen in NGF and BDNF (Fig. 2, A and C). In some species, the two COOH-terminal amino acids of NGF are also proteolytically removed. Unlike NGF, rat NT-3 does not have an obvious potential cleavage site at its COOH-terminus (Fig. 2, A and C), and we infer that, as with BDNF in all species examined, there is no proteolytic modification at the COOH-terminus of NT-3 (*19*).

Based on these considerations, the predicted size of the mature NT-3 polypeptide is 119 amino acids, with a computed isoelectric point (*p*I) of 9.5. Thus, in size and charge, NT-3 closely resembles NGF and BDNF. The seven NH_2-terminal amino acids of mature NT-3 differ completely from NGF and BDNF. Starting from amino acid eight of mature NT-3, optimal alignment required a single gap of two amino acids relative to BDNF and a single insertion of one amino acid relative to NGF (Fig. 2D). The mature rat NT-3 displays 57 percent amino acid identity with rat NGF, and 58 percent amino acid identity with rat BDNF; 57 of the 119 residues (48 percent) are shared by all three proteins (Fig. 2D). The six cysteine residues found in NGF and BDNF are absolutely conserved in NT-3, and regions of greatest homology between the three proteins are mainly clustered around these cysteine residues.

Just upstream to the presumptive cleavage site that releases mature NT-3, there is a universal glycosylation acceptor site (Asn-X-Thr or Ser; Fig. 2, A and C), which also has been found at the same position in NGF and BDNF (*5, 10*). Whether this glycosylation site plays a role in processing of the NT-3, NGF or BDNF precursors remains unknown.

Further comparison of the NT-3 sequence with the NGF and BDNF precursors reveals two regions of amino acid homology upstream of the mature NT-3 sequence (regions I and II in Fig. 2, B and C). The region I homology leads us to predict the existence for rat NT-3 of a B start site (defined for NGF above), which would yield a short precursor of 258 amino acids, similar in size to the short precursors for NGF (241 amino acids) and BDNF (249 amino acids); the apparent methionine initiation codon, secretory signal

