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The neurotrophin family of NGF-related neurotrophic factors

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Abstract

The recent molecular cloning of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) has established the existence of an NGF-related family of neurotrophic factors – the neurotrophins. Purification and recombinant production of BDNF and NT-3 has allowed the initiation or extension of in vitro studies of the neuronal specificity of each of these factors. We have found that NT-3, like NGF and BDNF, promotes survival and neurite outgrowth from certain populations of sensory neurons. There appear to be both distinct and overlapping specificities of the 3 neurotrophins towards peripheral neurons – sympathetic neurons and subpopulations of neural crest and neural placode-derived sensory neurons. Using cultures of central nervous system neurons, we have recently established that BDNF: (i) promotes the survival and phenotypic differentiation of rat septal cholinergic neurons, a property consistent with the discovery of high levels of BDNF mRNA expression within the hippocampus; (ii) promotes the survival of rat nigral dopaminergic neurons and furthermore protects these neurons from two dopaminergic neurotoxins, 6-hydroxydopamine (6-OHDA) and MPTP. Thus the neurotrophic effects of these factors towards peripheral neurons and neuronal populations known to degenerate in two of the major human neurodegenerative diseases – Alzheimer's and Parkinson's disease – provokes the question of whether neurotrophic factors may have therapeutic potential in halting the progression and ameliorating the symptoms of devastating neurological disorders of the CNS or PNS, or improving regeneration of neurons of CNS or PNS after traumatic injury.

Introduction

Nerve growth factor – prototypical neurotrophic factor

Until recently nerve growth factor (NGF) was the only fully characterized neurotrophic factor which had been shown both in vitro and in vivo to be essential for the survival of specific populations of neurons during development and to be important for maintenance of the differentiated phenotype of mature neurons [10–12]. The most widely known action of NGF, is undoubtedly the well-established role of this prototypical neurotrophic factor as a survival factor for developing sympathetic neurons and neural crest-derived sensory neurons [10–12]. However, two other important functions of NGF have emerged in the last few years: (a) it is now clear that NGF is not only a neurotrophic factor for peripheral neurons – in vitro and in vivo studies have shown survival promoting effects of NGF on cholinergic neurons of the basal forebrain [14–18], a role consistent with the demonstration of high levels of NGF mRNA in the targets of these neurons – the hippocampus and cortical regions [2,19–21], (b) the actions of NGF are not confined to development. Although mature neurons may no longer require NGF as a survival factor, it has been shown for example that NGF is essential for maintenance of the fully differentiated phenotype of adult rat dorsal root ganglion neurons [22,23].

Potential therapeutic use of neurotrophic factors

The emerging, broader role of NGF as a neuronal maintenance factor and/or regulator of neuronal function in mature neurons has prompted a great deal of new interest and speculation in the possible thera-

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peutic applications of neurotrophic factors in human neurodegenerative diseases [24]. Much of this speculation has been driven by the finding that basal forebrain cholinergic neurons, one of the major population of neurons to degenerate in Alzheimer's disease, are responsive to NGF both in tissue culture and in vivo [13–18]. It has now been demonstrated both in rats and primates that axotomy-induced degeneration or atrophy of septal cholinergic neurons can be reversed by intraventricular infusion of NGF [16,17,24,25]. While great caution should be exercised in drawing any conclusions from such animal studies regarding the possible efficacy of NGF in Alzheimer's disease, these studies have been instrumental in awakening the notion that neurotrophic factors may have therapeutic potential in a broad spectrum of CNS and PNS neurodegenerative diseases and nerve trauma.

Novel neurotrophic factors

The limited neuronal specificity of NGF (sympathetic, neural crest-derived sensory and basal forebrain cholinergic neurons) has long predicted the existence of other neurotrophic factors with different neuronal specificities. Although a number of distinct and potent neurotrophic activities have been described, purification and molecular characterization of these molecules has been hampered by their extremely low abundance [27]. The low abundance has also severely limited characterization of the neuronal specificities of these activities either in tissue culture or animal studies. The cloning of 3 novel neurotrophic factors in the last two years – brain-derived neurotrophic factor (BDNF) [1], neurotrophin-3 (NT-3) [2–4] and ciliary neurotrophic factor (CNTF) [28,29] has opened a new era in the biology of neurotrophic factors.

Brain-derived neurotrophic factor (BDNF)

Brain-derived neurotrophic factor was first described as a neurotrophic factor with survival-promoting effects on dorsal root ganglion (DRG) sensory neurons [30–32]. Although this activity of BDNF was similar to that of NGF towards DRG neurons, antibodies known to neutralize the biological activity of NGF did not block BDNF activity. Using sensory neurons as a bioassay, Barde and colleagues achieved purification of BDNF from pig brain, a feat which required an enrichment of almost $5 \cdot 10^6$-fold to achieve homogeneity [31]. A further 7 years were required to purify enough BDNF to yield sufficient protein for microsequencing and eventual molecular cloning of the gene encoding porcine BDNF [1]. The cloning of BDNF has had at least two very important immediate consequences: (a) it has allowed the production of recombinant BDNF, which will in turn permit the full characterization of the biological actions of BDNF, especially its neuronal specificity; (b) it has led directly to the discovery of a completely novel neurotrophic factor – neurotrophin-3 or NT-3 [2–4].

