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Neurotrophins and neurodegeneration

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Neurotrophins and neurodegeneration

There is growing evidence that reduced neurotrophic support is a significant factor in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). In this review we discuss the structure and functions of neurotrophins such as nerve growth factor, and the role of these proteins and their tyrosine kinase (Trk) receptors in the aetiology and therapy of such diseases. Neurotrophins regulate development and the maintenance of the vertebrate nervous system. In the mature nervous system they affect neuronal survival and also influence synaptic function and plasticity. The neurotrophins are able to bind to two different receptors: all bind to a common receptor $p75^{NTR}$, and each also binds to one of a family of Trk receptors. By dimerization of the Trk receptors, and subsequent transphosphorylation of the intracellular kinase domain, signalling pathways are activated. We discuss here the structure and function of the neurotrophins and how they have been, or may be, used therapeutically in AD, PD, Huntington's diseases, ALS and peripheral neuropathy. Neurotrophins are central to many aspects of nervous system function. However they have not truly fulfilled their therapeutic potential in clinical trials because of the difficulties of protein delivery and pharmacokinetics in the nervous system. With the recent elucidation of the structure of the neurotrophins bound to their receptors it will now be possible, using a combination of *in silico* technology and novel screening techniques, to develop small molecule mimetics with much improved pharmacotherapeutic profiles.

Keywords: ALS, Alzheimer's, Huntington's, mimetics, neuropathy, neurotrophins, NGF, Parkinson's

The neurotrophins and their receptors

Over the last two decades much progress has been made in understanding the relationship between neurotrophins, their receptors and neurodegenerative diseases. In particular nerve growth factor (NGF) and its receptor TrkA have a prominent role in both the aetiology and the treatment of Alzheimer's disease (AD). In Huntington's disease the mutant protein huntingtin leads to a down-regulation of brain-derived neurotrophic factor (BDNF) in the basal ganglia leading to neuronal loss, opening up the possibility of BDNF therapy. Moreover, startling clinical results have been found using intraparenchymal injection of a related neurotrophic factor, glial-derived neurotrophic factor (GDNF), for the treatment of Parkinson's disease (PD). This review discusses in depth the involvement of the neurotrophins in neurodegenerative diseases.

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Neurotrophins, a historical perspective

The first neurotrophin to be discovered was NGF. In 1948 it was shown that, when fragments of a mouse sarcoma were grafted into the body wall of chick embryos, the sensory nerve fibres from the chick dorsal root ganglion (DRG) penetrated into the sarcoma [29]. Later sympathetic fibres were also shown to penetrate the sarcoma, and the sympathetic ganglia innervating the tumour increased in volume [120]. Subsequent purification studies led to the isolation of a nucleoprotein fraction with nerve growth-promoting activities. A systemic search of mammalian tissues showed that male mouse salivary glands contained relatively large amounts of NGF [42]. Further work established that NGF is released by the target tissues of sympa-

thetic and sensory fibres, and is taken up by the fibres and retrogradely transported to the cell body. From this work the Neurotrophic Factor Hypothesis is developed . This proposed that a developing neurone, having extended processes into its target organ, would be in competition with other neurones for limited supplies of target-produced neurotrophic factors. Those that obtain the growth factor survive; those that do not die.

The sequencing of mouse salivary gland NGF, in 1971 [93], provided information on its primary structure but more importantly also paved the way for the cloning of the mouse and human genes. The human NGF gene is located on the short arm of chromosome 1 (1p22) [66] and codes for a polypeptide of 307 amino acids which, when the signal peptide and pro region are cleaved, gives rise to a mature protein of 118 amino acids which naturally exists as a noncovalently bound homodimer.

The neurotrophin family

In addition to NGF, another factor was purified from pig brain that demonstrated neuronal growth-promoting activities [13]; this was later called BDNF. Cloning of the gene [119] (present on human chromosome 11: 11p13 [128]) revealed close homologuey to NGF with 51 identical amino acids; such similarity suggested that it also existed as a homodimer. By using the contiguous regions between NGF and BDNF to design oligonucleotides for polymerase chain reaction cloning, a third related protein called neurotrophin 3 (NT-3) was cloned [59,94,127]. This is located on human chromosome 12 (12p13) [128]. Finally after an exhaustive search the last of the neurotrophin family was identified and cloned in Xenopus (NT-4) [80]. The equivalent human cDNA was sufficiently different from Xenopus NT-4 for it to be thought a separate gene and called NT-5 [19]. Subsequently it was realized that they are homologueous genes and this neurotrophin is often now termed NT-4/5. NT-4/5 is present on human chromosome 19 (19q13.3) [18]. NT-6 has only been found in fish [75]. All the neurotrophins are synthesized as precursor proteins (approximately 30 kDa in size) and are cleaved to form the mature form, and all form noncovalent-linked homodimers where each monomer is approximately 13 kDa. They are all basic proteins with isoelectric points above 9.0. Because of the characteristic formation of a double loop formed by two disulphide bonds, penetrated by a third disulphide bond, they are classed as part of the Cysteine Knot Superfamily. The X-ray crystal structures of the neurotrophins have all been elucidated and are shown in Figure 1. Shown for comparison is the structure of a related protein, GDNF, which is also a member of the Cysteine Knot Superfamily. This protein is briefly mentioned in this review because of its recent success in PD trials.

Neurotrophin receptors: identification and structure

$P75^{NTR}$ receptor The receptors for NGF were first identified on chick sensory ganglia and DRG using receptor-binding techniques with ^{125}I-labelled NGF [194]. Originally this receptor was thought to be specific for NGF, although its method of signalling was unclear. It was eventually established, by analysis of the binding kinetics, that there were two receptors present: one with a dissociation constant (Kd) of approximately 10^{-11} M and the other with a Kd of approximately 10^{-9} M. The molecular weights were subsequently determined by cross-linking experiments and found to be approximately 140 kDa and approximately 75 kDa, respectively. The lower-affinity receptor was cloned from rat [161] and human [103] and was usually referred to as $p75^{NGFR}$. It is now known to be a common receptor for all of the neurotrophins ($p75^{NTR}$) [169]. The 3.8 kb mRNA for $p75^{NTR}$ encodes a 427 amino acid protein containing a 28 amino acid signal peptide, a single transmembrane domain and a 155 amino acid cytoplasmic domain. The gene is present on the human chromosome 17 (17q21–22) [97]. A schematic of the structure of the $p75^{NTR}$ and that of the tyrosine kinase (Trk) receptors is given in Figure 2. The $p75^{NTR}$ receptor was shown to be a member of the tumour necrosis superfamily of receptors [26,35] and contains a cytoplasmic 'death' domain [122], a domain known to be involved in apoptosis.