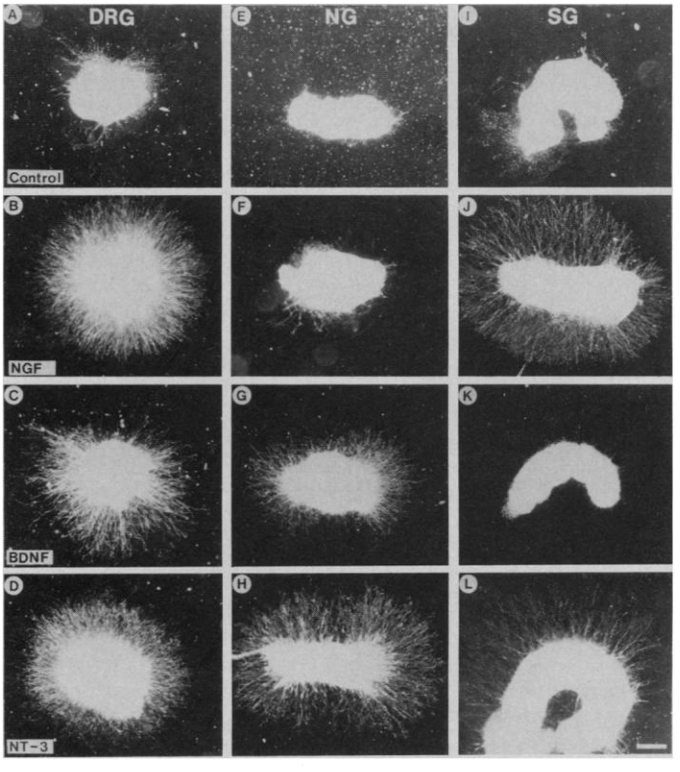

Fig. 3. Comparison of NGF, BDNF and NT-3 activities as assayed on explanted embryonic (day 8) chick ganglia. Photomicrographs of DRG (**A** to **D**), NG (**E** to **H**), and SG (**I** to **L**) cultured for 24 hours (DRG and NG) or 48 hours (SG) either in the absence of any neurotrophic factor (Control; A, E, I) or in the presence of COS cell supernatants (*34*) containing NGF (B, F, J), or BDNF (C, G, K) or NT-3 (D, H, L). In each case, representative ganglia are shown in the presence of the amount of supernatant that elicits the maximum fiber outgrowth. There is almost no neurite outgrowth in control cultures (500 μl of COS cell supernatant from mock-transfected cells). NGF (10 μl of COS cell supernatant shown) produced profuse fiber outgrowth from DRG and SG but not NG. Increasing the NGF COS cell supernatant from 20 to 500 μl produced no effect on NG. BDNF (10 μl of COS cell supernatant) produced fiber outgrowth from DRG and NG but not SG; higher amounts (20 to 500 μl) had no effect on SG. NT-3 (20 μl of COS cell supernatant on DRG and NG, 200 μl on SG) produced fiber outgrowth from all three types of ganglia although initiation of growth was slower and less profuse from SG. Ganglia were cultured as explants in collagen gel (*50*) in 2 ml of F14 medium supplemented with 5 percent horse serum as described previously (*31*). In three separate experiments more than five ganglia were treated at varying concentrations (1 μl to 500 μl for each growth factor) and compared to the NGF dose response as originally described (*50*). Scale bar, 200 μm.

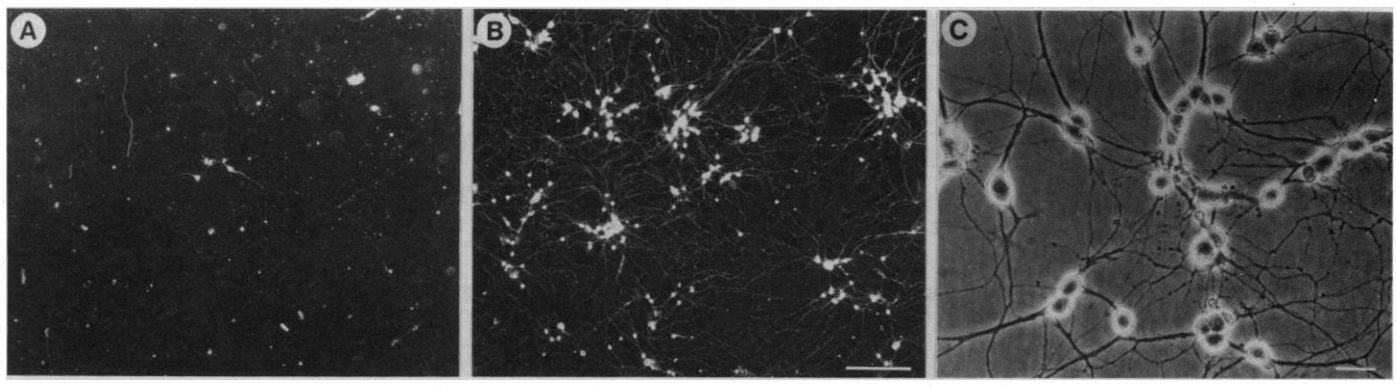

Fig. 4. NT-3 promotes survival and neurite outgrowth in highly enriched cultures of DRG neurons. Photomicrographs of neuron-enriched (>95 percent neurons) cultures of dissociated chick embryonic (day 8) DRG-treated for 48 hours with either: (**A**) supernatant (500 μl) from mock-transfected COS cells or (**B** and **C**) supernatant (50 μl) from NT-3–transfected cells. (A) and (B) are dark-field micrographs; in (A) (control culture) fewer than 5 percent of the neurons plated survived; in (B) the number of process-bearing neurons was approximately 60 percent of the neurons plated. In three separate dose-response experiments performed as previously described for NGF (*50*), NT-3 was found to consistently support survival of 50 to 60 percent of chick E8 DRG neurons. (C) A higher magnification phase contrast micrograph of the same culture as shown in (B). Note the large number of phase bright neuronal cell bodies and the virtual absence of any non-neuronal cells. Cultures were established as previously described (*31*). Scale bar, 150 μm (A and B); 15 μm (C).

sequence and signal sequence cleavage site for the short precursors of all three factors are conserved (Fig. 2C). Because the region I homology extends upstream of the B start site, we also predict that the existence of a long precursor for NT-3 would initiate from an A start site (Fig. 2, A to C) (*25*). As has been seen with NGF (*5, 23*), and proposed for BDNF (*19*), such a start site presumably would be encoded on additional exons upstream to the single exon which encodes the entire short precursor (Fig. 2C) (*25*).