Discovery of neurotrophin-3 (NT-3)

The molecular cloning of BDNF led to the realization that NGF and BDNF are members of a gene family. Although similarities in the physico-chemical properties of NGF and BDNF (similar monomer molecular mass of 12–14 kDa, highly basic proteins of pI 9–10) had suggested that the two factors might be related, only upon cloning was it clear that amino acid sequences of the two mature proteins were highly homologous (55% identity). Of particular note was the absolute conservation of the 6 cysteine residues in both proteins and a high degree of conservation of amino acids flanking these residues. Although not yet proven, this would strongly predict that the 3 disulfide bridges found in NGF are identical in BDNF.

Discovery of the striking structural similarity of BDNF to NGF immediately suggested that other members of an even larger NGF-related family might exist. Using two slightly different strategies, but both employing polymerase chain reaction (PCR) to amplify novel related sequences with oligonucleotide primers corresponding to several of the most highly conserved regions of NGF and BDNF, Maisonpierre et al. and Hohn et al. almost simultaneously [2–3] discovered a third member of the NGF family, a gene which encodes a neurotrophic factor which is now known as neurotrophin-3 or NT-3. (Fig. 1). The same gene has now been cloned by other groups [4], and has in one instance been referred to as hippocampal-derived neurotrophic factor (HDNF) [33]. As with NGF and BDNF, NT-3 is generated from a pre-pro NT-3 species which yields a mature protein of 119 amino acids [2]. Mature NT-3 from the rat shares 57% amino acid identity with rat NGF and 58% amino acid identity with rat BDNF. The 6 cysteine residues which form 3 disulfide bridges in NGF are completely conserved among all 3 neurotrophins, and the regions of greatest homology between NT-3 and either NGF or BDNF are mostly localized around these cysteine residues. NT-3 and BDNF have now been cloned from a variety of vertebrate species. Remarkably there is 100% conservation of the amino acid sequence of NT-3 and BDNF among all mammalian species examined (human, rat, mouse, pig) to date [34]. Such a degree of sequence conservation is virtually unprecedented among protein families.

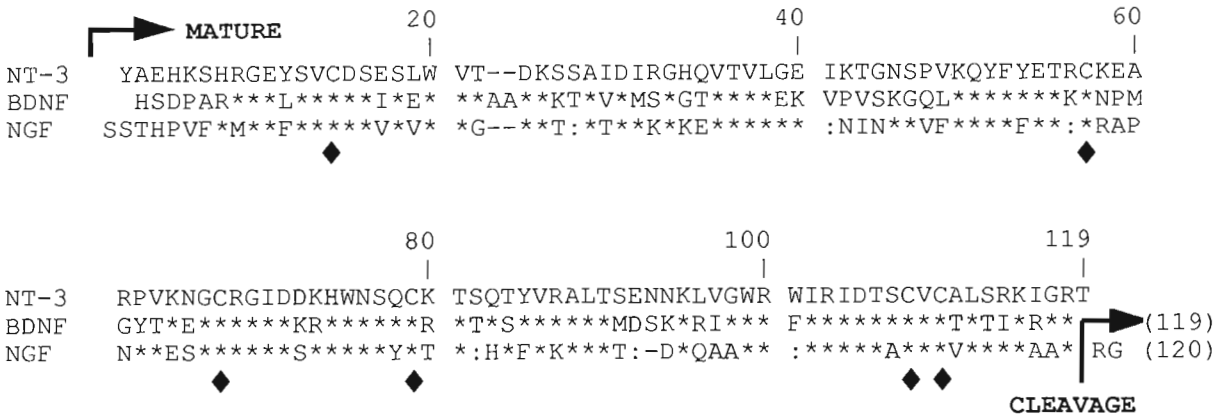

Fig. 1. Amino acid sequences of the rat neurotrophins. The amino acid sequence of the mature form of rat NT-3 is shown aligned with sequences for the mature forms rat BDNF and rat NGF. The diamond symbols (◇) indicate the 6 cysteine residues which are entirely conserved in all three neurotrophins. Sequences have been aligned to maximize homology. Gaps resulting from this alignment are shown by a dash (–). Identities of either NGF or BDNF with NT-3 sequence are shown by the asterisks. Identities of BDNF with NGF are shown by colons (:). Data from Maisonpierre et al. [2].