Neurotrophin binding to $p75^{NTR}$ has been shown to affect cell survival [14] and axonal outgrowth [17,207]. Independent of Trk, the binding of neurotrophins to the $p75^{NTR}$ receptor results in activation of NFκβ, a transcription factor [33], and c-Jun N-terminal kinase kinase [34]. Recently $p75^{NTR}$ has been shown to interact with the Nogo receptor which is involved in myelin-associated inhibition of axonal elongation [53].

Trk receptors: discovery and structure In 1986 a human oncogene was isolated from colon carcinoma and was called *trk* (tyrosine receptor kinase) [130]. The gene consisted of a fusion between a known muscle protein, tro-

pomyosin, and a receptor kinase domain from an unknown protein. Use of the sequence of the Trk domain to screen cDNA libraries led to the identification of the proto-oncogene *trkA* [131]. The fact that the expression profile for *trkA* correlated with the location of those neurones that respond to NGF and that the size of the TrkA protein was approximately 140 kDa led to the breakthrough by Klein [107] that TrkA was the receptor for NGF. Screening of cDNA libraries had led to the identification of the related genes *trkB* [110] and *trkC* [118]. TrkB protein was shown to be a receptor for BDNF [109] and NT-4/5 [108] and TrkC was found to be a receptor for NT-3 [118]. The protein Trk domains of TrkA, TrkB and TrkC were found to be 87% homologueous at the amino acid level. TrkA, like NGF, is located on chromosome 1 (1q21–22), TrkB is on chromsome 9 (9p22.1) and TrkC is on chromosome 15 (15q25). Northern analysis using probes for *trkA*, *trkB* and *trkC* demonstrated that their expression is restricted primarily to the nervous system.

These Trk receptors form a homologueous family of proteins, each comprising about 800 amino acids with half of the residues at the amino terminus forming the extracellular portion of the receptor. Examination of sequence motifs in the extracellular region of the Trk receptors showed that there are five distinct domains [180] (Figure 2). Mutational and domain-swapping

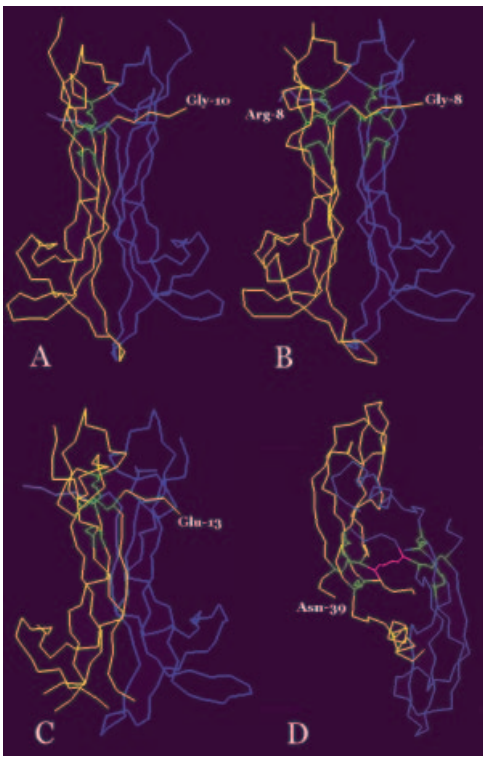

Figure 1. X-ray crystal structures of (**A**) NGF homodimer; (**B**) BDNF:NT-3 heterodimer, a recombinant protein which may not exist naturally, made for the structural determination of both proteins simultaneously [168]; (**C**) NT-4 homodimer; and (**D**) GDNF homodimer. One chain is shown in yellow, the other in blue. The disulphide bonds which form the cysteine knot motif are shown in green and are only shown for one chain for **A**, **C** and **D**. For **B** they are shown for both monomers because this comprises of two different neurotrophins. The two monomers of GDNF are connected by a disulphide bond, which is shown in red. The N-terminal amino acid for each of the chains is indicated. Note that in each case several amino acids are missing in the structures. GDNF is not a neurotrophin but is of the cysteine knot superfamily, which all share a similar overall monomer fold.
NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT, neurotrophin; GDNF, glial-derived neurotrophic factor.

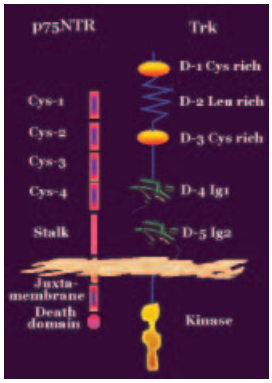

Figure 2. Schematic representation of the $p75^{NTR}$ and a generic tyrosine kinase receptor showing individual domains.

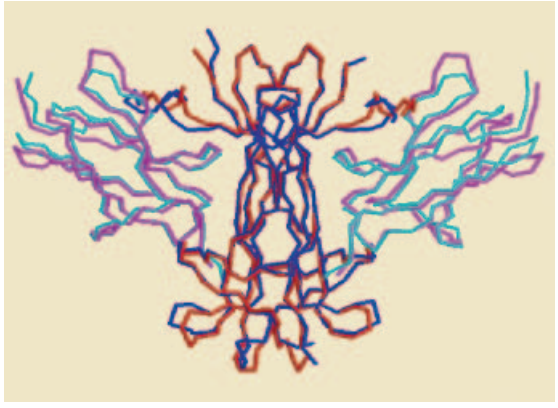

Figure 3. Comparison of the X-ray crystal structures of known Trk-neurotrophin complexes. Cα traces of the TrkBIg2:NT-4/5 (cyan and blue, respectively) and TrkAIg2:NGF (pink and red, respectively). Note that in each case the N-terminal of each neurotrophin is now fully visible and forms intimate contact with the Trk receptors.
Trk, tyrosine kinase; Ig, immunoglobulin; NT, neurotrophin; NGF, nerve growth factor.

experiments suggested that the immunoglobulin (Ig)-like domains were involved in ligand binding [153]. The expression and purification of recombinant Ig domains in *Escherichia coli* allowed the determination of the dissociation constants for their ligands, the neurotrophins [95,167]. These studies conclusively showed that the Ig domains bind to the neurotrophins with similar affinity to the wild type membrane-bound receptors [95,167]. The X-ray crystal structures of TrkAIg2, TrkBIg2 and TrkCIg2 domains have been determined [167,203] but in each case, the structures generated were those of artificial strand-swapped dimers where the A strand from each monomer sits next to the B strand of the other monomer. The correct structures were generated using molecular modelling and showed that the Ig2 domains adopt an I-set fold. Subsequently the crystal structures of NGF bound to TrkAIg2, NT-4/5 bound to TrkBIg2 (Figure 3) and a model of BDNF bound to TrkBIg2 showed that the original models of the Ig2 domains were indeed correct [12,149,210].