In addition to the region I and II amino acid homologies, comparisons of hydrophilicity plots for NT-3, NGF, and BDNF reveal a similarity of structure in the precursors, upstream of the mature products. Altogether, the apparent conservation of long and short precursor versions for NT-3, NGF, and BDNF supports the idea that the upstream regions play important and specific roles in the folding, processing or transport of these neurotrophic factors (*19*).

Neurotrophic activity of NT-3. The striking homology in the protein sequences of NT-3, NGF, and BDNF strongly suggested that NT-3 might have neurotrophic activity. Both NGF and BDNF can promote the survival of selected populations of peripheral and central nervous system neurons in vivo and in vitro (*26—28*). For example, the administration of either factor to developing avian embryos prevents naturally occurring neuronal death in specific peripheral ganglia (*29*). When added to explanted ganglia, NGF and BDNF induce neurite outgrowth (*30*); when added to cultures of dissociated ganglionic neurons, these factors promote neuronal survival and differentiation (*31*). Such in vitro assays, on several types of chick peripheral ganglia, have been used to distinguish between the neurotrophic activities of NGF and BDNF. Whereas both factors act on populations of sensory neurons found in dorsal root ganglia (DRG), derived from neural crest, only BDNF supports the sensory neurons of the neural placode–derived nodose ganglion (NG) (*31*). In contrast, NGF, but not BDNF, can support the survival and growth of neurons of the paravertebral chain sympathetic ganglia (SG) (*32*).

In order to assess the potential biological activity of NT-3, we inserted the rat NT-3 gene into a vector, pCDM8 (*33*), which was previously used to transiently express BDNF and NGF in mammalian cells (*19*). This construct was designed to express the short precursor form of NT-3 (*34*); expression of the short precursor forms of NGF and BDNF has yielded biologically active material (for example, *10, 19, 24*). The NT-3, NGF, and BDNF constructs were transfected into COS cells (*34*); culture supernatants were harvested and first assayed at varying concentrations for their ability to induce neurite outgrowth from DRG explants. As expected, NGF and BDNF promoted neurite outgrowth in this assay (Fig. 3). In the first demonstration that the NT-3 gene actually encodes a neurotrophic activity, the product of this gene induced profuse neurite outgrowth from the DRG explants (Fig. 3).

To establish that NT-3 acts directly on neurons, we assayed this factor in highly enriched cultures of dissociated DRG neurons (Fig. 4). In the virtual absence of Schwann cells and fibroblasts, NT-3 promoted survival and neurite outgrowth of approximately 60 percent of these DRG neurons. Given that NGF and BDNF together support virtually 100 percent of DRG neurons in culture (*31*), it must be assumed that NT-3 promotes survival of cells that are also responsive to at least one of the other two factors.

Neurotrophic activity of NT-3 is distinct from NGF and BDNF. To further explore the neuronal specificity of NT-3, we assayed the factor on NG and SG explants. As expected, control experiments verified that NGF induced neurite outgrowth from SG but not NG explants, whereas BDNF induced neurite outgrowth from NG but not SG explants. Interestingly, NT-3 promoted neurite outgrowth from both NG and SG explants (Fig. 3), suggesting a broader specificity than either NGF or BDNF. However, NT-3, like NGF and BDNF, failed to promote survival or promote neurite outgrowth from explants or dissociated, neuron-enriched cultures of the chick ciliary ganglion (*35*). As has been previously shown, the parasympathetic neurons that comprise this ganglion did respond to rat CNTF (*35*), a neurotrophic factor unrelated to the NGF–BDNF–NT-3 family (*11, 36*). No response was seen in any of these assays with supernatants from COS cells transfected with control vectors (Fig. 3).

While the DRG, NG, and SG explants each responded to at least two of the three related neurotrophic factors, the maximal response exhibited by a given ganglion depended on the factor used. In the case of DRG, the response to saturating levels of NGF, BDNF, and NT-3 was relatively equivalent. However, with NG, the maximal response to NT-3 was greater than to BDNF, while with SG, the maximal response to NT-3 was substantially lower and somewhat delayed compared to NGF. Because neuronal populations differ in their temporal requirements for neurotrophic factors (*30*), it would be interesting to see how the magnitude and specificity of the responses seen here may vary if ganglia from embryos of different ages are used. Further experiments will be required to establish whether multiple neurotrophic factors that act on the same ganglion

address distinct neuronal subpopulations within that ganglion, and whether a particular neuron may respond differently to the various factors.