Neurotrophic properties of NT-3 towards PNS neurons: comparison with NGF and BDNF

Effects of NT-3, BDNF and NGF on chick embryo dorsal root ganglion neurons

To compare the putative biological activity of NT-3 to the known neuronal specificities of NGF and BDNF towards PNS neurons, recombinant rat NT-3, BDNF and NGF were prepared. In the first instance recombinant proteins were obtained by transiently expressing in COS cells the rat genes for each of the neurotrophins [2]. The culture supernatants from transfected COS cells were assayed for neurite promoting activity in both explant and dissociated, neuron-enriched cultures of chick embryo dorsal root ganglion (DRG) sensory neurons. As expected, both NGF and BDNF promoted fiber outgrowth from explants of E8 chick embryo DRG (Fig. 2), with BDNF being less potent than NGF. Both NGF and BDNF also promoted survival of E8 chick DRG neurons in dissociated neuron-enriched cultures, where the effects of the two growth factors are known to be additive [35]. NT-3 was found to be equipotent with NGF in promoting fiber outgrowth from E8 chick DRG explants (Fig. 2D). In dissociated neuron-enriched cultures, NT-3 promoted survival of 50–60% of E8 DRG neurons [2], an effect greater than that of BDNF (30–40%) but similar to that of NGF. Thus each of the 3 neurotrophins exert a neurotrophic action towards a significant proportion of chick DRG neurons. Given that each factor alone can sustain the survival of 30–60% of E8 DRG neurons, it is likely that there is some overlap in their specificities towards subtypes of DRG neurons. However, the physiological relevance of the ability of all 3 factors to act upon DRG neurons is not immediately obvious. Because there are significant differences in the spatiotemporal patterns of NGF, BDNF and NT-3 mRNA expression in the peripheral targets of sensory neurons (see below) it is possible that each factor acts either on distinct subtypes of peripheral neurons and/or possibly on overlapping subpopulations but at different times in development.

Comparison of the effect of NGF, BDNF and NT-3 on sympathetic neurons and neural placode-derived sensory neurons

Whereas NGF and BDNF both promote fiber outgrowth from explants of chick DRG, NGF alone promotes survival and neurite outgrowth from sympathetic neurons either in explant or dissociated neuron-enriched cultures [36]. Conversely, BDNF, but not NGF, promotes survival and neurite outgrowth of neural placode-derived sensory neurons, such as those of the nodose ganglion [35,37,38]. As shown in Fig. 2, NT-3 appears to promote neurite outgrowth from both neural-crest (DRG) and neural placode-derived (nodose; NG) sensory neurons. The effect of NT-3 towards NG neurons was found to be more potent than BDNF (compare 2G and 2H), and separate experiments indicate that the effects of BDNF and NT-3 are additive [3], suggesting that each factor acts upon different subpopulations of NG neurons. In addition to effects upon sensory neurons, NT-3 produced limited fiber outgrowth from explants of sympathetic neurons. The effects of NT-3 on sympathetic neurons were very small compared to NGF, but consistent with effects upon a small population of sympathetic neurons. In agreement with earlier findings, BDNF had no effect upon explants of paravertebral sympathetic chain ganglia.