Differential splice variants of TrkA, TrkB and TrkC, producing additional short amino acid sequences in the juxtamembrane domains of the receptors, may have effects on ligand interactions [40,182,190]. There is also differential splicing of exons coding for the intracellular portion of the TrkB and TrkC receptors. Both these receptors have forms that do not have kinase domains, the functions of which are thought to include clearance of ligand, and perhaps inhibition of dimerization thus reducing response [57].

Signalling through the Trk receptors

Binding of the neurotrophins to the Trk receptors initiates signalling cascades, by means of phosphorylation of tyrosine residues on the cytoplasmic domains of the receptors. Phosphorylation induces docking of adapter proteins with phosphotyrosine-binding or src-homologuey-2 motifs. These adapter proteins couple the receptors to intracellular signalling cascades, which include the phosphatidylinositol-3-kinase/Akt kinase pathway, phospholipase Cγ and the Ras kinase pathway [106] ultimately leading to activation of gene expression, neuronal survival and neurite outgrowth.

Interactions of Trk and p75^{NTR}

Binding of neurotrophins to their Trk receptors causes signalling events which promote neurone survival, whereas activation of the p75^{NTR} pathway triggers apoptosis and cell death [20]. The capacity of NT-3 to activate TrkA, and of NT-3 and NT-4/5 to activate TrkB is regulated by p75^{NTR} [21]. It is likely that neurotrophins can bind both Trk and p75^{NTR} simultaneously, and that Trk receptors and p75^{NTR} may interact with each other [20,21].

Distribution of the neurotrophins and their receptors in the brain

All of the neurotrophins show different distributions through the peripheral nervous system and central nervous system (CNS) with distinct and overlapping specificity towards subpopulations of sensory neurones of both neural crest and neural placode origin. Highest levels of BDNF mRNA, NGF mRNA and NT-3 mRNA in the adult mouse brain [91] are found in the hippocampus, with 50 times more BDNF mRNA being present than NGF mRNA. NT-3 is by far the most highly expressed in the immature CNS and expression dramatically decreases with maturation. BDNF expression is low in developing regions of the CNS and increases as these regions mature, whereas NGF expression varies during the development dependant on the region [126]. Cholinergic neurones project from the basal forebrain nucleus (nucleus basalis of Meynert, nbM) to the hippocampus and neocortex with 80–90% of the neurones in the human/primate nbM being cholinergic [137,138]. If ^{125}I-NGF is injected into the hippocampus or the cortex, it is taken up by the terminals of cholinergic cells and retrogradely transported back to the cell bodies in the basal forebrain [181]. Thus levels of NGF mRNA in various regions of the brain were found to correlate with the degree of basal forebrain cholinergic innervation [114] with highest levels of NGF and NGF mRNA in the hippocampus and cerebral cortex. Figure 4 shows a human basal forebrain neurone stained with anti-p75^{NTR}.

Immunohistochemical and *in situ* analysis of the distribution of p75^{NTR} in rat and primate brain [4,49,111,165] and colocalization of choline acetyltransferase (ChAT) and p75^{NTR} immunoreactivity [50,111] show that p75^{NTR} is almost exclusively located on basal forebrain cholinergic neurones. More recently, examination of the distribution of TrkA protein and mRNA in rat and human brain shows that TrkA is also expressed on basal forebrain cholinergic neurones [186,189] although there are some TrkA-positive, p75^{NTR}-negative neurones in the striatum [4,50,189]. By contrast TrkB and TrkC are much more widely distributed [36].

Alzheimer's disease

Overview of symptoms and pathogenesis

Alzheimer's disease is the most common form of dementia in the elderly. It is characterized by memory loss and confusion, together with neuropathological changes including neuronal loss, extracellular deposits of amyloid plaques and intracellular deposition of hyperphosphorylated tau ('tangles') [2]. Rarely, cases are familial, with autosomal dominance, because of mutations in the proteins amyloid precursor protein (APP), or presenilin 1 or 2 (PS1, PS2); but most cases are sporadic, of unknown aetiology, although the presence of a particular isoform of the protein apolipoprotein E, apoE4, results in an increased risk of AD in the general population. It is widely thought that pathology is related to increased production of a 4-kDa peptide called amyloid or Aβ, processed from APP by enzymes known as β-secretase (BACE: β site APP cleaving enzyme) and γ-secretase (associated with PS1/2) and recent evidence shows that enzymes associated with the processing of APP may be affected in sporadic AD [96,202].

The cholinergic deficit and current treatment

The earliest symptom of AD is a loss of short-term memory. This has been associated with the early and substantial decline in cholinergic function in the cortex and hippocampus [27,48,156]. The majority of the cholinergic neurones innervating the cortex and hippocampus originate from the basal forebrain nuclei including the medial septal nucleus and the nbM [137]. Lesions of the innervating cholinergic pathways or administration of anticholinergics such as scopolamine result in impaired ability to perform certain memory tasks [56,164]. Currently the only successful drug therapies for AD are acetylcholinesterase inhibitors, which inhibit the breakdown of acetylcholine and thus potentiate its action. Early trials [193] showed great improvements in patients using the anticholinesterase tacrine (Cognex). Now large multicentre studies have shown that tacrine treatment is of some benefit in some AD patients, and although not as dramatic as in the original study it is able to improve cognitive function significantly [11]. Side-effects were cause for concern, however, second-generation cholinesterase inhibitors, donepezil (Aricept), rivastigmine (ENA 713, Exelon), and galantamine (Reminyl), are better tolerated resulting in cognitive improvement in over half the patients, often for 6–9 months or even longer.