Exploring the sites of NT-3 synthesis. During development neuronal survival depends on target-derived neurotrophic molecules. Continued survival, even in the adult, may require the persistence of a neurotrophic influence (*37*). In other cases, survival of mature neurons may no longer depend on a neurotrophic factor; nevertheless such factors profoundly affect the differentiated phenotype of neurons (*38*). Determining the sites of synthesis of a neurotrophic molecule may therefore help to elucidate its physiological roles.

To explore the sites of NT-3 synthesis and to compare NT-3 expression with that of NGF and BDNF, triplicate RNA (Northern) blots of RNA samples prepared from a variety of adult rat tissues were hybridizided to probes specific for each of these genes (Fig. 5). As previously demonstrated (*39, 40*), expression of NGF mRNA was highest in the brain, heart, and spleen; at least trace levels were detectable in all other tissues examined. BDNF displayed a more restricted pattern of expression; highest levels were found in brain (*10*), and significant levels were seen in heart, lung, and muscle (*19*). As with NGF, the NT-3 transcript (1.4 kb) was detectable in all adult tissues surveyed. However, in all peripheral tissues surveyed the level of expression of NT-3 mRNA was at least comparable to that seen in the adult brain, and in some cases (for example, kidney and spleen) was substantially higher.

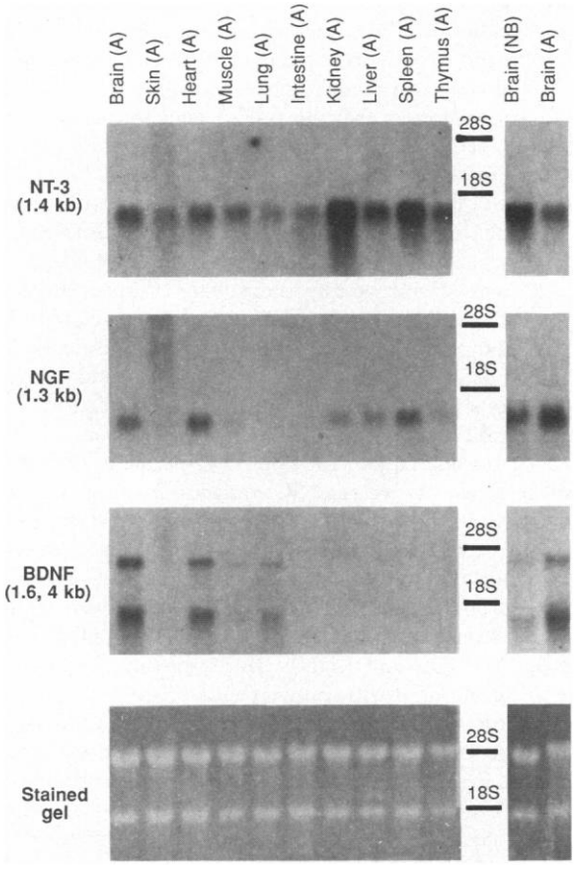

Fig. 5. Northern (RNA) blot comparisons of NT-3, NGF, and BDNF expression in rodent tissues. RNA was prepared (*51*) from the indicated tissues of rat (left panels) or mouse (right panels). RNA (10 μg) from the indicated sources was then fractionated on 1 percent formaldehyde-agarose gels and transferred to nylon membranes in 10× SSC; triplicate Northern blots were hybridized (*47*) at 68°C with ^{32}P-labeled (*52*) rat NT-3, rat BDNF, and rat NGF DNA fragments, and then washed at 68°C in 2× SSC, 0.1 percent SDS. DNA fragments for NT-3, NGF, and BDNF were derived from the expression constructs containing these genes in pCDM8 (*34*); the approximately 775-bp Xho I inserts in these constructs were gel-purified prior to labeling. The BDNF probe identifies two distinct transcipts at high stringency; the structure of these two transcripts, which are always coexpressed, has not been further explored (*19*). A picture of the ethidium bromide–stained gel, allowing comparison of the total amount of RNA per sample, is included. An NT-3 expression pattern, similar to that depicted here in rat tissues was found in a study of murine peripheral tissues (*41*).