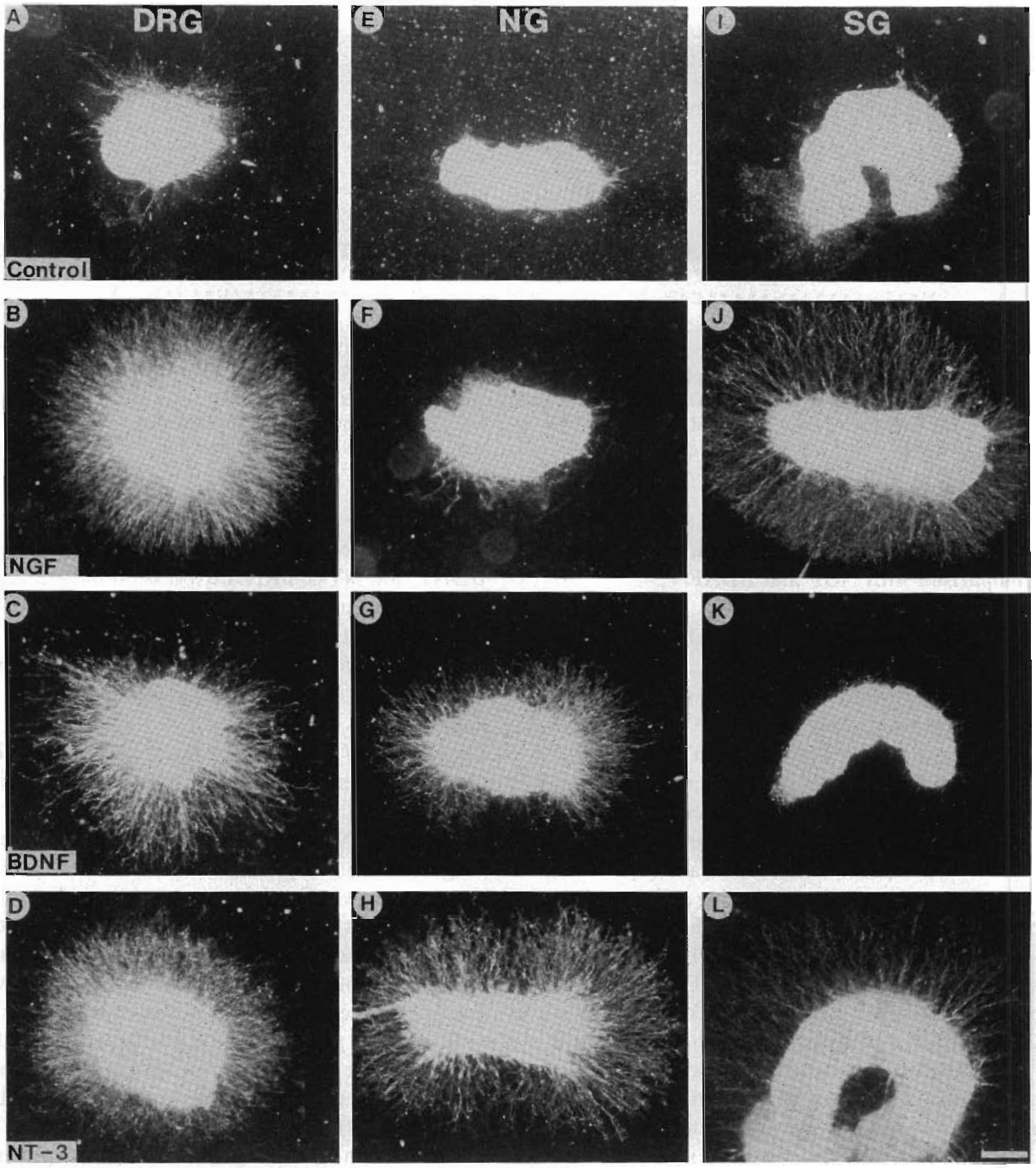

Fig. 2. Neuronal specificity of NGF, BDNF and NT-3 towards explants of chick embryo peripheral ganglia. Photomicrographs of E8 chick dorsal root ganglia (DRG; A–D), nodose ganglia (NG; E–H) and paravertebral sympathetic ganglia (SG; I–L) cultured for 24 h (DRG and NG) or 48 h (SG) either in the absence of any neurotrophic factor – upper panel – Controls, (A,E,I) – or in the presence of NGF (B,F,J), BDNF (C,J,K) or NT-3 (D,H,L). In each case the photographs are representative of the maximum fibre outgrowth from each type of ganglia seen in response to a saturating level of each neurotrophin. Data are from Maisonpierre et al. [2]. Scale bar = 200 μm.

Expression of NT-3, BDNF and NGF mRNA in peripheral tissues

The levels of expression of NT-3 mRNA in most peripheral tissues of the adult rat, with the exception of heart, are much higher than either NGF or BDNF [2,6]. Although BDNF is predominantly found in adult brain tissue, significant levels of BDNF are also present in both heart and lung. Whereas detectable levels of NGF are found in many peripheral tissues including kidney, liver and thymus, abundant levels of NGF mRNA are only seen in heart and spleen [2,6]. Although directly comparing the relative levels of mRNA for each neurotrophin in a tissue may not accu-

rately reflect the relative abundance of NGF, BDNF and NT-3 protein within that tissue, the much higher levels of NT-3 mRNA compared to NGF mRNA within peripheral tissues may suggest that NT-3, rather than NGF, is the physiologically important neurotrophic factor for many sensory neurons. This raises the possibility that we may need to re-evaluate our views of the precise role of NGF in development and maintenance of sensory neurons. Furthermore, the observation that NT-3 mRNA levels tend to be higher during development than in the adult, while conversely, BDNF levels are low during development but high in the adult [2,6], may suggest that some neurotrophic factors are primarily involved in neuronal survival and specification of neuronal phenotype during development while other neurotrophic factors have as their key role maintenance and regulation of neuronal function in the adult.