Cholinergic cell loss in Alzheimer's disease

In AD, cholinergic cells of the basal forebrain may be either lost or atrophied, although, in general, the loss of cortical cholinergic activity far exceeds the neuronal loss in the nbM. There is a variance as to extent of loss reported, probably mainly because of age of patients, as brain samples from younger patients often show more extreme losses [3,72,87,143]. Analysis of cholinergic cells in brains from severely demented patients who died in their late 70s and 80s, visualized using anti-$p75^{NTR}$, showed only moderate cell loss in most areas of the nbM [3]. Dementia (Kew) score correlated best with accumulated neuronal losses in the intermediate and posterior regions, areas reported by others as most affected [143,201]. In normal and AD basal forebrain, similar amounts of mRNA for $p75^{NTR}$ were found by Northern blotting [72] and with ligand binding studies of $p75^{NTR}$ [143,201]; although studies examining the number of TrkA positively staining cells in AD basal forebrain have generally shown a marked reduction in AD [25,145].

It has been suggested that the observed degeneration of these cholinergic cells in AD may be caused by reduced trophic support; that is, a lack of production of NGF in the cerebral cortex and hippocampus. However, measurements of the content of NGF protein in brain have shown nonsignificant [5] or significant [47] increases, not decreases as expected, in cerebral cortex in AD. Recent evidence suggests that the situation is not quite so straightforward. By use of Western blotting, pro-NGF rather than mature NGF was seen to be doubled in AD parietal cortex [61]. Thus the balance between pro-NGF and mature NGF may be critical [36]. In addition, it is possible that there is a dysfunction in retrograde transport of NGF from the target tissues to the cell bodies. Support for this hypothesis comes from a study that shows defective transport in NGF in a model of Down's syndrome or trisomy 21. Down's syndrome invariably results in the symptoms and pathology of AD by the fourth decade of life. This is thought to be caused by the presence of an extra copy of the APP gene in Down's syndrome patients, possibly leading to an increased amyloid burden. In a study using a mouse model of this disorder (trisomy 16), there was no retrograde transport of radio-iodinated NGF from the hippocampus to the septum in trisomic mice, compared with almost complete transport in wild-type littermates [45]. If this were also true of

AD, it would explain why NGF levels are not diminished in target areas of the cholinergic basal forebrain cells. In addition, we know that NGF content, whilst not reduced in the cerebral cortex and hippocampus, is decreased in the basal forebrain [144].

NGF restores cholinergic function

Numerous studies have shown that administration of NGF to the basal forebrain cholinergic neurones *in vitro* results in increased survival and up-regulation of ChAT (the enzyme involved in synthesis of acetylcholine) activity [71,83–85,88,132]. This is also true *in vivo*. Lesion of the connection between the septum and the hippocampus, involving severing of the fimbria-fornix in rats, prevents the retrograde transport of NGF down cholinergic fibres to the cell bodies, resulting in cholinergic cell atrophy. Intracerebroventricular (icv) administration of NGF has been shown to abolish this degeneration [68,82,86,116,213] and the cognitive deficits arising from these lesions [129,212,213]. In addition, a subpopulation of aged rats which perform poorly in the Morris water maze and have reduced cholinergic function is able to function normally after icv infusion of NGF [63–65].

Clinical trials with NGF protein

Intracerebroventricular administration of NGF In clinical trials in the early 90s, three AD patients in Sweden were administered mouse NGF icv [104,151] continuously for up to 3 months. Two of these patients were given up to a total of 6.6 mg. The results were different in each patient. In general, some improvements were seen in a few of the cognitive tests: in one patient cortical blood flow was increased, and another showed increased glucose metabolism; ^{11}C nicotine binding in the cortex was increased in the two patients given the higher dose. Nevertheless, there were side-effects. The two patients with higher doses showed loss of weight and appetite, and all three patients reported pain. These symptoms all ceased when the NGF infusion was stopped. However, in addition, a dose-related Schwann cell hyperplasia after icv administration of rhNGF in rats [52,214] and primates [214] has been observed. Histological examination showed atypical hyperplastic tissue, attached to the dorsal and lateral surface of the medulla and the spinal cord. This hyperplasia was reversible: in the Day-Lollini study [52] it was significantly reduced after 8 weeks and completely abolished after 52 weeks. These studies suggest that infusion with icv administration of NGF will probably result in a similar hyperplasia in man and, although probably reversible and nonmalignant, it may be partly responsible for pain. It is also worthy of note that this is caused by the presence of $p75^{NTR}$ receptors, not TrkA, because these hyperplastic cells were found to be immunoreactive for $p75^{NTR}$ but not TrkA. Thus it may be better to administer NGF by some other route to avoid side-effects associated with icv administration.

Other routes of administration Studies have shown that retroviral transfected fibroblasts, expressing NGF, rescue cholinergic neurones after a fimbria fornix lesion in the rat [23]. In addition, aged rhesus monkeys, with atrophy in the cholinergic basal forebrain, injected with NGF-expressing fibroblasts into the tissue adjacent to the basal forebrain, restored numbers of ChAT-positive subcortical neurones after 3 months [185]. In April 2001, a 60-year-old Caucasian woman, in the early stages of AD, was surgically implanted with her own modified fibroblasts, transfected with a virus-expressing NGF. The patient received five implants of modified cells near the nbM. We await results of this trial by researchers at the University of California at San Diego and the Salk Institute for Biological Sciences. In addition, reports have shown that following injection of ^{125}I-NGF into the olfactory bulb of rats, radio-labelled NGF was found to be retrogradely transported specifically to basal forebrain cholinergic nuclei [7]. Similarly it has been shown that radio-labelled NGF can be transported into the brain following administration as nose drops [39,197]. This may be a feasible mode of administration for the future.

A link between NGF and the neuropathology of Alzheimer's disease

In the phenotypes of knockouts of both NGF and TrkA [46,184] there are large reductions in ChAT immunoreactivity in the basal forebrain and loss of cholinesterase activity in both the hippocampal and cortical issues. Mice that are heterozygous for the NGF deletion have deficits in spatial memory function in the Morris water maze [37,38] that correlate with losses in ChAT-positive basal forebrain neurones.