We also compared the relative abundance of the NGF, BDNF, and NT-3 transcripts in the brains of newborn and adult mice. In contrast to both NGF and BDNF, the level of NT-3 mRNA in newborn brain was higher than in adult brain (Fig. 5). A more detailed analysis has revealed that NT-3 mRNA levels in the central nervous system are dramatically higher during fetal development and then decrease to adult levels (*41*).

Structural comparisons among NGF, BDNF, and the newest member of this gene family, NT-3, highlight several conserved regions, and lead to the suggestion that the functional differences among these proteins are determined by sequences lying outside of these conserved regions. The predicted existence of long and short precursor forms of all three proteins supports the biological importance of both precursor forms in vivo. Nevertheless, vectors expressing the short precursor forms of these factors yield biologically active material in COS cells.

Our finding that these three neurotrophic factors display distinct stage-specific and tissue-specific patterns of expression supports the notion that neural development depends on the temporally and spatially distinct expression of discrete neurotrophic activities. The relatively higher expression of NT-3 in the brain of the newborn mouse, compared to that of NGF and BDNF, is intriguing. Our initial characterization of NT-3 neurotrophic activity in vitro, coupled with the generalized prevalence of NT-3 mRNA in both the adult brain and adult peripheral tissues, further suggest that NT-3 may have a widespread influence on neuronal function or survival in the adult. The more widespread expression of NT-3 also raises the possibility that this factor acts on cells other than those of the nervous system, as has been proposed for NGF (*42*).

Although it has not yet been clearly demonstrated that neurons can be simultaneously responsive to more than one neurotrophic factor, NGF and BDNF may act on overlapping neuronal populations. For example, the administration of either NGF or BDNF can rescue most of the DRG neurons that would otherwise die during normal avian development (*29*). Our observations on the effects of NT-3 on chick peripheral ganglia lend support to the possibility that individual neurons can respond to multiple, related factors. So, both the mediation and physiological relevance of simultaneous responsiveness would merit examination. For example, components of the receptors or signal transduction mechanisms for the three structurally related neurotrophic factors may be shared. In principle, simultaneously responsive neurons could have multiple receptors, each specific for a given neurotrophic factor, or a single receptor that can mediate a response to several neurotrophic factors. In vivo, these various factors may be simultaneously presented to all responsive neurons. More likely, there are spatio-temporal differences in the relative availability of individual factors (*43*). It may even be possible that different factors are available to different sites of the same neuron; for example, a sensory neuron may receive distinct factors from its peripheral and central terminals (*44*). If several factors are simultaneously available to some neurons, their actions might be either redundant or complementary.

Elucidating the individual and potentially complementary roles of NGF, BDNF, and NT-3 should provide information crucial for understanding normal development and maintenance of the nervous system. Animal studies have suggested that NGF may be of value in the treatment of degenerative neurological conditions (*9, 45, 46*).

The cloning of a new member of the NGF-BDNF gene family, and its potential interactions with the other members of the family, raise new considerations for use of these proteins in overcoming neuronal degeneration.