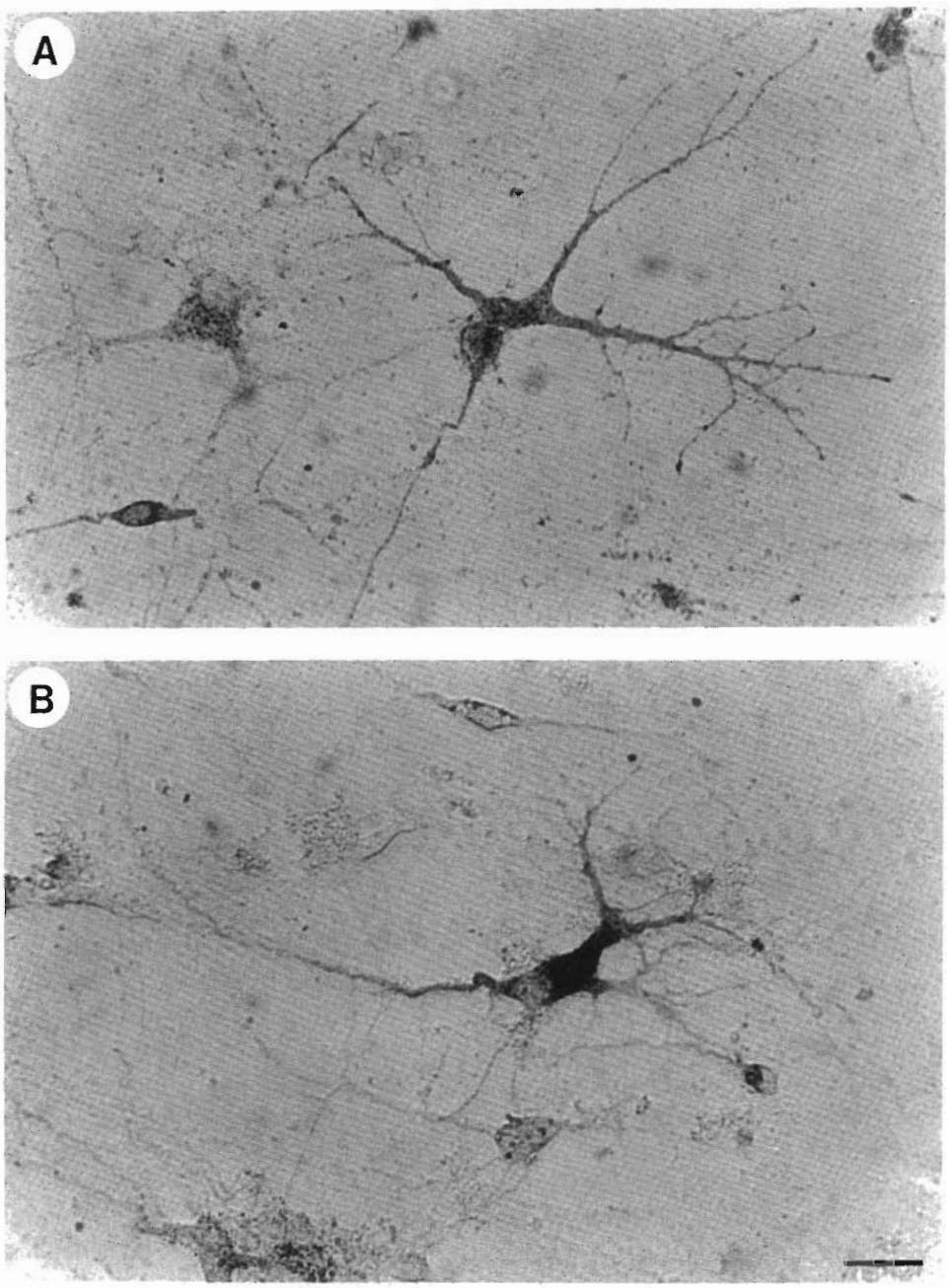

Fig. 3. BDNF increases the survival of cholinergic neurons in septal cultures as identified by markers for choline acetyltransferase and acetylcholinesterase containing neurons. Cultures derived from E18 rat brain septum (Alderson et al. [5]) were stained with antibodies to choline acetyltransferase (ChAT; A) or histochemically for acetylcholinesterase (AChE; B). These markers indicate that in the presence of BDNF there is a 2–3-fold increase in the number of cholinergic neurons which survive after 12 days in culture. Scale bar = 25 μm.

Effects of BDNF on survival and phenotypic expression of septal cholinergic neurons in culture

Although NGF was at first thought to increase choline acetyltransferase activity but not survival of cultured fetal rat septal cholinergic neurons [13], it has now been established that NGF promotes survival as well as differentiation of developing forebrain cholinergic neurons [13–15]. The numerous reports of NGF effects on basal forebrain cholinergic neurons in culture have now been corroborated by a growing literature of related in vivo studies. It has now been demonstrated by many groups that intraventricular infusion of NGF in adult rats or monkeys can prevent the atrophy and loss of phenotypic makers of septal cholinergic neurons that normally results from axotomy of the septo-hippocampal pathway following a fimbria-fornix lesion [16–18,24–26]. These studies are interesting not only because they provide a clear indication that neurotrophic factors can act to sustain the survival of mature neurons that would otherwise rapidly degenerate as a consequence of axotomy, but specifically because basal forebrain cholinergic neurons have been identified as one of the major neuronal populations which degenerate in Alzheimer's disease.