Recently, a transgenic mouse (AD11) has been established which produces antibodies to NGF [32,175]. These antibodies accumulate throughout the adult life of the animal thus obviating the developmental problems

associated with the NGF knockout. The mice acquire an age-dependent pathology reminiscent of human AD, including amyloid plaques, and hyperphosphorylated tau in cortical and hippocampal neurones [32]. In addition they display extensive cortical cell loss, cholinergic deficit in the basal forebrain and behavioural deficits. Recently the effects of intranasal administration of NGF and intraparenchymal injection of galantamine have been studied in these mice [31]. A variety of regimes at 2, 6 and 6.5 months of age were assessed with striking results. Administration of NGF (1.2–12 μg on alternate days) or galantamine (3.5 mg/kg daily) completely restored the number of ChAT-immunopositive neurones to normal values. In addition, NGF reversed deposition of hyperphosphorylated tau in 2- and 6-month-old and markedly reduced amyloid plaques in the 6- and 6.5-month-old rats. It had no effect, however, on deposited APP in cerebral vessels. By contrast galantamine removed APP deposition but had no effect on hyperphosphorylated tau. It did, however, remove amyloid plaques almost as effectively as NGF. Surprisingly the acetylcholinesterase inhibitors tacrine and physostigmine had no effect on any of these parameters and it was suggested by the authors that it is the nicotinic receptor modulating effects of galantamine that are important here. These studies suggest that it is a lack of available NGF that begins the process of plaque and tangle formation. It seems that galantamine is able to reverse the plaque deposition but not tangle formation. In the future it will be interesting to note if AD patients, on a regime of galantamine, have lower plaque numbers than expected.

BDNF in Alzheimer's disease

In addition to the link between NGF and AD there is some evidence to implicate changes in BDNF and TrkB in the disease process. Firstly, BDNF mRNA has been reported as decreased in AD hippocampal samples by *in situ* hybridization [157], and by immunocytochemical techniques; BDNF protein levels are reported as reduced in neurones of the hippocampus and temporal cortex [44]. There is also some evidence to suggest a change in the TrkB receptor. Immunopositive, truncated (95 kDa) TrkB receptors are reported as present in amyloid plaques of the hippocampus [43]. In the basal forebrain, immunostaining showed a large reduction in TrkA, TrkB and TrkC expression in AD [176], whereas *in situ* hybridization showed no change in TrkB [24]. In parietal cortex mRNA of TrkB was reported as unchanged whereas TrkA was reduced to less than 50% of normal value [90]. However, Western blotting showed a selective reduction of TrkB catalytic form (145 kDa), not the truncated form, in both frontal and temporal cortex [6], with blots showing a preponderance of truncated over catalytic form of TrkB. Interestingly, studies on PS1 knockouts have shown that the maturation of TrkB and BDNF-inducible TrkB autophosphorylation is compromised in neurones lacking PS1 [148]. The inference here is that mutations in PS1 (as seen in familial AD) may be having an effect on neurotrophin transport.

If there is loss of, or aberrant processing or defective transport of, the Trk receptors in AD or other disease states, this may have important consequences for specific groups of neurones, some being more vulnerable than others.

Huntington's disease

Symptoms and neuropathology

Huntington's disease is an autosomal dominant neurodegenerative disease characterized by a progressive choreic moment disorder. In addition, there are cognitive abnormalities and emotional disturbance, with symptoms often beginning in mid-adulthood. Neuropathology includes the loss of neurones particularly from the striatum and cerebral cortex. In the striatum there is selective degeneration of medium-sized spiny projection neurones [51,163,206] (Figure 5). The protein huntingtin is central to the pathogenesis of this disease, in which huntingtin is mutated by the presence of a polyglutamine expansion at the N-terminus [152]. It is a large cytoplasmic protein (over 3140 amino acids) and is highly expressed in the brain. In almost every case, proteins with repeats of fewer than 38 glutamine residues are harmless, but those with repeats of more than 41 glutamine residues form toxic neuronal nuclear aggregates in the affected neurones, and it has been shown that exon I of the *huntingtin* gene, with an expanded CAG repeat, is sufficient to cause a progressive neurological phenotype in transgenic mice [15].

Protective effects in animal models

The striatal spiny neurones, so vulnerable in Huntington's disease, require BDNF for survival and differentia-

tion [99,142,205], and BDNF protects the majority of striatal projection neurones from excitotoxin lesions [1,30]. Other trophic factors are able to protect certain subsets of these neurones [1]. Overexpression of wild-type huntingtin protein in cell lines and 'knock-in' transgenic mice leads to increased levels of BDNF mRNA and protein [218]. By contrast it has been shown that mutant huntingtin down-regulates BDNF production [218]: thus a reduction in BDNF expressed was seen in cells lines established from a 'knock-in' mouse where a 109 CAG triplet repeat had been inserted into the endogenous mouse *huntingtin* gene. In addition BDNF protein level was reduced in striatum and cortex of transgenic mice overexpressing mutant huntingtin. Most importantly BDNF levels in cortical tissue are reduced by 45% in brains from Huntington's disease patients [218]. Various studies on animal models suggest neurotrophic factors may be of benefit in promoting the survival of striatal neurones. In animals treated with the excitotoxin quinolinic acid, cells genetically engineered to release the neurotrophins BDNF, NT-3, or NT-4/5 were implanted into the striatum. All these neurotrophins were shown to promote the survival of striatal projection neurones, with BDNF being most effective as a survival agent [133,155] and NT-3 most successful at initiating differentiation [154]. Whether this therapeutic potential of the neurotrophins will extend to the clinic remains to be seen.

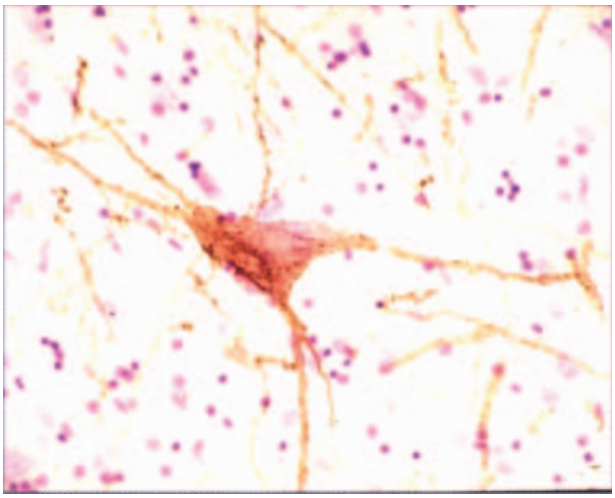

Figure 4. Photomicrograph of a human basal forebrain magnocellular, hyperchromic, cholinergic neurone stained using an antibody to the $p75^{NTR}$ receptor. Note perinuclear, dendritic and axonal staining.