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18. Completely or partially degenerate oligonucleotides corresponding to segments of four protein sequences highly conserved between NGF and BDNF were synthesized; the protein sequences (which can be found within the NGF-BDNF sequences presented in Fig. 2D) were (1) Gly-Glu-(Tyr/Phe)-Ser-Val-Cys-Asp-Ser (2) Lys-Gln-Tyr-Phe-(Tyr/Phe)-Glu-Thr-Lys-Cys (3) Gly-Cys-Arg-Ile-Asp, and (4) Trp-Arg-Phe-Ile-Arg-Ile-Asp-Thr-(Ser/Ala)-Cys-Val-Cys. A series of degenerate sense and antisense oligonucleotides (containing a degenerate portion 15 to 26 nucleotides long, corresponding to five to nine amino acids of the indicated protein sequences in either the sense or antisense direction, as well as a nondegenerate tail encoding restriction enzyme recognition sites) were used in PCR reactions. All potential amplification reactions (12 total reactions performed on the genomic DNA of each species tested) between pairs of upstream sense and downstream antisense primers were carried out according to the conditions recommended by Perkin-Elmer–Cetus, except that the annealing temperature, Mg^{2+} concentration, and extension time were varied to determine optimal conditions for each pair. The exact sequence of the 1B sense primer (encoding a portion of the protein sequence 1 above) was 5′-GACTCGAGTCGACATCG-GTG-TG(C,T)-GAC-AG(C,T)-(A,G)T(A,C,T)-AG-3′ and the 2C antisense primer (corresponding to the antisense codons of a portion of protein sequence 2 above) was 5′-CCAAGCTTCTA-GAATTC-CA-(C,T)TT-(A,G,C,T)GT-(C,T)TC-(A,G,)(A,T)A-(A,G)AA-(A,G)-TA-(C,T)TG-3′. Subsequent sequence analysis showed that the 1B oligonucleotide had a two-nucleotide mismatch with the NT-3 sequence, whereas the 2C oligonucleotide had a one-nucleotide mismatch with the NT-3 sequence.
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25. The NGF gene can utilize an upstream initiation codon, start site A (*23, 24*), to produce a substantially longer precursor than that initiating from start site B. Sequencing of the NGF gene from several species has revealed that most of this additional NH_2-terminal sequence is found on separate exons, except for four codons (Val-His-Ser-Val) that are included at the 5′ end of the exon that encodes the entire short (start site B) precursor. We have shown that two of these four codons for (Val-X-X-Val), as well as the RNA splice acceptor site that precedes them, are conserved just upstream to a conserved B start site in BDNF genes isolated from several species; this finding led us to propose that both long and short precursor forms of BDNF exist (*19*). There is conservation of the Val-X-X-Val codons, as well as the splice acceptor site, in the rat NT-3 gene (Fig. 2A). These sequence considerations lead us to predict the existence of upstream coding exons for the NT-3 gene, which would encode a long precursor form. The finding of the conserved sequence upstream of the putative NT-3 B start site further reinforces our prediction of the existence of a long BDNF precursor and suggests a conserved role of this long precursor for all the members of the NGF family.
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34. The rat NT-3 expression construct was made by PCR to amplify the coding region of the presumed short precursor of NT-3 from a plasmid containing the 3.2-kb Sst I rat genomic fragment (Fig. 1B) that spans the NT-3 gene; the oligonucleotides used in the PCR reaction contained synthetic Xho I recognition sites at their ends, to permit insertion of the amplified coding region into the Xho I site in the polylinker of the pCDM8 expression vector (*33*). The exact oligonucleotides used to amplify the rat NT-3 gene coding region were the upstream sense primer 5′-CGG TAC CCT CGA GCC ACC ATG TCC ATC TTG TTT TAT GTG-3′ (the underlined ATG corresponds to the B start site initiation codon, with sequence downstream of the ATG matching the NT-3 sequence exactly; upstream of the ATG the primer contains a synthetic Xho I site), and the downstream antisense primer 5′-CGG TAC CCT CGA GAT GCC AAT TCA TGT TCT TCC G-3′ (the underlined triplet is complementary to the termination codon for the NT-3 gene; this triplet is flanked by exact antisense NT-3 sequence, and there is an Xho I site at the 5′ end of this primer). Similar strategies had been used to insert the coding regions of rat NGF and BDNF into the Xho I site of the same pCDM8 vector (*19*). The NT-3, NGF, and BDNF expression constructs were transfected [C. Chen and H. Okayama, *Mol. Cell. Biol.* 7, 2745 (1987)], into COS-M5 cells seeded at 5×10^5 cells per 60-mm plate and cultured in 2.5 ml of Dulbecco's modified Eagle's medium containing glucose (4500 μg/ml) and 10 percent fetal bovine serum; cells were harvested 72 hours after transfection and supernatants were analyzed.
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48. Isotopic labeling by PCR was done according to the recommended gene amplification reaction by Perkin-Elmer–Cetus, with the following modifications: 1 to 10 ng of DNA template in low-melting-point agarose was added to a reaction mixture containing unlabeled dATP, dGTP, and dTTP at a final concentration of 50 μ*M*; 50 μCi of [α-^{32}P]dCTP (3000 Ci/mmol) was added per 50-μl reaction, and the reaction was subjected to seven amplification cycles. Amplification primers were identical to the degenerate primers used in the original PCR reaction.
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