The homology of BDNF to NGF prompted us to investigate whether or not BDNF might also influence the survival or phenotypic differentiation of cholinergic neurons cultured from the embryonic rat basal forebrain. Using a histochemical stain for acetylcholinesterase (AChE) and antibodies to choline acetyltransferase (ChAT) to identify cholinergic neurons (Fig. 3), we have found that BDNF has similar effects to NGF in increasing the survival of septal cholinergic neurons [5]. After 12 days in culture there were 2.4-fold more AChE-positive cells in BDNF-treated cultures as compared to controls. As observed with NGF, the greatest effects of BDNF on cholinergic neuron survival were seen at low cell densities [5,14]. Although there were some clear differences in the dose – response curves to NGF and BDNF, we found that effects of BDNF on the expression of phenotypic markers in septal cultures were broadly similar to NGF [5]. BDNF-induced increases in choline acetyltransferase activity (ChAT), acetylcholinesterase activity, high-affinity sodium-dependent choline uptake and the number of NGF-receptor immunopositive cells were essentially of the same magnitude (2-3-fold) as previously reported following NGF treatment.

Whereas there were no additive or synergistic effects of NGF and BDNF in terms of survival of AChE-positive cells, indicating that both growth factors probably act upon the same neuronal population, BDNF and NGF clearly act synergistically in stimulating maximal expression of ChAT activity (Fig. 4). Although it is not immediately obvious as to why these cholinergic neurons should be responsive to two members of the neurotrophin family, it is noteworthy that BDNF mRNA is as abundant as NGF mRNA in the adult hippocampus, the target field of septal cholinergic neurons [6–8]. NT-3 levels are also exceptionally high in the developing and adult hippocampus [6]. It will be interesting to determine whether or not this third member of the neurotrophin family has any effect upon the survival or differentiated phenotype of septal cholinergic neurons.

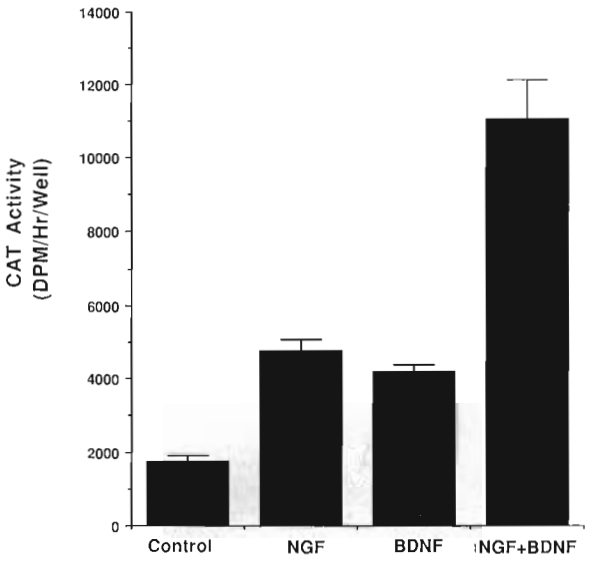

Fig. 4. Synergistic effect of co-addition of BDNF and NGF on the level of choline acetyltransferase activity in septal cultures. Saturating concentrations of NGF (50 ng/ml) and BDNF (25 ng/ml) individually produced increases of 2.5–3.0-fold in the level of ChAT activity detected in septal cultures after 12 days. Co-addition of BDNF and NGF produced a level of ChAT induction (5–6-fold) that was greater than the sum of the induction produced by each growth factor alone. Values are the mean ± S.E.M. of $n = 4$ or 5. Methods as described in Alderson et al. [5].

Effects of BDNF on survival of nigral dopaminergic neurons in culture

Degeneration of dopaminergic neurons of the human substantia nigra is the principal cause of the onset and progressively debilitating course of Parkinson's disease. As yet there have been no reports of a fully characterized growth factor having direct neurotrophic action towards dopaminergic neurons of the developing or mature substantia nigra. NGF has been shown to have no effect upon the survival of nigral dopaminergic neurons. There have been some reports of effects of basic fibroblast growth factor (bFGF) on these neurons [39,40], but it now appears that the effects of bFGF