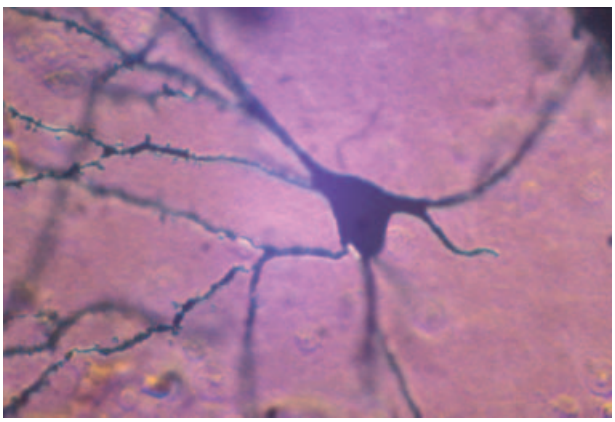

Figure 5. Golgi-stained section from human striatum showing a medium-sized spiny neurone. These neurones degenerate in Huntington's disease.

Parkinson's disease

Overview of symptoms and neuropathology

Parkinson's disease is a slow, progressive disease involving rigidity and tremor of the limbs, postural instability and bradykinesia of the limbs and body. It has a peak onset after the age of 60 years and affects about 1% of people over this age. The motor symptoms of PD are directly related to the loss of pigmented cells in the substantia nigra, a nucleus in the ventral midbrain, and to the reduction of the neurotransmitter dopamine in the striatum. The characteristic hallmark is also the presence of the Lewy body, containing the proteins ubiquitin and alpha-synuclein, within the cytoplasm of dying nerve cells. The neuronal loss has been proposed to occur by the action of free radicals and environmental toxins. Genetic linkages have been found in several families [69,158]; the two best described are *alpha-synuclein* on chromosome 4 and *parkin* on chromosome 6.

Current treatment

Most symptoms arise from a deficiency of dopamine and most anti-Parkinson drugs restore or mimic the actions of dopamine. Most commonly levodopa (Atamet or Sinemet) is given to improve motor symptoms, in combination with carbidopa that inhibits peripheral breakdown. The effects of levodopa are transient, because it does not slow down the continuing loss of dopaminergic cells.

Implantation of dopamine-rich tissue into the striatum aims to replace the lost nigral cells. Possible sources are dopamine-like cells from the patient's adrenal gland or carotid body. The results of such operations have been largely unsuccessful because of the low percentage of dopaminergic cells and low neuronal survival rate. Nigral cells from substantia nigra may be an alternative, possibly from pig brain, and embryonic stem cells implanted in the brains of mice and rats have been shown to transform into dopaminergic cells and connect with nearby brain cells [22].

Another alternative is the use of transplanted embryonic neural tissue. Small open-label (no control group) studies have reported long-term improvement in PD patients [55]. However, the first placebo-controlled study using transplanted embryonic neural tissue reported less successful results, with some transplants producing severe side-effects [67] which have been attributed to some aspects of the protocol used, such as tissue-storage and surgical methods.

Trophic factors

Combination of implants and trophic factors Studies have been carried out on implanted cells and tissue, which suggest that pretreatment of cells with trophic factors may improve cell survival [60,81,134]. In mesencephalic primary cultures, addition of NT-4/5 increases survival of dopaminergic neurones, size of the neuronal soma and the complexity of dendritic branching [191]. NT-4/5 infusions can increase the efficacy of nigral grafts in rat models of PD [81,98]. In addition, BDNF treatment has been shown to increase number of dopaminergic neurones and the release of dopamine in cultures before grafting into rat models [92,187].

GDNF (see Figure 1) is a survival factor for mesencephalic dopaminergic neurones [123]. It is able to improve motor deficits in animal models of PD, lesioned by 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [77]. Various methods of administration of GDNF have been employed including use of viral vectors, implantation of cells and protein infusion [112,219]. In cultures of foetal midbrain GDNF increases survival and differentiation of dopaminergic neurones [124,139,208] and promotes cell survival and fibre growth in nigral grafts [76,102,172,183,217]. Beneficial effects have been seen in a combined treatment of BDNF and GDNF on foetal nigral tissue [140,178]. Two studies showed benefit from chronic delivery of GDNF in nonhuman primates. Kordower and colleagues [112] used a lentiviral vector to transfect the striatum and substantia nigra of Parkinson rhesus monkeys. The infusion was carried out 1 week after MPTP toxicity and therefore it is possible that its effects were partly caused by neuroprotective effects of GDNF. Recently Grondin and colleagues [77] showed that infusion of GDNF into the lateral ventricle or the dorsal putamen in a primate MPTP model of PD reversed a 3-month motor dysfunction. During the first month of treatment both methods produced a marked reduction in bradykinesis and rigidity, and an increased number of nigro-striatal dopaminergic fibres and cell bodies in the substantia nigra.

Clinical trials with GDNF Clinical trials with GDNF are so far only in their early stages. In 1999 a report was made of the *post-mortem* results from a patient given monthly intraventricular GDNF injections for over 1 year [113]. No restoration of dopaminergic cells and no improvement in symptoms were observed. However, side-effects, including nausea and psychiatric symptoms, were reported. The authors suggested that other forms of delivery of GDNF should be explored, and it is possible that, as with administration of NGF in AD, the intraventricular route of administration may not be of most benefit. Recently GDNF was administered directly into the parenchyma of the dorsal putamen via a pump and catheter, in five PD patients. After 1 year of treatment the patients are showing a marked reduction of symptoms with no notable side-effects [70]. These exciting results pave the way for a larger controlled trial of GDNF.