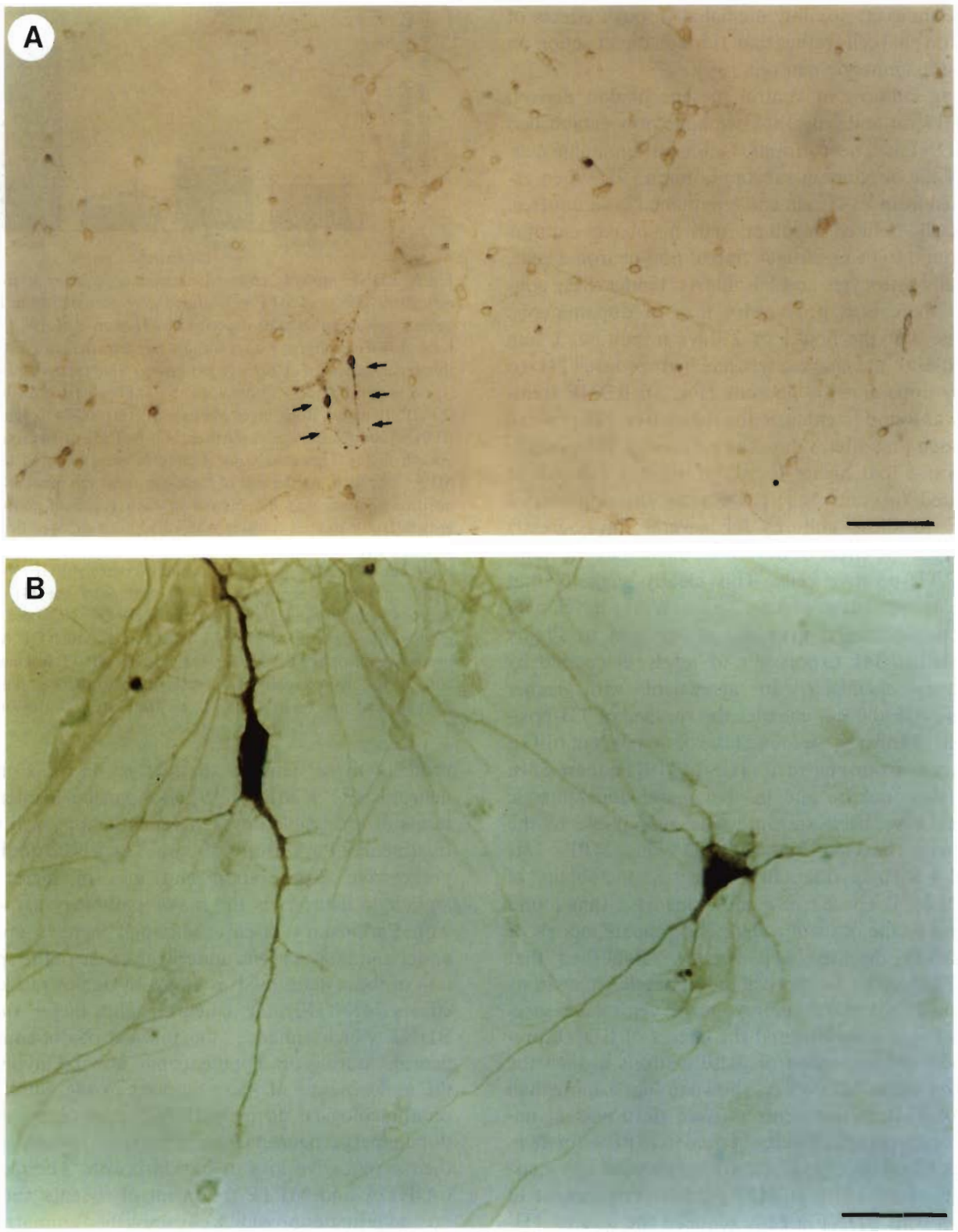

Fig. 5. BDNF promotes survival of dopaminergic neurons of the developing rat substantia nigra. Photomicrographs of neuronal cultures derived from E14 rat ventral mesencephalon. Dopaminergic cells were identified using antibodies to tyrosine hydroxylase (TH). In BDNF treated cultures there was a 5-fold greater number of TH positive neurons compared to untreated cultures after 8 days. Cultures were prepared as described in Hyman et al. [9]. Scale bars = 100 μm (A) and 25 μm (B).

may be indirect, possibly mediated through effects of bFGF on glial cells rather than through direct action on nigral dopaminergic neurons [39].

Using cultures of ventral mesencephalon derived from E14 rat embryo brain, we have now established that BDNF is a neurotrophic factor for dopamine neurons of the developing substantia nigra [9]. When established from E14 brain and maintained in serum-free, chemically defined medium, mesencephalic cultures were found to be essentially free of non-neuronal cells, especially astrocytes and fibroblasts. Under these conditions there is a progressive loss of dopaminergic neurons after the first 1 or 2 days in culture. Using antibodies to the enzyme tyrosine hydroxylase (TH) to identify dopaminergic neurons (Fig. 5), BDNF treatment was found to enhance the survival of TH-positive cells, such that after 8 days the number of TH-positive cells was 5-fold higher in BDNF-treated cultures as compared to controls [9]. Delaying the addition of BDNF to these cultures for several days, greatly diminished the effect of BDNF on sustaining the number of TH-positive cells. This clearly suggests that BDNF is required to sustain the survival of developing nigral dopaminergic neurons, as opposed to simply upregulating TH expression to levels detectable by immunocytochemistry. In agreement with earlier studies NGF did not enhance the survival of TH-positive cells. Similarly, we found little or no effect of bFGF or ciliary neurotrophic factor (CNTF) [9] on these cells.