Amyotrophic lateral sclerosis

Symptoms and pathology

The most common form of adult onset motor neurone disease is amyotrophic lateral sclerosis (ALS, or Lou Gehrig's disease). ALS is a condition in which motor neurones in the spinal cord, brain stem and motor cortex progressively die. The symptoms, limb and bulbar muscle weakness, usually begin in early to mid-50s and rapidly progress towards paralysis and eventual death, usually within 3–5 years of diagnosis. About 30 000 people suffer from this in the USA, and approximately 5–10% of cases are familial. Of the latter, around 20% are caused by dominant mutations in the free-radical-scavenging enzyme, Cu/Zn

superoxide dismutase gene, *SOD1* [166,171], and nearly 100 mutations have now been identified. *SOD1* knockouts are essentially normal [162] but mutant *SOD1* transgenic mice [215] develop adult-onset motor neurone disease with similar neuropathology to human ALS. Riluzole, an antiexcitotoxic agent, extends life by 2–3 months [16,141] and is the major drug approved for treatment. Neuronal loss is accompanied by astrocytosis and the presence of abnormal accumulation of phosphorylated neurofilaments in the cytoplasm [146]. There are many theories as to the mechanism of pathology including oxidative stress, neurofilament abnormalities, aberrant mitochondrial function and glutamate excitotoxicity [41,105,173,174,188]. It is suggested that the neuronal cell death may result from an apoptotic mechanism [115,121,177]. Mitochondrial function is compromised in ALS tissues [135,209] in hybrid cell lines containing mitochondria from ALS patients [195], and in SOD1 mice [78,100].

Neurotrophins and other trophic factors as therapeutics

Alterations in neurotrophins and their receptors have been found in ALS. In one study in ALS muscle biopsies at *post-mortem* [192], NGF concentrations were 140% higher than normal. In another, mRNA and protein levels of NGF, BDNF, NT-3 and NT-4/5 were all found to be increased [117]. BDNF was strongly up-regulated in the early stage of the disease, whereas levels of NGF, NT-3 and NT-4/5 gradually increased during the course of the disorder. In a study of ALS spinal cords TrkB mRNA was up-regulated [147] but the receptor was found to be much less phosphorylated on tyrosine residues than was that of controls. In an organotypic motor neurone culture model of mitochondrial inhibition [204], NT-4/5 completely prevented malonate-induced toxicity, whereas BDNF or NT-3 had no neuroprotective effect. It was suggested from this that it may be better to explore the use of NT-4/5, as a therapeutic for ALS, than BDNF. Adenovirus-mediated intramuscular gene transfer of NT-3 produced a 50% increase in life span, reduced loss of motor axons and improved neuromuscular function in the mouse mutant pmn (model of progressive motor neuropathy) [79].

However, to date clinical trials have not been particularly successful. In an initial phase I/II, trial BDNF appeared to increase survival rate and retard loss of pulmonary function in ALS patients [28]. These findings were not replicated, however, in a multicentre placebo-controlled 9-month trial of 1135 ALS patients administered 25 or 100 μg/kg BDNF [196], although the 20% of patients treated with the higher dose did show a nonsignificant trend toward increased survival over a 9-month period. In a later double-blind, sequential, dose-escalation study, recombinant human methionyl BDNF was infused intrathecally for 12 weeks, by means of an implanted pump, into 25 patients with ALS (25–1000 μg/day). The majority of patients reported only mild sensory symptoms usually confined to the lower limbs, which mostly declined or disappeared over several weeks. However, the small number of patients did not allow conclusions to be drawn about the efficacy of the treatment [150], although from the results it would seem that BDNF can be given safely by this method.

Peripheral neuropathy

Causes, symptoms and current therapy

Peripheral neuropathy encompasses a range of neurological disorders resulting from damage to the peripheral nerves. The most common cause is diabetes, in which symptoms are predominantly related to the degeneration of sensory fibres and include numbness, paresthesiae and limb pain, as well as weakness because of motor fibre involvement. In general, drugs are of limited benefit.

NGF implicated in neuropathy and replacement in animal models

NGF is trophic for small fibre sensory and sympathetic neurones, which are known to be affected at an early stage in diabetes. Over two decades ago, changes in retrograde transport of NGF were reported in streptozotocin (STZ)-induced diabetes in rats [101], a model of diabetic neuropathy. In the early 1990s, evidence began to accumulate of an involvement of NGF in diabetic neuropathy. Serum levels of NGF in patients with diabetic neuropathy were found to be lower than in controls, the reduction in NGF levels correlating with the decrease of motor nerve conduction velocity [62]. In rats with STZ-induced sensory neuropathy, NGF levels were doubled in most peripheral targets, perhaps reflecting impaired removal, whereas levels were decreased to roughly half in sympathetic ganglia [89]. Experiments showed that in young diabetics there was an early dysfunction of small-diameter sensory fibres, with

depletion of skin NGF and the sensory neuropeptide, substance P [8]. In rodent models of diabetes, deficits in NGF and TrkA were reported, presumably resulting in decreased support of NGF-dependent sensory neurones [199]. Treatment with exogenous NGF normalized these deficits [200]. In a rat model of hypoinsulinaemic diabetes, sensory and motor conduction velocities are significantly reduced at 7 months, indicative of a polyneuropathy. However administration of NGF (subcutaneous 1 mg/kg, 3× week) was able to restore the diabetes-related deficit in sensory C-fibre function [58].

In cancer patients, NGF plasma levels were shown to decrease dramatically during chemotherapy. The decrease in NGF levels seemed to correlate with the severity of neurotoxicity and suggested that NGF might prevent antineoplastic drug-induced neuropathies [54]. Gene transfer of herpes simplex virus vector, expressing NGF, into the DRG of mice with STZ-induced sensory neuropathy [74] protected against visible measurements of neuropathy.

Studies in animal models have suggested that NT-3 may also be useful therapeutically in several neuropathies. NT-3 mRNA was found to be deficient in leg muscle of diabetic rats [199] and administration of rhNT-3 was able to restore conduction velocity [200]. Furthermore, intramuscular injection of a recombinant adenovirus encoding NT-3 to deliver sustained low doses of NT-3 [159] prevented or partially prevented the slowing of motor and sensory nerve conduction velocities in STZ-induced diabetes and acrylamide experimental neuropathy.

More recently, with the new interest in pro-NGF, the levels of NGF in diabetes have been re-examined. In a study of human and rat skin and nerve extracts [216], using Western blotting, prepro-NGF was seen to be markedly reduced in skin extracts from patients with subclinical diabetic neuropathy. The overall results of the study suggested that prepro-NGF may be preferentially taken up and transported by $p75^{NTR}$.