In tissue culture and in vivo nigral dopaminergic neurons have been shown to be susceptible to the neurotoxic effects of both 6-OHDA and MPP+ (1-methyl-4-phenylpyridinium; the active metabolite of MPTP) [41]. These two neurotoxins have thus found wide use as the basis of rodent and primate models of Parkinson's disease [41]. Having established that BDNF enhances the survival of dopaminergic neurons in cultures derived from embryonic rat ventral mesencephalon [9], we investigated the effects of BDNF pretreatment on protection of these neurons against the neurotoxicity of MPP+. As shown in Fig. 6, more than 80% of TH-positive neurons were destroyed in untreated cultures which were exposed to MPP+ for 48 h. Neither bFGF nor NGF treatment induced any resistance to the toxicity of MPP+, but pretreatment of cultures with BDNF greatly reduced the loss of TH-positive neurons after 48 h exposure to MPP+. Not only was BDNF treatment found to protect dopaminergic neurons against MPP+, but also against 6-OHDA toxicity, as measured by a reduced loss of TH-positive neurons or dopamine uptake.

The finding that BDNF enhances the survival of developing nigral dopaminergic neurons in culture and protects these neurons to a large degree from the neurotoxicity of MPP+ [9] is of particular interest in terms of a possible novel therapeutic approach to the treatment of Parkinson's disease. There is no doubt that progressive degeneration and loss of nigral dopaminergic neurons is the major pathology associated with Parkinson's disease, although there is no clear understanding as to the underlying cause of the specific loss of these neurons. It is likely, by analogy to known effects of NGF, that a neurotrophic factor such as BDNF which enhances the survival of dopaminergic neurons during development may also be involved in the maintenance of these neurons in the adult. Thus pharmacological doses of BDNF may rescue mature dopaminergic neurons from the insult(s) which leads to their progressive loss in Parkinsonism. The fact that 6-OHDA and MPTP treatment of rodents and non-human primates provide well established animal models of Parkinsonism [41] will allow testing of this hypothesis in the near future.

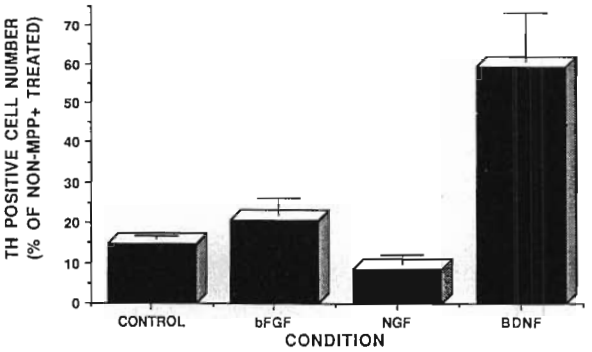

Fig. 6. BDNF protects cultured dopaminergic neurons from the neurotoxic effects of MPP+. Cultures were prepared from E14 rat ventral mesencephalon as described by Hyman et al. [9]. Cultures were grown initially for 3 days without any growth factor. Following this separate sets of cultures (6 per group) were treated either with (i) no growth factor – Control, (ii) basic fibroblast growth factor (bFGF, 10 ng/ml; Boehringer-Mannheim), (iii) NGF (50 ng/ml), (iv) BDNF (50 ng/ml). After a further 24 h half (3) of each group of growth factor-treated or control cultures were exposed to 1 μM MPP+ for 48 h. At the end of the experiment the number of TH-positive neurons was determined in each group of control and growth factor-treated cultures which had been exposed or not exposed to MPP+. In this bargraph the number of TH-positive neurons counted in MPP+ exposed cultures (control, bFGF, NGF and BDNF groups) is expressed as a percentage of the TH-positive neurons found in similar cultures (control, bFGF, NGF and BDNF groups) not exposed to MPP+. Thus bFGF and NGF have no protective effect against MPP+ toxicity, but BDNF rescues about 50% of the neurons which are susceptible to MPP+ poisoning. Results are the mean ± S.E.M. of triplicate cultures in each condition.

Summary

Until recently almost all our knowledge of neurotrophic factor biology – their role in development,

their role in maintenance and regulation of function of mature neurons and their role in regeneration – was based on studies with NGF. The wealth of information on NGF and the paucity of data on other neurotrophic factors is undoubtedly due to there being a rich biochemical source of NGF (in the mouse salivary gland) versus extremely low abundance of neurotrophic factors in general. The recent cloning of BDNF, NT-3 and CNTF and the likelihood of other neurotrophic molecules being cloned in the near future opens up the way to producing these rare molecules by recombinant techniques. The more widespread availability of neurotrophic factors, antibodies to neurotrophic and probes to detect the sites and levels of synthesis of neurotrophic factors should soon demonstrate that NGF is indeed only part of the neurotrophic story, not all of it. As greater understanding of the specificity of novel neurotrophic factors emerges, the potential utility of such molecules as therapeutic agents will increase. It is already envisaged that neurotrophic factors may be useful in the treatment of neurodegenerative diseases of both the peripheral and central nervous system. It is likely in the longer term that specific neurotrophic factors, possibly already known molecules, will be found that will enhance the survival and regeneration of axotomized spinal cord neurons. Such factors may well form part of a 'cocktail' of agents that will aid in improving the outcome of human spinal cord trauma.

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