Neurotrophin trials

In the mid-1990s the large-scale production of NGF and other trophic factors made therapeutic trials feasible for various neurodegenerative disorders [170]. Initial Phase I clinical studies in humans indicated that rhNGF produced mild to moderate myalgias shortly following single intravenous or subcutaneous doses of 1 μg/kg; in general, these effects were not observed at lower doses. Following multiple dosing, some patients with peripheral neuropathies reported an improvement in clinical symptoms, which in some cases correlated with improvement on neurological examinations [10]. Overall, rhNGF was given to 250 patients with diabetic polyneuropathy for 6 months with significant improvement in sensory tests. The impression of most subjects was that their neuropathy had improved. The treatment was well tolerated, with injection site discomfort reported as the most frequent adverse event.

Subsequently, rhNGF was tested in a phase II trial of 270 patients with HIV-associated sensory neuropathy within a multicentre, placebo-controlled, randomized trial [125]. Subcutaneous injection of 1 μg/kg, or 0.3 μg/kg rhNGF twice weekly was given for 18 weeks. Both doses produced significant relief of pain. Long-term evaluation (48 weeks) [179] in an open-label study of 200 subjects with HIV-associated neuropathy gave similar results, confirming that NGF was safe, well-tolerated and significantly reduced pain, although no improvement was reported in sensory function or epidermal nerve fibre density. This led to a phase III trial lasting 48 weeks, in which 1019 patients received rhNGF or placebo. Unfortunately the earlier suggestion of efficacy was not replicated. This was blamed on a variety of differences between the phase II and III trials. However, the end result was that Genentech, the producer of the rhNGF, decided not to proceed further with clinical trials [9].

Neurotrophin agonists/antagonists as therapeutic agents

The pharmacokinetic properties of the neurotrophins are probably the key factor in the therapeutic success or failure of these proteins. The production of small molecule agonists would help to circumvent this problem. Until now the production of such drugs has required the mass screening of large libraries of synthetic chemicals. However, recently small molecule mimetics have been designed which are able to act at the erythropoietin receptor [73]. This has been achieved largely through a detailed knowledge of the interactions between erythropoietin with its receptor. Like NGF, erythropoietin dimerizes two receptors to induce signal transduction. The general principle used was to identify a compound that would bind to one receptor, thereby acting as an antagonist, and to then synthesize that molecule in an oligomeric form such that it was able to bind to two receptors, induce dimerization and initiate signal transduction [160]. Similarly, a small molecule has been

shown as able to mimic the actions of the protein granulocyte colony stimulating factor [198].

Examinations of the structures of the NGF-TrkAIg2 and NT-4/5- and BDNF-TrkBIg2 complexes (Figures 3 and 6) show that the Ig2 domains consist of two β-sheets with one exposed disulphide bond. Two contact regions between each receptor domain and the neurotrophin have been identified. One of these comprises residues from the central β-sheet of NGF in contact with the loops at the carboxy-terminal pole of the TrkAIg2 monomer. As many of the amino acids forming these surfaces are conserved between each of the neurotrophins and their Trk receptors, interactions in this region can be termed the 'conserved' patch, with similar contacts for each neurotrophin:Trk receptor pair. The second contact region comprises the N-terminal residues of NGF and the face of one β-sheet of TrkAIg2. This can be termed the 'specific' contact patch, as amino acids forming these areas vary between both neurotrophins and the Trk receptors. This region will therefore contribute most of the specificity of binding of the neurotrophins with their receptors. If we examine the interactions formed between NGF and TrkAIg2 (Figure 6**A**) and NT-4/5 with TrkBIg2 (Figure 6**B**), we can see that in the former case there are key residues which contribute to a hydrophobic environment compared with the NT-4/5:TrkBIg2 interaction, which is more ionic, in particular, the salt bridge between Arg-11 (NT-4/5) and Asp-298 (TrkBIg2). This is somewhat of an oversimplification but serves to illustrate how the X-ray crystal structures of the Trk-binding domains, in combination with state-of-the-art computing power, will allow the *in silico* screening of very large virtual libraries (of the order of 10^{11} molecules), hopefully to produce useful therapeutic compounds.

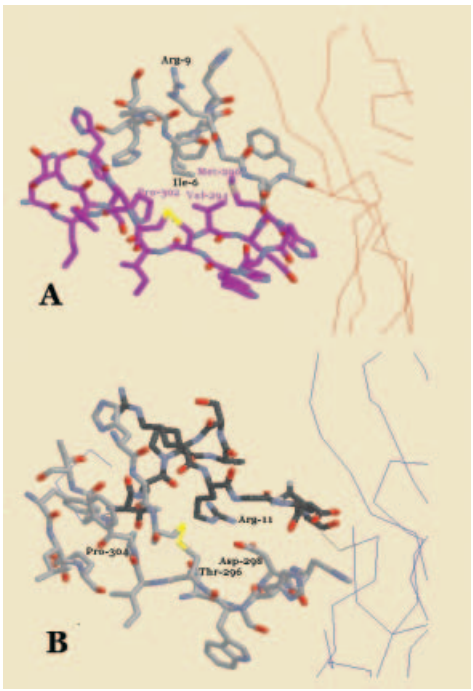

Figure 6. **(A)** Stick representation of the N-terminus of NGF (grey) binding to a pocket in the face of the TrkA molecule (purple). At the base of the pocket is a disulphide bond between two opposing strands of the sheet. The amino acids forming the sides of the pocket are Pro-302, Val-294 and Met-296. The main contact from the NGF N-terminal is from the side chain of Ile-6 which sits in and forms hydrophobic contacts. **(B)** Stick representation of the N-terminus of NT-4/5 (black) binding to a pocket in the face of the TrkB molecule (grey). At the base of the pocket is a disulphide bond between two opposing strands of the sheet. The amino acids forming the sides of the pocket are Pro-304, Thr-296 and Asp-298. The main contact from the NT-4/5 is from Arg-11, which forms a salt bridge with Asp-298.
NGF, nerve growth factor; Trk, tyrosine kinase; NT, neurotrophin.

Summary

Neurotrophic proteins have been used in clinical trials for neurodegenerative disorders such as AD, PD, Huntington's disease, ALS and peripheral neuropathy. In general, the results of experiments with animal models and many phase I trials looked promising. However, because of poor pharmacokinetic profiles and difficulties in the delivery of proteins into the brain, the initial promise has not been upheld in larger, placebo-controlled trials. Recent breakthroughs from X-ray crystallography of the neurotrophins and their receptors should allow the development of small molecule agonists which will hopefully fulfil the clinical potential of the neurotrophin approach to the treatment of several neurodegenerative diseases.

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